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Phenotypic Characterization and Regulation of the *nolA* gene of *Bradyrhizobium japonicum*

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Two *Bradyrhizobium japonicum nolA* mutants were constructed and used to test the functional role of NolA in nodulation. Contrary to the previous hypothesis that NolA acts as a repressor of *nod* gene transcription, the expression of a *nodD1-lacZ* or *nodY-lacZ* fusion in the *nolA* mutant strains was similar to that found in the wild type. However, NolA does appear to act as a transcriptional regulatory protein since it is required for its own expression, as well as that of *nodD2*. Expression of NodD2 from a constitutive promoter led to a significant reduction in *nodC-lacZ* activity. Therefore, the repression of *nod* gene expression by NolA is likely an indirect effect, perhaps mediated by other genes (e.g., *nodD2*) that are regulated by NolA. When inoculated onto soybean roots, the *nolA* mutant strains showed only a slight delay in nodulation as compared to the wild type. However, the mutant strains were grossly defective in nodulation and nitrogen fixation on cowpea plants. Microscopic examination of soybean nodules induced by the *nolA* mutant strains showed developmental and morphological characteristics similar to nodules formed by the wild type with only a slight delay in bacteroid maturation. In contrast, cowpea nodules induced by the *nolA* mutant strains contained fewer infected cells and bacteroids were not found in a typical symbiosome structure. These results indicate that NolA is a transcriptional activator required for the expression of genes that play a role not only in the early stages of infection, but also during the later stages of bacteroid development and maintenance.

Additional keywords: rhizobia, symbiotic nitrogen fixation.

Bacterial species belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* are Gram-negative soil bacteria that can infect the roots of leguminous plants and establish a nitrogen-fixing symbiosis. The site of nitrogen fixation is a specialized plant organ, called the nodule. Early events of nodule formation involve the attachment of bacteria to the root hair and subsequent curling of the hair. Subsequently, a meristem forms within the root that enlarges and forms the nodule. The bacteria invade the developing nodule through an infection thread that is formed by the invagination of the root hair cell wall at the site of infection. The infection thread ramifies and penetrates the cells of the developing nodule into which the bacteria are released. The bacteria, now termed bacteroids, enlarge and differentiate into their mature form capable of nitrogen fixation. The mature bacteroid is able to express *nif* and *fix* genes that encode the enzymes necessary for nitrogen fixation.

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The early events of the infection process, such as root hair curling and induction of meristem formation, involve the production and recognition of signal molecules by the respective symbiotic partners. The bacteria produce lipo-chitin oligosaccharide signal molecules, called Nod signals, in response to plant-produced flavonoid compounds (reviewed in Carlson et al. 1994; Denarie and Cullimore 1993). The production and excretion of Nod signals is dependent on the expression of nodulation (*nod/nol*) genes. In *Bradyrhizobium japonicum*, the regulation of *nod* gene expression is mediated by at least three regulatory proteins (i.e., NodD1, NodW, and NolA; reviewed in Stacey et. al. 1995). A LysR-type regulator, NodD1, and a two-component regulatory system, NodV/NodW, are capable of activating *nod* gene expression in response to plant isoflavone signals. The NodD1 and NodV/NodW systems can functionally complement one another with regard to soybean nodulation, but the NodV/NodW system is essential for the nodulation of other legume hosts, such as cowpea and siratro (Sanjuan et al. 1994; Gottfert et al. 1990). A homolog of the *nodD1* gene, *nodD2*, is also present in *B. japonicum*. Although the predicted NodD1 and NodD2 proteins share 62% amino acid identity, NodD2 cannot complement the nodulation defect caused by a *nodD1* mutation (Gottfert et al. 1992). The *nolA* gene was first identified by its ability to extend the host range of *B. japonicum* serogroup 123 strains to specific soybean genotypes (Sadowsky et al. 1991). For this reason, *nolA* was termed a genotype-specific nodulation gene. NolA contains a N-terminal, helix-turn-helix DNA-binding motif which shares sequence homology to the MerR-type regulatory proteins. Recently, Dockendorff et al. (1994) showed that expression of NolA from a multicopy plasmid in *B. japonicum* resulted in a marked reduction in *nod* gene expression. These data supported the hypothesis that NolA is a repressor of *nod* gene expression.

Although much is known about bacterial determinants required during the early (i.e., *nod/nol* genes) and late (i.e., *nif/fix* genes) events of the symbiotic process, relatively little is known about functions required for the intermediate steps of nodule development (e.g., infection thread elongation, release into plant cells, and bacterial differentiation into mature bacteroids). Studies on various *Rhizobium* species have identi-

fied several genes that are required for bacteroid maturation. These include genes for exopolysaccharide biosynthesis (reviewed in Leigh and Walker 1994), synthesis and transport of cyclic β–(1,2) glucan (Dylan et al. 1986; Geremia et al. 1987), transport of C4-dicarboxylic acids (Bolton et al. 1986; Ronson et al. 1981), and leucine biosynthesis (Truchet et al. 1980). Rhizobia that carry mutations in any of these genes are able to initiate nodules but are defective in the formation of mature bacteroids. In *Bradyrhizobium japonicum*, mutations in the nitrogen-fixation regulatory genes *nifA* (Fischer et al. 1986), *rpoN1/2* (Kullik et al. 1991), or *fixLJ* (Anthamatten and Hennecke 1991) resulted in pronounced, premature bacteroid degradation. Metabolic mutants, such as those defective in C4-dicarboxylate transport (Humbeck and Werner 1989) and molybdate uptake (Maier et al. 1987), have also been reported to be defective in bacteroid maturation. Very recently, a host-specific nitrogen fixation gene, *hsfA*, has been reported to be required for normal *B. japonicum* bacteroid development in cowpea but not in soybean plants (Chun et al. 1994).

In this study, we constructed two *nolA* mutations by interposon mutagenesis and subsequently analyzed the function of NolA in symbiotic nitrogen fixation. Mutations in *nolA* do not lead to elevated expression of a *nodD1-lacZ* or *nodY-lacZ* fusion, suggesting that NolA does not act directly in the repression of *nod* gene expression. However, it is likely that NolA is a transcriptional regulatory protein since it positively regulates its own expression, as well as *nodD2*. Since, elevated expression of NodD2 represses *nod* gene expression; therefore, it is possible that NolA mediates the repression of *nod* gene transcription through NodD2 or via some other unidentified regulatory protein. Lastly, we present data showing that NolA is required not only in the early events of nodule initiation but also in later stages of nodule development. This requirement for *nolA* in nodule development appears to be host specific.

RESULTS

Effect of NolA on *nod* gene expression.

Previous reports indicated that maximum induction of a *nodD1-lacZ* or *nodY-lacZ* fusion in *B. japonicum* requires both NodD1 and NodW (Sanjuan et al. 1994). However, strains deleted for *nodD1*, *nodD2*, and *nolA* (i.e., strains Bj1267 and Bj329; Sanjuan et al. 1994) showed wild-type levels of *nod* gene induction. These results led to the suggestion that a gene encoding a repressor (i.e., *nolA*) had been deleted in these strains, which allowed for *nod* gene induction mediated by NodW alone (Dockendorff et al. 1994, Sanjuan et al. 1994). Subsequently, it was found that the expression of NolA from a multicopy plasmid inhibited the isoflavone-mediated induction of a *nodC-lacZ* fusion, suggesting that *nolA* encodes a

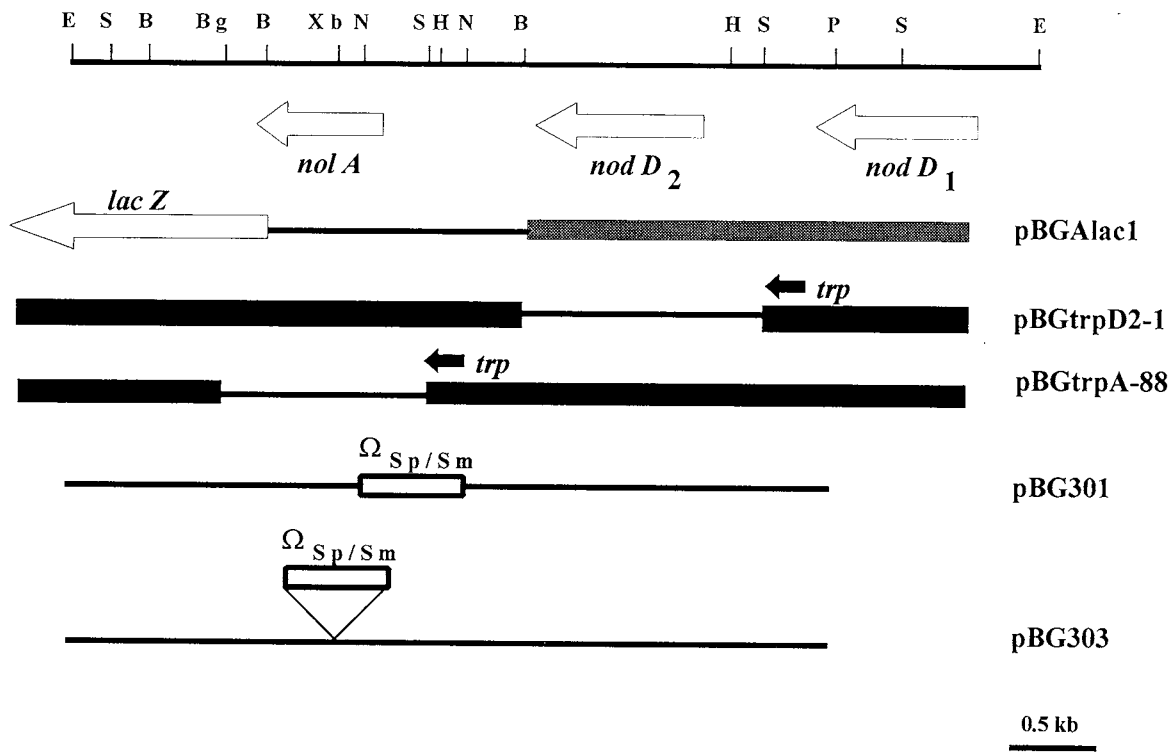

Fig. 1. Restriction map of *nod* gene region of the *Bradyrhizobium japonicum* chromosome under study (top). The position of the *nolA*, *nodD1*, and *nodD2* genes are shown immediately below the map. (Only the pertinent restriction sites are shown. E = *Eco*RI; S = *Sal*I; B = *Bam*HI; Bg = *Bgl*II; Xb = *Xba*I; N = *Nhe*I; P = *Pst*I). Below the restriction map are shown the various *nolA* and *nodD2* constructs used in the study. pBGAlac1; the thick, hatched line represents the pNM480 vector into which the *nolA* promoter and coding sequence (thin black line) was cloned to produce an in-frame fusion with *lacZ* (open arrow, labeled). pBGtrpD2-1; the thick, solid line represents the pTE3Ω vector into which the *nodD2* gene (thin black line) was cloned so as to be expressed from the consitutive *trp* promoter (black arrow, labeled). pBGtrpA-88; the thick, solid line represents the pTE3 vector into which the *nolA* gene (thin black line) was cloned so as to be expressed from the consitutive *trp* promoter (black arrow, labeled). pBG301; pSUP202 plasmid (not shown) containing a 4.5-*Eco*RI-*Pst*I fragment (thin black line) into which the Ω Sp/Sm resistance cassette (open box, labeled) was cloned so as to delete the *nolA* promoter and 5′ region of the coding sequence. pBG303; pSUP202 plasmid (not shown) containing a 4.5-*Eco*RI-*Pst*I fragment (thin black line) with the Ω Sp/Sm resistance cassette (open box, labeled) inserted into the *Xba*I site so as to disrupt the *nolA* coding sequence.

repressor (Dockendorff et al. 1994). To further study the role of NolA in *nod* gene regulation, two *nolA* mutant strains were constructed (Fig. 1). The mutation in strain BjD21 (constructed using pBG303, Fig. 1) was made by an interposon insertion into the *nolA* coding region. However, the site of this mutation did not preclude the possibility that a C-terminal truncated protein containing the helix-turn-helix DNA binding region could be made in this strain. Therefore, a second mutant strain, BjB3 (constructed using pBG301, Fig. 1), was constructed in which the 5′ end of the *nolA* coding sequence, as well as sequences upstream of the putative start codon, were deleted. Plasmids encoding a *nodD1-lacZ*, *nodY-lacZ*, or *nodD2-lacZ* fusion were mobilized into these mutant strains and the β-galactosidase activity was assayed. As shown in Table 1, the basal and induced expression of *nodD1* and *nodY* in both mutant strains was comparable to that of the wild type. Therefore, contrary to our previous hypothesis (Dockendorff et al. 1994), it seems unlikely that NolA acts directly to repress *nod* gene expression. Surprisingly, the expression of the *nodD2-lacZ* fusion was significantly lower in the mutant strains as compared to the wild type. Therefore, NolA may be involved in the positive regulation of *nodD2* expression.

The lack of elevated expression of *nodD1* and *nodY* in the *nolA* mutant backgrounds is unexpected in view of the reported role of NolA in the repression of *nod* gene expression. One possible explanation for our previous results is that NolA mediates *nod* gene repression indirectly through another protein. A likely candidate is NodD2, a homolog of which has been shown to be involved in the repression of *nod* gene expression in *Bradyrhizobium* sp. (*Arachis*) NC92 (Gillette and Elkan 1996). To test whether *B. japonicum* NodD2 has a repressive effect on *nod* gene expression, NodD2 was expressed from the *trp* promoter of pTE3 and its effect on the expression of a chromosomal *nodC-lacZ* fusion was tested. As shown in Table 2, the expression of NodD2 from the constitutive *trp* promoter results in a drastic reduction of *nodC-lacZ* expression.

Table 1. Expression of *nodY, nodD2* and *nodD1* in wild type and *nolA* mutant strains

	β–Galactosidase activity[a,b] (U)		
	Uninduced	**+SSE[c]**	**+Genistein**
nodY-lacZ			
USDA110 (wild type)	31 ± 2	1,268 ± 21	650 ± 43
BjD21 (*nolA*)	36 ± 9	1,623 ± 23	786 ± 19
BjB3 (*nolA*)	29 ± 9	1,488 ± 109	724 ± 98
nodD1-lacZ			
USDA110 (wild type)	7 ± 1	36 ± 5	19 ± 4
BjD21 (*nolA*)	6 ± 1	31 ± 4	21 ± 5
BjB3 (*nolA*)	5 ± 1	34 ± 9	22 ± 3
nodD2-lacZ			
USDA110 (wild type)	178 ± 20	184 ± 16	160 ± 11
BjD21 (*nolA*)	23 ± 5	36 ± 3	19 ± 4
BjB3 (*nolA*)	31 ± 4	29 ± 5	25 ± 5

[a] Units using CPRG as a substrate
[b] Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.
[c] Soybean seed extract

Table 2. Expression of a chromosomal *nodC-lacZ* in the presence or absence of NodD2

	β-Galactosidase activity[a,b] (U)		
Strain	**Uninduced**	**+SSE[c] (–tryptophan)**	**+SSE (+tryptophan)[d]**
Bj1221 (*nodC-lacZ*; pTE3Ω	13 ± 6	400 ± 6	400 ± 12
BjtrpD2-1 (*nodC-lacZ*; pBGtrpD2-1)	21 ± 3	44 ± 5	47 ± 7

[a] Units using CPRG as a substrate.
[b] Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.
[c] Soybean seed extract.
[d] added at 20 ug/ml of culture.

nolA expression is positively autoregulated.

The *nolA* DNA sequence predicts a translation product possessing a N-terminal, helix-turn-helix DNA binding motif which shares sequence similarity to the MerR-type regulatory proteins (Sadowsky et al. 1991). Studies on MerR and TipA, two proteins belonging to this family of regulatory proteins, have shown that these proteins autoregulate their own expression (Lund et al. 1986; Holmes et al. 1993). Therefore, the possibility that *nolA* expression is autoregulated was investigated. A plasmid encoding a *nolA-lacZ* fusion was constructed and is shown in Figure 1 (i.e., pBGAlac1). In addition to this

Table 3. Expression of *nolA-lacZ* in *E. coli* JM 109 in the presence or absence of NolA

	β-Galactosidase activity[a,b] (U)	
Plasmid/fusion	**+Tryptophan[c]**	**–Tryptophan**
pBGAlac1 (*nolA-lacZ*)		
+pTE3	26 ± 11	28 ± 9
+pBGAtrpA-88	30 ± 3	351 ± 43
pNM480 (vector)		
+pTE3	0	0
+pBGtrpA-88	0	0

[a] Units using CPRG as a substrate
[b] Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.
[c] tryptophan was added at 20 ug/ml growth culture.

Table 4. Expression of a *nolA-lacZ* in *Bradyrhizobium japonicum* wild type and mutant strains

Strain (Phenotype)	**β-Galactosidase activity[a,b] (U)**
USDA110 (wild type)	595 ± 13
BjD21 (*nolA*)	108 ± 34
BjB3 (*nolA*)	96 ± 12
Bj586 (*nodD1*)	274 ± 8
Bj613 (*nodW*)	550 ± 62

[a] Units using CPRG as a substrate
[b] Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.

nolA-lacZ fusion, the *nolA* gene was cloned into plasmid pTE3 in order to express the NolA protein from the *trp* promoter (i.e., pGBtrpA-88, Fig. 1). This promoter can be regulated in *E. coli* by the presence or absence of tryptophan, but is constitutively expressed in *B. japonicum*. *E. coli* cells were cotransformed with pBGlac1 (containing the *nolA-lacZ* fusion) and pBGtrpA-88. As controls, cotransformations were also done using the vectors pTE3 or pNM480 (see Methods). β-Galactosidase activity assays were done on logarithmically growing cells in the presence or absence of tryptophan in the growth medium (Table 3). High expression of the *nolA-lacZ* fusion required the presence of NolA (i.e., in the presence of pBGtrpA-88 and the absence of tryptophan in the growth medium), suggesting that NolA acts as a positive regulator of its own expression.

To confirm *nolA* autoregulation in *B. japonicum*, plasmid pBGAlac1 was cointegrated with the conjugative plasmid pRK290 and mobilized into different *B. japonicum* backgrounds. Consistent with the notion that *nolA* is positively autoregulated, *nolA-lacZ* expression was reduced sixfold, as compared to the wild type, in the *nolA* mutant strains BjD21 and BjB3 (Table 4). The expression of the *nolA-lacZ* fusion in the *nodW* mutant strain Bj613 was comparable to that of the wild type, suggesting that NodW is not involved in the regulation of *nolA*. However, the expression of *nolA* in the *nodD1* mutant strain Bj5686 was reduced approximately twofold compared to the wild type. Therefore, in addition to NolA, maximum expression of *nolA* requires NodD1.

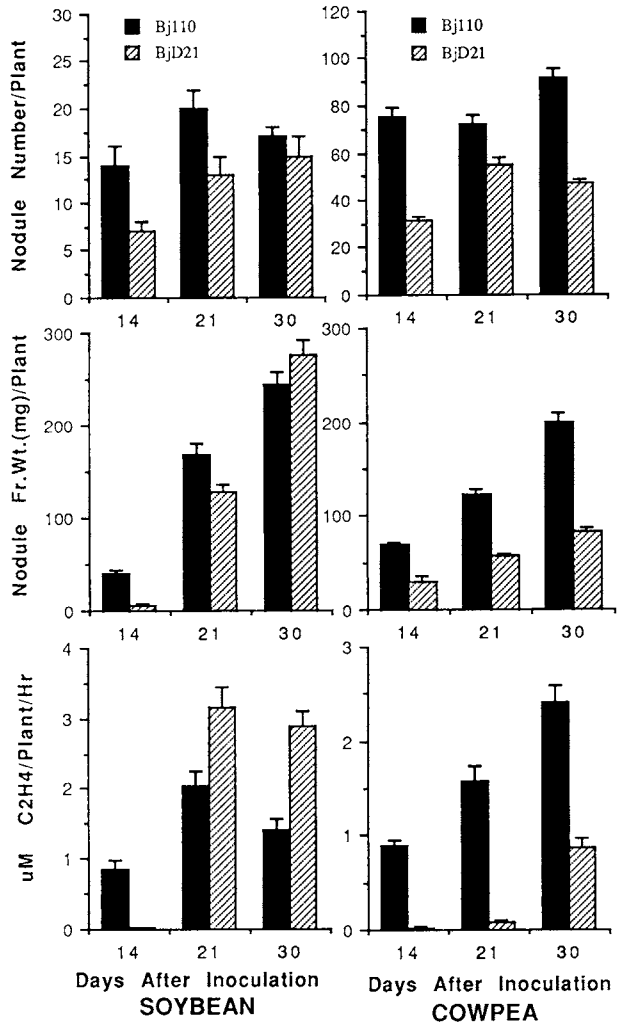

Fig. 2. Nodulation and nitrogen fixation phenotype of *Bradyrhizobium japonicum* strain USDA110 (wild type, solid bar) and the *nolA* mutant strain, BjD21 (hatched bar), on soybean (left) and cowpea (right) plants. Top panel, nodule number per plant at 14, 21, and 30 days post inoculation (PI). Middle panel, nodule fresh weight (mg) per plant at 14, 21, and 30 days PI. Lower panel, acetylene reduction activity (μM C_2H_4 formed per plant per hour) at 14, 21, and 30 days PI. Results presented are the means of 18 plants assayed over two trials (standard error is shown above each bar).

Nodulation and nitrogen fixation phenotype of *nolA* mutants.

The previous report by Sadowsky et al. (1991) indicated that NolA was involved in mediating genotype-specific nodulation of soybeans. In addition, Dockendorfff et al. (1994) have reported that conjugation of a plasmid encoding *nolA* into a *B. japonicum* strain deleted for *nodD1*, *nodD2*, and *nolA* significantly improved nodulation ability. These reports point to the importance of *nolA* in the ability of *B. japonicum* to effectively nodulate its plant hosts. To further study the function of NolA in nodule development, the nodulation and nitrogen fixation phenotypes of the *nolA* mutant strains BjD21 and BjB3 were tested on *Gycine max* (soybean) cv. Essex and *Vigna unguiculata* (cowpea) cv. Caloona. Nodulation efficiency was examined by determining the nodule number and fresh weight produced per plant, whereas nitrogen fixation was measured by acetylene reduction activity. As shown in Figure 2, the *nolA* mutant strain BjD21 showed grossly defective nodulation and nitrogen fixation on cowpea plants. Similar results were obtained for mutant strain BjB3 (data not shown). Nodulation efficiency of the mutant strain was about half that of the wild type, even at 30 days postinoculation (PI). The nitrogen fixation activity shown by the mutant strain was only 10% of the wild type at 14 and 21 days PI, and increased to approximately 30% of the wild-type level at 30 days PI. Cowpea plants inoculated with the mutant strain resembled uninoculated control plants in that their leaves turned chlorotic and the plants stopped growing. This gross defect in nodulation and nitrogen fixation by the mutant strain was not observed in soybean, which showed only a slight delay in nodulation, and even a higher degree of nitrogen fixation than the wild type at 21 and 30 days PI.

Nodules formed by mutant strain BjD21 were examined by light and electron microscopy and compared to those formed by the wild type. Light microscopy revealed that soybean nodules induced by the the wild type at 14 days PI contained larger infected cells and a higher ratio of infected to uninfected cells than nodules formed by the mutant strain (Fig. 3A and C). When examined by electron microscopy, plant cells infected by the wild type were typically shown to contain symbiosomes with two to three bacteroids. These bacteroids had significant internal deposits of polyhydroxybutyrate (Fig. 3B). In contrast, plant cells infected by the mutant had symbiosomes that typically contained a single bacteroid. The mutant bacteroids showed little internal polyhydroxybutyrate (Fig. 3D).

Soybean nodules induced by strain BjD21 at 21 days PI showed a similar ratio of infected to uninfected cells to that found in nodules formed by the wild type (Fig. 4A and C).

The bacteroids formed by both the wild type and mutant contained significant amounts of poyhydroxybutyrate (Fig. 4B and D).

Cowpea nodules formed by the wild type and mutant strain BjD21 were also analyzed by light and electron microscopy at 14 and 21 days PI. Light microscopy revealed that nodules formed by the mutant strain had a lower ratio of infected to uninfected cells at both 14 and 21 days PI as compared to nodules induced by the wild type (Figs. 5A and C and 6A and C). Electron microscopy revealed that nodule cells infected by the wild-type strain at 14 days PI exhibited symbiosomes typically containing multiple bacteroids (Figs. 5B and 6B). In contrast, cells infected by strain BjD21 at 14 days PI contained only a few apparent symbiosomes that typically contained only a single bacteroid (data not shown). Most of the mutant bacteroids were found embedded in a large vesicular-like structure containing fibrous material (Fig. 5D). The origin of these structures is unknown, but they clearly do not resemble normal symbiosomes.

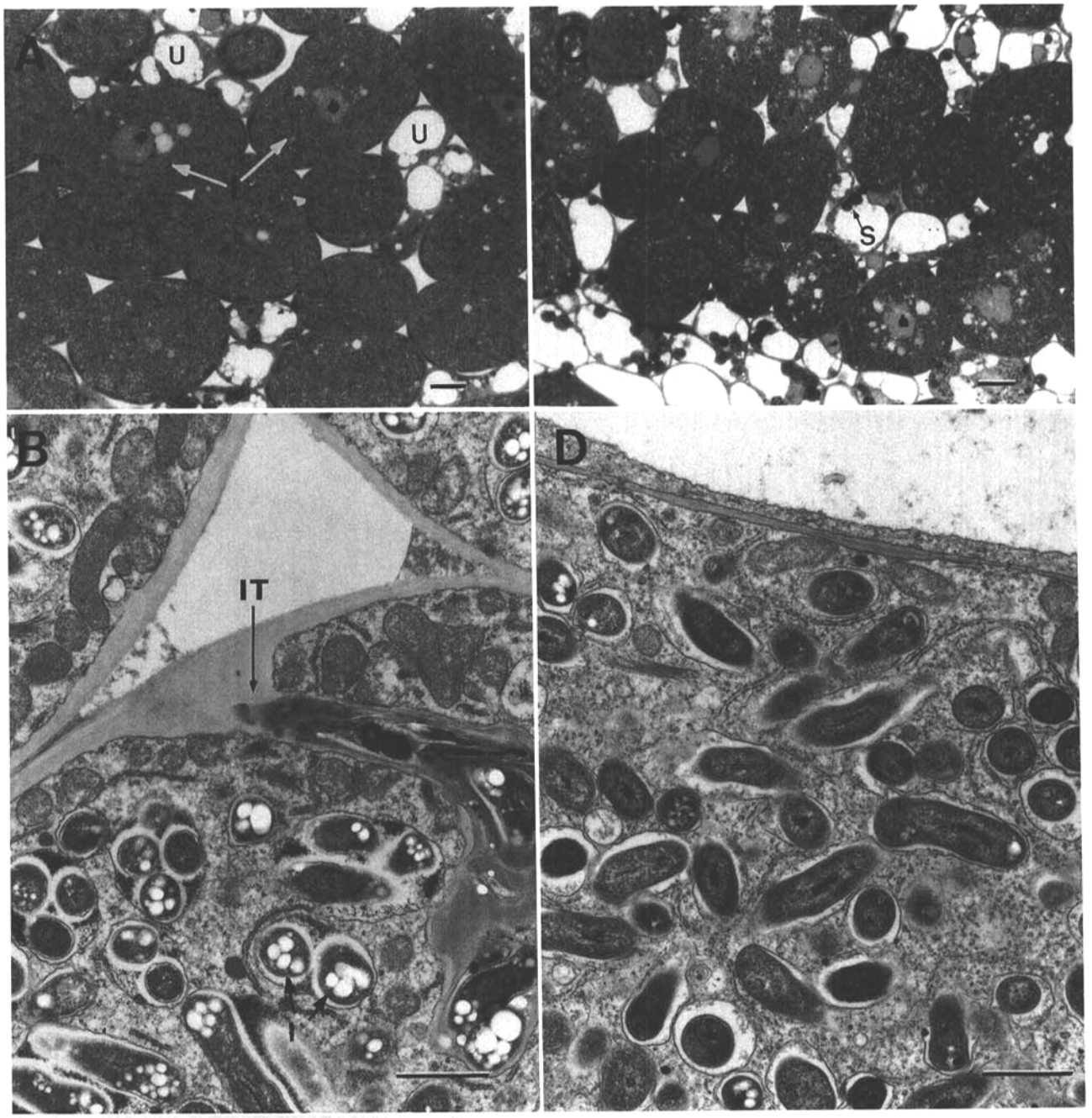

Fig. 3. Light **(A and C)** and electron **(B and D)** micrographs of 14 days PI soybean nodules induced by *Bradyrhizobium japonicum* strain USDA110 (A and B) and *nolA* mutant strain BjD21 (C and D). Magnification: light micrographs A and C;bar =10 µm: electron micrographs B and D; bar = 1 µm. Abbreviations: I = infected cell; U = uninfected cell; P = polyhydroxybutyrate; IT = infection thread; V = vesicle; S = starch; FB = fibrous material; FM = fibrillar material.

At 21 days PI, nodule cells infected by the wild-type strain were filled with symbiosomes typically containing several bacteroids (Fig. 6B). In contrast, cells infected by the mutant strain contained fewer bacteroids. As was the case at 14 days PI, no typical symbiosomes were present in the mutant infected nodule cells (Fig. 6D and E). Fine fibrillar material was loosely distributed throughout the mutant infected cells (Fig. 6D). Even though the mutant bacteroids were not found within symbiosomes, they did not appear to be in a state of degradation (Fig. 6E).

DISCUSSION

Previously, Dockendorffff et al. (1994) suggested that NolA is a repressor of *nod* gene expression in *B. japonicum*. This suggestion was based on the fact that a strain deleted for *nodD1, nodD2,* and *nolA* showed elevated *nod* gene expression and that expression of *nolA* from a multi-copy plasmid resulted in a significant reduction in *nodC-lacZ* activity. As part of this study, repeated attempts were made to isolate mutations in *nolA* but were unsuccessful. This was discussed by Dockendorffff et al. (1994) and it was suggested that the insta-

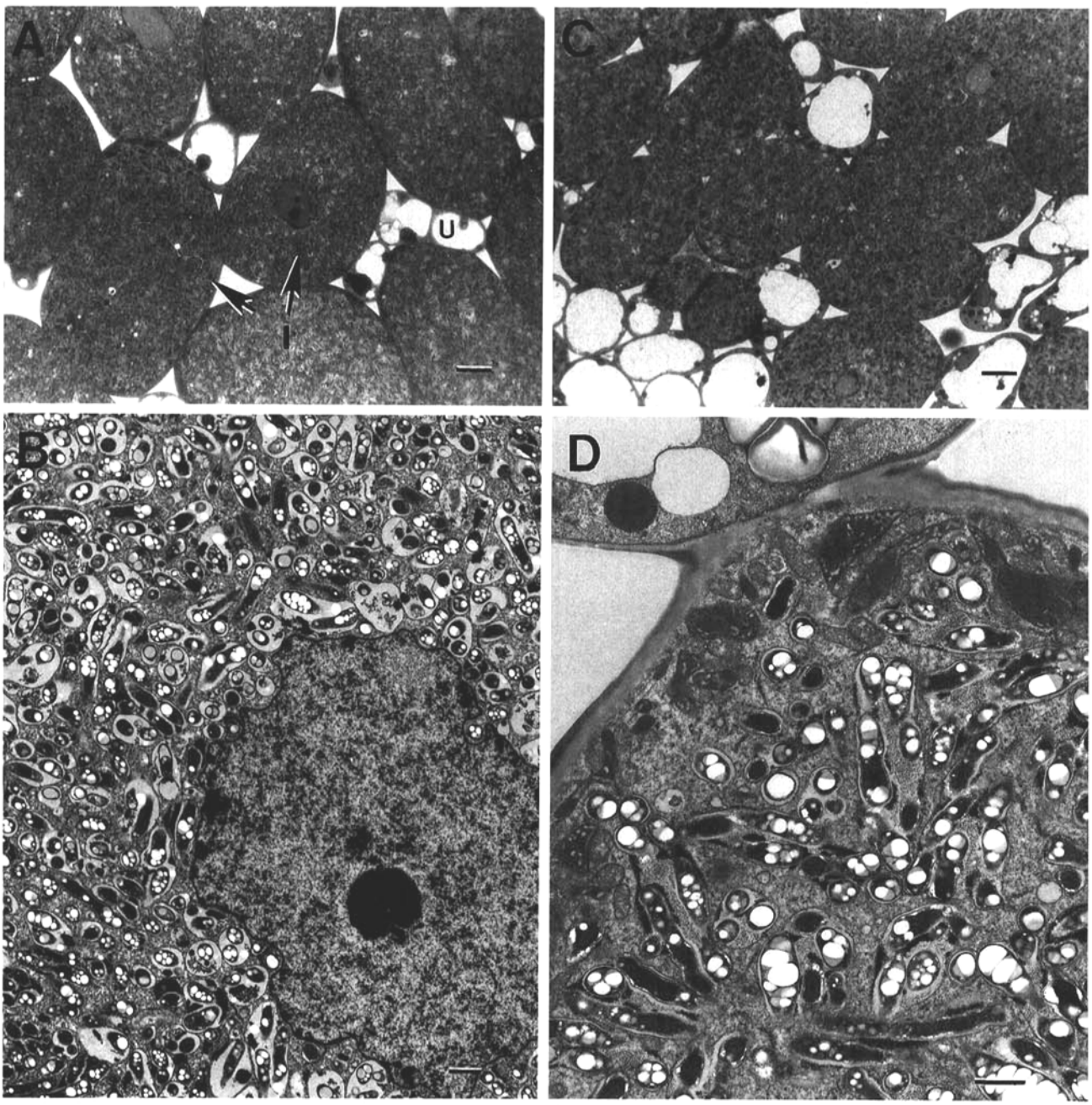

Fig. 4. Light (A and C) and electron (B and D) micrographs of 21 days PI soybean nodules induced by Bradyrhizobium japonicum wild-type strain USDA 110 (A and B) and nolA mutant strain BjD21 (C and D). Magnification: light micrographs A and C, bar = 10 µm; electron micrographs B and D, 1 µm. Abbreviations listed in caption to Figure 3.

bility noted in this region by Gottfert et al. (1992) might make such mutations unstable or lethal. In this study, we have succeeded in isolating two *nolA* mutants (i.e., BjD21 and BjB3). We have no explanation for our prior failure to isolate such mutants. Contrary to the predicted role of NolA as a repressor, mutations in *nolA* did not result in higher expression of *nodD1* or *nodY*. Therefore, NolA likely does not act directly on the promoters of these genes to mediate repression. Since NolA appears to be required for higher expression of *nodD2* (Table 1) and elevated NodD2 expression represses *nod-lacZ* activity (Table 2), it is possible that NolA mediates repression through NodD2. The idea that NodD2 can act as a repressor is not unique to our work since a homologous protein has been shown to repress *nod* gene expression in *Bradyrhizobium* sp. (*Arachis*) (Gillette and Elkan 1996). However, it is becoming obvious that *nod* gene regulation in *B. japonicum* is very complex and likely involves the interplay of different regulatory proteins. The role of NolA and NodD2, as well as other regulatory proteins, in *nod* gene expression is likely more complex than currently envisioned. Therefore, we are cautious with regard to attributing the repressive effects of NolA expression simply to an effect on *nodD2*.

The amino-terminus of NolA contains a helix-turn-helix DNA binding motif that shares sequence similarity to the MerR-type regulatory proteins (Sadowsky et al. 1991). Members of this family of regulatory proteins have been shown to

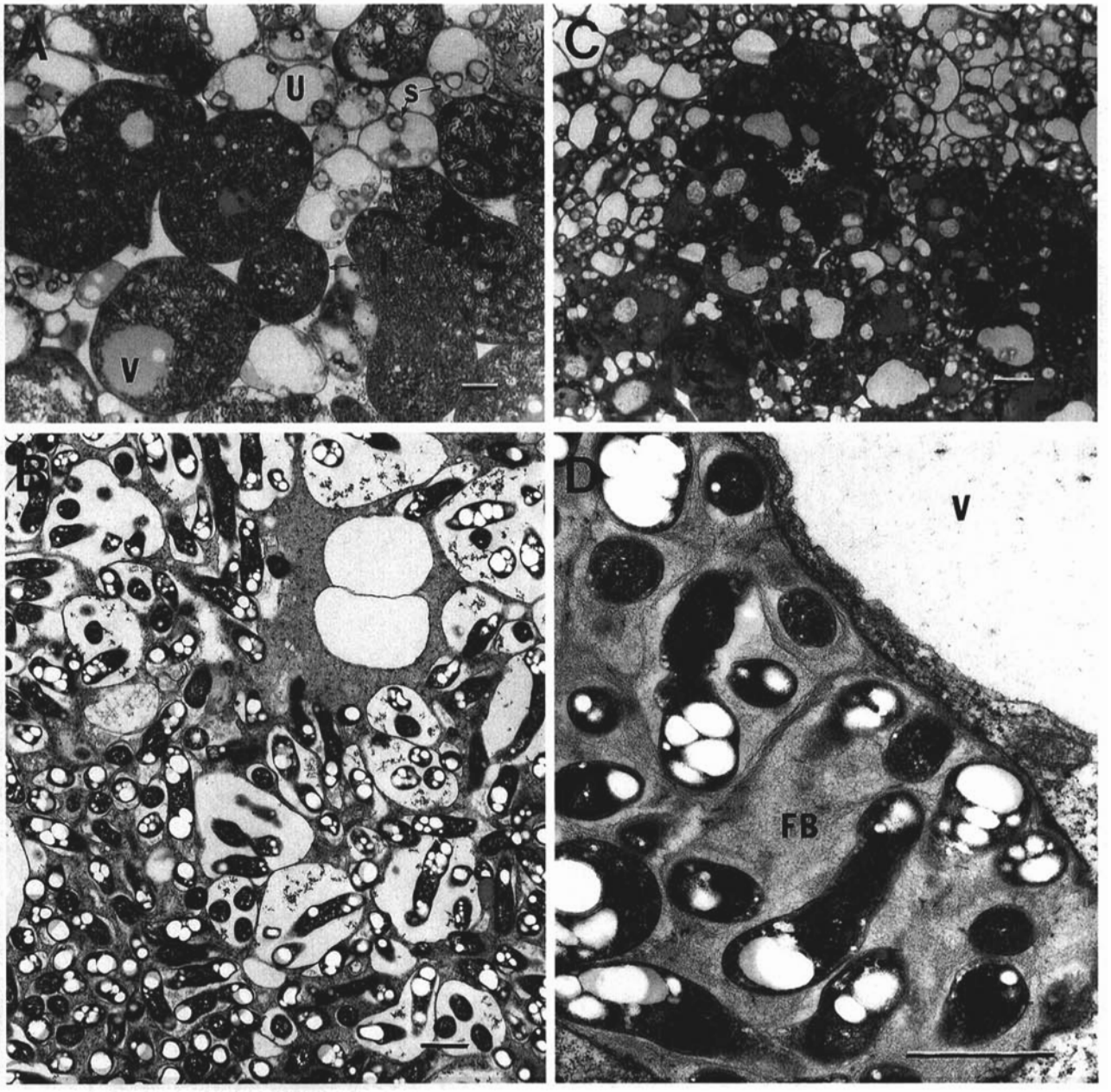

Fig. 5. Light (A and C) and electron (B and D) micrographs of 14 days PI cowpea nodules induced by *Bradyrhizobium japonicum* wild-type strain USDA110 (A and B) or *nolA* mutant strain BjD21 (C and D). Magnification: light micrographs A and C, bar = 10 μm; electron micrgraphs B and D, bar = 1 μm. Abbreviations listed in caption to Figure 3.

be positive activators of genes involved in important cellular processes (Lund et al. 1986; Holmes et al. 1993; Ahmed et al. 1994; Amabile-Cuevas and Demple 1991). Since two members of this family are autoregulated (i.e., MerR and TipA; Lund et al. 1986; Holmes et al. 1993), the possibility that NolA expression is autoregulated was tested. Results obtained in *E. coli* and *B. japonicum* indicate that NolA positively activates its own expression. NolA also appears to be essential for elevated expression of NodD2 (Table 1). There are areas of DNA sequence similarity in the *nolA* and *nodD2* promoter regions that could identify putative NolA binding sites (data not shown). However, the transcriptional start sites for *nolA* and *nodD2* have not been identified and additional work will be required to confirm that NolA interacts directly with both promoters.

B. japonicum strains which are mutated in *nolA* showed a slight delay in soybean nodulation, but were strongly affected for nodulation of cowpea. It is unlikely that this reduced nodulation is due to an effect on Nod signal production since *nodY-lacZ* or *nodD1-lacZ* fusion expression was not affected

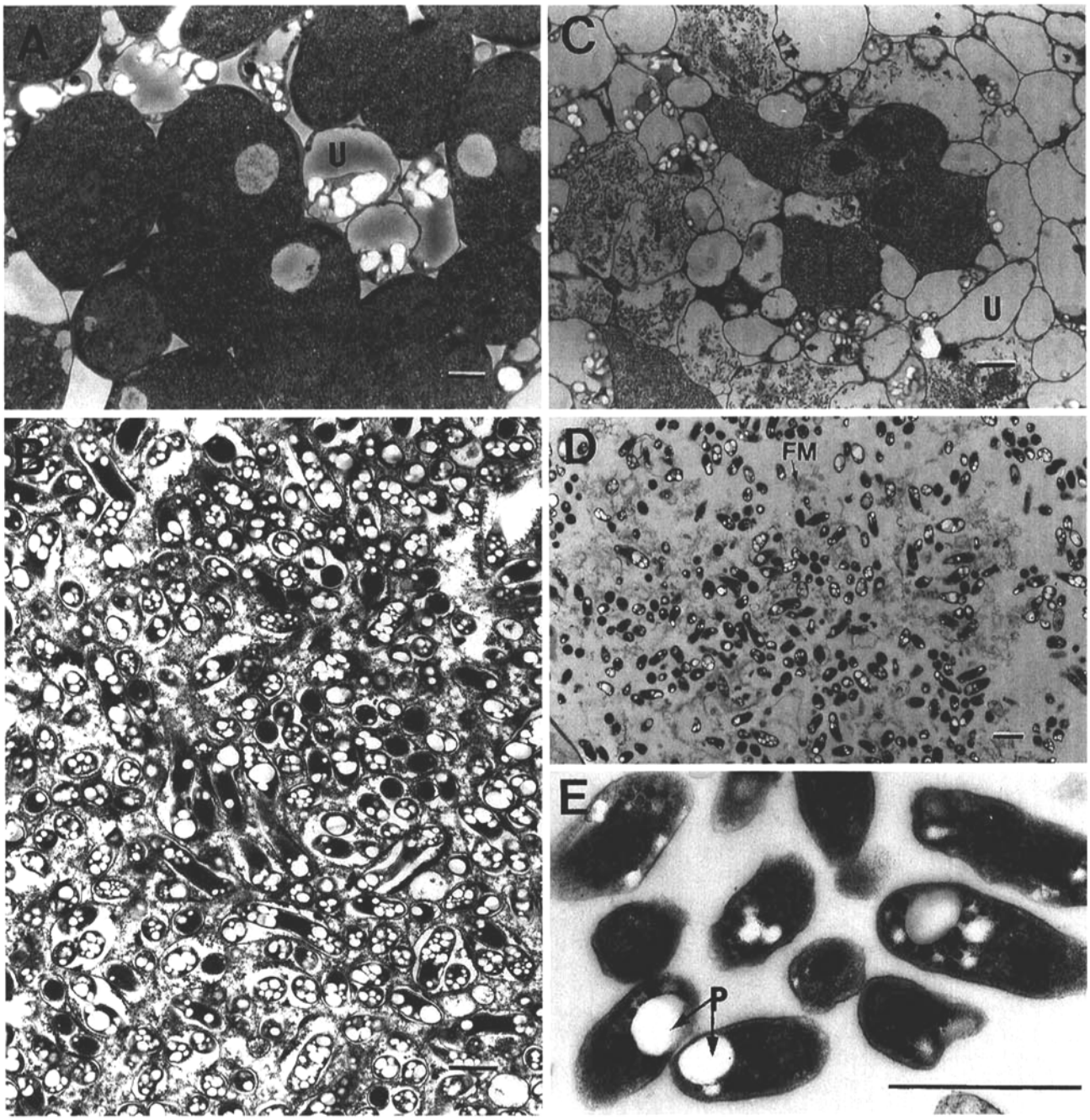

Figure 6. Light (A and C) and electron (B,D, and E) micrographs of 21 days PI cowpea nodules induced by *Bradyrhizobium japonicum* wild-type strain USDA110 (A and B) or *nolA* mutant strain BjD21 (C, D, and E). Magnification: light micrograph A and C, bar = 10 μm; electron micrographs B, D, and E, bar = 1 μm. Abbreviations listed in caption to Figure 3.

in the *nolA* mutants. Moreover, analysis of the level of lipochitin Nod signals produced by the *nolA* mutant strains by thin-layer chromatography (Spaink et al. 1992) showed levels similar to that of the wild type (data not shown). It seems more likely that NolA plays an important role in nodulation by regulating other, as yet, unidentified genes. Judging from the nodule ultrastructure of plants inoculated by the *nolA* mutants, such genes are likely important not only for nodule initiation, but also for bacteroid development and maintenance.

Electron microscopy of soybean nodules induced by the mutant strains showed well-developed bacteroids similar in morphology to those formed by the wild type. The mutant nodules appeared to have a reduced ratio of infected to uninfected cells at 14 days PI, but this phenotype was not apparent at 21 days PI. These data suggest a slight delay in the ability of strain BjD21 to fully infect soybean. In contrast to soybean plants infected by the *nolA* mutants strains, cowpea plants nodulated by the mutant strains showed a drastic reduction in nitrogen fixation activity. Nitrogen fixation in cowpea nodules was only 10% of the wild-type level at 21 days PI. Microscopic examination of cowpea nodules infected by strain BjD21 showed the lack of clearly identifiable symbiosomes. At 14 days PI, bacteroids were in large vesicular-like structures filled with fine granular material. The nature of this material is unknown, as is the origin of these structures. By 21 days, it appeared as if these structures had disintegrated, leaving the bacteroids free in the cytoplasm. In some respects this resembles the effects of early nodule senescence (c.f., Fischer et al. 1986; Kullik et al. 1991; Anthamatten and Hennecke 1991). However, contrary to such reports, the morphology of the mutant bacteroids did not suggest a state of degradation. This is also suggested by the fact that these nodules showed low, but measurable levels of nitrogen fixation (i.e., 10% of the wild-type level). Thus, it may be that the *nolA* mutants induce the desolution of infected cell structure (e.g., symbiosomes), but without inducing a rapid onset of senescence. Nodule morphology was not followed beyond 21 days to see if bacteroid degradation did eventually occur.

Previously, we reported that mutations in the *hsfA* gene led to a loss of nitrogen fixation ability on cowpea, but not on soybean (Chun et al. 1995). However, ultrastructural analysis of soybean nodules infected by the *hsfA* mutant did reveal minor differences from wild-type nodules. Thus, the phenotype of both the *nolA* and *hsfA* mutants is more severe on cowpea, than on soybean. This indicates clearly that there are differences in the response of soybean and cowpea plants to *B. japonicum* infection. A difference in the response of soybean and cowpea plants to *B. japonicum* inoculation was also reported by Bauer et al. (1985). The ability of different *B. japonicum* strains to nodulate cowpea and soybean plants was tested and it was found that cowpea required 100-fold-higher inoculum levels to initiate early nodulation responses. Likewise, although the regulatory proteins NodD1 and NodW can functionally complement one another with regard to soybean nodulation, only NodW is essential for cowpea nodulation (Sanjuan et al. 1994; Gottfert et al. 1990). This type of variation seen within plant hosts, as well as between host genotypes (e.g., Sadowsky et al. 1991), further complicates the analysis of the functional role of nodulation gene products.

Our current model for *B. japonicum nod* gene regulation predicts that transcription is controlled through the concerted action of specific members of three families of global regulators. Induction of *nod* gene expression occurs in response to plant-produced isoflavones mediated by a LysR-family protein, NodD1, and a member of the two-component regulator family, NodW. It is a unique and rather curious feature of *B. japonicum* that these two, apparently redundant systems function to activate *nod* gene expression. One explanation is that NodW is required for the nodulation of alternative hosts, such as cowpea, for which NodD1-mediated *nod* gene expression is insufficient. A member of the MerR-family of transcriptional regulators, NolA, does not appear to directly repress *nod* gene expression. Instead, NolA may activate the expression of additional genes, perhaps NodD2, that, in turn, repress *nod* gene expression.

MATERIALS AND METHODS

Media and bacterial growth conditions.

B. japonicum strains were grown at 30°C in modified RDY medium (So et. al. 1987) for routine growth and plant tests. HM salt medium (Cole and Elkan 1973) supplemented with 0.1% arabinose was used for growth of *B. japonicum* when conducting matings and extracting nucleic acids. Minimal medium (Bergersen 1961) was used for β–galactosidase activity assays. *E. coli* strains were cultured in LB or M9 medium (Sambrook et al. 1989) at 37°C. The following antibiotics were used for plasmid selection: with *E. coli*, ampicillin (Ap, 200 μg/ml), kanamycin (Km, 30 μg/ml), tetracycline (Tc, 25 μg/ml), streptomycin (Sm, 30 μg/ml), and spectinomycin (Sp, 100 μg/ml); with *B. japonicum*, kanamycin, spectinomycin, and tetracycline (100 μg/ml), and chloramphenicol (Cm, 30 μg/ml).

Bacterial strains and plasmids.

All strains and plasmids used in this study are listed in Table 1. The different *nolA* constructs and mutations are shown in Table 5.

The *nolA-lacZ* fusion was constructed as follows: A 1.5-kb *Bam*HI fragment containing *nolA* was cloned into the *Bam*HI site of pNM480 to give pBGAlac1. To conjugate pBGAlac1 into *B. japonicum*, this plasmid was cointegrated with the conjugative plasmid pRK290 by restricting pBGAlac1 with *Eco*RI and ligating into the *Eco*RI site of pRK290. The resulting cointegrate was transformed into *E. coli* S17-1 and then mobilized into different *B. japonicum* strains by biparental mating as described by Banfalvi et al. (1988). The in-frame fusion site in pBGAlac1 was confirmed by DNA sequence analysis using the dideoxynucleotide chain termination method of Sanger et al. (1977).

Plasmid pBGtrpA-88 was constructed to express *nolA* from the *trp* promoter of pTE3. This plasmid was constructed by isolating a 1.2-kb *Sal*I-*Bgl*II fragment containing *nolA*, filling-in the 5′ overhanging ends with Klenow DNA polymerase and then adding *Nsi*I linkers (5′-d(TGCATGCATGCA)-3′, United States Biochemical, Cleveland, OH). After digestion with *Nsi*I, this fragment was ligated into the *Pst*I site of pTE3. Plasmid pBGtrpD2-1 expresses *nodD2* from the *trp* promoter of pTE3 and was constructed as follows: A 1.4-kb *Bam*HI-*Sal*I fragment encompassing *nodD2* was cloned into the *Bam*HI-*Sal*I site of pUC19. The resulting plasmid was cut with *Bam*HI and *Pst*I, and the fragment containing *nodD2* was

cloned into the *Bam*HI-*Pst*I site of pTE3Ω. pTE3Ω was constructed by inserting the Sp/Sm cassette of pHP45Ω into the *Bgl*II site of pTE3. Gene expression from the *trp* promoter was determined by RNA slot blot hybridization.

The *nolA* mutant strain BjD21 was constructed as follows: A 4.5-kb *Eco*RI-*Pst*I fragment encompassing *nolA* was cloned into pUC19. This plasmid (pBG101) was cut at the *Xba*I site internal to the *nolA* coding region, and the 5′ overhanging ends were blunted with Klenow DNA polymerase. A 2.0-kb *Sma*I fragment containing the Sp/Sm resistance cassette of pHP45Ω was ligated to the *Xba*I site. The resulting plasmid was cut with *Eco*RI-*Pst*I to release the pUC19 vector, and the fragment carrying the mutated *nolA* was ligated to the *Eco*RI-*Pst*I site of pSUP202. The resulting plasmid (pBG303) was transformed into *E. coli* S17-1 and conjugated into *B. japonicum* USDA 110. Transconjugants expressing Sp/Sm resistance and Tc sensitivity, indicating double cross-over events, were selected. The mutation in BjD21 was confirmed by Southern blot hybridization.

The second *nolA* mutant strain, BjB3, was constructed as follows: pBG101 was cut with *Nhe*I, releasing a 600-bp fragment containing sequences 5′ to the *nolA* start codon and the 5′ terminus of the *nolA* coding region. The larger *Nhe*I fragment was filled in with Klenow DNA polymerase and ligated to the 2.0-kb *Sma*I fragment containing the Sp/Sm resistance cassette of pHP45Ω. The resulting plasmid was cut with *Pst*I-*Eco*RI to release the pUC19 vector, and the fragment containing the Ω insertion was ligated to the *Pst*I-*Eco*RI site of pSUP202. The resulting plasmid (pBG301) was transformed into *E. coli* S17-1 and conjugated into *B. japonicum* USDA 110. Marker exchange events were selected as above and confirmed by Southern hybridization.

Genetic techniques and nucleic acid manipulations.

Recombinant DNA techniques were done following established protocols as compiled by Sambrook et al. (1989). RNA was isolated using the hot phenol method described by Wang and Stacey (1991). Southern blot hybridizations were done following protocols described by Amersham Corporation, Arlington Heights, IL.

β–Galactosidase activity assays.

The β–galactosidase activity of *B. japonicum* strains harboring *lacZ* gene fusions was assayed as described by Banfalvi et al. (1988). The induction of *nod-lacZ* expression by the isoflavone genistein (2 μM) or soybean seed extract was done as described by Yuen and Stacey (1995). Soybean seed extract was prepared as described by Smit et al. (1992) and was added at 20 μl/ml. The β-galactosidase activity of *E. coli* cells was assayed as follows: Cells were grown overnight in M9 medium (Sambrook et al. 1989) supplemented with 0.5% (w/v) Casamino Acids, 20 μg/ml tryptophan and the appropriate antibiotics. Overnight cultures were diluted 10-fold into fresh M9 medium in the presence or absence of tryptophan. β–Galactosidase activity were measured 2 h after subculture following the method described by Banfalvi et al (1988).

Plant infection tests.

Seeds of *Glycine max* (soybean) cv. Essex and *Vigna unguiculata* (cowpea) cv. Caloona were surface-sterilized as de-

Table 5. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
B. japonicum		
USDA 110	Wild type	USDA, Beltsville, MD
BjD21	*nolA*, Sp^r, Sm^r	This study
BjB3	*nolA*, Sp^r, Sm^r	This study
Bj110-573	USDA110, chromosomal *nodC-lacZ* fusion, Tc^r	Dockendorff et al. 1994
Bj613	*nodW*, Sp^r, Sm^r	Gottfert et al. 1990
Bj586	*nodD1*, Km^r	Gottfert et al. 1992
E. coli		
JM109	*endA1, recA1, gyrA96, thi, hsdR17* (r_k^-, m_k^+), relA1, supE44, (lacproAB) [F′, traD36, proAB, lacIqZ M15]	Promega
S17-1	RP4 2-Tc::Mu-Km::Tn7 *pro, hsdR, recA*	Simon et al. 1983
Plasmids		
pRK2013	RK2, Tra^+, Km^r	Figurski and Helinski 1979
pRK290	RP4, Mob^+ Tc^r	Ditta et al. 1980
pTE3	Tc^r	Egelhoff and Long 1985
pTEΩ	Tc^r, Sp^r/Sm^r cassette	This study
pUC19	Ap^r	Yanisch-Perron et al. 1985
pBluescriptII SK+	Ap^r	Stratagene
pNM480	Ap, promoterless *lacZ*	Minton 1984
pHP45Ω	Sp^r/Sm^r,Ω cassette	Prentki and Krisch 1984
pSUP202	RP4 *mob*, Tc^r, Ap^r, Cm^r	Simon et al. 1983
pZB22	$nodD_1$-*lacZ*, Tc^r, Ap^r	Banfalvi et al. 1988
pZB32	*nodY-lacZ*, Tc^r, Ap^r	Banfalvi et al. 1988
pRJ1248	$nodD_2$-*lacZ*, Tc^r	Gottfert et al. 1992
pBGAlac1	*nolA-lacZ*, Ap^r	This study
pBG301	4.5-kKb *EcoR1-Pst1* fragment with Ω insertion in *nolA* cloned into pSUP202, Sm^r, Sp^r, Tc^r	This study
pBG303	4.5-kb *EcoR1-Pst1* fragment with deletion and Ω insertion in *nolA* cloned into pSUP202, Sm^r, Sp^r, Tc^r	This study
pBGtrpA-88	1.2-kKb *Sal1-BglII* clone of *nolA* in pTE3, Tc^r	This study
pBGtrpD2-1	1.4-kb *Bam*H1-*Sal*1 clone of *nodD2* in pTE3Ω, Tc^r, Sm^r, Sp^r	This study
pBG101	4.5-kb *Eco*RI-*Pst*I clone of *nodD2* and *nolA* in pUC19, Ap^r	This study

scribed by Nieuwkoop et al. (1987). After germination, seedlings were transferred into sterile Leonard jars containing vermiculite and half-strength plant nutrient solution (Wacek and Brill 1976). Plants were grown in a greenhouse under 16-h daylight. Nitrogen-fixation activity was detected by the acetylene reduction activity assay (Wacek and Brill 1976) with a Shimadzu GC-8A gas chromatograph equipped with a 6-ft (approximaely 2 m) Poropak R column. The detector was maintained at 100°C and the column at 75°C.

Light and electron microscopy.

Nodules formed on soybean and cowpea were excised at 14 and 21 days postinoculation, and 1-mm disks were taken from the center of each nodule. Tissue samples were prepared as described by Roth and Stacey (1989). For light microscopy, 1-μm thick sections were stained with toluidine blue and photographed with a Nikon microphot optical microscope. For electron microscopy, 150-nm thick sections were stained and then examined and photographed with a Hitachi H-600 electron microscope operating at 75 kV.

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