Identification and Characterization of the *nolYZ* Genes of *Bradyrhizobium japonicum*

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Characterization of an isoflavone-inducible locus closely linked to the common *nod* genes of *Bradyrhizobium japonicum* USDA110 led to the discovery of two open reading frames, designated *nolY* and *nolZ*. These open reading frames are preceded by a sequence with strong similarity to a consensus NodD-binding site (*nod* box). Studies utilizing a *nolZ'-'lacZ* fusion indicated that inducible expression is dependent upon both NodD1 and NodW, transcriptional regulators that are required for the expression of the common nodulation genes (e.g., *nodYABC*) of *B. japonicum*. A deletion mutation within *nolY* produced only slight defects in nodulation of soybeans, siratro, and cowpeas, but stronger defects were observed in nodulation of mung beans. An insertion mutation within *nolZ* showed no nodulation defects in the host plants tested. Competition assays for nodule occupancy in soybeans did not show any decrease in the competitiveness of a *nolY* mutant, nor did a *nolY* mutant show any detectable alteration in the production of lipooligosaccharide nodulation signals.

Additional keywords: gene regulation.

Members of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* (collectively termed rhizobia) can infect appropriate host plants and develop nitrogen-fixing symbioses. In both plant and bacteria, specific genes are expressed during events leading to the development of symbiosis (reviewed by Fisher and Long [1992]). The *nod/nol* genes are a class of rhizobial genes involved in symbiotic development. These genes, as a whole, are essential for initiating the development of the nitrogen-fixing nodule. The *nod* genes are divided into two classes: common and host-specific. Common *nod* genes (e.g., *nodABC*) are found in all rhizobia and are essential for nodulation, and those from one species can complement mutations in homologous genes in other species. Host-specific *nod* genes are limited to certain species of rhizobia. Examples include the *nodFEG*, *QP*, and *H* genes from *R. meliloti* (Debellé *et al.* 1986; Horvath *et al.* 1986; Schwedock and Long

1989), the *nodFEL*, *nodMNT*, and *nodO* genes from *R. leguminosarum* bv. *viciae* (Shearman *et al.* 1986; Surin and Downie 1988; Surin *et al.* 1990; de Maagd *et al.* 1989), and the *nodZ* gene of *B. japonicum* (Stacey *et al.*, in press). Mutations in these genes tend to inhibit or abolish nodulation of normal host plants. Certain *nod* gene products are now known to be responsible for the synthesis of lipooligosaccharide factors (*nod* factors) that induce root-hair curling and cortical cell division in host plants, early stages of nodule development (reviewed by Spaink [1992] and Dénarié *et al.* [1992]).

The transcription of *nod* genes in rhizobia is induced by flavonoid molecules that are released from host plant roots (Peters *et al.* 1986; Redmond *et al.* 1986; Kosslak *et al.* 1987). The NodD protein, a transcriptional regulator, is thought to interact with flavonoid molecules and subsequently activate the transcription of other *nod* genes (reviewed by Györgypal *et al.* [1991] and Schlaman *et al.* [1992]). NodD is a member of the large LysR-type family of transcriptional regulatory proteins (Schell, 1993). Recently, the NodW protein from *B. japonicum* USDA110 was shown to be essential for isoflavone-induced transcription of the *nodD₁* and *nodYABCSUIJ* operons (Sanjuan *et al.*, in press). NodW has significant similarity to the transcriptional regulator subclass of two-component regulators, and its gene is co-transcribed with *nodV*, a gene encoding a putative membrane-associated sensor protein (Göttfert *et al.* 1990a). NodD has been demonstrated to interact with a conserved *cis*-acting sequence termed the *nod* box (Hong *et al.* 1987; Fisher *et al.* 1988; Kondorosi *et al.* 1989; Fisher and Long 1989), which has been found upstream of many NodD-dependent, flavonoid-induced operons in rhizobia (Rostas *et al.* 1986). Direct interaction of NodW with a *nod* gene promoter has yet to be demonstrated.

In *B. japonicum* USDA110, all isoflavone-inducible, NodD-dependent genes so far reported have been localized near the common *nod* genes. One inducible operon includes *nodYABCSUIJ* (Kosslak *et al.* 1987; Banfalvi *et al.* 1988; Göttfert *et al.* 1990b) (see Fig. 1 for a linkage map). Gene *nodD₁* in USDA110 is also inducible and positively autoregulates its synthesis (Banfalvi *et al.* 1988). The tightly linked *nodD₂* gene does not have any apparent dependence upon NodD₁, NodD₂, or isoflavones for its expression (Göttfert *et al.* 1992). The genotype-specific nodulation gene *nolA* shows a slight inducibility by isoflavones (Sadowsky *et al.* 1991), but the placement and nature of *nolA* regulatory sequences remain unknown. Göttfert *et al.* (1989), using an oligonucleotide probe with similarity to a consensus *nod* box, isolated several clones from a USDA110 library in which

nod box–like sequences were present. Deletions of these putative *nod* boxes and surrounding DNA had, with one exception, no discernible effect on nodulation of soybean and other hosts of *B. japonicum* USDA110. It was not determined whether any of the putative *nod* boxes found were functional. In this study we report the identification and characterization of a locus closely linked to the common *nod* genes of *B. japonicum* USDA110. This locus is regulated by isoflavones, NodD$_1$, and NodW—a regulatory pattern similar to that of the *nodD$_1$* and *nodYABCSUIJ* operons in USDA110.

RESULTS

Identification and sequence analysis of *nolYZ*.

Random mini-Mu::*lacZ* mutagenesis of a 5.3-kb *Eco*RI fragment adjacent to the *nodD* genes of USDA110 led to the identification of an isoflavone-inducible fusion (Deshmane and Stacey 1989) (see Fig. 1 for a genetic map). DNA flanking this insertion was subsequently sequenced and analyzed to reveal two open reading frames (Fig. 2). The two open reading frames, designated *nolY* and *nolZ*, consist of 726 and 225 bp and produce predicted translation products of 26,581 and 8,604 Da, respectively. Computer comparison of the predicted translation products of *nolY* and *nolZ* to sequences within the GenBank database did not reveal any significant similarities. In addition, no notable motifs that could suggest a possible biochemical function (e.g., membrane spanning, helix-turn-helix, or nucleotide binding) were seen.

A sequence was detected upstream of *nolYZ* that had similarity to *nod* boxes sequenced from other rhizobia (Fig. 2). Alignment of this putative *nod* box with a consensus *nod* box sequence showed 70% nucleotide similarity. Two models have been proposed to define functional sequences/motifs in rhizobial *nod* boxes. One (Wang and Stacey 1991) proposes a tetramer of 9-bp direct repeats as a functional motif. Another, (Goethals *et al.* 1992) proposes two motifs of ATC(N$_9$)GAT as being necessary for function. A consensus motif that consists of the sequence T(N$_{11}$)A has been proposed for LysR-type regulators (Goethals *et al.* 1992). A comparison of the putative *nolYZ nod* box with the proposed models shows that it has similarity to all of these models.

Expression of *nolYZ*.

A translational *lacZ* fusion was mapped to *nolZ* by cloning the junction of the fusion insertion and surrounding DNA and sequencing to assess the point of insertion. Mobilization of this fusion plasmid (pND228) into wild-type USDA110 showed that induction of the fusion was dependent upon the addition of the isoflavone genistein or soybean seed extract (Table 1). This fusion was mobilized to various regulatory mutants of USDA110 to test the effect on *nolZ* expression. The results presented in Table 1 show that induction of this fusion is dependent upon the NodD1 protein, similar to the *nodYABC* and *nodD$_1$* operons (Banfalvi *et al.* 1988). In addition, the fusion was not induced in a *nodW* mutant of USDA110. The *nodVW* genes have similarity to the prokaryotic family of two-component regulatory systems (Göttfert *et al.* 1990a). Recently, it was shown that NodW is essential for isoflavone-mediated induction of both the *nodYABC* and *nodD$_1$* operons in USDA110 (Sanjuan *et al.*, in press). The lack of inducibility of a *nolZ'-'lacZ* fusion in a *nodW* mutant suggests that NodW may be a common component in the transcription of all isoflavone-inducible, NodD-dependent genes in B. *japonicum* USDA110. Despite several efforts, an inducible *nolY'-'lacZ* fusion was not obtained. Therefore, *nolY* expression was quantitated by the use of RNA dot blots, with RNA from uninduced and isoflavone-induced cultures and a *nolY*-specific probe. The results obtained indicated a 40- to 50-fold increase in expression over that of uninduced controls (Fig. 3).

Mutational analysis of *nolYZ*.

Deletion and/or insertion mutations were constructed in the *nolY* and *nolZ* genes to test what effect these mutations would have upon nodulation proficiency. An internal deletion that removed 65% of the *nolY* coding sequence was constructed (see Materials and Methods), and this mutant (TCD520) was tested on various plant hosts. Only slight alterations in nodule numbers were seen in soybean, cowpea, or siratro inoculated with this mutant (Fig. 4). However, a stronger reduction in nodule number and a reduction in the percentage of plants nodulated during the early stages of the nodulation process were noted in mung bean plants inoculated with strain TCD520 (Fig. 5). A Tn*5* insertion was mapped to *nolZ* (strain TCD530); however, no detectable symbiotic defects were noted in any plant host tested.

Competition studies based upon nodule occupancy of mutant versus wild-type strains have been used to detect otherwise unnoticeable phenotypic alterations of nodulation proficiency in *R. meliloti* (Sanjuan and Olivares 1991). A recent
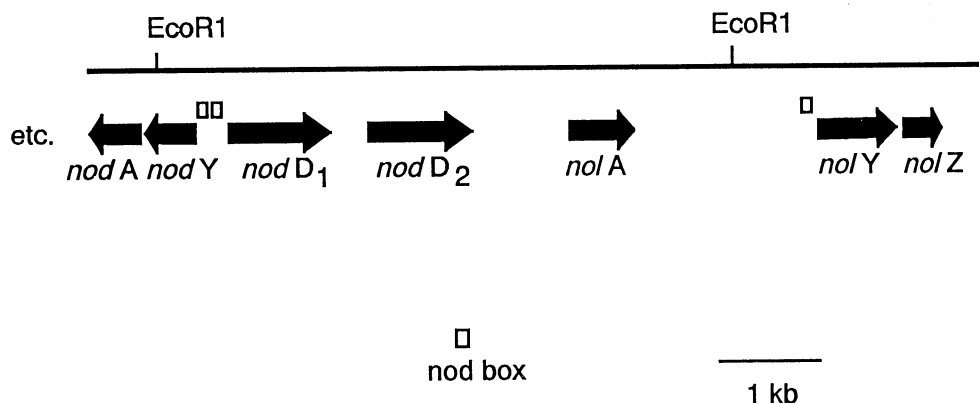

Fig. 1. Linkage map of the common *nod* genes and *nolYZ* of *Bradyrhizobium japonicum* USDA110.

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  1    CCCGGGCATTCCCTATGCTTCCTAGCCCGGATAGCAGGAGAACCAATCAACCGGCTCCGA

 61    TGAAGGCGGTGAGATTGCTCGCCTGCGCTATCAACGAACTGGATGCATCCCATCAACACT

121    TTCTATTTTACTAATTGAAGCCAAACACCGCATAAGTCGATCGCCGGCTTACGGAAGGCA

181    GTGTTCACGAATGTCGCGTGATAATGGATCACTTGGTCGCAGAGCAAATCACGTGCTTCT

241    TGGCTGCTTCGAGCAATGTTTGACGGCAAGACACGTGGGGGCACGAATGCAGTGCGGTGA

301    CCAGTCGCAGTTGGCGCGCAATCTACACGACGTTGCCTGCCCCTTTGGGCATGATACTTG

361    ATTGCTCTAAGAAGACAGCTATCACGCACACTCGGCGATGGTAAATGCCTCCGCAGCGTA
                                                    M  P  P  Q  R

421    GAACTCGGGCAGGTGAGTTGGCCTTCGCAGGGGTGCGGGCTGGACCTTTCGCTCTGACCA
        R  T  R  A  G  E  L  A  F  A  G  V  R  A  G  P  F  A  L  T

481    GGCGGGTCGCCTCAACAGTCCGGCGCTGTTGCCGCGGCTCCAAGCAGAGCAGTACAATCA
        R  R  V  A  S  T  V  R  R  C  C  R  G  S  K  Q  S  S  T  I

541    GCTGTGTGGCCCCGAACAGAGTCACCGCTGTTGCCGAACGCACTGGGCGGCTCGAGAATC
        S  C  V  A  P  N  R  V  T  A  V  A  E  R  T  G  R  L  E  N

601    CATTCAAATCCCGCGCCATCGGCAACGCTTTTTCGTTTTTCCCTAGACGCTCATTGCACTT
        P  F  K  S  R  A  I  G  N  A  F  R  F  S  L  D  A  H  C  T

661    TTGCAATCCTTGCAGCAGTCATTTGTCCAGCGAAGCGTCCGCCGTTTGGCGAACGGGAAA
        F  A  I  L  A  A  V  I  C  P  A  K  R  P  P  F  G  E  R  E

721    CGATACCTCTACTCCGCCTGTCTTGCATTTCACCACTTTGGAGAAATACCGTGGAACTTG
        T  I  P  L  L  R  L  S  C  I  S  P  L  W  R  N  T  V  E  L

781    ACCCTTCCAATTCAAGAATACAAAACCTCGAGGATCGCGTCGACCTGCAACTCGACATCG
        D  P  S  N  S  R  I  Q  N  L  E  D  R  V  D  L  Q  L  D  I

841    TGGCTGGGCTCATGCGCGAAGAAGGCAACGAGCTGGAAGCGATCCGATTACTTAATGCCC
        V  A  G  L  M  R  E  E  G  N  E  L  E  A  I  R  L  L  N  A

901    TCATAGGCCAAATGATCACCGCGGAACTGGAGCTCGGCCAGCTTGGCGATACAAGAACCG
        L  I  G  Q  M  I  T  A  E  L  E  L  G  Q  L  G  D  T  R  T

961    GCCACGGACGAATGCGATTGTCGCAAGACCGTGGCGTTTACCACCCCGAGGACCTTCAGA
        G  H  G  R  M  R  L  S  Q  D  R  G  V  Y  H  P  E  D  L  Q

1021   TTCTGGGACGCCTTTTTGATCAGACTGTGGCGGCATTACCGGCCGCGCTGCGGACTTCCG
        I  L  G  R  L  F  D  Q  T  V  A  A  L  P  A  A  L  R  T  S

1081   CCAATCGATTGACGATCGCCAAGCTCATTCTTTCACGTGCGGCAGCGGACTGAGATTTCA
        A  N  R  L  T  I  A  K  L  I  L  S  R  A  A  A  D  U

1141   TTTGCGCTTTTTGGAGATTGATGGCGTTGAGCGGACGTTTATGGCTGGGCTGACAGTTAC

1201   ACTTAGGCCGCACACACCAGGGATCGACATTTGCTGTTTATGGGCTCACCGTCCGTAAGC
                                                  M  G  S  P  S  V  S

1261   GCGCGGCTTCCGCTGGCCTTTCATATGGCAACAGTTCGCCACCACTTTCGAAATCAGGCT
        A  P  L  P  L  A  F  H  M  A  T  V  R  H  H  F  R  N  Q  A

1321   TGCTCTATCTCACGCCGCCGGCGACGCATTCGATCTAGCTGGGCCTTGCTGTCCCTCGAC
        C  S  I  S  R  R  R  R  R  I  R  S  S  W  A  L  L  S  L  D

1381   GCCTTTCACGAGCTGCAAGTATCCAAAGCTCGGAAGCGCGCCGCCGATCCCACGCTGCGC
        A  F  H  E  L  Q  V  S  K  A  R  K  R  A  A  D  P  T  L  R

1441   CGGCTTTCCTACGCAACTCCGTATTGAACCCACACCGACGGCGCCCGGCCCTTCTTTGCC
        R  L  S  Y  A  T  P  Y  U
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Fig. 2. Nucleotide sequence of the *nolYZ* genes. Translated open reading frames *nolY* and *nolZ* are delineated by the single-letter amino acid code. The underlined DNA sequence upstream of *nolY* has similarity to rhizobial *nod* boxes. The arrow shows where a *lacZ* fusion maps within *nolZ*; the arrowhead shows where a Tn*5* insertion maps within *nolZ*. Amino acids underlined in NolY denote the extent of a *nolY* deletion mutant (strain TCD520).

study by Bhagwat and Keister (1992) reported the isolation of clones from a *B. japonicum* USDA438 subtraction library that enhanced the competitiveness of *B. japonicum* USDA110. Moreover, these clones were found to encode genes selectively expressed in a medium that contained soybean seed extract, suggesting that such genes may be transcriptionally regulated by flavonoids. To see if a competitively altered phenotype could be noted for a *nolY* mutant, competition assays with USDA110 and the *nolY* deletion mutant TCD520 were performed; a nifD mutant of USDA110 was used as a control (strain B.j.A3) (Hahn *et al.* 1984) and soybean as a host. It was hypothesized that the mutant might be less competitive for nodule occupancy than the wild-type strain. The results indicated that TCD520 is at least equally as competitive with the wild-type USDA110. For example, with USDA110 and TCD520 inocula in a 1:1 ratio, TCD520 occupied about 80% of the nodules. With a 10:1 inoculum ratio, USDA110 occupied about 70% of the nodules. With a 1:10 inoculum ratio, strain TCD520 occupied about 95% of the nodules.

Recently, a protocol was developed to analyze the production of *nod* factors from rhizobia by the use of thin-layer chromatography (Spaink *et al.* 1992). Isoflavone-induced cul-

ture supernatants of the TCD520 mutant and of USDA110 were analyzed by this technique. No detectable alteration was found in the R_f of the factors from the mutant compared to those from the wild type. In addition, the amount of factor produced by the mutant appeared to be similar to that of the wild type, which would suggest that NolY is neither a regulator of common *nod* gene expression nor a transporter of the factor (data not shown).

DISCUSSION

Results presented here describe the sequence and characterization of two open reading frames from *B. japonicum* USDA110, designated *nolY* and *nolZ*. The transcription of these genes is dependent upon isoflavones and the $NodD_1$ and NodW proteins. This is only the third isoflavone-inducible, NodD-dependent operon reported from *B. japonicum* USDA110.

Mutants defective in *nolY* or *nolZ* exhibited no truly significant defects in nodulation of soybean, siratro, or cowpea plants. A modest reduction in the number of nodules and the percentage of plants nodulated was noted in mung bean plants inoculated with the *nolY* deletion mutant strain TCD520 (Fig. 5). The lack of a strongly altered nodulation phenotype is surprising, because of the substantial increase in *nolYZ* tran-

Table 1. Expression of a *nolZ'-'lacZ* fusion plasmid in wild-type *Bradyrhizobium japonicum* USDA110 and regulatory mutants

Strain	Units of activity[a]		
	Control	2 μM Genistein	SSE[b]
NAD2021 (wild type)	7 ± 1	110 ± 10	142 ± 20
TCD1050 (*nodD₁*)	4 ± 1	6 ± 2	12 ± 1
TCD3000 (*nodW*)	6 ± 2	7 ± 2	10 ± 1

[a] Units using CPRG (chlorophenol red–β-D-galactopyranoside) as a substrate.
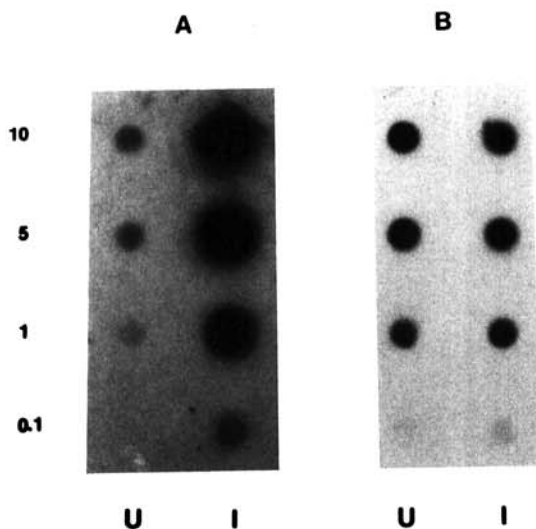[b] Soybean seed extract.

Fig. 3. Analysis of *nolY* transcription via RNA dot blots. Total RNA from uninduced cultures of *Bradyrhizobium japonicum* USDA110 and from cultures induced with soybean seed extract was spotted onto a filter and hybridized with a *nolY*-specific probe. **A,** USDA110 RNA from uninduced (U) and induced (I) cultures hybridized with *nolY*. **B,** The same filter hybridized with a 23S rRNA gene probe as a control for RNA quantity on the filter. The numbers on the left indicate the quantity of RNA spotted (in μg).
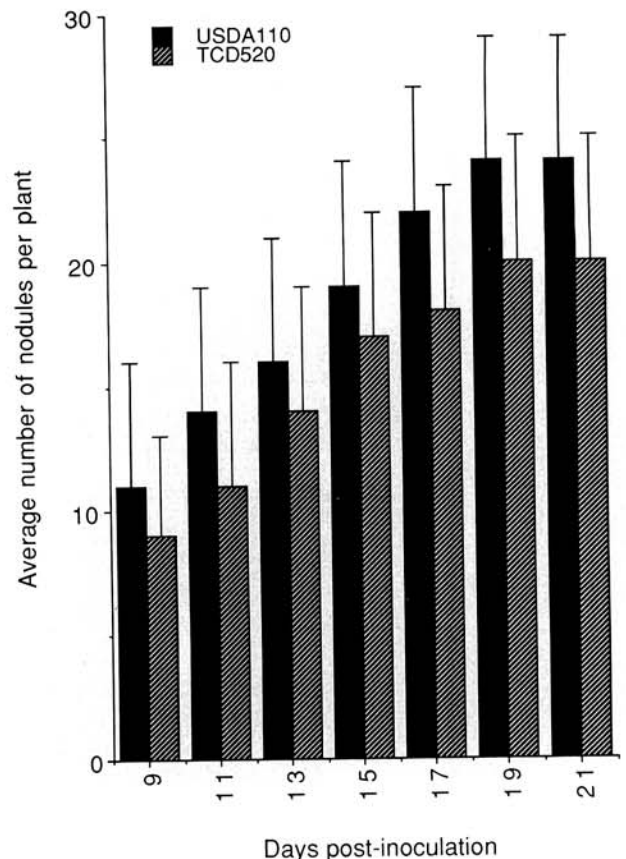

Days post-inoculation

Fig. 4. Nodulation kinetics of *Bradyrhizobium japonicum* USDA110 and TCD520 (a *nolY* mutant derivative) on soybean plants. The average number of nodules per plant and the standard deviation at various times are presented. Similar results were obtained when siratro and cowpea plants were inoculated with the mutants. A minimum of 10 plants were used for each assay.

scription in wild-type strains upon the addition of isoflavones. However, such a result is not without precedent. For example, although regulated by NodD, mutations in the *nodT* gene in *R. leguminosarum* bv. *viciae* and *R. l.* bv. *trifolii* ANU843 (Surin *et al.* 1990) and the *nodY* and *nodSU* genes of *B. japonicum* USDA110 (Göttfert *et al.* 1989, 1990b) do not produce a measurable nodulation phenotype. These results are unexplained, but it is possible that laboratory assays used to monitor nodulation efficiency do not have a high enough level of sensitivity to detect subtle nodulation defects. It is also possible that certain strains or cultivars of plants are relatively insensitive to certain types of symbiotic defects present in the infecting bacteria. Another possible explanation is that the *nolYZ* gene products are critical for the nodulation of certain plant species or cultivars not used in our assays. A variety of genes involved in cultivar-specific nodulation have been reported (Davis *et al.* 1988; Heron *et al.* 1989; Lewis-Henderson and Djordjevic 1991). The *nodT* gene from *R. l.* bv. *trifolii* ANU843 is not important in the nodulation of the subterranean clover cultivar Woogenellup, but its transfer to *R. l.* bv. *trifolii* strain TA1 allows TA1 to nodulate Woogenellup, a cultivar that TA1 otherwise nodulates poorly (Lewis-Henderson and Djordjevic 1991). *nodX*, from *R. l.* bv. *viciae* strain TOM, is required for efficient nodulation of the primitive pea cultivar Afghanistan (Davis *et al.* 1988), but not other cultivars. A similar function for the *nolYZ* products is quite possible.

Functional reiteration of *nolYZ* could also explain the lack of a strong mutant phenotype. This has been shown to be the basis for the relatively weak nodulation phenotypes seen in *nodPQ* mutants of *R. meliloti* (Schwedock and Long 1989, 1990, 1992) and *nodM* mutants of *R. l.* bv. *viciae* (Marie *et al.* 1992). However, Southern hybridization of chromosomal DNA with a *nolY*-specific probe failed to detect any strongly hybridizing bands, even at low stringency (data not shown). However, it should be noted that the two copies of glutamine synthetase in *B. japonicum* USDA110 have no significant similarity with each other, and yet both copies must be inactivated to see glutamine auxotrophy and a Fix⁻ phenotype (Scott-Craig and Chelm 1992). Thus the possibility of functional reiteration of *nolY*, and perhaps *nolZ*, remains as an explanation for the lack of a significant nodulation phenotype associated with these mutations.

In addition to NodD₁, NodW was found to be essential for isoflavone induction of a *nolZ'-'lacZ* fusion. NodW was previously shown to be necessary for isoflavone induction of the *nodYABC* and *nodD₁* operons in *B. japonicum* USDA110 (Sanjuan *et al.*, in press). These results suggest that NodW may be a general component involved in the activation of isoflavone-induced *nod* genes in *B. japonicum* USDA110.

A sequence that has similarity to previously characterized rhizobial *nod* boxes was identified 5′ to *nolY*. This sequence also contained the various proposed motifs necessary for *nod* box function. The dependence of *nolZ* expression on NodD₁
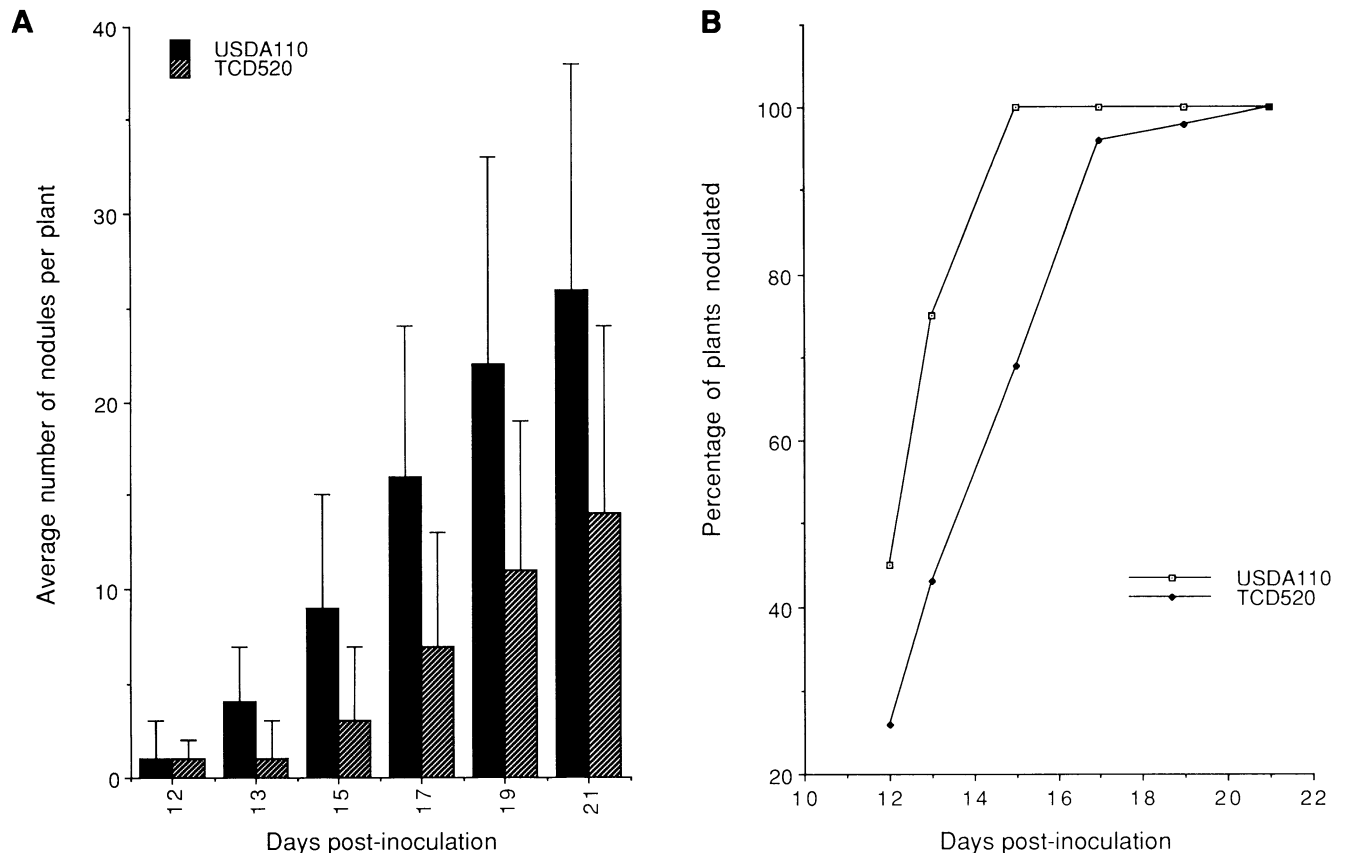

Fig. 5. Nodulation kinetics of *Bradyrhizobium japonicum* USDA110 and TCD520 (a *nolY* mutant derivative) on mung bean plants. **A,** Average number of nodules per plant and standard deviation at various times. **B,** Percentage of plants nodulated. The data are results of tests of 40 plants, spread over three assays.

and isoflavones suggest that this putative *nod* box is functional. In a study by Göttfert *et al.* (1989), a synthetic oligonucleotide that had sequence similarity to rhizobial *nod* boxes was used to probe for cross-hybridizing clones in a *B. japonicum* USDA110 chromosomal library. Interestingly, none of the clones obtained in that study correspond to the *nolYZ* region. Although the *nolYZ* operon does not appear to be critical for nodulation, it is induced to a significant extent by the addition of isoflavones and appears to have a functional *nod* box. These observations raise the obvious possibility that there may be other undiscovered loci present in *B. japonicum* USDA110 whose transcription is isoflavone-and NodD-dependent and that may be involved in nodulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

All strains and plasmids used in this study are listed in Table 2. *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 and ex-

tracting nucleic acids. Minimal medium (Bergersen 1961) was used to assay for *nod* factor production. *B. japonicum* strains were grown at 30° C. *Escherichia coli* strains were cultured on Luria-Bertani (LB) or M9 medium (Sambrook *et al.* 1989) at 37° C. Antibiotics for selective markers were used in the following concentrations: with *E. coli*, ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 20 μg/ml; and with *B. japonicum*, kanamycin, streptomycin, and tetracycline at 150 μg/ml each.

A *nolY* deletion mutant was constructed as follows. A 3.2-kb *Eco*RI-*Bam*HI fragment encompassing *nolYZ* was cloned into pUC18. This plasmid was digested with *Xho*I and *Cla*I, releasing a 490-bp fragment from *nolY*. The resulting 5.4-kb fragment was recovered, and the 5' overhanging ends were blunted with the Klenow fragment of DNA polymerase I. A 1.6-kb kanamycin resistance cassette, pUC4-KIXX (Pharmacia, Uppsala, Sweden) was digested with *Xho*I, and its 5' overhangs were blunted with Klenow. The 5.4-kb fragment and the KIXX cassette were ligated, and ampicillin-resistant, kanamycin-resistant transformants were selected. A clone in which transcription of the kanamycin resistance gene read towards *nolZ* was selected. This clone was then digested with *Eco*RI and *Bam*HI to release the pUC18 vector. The 4.3-kb fragment with *nolYZ* and the KIXX cassette was then ligated to pSUP203 DNA that had been cut with *Eco*RI and *Bcl*I. pSUP203 is a high frequency of mobilization suicide vector (Simon *et al.* 1983). *E. coli* transformants that were ampicillin-, tetracycline-, and kanamycin-resistant were selected and checked for integrity by restriction analysis. The resulting clone (pTD623) was then mobilized to *B. japonicum* USDA110, and kanamycin-resistant, tetracycline-susceptible strains, indicating double-crossover events, were selected. The integrity of the strains was confirmed by Southern hybridization.

Genetic techniques.

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

Nucleic acid purifications and manipulations.

Restriction endonucleases, DNA polymerases, and DNA ligase were used following standard protocols (Sambrook *et al.* 1989). DNA-DNA hybridizations were performed following protocols described by Amersham Corporation (Arlington Heights, IL). Low-stringency washes used for detecting possible reiterated sequences were performed at 45° C but were otherwise similar to high-stringency washes. RNA from uninduced cultures and cultures induced with soybean seed extract was prepared as follows. Cells were grown to log phase in RDY medium and then diluted to an OD_{600} of 0.05–0.1 in 150 ml of RDY medium. In one culture, 3 ml of soybean seed extract (Banfalvi *et al.* 1988) was added for induction. The cultures were grown for 8 hr. Cells were pelleted and resuspended in 5 ml of 20 mM Na^+ acetate, 1 mM EDTA, and 0.5% sodium dodecyl sulfate (pH 5.5). An equal volume of hot phenol (65° C) equilibrated with 20 mM Na^+ acetate (pH 5.5) was added and mixed. The aqueous phase was mixed with phenol/chloroform, and upon recovery of the aqueous

Table 2. Bacterial strains, plasmids, and phages

Strain	Relevant characteristics	Source
Bradyrhizobium japonicum		
USDA110	Wild type	USDA, Beltsville, MD
AN314	Tn*5* in *nodD*₁, Km^r, Sm^r	Banfalvi *et al.* 1988
B.j.613	*nodW*, Sm^r, Spc^r	Göttfert *et al.* 1990a
TCD520	*nolY* deletion, Km^r	This study
TCD530	Tn*5* in *nolZ*, Km^r, Sm^r	This study
NAD2021	pND228 in USDA110, Tc^r	Deshmane and Stacey 1989
TCD1050	pND228 in AN314, Tc^r, Km^r, Sm^r	This study
TCD3000	pND228 in B.j.613, Tc^r, Sm^r, Spc^r	This study
B.j.A3	Tn*5* in *nifD*, Km^r, Sm^r	Hahn *et al.* 1984
Escherichia coli		
JM101	*supE, thi-1*, Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q} Z*Δm15*]	Messing 1983
Plasmids and phages		
M13mp18	M13 sequencing vector	Norrander *et al.* 1983
M13mp19	M13 sequencing vector	Norrander *et al.* 1983
pUC18	Ap^r	Yanisch-Perron *et al.* 1985
pSUP203	RP4 *mob*, Tc^r, Ap^r, Cm^r	Simon *et al.* 1983
pUC4-KIXX	Km^r cassette	Pharmacia, Uppsala, Sweden
pRK2013	RP4 tra$^+$, Km^r	Figurski and Helinski 1979
pND228	*nolZ-lacZ* fusion, Tc^r	Deshmane and Stacey 1989
pTD620	3.2-kb *Eco*RI-*Bam*HI clone of *nolYZ* in pUC18	This study
pTD623	*Xho*I-*Cla*I deletion from *nolY*, replaced with KIXX cassette, cloned into pSUP203, Km^r, Tc^r	This study

phase, 0.1 volume of 3 M Na⁺ acetate was added, followed by two volumes of ethanol. RNA dot blots and RNA-DNA hybridizations were carried out following a protocol from Sambrook et al. (1989). The 23S rRNA gene probe was from Festl et al. (1986). Quantitative scanning of filters was carried out by the use of a radioanalytic imaging scanner (AMBIS Corporation, San Diego, CA).

DNA sequencing and sequence analysis.

DNA sequencing was performed by cloning various restriction fragments into M13mp18 or M13mp19. Protocols for phage-handling techniques and the generation of single-stranded phage DNA template were as described in Sambrook et al. (1989). Sequencing reactions were done according to the chain termination method of Sanger et al. (1977), using either Sequenase or Taquence polymerases (United States Biochemical, Cleveland, OH). DNA and protein sequence analyses were done with the use of programs from the Genetics Computer Group, University of Wisconsin (Madison). Mapping of Tn5 insertions from plasmid clones of USDA110 DNA was done through the use of a primer homologous to the end of IS50L with the sequence 5'CAGGACGCTACTT-GT3', and by double-strand sequencing.

Plant tests.

Seeds of Glycine max cv. Essex, Macroptilium atropurpureum (siratro), Vigna unguiculata (cowpea) cv. Caloona, and V. radiata (mung bean) cv. King 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). Each seedling was inoculated with approximately 10⁶ bacterial cells. Assays for competition for nodule occupancy were performed by growing appropriate cultures in RDY broth and then diluting them to an OD_{600} of 0.001 (i.e., about 10⁶ cells per milliliter). Cultures were mixed in 1:1, 1:10, and 10:1 ratios. The ratios were subsequently confirmed by viable counts of the respective parent cultures. Each seedling was inoculated with a total of 2 × 10⁶ bacterial cells. At 18 days post-inoculation, 20–40 nodules from each treatment were picked, surface-sterilized, crushed, and plated on RDY medium. The resulting colonies were then transferred to RDY plus kanamycin to score for antibiotic resistance. Double occupancies, in which both bacterial genotypes were present in the nodule, occurred at a low frequency (<15%); these were discarded from analysis.

β-Galactosidase assays.

The β-galactosidase activity of strains harboring lacZ fusions was assayed following protocols described by Banfalvi et al. (1988). CPRG (chlorophenol red–β-D-galactopyranoside, Boehringer Mannheim) was used as a substrate. The numbers presented in Table 2 are averages and standard deviations from three or more independent assays.

Assay for nod factor metabolites.

A general protocol developed by Spaink et al. (1992) was used. ¹⁴C-labeled acetate used for the assay was obtained from New England Nuclear.

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