The *Bradyrhizobium japonicum noeD* Gene: A Negatively Acting, Genotype-Specific Nodulation Gene for Soybean

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Bradyrhizobium japonicum strain USDA 110 is restricted for nodulation by soybean genotype PI 417566. We previously reported the identification of a USDA 110 Tn5 mutant, strain D4.2-5, that had the ability to overcome nodulation restriction conditioned by PI 417566 (S. M. Lohrke, J. H. Orf, E. Martínez-Romero, and M. J. Sadowsky, Appl. Environ. Microbiol. 61:2378-2383, 1995). In this study, we report the cloning and characterization of the negatively acting DNA region mutated in strain D4.2-5 that is involved in the genotype-specific nodulation of soybean. The Tn5 integration site was localized to a 5.2-kb *EcoRI* fragment isolated from wild-type USDA 110 genomic DNA. Saturation Tn5 mutagenesis of this 5.2-kb region and DNA homogenization studies indicated that a 0.9-kb DNA region was involved in the genotype-specific nodulation of PI 417566. A single open reading frame (ORF) of 474 nucleotides, encoding a predicted protein of 158 amino acids, was identified within this region by DNA sequencing. This ORF was named *noeD*. Computer comparisons with available data bases revealed no significant similarities between the *noeD* DNA or predicted amino acid sequence and any known genes or their products. However, comparisons done with the region upstream of *noeD* revealed a high degree of similarity (about 76% similarity and 62% identity) to the N-terminal regions of the *Rhizobium leguminosarum* bv. *viciae* and *R. meliloti nodM* genes, which have been postulated to encode a glu-

cosamine synthase. Southern hybridization analysis indicated that *noeD* is not closely linked to the main or auxiliary nodulation gene clusters in *B. japonicum* and that both nodulation-restricted and -unrestricted *B. japonicum* serogroup 110 strains contain a *noeD* homolog. High-performance liquid chromatography and fast atom bombardment-mass spectrometry analyses of the lipo-chitin oligosaccharide (LCO) nodulation signals produced by an *noeD* mutant showed a higher level of acetylation than that found with wild-type USDA 110. These results suggest that specific LCO signal molecules may be one of the factors influencing nodulation specificity in this symbiotic system.

Additional keywords: Nod factors.

Bradyrhizobium japonicum strains form nitrogen-fixing nodules on the roots of soybean (*Glycine max* L. Merr.). Genes in a number of rhizobial genera (i.e., *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium*) have been shown to be involved in the genotype-specific nodulation (GSN) of legumes (Davis et al. 1988; Djordjevic et al. 1985; Krishnan and Pueppke 1991; Lewis-Henderson et al. 1991a, 1991b; Lohrke et al. 1995, 1996; Meinhardt et al. 1993; Sadowsky et al. 1991). These genes can act in either a positive or negative manner to control nodulation specificity (Triplett and Sadowsky 1992). *B. japonicum* serogroup 123 strains are restricted for nodulation by soybean genotype PI 377578 (Cregan and Keyser 1986; Cregan et al. 1989a, 1989b; Sadowsky et al. 1987). The *B. japonicum nola* gene, identified in strain USDA 110, is a positively acting determinant that allows serogroup 123 strains to nodulate PI 377578 (Sadowsky et al. 1991). *NolA* has been postulated to encode a transcriptional regulatory protein involved in *nod* gene expression (Garcia et al. 1996). Another positively acting GSN gene, *nodX*, has been found in *Rhizobium leguminosarum* bv. *viciae* strain TOM (Davis et al. 1988). Nodulation of *Pisum sativum* cv. Afghanistan by *R. leguminosarum* bv. *viciae* strains requires the presence of *nodX*. The *nodX* gene product mediates the O-acetylation of the lipo-chitin oligosaccharide (LCO)

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signals produced by *R. leguminosarum* (Firmin et al. 1993). Negatively acting GSN genes have also been described (Krishnan and Pueppke 1991; Lewis-Henderson et al. 1991b; Meinhardt et al. 1993). *Sinorhizobium fredii* strain USDA 257 nodulates primitive lines of soybean, but is unable to nodulate improved varieties, such as cv. McCall (Heron et al. 1989). The *S. fredii nolC* (Krishnan and Pueppke 1991) and *nolXWBTUV* (Meinhardt et al. 1993; Bellato et al. 1997) genes have been shown to prevent nodulation of cv. McCall. Both positively and negatively acting genetic determinants have been shown to control the interaction between *Trifolium subterraneum* cv. Woogenellup and *R. leguminosarum* bv. *trifolii* strain TA1 (Lewis-Henderson et al. 1991a, 1991b).

Transcription of the nodulation genes (*nod*, *nol*, and *noe*) in *Bradyrhizobium* strains requires the NodD protein and plant-produced flavonoid (isoflavones) signal molecules (Dénarie et al. 1992; Göttfert 1993; Kossak et al. 1987; Pueppke 1996; Schlaman et al. 1992). Several of the *nod* gene products are essential for the synthesis of substituted LCO nodulation signals (Carlson et al. 1993, 1994). The *nodABC* genes are involved in synthesizing the basic lipo-chitin backbone, while other host-specific nodulation genes encode enzymes that modify this basic backbone structure (Dénarie et al. 1992; Göttfert 1993; Spaink et al. 1991; Stacey et al. 1994). The LCO nodulation signals are essential for initiating many of the early nodulation events (Carlson et al. 1994; Dénarie et al. 1992; Relić et al. 1994). For example, LCO signals are capable of eliciting root hair deformation (Lerouge et al. 1990; Price et al. 1992; Sanjuan et al. 1992; Schultze et al. 1992; Spaink et al. 1991; Stokkermans et al. 1995) and curling (Relić et al. 1993) at pico- to nanomolar concentrations.

The LCO nodulation signals from three *Bradyrhizobium* strains have been characterized (Carlson et al. 1993, 1994; Sanjuan et al. 1992). These molecules when applied to *Glycine soja* roots will induce the development of nodule structures similar to those produced by bacterial inoculation (Minami et al. 1996; Stokkermans et al. 1996). The basic structure of the *B. japonicum* nodulation signal is five β -1,4-linked *N*-acetylglucosamine residues adorned with a 2-*O*-methylfucose residue and a fatty acid side chain. The ability of *B. japonicum* to nodulate siratro, but not soybean, depends on the presence of the 2-*O*-methylfucose moiety on the terminal, reducing *N*-acetylglucosamine residue (Sanjuan et al. 1992; Stacey et al. 1994). The *nodZ* gene encodes a fucosyl-transferase that is essential for this modification (Lopez-Lara et al. 1996; Stacey et al. 1994). The fatty acid chains present on the LCO signals produced by *B. japonicum* and *B. elkanii* are either C16:0, C16:1, or C18:1. Acetyl groups are present at the terminal, reducing end of some *Bradyrhizobium* nodulation signals, and some *B. elkanii* strains have a carbamoyl substituent on the terminal, nonreducing *N*-acetylglucosamine residue (Carlson et al. 1993; Stacey et al. 1994; Stokkermans and Peters 1994). The acetyl or carbamoyl residues have not been shown to be involved in host specificity.

Previous studies have shown that soybean genotypes are differentially nodulated by strains of *B. japonicum* (Cregan et al. 1989a, 1989b; Sadowsky et al. 1987; Vest et al. 1973). Soybean genotype PI 417566 restricts nodulation by strain USDA 430, as well as strains in serogroups 129 and 110 (Cregan et al. 1989b; Lohrke et al. 1995, 1996; Sadowsky et al. 1995). Microscopic analyses indicated that the nodulation-

restricting soybean genotypes prevent nodulation at a symbiotic stage after initial root hair curling events (Sadowsky et al. 1995). Subsequent studies have shown that restriction of nodulation by serogroup 110 isolates is strain- and temperature-dependent (Lohrke et al. 1995; Sadowsky et al. 1995), is determined by the root genotype (Sadowsky et al. 1995), and is conditioned by a single, recessive host gene (Lohrke et al. 1996). Taken together, these results indicate that root and environmental factors influence host-controlled nodulation restriction and that such restriction occurs at a symbiotic stage subsequent to the initial recognition of the microsymbiont and its host.

We previously reported the isolation of a Tn5-induced mutant of *B. japonicum* strain USDA 110 that had the ability to overcome nodulation restriction conditioned by soybean genotype PI 417566 (Lohrke et al. 1995). This mutant, strain D4.2-5, was unchanged in its nodulation host range, relative to the wild-type strain, with the exception of the ability to nodulate genotype PI 417566. In this study, we present a detailed characterization of the negatively acting *B. japonicum noeD* gene, identified by the mutation in strain D4.2-5. Mutations in *noeD* lead to the formation of LCO nodulation signals with an increased level of O-acetylation. These effects may play a prominent role in the ability of *noeD* to mediate GSN of PI 417566.

RESULTS

Isolation of Tn5 insertion site in mutant D4.2-5.

To isolate the gene region in mutant D4.2-5 that contained the Tn5 insertion site, genomic DNA from mutant strain D4.2-5 was digested with *EcoRI* and size-fractionated, and fractions were analyzed by Southern hybridization with Tn5 as a probe. The hybridizing fraction was cloned into pACYC184; a single, kanamycin-resistant colony was isolated and was subsequently designated pSL5. Southern hybridization analysis with a ³²P-labeled Tn5 probe indicated that pSL5 contained a single copy of Tn5 and wild-type genomic DNA that flanked the transposon insertion site. However, restriction enzyme mapping and Southern hybridization analysis with a Tn7 gene probe indicated that pSL5 contained all of Tn5, 9 kb of Tn7, plus approximately 1.6 kb of wild-type flanking DNA. The source of Tn7 was most likely pBLK1-2 (Kim et al. 1988), which was previously used to generate mutant D4.2-5 (Lohrke et al. 1995).

Identification of a wild-type region corresponding to the site of Tn5 insertion in D4.2-5.

To isolate the wild-type DNA region that corresponded to the Tn5 insertion site in mutant D4.2-5, a ³²P-labeled pSL5 probe was hybridized to a wild-type gene library from *B. japonicum* strain USDA 110. A single, hybridizing cosmid was identified and was designated pSL23. A physical map of pSL23 was determined by restriction enzyme analysis (Fig. 1). Subsequent hybridization studies indicated that pSL23 contained a 5.2-kb *EcoRI* fragment that hybridized to pSL5. This fragment was subcloned into pUC18 and designated pSL5.2 (Fig. 1). The location of the Tn5 insertion in mutant D4.2-5 was determined by Southern hybridization, restriction enzyme, and sequence analyses. Results of these studies indicated that the initial Tn5 insertion site in mutant

D4.2-5 corresponded to the 0.9-kb *Hind*III-*Pst*I fragment of pSL5.2 (Fig. 1).

To determine whether the isolated cosmid contained any of the previously identified nodulation genes, ³²P-labeled pSL23 was hybridized to *Hind*III-digested cosmids pRJUT10, pRJUT14, and pRJUT24, which overlap the nodulation gene cluster in *B. japonicum* strain USDA 110. The cosmid pRJUT10 contains the *B. japonicum* *nolA*, *nodD2*, *nodD1*, *nodYABCSUIJnolMNO*, and *nodZ* genes (Dockendorff et al. 1994; Nieuwkoop et al. 1987; Russell et al. 1985), while cosmids pRJUT14 and pRJUT24 correspond to approximately 65 kb of DNA flanking one side of pRJUT10. No detectable hybridization was observed to any of the tested DNAs, indicating that pSL23 does not constitute any part of one of the previously identified main nodulation gene clusters (data not shown).

Mutant reconstruction in wild-type USDA 110.

In order to verify that the Tn5 insertion in mutant D4.2-5 was responsible for the extended host range phenotype, the mutation was again constructed in wild type USDA 110. To do this, pSL5.2 was subjected to random λ::Tn5 mutagenesis, independent mutations in pSL5.2 were subcloned into pSUP202, marker-exchanged into wild-type USDA 110, and the new mutants were tested for their ability to nodulate PI 417566. Nine independent Tn5 insertions that mapped to the insert DNA in pSL5.2 (Fig. 1) were marker exchanged into wild-type USDA 110. Genomic DNA from several kanamycin-resistant and tetracycline-sensitive USDA 110 colonies were shown to contain a single, 11-kb, *Eco*RI-hybridizing fragment, corresponding to the wild-type fragment (5.2 kb) and Tn5 (5.8 kb). Results in Table 1 show that only one inser-

tion mutant, 110-36, had gained the ability to overcome nodulation restriction conditioned by PI 417566. This insertion was located in the 0.9-kb *Hind*III-*Pst*I fragment, approximately 100 bp upstream of the *Pst*I site. The site of integration was similar to the location of Tn5 in the original insertion in D4.2-5 (data not shown). Insertion mutants 110-13, 110-16, and 110-1, which had Tn5 inserted in DNA flanking the mutation site in 110-36, and were located in the 0.9-kb fragment, did not nodulate PI 417566 (Table 1). Moreover, none of the other tested mutants acquired the ability to nodulate PI 417566. Since the distance between mutations 110-1 and 110-35, which flank mutation 110-36, is 0.5 kb, our results indicate that a relatively small DNA region is involved in the GSN of PI 417566.

Table 1. Nodulation response of selected USDA 110 marker exchange mutants on *Glycine max* PI 417566

Strain	Mean nodule number ^a
USDA 123	28.3 ± 2.0
D4.2-5	37.8 ± 7.7
110-36	34.5 ± 6.2
110-1	2.0 ± 0.5
110-5	0.3 ± 0.2
110-9	1.8 ± 0.9
110-10	1.2 ± 0.5
110-13	1.7 ± 0.7
110-16	2.2 ± 0.5
110-20	1.3 ± 0.4
110-35	1.3 ± 0.5
USDA 110	1.0 ± 0.4
Uninoculated	0.0 ± 0.0

^a Values are mean nodule number per plant ± standard error. Six plants per strain were analyzed.

Fig. 1. Physical and genetic map of pSL5.2. Plasmid pSL5.2 consists of the *Eco*RI fragment from pSL23 cloned into pUC18 DNA. Location and transcriptional direction of *noeD* gene indicated by horizontal arrow. Region sequenced indicated by solid line. Relative locations of Tn5 insertions in pSL5.2 indicated by vertical arrows. Filled arrow denotes location of the Tn5 insertion in mutant 110-36 that has the ability to nodulate PI 417566. Hollow arrows mark location of Tn5 insertions in mutants that remained restricted for nodulation by PI 417566. Numbers below arrows refer to Tn5 mutant designations in *Bradyrhizobium japonicum*.

DNA sequence analysis.

The nucleotide sequence of the approximately 3.7-kb *EcoRI-XbaI* DNA fragment, cloned in pSL5.2, was determined on both strands. Nucleotide sequence was determined initially by subcloning fragments from pSL5.2 into pUC18, and subsequently with primers designed based on sequence information obtained from subcloned DNA fragments. The nucleotide sequence is shown in Figure 2. DNA sequence analysis revealed several possible open reading frames (ORFs) beginning with ATG. One ORF, beginning at base number 1136, was designated as the *noeD* gene. Gene assignment was based, in part, on the length of the potential ORFs, and codon preference and rare codon analysis. The *noeD* gene consists of 474 nucleotides that encode a polypeptide of 158 amino acids with a deduced M_r of 17,140 and a pI of 9.5. The protein sequence derived from a translation of the ORF is shown in Figure 2. Sequence analysis indicated that the *noeD* sequence contained few rare codons. Other ORFs that were identified were of insufficient length or contained a large number of rare codons and were postulated as to not encode genes. Restriction enzyme mapping of the Tn5 insertion site in mutants 110-36 and D4.2-5 indicated that the insertions were located within the *noeD* ORF, suggesting its possible involvement in the GSN of soybean. The location of the Tn5 insertion in mutant D4.2-5 was verified by DNA sequencing and was found to be located in the 3' end of the gene (Fig. 2).

Nucleotide and protein searches of the GenBank, EMBL, Swiss-Prot, and PIR data bases indicated that *noeD* or NoeD had no significant similarity, at the nucleotide or amino acid level, to any previously described nodulation or other genes. In addition, the upstream region of *noeD* did not contain a canonical *nod* box, or other potential promoter sites. However, a consensus binding site for 30S ribosomal RNA was found to be located 22 nucleotides upstream from the proposed *noeD* translation initiation codon. Taken together, these results indicate that *noeD* represents a previously undescribed, negatively acting, gene that is involved in the GSN of soybean PI 417566.

A region upstream of *noeD*, beginning at the *EcoRI* site and ending at base position 841, contained significant nucleotide and amino acid homology to *nodM*, a nodulation gene found in several fast-growing rhizobia. This region had 75.2% sequence similarity and 61.6% identity, at the amino acid level, to NodM from *R. meliloti* (GenBank accession no. X58632), and 76.0% similarity and 63.5% identity to NodM from *R. leguminosarum* bv. *viciae* (GenBank accession no. Y00548) (Fig. 3). It seems likely, therefore, that this region constitutes the *B. japonicum nodM* gene, a nodulation gene that has not been previously identified in this organism. In addition, the same gene region contained significant nucleotide homology, 68.0% over 147 nucleotides, to the left end and attachment site for transposon Tn7. The high degree of homology suggests that this region could act as a preferential site for the integration of Tn7, and most likely explains the presence of Tn7, introduced from plasmid pBLK1-2, in the original mutated locus in D4.2-5.

Presence of *noeD* in other serogroup 110 strains.

EcoRI-digested genomic DNAs from *B. japonicum* serogroup 110 strains that are restricted or not restricted for nodulation by PI 147566 (Lohrke et al. 1995) were examined for

hybridization homology to a 750-bp *noeD* gene internal fragment. Results of this analysis indicated that the probe hybridized to a 5.2-kb *EcoRI* fragment in all strains, with the exception of the nodulation-restricted strains USDA 454 and USDA 444 and the nodulation-unrestricted strains WI3728 and USDA 451, which failed to hybridize to the probe (data not shown).

B. japonicum USDA 110 and mutant D4.2-5 produce different nodulation factors.

One- and two-dimensional thin-layer chromatography (TLC) analyses of ^{14}C -acetate-labeled nodulation (Nod) factors from genistein-induced and -uninduced cultures of wild-type USDA 110 and mutant strain D4.2-5 indicated that both strains had similar TLC profiles (data not shown). However, when the Nod factors from both strains were isolated and purified by high-performance liquid chromatography (HPLC), there were significant differences in HPLC elution profiles (Fig. 4A and B). Identical results were obtained with the reconstructed *noeD* mutant 110-36. The profiles from strain USDA 110 and the *noeD* mutant differed in respect to the retention times and quantity of eluting material. While the elution profile of partially purified Nod factors from strain USDA 110 had five major peaks with peak 2 in the largest amount, the *noeD* mutants had an elution profile with five major peaks, with peaks 2, 3, and 5 in the largest amounts. The elution profiles also differed with respect to the relative amounts of eluting material. While there was more of the peak 2 than the peak 3 compound in the USDA 110 elution profiles, the profile from the *noeD* mutants contained more of the peak 3 than peak 2 material. In addition, the *noeD* mutant had a greater amount of peak 5 compound than did USDA 110.

B. japonicum strain USDA 110 produces one major LCO signal molecule, NodBj-V(C18:1,MeFuc) (Carlson et al. 1993, 1994), which is reflected in the HPLC elution profile in Figure 4A as peak 2. Peaks 1, 2, 3, and 5 were purified from USDA 110 and the *noeD* mutant and subjected to gas chromatography-mass spectrometry (GC-MS), fast atom bombardment (FAB), and MS-MS analysis. The remaining peaks were not present in sufficient amounts to obtain MS-MS data. Table 2 gives the results of FAB-MS analysis of the purified HPLC fractions. Peaks 1, 2, and 3 from strain USDA 110 contain NodBj-V(C16:0,Mefuc), NodBj-V(C18:1,Mefuc), and NodBj-V(Ac,C18:1,Mefuc), respectively. FAB-MS for Nod factors obtained from the *noeD* mutant indicated that fractions 1, 2, and 3 contain the same LCOs found in those respective fractions from USDA 110.

FAB-MS analysis of peak 5 gave ions of $m/z = 1,458$ and $1,480$, with the latter ion being of greatest intensity. These are the likely $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{Na}]^+$ ions, respectively. Tandem MS-MS analysis of the $m/z = 1,458$ ion gave a clean fragmentation pattern of m/z 468, 671, 874, and 1,077, while MS-MS of the m/z 1,480 ion gave a very complex fragmentation pattern due to intra-ring cleavage of the glycosyl residues, a result that is commonly observed for MS-MS of $[\text{M}+\text{Na}]^+$ ions. This result showed that the LCO in peak 5 had the same molecular weight as that of peak 3, NodBj-V(Ac,C18:1,MeFuc). Furthermore, MS-MS of the peak 5 was identical to that for NodBj-V(Ac,C18:1,MeFuc). However, the fact that peak 5 eluted from the HPLC column several minutes later than peak 3 suggested that it was more hydrophobic.

nodM →

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1   GGAATTCCGT AACAGGGAGG CCCTTGC GCC GAGGCAATTT GCAATAGTCA TTCTGCAATC
61  GGGCGAAACC GCTGACACAT TAGCTGCCTT GCGATACGCC AAGGGCAAGG GCTTGCATAC
121 GATATCTGTG GTTAATGTAC CGACGTCGAC GATTGCGCGC GAGAGCGAAT CCGTTTTGCC
181 TACCCTGGCA GGGCCAGAAA TCGGCGTCGC TTCCACCAAG GCATTTATCT GCCAGCTGAT
241 GGTATTGGGG GTGCTTGCCG TCAGAGCAGC TAAGGAGCGG GGTAAGCTGT CTGAAATCGA
301 TGAATCGCAG CTCGTGCGCG AGCTCATTGA GGTGCCGCGA TTGATTGCTG CGGCCCTGTT
361 GGTGAGAGCC CAGATTGAGA AGCTGCGCGC TTATATCGCC GGCGCGAGGA CAGTACTCTA
421 TCTCGGTCGT GGCACATCGG CCCCTTGGC CTTGGAGGGC GCGCTCAAGT TGAAGGAAAT
481 TTCTATATC CACTCAGAGG GCTACGCCGC CGGCGAGCTC AAGCACGGCC CGATCGCACT
541 GATCGATGAG GCTGTGCCTG TGGTGGTGAT CGCGCCTTAC GACGAGGTCT TCGAGAAGAC
601 CGTCTCTAAC ATGCAAGAGG TGGCCGCTCG GGGCGGCAAG ATCATCCTGA TCACCGATGC
661 GAAGGGTTCG TCTGAAGCGA TGCTAGACAC GCTCCTGACC ATCGTGCTGC CGGCAATGGT
721 CGCAAGCTTT ACGCCGCTGG TCTATGCCAT TCCGGTCCAG CTCTTGGCTT ACCATACCGC
781 GGTGCGAAGG GGGGCGGACG TGGGATCAGC CGCGTAATCT TGCAAAATCG GTCACGGTGG
841 AATGAGGCTG GAGAAGTTAG TATCAAAGCA TTGAGGTCTC GGCCTCGAGC CATTCGGCGC
      *
901 GGGTTTGGTG GCCTAGGTTT TCGGGGACG GAATCCGACA AGTTCCCAAT CCGGGTCGCT
961 CGTAGCTGTC GTGCGGTTTT GGTGAACCTA GCTGATGCTG CCATCAGCCT CGTATAGCAG
1021 AATCGACTAG CCTAGGGGCG ATCCGGGATG TTGCACGACT GGGACGGCGT TCATCAGGCC
1081 CGTCCACAGC CCTGTTGCCC TGGTCGCCAT CGACCGGAAC AGGTGGTCGT CATCGATCAG

      *
1141 AAATGGTGGT CGCCATCAAT CGGATTCACT GGTCGGCGTG CCGCGGAGCG CGCAGCCTGA
      M V V A I N R I H W S A C P G A R S L
1201 CTTGACGGC TCTGTACTTC GAGCGCTGTT TATTGAAACG AATACCGAAA GTCACGCGCG
      T S T A L Y F E R C L L K R I P K V T R
1261 TGCCGGACAC CAGATCAGCC CCTGCGCAGC CGCGTAAGGT CCGATATGTG CACGAACAAA
      V P D T R S A P A Q P R K V R Y V H E Q
1321 TCCTTGCCGT CGCCATAGTC AGGCCGAATG AACTCCCAGC CCTTTTCAAC GCTGTACGAC
      I L A V A I V R P N E L P A L F N A V R
1381 ACTACTGTTC CGGTGCGCAT GTTTGTTCCA CGGTCTTGCG GCTCGATATC GCCGCTACT
      H Y C S G A H V C S T V L R L D I A A Y
1441 ACTCCGGAGC TGCGGGTGCA CTTTCCGTTC CTAATCCGGC GCGACGAGC GCGAGCGAAG
      Y S G A A G A L S V P N S A A T S A S E
1501 ATGAGCGCCA CCACTTGAAC ATCCTGACTC ACCTCGCCTC ACAGCAAACC GCGGGGCGG
      D E R H H L N I L T H L A S Q Q T G G A
1561 ACCAGGCGCT AGAAGCGACG AGCCCGCCCC TAACATTACC GCATCTGGAG ACTCGGTGAT
      D Q A L E A T S P P L T L P H L E T R *
1621 GCTGCCCAGG ACCGAGCAAG GGTCGTGCCG AATCGAGAAT CGGAGATTCT GCAGGCGCTT
1681 CGTGGCGGGC TACAGGGTCA AGGTGTTGCG TTTGGTCTGA AAATGATGG CCGACGGGGG
1741 CACTGTCCGT CCCAGTTGGT CCCGCCGAAC TTCGACTGCG AGGCTCACGC TCGCGCCGCC
1801 AAATTGCCCC GACAACGTGC TGCCAGCGC ATTCTCTTGC ACGCCCGGCC TGTACCGCAT

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noeD →

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1141 AAATGGTGGT CGCCATCAAT CGGATTCACT GGTCGGCGTG CCGCGGAGCG CGCAGCCTGA
      M V V A I N R I H W S A C P G A R S L
1201 CTTGACGGC TCTGTACTTC GAGCGCTGTT TATTGAAACG AATACCGAAA GTCACGCGCG
      T S T A L Y F E R C L L K R I P K V T R
1261 TGCCGGACAC CAGATCAGCC CCTGCGCAGC CGCGTAAGGT CCGATATGTG CACGAACAAA
      V P D T R S A P A Q P R K V R Y V H E Q
1321 TCCTTGCCGT CGCCATAGTC AGGCCGAATG AACTCCCAGC CCTTTTCAAC GCTGTACGAC
      I L A V A I V R P N E L P A L F N A V R
1381 ACTACTGTTC CGGTGCGCAT GTTTGTTCCA CGGTCTTGCG GCTCGATATC GCCGCTACT
      H Y C S G A H V C S T V L R L D I A A Y
1441 ACTCCGGAGC TGCGGGTGCA CTTTCCGTTC CTAATCCGGC GCGACGAGC GCGAGCGAAG
      Y S G A A G A L S V P N S A A T S A S E
1501 ATGAGCGCCA CCACTTGAAC ATCCTGACTC ACCTCGCCTC ACAGCAAACC GCGGGGCGG
      D E R H H L N I L T H L A S Q Q T G G A
1561 ACCAGGCGCT AGAAGCGACG AGCCCGCCCC TAACATTACC GCATCTGGAG ACTCGGTGAT
      D Q A L E A T S P P L T L P H L E T R *
1621 GCTGCCCAGG ACCGAGCAAG GGTCGTGCCG AATCGAGAAT CGGAGATTCT GCAGGCGCTT
1681 CGTGGCGGGC TACAGGGTCA AGGTGTTGCG TTTGGTCTGA AAATGATGG CCGACGGGGG
1741 CACTGTCCGT CCCAGTTGGT CCCGCCGAAC TTCGACTGCG AGGCTCACGC TCGCGCCGCC
1801 AAATTGCCCC GACAACGTGC TGCCAGCGC ATTCTCTTGC ACGCCCGGCC TGTACCGCAT

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(Continued on next page)

Fig. 2. Nucleotide sequence of *Bradyrhizobium japonicum noeD*. Complete nucleotide sequence of approximately 3.7-kb *EcoRI-XbaI* DNA fragment, cloned in pSL5.2, was determined on both strands, three times, by the primer walking method and polymerase chain reaction. The *noeD* open reading frame (ORF) is indicated by arrow. Underlined sequence marks proposed *noeD* translation initiation codon; * marks stop codon. Site of Tn5 insertion in mutant D4-2.5 indicated by filled-in triangle. *B. japonicum nodM* ORF begins upstream of leftmost *EcoRI* site and ends at the *.

Both peaks 3 and 5 were analyzed for their glycosyl and fatty acyl components. Only very small amounts of peak 5 were available for this analysis; however, the results showed that peak 3 contained 2-*O*-methylfucose, *N*-acetylglucosamine, and a C18:1 fatty acyl residue. Small contaminating amounts of glucose, C16:0, and C18:0 were also present in peak 3. These same contaminants were also found in fraction 5. Fraction 5, as did peak 3, contained 2-*O*-methylfucose, and *N*-acetylglucosamine. However, unlike peak 3, analysis of peak 5 showed only small amounts of C18:1 methyl ester, an additional C20:1 fatty acyl methyl ester, and the alditol acetate of *N*-methyl-*N*-acetylglucosamine. If the small amount of C18:1 is due to contamination, these results suggest that the Nod factor in fraction 5 consists of 2-*O*-methylfucose, *N*-acetylglucosamine, *N*-methylglucosamine, and C20:1. Therefore, it is proposed that this Nod factor is NodBj-V(C20:1,NMe,MeFuc). Such a molecule would be identical in mass, and would give the same MS-MS fragmentation pattern as that for NodBj-V(Ac,C18:1,MeFuc). Additionally, the

presence of the longer fatty acyl chain and the *N*-methyl group is consistent with its more hydrophobic character. Future work is in progress to determine the exact structure of the peak 5 LCO.

Taken together, these results show (i) that the *noeD* mutants produce a much greater proportion of acetylated LCOs than the parent USDA110, and (ii) that the *noeD* mutants produce an increased level of a more hydrophobic LCO (i.e., that in peak 5), which is proposed to be NodBj-V(C20:1,NMe,MeFuc). These results suggest that changes in the ratio, or type, of LCOs are correlated with the ability of the *noeD* mutant to nodulate PI 417566. At present, however, we cannot discount the possibility that mutations in *noeD* affect traits in addition to Nod factor synthesis.

DISCUSSION

Although much is known about the genetic interaction of *B. japonicum* and soybean and numerous incompatible host-

Fig. 2. (Continued from previous page)

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1861      GGGGGGATTC ATCCGACCTG CTAAGCACAC AGAAATTTCG CCGGCGTATT GAACAGCAGG
1921      CAAGCGGGCT TTTAATCGAT CGCTCCCTT CGGACGTGTA CCCCCTACTC CAACACGCTG
1981      TGCGCGATAG AGGCTCGAAA ACTACGCAAT CACGAGCTCG ACCATCTCCA TCTCAGAATC
2041      ACTGAGCCTC ACGAGGAGGA GCATTGCGAT GGGTAGTCCG AGCAAGTACG CAAGCTGGAC
2101      TGGGGTGATT GTTAGGCTTG CCAAAGGTGC GTTCACCTCG GAGTTAATAG AGCGCCATCG
2161      ACCCTATTTC ACGCTTGGCG AATTGCTCAG CTAGCGCAGA TGACGGCCCG CTCTAAATGA
2221      TCTTATCTCT CAGCCTTTGC TGAACGATTT CGACCAGAAC TGCTCCGCGA AATCCAGATG
2281      GCACTTCATC ACGTCCGAGG GAGTTTTGGA ATTGGCAAGA TTCTTCAGAA GATTGACCTG
2341      AGCGTGCAGA GAGGAAGCAG CCAGCTTTGA CCGTCCTTCA ACGCCGCAGC GTAACAGGTC
2401      TGTTGTCTCT TCGAGGGATT TTTTCCATTC CGGAGACGCA AATGAATGGC TTGATGTAGC
2461      CCTCGCTGAT TCTCCAACAT CGTGCGTGGT GCCTCCCGCT TCAGAGGCTG AAGCCTGGGC
2521      GGGCCCGTTC GGGCTCTGGA TGGGTCTTCC GTTCGCGTTT CTCGTATTCTG GGACCTCTCG
2581      CGGTCAATCA ACGTCTCTCC GTCTAAACAG GATCGCAGCT CGCTGCGTGG TCTAGATCCG
2641      ACCAAGAGCT TCGGAAAGTA CCACAACCGG CAACATGTCG AACCTATCTC ATTTGGTAGC
2701      ACTTGCTCAT CGGGATTGCT TAAGCGGATA TCCGCACCAT TTGCGGGGTA TGCGAACGAC
2761      CGCGAGATTT GACTGAGAAT TTCTGCACG GCATGAAAAG AAAGTTGGTG GCAAGGAAGT
2821      CGAGAAGGAC CGCGTTCTAA CACTCGACGA ACTGAAGCCT TCTTTGCGAT CGTCAACGAT
2881      GAGGAAGCGG GCATCACGGA TGGAACGCGC TTGGCGCTGA AAGTCCGTAT TGCTGACAGC
3941      CCAGCGCCGG GCGAAGTCG CGAGCATGAT GCGTTCCGAG CTTACAGGCC TACACGGGGA
3001      AAAGCCCCAC TGGATCATTC CCGCTGTACG GACGAAGAAC AAGAAGGCGG AGCACACAGT
3061      CCCGCTCTTC CCGACTGCCG TCAAGTTGAT CGAGGCGGCC TTGGAGATCG GCAAGCCGAG
3121      AAGGGAGAGG ACCAAGGCAA CAGACCCGTC GTTGCCAGTC GCTTCGAAAAG CGTTGGGGTG
3181      CTTGCGAGCC ACTGTATGAG CCAGGCTGTG CGGCGCTTGT TGGAAGACAA TGAGCTGGTA
3241      GCCTTTACGC CCCATGACCT CAGGCGTACA GCCGCGACCA TCGTTTCATGC GTCTATGCGC
3301      GATGGCATAT GTTCGAAGAG AAGCGAGAAG CGATTCCAGC GGATTGAAAA GCATTTAATC
3361      ACAATCACCG GTTAGCAAAC TTCTGCGACA CTTAGCTCC CCTCTCGTCT GTATCGAGCC
3421      AGTGCTGTCC GCTCTCTGCG GCTAATGCCC ATGAATGTTA CGCCGTTGTA CCCGCGGTTT
3481      GAAAGGGCAG ATCGCACGTC ATCCGTCGAA GCGCCGATAT CGTAAGCGAT GTTAGCGAAC
3541      ATATATTGCT GGGTGTGCT CTATTGTTT GCGATCTGGG TATTAAGGTG ATTGGCGGCG
3601      CGATGCGCAA CCGCGTTATA AACCCTGTTT TTTTGCCTG CAACTTTCGG TTCACGCCAC
3661      TGTAGTATCT GAGTTTTGGT TTGACGGGGA ACCGAATCAT GATCAGGCAC CAAACACGCC
3721      AGCGCCACAG AGGCGCCCTC TAGA

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strain combinations have been described, little information is available concerning the genetics of host-controlled nodulation restriction. Bacterial genes involved in the GSN of soybean can act in either a positive or negative manner to control nodulation specificity (Triplett and Sadowsky 1992). We previously identified the *B. japonicum nola* gene, in strain USDA 110, which was shown to be a positively acting determinant that allows serogroup 123 strains to nodulate PI 377578 (Sadowsky et al. 1991). Subsequently, *nola* has been shown to regulate *nod* gene expression with elevated Nola expression leading to reduced production of LCO nod signals (Dockendorff et al. 1994; Garcia et al. 1996). Negatively acting GSN genes have also been described in *S. fredii* (Krishnan and Pueppke 1991; Meinhardt et al. 1993; Pueppke 1996). The *S. fredii nolC* gene (Krishnan and Pueppke 1991) and *nolXWBTUV* cluster (Bellato et al. 1997; Meinhardt et al. 1993) are negatively acting genes that have been shown to prevent nodulation of soybean cv. McCall.

The *noeD* gene represents the first example of a bona fide, negatively acting GSN gene identified from *B. japonicum*. Mutant strains D4.2-5 and 110-36 containing a *noeD*::Tn5 mutation can overcome the nodulation restriction conditioned by soybean genotype PI 417566. The results definitively show that the Tn5 mutation is responsible for this phenotype. This is important since in a previous study we demonstrated that the extended host range phenotype of a USDA 438 mutant was

not due to the Tn5 insertion in the identified locus (Judd et al. 1993). Moreover, since insertion mutations in strain USDA 110 that were immediately upstream and downstream of the Tn5-36 mutation do not allow nodulation of PI 417566, our results indicate that the Tn5 insertion was not acting in a polar manner on adjacent genes.

Southern hybridization experiments clearly showed that *noeD* is not contained within the major *nod* gene clusters in *B. japonicum* strain USDA 110 (Göttfert 1993; Nieuwkoop et al. 1987). However, the discovery of the closely linked *nodM* gene suggests that *noeD* and *nodM* may collectively identify a third cluster of *nod* genes. Therefore, further genetic analysis of this region may be warranted. NodM is homologous to D-glucosamine synthase (*glmS* from *Escherichia coli*) and has been postulated to provide an increased supply of D-glucosamine monomers for the synthesis of the LCO nodulation signals (Carlson et al. 1994; Marie et al. 1992). However, a Tn5 insertion in the end of the *nodM* gene of *B. japonicum* had no apparent effect on nodulation ability. This is not too surprising, as other studies have indicated that the presence of a second "housekeeping" copy of a gene with GlmS activity may be sufficient to allow for LCO synthesis (Marie et al. 1992). A second *nodM/glmS* gene copy in *B. japonicum* can be inferred since cell wall synthesis requires D-glucosamine and a mutant incapable of such synthesis would likely be nonviable.

BJnodM	EFRNRE	ALA	PRQFAIVILQ	SGETADTLAA	LRYAKGKGLH	TISVNVPTS	49
rlnod	EFYRIEIHLS	PRSAALFISQ	SGETADTLAS	LRYCKAHGLR	IGAVVNTRES		376
rmprme41b	EFYRIEIHLS	PQS	ALFISQ	SGETADTLAS	LRYCKAHGLR	IGAVVNARES	374
BJnodM	TIARESESVL	PILAGPEIGV	ASTKAFTICQL	MVLGVLA VRA	AKERGLKSEI		99
rlnod	TMAREADAIF	PILAGPEIGV	ASTKAFTICQL	AVLAALRIGA	GKARGTITDD		426
rmprme41b	TMARESDAVF	PILAGPEIGV	ARTKAFTICQL	AVLAALRAGA	GKARGTISGD		424
BJnodM	DESQLVRELI	EVERLIAAAL	.LVEFQIEKL	ARYIAGARTV	LYLGRGTSAP		148
rlnod	EEQVLVQSLA	TLEGVMRQVL	NDITPEIEILL	SRELSHYRDV	LYLGRGTSFP		476
rmprme41b	EEQALIKSLA	EMFAIMGQVL	NSIQPEIEVL	SRELSNCRDV	LYLGRGTSFP		474
BJnodM	LALEGALKLK	ETSYIHSEGY	AAGELKHGPI	ALIDEAVPVV	VIAPYDEVFE		198
rlnod	LAMEGALKLK	EVSYIHAEGY	AAGELKHGPI	ALIDENMPVI	VIAPHDRFFD		526
rmprme41b	LAMEGALKLK	ETSYIQPKSY	AAGQLKHGPY	ALIDENMPVI	VIAPHDRFFD		524
BJnodM	KTVSNMQEVA	ARGGKIILIT	DAKGASEAMV	DTLLTIVLPA	MVASFTPLVY		248
rlnod	KTVSNMQEVA	ARGGRIILIT	DETGASMSKL	PIMHTIVLPD	VAEIIAPMIF		576
rmprme41b	KTVINMQEV.	ARGGRIILIT	DEKGAAASKL	DTMHTIVLPE	VDEIIAPMIF		573
BJnodM	AIENVQLLAYH	TAVARGADVD	QPRNLAKSVT	VE			280
rlnod	SLFLQLLAYH	TAVVMGADVD	QPRNLAKSVT	VE			608
rmprme41b	SLFLQLLAYH	TAVFMGIDVD	QPRNLAKSVT	VE			605

Fig. 3. Amino acid sequence alignment of *Bradyrhizobium japonicum* NodM (top) and the NodM proteins from *Rhizobium meliloti* (middle) and *R. leguminosarum* bv. *viciae* (bottom). Identical amino acids indicated by solid letters; similar amino acids denoted by shaded boxes. Gaps have been introduced to optimize the alignment.

A curious aspect of this study is the detection of a region near the *Hind*III site in pSL23 with significant DNA sequence homology to the left end and attachment site for Tn7. The high degree of homology, 71% over 113 nucleotides, suggests that this site might be a "hot spot" for Tn7 integration and likely accounts for the insertion of Tn7 and Tn5 near the same locus in mutant D4.2-5. Interestingly, sequences located near

the carboxy terminus of the *E. coli glmS* gene, encoding glucosamine synthase, have also been shown to be required for transposition of Tn7 (Qadri et al. 1989).

The lack of detectable nucleic acid or protein sequence similarity of *noeD* to other *nod* genes or other known genes makes it difficult to predict a possible biochemical function. Mutations in several nodulation genes are known to result in

Fig. 4. High-performance liquid chromatography elution profiles of flavonoid-induced lipo-chitin oligosaccharide nodulation factors produced by *Bradyrhizobium japonicum* USDA 110 (A) and the *noeD* mutants, 110-36 or D4.2-5 (B). Peak 1 contains NodBj-V(C16:0, MeFuc); peak 2 contains NodBj-V(C18:1,MeFuc); and peak 3 contains NodBj-V (Ac,C18:1,MeFuc). Peak 5 contains the sodium adduct of NodBj-V (Ac,C20:1,NMe,MeFuc).

alterations in the structure of the LCO nod signals (e.g., Demont et al. 1993; Spaink et al. 1991). Indeed, the data show that the profile of LCO molecules isolated from strains 110-36 and D4.2-5 differs significantly from that found for the wild-type strain USDA 110. Most notably, the major LCO molecule produced by strains 110-36 and D4.2-5 is NodBj-V (Ac,C18:1,MeFuc) (Fig. 4B, peak 3). This molecule is only a minor constituent of the LCO molecules produced by the wild type. These data suggest that the ability of strain D4.2-5 to overcome restricted nodulation may be due to either an increased synthesis of an acetylated nod signal or the ratio of acetylated:nonacetylated Nod factors. This result, in part, is similar to that reported for the interaction between *R. leguminosarum* bv. *viciae* strain TOM and *P. sativum* cv. Afghanistan. In this case, the ability to nodulate the restricting cultivar is dependent on the *nodX* gene product that encodes a transacetylase that modifies the LCO Nod factor structure (Firmin et al. 1993). It should be noted, however, that at this time we cannot rule out the involvement of the proposed new Nod factor, NodBj-V(C20:1,NMe,MeFuc), in the nodulation of PI 417566 by mutants 110-36 and D4.2-5. If the chemical identity of this new Nod factor is borne out by additional studies, then this would be the first report of an N-methylated Nod factor produced by *B. japonicum* strain USDA 110. Such a Nod factor is consistent with the previous localization of a *nodS* gene (encoding N-methyltransferase) in this strain.

The proximity of the *nodM* gene to *noeD* raises the possibility that these two genes could constitute a single operon. Examination of gene expression by Northern (RNA) hybridization analyses indicated that *noeD* expression was elevated by the addition of soybean seed extract to cells of *B. japonicum* strains AN314 (*nodD1* mutant) and 913 (*nodD1* and *nodW* mutants). In contrast, *noeD* expression was significantly higher in cells of the wild type and strain 613 (*nodW* mutant) in the absence of soybean seed extract (data not shown). These results suggest that *noeD* expression is suppressed in the presence of a functional NodD1, but only in the presence of soybean seed extract. However, the lack of a consensus *nod* box 5' of the *noeD* gene suggests that NodD1 does not act directly to regulate *noeD* expression. In contrast, Northern hybridization analysis indicated that *nodM* was expressed at a low, constitutive level in seed-extract-induced and -uninduced cells of all strains examined. Therefore, it appears unlikely that *nodM* and *noeD* are co-regulated. These interesting observations require further study.

Previous studies have shown that nodulation restriction by soybean cv. PI 417566 is controlled by a single, recessive gene (Lohrke et al. 1996). In this study, we have shown that

noeD is a critical bacterial gene controlling the ability to overcome this GSN restriction. These data suggest that plant and bacterial genes may be interacting in a gene-for-gene manner similar to that of those examples studied extensively in plant pathogens (Keen and Staskawicz 1988). Unfortunately, we have no data on the possible function of the plant gene controlling nodulation restriction in PI 417566. An interesting idea suggested from the work with the *R. leguminosarum* bv. *viciae* *nodX* gene is that pea cultivars, other than Afghanistan, may possess plant enzymes capable of acetylating the LCO Nod signals (Firmin et al. 1993). It is postulated that such acetylation may be necessary to avoid degradation or recognition by the plant host. The discovery that *noeD* mutants have an elevated acetylation of the LCO Nod signals suggests that a similar mechanism could be operating in soybean. However, at this point we are unsure whether a specific, acetylated Nod factor, or the ratio of various Nod factors produced by a strain, is controlling nodulation restriction. In any case, such hypotheses are only speculative and much work remains to be performed. The availability of well-defined GSN genes should aid such future experimentation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The strains and plasmids used in this study are shown in Table 3. *B. japonicum* cultures were grown at 28°C on arabinose-gluconate (AG) medium (Sadowsky et al. 1987). Strains containing Tn5 were grown on AG medium supplemented with 200 µg of kanamycin and 200 µg of streptomycin per ml. *E. coli* cultures were grown at 37°C in Luria-Bertani (LB) medium (Sambrook et al. 1989). Antibiotics were added, when appropriate, to *E. coli* cultures at the following concentrations: ampicillin (50 µg/ml), kanamycin (50 µg/ml), and tetracycline (30 µg/ml). For liquid cultures, concentrations of antibiotics were reduced by one-half.

Isolation and subcloning of wild-type locus.

Genomic DNA from mutant D4.2-5 was digested to completion with *EcoRI* and size fractionated by sucrose density ultracentrifugation (Sadowsky et al. 1987; Sambrook et al. 1989). Plasmid DNA from *E. coli* cultures was isolated by the alkaline lysis procedure, while cosmids were isolated by the sodium dodecyl sulfate-NaCl procedure (Sambrook et al. 1989). Fractions were analyzed by Southern hybridization (Sambrook et al. 1989) with Tn5 DNA as a probe. Fractions that hybridized to Tn5 were subcloned into plasmid pACYC184 (Chang and Cohen 1978) and transformed into *E. coli* strain DH5α as described (Sambrook et al. 1989). Colonies were selected on LB medium containing 50 µg of kanamycin per ml. A plasmid, pSL5, was isolated from a single kanamycin-resistant colony. Southern hybridization analysis with a ³²P-labeled Tn5 probe and restriction enzyme mapping indicated that pSL5 contained Tn5 and wild-type genomic DNA that flanked the transposon insertion site. Plasmid pSL5 was used as a hybridization probe to a wild-type USDA 110 gene library, cloned as a partial *HindIII* digest in pHC79. A colony that hybridized to the probe was designated pSL23. Cosmic pSL23 was digested to completion with *EcoRI*; fragments were separated by gel electrophoresis in a 0.7% agarose gel and Southern hybridized to a ³²P-labeled pSL5 probe as

Table 2. Fast atom bombardment-mass spectrometry results of lipochitin oligosaccharides (LCOs) isolated from wild-type *Bradyrhizobium japonicum* strain USDA 110 and the *noeD* mutant, strain D4.2-5

Peak no. ^a	Proposed Nod factor structure	[M+H] ⁺ ion m/z
1	NodBj-V(C16:0,MeFuc)	1,390
2	NodBj-V(C18:1,MeFuc)	1,416
3	NodBj-V(Ac,C18:1,MeFuc)	1,458
5 ^b	NodBj-V(C20:1,NMe,MeFuc) ^c	1,458, 1,480

^a Peak designations defined in Figure 4 caption.

^b Not detected in strain USDA 110.

^c This is the [M+Na]⁺ ion, m/z = 1,458, for an LCO with the indicated proposed structure.

described (Sambrook et al. 1989). A single, hybridizing, 5.2-kb *EcoRI* fragment was subsequently isolated from a 0.7% agarose gel with the Prep-a-Gene DNA Isolation System (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. The 5.2-kb *EcoRI* fragment was ligated into *EcoRI*-digested pUC18 DNA (Sambrook et al. 1989), transformed into *E. coli* DH5 α (Miller 1972), and selected on LB medium containing 50 μ g of ampicillin per ml. The resulting plasmid was designated pSL5.2.

Tn5 saturation and marker exchange mutagenesis.

Random Tn5 mutagenesis of plasmid pSL5.2 was done with λ ::Tn5 (*b221, rex::Tn5, cI857, Oam29, Pam80*) as described (de Bruijn and Lupski 1984). *E. coli* strain SE5000 was used as the host for pSL5.2. Tn5 insertions in cloned, insert DNA were identified and mapped by restriction enzyme and by Southern hybridization analyses. Individual Tn5 insertions in pSL5.2 that were located in and around the original Tn5 insertion site in mutant D4.2-5 were homologously recombined into wild-type USDA 110. Insert DNA from plasmids containing Tn5 were digested with *EcoRI*, and the 11-kb fragments were gel purified as described above. The gel-purified fragments were ligated into *EcoRI*-digested pSUP202 DNA and transformed into *E. coli* strain S17-1 (Simon et al. 1983) and recombinant plasmids were conjugated to *B. japonicum* strain USDA 110. Transconjugants were selected on AG minimal medium (Sadowsky et al. 1991), containing 200 μ g of kanamycin per ml, and were tested for loss of pSUP202 by plating on AG minimal medium containing 60 μ g of tetracycline per ml. Genomic DNA was isolated from several kanamycin-resistant and tetracycline-sensitive transconjugants and digested with *EcoRI*. Fragments were separated by electrophoresis in 0.7% agarose gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) as described (Sadowsky et al. 1987). Southern blots were hybridized with ³²P-labeled 5.2-kb insert DNA from wild-type USDA 110 and Tn5. Transconjugants that had undergone successful marker exchange contained a single hybridizing fragment of 11 kb and a single copy of Tn5.

DNA sequencing.

The nucleotide sequence of the approximately 3.7-kb *EcoRI-XbaI* DNA fragment was determined on both strands. DNA was sequenced, three times, with a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Norwalk, CT) and an ABI Model 373A DNA Sequencer (Applied Biosystems, Foster City, CA). DNA sequencing was done at the Microchemical Facility of the University of Minnesota. Nucleotide sequence was determined initially by subcloning and subsequently with primers designed based on sequence information obtained from subcloned DNA fragments. Primers utilized include the M13/pUC universal forward and reverse primers, along with custom synthetic primers from Life Technologies (Grand Island, NY). The GCG sequence analysis software package (Genetics Computer Group, Madison, WI) and the Eugene software package (Department of Microbiology, University of Minnesota) were used for all DNA and protein sequence analyses. DNA and protein sequences were compared with entries in the GenBank, EMBL, PIR, and Swiss-Prot sequence data bases. The nucleotide sequence of the *B. japonicum noeD* and *nodM* genes have been deposited in GenBank and assigned accession numbers U67277 and U67278, respectively.

Analysis of Nod factors.

The Nod metabolites from *B. japonicum* strains USDA 110 and D4.2-5 were induced and purified as described (Sanjuan et al. 1992), except that a Vydac 5 μ 300A (Vydac, Hesperia, CA) HPLC column was used, and the gradient was lengthened to 90 min in order to enhance separation and resolution. Cultures were induced with 2 μ M genistein prior to isolation of Nod metabolites.

The structures of Nod factors were determined by chemical composition analysis with nuclear magnetic resonance (NMR) spectroscopy and GS-MS analysis (Carlson et al. 1993). All MS were obtained with a JEOL (Tokyo, Japan) SX/SX 102A tandem, four-sector mass spectrometer, operated at a 10-kV

Table 3. Strains and plasmids used in this study

Strain, plasmid, or phage	Relevant characteristic(s)	Source or reference
<i>Bradyrhizobium japonicum</i>		
USDA 16, USDA 17, USDA 20, USDA 30, USDA 110, USDA 141, USDA 335, USDA 448, USDA 450, USDA 451, USDA 452, USDA 454, USDA 455, USDA 456, USDA 460, USDA 461, USDA 222	Wild type, serogroup 110 strains	USDA-ARS, Beltsville, MD
USDA 123	Wild type, serogroup 123	USDA-ARS, Beltsville, MD
D4.2-5	Original 110 Tn5 mutant, Nod ⁺ on PI 417566	Lohrke et al. 1995
110-1, 110-5, 110-9, 110-10, 110-13, 110-16, 110-20, 110-35	110::Tn5, Nod ⁺ on PI 417566	This study
110-36	110::Tn5, Nod ⁺ on PI 417566	This study
Plasmids		
pSUP202	Suicide vector	Simon et al. 1983
pRJUT10	pHC79 clone of <i>B. japonicum nod</i> region	Nieuwkoop et al. 1987; Russell et al. 1985
pRJUT14	pHC79 clone flanking <i>B. japonicum nod</i> region	Nieuwkoop et al. 1987; Russell et al. 1985
pRJUT24	pHC79 clone flanking <i>B. japonicum nod</i> region	Nieuwkoop et al. 1987; Russell et al. 1985
pSL23	29-kb clone from USDA 110 containing <i>noeD</i> and <i>nodM</i>	This study
pSL5.2	5.2-kb subclone from USDA 110 containing <i>noeD</i> and <i>nodM</i>	This study
pSL5	Tn5-containing pACYC184 clone from mutant D4.2-5	This study
Bacteriophage		
λ ::Tn5 (<i>b221, rex::Tn5, cI857, Oam29, Pam80</i>)		de Bruijn and Lupski 1984

accelerating potential as described (Carlson et al. 1993). Ions were produced by FAB with xenon, using a JEOL FAB gun operated at 6 kV in a conventional FAB ion source. Spectra acquired for the first MS are averaged profile data of 3 scans as recorded by a JEOL complement data system. These spectra were acquired from 200 to 2,000 m/z , at a rate that would scan from 0 to 2,500 m/z in 1 min. A filtering rate of 100 Hz and an approximate resolution of 1,000 (with a 10% valley) were used in acquiring these spectra. The resolution was sufficient to resolve the isotope peaks for all the samples. Samples were dissolved in dimethyl sulfoxide (DMSO) and 1- μ l aliquots were mixed with an equal volume of the FAB matrix, thioglycerol (TG), on the probe tip. Tandem MS-MS was performed on the ^{13}C isotope peak of the samples. Collisional activation was performed in the third field region, with helium as the collision gas. Helium pressure was sufficient to attenuate the primary ion beam by 75%. The collision cell was floated to 3 kV, providing a collision energy of 7 kV. Acquired MS/MS spectra are averaged profiles of four scans as recorded by the JEOL complement data system.

For composition analysis, samples were hydrolyzed in 2 M trifluoroacetic acid at 121°C for 2 h. After cooling, fatty acids were extracted to hexane. The aqueous and hexane layers were independently evaporated to dryness under a stream of dry nitrogen. The glycosyl residues from the aqueous layer were converted to their alditol acetates as previously described (Carlson et al. 1993) and analyzed by combined gas liquid chromatography (GLC)-MS. The fatty acids from the hexane layer were converted to methyl esters by methanolysis in 1 M methanolic HCl at 80°C for 2 h. The solvent was evaporated and the resulting fatty acid methyl esters were dissolved in hexane and analyzed by combined GC-MS. The location of the double bond in the fatty acyl residue was determined by oxidation of the double bond to a diol and acid-catalyzed methanolysis and trimethylsilylation (Yruea et al. 1990).

Nodulation assays.

The nodulation phenotype of wild-type and marker exchange mutants were determined in modified Leonard jar assemblies as described (Sadowsky et al. 1987). Leonard jars were planted with three surface-sterilized seeds of *G. max* PI 417566 and thinned to two seedlings per Leonard jar, 3 days after emergence (Sadowsky et al. 1991). Seeds were inoculated, at planting, with 1.0 ml (about 10^8 cells) of AG-grown *B. japonicum* strains. Isolates were inoculated in triplicate Leonard jars. Uninoculated plants served as negative controls, *B. japonicum* strain USDA 123 served as a positive control, and strain USDA 110 served as a baseline against which the mutants were compared.

Southern and Northern hybridization analyses.

Total genomic DNA from nodulation restricted (USDA 16, USDA 17, USDA 30, USDA 110, USDA 141, USDA 454, USDA 460, and USDA 461) and unrestricted (USDA 20, USDA 335, USDA 448, USDA 450, USDA 451, USDA 452, USDA 455, and USDA 456) *B. japonicum* serogroup 110 strains was isolated as previously described (Sadowsky et al. 1987). DNAs were digested with *Eco*RI, restriction fragments were separated on 0.8% agarose gels, fragments were Southern transferred to Nytran Membranes (Schleicher & Schuell,

Keene, NH), and blots were hybridized to a ^{32}P -labeled, 750-bp, *Xho*I-*Pst*I fragment from pSL5.2 that was internal to the *noeD* gene as described (Sadowsky et al. 1987).

Total RNA was extracted from induced and uninduced cultures of *B. japonicum* strains USDA 110, AN314 (*nodD1* mutant), 613 (*nodW* mutant), and 913 (*nodD1* and *nodW* mutants) by a hot-phenol extraction method (Stacey et al. 1994). Cells were grown in HM medium (Nieuwkoop et al. 1987) and induced with 40 μ l of 10 $\times$ soybean seed extract (Smit et al. 1992) per ml of culture at $\text{OD}_{600} = 0.5$. Slot blots were prepared by loading 5, 10, and 20 μ g of RNA of each sample on a Bio-Dot SF microfiltration apparatus (BioRad, Hercules, CA). The blot was subsequently hybridized with a 500-bp polymerase chain reaction (PCR) internal fragment of *noeD*, a 600-bp PCR internal fragment of *nodM*, and a 360-bp DNA fragment coding for the 23S rRNA from *Pseudomonas aeruginosa* as positive control (Festl et al. 1986). Procedures for blotting, prehybridization, hybridization, and filter washings were done as described by Sambrook et al. (1989). Autoradiography was done for 5 days for the *noeD* and *nodM* probe, and 30 min for the 23S rRNA probe.

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