Regulation of *syrM* and *nodD3* in *Rhizobium meliloti*

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ABSTRACT

The early steps of symbiotic nodule formation by Rhizobium on plants require coordinate expression of several *nod* gene operons, which is accomplished by the activating protein NodD. Three different NodD proteins are encoded by Sym plasmid genes in *Rhizobium meliloti*, the alfalfa symbiont. NodD1 and NodD2 activate *nod* operons when Rhizobium is exposed to host plant inducers. The third, NodD3, is an inducer-independent activator of *nod* operons. We previously observed that *nodD3* carried on a multicopy plasmid required another closely linked gene, *syrM*, for constitutive *nod* operon expression. Here, we show that *syrM* activates expression of the *nodD3* gene, and that *nodD3* activates expression of *syrM*. The two genes constitute a self-amplifying positive regulatory circuit in both cultured Rhizobium and cells within the symbiotic nodule. We find little effect of plant inducers on the circuit or on expression of *nodD3* carried on pSyma. This regulatory circuit may be important for regulation of *nod* genes within the developing nodule.

RHIZOBIUM meliloti elicits development of nodules on its host, alfalfa (*Medicago sativa* L.). Formation of nodules in this and other Rhizobium-host systems is a multistage process involving the progressive differentiation of both bacterial and host cells. Phenotypic analysis shows that specific bacterial and host genes are required for individual developmental stages (LONG 1989b). Systems in both bacteria and their host plants must ensure timely and appropriate gene regulation in order to coordinate this developmental process. The exchange of molecular signals between plant and bacterium is now known to be the initiating event of the symbiosis (FISHER and LONG 1992).

There are numerous nodulation (*nod*) genes in at least five operons on symbiosis plasmids in *R. meliloti*. The *nod* promoters contain highly conserved regions termed *nod* boxes (ROSTAS *et al.* 1986), which are about 50 bp in size and lie about 26–28 bp upstream from defined +1 start sites of *nod* mRNA (FISHER *et al.* 1987a, 1987b; MULLIGAN and LONG 1989; SPAINK *et al.* 1989). Expression of *nod* operons is induced by plant flavonoids and requires the presence in the bacterium of an activator gene, *nodD* (reviewed by LONG 1989a). The sequence of NodD places it in the LysR family of bacterial activating proteins (HENIKOFF *et al.* 1988). The LysR-type proteins are autonomous activators, unlike two-component regulators such as NtrB/NtrC. They are typically encoded by genes transcribed divergently from the regulated operon, the proteins have a helix-turn-helix motif near the N-terminus, and in many cases the protein levels are autogenously regulated.

Some bacterial species have multiple *nodD* genes. In *R. meliloti*, the *nodD1* and *nodD2* genes encode products that activate *nod* promoters when cells are exposed to various plant inducers (HONMA and AUSUBEL 1987; HORVATH *et al.* 1987; MULLIGAN and LONG 1985). The *nodD3* gene was cloned on a fragment in plasmid pRmJT5 that also bore a gene termed *syrM* (for *sy*mbiotic *r*egulator). This multicopy plasmid caused constitutive expression of all *nod* operons tested (MULLIGAN and LONG 1989). The transcription start sites for *nod* mRNA were the same whether activation was caused by NodD1 or by NodD3-SyrM. Because constitutive expression of the target *nod* operons required both *nodD3* and *syrM*, we hypothesized that the expression of the *nodD3* gene or the activity of the NodD3 protein might depend on SyrM.

The protein encoded by *nodD3* is similar to that encoded by *nodD1* and *nodD2*, although those two activators are more similar to each other (87% identical amino acids) than either is to *nodD3* (77% and 79% identical amino acids, respectively) (RUSHING, YELTON and LONG 1991). The sequence of SyrM reveals lesser but still significant similarity to the NodD family (30% similar including conservative substitutions). This places SyrM in the LysR family as a probable gene activator (BARNETT and LONG 1990). We began the present study with the hypothesis that *syrM* might activate expression of *nodD3*.

We have examined the relationship between *syrM* expression and *nodD3* expression using gene fusions marker-exchanged into the pSyma plasmid. We have obtained results that differ in several respects from concurrent studies based on multicopy plasmid-borne fusions (DUSHA *et al.* 1989; KONDOROSI *et al.* 1991; MAILLET, DEBELLE and DENARIE 1990). We find no

TABLE 1

Strains and plasmids

Strain name	Description	Reference
Rhizobium meliloti[a]		
1021	Wild-type; SU47, Sm^r	MEADE *et al.* (1982)
JAS105	1021, *syrM*::Tn*5*-233	This work
JAS132	*syrM*::Tn*5*, *nodD3-lacZ* 4-5 fusion	This work
JAS133	*syrM*::Tn*5*, *nodD3-lacZ* 5-1 fusion	This work
JAS134	*syrM-gusA* fusion, *nodD3-lacZ* 4-5 fusion	This work
JAS135	*syrM-gusA* fusion, *nodD3-lacZ* 5-1 fusion	This work
JAS136	*syrM*::Tn*5*, *nodD3-gusA* 4-3 fusion	This work
JAS153	1021, *nodC-gusA* fusion	This work
JAS300	1021, *nodD3-lacZ* 4-5 fusion	This work
JAS301	1021, *nodD3-lacZ* 5-1 fusion	This work
JAS302	1021, *nodD3-gusA* 4-3 fusion	This work
JM57	1021, *nodC-lacZ* fusion	MULLIGAN and LONG (1985)
JM142	1021; *syrM-gusA* fusion	This work
JM200	1021; *syrM-gusA* fusion; *nodD3*::Tn*5*#303	This work
JT303	1021, *nodD3*::Tn*5*#303	SWANSON *et al.* (1987)
JT701	1021, *syrM*::Tn*5*	SWANSON *et al.* (1987)
E. coli		
C2110	*polA*	LEONG, DITTA and HELINSKI (1982)
Plasmids[b]		
pHoHoGUS, pSShe	Tn*3-gusA* delivery system	B. STASKAWICZ, unpublished data
pHoKm, pSShe	Tn*3-lacZ* delivery system	GLAZEBROOK and WALKER (1989)
pMB2	ColEI; *syrM* in pUC119	BARNETT and LONG (1990)
pPH1JI	IncP, Sp^{r}Gm^r	HIRSCH and BERINGER (1984)
pRK607	ColEI, pRK2013::Tn*5*-233	DE VOS, WALKER and SIGNER (1986)
pRK2013	ColEI, provides RK2 transfer functions	FIGURSKI and HELINSKI (1979)
pRmD3-25	*nodD3*	L. ZUMSTEIN and S. LONG, unpublished data
pRmE43	*nodD1* expressed from the *trp* promoter in pTE3	FISHER *et al.* (1988)
pRmE65	*nodD3* expressed from the *trp* promoter in pTE3	FISHER *et al.* (1988)
pRmE65::GUS 4-3	*nodD3-gusA* 4-3 fusion	This study
pRmE65::lac 4-5	*nodD3-lacZ* 4-5 fusion	This study
pRmE65::lac 5-1	*nodD3::lacZ* 5-1 fusion	This study
pRmJT5	Large cosmid clone covering *syrM* and *nodD3* region	SWANSON *et al.* (1987)
pRmM113	*syrM*	MULLIGAN and LONG (1989)
pRmM136	*nodD3*	MULLIGAN and LONG (1989)
pRmM142	pRmJT5, *syrM::gusA* fusion	MULLIGAN and LONG (1989)
pRmS73	*syrM* expressed from the *trp* promoter in pTE3	This study
pRmS303	pRmJT5, *nodD3*::Tn*5*#303	SWANSON *et al.* (1987)
pRmS701	pRmJT5, *syrM*::Tn*5*	SWANSON *et al.* (1987)
pTE3	Broad host range expression vector, IncP	EGELHOFF and LONG (1985)
pUC1813	pUC vector containing symmetric polylinker	KAY and MCPHERSON (1987)

[a] All *Rhizobium* strains carry pSyma.
[b] All plasmids are IncP in approximately three to five copies per cell unless otherwise indicated.

effect of *nodD3* on its own expression and little effect of plant inducers on *nodD3-syrM* interaction. We find that the *syrM* product is required for *nodD3* gene expression, and also that the *nodD3* product activates expression of *syrM*. Thus, *nodD3* and *syrM* constitute a self-sustaining, positive regulatory circuit.

MATERIALS AND METHODS

Strains and media: Bacterial strains and plasmids used in this study are listed in Table 1. Growth conditions and media were as described (SWANSON *et al.* 1987).

Bacterial genetic techniques: Triparental conjugations to transfer pRK290-based plasmids into Rhizobium were carried out using the helper plasmid pRK2013 (DITTA *et al.* 1980). Replacement of the Nm^r of Tn*5* with Sp^r/Gm^r of Tn*5*-233 was performed by the method of DE VOS, WALKER and SIGNER (1986). Mutagenesis with the transposons on plasmids pHoHoGUS and pHoKm was performed as described (STACHEL *et al.* 1985). Fusions were homogenotized into *R. meliloti* pSyma using pPH1JI, as reported previously (JACOBS, EGELHOFF and LONG 1985). Phage N3 transduction was performed as described (FINAN *et al.* 1984).

Construction of *nodD3* fusion strains: Transposons containing fusion cartridges for *lacZ* and *gusA* were used to construct fusions to *nodD3*. Insertion of these transposons into *nodD3* was detected by a screen for loss of *nodD3* function. We used a fusion to *nodC* as a reporter gene whereby a mutation in the regulator (*nodD3*) was detected by its effect on the reporter fusion in *nodC*.

Construction of nodD3-gusA fusion strains: A clone containing *nodD3* expressed from the *trp* promoter in pTE3 (pRmE65) was chosen as the target for mutagenesis with the

transposon on the plasmid pHoHoGUS, which can generate transcriptional or translational fusions to *gusA*. Competent cells were made from *E. coli* containing pHoHoGUS and pSShe and transformed with pRmE65 DNA. Potential insertions in pRmE65 were selected by mobilization into C2110, a *polA E. coli*, using pRK2013 as the mobilizing plasmid. The plasmids were then mobilized into JM57. Transconjugants were screened on LB SpNmTc with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal). JM57 with pRmE65 exhibits high-level constitutive expression of the *nodC-lacZ* fusion due to the overexpression of *nodD3* (J. SWANSON and S. LONG, unpublished data). Plasmids with an insertion in *nodD3* would no longer activate *nodC-lacZ* expression in JM57 and would therefore give rise to white colonies on plates containing Xgal. Such colonies were tested for β-glucuronidase activity using a fluorescent assay adapted for single colonies. Plasmid DNA was isolated from potential *nodD3* insertion mutants and transformed into *E. coli*. Two independent fusions were isolated and their location within the *nodD3* coding region verified by restriction mapping.

Construction of nodD3-lacZ fusion strains: The plasmid pRmE65 was also used as the target for mutagenesis with the transposon on the plasmid pHoKm, which carries a *lacZ* reporter gene. Competent cells were made from *E. coli* containing pHoKm and pSShe and transformed with pRmE65 DNA. Potential insertions in pRmE65 were selected by mobilization into C2110 using pRK2013 as the mobilizing plasmid. The plasmids were then mobilized into JAS153, a *nodC-gusA* fusion strain. JAS153 with pRmE65 exhibits high level constitutive expression of the *nodC-gusA* fusion due to overexpression of *nodD3* (J. SWANSON and S. LONG, unpublished data). Transconjugants were screened for β-glucuronidase activity using a fluorescent assay modified for single colonies. Eight colonies were negative for β-glucuronidase activity, indicating the lack of *nodD3*-mediated activation of *nodC-gusA* expression. Plasmid DNA was isolated from these colonies and transformed into *E. coli*. Two independent fusions were isolated and their positions within the *nodD3* coding region verified by restriction mapping.

Construction of genomic (pSyma) fusion strains: nodD3-lacZ fusions and *nodD3-gusA* fusions were homogenotized into strain 1021 using pPH1JI, selecting for SmNmSp. *nodD3* fusions were transduced into a *syrM*::Tn*5* background using *R. meliloti* transducing phage N3. JAS105 was constructed by homologous recombinaton and replacement of the Nm^r of Tn*5* with Sp^r/Gm^r of Tn*5*-233. JAS132 was constructed by transduction of the *nodD3-lacZ* 4–5 fusion from JAS300 into JAS105. JAS133 was constructed by transduction of the *nodD3-lacZ* 5–1 fusion from JAS301 into JAS105. JAS136 was constructed by transduction of the *nodD3-gusA* fusion from JAS302 into JAS105.

Double fusion strains were constructed by transduction of the *nodD3-lacZ* fusions into a *syrM-gusA* fusion strain. JAS134 was constructed by transduction of the *nodD3-lacZ* 4–5 fusion from JAS300 into JM142. JAS135 was constructed by transduction of the *nodD3-lacZ* 5–1 fusion from JAS301 into JM142. JM142 was constructed by homogenotization of the *syrM-gusA* fusion from pRmM142 into 1021 using plasmid incompatibility. JM200 was constructed by homogenotization of the *syrM-gusA* fusion from pRmM142 into JT303 using plasmid incompatibility.

Construction of *syrM* expression clone: A clone containing *syrM* expressed from the *trp* promoter was constructed as follows. A 1.2-kb *Sma*I fragment containing *syrM* was isolated from pMB2. The *Sma*I ends were filled in with Klenow and the fragment was ligated into pUC1813 digested with *Sma*I and phosphatased. The symmetrical *Bam*HI sites in the polylinker were used to cut out the insert as a *Bam*HI fragment. pTE3 was digested with *Bam*HI and phosphatased. The *syrM* insert was cloned in both orientations to create pRmS73 (correct orientation with respect to the *trp* promoter) and pRmS74 (opposite orientation).

Assay of reporter gene fusions: β-galactosidase assays were performed as described by MILLER (1972) as modified by MULLIGAN and LONG (1985). β-glucuronidase activity was determined by a spectrophotometric assay as described (JEFFERSON, BURGESS and HIRSCH 1986) with the modification that 50 μl of chloroform was added to each sample; 200 μl of cells were typically used. Assays were incubated overnight. For screening purposes, β-glucuronidase activity was measured using a fluorescent assay adapted for 96-well microtiter dishes. Single colonies were transferred to individual wells of microtiter dishes containing 50-μl MUG lysis buffer (50 mM sodium phosphate, pH 7.0; 10 mM β-mercaptoethanol, 0.1% Triton X-100, and 1 mM 4-methyl umbelliferyl glucuronide). The assay plates were incubated at 37° for 2 hr; 25 μl 1 M Na_2CO_3 was added to stop the reactions. The plates were visualized with a hand-held UV lamp and scored for presence or absence of fluorescence.

Nodule staining: Nodules were excised from alfalfa roots and sectioned by hand without fixation. Sections were stained for β-glucuronidase activity with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) + 0.02% SDS in 50 mM sodium phosphate buffer, pH 7.0, for 16 hr at 37° (JEFFERSON 1987). Nodules used in this study were excised and stained 23 days post-inoculation.

RESULTS

We had previously shown that *nodD3* on the multicopy plasmid pRmJT5 stimulated high level constitutive expression of the *nodC-lacZ* fusion in JM57. Further experiments showed that the stimulatory effect of *nodD3* on the *nodC-lacZ* fusion required the presence of *syrM* on pRmJT5 (MULLIGAN and LONG 1989). We initiated the present study to investigate more fully the interactions between *syrM* and *nodD3*.

***nodD3* fusion activity:** We assayed the response of *nodD3* gene expression to the presence of various genes carried in *trans* or in *cis* on multicopy plasmids. To correct for possible effects of position, we constructed two *nodD3-lacZ* fusions and one *nodD3-gusA* fusion and recombined each into pSyma. Thus, these starting fusions were in an otherwise wild-type genetic background. We found similar results with all three fusions (Table 2). pRmJT5, containing *nodD3* and *syrM*, stimulated *nodD3* expression (Table 2, compare lines 1 and 2). Smaller subclones were used to identify which loci on pRmJT5 were responsible for this stimulation: *syrM* (pRmM113) stimulated *nodD3* expression but *nodD3* controlled by its own flanking sequences did not (Table 2, lines 3, 4 and 5). *nodD3* expression in *trans* from the more active *trp* promoter did stimulate *nodD3* expression (Table 2, line 6).

Effect of *syrM* background on expression of the *nodD3* fusion: The previous set of results suggests that *syrM* is required for *nodD3* expression in most cases. The effects of *trp*-promoter driven NodD3 syn-

TABLE 2

Genetic control of *nodD3* expression

Plasmid	Relevant characteristics	JAS300	JAS132 *nodD3-lacZ* 4-5	JAS134	JAS301	JAS133 *nodD3-lacZ* 5-1	JAS135	JAS302 *nodD3-gusA* 4-3	JAS136
		syrM$^+$ βgal	*syrM*::Tn*5* βgal	*syrM-gusA* βgal	*syrM*$^+$ βgal	*syrM*::Tn*5* βgal	*syrM-gusA* βgal	*syrM*$^+$ βgluc	*syrM*::Tn*5* βgluc
1. None		6	6	10	6	7	11	7	10
2. pRmJT5	*nodD3*$^+$, *syrM*$^+$	27	18	38	47	30	46	149	141
3. pRmM113	*syrM*$^+$	23	47	48	49	87	69	85	155
4. pRmM136	*nodD3*$^+$	5	6	8	7	6	9	14	12
5. pRmD3-25	*nodD3*$^+$	14	8	9	12	11	10	14	7
6. pRmE65	*nodD3* expressed from *trp* promoter	30	4	8	39	4	12	85	21
7. pRmS701	pRmJT5, *syrM*::Tn*5*	6	7	8	7	7	10	13	16
8. pRmS303	pRmJT5, *nodD3*::Tn*5*#303	8	10	12	13	12	12	18	25
9. pRmS73	*syrM* expressed from *trp* promoter	35	55	53	66	102	82	140	129

Expression of *nodD3* as measured by three different reporter gene fusions. *syrM* genotype was varied as indicated. Each strain was assayed three to five times in duplicate. β-galactosidase and β-glucuronidase were measured as in MATERIALS AND METHODS.

thesis on expression of a *nodD3* fusion (Table 2, line 6), and other results (MAILLET, DEBELLE and DENARIE 1990) suggested that *nodD3* stimulates its own expression. To determine whether this stimulation required *syrM*, we tested the same multicopy plasmids in backgrounds containing the *nodD3* fusions with or without a genomic (pSyma) copy of *syrM*. The presence or absence of a genomic copy of *syrM* made little difference on the stimulation of *nodD3* expression by pRmJT5, as expected since there is a copy of *syrM* present on this plasmid (Table 2, line 2). The stimulatory effect of pRmJT5 was abolished when its copy of *syrM* was inactivated by a Tn*5* insertion (Table 2, line 7). Consistent with this, *nodD3* under the control of its own promoter still failed to stimulate expression of a pSyma *nodD3* gene (Table 2, lines 4 and 5). The background genotype for *syrM* did affect the stimulation of *nodD3* expression by pRmE65. Specifically, we found that when *syrM* was inactivated in pSyma, the stimulation of *nodD3* expression by pRmE65 was abolished (Table 2, line 6, compare column 1 to columns 2 and 3, column 4 to columns 5 and 6, and column 7 to column 8).

When *syrM* was under the control of its own promoter in pRmM113 or under the control of the *trp* promoter in pRmS73, it stimulated maximal expression of the *nodD3* fusion (Table 2, lines 3 and 9), even though *nodD3* itself was inactivated. *syrM* stimulation of *nodD3* expression is thus independent of NodD3 protein.

We also tested the effect of mutating the *nodD3* locus in pRmJT5 (Table 2, line 8). A *nodD3*::Tn*5* derivative of pRmJT5 did not stimulate expression of *nodD3* fusions, despite the copy of *syrM* on this plasmid. It is not clear why the *syrM* on pRmJT5 or pRmS303 is not as effective as the *syrM* on the smaller pRmM113 clone (Table 2; compare lines 2, 3, and 8). One possibility is that *syrM* on pRmM113 is transcribed at an elevated level due to vector sequences. It is also possible that the sequences upstream of *nodD3* in pRmJT5 may constitute regulatory sites that interact with SyrM, thereby reducing its ability to stimulate the pSyma fusion.

***syrM* fusion activity:** The overall amplification of *nod* gene expression by the *nodD3*$^+$, *syrM*$^+$ multicopy plasmid pRmJT5 suggests that each locus may affect expression of the other. We tested this systematically, using appropriate constructs that disrupted and reconnected the possible circuits for gene regulation.

We used the *gusA* fusion to *syrM*, JM142, to assay *syrM* expression (Table 3, column 1). The *nodD3*$^+$, *syrM*$^+$ plasmid pRmJT5 stimulated the *syrM* fusion to a high level (Table 3, column 1, line 2). A subclone from pRmJT5 with *syrM* under the control of its own promoter also stimulated genomic (pSyma) *syrM* expression, although to a lesser extent than pRmJT5 (Table 3, line 3). *nodD3* under its own promoter stimulated *syrM* expression only slightly (Table 3, lines 4 and 5). However, this reflects low *nodD3* gene expression, not properties of the NodD3 protein: we found that *nodD3* under the control of the *trp* promoter in pRmE65 stimulated *syrM* expression to a high level (Table 3, line 6). The SyrM protein is not as effective: when expression of *syrM* was forced from the *trp* promoter in pRmS73, a target genomic *syrM* fusion was expressed at an intermediate level (Table 3, line 9).

To determine the effect of the genomic (pSyma) copy of *nodD3* on the expression of *syrM*, we measured the activity of the *syrM* fusion in strains with a wild-

TABLE 3

Genetic control of *syrM* expression

Plasmid	Relevant characteristics	JM142	JM200	JAS134	JAS135
		syrM::gusA			
		nodD3$^+$	*nodD3*::Tn*5*#303	*nodD3-lacZ* 4-5	*nodD3-lacZ* 5-1
		βgluc	βgluc	βgluc	βgluc
1. None		13	9	13	9
2. pRmJT5	*nodD3*$^+$, *syrM*$^+$	712	586	452	437
3. pRmM113	*syrM*$^+$	244	42	63	45
4. pRmM136	*nodD3*$^+$	37	24	31	32
5. pRmD3-25	*nodD3*$^+$	21	NT	26	24
6. pRmE65	*nodD3* expressed from *trp* promoter	838	871	719	424
7. pRmS701	pRmJT5, *syrM*::Tn*5*	40	25	36	31
8. pRmS303	pRmJT5, *nodD3*::Tn*5*#303	170	32	37	24
9. pRmS73	*syrM* expressed from *trp* promoter	495	354	362	233

Expression of *syrM* as measured by a *syrM-gusA* fusion. *nodD3* genotype was varied as indicated. A clone with *syrM* in the opposite orientation with respect to the *trp* promoter, pRmS74, gave background levels of β-glucuronidase when assayed in JM142 (18 units) and JAS134 (23 units).

type copy of *nodD3*, or with the genomic copy of *nodD3* mutated either by a Tn*5* insertion or by a *lacZ* fusion. *syrM* stimulation of its own expression was reduced by an insertion in the genomic copy of *nodD3* (Table 3, lines 3 and 8; compare column 1 with columns 2, 3 and 4). *syrM* under the control of the *trp* promoter in pRmS73 still stimulated its own expression in the absence of *nodD3* (Table 3, line 9; compare column 1 with columns 2, 3 and 4). In summary, SyrM protein can stimulate its own synthesis to a low level without NodD3; but NodD3 is required for amplification of *syrM* expression. This requirement for NodD3 can be obviated in a construct where *syrM* is overexpressed under control of an exogenous promoter in *trans*.

Absence of flavonoid inducibility of most *nodD3* fusions: There have been two recent reports that *nodD3* expression is affected by plant flavonoid inducers such as luteolin (DUSHA *et al.* 1989; MAILLET, DEBELLE and DENARIE 1990). We tested this with both of our *nodD3-lacZ* fusions and our *nodD3-gusA* fusion, but did not observe significant induction of *nodD3* by luteolin using our standard assay conditions (luteolin induction for 4 hr beginning with cells in rich medium at OD600 of 0.2–0.4). Other studies involved much lower cell densities and longer induction periods (MAILLET, DEBELLE and DENARIE 1990). A slight (less than fourfold) increase in *nodD3* expression was seen under those conditions for one of our fusions (Table 4), but not for the other two. We therefore suggest that luteolin is not a significant direct inducer of *nodD3* expression in *R. meliloti* 1021.

***In situ* expression of *syrM* and *nodD3*:** We used Rhizobium strains carrying genomic (pSyma) β-glucuronidase fusions to *syrM* or to *nodD3* (without extra copies of *nodD3* or *syrM*) to observe the position of

TABLE 4

Expression of *nodD3* in the presence and absence of luteolin

	No inducer	10 μM luteolin
β-gal activity		
JAS300 *nodD3-lacZ* 4-5 fusion		
4 hr	4	4
16 hr	4	4
JAS300/pRmJT5		
4 hr	22	24
16 hr	23	22
JAS301 *nodD3-lacZ* 5-1 fusion		
4 hr	4	5
16 hr	6	8
JAS301/pRmJT5		
4 hr	66	65
16 hr	40	159
β-gluc activity		
JAS302 *nodD3-gusA* 4-3 fusion		
4 hr	1	3
16 hr	4	7
JAS302/pRmJT5		
4 hr	73	71
16 hr	87	93

Cells were induced for 4 hours or 16 hours in TY. The OD600 of the culture when inducer was added was 0.02; 16-hr inductions were also tried in M9 (initial OD600 = 0.02) with comparable results.

activity in developing nodules. We observed strong activity of *syrM* that was dependent on *nodD3* (Figure 1, compare A and B). Furthermore, expression of *nodD3* was weak in a *syrM*$^+$ background and was abolished in a *syrM*$^-$ background (Figure 1, compare C and D). The interdependence of *nodD3* and *syrM* thus is evident both in free-living cells and late in symbiosis.

DISCUSSION

Initial studies suggested that *Rhizobium nod* genes were controlled by a single constitutively produced

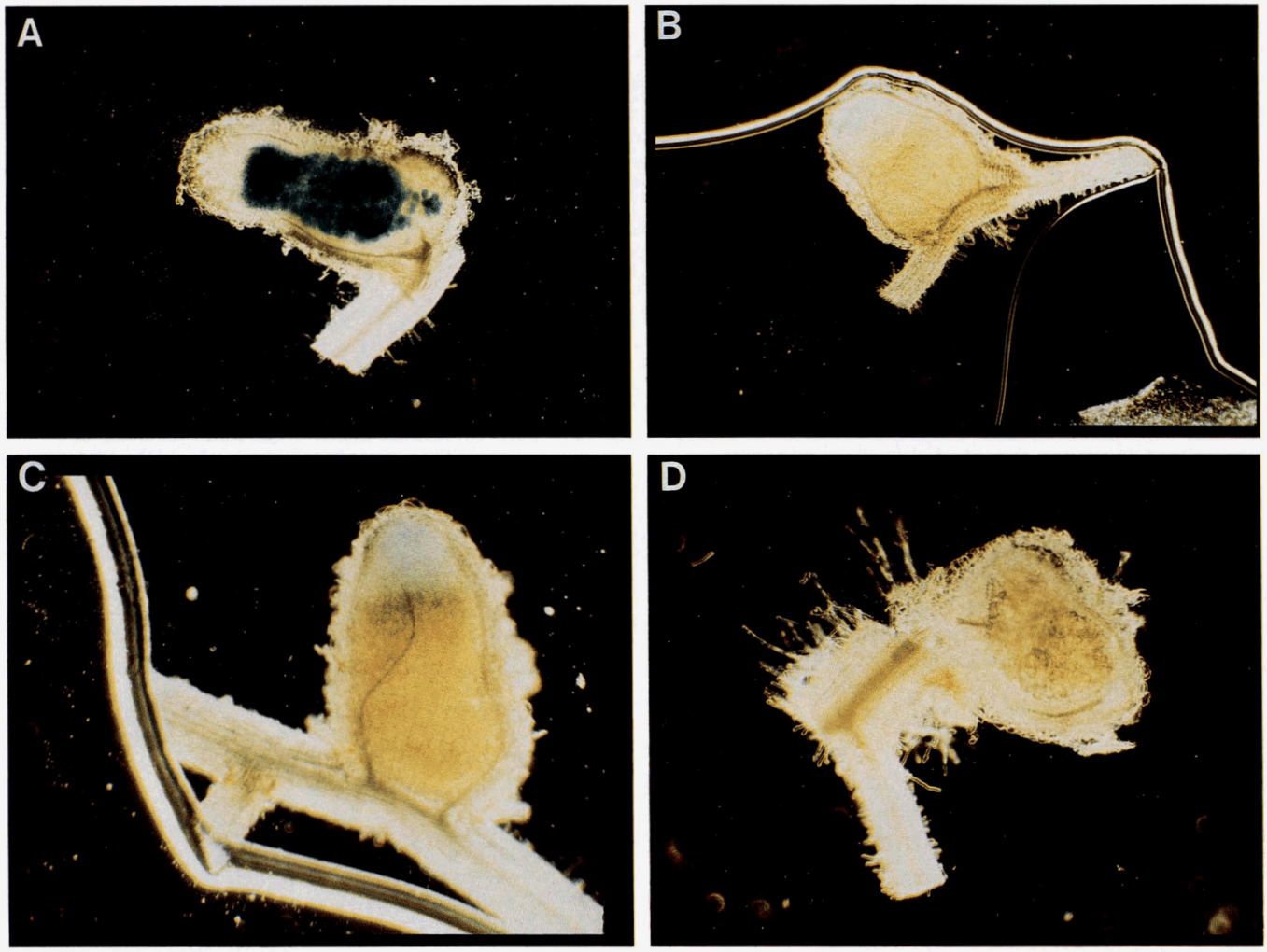

FIGURE 1.—In situ staining for *syrM-gusA* and *nodD3-gusA* fusion activities in wild-type and mutant backgrounds. All sections were stained for β-glucuronidase activity (see MATERIALS AND METHODS). (A) JM142 (*syrM-gusA*, *nodD3*$^+$); (B) JM200 (*syrM-gusA*, *nodD3*::Tn*5*); (C) JAS302 (*nodD3-gusA*, *syrM*$^+$); (D) JAS136 (*nodD3-gusA*, *syrM*::Tn*5*)

NodD regulator, acting with an inducer. Subsequent work has shown that *nod* gene control in many Rhizobium and Bradyrhizobium species involves multiple *nodD* genes, showing diverse properties and regulation (reviewed by FISHER and LONG 1992; SCHLAMAN, OKKER and LUGTENBERG 1992; see also DAVIS and JOHNSTON 1990a, 1990b; GOTTFERT *et al.* 1986, 1992; HONMA and AUSUBEL 1987; MULLIGAN and LONG 1989). For example, in *R. meliloti* expression of *nodD1* is constitutive (MULLIGAN and LONG 1985), as is expression of *nodD2* and *nodD3* of *R. leguminosarum* bv. *phaseoli* (DAVIS and JOHNSTON 1990a). In *R. leguminosarum* bv. *viciae*, expression of the single *nodD* is negatively autoregulated (ROSSEN *et al.* 1985). In *Bradyrhizobium japonicum* and in *R. leguminosarum* bv. *phaseoli*, expression of *nodD1* is positively activated by NodD1 protein and is induced or increased by flavonoids (BANFALVI *et al.* 1988; DAVIS and JOHNSTON 1990a; GOTTFERT *et al.* 1992; SMIT *et al.* 1992; WANG and STACEY 1991). Multiple forms of NodD protein may allow the bacteria to interact with diverse signal molecules (for example, GOTTFERT *et al.* 1992; GYORGYPAL, KONDOROSI and KONDOROSI 1991; HARTWIG *et al.* 1990; HONMA, ASOMANING and AUSUBEL 1990; HUNGRIA, JOHNSTON and PHILLIPS 1992). It will clearly be of importance to understand the diverse control of *nodD* genes themselves in these systems.

The *nodD3* gene product of *R. meliloti* has two unique features. First, it is highly active as a flavonoid-independent activator of *nod* genes. Other NodD proteins require flavonoids for maximal activity (DAVIS and JOHNSTON 1990b; HUNGRIA *et al.* 1992; GOTTFERT *et al.* 1992; BANFALVI *et al.* 1988). Second (present report), *nodD3* forms a positively amplifying circuit with *syrM*. *syrM* stimulates its own expression at a low level in a *nodD3*$^-$ background. *syrM* expression in a cell is proportionally related to the copy number of *nodD3*. Therefore, *nodD3* may function in modulating the level of *syrM* expression. *syrM* is required more stringently for any level of *nodD3* expression in

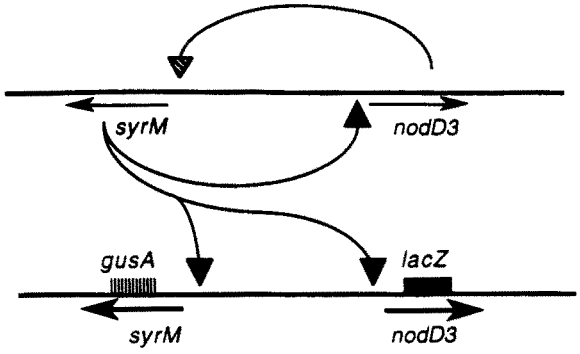

FIGURE 2.—Model of the NodD3, SyrM regulatory circuit. pSyma, shown on the lower line, carries fusions (boxes) to *nodD3* and *syrM* (bold arrows). JAS134 and JAS135 are two such examples from our work. pRmJT5 is added to this strain, represented by the upper line. This plasmid carries wild-type copies of *nodD3* and *syrM*, as shown by the arrows on the upper line. The plasmid-borne copy of *nodD3* stimulates an increase in expression of *syrM*; *syrM* then acts to increase the expression of *nodD3* and of itself, resulting in its own amplification.

Rhizobium grown in culture. This kind of regulatory relationship has not been observed in any other Rhizobium species.

Several previous studies have also found that *nodD3* and *syrM* interact in some way (HONMA, ASOMANING and AUSUBEL 1990; KONDOROSI *et al.* 1991; MAILLET, DEBELLE and DENARIE 1990). In these three studies, the combined effect of the two genes on *nodC* expression was measured. It was found that clones containing *nodD3* under control of its own promoter did not strongly stimulate *nodC* expression unless *syrM* was also present.

However, the present study comes to very different conclusions about the details of this interaction. First, whereas KONDOROSI *et al.* (1991) inferred that the *syrM* effect is direct, we propose that the SyrM effect on *nod* gene expression is not direct, but is mediated by NodD3, the expression of which depends on SyrM. Second, whereas others reported that *nodD3* positively autoregulates its expression (KONDOROSI *et al.* 1991; MAILLET, DEBELLE and DENARIE 1990), we find that NodD3 does not affect expression of the *nodD3* gene. Third, unlike two other reports (DUSHA *et al.* 1989; MAILLET, DEBELLE and DENARIE 1990), we found no evidence for significant induction of the *nodD3* gene by luteolin. We ascribe the differences in our findings in part to our experimental strategy: we constructed strains with genomic (pSyma) fusions to both *syrM* and *nodD3*, and tested the effect of both *nodD3* and *syrM* in *trans*. To control for expression of *syrM* and *nodD3*, we tested their effects when placed under control of constitutive promoters. Figure 2 presents an overview of the regulatory circuits, showing the complications that occur in the case where additional genes are carried in *trans* on a plasmid.

The effect of *syrM* on nod boxes is indirect and depends on *nodD3*: HONMA, ASOMANING and AUSUBEL (1990) and KONDOROSI *et al.* (1991) found no stimulation of a *nodC-lacZ* fusion by *syrM* in a *nodD1⁻nodD2⁻nodD3⁻* triple mutant. This is consistent with our results and with the interpretation that *syrM* does not directly stimulate *nodC* expression but rather appears to act through *nodD3*. KONDOROSI *et al.* (1991) observed that the ability of a *syrM-nodD3* containing plasmid (pNID6) to cause high constitutive *nodC-lacZ* expression was more affected by a mutation in *syrM* than in *nodD3*, and that this same plasmid carried in a triple *nodD1⁻nodD2⁻nodD3⁻* mutant caused modest constitutive *nodC-lacZ* expression, which was again more affected by a *syrM* mutation in the plasmid than by a leaky Tn*5* mutation in *nodD3*. They proposed that SyrM directly causes flavonoid-independent *nod* promoter expression, and that *nodD3* participates primarily as an activator of *syrM* expression.

However, these data came from constructs that did not distinguish between the expression of a gene and the intrinsic properties of its protein product. Our experiments tested the properties of SyrM and NodD3 by using constructs in which high expression ensured that none of the proteins was limiting. We found that high expression of *syrM* did not cause significant expression of *nod* genes, unless a normal *nodD3* gene was also present. We thus propose that SyrM affects *nod* box promoters only through its regulatory effect on the abundance of NodD3. Future experiments should test this proposal at the biochemical level by examining SyrM-promoter interactions.

No autoregulation of *nodD3*: Studies differ on the relationship of *nodD3* to its own expression. MAILLET, DEBELLE and DENARIE (1990) observed that extra *nodD3* genes on a multicopy plasmid increased expression of a *nodD3-lacZ* fusion on pSyma, and interpreted this as evidence for autoregulation of *nodD3*. However, the strain background used for their study was wild-type for *syrM*. We found that a clone with *nodD3* under control of the *trp* promoter (pRmE65) stimulated *nodD3* expression when a wild-type copy of *syrM* was present, but not when *syrM* was mutant. Thus, our results indicate that *nodD3* effects on *nodD3* expression are mediated by *syrM* (see Table 2, line 6).

The interdependence of *syrM* and *nodD3* expression also accounts for the results of KONDOROSI *et al.* (1991), who propose autoregulation of *nodD3* based on the observation that the degree of gel shift on DNA fragments upstream of *nodD3* depended on the *nodD3* genotype of the cells from which cell extracts were made. Thus, previous results suggestive of NodD3 autoregulation can be explained by the experimental design. We ruled out the autoregulation model by a direct test: constitutively expressed NodD3 does not affect expression of a *nodD3* gene fusion, unless there is a normal *syrM* gene in the cell.

No significant induction of *nodD3* by luteolin: The third major difference between our work and that of other groups is the report that *nodD3* is inducible by luteolin. MAILLET, DEBELLE and DENARIE (1990) reported that a *nodD3* fusion, carried on a plasmid, was inducible by luteolin or plant exudate. DUSHA *et al.* (1989) reported that their *nodD3-lacZ* fusion was inducible by luteolin under low nitrogen growth conditions. By contrast, our fusions showed little or no response to luteolin in standard assay conditions, alone or in combination with various multicopy plasmids. Those strains that carried *syrM* and *nodD3* on the same multicopy plasmid showed very high inducer-independent expression, as indicated above, and displayed no induction in response to inducers.

Why did luteolin seem to affect *nod* gene expression in other studies? One possibility is a recently demonstrated effect of luteolin on cell growth. HARTWIG, JOSEPH, and PHILLIPS (1991) found that *R. meliloti* shows severe growth inhibition when it is diluted to very low culture densities following growth to mid-log phase. Luteolin reverses this growth inhibition in a NodD-independent manner. MAILLET, DEBELLE and DENARIE (1990) and DUSHA *et al.* (1989) used protocols for cell preparation that involved dilution to low culture densities prior to luteolin induction. They did not report data for the growth rate of the cultures with and without luteolin; we repeated the experimental conditions of DUSHA *et al.* and confirmed that cells are severely inhibited in their growth following dilution to low cell density, and that luteolin reverses this effect (J. SWANSON and G. KALINOWSKI, unpublished data). The pronounced effect of luteolin on *nodD3* fusions seen by MAILLET, DEBELLE and DENARIE (1990) and by DUSHA *et al.* may thus reflect a general requirement for luteolin for growth from low cell densities, and perhaps an effect on *nodD3* expression that requires several cell generations to be evident.

Developmental expression of *nodD3* and *syrM*: Regulation of symbiotic genes within nodules may give clues about the physiological or developmental requirement for the gene functions. We studied the expression and interdependence of *syrM* and *nodD3 in situ*. As previously reported by SHARMA and SIGNER (1990), we found that the *nodD3-gusA* fusion was expressed in the meristematic region and the infectible zone of the nodule, and that the *syrM* fusion was expressed in the central tissue of the nodule on the proximal side of the meristem.

Our *in situ* observations showed interdependence of *nodD3* and *syrM* expression in nodules. *nodD3-gusA* expression was very low in nodules formed by an otherwise wild-type strain, possibly because the lack of an active *nodD3* locus results in low overall expression of both *syrM* and *nodD3*. No *nodD3-gusA* expression occurred if *syrM* was mutated, and conversely no *syrM-gusA* expression occurred if *nodD3* was mutated (Figure 1). These results suggest that *nodD3* and *syrM* are each required for the continued expression of the other in nodules.

Further study is needed to define the signals controlling *syrM* expression and to determine whether other regulators are involved. Strains with *syrM* and *nodD3* in single copy do not show expression of either gene in cultured cells, but strains with a higher copy number (for example, carrying clone pRmJT5) show constitutive expression of both. This suggests that one or both genes might be repressed in the free-living state, and that extra copies of the promoters titrate away hypothesized repressor(s). We have identified a locus that, when mutated, allowed greater expression of *syrM* in the free-living state, a phenotype consistent with that of a mutated repressor (M. BARNETT and S. LONG, unpublished data).

The high activity of the *syrM* fusion in the symbiotic state (Figure 1) suggests the possibility that its expression is stimulated by plant signal molecules other than luteolin, or by general physiological conditions in the nodule. The fact that *syrM* is expressed late in nodule development also suggests that it may have some regulatory function at these later stages. For example, the SyrM-NodD3 inducer-independent circuit could be useful in sustaining *nod* gene expression if inducers become limiting during later stages of invasion. However, the activity of the *syrM-nodD3* circuit in the bacteroid zone itself (Figure 1) presents a puzzle. SHARMA and SIGNER (1990) found that *nodF* and other *nod* genes were only expressed in the infectible zone, which corresponds to a distinct, earlier developmental stage. We have observed the same two patterns using *nodE* and *nodH-gusA* fusions (J. SWANSON and S. LONG, unpublished data). Lack of *nod* gene expression in bacteroids was also reported by SCHLAMAN *et al.* (1991), based on analysis of proteins and RNA. This was true even when a mutant *nodD* gene that activates without inducer was carried in the bacterium (SCHLAMAN *et al.* 1991). Therefore, the presence of NodD, even an inducer-independent form such as NodD3, is not sufficient to assure expression of *nod* genes in bacteroids. Thus, other regulatory mechanisms are likely to exist in bacteroids, in addition to SyrM and NodD3. Details of such mechanisms may be revealed by biochemical study of *nod* gene transcription initiation and of *nod* mRNA elongation and stability in early and late stages of symbiosis.

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