Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn5–gusA

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Tn5–gusA promoter/probe transposons have been constructed that fuse the *Escherichia coli* gusA reporter gene transcriptionally or translationally with a target promoter. These have been used to monitor expression of *Rhizobium meliloti* symbiotic genes within alfalfa nodules. Fusions in all 11 *nod* genes studied show the same pattern of expression: first on the root surface, then throughout the developing nodule, then mainly in the nodule meristem, falling off progressively through the central region, and then disappearing. In contrast, fusions in all five *nif* genes studied, all four *fix* genes, and *syrM* show a second, different pattern: expression beginning later, first throughout the nodule except for the meristem, strongest just behind the meristem, and falling off progressively through the central region. Novel features revealed by these studies include *nod* expression in the meristem, regulated in planta expression of control genes *nodD1* and *nodD3*, disappearance of *nod* expression late in organogenesis, and properties of *syrM*.

[Key Words: *Rhizobium meliloti*; Tn5–gusA; *nod* genes]

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Rhizobia elicit the formation of root nodules on leguminous hosts by means of a complex series of developmental changes [Newcomb 1981]. The invading bacteria first induce root hairs to curl into “shepherd’s crooks” and stimulate cell divisions in the plant root cortical layer, giving rise to a new meristem [Callaham and Torrey 1981]. Then, bacteria penetrate the curled root hair and invade the growing meristem through a tubular polysaccharide structure called the infection thread. Finally, the bacteria surrounded by a plant-derived membrane are released into the interior of plant cells, where they differentiate into morphologically distinct forms called bacteroids that can fix atmospheric nitrogen into ammonia.

Both nodule formation and symbiotic nitrogen fixation require the developmentally regulated expression of bacterial genes. One group, the *nod* genes (Fig. 1), is involved in nodule formation. The “common” *nod* genes, *nodA*, *nodB*, and *nodC*, are functionally and structurally conserved among different rhizobia [Kondorosi et al. 1984; Fisher et al. 1985]. In *Rhizobium meliloti* three regulatory genes, *nodD1*, *nodD2*, and *nodD3*, activate these and other *nod* genes in the presence of plant root exudates [Mulligan and Long 1985, 1989; Peters et al. 1986; Honma and Ausubel 1987], and help to determine host range by their differential response to various exudate flavonoids [Spaink et al. 1987; Gyorgypal et al.

1988]. The host-specific nodulation (*hcn*) genes, *nodH*, *nodF*, *nodE*, *nodG*, *nodP*, and *nodQ*, in which mutations cannot be complemented by cloned genes from other rhizobia [Kondorosi et al. 1984; Debelle and Sharma 1986; Debelle et al. 1986; Cervantes et al. 1989], also help determine host range, at least in part, by modifying the growth-promoting substance synthesized by *nodA* and *nodB* [Schmidt et al. 1988; Faucher et al. 1989]. Two additional genes, *nodI*, and *nodJ* (*nod* region IIa; Debelle et al. 1986; Evans and Downie 1986), which are probably part of the *nodABC* operon, affect the kinetics of nodulation.

Another group of genes, pivotal to symbiotic nitrogen fixation, includes those coding for nitrogenase (*nif*) and others required for fixation whose precise functions are not known (*fix*). In *R. meliloti*, *nif* genes include *nifH*, *nifD*, and *nifK* [Ruvkun et al. 1982], *nifN* [Aguilar et al. 1987], and *nifA* and *nifB* [Buikema et al. 1985, 1987; and the cotranscribed *fdxN* gene for ferredoxin; Klipp et al. 1989], whereas *fix* genes include *fixA*, *fixB*, *fixC*, and *fixX* [Earl et al. 1987], *fixL* and *fixJ* [David et al. 1988], *fixK* and *fixN* [Batut et al. 1989], and *fixG*, *fixH*, and *fixI* [Kahn et al. 1989]. These genes are induced in bacteroids by the FixL and FixJ proteins, which belong to a conserved family of two-component regulators [David et al. 1988]. FixL recognizes a signal present in the low oxygen symbiotic environment and modulates the activity of FixJ which, in turn, activates *nifA* and *fixK* to produce positive activators of other *nif* and *fix* genes. Both *nifA* and *fixK* can also be induced by microaerobic culture

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Our knowledge of how these symbiotic genes are regulated in planta is fragmentary. In this paper we describe an easy and rapid system for in planta studies based on Tn5-*gusA* transposons that fuse target operons or genes with the promoterless reporter *gusA* gene of *Escherichia coli*. The *gusA* gene product, β -glucuronidase, can be easily and accurately assayed by spectrophotometric, fluorometric, and histochemical techniques [Jefferson et al. 1986, 1987]. Because neither *R. meliloti* nor alfalfa have detectable β -glucuronidase activity, *gusA* fusions can be used to monitor in planta expression of *R. meliloti* genes during nodule development. Using Tn5-*gusA* fusions, we show that the expression of symbiotic genes is temporally regulated and spatially restricted during nodule development with genes that act early (*nod*), showing a different pattern from genes that act late (*nif*, *fix*, *sydM*).

Construction of Tn5-*gusA1*, a transcriptional operon fusion transposon, is shown in Figure 2. Tn5-*gusA1* includes the first 25 bp of IS50L, which is sufficient for

Plasmid pRS475 carrying the *E. coli lac* operon was mutagenized with $\lambda \Omega :: \text{Tn5-gusA1}$, and colonies that were white on X-gal [5-bromo-4-chloro-3-indolyl β -D-galactopyranoside] + IPTG (isopropyl β -D-thiogalactopyranoside), but deep blue on X-Glc [5-bromo-4-chloro-3-indolyl β -D-glucuronide] + IPTG, were analyzed. The results (Fig. 3) demonstrate that Tn5-gusA1 fusion inserts are regulated by IPTG, as expected.

Figure 2. Construction and map of Tn5-gusA transposons. Operon and gene fusion transposons are represented by odd and even numbers, respectively. Open and hatched boxes depict Tn5-341:25 DNA (Johnson and Reznikoff 1983) and additional DNA fragments inserted into it, respectively; the wavy line represents ColE1 plasmid DNA. The DNA sequence above Tn5-gusA1 shows the *gusA*-coding strand, including the first 25 bp of IS50L fused to a synthetic DNA fragment; the sequence above Tn5-gusA2 includes the first 25 bp of IS50L but not the fragment; the arrow and the asterisks (*) indicate the junction points and the translation terminators, respectively; the Shine-Dalgarno sequence, initiation codon, and HindIII and EcoRI sites are underlined; letters below the sequence represent amino acids; the final ATG shown is the initiation codon of *gusA*; (E) EcoRI; (H) HindIII; (P) PstI; (S) SalI; (B) BglII; (X) XbaI. Tn5-gusA1 was constructed by ligation of an ~13-kb BamHI-SalI fragment from pRZ465 (ColE1 Ω :: Tn5-341:25) with a synthetic DNA fragment carrying Sau3AI and HindIII cohesive ends (see Experimental procedures), a 1.8-kb HindIII-SauI (*gusA* gene) fragment from pRAJ260, and a 1.3-kb SalI (Km^r, Tn903) fragment from pKAN. Tn5-gusA2 was constructed by ligation of an ~13-kb BamHI-SalI fragment from pRZ465 (with the BamHI site filled in by Klenow polymerase) with a 1.8-kb HindIII-SalI fragment from pRAJ260 (with the HindIII site filled in by Klenow polymerase) and a 1.3-kb SalI fragment from pKAN. Tn5-gusA3 and Tn5-gusA4 were made by deletion of the 1.3-kb SalI (Km^r, Tn903) fragments of Tn5-gusA1 and Tn5-gusA2, respectively, and religation. Similarly, Tn5-gusA5 and Tn5-gusA6 were constructed by deletion of the BglII-XbaI (Tc^r) fragments of Tn5-gusA1 and Tn5-gusA2, respectively, and religation after filling in of the noncohesive ends with Klenow polymerase. Tn5-gusA7 was made by deletion of the SalI-BglII (Km^r, Tc^r) fragment of Tn5-gusA1, and blunt-end ligation of the 2.2-kb EcoRI (Ω -Km^r) fragment of pHP45 Ω -Km (after filling in with Klenow polymerase). Tn5-gusA9 and Tn5-gusA10 were constructed like Tn5-gusA7 but with the 2-kb EcoRI (Ω -Sm^r/Sp^r) fragment of pHP45 Ω instead of Ω -Km^r.

Regulation of nod :: Tn5-gusA fusions in culture by luteolin

Tn5-gusA fusions were made in *nod* genes and exchanged to the *R. meliloti* genome. Insertions in *nodD1*, *nodD3*, *nodJ*, and *syrM* were verified by Southern hybridization (data not shown). Genomic insertions in *nodB* and *nodC* showed no hair curling (Hac⁻), and those in *nodI*, *nodJ*, *nodP*, *nodQ*, and *nodE* were delayed

in nodulation (Nod^d), as expected. Genomic insertions in *nodB*, *nodC*, *nodE*, *nodI*, *nodJ*, *nodP*, and *nodQ* were induced manyfold by luteolin (Table 2). However, the same insertions located on plasmid pGMI149 had high basal levels, whereas insertions in *nodH*, *nodE*, and *nodG* located on plasmid pGMI290 had comparatively low expression, which was also not stimulated by luteolin (Table 2). This may be due to *syrM*, *nodD3*, or both on pGMI149, which, on a plasmid, have been found

Table 1. Bacterial strains, plasmids, and phages

Name	Relevant markers	Source
<i>Escherichia coli</i>		
C2110	<i>polA</i>	lab collection
HB101	<i>hsdS20</i> ($x_B^- m_B^-$) <i>recA13 ara-14</i> <i>lacY1 galK2 rpsL20</i> (Sm^r) <i>xy1-5</i> <i>mtl-1 supE44</i>	Maniatis et al. (1982)
JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> λ^- <i>supE44 relA1</i> Δ <i>lac-proAB</i> (F' <i>traD36</i> <i>proAB lacI^Q lacZ</i> Δ M15)	Yanisch-Perron et al. (1985)
LE392	F^- <i>hsdR514 supE44 supF58 lacY1 galK2</i> <i>galT22 metB1 trpR55</i> λ^-	Maniatis et al. (1982)
MM294A	<i>pro-82 thi-1 endA1 hsdR17 supE44</i>	G.C. Walker
MRO	F^- <i>lac</i> Δ X74 <i>his</i>	lab collection
MT607	MM294 <i>recA56</i>	T.M. Finan
Q5137	(λ imm434)	lab collection
<i>Rhizobium meliloti</i>		
JM83	JM57 <i>syrM</i> :: Tn5	Mulligan and Long (1985)
JT701	RM1021 <i>syrM</i> :: Tn5	Mulligan and Long (1985)
Rm1021	SU47 <i>str-7</i>	F. Ausubel
Rm1947	Rm1021 <i>fixJ</i> :: Tn5	L. Albright
RmS89	Rm1021 <i>nodB</i> :: Tn5- <i>gusA1.3</i>	this work
RmS90	Rm1021 <i>nodC</i> :: Tn5- <i>gusA1.9</i>	this work
RmS91	Rm1021 <i>nodQ</i> :: Tn5- <i>gusA1.11</i>	this work
RmS92	Rm1021 <i>nod</i> region IIa (<i>nodJ</i>) :: Tn5- <i>gusA1.20</i>	this work
RmS94	Rm1021 <i>nodQ</i> :: Tn5- <i>gusA1.54</i>	this work
RmS95	Rm1021 <i>nodE</i> :: Tn5- <i>gusA1.56</i>	this work
RmS98	Rm1021 <i>nod</i> region IIa (<i>nodI</i>) :: Tn5- <i>gusA2.440</i>	this work
RmS99	Rm1021 <i>nodP</i> :: Tn5- <i>gusA2.475</i>	this work
RmS102	Rm1021 <i>nodC</i> :: Tn5- <i>gusA2.527</i>	this work
RmS163	Rm1021 <i>syrM</i> :: Tn5- <i>gusA2.444</i>	this work
RmS225	Rm1021 <i>nodD1</i> :: Tn5- <i>gusA1.17</i>	this work
RmS227	Rm1021 <i>nodD1</i> :: Tn5- <i>gusA1.35</i>	this work
RmS229	Rm1021 <i>nodD1</i> :: Tn5- <i>gusA6.45</i>	this work
RmS239	Rm1021 <i>nodD3</i> :: Tn5- <i>gusA1.16</i>	this work
RmS238	Rm1021 <i>nifH</i> :: Tn5- <i>gusA2.774</i>	this work
RmS253	Rm1021 <i>fixX</i> :: Tn5- <i>gusA2.98</i>	this work
RmS255	Rm1021 <i>nifA</i> :: Tn5- <i>gusA2.33</i>	this work
RmS257	Rm1021 <i>fixA</i> :: Tn5- <i>gusA2.38</i>	this work
RmS261	Rm1021 <i>nifB</i> :: Tn5- <i>gusA2.68</i>	this work
RmS263	Rm1021 <i>nifD</i> :: Tn5- <i>gusA2.71</i>	this work
RmS264	Rm1021 <i>fixB</i> :: Tn5- <i>gusA2.72</i>	this work
RmS268	Rm1021 <i>fixC</i> :: Tn5- <i>gusA2.104</i>	this work
RmS318	Rm1021 <i>nodJ</i> :: Tn5- <i>gusA1.20</i> <i>syrM</i> :: Tn5	this work
Plasmids		
pGMI149	pRK290 prime carrying <i>nod</i> genes	Debelle et al. (1986)
pGMI290	pRK290 derivative carrying <i>nodHFEG</i>	Debelle and Sharma (1986)
pHP45 Ω	Carries Ω Sm^r /Spc r DNA fragment	Fellay et al. (1987)
pHP45 Ω -Km	Carries Ω Tn5 Km r DNA fragment	Fellay et al. (1987)
pKAN	pBR322 derivative carrying Tn903 Km r	W.S. Reznikoff
pRAJ260	pEMBL9 derivative carrying <i>gusA</i>	Jefferson et al. (1986)
pRK290.720	pRK290 prime carrying <i>nif</i> and <i>fix</i> genes	Corbin et al. (1982)
pRK600	pRK2013 <i>npt</i> :: Tn9 Cm r	lab collection
pRS475	pBR322 carrying <i>lac</i> operon	R. Simons
pRZ465	ColE1 plasmid carrying Tn5-341:25	Johnson and Reznikoff (1983)
pSB26	pRK290 prime carrying 8.7-kb <i>EcoRI</i> fragment with <i>nodABCD</i>	lab collection
pSB165	ColE Ω :: Tn5- <i>gusA1</i>	this work
pSB166	ColE Ω :: Tn5- <i>gusA2</i>	this work
pSB354	pRK600 Ω :: Tn5- <i>gusA2</i>	this work
pSB387	pRK600 Ω :: Tn5- <i>gusA1</i>	this work

(Continued on p. 348.)

Table 1. Continued

Name	Relevant markers	Source
pSB437	pGMI290 <i>nodH</i> :: Tn5- <i>gusA2.437</i>	this work
pSB445	pGMI290 <i>nodE</i> :: Tn5- <i>gusA2.445</i>	this work
pSB467	ColE Ω :: Tn5- <i>gusA3</i>	this work
pSB469	ColE Ω :: Tn5- <i>gusA4</i>	this work
pSB471	ColE Ω :: Tn5- <i>gusA5</i>	this work
pSB473	ColE Ω :: Tn5- <i>gusA6</i>	this work
pSB482	ColE Ω :: Tn5- <i>gusA7</i>	this work
pSB483	ColE Ω :: Tn5- <i>gusA9</i>	this work
pSB485	ColE Ω :: Tn5- <i>gusA10</i>	this work
pSB504	pRK600 Ω :: Tn5- <i>gusA7</i>	this work
pVL4	pBR322 carrying <i>nifA</i> under control of <i>Ptac</i> promoter	M.P.V. Lemley
Phages		
λ 58	λ b221 <i>Oam29 Pam80</i>	M.S. Fox
λ SB242	λ 58 Ω :: Tn5- <i>gusA1</i>	this work
λ SB247	λ 58 Ω :: Tn5- <i>gusA2</i>	this work
ϕ M12	transducing phage of <i>R. meliloti</i>	Finan et al. (1984)

to cause a high basal level of *nodC* :: *lacZ* expression (Mulligan and Long 1989). Our results indicate that the expression of the *nodE*, *nodG*, *nodP*, and *nodQ* genes are also regulated like *nodC* by *sydM* and *nodD3*. Fusions with *nodD1* and *nodD3* were constitutive, as expected (Table 2). Comparison of the data for Tn5-*gusA1* and Tn5-*gusA2* shows that the synthetic Shine-Dalgarno sequence is functional in *R. meliloti*.

In situ expression of nod genes

nod :: Tn5-*gusA* fusions were assayed in three genetic configurations: (1) fusions in *nodB*, *nodC*, *nodD1*, *nodD3*, *nodG*, *nodH*, *nodI*, *nodJ*, *nodP*, or *nodQ* on plasmid pGMI149 with a complementing wild-type allele in the genome; (2) fusions in each of these genes (except *nodG* and *nodH*) in the genome with the wild-type allele on complementing plasmid pGMI149; and (3) fusions in *nodD1*, *nodD3*, *nodE*, *nodI*, *nodJ*, *nodP*, and *nodQ* in the genome without a complementing wild-type allele. Expression in the different genetic configurations was generally similar, although nodule sections of fusions on the plasmids developed color faster than the same genomic fusions (Table 3). The exception was *R. meliloti* carrying *nodG* :: Tn5-*gusA2* on plasmid pGMI290, which had temporally and spatially aberrant expression that was found to correlate with loss of the

plasmid (data not shown), even though pGMI290 *nodH* :: Tn5-*gusA2* had typical expression; this was not pursued. The sections of fusions in *nodD1* and *nodD3* developed color slower than those of other *nod* fusions. In planta expression of all the *nod* operon and gene fusions showed the same temporal and spatial pattern in relation to the stages of alfalfa nodule development, illustrated for *nodC* :: Tn5-*gusA2.527* in Figure 4.

Expression on the surface of root hairs and of the nodule cortex (Fig. 4A,B) By 36 to 48 hr after inoculation, *nod* :: Tn5-*gusA* fusions were expressed in bacteria located ~0.5–1 cm away from the root tip. At 3–6 days post inoculation (dpi), expression was not distributed evenly over the length of the root but was scattered randomly with a slight preference for the point of emergence of secondary roots. By 6–7 dpi, small nodule-like structures began appearing, and expression was seen on the root hairs and on the surface of the nodule cortex. In some cases, there was a single spot at the tip of the nodule; in others, several spots were scattered over the nodule surface. Up to 3 days after nodule emergence (10 dpi), expression was limited to several peripheral cells of the nodule cortex. Later expression of *nod* genes in our system compared to others (e.g., Dudley et al. 1987) presumably reflects differences in experimental conditions.

Figure 3. Map of *lacZYA* and positions of Tn5-*gusA1* insertions. Shaded box, open box, and thin line represent *lac* promoter region, *lacZYA* structural genes, and plasmid pRS475 DNA, respectively. Vertical lines on *lacZYA* show the positions of Tn5-*gusA1* insertions. Inducibility of pRS475 *lacZ* :: Tn5-*gusA1* insertions in JM109 by 0.1 mM IPTG is given below the map.

<i>E. coli</i> strain	β -glucuronidase units	
	No IPTG	0.1 mM IPTG
JM109 (pRS475)	14	14
JM109 (pRS475::Tn5- <i>gusA1.1</i>)	33	1565
JM109 (pRS475::Tn5- <i>gusA1.4</i>)	35	1923
JM109 (pRS475::Tn5- <i>gusA1.5</i>)	21	1817

Table 2. Induction of *Tn5*–*gusA* fusions by luteolin

Strain	Insertion	β -Glucuronidase units	
		(–)	(+)
Genomic insertions			
Rm1021		0	0
RmS89	Rm1021 <i>nodB</i> :: Tn5– <i>gusA1.3</i>	3	69
RmS90	Rm1021 <i>nodC</i> :: Tn5– <i>gusA1.9</i>	6	81
RmS102	Rm1021 <i>nodC</i> :: Tn5– <i>gusA2.527</i>	2	132
RmS98	Rm1021 <i>nodI</i> :: Tn5– <i>gusA2.440</i>	3	92
RmS92	Rm1021 <i>nodJ</i> :: Tn5– <i>gusA1.20</i>	11	121
RmS91	Rm1021 <i>nodQ</i> ⁺ :: Tn5– <i>gusA1.11</i> ^a	12	124
RmS94	Rm1021 <i>nodQ</i> :: Tn5– <i>gusA1.54</i>	5	525
RmS99	Rm1021 <i>nodP</i> :: Tn5– <i>gusA2.475</i>	7	71
RmS95	Rm1021 <i>nodE</i> :: Tn5– <i>gusA1.56</i>	2	388
RmS225	Rm1021 <i>nodD1</i> :: Tn5– <i>gusA1.17</i>	16	19
RmS227	Rm1021 <i>nodD1</i> :: Tn5– <i>gusA7.35</i>	41	42
RmS239	Rm1021 <i>nodD3</i> :: Tn5– <i>gusA1.16</i>	5	8
RmS163	Rm1021 <i>syrM</i> :: Tn5– <i>gusA2.444</i>	2	3
Insertions on plasmids			
RmS32	Rm1021 pGMI149 <i>nodB</i> :: Tn5– <i>gusA1.3</i>	904	897
RmS34	Rm1021 pGMI149 <i>nodC</i> :: Tn5– <i>gusA1.9</i>	1660	1870
RmS68	Rm1021 pGMI149 <i>nodC</i> :: Tn5– <i>gusA2.527</i>	622	820
RmS60	Rm1021 pGMI149 <i>nodI</i> :: Tn5– <i>gusA2.440</i>	964	990
RmS38	Rm1021 pGMI149 <i>nodJ</i> :: Tn5– <i>gusA1.20</i>	1496	1942
RmS35	Rm1021 pGMI149 <i>nodQ</i> :: Tn5– <i>gusA1.11</i>	660	970
RmS45	Rm1021 pGMI149 <i>nodQ</i> :: Tn5– <i>gusA1.54</i>	212	686
RmS63	Rm1021 pGMI149 <i>nodP</i> :: Tn5– <i>gusA2.475</i>	916	990
RmS46	Rm1021 pGMI149 <i>nodE</i> :: Tn5– <i>gusA1.56</i>	310	612
RmS184	Rm1021 pGMI290 <i>nodH</i> :: Tn5– <i>gusA2.437</i>	41	62
RmS185	Rm1021 pGMI290 <i>nodG</i> :: Tn5– <i>gusA2.443</i>	7	8
RmS187	Rm1021 pGMI290 <i>nodE</i> :: Tn5– <i>gusA2.445</i>	40	45
RmS37	Rm1021 pGMI149 <i>nodD3</i> :: Tn5– <i>gusA1.16</i>	737	796
RmS212	Rm1021 pSB26 <i>nodD1</i> :: Tn5– <i>gusA1.17</i>	784	794
RmS214	Rm1021 pSB26 <i>nodD1</i> :: Tn5– <i>gusA1.35</i>	260	279
RmS62	Rm1021 pGMI149 <i>syrM</i> :: Tn5– <i>gusA2.444</i>	3	4
		(1) ^b	(1) ^b
RmS183	Rm1021 <i>fixJ</i> :: Tn5 (pGMI149 <i>syrM</i> :: Tn5– <i>gusA2.444</i>)	3	3
RmS25	Rm1021(pRK290.720 :: Tn5– <i>gusA2.774</i>)	7 (515) ^b	12 (941) ^b

^aInsert is 3' to *nodQ*-coding sequence.^bUnits in parentheses represent β -glucuronidase activity in microaerobic culture (oxygen, 0.4%)**Table 3.** Approximate time required for nodules carrying *gusA* fusions to show distinct blue color

Genotype	Time (min)
Rm1021 pRK290.720 <i>nifH</i> :: <i>Tn5</i> – <i>gusA2.774</i>	5–15
Rm1021 pRK290.720 <i>fixB</i> :: <i>Tn5</i> – <i>gusA2.72</i>	20–30
Rm1021 pRK290.720 <i>nifA</i> :: <i>Tn5</i> – <i>gusA2.33</i>	25–35
Rm1021 pGMI149 <i>nodC</i> :: <i>Tn5</i> – <i>gusA2.527</i>	40–60
Rm1021 pGMI149 <i>nodE</i> :: <i>Tn5</i> – <i>gusA1.56</i>	40–60
Rm1021 pGMI149 <i>nodD1</i> :: <i>Tn5</i> – <i>gusA7.35</i>	180–300
Rm1021 pGMI149 <i>syrM</i> :: <i>Tn5</i> – <i>gusA2.444</i>	180–600
Rm1021 <i>nifH</i> :: <i>Tn5</i> – <i>gusA2.774</i> (pRK290.720)	15–25
Rm1021 <i>fixB</i> :: <i>Tn5</i> – <i>gusA2.72</i> (pRK290.720)	40–60
Rm1021 <i>nodC</i> :: <i>Tn5</i> – <i>gusA2.527</i> (pGMI149)	60–90
Rm1021 <i>nodE</i> :: <i>Tn5</i> – <i>gusA1.56</i> (pGMI149)	60–90
Rm1021 <i>nodD1</i> :: <i>Tn5</i> – <i>gusA7.35</i> (pGMI149)	480–720
Rm1021 <i>syrM</i> :: <i>Tn5</i> – <i>gusA2.444</i> (pGMI149)	180–600

Uniform expression within the nodule (Fig. 4C,D) From 10 to 12 dpi, expression was visible both in several peripheral cells of the nodule cortex and in cells of the central region proximal to the cortex; slightly older (11–17 dpi) nodules showed more or less uniform expression throughout the central region and a limited area of the nodule cortex.

Polarity of expression toward the nodule meristem (Fig. 4E–G) Between 16 and 22 dpi, expression began to show polarity, appearing highest in the nodule meristem and falling off in the central region. From 20 to 25 dpi, the region of the nodule between the meristematic region and the central zone, where leghemoglobin is most prominent, showed distinctly lower expression than the meristem, and by 30 dpi, expression was limited to the

Figure 4. Typical in planta expression of *nodC* :: Tn5-*gusA2.527* [RmS102(pGMI149)] revealed by staining without prior fixation. (A) 5 dpi, expression on root hairs and at the point of emergence of a secondary root; (B) 9 dpi, expression at the tip of a newly emerging nodule; (C) 11 dpi, expression beginning to appear in the nodule tissue; (D) 15 dpi, expression throughout the nodule except for the uninfected cortex, epidermis, and endodermis; (E) 17 dpi, higher expression in the meristematic zone; (F) 27 dpi, expression high in the meristem and low in the central region (note secondary infection near the root); (G) 30 dpi, expression remaining only in the meristem; (H) 38 dpi, expression mostly gone (note secondary infection and outgrowth of the region proximal to the root); (I) 45 dpi, expression gone.

nodule meristem only. Occasional nodules had two meristems; in some of these, both had equal expression, whereas in others one meristem predominated (not shown). In some nodules, expression was unusually low in the region between the meristem and the central region (not shown).

Disappearance of expression (Fig. 4H–I) In general, expression began disappearing progressively from the meristematic zone between 31 and 37 dpi and had disappeared in most (~80%) of the nodules by 45 dpi. However, several (~10%) of the long, large, pink nodules showed expression in the meristematic zone even after 75 dpi. We have some indication that *nodD1* fusions may differ slightly from other *nod* fusions, in having the remaining expression just proximal to the nodule meristem rather than to the meristem itself (data not shown). To test whether disappearance resulted from depletion of *nod* gene inducers, 42–47 dpi nodules induced by rhizobia carrying *nodB*, *nodC*, *nodI*, *nodJ*, *nodE*,

nodP, or *nodQ* were aseptically sectioned into halves longitudinally; one half was incubated in H₂O, and the other half was incubated in 25 μ M luteolin in either M9 succinate or M9 sucrose. After a 16-hr incubation at 30°C, neither half regained expression, even though with younger nodules (26–27 dpi) both halves showed expression under similar conditions, suggesting that inducer depletion is not the reason *nod* expression disappears.

syrM in concert with *nodD3* has been implicated in regulating the expression of *nodC* in culture (Mulligan and Long 1989). Nevertheless, in planta expression of fusions *nodJ* :: Tn5-*gusA1* [genomic, in RmS318], *nodE* :: Tn5-*gusA2* [in JT701 (pSB445)], and *nodH* :: Tn5-*gusA2* [in JT701 (pSB437)] is not altered by the additional mutation *syrM* :: Tn5 (not shown).

Nodules are subject to multiple infection

Because the nodule cortex shows early expression of *nod* :: Tn5-*gusA* fusions only at the point of infection,

reinfection of a nodule is easily seen. This is particularly true after 27 dpi (Fig. 4F), when expression is seen mainly in the meristematic zone, and after 42 dpi (Fig. 4H), when most of the nodules are not expressing at all. Secondary infection was seen in nodules at all of the stages noted above and was more common in the part of the nodule proximal to the root. Reinfection spread into the previously infected central zone in some cases (Fig. 4F), and was limited to the nodule cortex in others (Fig. 4H). This result suggests two conclusions: (1) The nodule may continue to secrete *nod* gene inducer(s) even after expression within the nodule has disappeared; and (2) the previously infected central zone can be infected a second time.

Expression of nod genes per se is not required for nitrogen fixation

To test nitrogen fixation, duplicate 42- to 47-dpi nodules induced by rhizobia carrying fusions in *nodB*, *nodC*, *nodE*, *nodI*, *nodJ*, *nodP*, and *nodQ* were sectioned into halves longitudinally; one half was assayed for acetylene reduction, and the other for *nod* gene expression by staining with X-Glc. All of the nodule halves reduced acetylene significantly, even though the corresponding halves did not show any staining after 24 hr incubation, as expected (with the exception of *nodE* and *nodI* fusions, each of which showed some expression in the nodule meristem in one of the replicates but not the other). Similar results were obtained when 42- to 47-dpi nodules were first assayed for acetylene and then sec-

tioned for expression. Thus, *nod* expression per se does not appear to be required for nitrogen fixation.

nif :: Tn5-gusA and fix :: Tn5-gusA expression in culture

nif and *fix* genes are known to be induced under microaerobic conditions either in culture or in the nodule (Ditta et al. 1987; David et al. 1988; Batut et al. 1989). Consistent with this, we found that rhizobia carrying *nif* or *fix* fusions formed white or pale blue colonies on X-Glc agar but elicited nodules that stained blue (Fig. 5). Table 2 shows induction of a *nifH* fusion in microaerobic culture and slight stimulation by luteolin.

In situ expression of nif and fix genes

Tn5-*gusA* fusions in *nif* and *fix* genes were analyzed in three configurations: (1) genomic fusions with *nifA*, *nifD*, and *nifH* and with *fixA*, *fixB*, *fixC*, and *fixX*; (2) the same genomic fusions with plasmid pRK290.720, which carries the wild-type allele for all of the genes except *nifB* and *nifK*; and (3) plasmid fusions with *nifA*, *nifB*, *nifH*, *nifD*, and *nifK* and *fixA*, *fixB*, *fixC*, and *fixX*, complemented by the genomic wild-type allele. In contrast with the *nod* fusions (Fig. 4), in situ expression of fusions with *nif* and *fix* genes (Fig. 5) showed a different pattern, illustrated for *nifH :: Tn5-gusA2.774* in Figure 5. (1) At 11–12 dpi, when the central region had the pink color of leghemoglobin, expression began appearing throughout the nodule, except in the meristematic zone (Fig. 5A–C). (2) After ~37 dpi, most Fix⁺ nodules

Figure 5. Typical in planta expression of *nifH :: Tn5-gusA2.774* [RmS238[pRK290.720]] revealed by staining without prior fixation. (A) 12 dpi; (B) 15 dpi; (C) 17 dpi, expression in the central zone of the nodule (strongest proximal to the meristem); (D) 30 dpi, expression disappearing in the central zone distal to the meristem; (E) 37 dpi, expression remaining in the central zone only proximal to the meristem; (F) 55 dpi, expression gone.

showed expression only in the central region proximal to the meristem, presumably because of senescence of the distal region (Fig. 5D,E). Fusions that were *Fix*⁻ (i.e., those not complemented by the wild-type allele) began to lose distal expression earlier, at ~22 dpi, presumably because of earlier senescence. (3) By ~55 dpi, all nodules had very low expression (Fig. 5F).

Expression of *syrM*

Expression of a *syrM* fusion was studied in the same three configurations as above. Expression in either aerobic or microaerobic culture was low (Table 2). The in situ expression pattern of the *syrM* fusion was like that of the *nif* and *fix* fusions, although staining was slower (Table 3). A doubly mutant strain carrying pGMI149 *syrM* :: Tn5-*gusA2*, as well as *fix* :: Tn5, had the same temporal expression pattern (although at ~22 dpi, most of the nodules showed expression only in the central region of the nodule proximal to the meristem, as with other *Fix*⁻ strains; see above). Thus, although we have not tested other mutations in combination with *fix*, expression of *syrM*, in contrast to that of other genes expressed late (*nif* and *fix*), appears not to be regulated by *fixLJ*.

Discussion

In this paper we describe the construction and use of Tn5-*gusA* promoter/probe transposons that fuse the *gusA* reporter gene either transcriptionally or translationally with a target promoter. In situ assay of *gusA* gene and operon fusions has proved to be of enormous value for the study of gene expression in transgenic plants, particularly with regard to cell and tissue specificity (Jefferson 1987; Jefferson et al. 1987; Willmitzer 1988). Similarly, Tn5-*gusA* fusions, which can be constructed in bacteria in vivo by simple transpositional mutagenesis, allow in situ monitoring of target gene expression during plant/bacterial interaction.

We have used these constructs to study the expression of *R. meliloti* genes involved in symbiosis with alfalfa during organogenesis of nitrogen-fixing root nodules. Our results delineate the overall patterns of expression of 21 such symbiotic genes and reveal a number of new and unexpected aspects.

The principal result is that each of the 21 genes can be assigned to one of only two overall patterns of temporal and spatial expression during alfalfa root nodule development. The Nod pattern is displayed by all *nod* genes studied (*nodB*, *nodC*, *nodD1*, *nodD3*, *nodE*, *nodG*, *nodH*, *nodI*, *nodJ*, *nodP*, and *nodQ*). This pattern consists of expression first on the root surface and nodule cortex (36 hr; 10 dpi); next throughout the developing nodule, including the nodule meristem (10–17 dpi); then mainly in the nodule meristem, progressively falling off distally through the central region toward the root (16–41 dpi); and, finally, little, if at all, throughout, except in several unusual nodules (42–75 dpi). In contrast is the Nif pattern, displayed by all *nif* genes studied (*nifA*, *nifB*, *nifD*,

nifH, and *nifK*), all *fix* genes studied (*fixA*, *fixB*, *fixC*, and *fixX*), and *syrM*. This pattern consists of expression that begins only later (~10 dpi), first throughout the central region but strongest in the “active symbiotic zone” just behind the nodule meristem, where leghemoglobin is most prominent, and not at all in the meristem itself (11–35 dpi); then behind the meristem but progressively falling off distally through the rest of the central region (35–55 dpi); and, finally, little, if at all, throughout (55–75 dpi).

Others have shown that genes of the Nod pattern are regulated by the NodD1, NodD2, and/or NodD3 products, which induce the remaining *nod* genes in response to plant flavonoids (Mulligan and Long 1985, 1989; Peters et al. 1986; Spaink et al. 1987). Likewise, genes of the Nif pattern are regulated by the *FixLJ*/*NifA*/*FixK* cascade, which induces the remaining *nif* and *fix* genes in response to low oxygen tension (Ditta et al. 1987; David et al. 1988; Batut et al. 1989). Our results extend this work by revealing some of the temporal and spatial dimensions of these regulatory interactions.

Nod pattern

In cylindrical nodules like those of alfalfa, the meristem located at the nodule tip contains small nonvacuolate actively proliferating cells. These are usually considered to be uninfected, with infection accompanying a transition from meristematic to the larger vacuolate nitrogen-fixing cells (e.g., Newcomb 1981). A striking and unexpected component of the Nod pattern is expression of all *R. meliloti nod* genes in the meristematic zone. This has not been reported previously. Our data do not show whether invading bacteria have infected the meristematic cells or are only present between them. Because morphological studies have not revealed bacteria within meristematic cells, the latter may be more likely.

The timing of *nod* gene expression and its prevalence in the meristem is consistent with evidence that the products of *nodA* and *nodB* appear to stimulate cell growth (Schmidt et al. 1988), and other aspects of the Nod pattern show this correlation as well. Thus, the later disappearance of *nod* gene expression coincides in time with cessation of nodule elongation; unusual nodules in which *nod* expression continues at late times are also unusually long; late secondary infection, which normally occurs at the base of the nodule, is usually accompanied by a small outgrowth; and young nodules that abort and fail to develop further have no *nod* expression (data not shown). Continued synthesis of *nod* gene products, therefore, seems necessary for continued nodule growth and development.

The later disappearance of *nod* gene expression, which incidentally implies turnover of enzyme expressed earlier, is also unexpected. Disappearance is not due to depletion of *nod* inducers, because expression cannot be restored with luteolin. This is also shown by the ability of rhizobia expressing *nod* to reinfect nodules in which prior *nod* expression has already ceased, although we cannot tell whether the individual cells in a region in-

ected secondarily are the same ones that were infected previously. Disappearance could reflect a specific signal, which may be part of the nodule cells senescence program but cannot reflect general cell death because genes of the Nif pattern continue to be expressed at this time. Interestingly, the early pea nodulin ENOD2, which seems to be induced by a signal based on *nod* expression (Govers et al. 1986; Dickstein et al. 1988), also declines later in nodule development (Franssen et al. 1987). Such observations remain to be reconciled with indirect evidence suggesting effects of *nod* expression later in development on *exo* gene function in *R. meliloti* (Klein et al. 1988) and on induction of vetch nodulins by *Rhizobium leguminosarum* (Nap et al. 1989).

In any case, our results show the same Nod pattern for the regulatory *nodD1* and *nodD3* genes as for the other, presumed structural *nod* genes, suggesting that the *nodD* genes, themselves, are subject to regulation in planta as well. Whether this regulation is related to the regulation of *nodD* found in derivatives of *R. meliloti* strain Rm41 but not of strain SU47 (Kondorosi et al. 1989), which was used here, is not yet clear.

Nif pattern

The Nif pattern of expression differs markedly from the Nod pattern, both temporally and spatially. Temporally, expression takes place considerably later in the Nif than in the Nod pattern, as has long been known for the genes encoding nitrogenase (Bergersen and Goodchild 1973; Long 1989). Spatially, expression in the Nif pattern, unlike the Nod pattern, is completely absent from the meristematic zone but is most prominent just behind the meristem, like expression of leghemoglobin (Bergersen and Goodchild 1973; Verma et al. 1979), upon which nitrogenase function depends. Although expression of Nif pattern genes occurs later and is known to be regulated (by FixLJ–NifA), it does not seem to depend on continued expression of Nod pattern genes per se, as it continues well after *nod* expression has disappeared. As in the case of Nod pattern gene expression, Nif pattern expression disappears very late in nodule development, as a result of either regulation or general cell senescence.

Although little is known about the symbiotic role of *syrM*, our results for this gene are surprising. *syrM* has been implicated in the regulation of *nod* and *exo* genes (Mulligan and Long 1989), both of which are expressed early. Yet, we find that mutation of *syrM* does not detectably affect in situ expression of Nod pattern genes; that expression of *syrM*, itself, follows the later Nif pattern rather than the early Nod pattern, but that unlike other Nif pattern genes (*nif*, *fix*), expression of *syrM* depends neither on microaerobic conditions nor on *fixJ*. Thus, *syrM* remains a puzzle.

Final comments

Our findings support the well-established notion of nodule organogenesis as a coordinated developmental pathway involving both symbiotic partners. Temporally,

rhizobial symbiotic gene expression resembles that of other prokaryotic sequential differentiated systems, such as sporulation and phage growth, particularly in its dependence on regulatory cascades that are now being elucidated. The in situ results clearly indicate considerable and complex signaling between plant cells and bacteria, regarding, for example, expression in the meristematic zone and disappearance late in nodule development.

These studies illustrate the utility of the Tn5–*gusA* transposons that we have constructed. Transposon fusions should continue to be useful in further unraveling of the regulatory interactions in nodule organogenesis. They should be similarly useful for studying other prokaryote/eukaryote systems, such as plant microbial pathogenesis. Finally, transposon mutagenesis in bacteria in vivo should simplify construction of fusions in cloned plant genes for subsequent introduction into transgenic plants.

Experimental procedures

Bacterial strains, plasmids, and media

Bacterial strains, plasmids, and phages are described in Table 1. Unless indicated otherwise, media and drug concentrations were as described by Finan et al. (1985) and Glazebrook and Walker (1989). For *R. meliloti*, M9 medium (Miller 1972) supplemented with 2 μ g/ml biotin, 0.2% (wt/vol) glucose or sucrose, 20 μ g/ml X-Glc, or 20 μ g/ml MUG (4-methyl umbelliferyl glucuronide) was used for assay of β -glucuronidase on plates. For *E. coli*, both M9 and LB supplemented with 20 μ g/ml X-Glc were used.

Genetic techniques

λ lysates were prepared according to de Bruijn and Lupski (1984). Transduction with ϕ M12 was as described in Finan et al. (1984). pGMI149 and various other plasmid derivatives were transferred from one strain to another by triparental mating with pRK600. Tn5 insertions in pRK290 plasmid derivatives were exchanged to the *R. meliloti* genome by introduction of the incompatible plasmid pPH1J1 (Ruvkun et al. 1982).

Plant and nodulation assays

Medicago sativa cv. Iroquois (Agway, Inc., Plymouth, Indiana) seeds were grown and inoculated as described by Glazebrook and Walker (1989). Root hair curling was examined according to Debelle et al. (1986). Date of emergence of each nodule induced by *R. meliloti* carrying a Tn5–*gusA* fusion was marked for temporal studies of expression. Nitrogen fixation was determined by plant growth and, in some cases, by acetylene reduction (Ruvkun and Ausubel 1981). Bacteria were recovered from nodules as described by Glazebrook and Walker (1989).

Spectrophotometric and histochemical enzyme assays

For β -glucuronidase enzyme assay, *R. meliloti* was grown in M9 supplemented with 0.4% (wt/vol) sodium succinate and 2 μ g/ml biotin to early log phase ($OD_{600} = 0.05$ to 0.1), and luteolin was then added to a 10 μ M final concentration. β -Glucuronidase was assayed according to Jefferson et al. (1986). For microaerobic studies, bacteria grown to early log phase were divided into four tubes; two of the tubes were then stoppered

tightly, flushed for 10 min with nitrogen, and injected with air to give the desired oxygen concentration; all four tubes were then shaken at 30°C for 1 hr, and luteolin was added to 10 μ M in one aerobic and one microaerobic tube; 1-ml aliquots were assayed for enzyme activity.

For histochemical assay of β -glucuronidase, nodules were embedded in 7% agarose and sectioned with a Sorval TC-2 tissue sectioner (DuPont Instruments). For fixation, nodules or nodule sections were incubated in 2% paraformaldehyde for 5 min at room temperature, washed several times with 50 mM phosphate buffer (pH 7), and stained. For staining, 100- to 250- μ m sections, as well as young nodules and roots, were incubated with 20 μ g/ml X-Glc + 0.02% SDS in 50 mM phosphate buffer (pH 7) with or without prior fixation at 30°C from 20 min to several hours and examined in the light microscope.

Construction of Tn5-gusA delivery vehicles

λ Tn5-gusA delivery vehicles were made as follows: A lysate of λ b221 cI857 Oam29 Pam90 (deleted for att λ) was made on LE392 (pSB165). λ derivatives carrying the transposon were isolated by infection of Q5137 (lysogenic for λ imm434 to provide homology for λ integration) at MOI = 5, and Km^r and Tc^r colonies were selected at 30°C. Colonies that grew at 30°C but not at 42°C (because of λ prophage induction) were used to make phage lysates. These colonies were then screened for plaques having imm λ and giving turbid centers at 30°C that contained Km^r bacteria, which carried prophage λ b221 cI857 Oam29 Pam80 Ω :: Tn5-gusA2 was constructed similarly with LE392 (pSB166).

pRK600 Ω :: Tn5-gusA1 derivatives were constructed by triparental mating of LE392 (pSB165), C2110, and MT607 (pRK600). Selected Nal^r Km^r colonies carried a Tn5-gusA1 insertion in the C2110 chromosome, as neither pRK600 nor pSB165 can replicate in this *polA* host. These colonies were then mated triparentally with HB101 and MT607 (pRK600). HB101 (pRK600 Ω :: Tn5-gusA1) colonies were selected as Sm^r Tc^r Km^r Cm^r, and the derivative that gave highest frequency of Tn5-gusA1 transfer into *R. meliloti* was chosen for further work. Other pRK600 Ω :: Tn5-gusA derivatives were constructed similarly.

Tn5-gusA mutagenesis

λ Tn5-gusA mutagenesis was performed as described by de Bruijn and Lupski (1984). Plasmids to be mutagenized were transformed into *E. coli* MR0, and colonies were selected on a high concentration of Km (200 μ g/ml) at 30°C to favor preferential insertion into the high copy plasmid. Plasmids isolated from these cells were transformed into *E. coli* and insertions were localized by restriction analysis. For delivery by pRK600, C2110 harboring the target plasmid was mated with *E. coli* (pRK600 Ω :: Tn5-gusA), and the mutagenized C2110 population was analyzed as above.

For isolation of Tn5-gusA insertions in *nod* genes, mutagenized *E. coli* populations grown on Km (200 μ g/ml) and Tc (20 μ g/ml) were mated en masse to *R. meliloti*, and colonies on M9 sucrose [0.2% (wt/vol)] plus 10 μ M luteolin that were Tc^r (10 μ g/ml) and Nm^r (100 μ g/ml) and blue on X-Glc (20 μ g/ml) were analyzed further.

Insertions in *nif* and *fix* genes were obtained by two methods. In the first method, a mutagenized population of pRK290.720 was transformed into JM109 (pVL4), in which *nifA* is under the control of the *P*_{tac} promoter. Transformants that were deep blue on M9 X-Glc with 1 mM IPTG, but pale blue without IPTG, should have insertions in *nif* or *fix* genes; of several such

insertions, one (*nifH* :: Tn5-gusA2) was analyzed further. *R. meliloti* carrying this insertion on a plasmid formed very pale blue colonies on M9 X-Glc agar but induced nodules that stained deep blue with X-Glc. However, induction of *nifA* with IPTG made the strain grow slowly. Consequently, a second method was used, in which 100 mutagenized populations of *E. coli* carrying pRK290.720 were mated into *R. meliloti*; from each mating, pale blue colonies were purified and inoculated on alfalfa individually. Of these colonies, 34 induced nodules that stained blue with X-Glc. Plasmids were isolated from these colonies, and the insertions were analyzed.

Recombinant DNA techniques

Plasmid isolation, DNA restriction with various enzymes, dephosphorylation of DNA ends, and purification of DNA fragments by electroelution, ligation, and transformation were all done according to Maniatis et al. (1982). Complementary DNA fragments with sequences 5'-GATCTAAGGAGGAAAAAAC-CATGGAA and 5'-AGCTTTCCATGGTTTTTCCTCCTTA, which include a third translation terminator, a Shine-Dalgarno sequence with properly spaced ATG (underlined), and *Bam*HI- and *Hind*III-compatible single-stranded ends, were custom synthesized at the Whitehead Institute (MIT).

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