NolA Represses *nod* Gene Expression in *Bradyrhizobium japonicum*

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Studies of *lacZ* fusions in mutant strains of *Bradyrhizobium japonicum* strain USDA110 show that the *nodD$_1$* and *nodYABCSUIJ* operons are expressed via NodW and isoflavones in the absence of NodD$_1$ and NodD$_2$ when the adjacent *nolA* gene is deleted. Such a result suggests that NolA exerts a repressive effect on isoflavone-induced expression of the *nodD$_1$* and *nodYABCSUIJ* operons. Indeed, conjugation of a plasmid encoding NolA into wild-type or mutant strains resulted in a marked reduction in *nod* gene expression. The *nolYZ* operon, whose regulation is also dependent on NodD$_1$ and NodW, is not expressed at a high level in the absence of NolA. These results indicate differential expression of nodulation operons by the action of NodD$_1$, NodW, and NolA. *B. japonicum* strains deleted for *nodD$_1$*, *nodD$_2$*, and *nolA* nodulate soybean very poorly and only after a substantial delay. Mobilization of a plasmid encoding *nolA* into such strains significantly improved nodulation. Fine-tuning of *nod* gene expression mediated by the interplay of positive regulators (i.e., NodD$_1$ and NodW) and negative regulators (i.e., NolA) appears to be important for efficient nodulation by *B. japonicum*.

Members of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* are able to infect leguminous plants to form nitrogen-fixing symbioses. Studies with rhizobia have revealed that a large number of genes are responsible for eliciting early stages of nodule development (Fisher and Long 1992). The products of several of these genes, termed nodulation (*nod*/*nol*) genes, are involved with the synthesis of a lipo-oligosaccharide signal molecule (*nod* factor) that can trigger early developmental stages of nodule formation (Dénarié *et al.* 1992; Spaink 1992).

Induction of *nod* gene expression is mediated by various flavonoid molecules exuded by host plant roots (Banfalvi *et al.* 1988; Peters *et al.* 1986; Redmond *et al.* 1986) and the products of rhizobial *nodD* genes, which act as transcriptional regulators (Gyorgypal *et al.* 1991; Schlaman *et al.* 1992). NodD proteins belong to the LysR family of prokaryotic transcriptional regulators (Schell 1993), having such properties as a helix-turn-helix motif associated with DNA binding and some conservation in DNA target site recognition (Goethals *et al.* 1992). Genetic studies suggest that NodD proteins from rhizobia interact with specific flavonoids produced by their respective plant hosts (Burn *et al.* 1989; Spaink *et al.* 1987). NodD binds to a conserved *cis*-acting sequence (*nod* box) found upstream of many flavonoid, NodD-dependent operons (Rostas *et al.* 1986), as demonstrated by gel-retardation assays and DNaseI footprinting (Fisher *et al.* 1988; Fisher and Long 1989; Hong *et al.* 1987; Kondorosi *et al.* 1989). Some rhizobial species have multiple copies of *nodD* (Davis and Johnston 1990; Honma and Ausubel 1987). For example, *R. meliloti* has three *nodD* genes, all of which must be mutated before the bacterium is incapable of nodulation (Honma and Ausubel 1987). The redundancy in *nodD* copies is apparently important in determining host range (Davis and Johnston 1990; Honma and Ausubel 1987). *B. japonicum* strain USDA110 has two copies of *nodD* (Göttfert *et al.* 1992). The product of *nodD$_1$* was reported to be essential for isoflavone induction of the *nodD$_1$* and *nodYABCSUIJ* operons (Banfalvi *et al.* 1988). No function has yet been observed for the NodD$_2$ product (Göttfert *et al.* 1992).

A puzzling aspect of the nodulation biology of *B. japonicum* is that strains deleted of both *nodD* genes, along with *nolA* and adjacent DNA, still nodulate host plants, albeit after a significant delay (Göttfert *et al.* 1992). It was hypothesized by Göttfert *et al.* (1992) that a cryptic promoter activity or a third copy of *nodD* might be responsible for the residual nodulation. However, the possibility of a third copy of *nodD* was judged to be unlikely, based upon hybridization analysis of chromosomal DNA (Göttfert *et al.* 1992). Therefore, until recently, there appeared to be no way to explain nodulation in the apparent absence of a means (i.e., NodD) to activate *nod* gene expression. This apparent paradox has been largely solved by the recent work of Sanjuan *et al.* (1994) in which *nod* gene expression in *B. japonicum* was also found to be controlled by the product of the *nodW* gene. In *B. japonicum*

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strain USDA110, *nodW* is cotranscribed with the *nodV* gene (Göttfert *et al.* 1990). These two genes have similarity to the prokaryotic family of two-component regulatory systems, with NodV being similar to the membrane-bound sensor component and NodW similar to the transcriptional regulators within this family (Göttfert *et al.* 1990). Sanjuan *et al.* (1994) showed that *B. japonicum* strains mutated in *nodW* behaved identically to mutants defective in $nodD_1$; that is, *nod* gene induction was essentially eliminated. However, whereas mutants defective in NodW lost the ability to nodulate hosts other than soybean (e.g., siratro, cowpea, and mungbean), $nodD_1$ mutants could nodulate such hosts (Sanjuan *et al.* 1994). Indeed, a completely nodulation-defective phenotype on soybean is found only in those mutant strains lacking $nodD_1$, $nodD_2$, and *nodW* gene function (see Results). Therefore, $NodD_1$ and NodW are essential for activating the expression of the $nodD_1$ and *nodYABCSUIJ* operons in *B. japonicum.* Recently, Dockendorff *et al.* (1994) identified the *nolYZ* operon in *B. japonicum* strain USDA110 and showed that its expression was also dependent on $NodD_1$ and NodW. Thus, all known isoflavone-induced nodulation operons in *B. japonicum* require both $NodD_1$ and NodW for their expression.

Table 1. Expression of *nodY, nodC, nolZ,* and $nodD_1$*-lacZ* fusions in wild-type *Bradyrhizobium japonicum* USDA110 and regulatory mutants

Strain/fusion (genotype)	Units of Activity[a,b] Control	2 μM Genistein	SSE[c]
A. Deletion of *nolA* affects *nod* gene expression			
TCD910/*nodY-lacZ* (wild type)	14	859	1,407
TCD1070/*nodY-lacZ* ($nodD_1$)	27	36	59
TCD1030/*nodY-lacZ* (BjΔ329)	9	897	1,533
Bj110-573/*nodC-lacZ* (wild type)	9	418	578
BjΔ1267-573/*nodC-lacZ* (BjΔ1267)	6	280	636
NAD2021/*nolZ-lacZ* (wild type)	7	110	142
TCD1000/*nolZ-lacZ* (BjΔ329)	4	5	8
ZB976/$nodD_1$*-lacZ* (wild type)	23	47	61
ZB1027/$nodD_1$*-lacZ* ($nodD_1$)	9	7	16
TCD4050/$nodD_1$*-lacZ* (BjΔ329)	13	60	84
TCD5000 /$nodD_1$*-lacZ* (WAj336)	10	58	89
B. NodW is essential for *nod* gene expression in strain BjΔ1267			
BjΔ1267/*nodY-lacZ* ($\Delta nodD_1$ $nodD_2$ *nolA*)	10	860	1,276
Bj912 /*nodY-lacZ* (Δ1267, *nodW*)	10	10	17
BjΔ1267/$nodD_1$*-lacZ* ($\Delta nodD_1$ $nodD_2$ *nolA*)	12	55	93
Bj912 ($nodD_1$*-lacZ*) (Δ1267, *nodW*)	12	10	16

[a] Units using CPRG as a substrate.
[b] Values are the means of three independent determinations with a standard deviation of less than 15%.
[c] Soybean seed extract.

Until the discovery of the involvement of NodW in *nod* gene regulation, the general model for transcriptional regulation of rhizobial *nod* genes was a positive regulatory mechanism; that is, NodD proteins were proposed to interact with both flavonoids and *nod* boxes to direct transcription of other *nod* genes (Gyorgypal *et al.* 1991). An exception to this model was the studies by Kondorosi *et al.* (1989, 1991) where specific *nod* gene operons in *R. meliloti* strain 41 were negatively controlled by the action of a repressor encoded by the *nolR* gene. The binding site for NolR was identified by footprint analysis and DNA sequence comparisons in the promoter regions of the *nodABC*, $nodD_1$, and $nodD_2$ operons. A *nolR* mutation led to a slight loss in nodulation proficiency compared with a wild-type strain and allowed for sevenfold higher expression of a *nodC-lacZ* fusion as compared with the wild type. Such results led to the proposal that fine-tuning of *nod* gene expression was important for optimal nodulation efficiency. A functional NolR repressor was not uniformly present in all *R. meliloti* strains tested; for example, the well-characterized strain 1021 did not produce an active repressor (Kondorosi *et al.* 1989).

In this paper, we continue our investigation of the regulation of *nod* gene expression in *B. japonicum* strain USDA110. In the course of our studies on NodW-mediated *nod* gene expression in *B. japonicum*, we noticed that a strain deleted for $nodD_1$, $nodD_2$, and *nolA*, in contrast to a $nodD_1$ mutant, gave wild-type levels of *nod* gene expression in the presence of isoflavone inducers. Under these conditions, NodW appears to be sufficient and essential for full *nod* gene expression. However, since such levels of *nod* gene expression are not found in a $nodD_1$ mutant that possesses a functional *nodW* gene, these results also suggest that the deleted region encodes a repressor of *nod* gene expression. Previously, *nolA* was implicated in genotype-specific nodulation of soybeans (Sadowsky *et al.* 1991). It was observed that transfer of *nolA* to *B. japonicum* strain SD6-1C allowed this strain to nodulate soybean genotypes that normally restrict nodulation. The translated sequence of *nolA* revealed a putative helix-turn-helix motif, suggestive of a DNA-binding function. Genes downstream of $nodD_2$ must also play some role in nodulation efficiency because strains missing *nolA* and downstream DNA nodulate soybeans and other plant hosts poorly (Göttfert *et al.* 1989, 1992), even though expression of the common nodulation genes (e.g., *nodYABCSUIJ*) is similar to that of the wild-type strain. Complementation studies show that NolA plays an important role in mediating the repression of *nod* gene expression. Furthermore, addition of *nolA* to strains deleted for this gene relieved to a significant extent the observed nodulation deficiencies observed with strains deleted of $nodD_1$, $nodD_2$, and *nolA*.

RESULTS

$NodD_1$ independent expression of $nodD_1$ and *nodYABCSUIJ*.

Previously, Banfalvi *et al.* (1988), had shown that $NodD_1$ from *B. japonicum* USDA110 was required for isoflavone-induced expression of both the $nodD_1$ and *nodYABCSUIJ* operons, a result consistent with those found from studies of

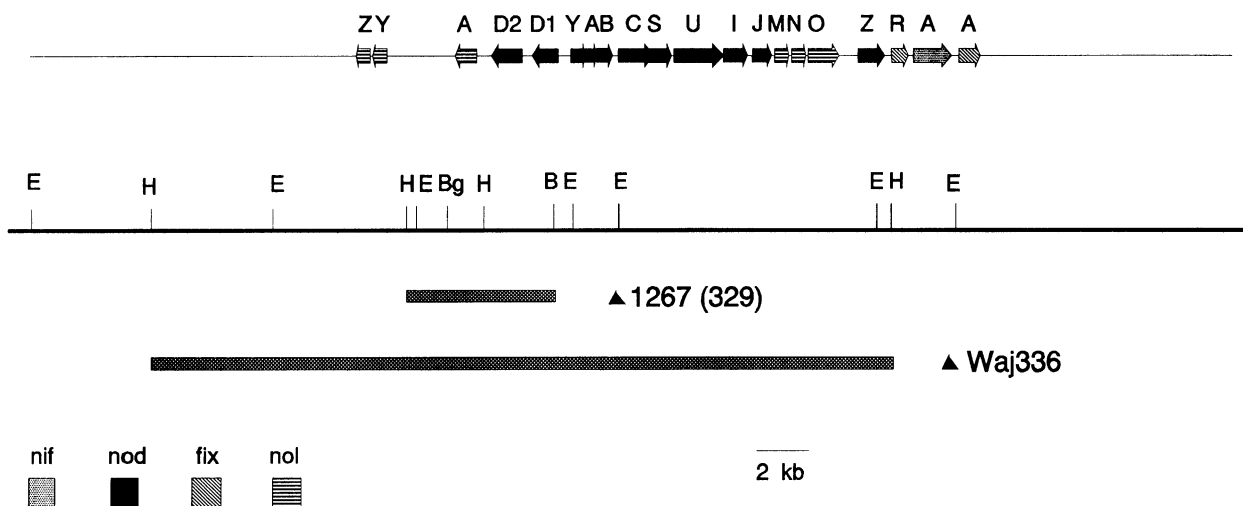

Fig. 1. A, Linkage map of the nodulation locus of *Bradyrhizobium japonicum* strain USDA110. Upper line shows the location of the (left to right) *nolZY*, *nolA*, *nodD*$_2$, *nodD*$_1$, *nodYABCSUIJnolMNO*, *nodZ*, *fixRnifA*, and *fixA* transcripts. The restriction map shown gives the location of all *EcoR*1 sites and other sites pertinent to this work. The hatched boxes indicate the extent of the deletions in strains Δ1267 (Δ329) and ΔWaj336. Strains Δ1267 and Δ329 are equivalent with the internal kanamycin cassette promoter directed toward the *Hin*dIII site in Δ1267 and toward the *Bsp*H*1* site in Δ329. Abbreviations: E, *EcoR1*; H, *Hin*dIII; B, *Bsp*H*1*; Bg, *Bgl*II; Bs, *Bss*H*1*; P, *Pst*I.

other rhizobial species (Goethals *et al.* 1990; Innes *et al.* 1985; Mulligan and Long 1985; Rossen *et al.* 1985). Therefore, it was of considerable surprise to find that a *nodY* or *nodD*$_1$-*lacZ* fusion was induced to levels similar to those seen in wild-type strains when these plasmids were mobilized into strain BjΔ329, a USDA110 derivative deleted of *nodD*$_1$, *nodD*$_2$, *nolA*, and about 1,300 bp of DNA downstream of *nolA* (Table 1A, Fig. 1). A similar result was noted when a chromosomally-encoded *nodC-lacZ* fusion was constructed in strain BjΔ1267 (i.e., BjΔ1267-573, Table 1A). BjΔ1267 is similar to BjΔ329, except the kanamycin resistance promoter within the inserted cassette directs transcription away from *nodYABC* (Göttfert *et al.* 1992). In contrast to results seen with the *nodD*$_1$ and *nodY* fusions, a *nolZ-lacZ* fusion plasmid was not induced in a BjΔ329 background (Table 1A). Previous work had shown that *nolYZ* expression is dependent on both NodD$_1$ and NodW (Dockendorff *et al.* 1994).

The BjΔ329 and BjΔ1267 strains have about 50% of the N-terminus of NodD$_1$ present. To show that these truncated NodD$_1$ peptides were not active, a *nodD*$_1$*′-′lacZ* fusion plasmid was mobilized to strain WAJ336. This strain has been completely deleted of the common *nod* gene cluster and surrounding DNA in USDA110 (see Fig. 1). Results from this construction show that *nodD*$_1$ expression is similar to that seen in strain BjΔ329 (Table 1A), which suggests that the truncated NodD$_1$ peptides from strains BjΔ329 and BjΔ1267 are not functional as transcriptional activators. These results also show that the factor(s) responsible for the induction of *nodD*$_1$ and *nodYABC* in strains BjΔ329, BjΔ1267, and WAJ336 is not tightly linked to the common *nod* genes of USDA110. We now know that *nod* gene expression in strains BjΔ329, BjΔ1267, and WAJ336 is due to the action of NodW. This can be seen by comparing the value for the expression of a *nodY-lacZ* fusion in strain BjΔ1267 to that of strain Bj912 (Table 1B). Similar results are also shown in Table 1B from these strains expressing a *nodD*$_1$-*lacZ* fusion. Strain Bj912 is a derivative of strain BjΔ1267 in which the *nodW* gene is also mutated. This strain shows no appreciable *nod* gene expression and is also completely defective in nodulation (data not shown). Thus, in the case of the BjΔ329, BjΔ1267, and WAJ336 mutants, NodW seems essential and sufficient to mediate wild-type levels of isoflavone-induced *nod* gene expression.

Table 2. Expression of a chromosomally integrated *nodC-lacZ* fusion in the presence and absence of *nolA*

Strain/fusion (genotype)	Units of Activity[a,b]		
	Control	2 μM Genistein	SSE[c]
Bj110-573 (wild type)	3	426	787
Bj1550 + *nolA* (wild type, pJS12S)	20	29	55
BjΔ1267-573 (BjΔ1267)	5	318	625
Bj1650 + *nolA* (BjΔ1267, pJS12S)	8	48	104

[a] Units using CPRG as a substrate.
[b] Soybean seed extract.
[c] Values are the means of three independent determinations with a standard deviation of less than 15%.

Complementation of deletion strains with *nolA*.

Results in Table 1A indicate that a *nodD*$_1$ mutant does not show appreciable *nod* gene expression even though a functional *nodW* gene is present. High levels of *nod* gene expression are only seen in the absence of NodD$_1$ when the DNA region downstream of *nodD*$_2$ is deleted. Thus, the results presented in Table 1A suggest that a repressor has been deleted in strains BjΔ329, BjΔ1267, and WAJ336 that allows induction of *nodD*$_1$ and *nodYABCSUIJ* by NodW in the absence of NodD$_1$. A possible candidate for such a repressor is the *nolA* gene product, which had previously been implicated as a factor involved with genotype-specific nodulation of soybeans (Sadowsky *et al.* 1991). The *nolA* DNA sequence predicts a translational product possessing a putative helix-turn-helix, DNA-binding motif (Sadowsky *et al.* 1991).

In order to test whether *nolA* encodes a repressor, plasmid pJS12S was constructed and conjugated into various mutant strains possessing a chromosomally integrated *nodC-lacZ*

fusion. The results of β-galactosidase assays using these strains are presented in Table 2. Under these conditions, *nolA* reduced expression of a *nodC-lacZ* fusion in both the wild-type USDA110 and BjΔ1267 backgrounds. The level of repression exerted by NolA is considerably stronger in the USDA110 background, which suggests that unknown genes deleted in BjΔ1267 could also contribute to the repressive function.

Nodulation assays with a complemented deletion strain. Previous results from Göttfert *et al.* (1989, 1992) had shown that strains BjΔ329 and BjΔ1267 were deficient in nodulation of soybeans and alternative plant hosts. This loss of nodulation proficiency was presumed to be due to the lack of efficient expression of the essential nodulation genes *nodABC*, and perhaps other unknown genes involved in the synthesis of the lipo-oligosaccharide signal molecules required for early stages of nodule development. However, as shown in Table 1A, strain BjΔ329 and BjΔ1267 show wild-type levels of *nod* gene expression but poor nodulation ability. This apparent paradox suggests that NolA might have an important effect upon nodulation proficiency. To test this hypothesis, pJS12S was mobilized into BjΔ1267 and the resulting transconjugants were tested for nodulation of soybean. Results showed that nodulation by a BjΔ1267 strain complemented with *nolA* (i.e., strain Bj1600) was significantly improved when compared with the same strain without *nolA* (Fig. 2). Nodules elicited by strain Bj1600 were delayed in appearance by about four days compared with those elicited by wild-type strain USDA110. By day 21, strain Bj1600 produced about 75% of the nodule numbers seen with USDA110, but approximately 80% of the nodules formed on these plants occurred on lateral roots, while only about 25% of nodules occurred on lateral roots of plants inoculated with USDA110. However, all of these values are a significant improvement over nodulation by BjΔ1267 or BjΔ1267 containing only the pVK100 vector. Thus, *nolA* can largely complement the nodulation defects associated with strain BjΔ 1267.

DISCUSSION

Results presented here show that the $nodD_1$ and *nodYABCSUIJ* operons, but not the *nolYZ* operon, are induced in a $nodD_1$ mutant background if the adjacent *nolA* gene is deleted. This induction has been shown to be mediated by NodW (Sanjuan *et al.* 1994; Table 1B). Furthermore, these results suggest that the *nolA* gene product has a repressive effect upon expression of the *nodYABCSUIJ* and $nodD_1$ operons. Negative regulation of *nodABC*, $nodD_1$, and $nodD_2$ by NolR in *R. meliloti* strain 41 has been previously reported (Kondorosi *et al.* 1989, 1991). Positive and negative regulation of *nod* gene expression has been proposed by Kondorosi *et al.* (1989) to act as a mechanism of fine-tuning of gene regulation. Overexpression of *nod* genes is known to be detrimental to efficient nodulation (Knight *et al.* 1986). *R. meliloti* strains lacking a functional NolR product are less effective at nodulation than strains expressing *nolR,* and strains containing an active *nolR* appear to predominate in nature (Kondorosi *et al.* 1989).

NolA has previously been implicated in mediating genotype-specific nodulation of soybeans (Sadowsky *et al.* 1991). Addition of *nolA* to specific *B. japonicum* strains allowed transconjugants to nodulate soybean genotypes that normally restricted nodulation by these strains. No mechanism of action for NolA was identified in the initial study, although a regulatory function was postulated based upon the presence of a putative helix-turn-helix, DNA-binding motif within the N-terminus of this protein.

Mobilization of pJS12S (encoding *nolA*) to either strain USDA110 or BjΔ1267 possessing a chromosomally-integrated *nodC-lacZ* fusion was found to inhibit isoflavone-mediated induction of gene expression, suggesting that NolA is acting as a repressor. The elevated expression of a $nodD_1$-

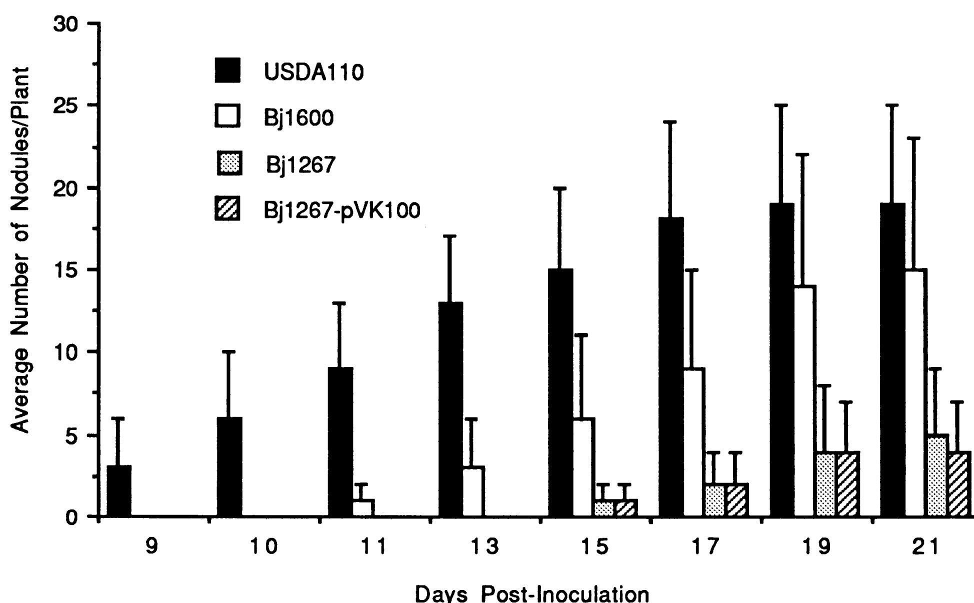

Fig. 2. Nodulation kinetics of USDA110, BjΔ1267, BjΔ1267 + pVK100 (vector control), and Bj1600 (i.e., BjΔ1267 + pJS12s) on soybean plants. Results presented are the means of two independent assays involving more than 20 plants.

lacZ fusion in strains BjΔ329 and BjΔ1267 suggests that NolA also negatively regulates $nodD_1$ expression. NolA has a putative helix-turn-helix, DNA-binding motif and, therefore, it is possible that NolA mediates this repressive effect by direct interaction with the *nod* promoter region.

In contrast, regulation of the *nolYZ* operon appears to differ from that of the $nodD_1$ and *nodYABCSUIJ* operons, although all three require both $NodD_1$ and NodW for expression (Dockendorff *et al.* 1994). This can be seen from the fact that a *nolZ-lacZ* fusion, unlike a $nodD_1$- or *nodY-lacZ* fusion, is not inducible in strain BjΔ329 (Table 1A). These results may indicate that *nolYZ* expression is controlled by a unique repressor and, therefore, is not relieved by the deletion of *nolA* or that NodW alone is not sufficient to induce this operon. Regardless of the explanation for these findings, it is clear that the various nodulation operons in *B. japonicum* can be regulated differentially. At present, the utility of such a mechanism is not apparent, especially considering that mutations in *nolYZ* have only a marginal effect on nodulation (Dockendorff *et al.* 1994).

Addition of *nolA* to strain BjΔ1267 was found to significantly improve nodulation competency. It is not clear if this increase in competency is due to the reduction in $nodD_1$ and *nodYABCSUIJ* expression, since the level of *lacZ* fusion activity in the deletion mutants in the absence of NolA was not higher than that found in the wild type. Therefore, it is possible that the effect of NolA could be as a regulator of other unknown genes necessary for nodule development. Göttfert *et al.* (1992) reported that nodulation of soybeans by strain BjΔ1267 could be complemented to near wild-type proficiency by addition of a plasmid that harbored $nodD_1$. Soybean nodulation by strain Bj1600 (BjΔ1267 with *nolA*) was delayed about 4 days in comparison with the wild type, and it seems likely that this delay was due to the lack of the $nodD_1$ gene. It is possible that $NodD_1$ is essential for expression of other unknown genes whose products contribute to nodulation efficiency.

It is not clear what correlation there is between the *nod* gene repression phenotype produced by NolA and its role in genotype-specific nodulation. An obvious model is that NolA regulates the expression of genes involved in genotype nodulation, for example, by reducing the expression of inhibitory nodulation signals. However, Sadowsky *et al.* (1991) noted no apparent relationship between the presence or absence of *nolA* in various *B. japonicum* strains and nodulation restriction. It is worth noting that a USDA110 $nodD_1$-*lacZ* fusion plasmid is induced 80-fold over background in *B. japonicum* USDA135 compared with a threefold induction in USDA110 (Banfalvi *et al.* 1988). Strain USDA135 appears to lack *nolA* as judged by DNA hybridization studies (Sadowsky *et al.* 1991). Nodulation restriction may be controlled by several factors, with modulation of *nod* gene expression via repression being only one of these factors.

An interesting and unexplained finding of our studies is the lack of correlation between nodulation ability and the level of *nod* gene expression. Clearly, *nodYABCSUIJ* expression is

Table 3. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Source
Bradyrhizobium japonicum		
USDA110	Wild type	USDA, Beltsville, MD
AN314	Tn5 in $nodD_1$Km^r Sm^r	Banfalvi *et al.* 1988
BjΔ329	$nodD_1$ $nodD_2$ *nolA* Km^r	Gottfert *et al.* 1989
BjΔ1267	$nodD_1$ $nodD_2$ *nolA* Km^r	Gottfert *et al.* 1992
Bj912	BjΔ1267, *nodW*::ω(sm/spc^r)	Sanjuan *et al.* 1994
WAj336	USDA110, Δ 10 kb common *nod* region each side of *nodA* Sm/Spc^r	P. Lauridsen
Bj110-573	USDA110, chromosomal *nodC′-′lacZ* fusion Tc^r	This study
BjΔ1267-573	BjΔ1267, chromosomal *nodC′-′lacZ* fusion Km^r Tc^r	This study
TCD910	USDA110 with pTD900 Tc^r	This study
TCD1030	BjΔ329 with pTD900 Km^r Tc^r	This study
TCD1070	AN314 with pTD900 Km^r Sm^r Tc^r	This study
ZB976	USDA110 with pZB22 Tc^r	Banfalvi *et al.* 1988
ZB1027	AN314 with pZB22 Km^r Tc^r	Banfalvi *et al.* 1988
TCD4050	BjΔ329 with pZB22 Km^r Tc^r	This study
TCD5000	WAj336 with pZB22 Sm/Spc^r Tc^r	This study
NAD2021	USDA110 with pND228 Tc^r	Deshmane and Stacey 1989
TCD1000	BjΔ329 with pND228 Km^r Tc^r	This study
Bj1550	Bj110-573, pJS12S Tc^r Sm/Spc^r	This study
Bj1650	BjΔ1267-573, pJS12S Km^r Tc^r Sm/Spc^r	This study
Bj1600	BjΔ1267,pJS12S Km^r Tc^r Sm/Spc^r	This study
Escherichia coli		
DH5α	*supE*44 Δ*lac*U169 (φ80 *lacZ*ΔM15) *hsd*R17 *recA*1 *endA*1 *gyrA*96 *thi-*1*relA*1	Bethesda Res. Lab.
S17-1	RP4 2-Tc::Mu-Km::Tn*7 pro hsdR recA*	Simon *et al.* 1983
Plasmids		
pRK2013	RK2 tra$^+$ Km^r	Figurski and Helinski 1979
pLAFR1	RK2 Mob$^+$ Tc^r	Friedman *et al.* 1982
pVK100	RK2 Mob$^+$ Km^r Tc^r	Knauf and Nester 1982
HP45Ω	Sm/Spc^r cassette	Prentki and Krisch 1984
pTD900	*nodY′-′lacZ* fusion Tc^r	This study
pZB22	$nodD_1$*′-′lacZ* fusion Tc^r	Banfalvi *et al.* 1988
pND228	*nolZ′-′lacZ* fusion Tc^r	Deshmane and Stacey 1989
pJS12S	*nolA*, HP45Ω cassette in pVK100 Tc^r Sm/Spc^r	This study
pRj573	6-kb *Nsi*I-*Bam*HI fragment containing a *nodC-lacZ* fusion cloned into the *Pst*I-*Bam*HI site of pRKPOL2 (Gottfert *et al.* 1992)	This study

required for nodulation, since the products of these genes (at least *nodABC*) are essential for the synthesis of the lipo-oligosaccharide nodulation factors (Dénarié *et al.* 1992). Therefore, it is a curious finding that strain BjΔ1267 can induce *nod* gene expression to wild-type levels and also produce a normal complement of the nodulation factors, but is grossly defective in nodulation (Sanjuan *et al.* 1994). An obvious conclusion from this information is that nodulation by this strain is limited by additional factors other than the nodulation signals. Regulating these hypothetical additional factors and integrating their activity with that of the lipo-oligosaccharide nodulation signals may be the rationale for the development of such a complex *nod* gene regulatory circuitry in *B. japonicum.*

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

All strains and plasmids used in this study are listed in Table 3. *B. japonicum* strains were cultured on modified RDY medium (So *et al.* 1987) for routine growth, β-galactosidase studies, and plant tests. HM salt medium (Cole and Elkan 1973) supplemented with arabinose at 0.1% was used for growth of *B. japonicum* when conducting matings. *B. japonicum* strains were cultured at 30° C. *E. coli* strains were cultured on LB medium (Sambrook *et al.* 1989) at 37° C. Antibiotics for selective markers were used in the following concentrations (in μg/ml): *E. coli*, ampicillin 100, kanamycin 50, tetracycline 20; *B. japonicum*; kanamycin 150, streptomycin 150, tetracycline 150, spectinomycin 150.

Plasmid pJS12S was constructed as follows: A 1.3-kb *Hin*dIII-*Bgl*II fragment encompassing *nolA*, and extending about 200 bp from the termination codon of *nolA* (Sadowsky *et al.* 1991), was cloned into pVK100. The HP45Ω streptomycin-spectinomycin resistance cassette (Prentki and Krisch 1984) was then cloned into the *Eco*RI site of pVK100 to form pJS12S. Plasmid pTD900 was constructed in the following manner: Plasmid pZB27, a *nodY′-′lacZ* fusion plasmid (Banfalvi *et al.* 1988) carries about 90% of the $nodD_1$ coding sequence. This plasmid was digested with *Sal*I, removing about 400 bp of the $nodD_1$ coding sequence, and religated. The subsequent plasmid was digested with *Eco*RI and ligated to pLAFR1 (Friedman *et al.* 1982) to form pTD900. Plasmid pRJ573 was constructed as follows: the 6-kb *Nsi*I-*Bam*HI fragment from pRJ458 (Göttfert *et al.* 1992) containing the *nodC-lacZ* fusion was subcloned into the *Pst*I-*Bam*HI site of pRKPOL2 (Göttfert *et al.* 1992). The resulting plasmid was digested with *Eco*RI and *Xba*I to yield plasmid pRJ573. To introduce the *nodC-lacZ* fusion into the chromosome, the plasmid pRJ573 was conjugated into the wild-type and the Δ 1267 mutant strain. Selection for co-integration yielded strains Bj110-573 and BjΔ1267-573. The correct genomic structures of the strains created were verified by appropriate Southern blot analyses.

Genetic techniques.

Transformation of plasmid DNA into *E. coli* was done following standard protocols (Sambrook *et al.* 1989). Triparental and biparental matings between *E. coli* donors and *B. japonicum* recipients were performed as previously described (Banfalvi *et al.* 1988; Ditta *et al.* 1980; Göttfert *et al.* 1989).

Plant tests.

Seeds of *Glycine max* 'Essex' were surface-sterilized, placed in plastic growth pouches (Vaughn's Seed Company, Downers Grove, IL) and cultivated following previously published procedures (Nieuwkoop *et al.* 1987). Approximately 10^6 bacterial cells were inoculated to each seedling. At 21 days postinoculation, nodules were surface-sterilized, crushed, and plated to RDY medium. Resulting colonies were then transferred to RDY plus kanamycin to ensure that nodules elicited on plants inoculated with mutant *B. japonicum* strains were indeed induced by such strains. All nodules picked were occupied by the appropriate bacterial strain.

β-galactosidase assays.

β-galactosidase activities of strains harboring *lacZ* fusions were assayed following protocols described earlier (Banfalvi *et al.* 1988). Numbers presented are averages and standard deviations of three or more independent assays. CPRG (chlorophenol red-β-D-galactopyranoside, Boehringer Mannheim) was used as a substrate for the assays.

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