Structural and Functional Analysis of Two Different *nodD* Genes in *Bradyrhizobium japonicum* USDA110

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Bradyrhizobium japonicum has two closely linked homologs of the nodulation regulatory gene, *nodD*; these homologs are located upstream of and in divergent orientation to the *nodYABCSUIJ* gene cluster. We report here the nucleotide sequence and mutational analyses of both *nodD* copies. The predicted NodD1 and NodD2 proteins shared 62% identical amino acid residues at corresponding positions and exhibited different degrees of homology with NodD proteins of other *Bradyrhizobium*, *Azorhizobium*, and *Rhizobium* strains. Induction of the *nodYABCSUIJ* operon, as measured by expression of a translational *nodC'-'lacZ* fusion, required the *nodD1* gene, but not *nodD2*. A *B. japonicum* mutant deleted for both *nodD* copies (strain Δ1267) still showed residual nodulation activity; however, nodulation of soybean was significantly delayed, and nodulation of mung bean and siratro resulted in strongly reduced nodule numbers. Fully efficient nodulation of mung bean and siratro by strain Δ1267 was restored by genetic complementation with the *nodD1* gene, but not with *nodD2*. We conclude from these data that *nodD1* is the critical gene that contributes to maximal nodulation efficiency, whereas the *nodD2* gene does not play any obvious role in nodulation of the host plants tested.

Additional keyword: gene regulation.

Nodulation (*nod*) genes enable bacteria of the genera *Rhizobium* and *Bradyrhizobium* to induce nodule formation on the roots of their legume host plants. The majority of the *nod* genes share a similar mode of transcriptional regulation. The product of the regulatory gene *nodD* binds to the *nod*-box, a conserved promoter region upstream of nodulation genes (Rostas *et al.* 1986), and acts as a transcriptional activator (Hong *et al.* 1987; Fisher and Long 1989; Kondorosi *et al.* 1989). Activation of *nod* gene transcription by the NodD protein requires the additional presence of phenolic compounds known as flavonoids and isoflavonoids (Firmin *et al.* 1986; Peters *et al.* 1986; Redmond *et al.* 1986; Kosslak *et al.* 1987; Banfalvi *et al.* 1988; Göttfert *et al.* 1988; Zaat *et al.* 1989). The decision about which flavonoid acts as an inducing molecule depends on the nature of the *nodD* copy (Horvath *et al.* 1987; Spaink *et al.* 1987). For example, the products of the three *nodD* homologs of *Rhizobium meliloti* Dangeard respond differently to the inducing molecules present: NodD1 induces the *nod* genes in the presence of luteolin (Peters *et al.* 1986); NodD2 interacts with methoxychalcone (Maxwell *et al.* 1989; Hartwig *et al.* 1990) and some, so far unknown compounds (Györgypal *et al.* 1988; Honma *et al.* 1990; Maillet *et al.* 1990); and NodD3 does not need an inducer molecule for gene activation, whereas transcription of *nodD3* itself

is subject to a complex regulatory circuit (Mulligan and Long 1989; Maillet *et al.* 1990). Based on these observations and because plant species produce different flavonoids and isoflavonoids, the *nodD* gene can be regarded as a determinant of host specificity.

The effect of a *nodD* mutation on nodulation is different depending on the rhizobial species examined. *R. leguminosarum* bv. *viceae* Jordan, *R. l.* bv. *trifolii* Jordan, and probably *Azorhizobium caulinodans* Dreyfus *et al.* have only one *nodD* gene, and *nodD* mutants are completely Nod⁻ (Downie *et al.* 1985; Innes *et al.* 1985; Goethals *et al.* 1990). Mutations in one of the three *nodD* homologs of *R. meliloti* affect nodulation efficiency in a host-dependent manner (Göttfert *et al.* 1986; Honma and Ausubel 1987; Györgypal *et al.* 1988; Honma *et al.* 1990). Three functional *nodD* genes were also identified in *R. l.* bv. *phaseoli* Jordan (Davis and Johnston 1990). *R. fredii* Scholla and Elkan USDA191 carries two *nodD* copies; in this species, NodD1 is the activator of *nod* gene transcription, whereas no such function could be assigned to the NodD2 protein (Appelbaum *et al.* 1988b). Similarly, NodD2 of *B. japonicum* Jordan strain USDA123 does not seem to be a transcriptional activator of *nod* gene expression (Appelbaum *et al.* 1988a). Apart from regulation by the *nodD* gene products, *nodABC* expression in *R. meliloti* strain AK631 is regulated additionally by a repressor protein (Kondorosi *et al.* 1989).

In *B. japonicum* strain USDA110, most of the characterized nodulation genes are part of a long operon covering *nodY*, *nodA*, *nodB*, *nodC*, *nodS*, *nodU*, and probably *nodI* and *nodJ* as well (Fig. 1; Lamb and Hennecke 1986; Nieuwkoop *et al.* 1987; Göttfert *et al.* 1990). Upstream of this operon (*nodYABCSUIJ*), two copies of *nodD* were located (Appelbaum *et al.* 1988a; Göttfert *et al.* 1989). Two isoflavonoids, daidzein and genistein, have been identified

as being the most potent inducers of *nod* gene induction in *B. japonicum* (Kosslak *et al.* 1987; Banfalvi *et al.* 1988; Göttfert *et al.* 1988). A mutation within *nodD1* drastically reduces the induction of a plasmid-coded translational *nodY'-'lacZ* fusion (Banfalvi *et al.* 1988). However, *nodD1* mutants still nodulate soybean and siratro with only a short delay (Nieuwkoop *et al.* 1987; Göttfert *et al.* 1989) and mung bean with a delay of about 4 days (Göttfert *et al.* 1989). It was proposed that in a *nodD1* mutant, the presence of *nodD2* sustains expression of the nodulation genes. In this study, we determined the DNA sequence of the *B. japonicum nodD1* and *nodD2* genes, which was a prerequisite for construction of defined insertion and deletion mutations, complementing clones, and for gene fusions to *lacZ*. With these tools at hand, we analyzed the function

and regulation of the two *nodD* genes to assess the relative contribution of each to nodulation of soybean and other host plants.

MATERIALS AND METHODS

Bacterial strains, vectors, and phages. *Escherichia coli* (Migula) Castellani and Chalmers RR28 (Hennecke *et al.* 1982) was used for maintenance and propagation of plasmids. *E. coli* S17-1 was used for the mobilization of pSUP202 derivatives (Simon *et al.* 1983) into *B. japonicum*. *E. coli* JM101 (Messing 1983) was the host for phages M13mp18 and M13mp19 (Norrander *et al.* 1983), which served as cloning vectors for the subsequent sequencing of cloned DNA. *B. japonicum* 110*spc*4, referred to as wild
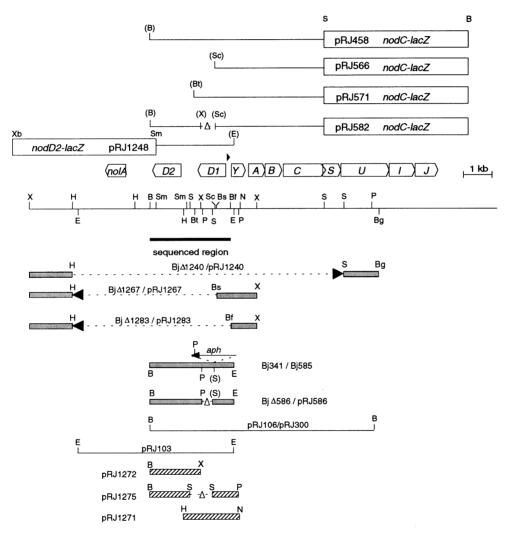

Fig. 1. Analysis of the *nodD1-nodD2* region of *Bradyrhizobium japonicum*. The horizontal open arrows indicate the extent and direction of the *nod* genes. The filled arrowhead between *nodD1* and *nodY* marks the location and orientation of the *nod*-box. The bold line below the restriction map marks the sequenced DNA fragment. For clarity, only the restriction sites relevant to this work are included. Restriction sites in parentheses were lost during the cloning procedure. Translational *nod'-'lacZ* fusions are shown above the genes. The *lacZYA* fragment is shown as a box. The *Bam*HI or *Xba*I site at the end of the *lacZYA* fragment originates from linker addition into the *Stu*I site located downstream of *lacA*. Mutants are indicated by designations starting with Bj, and plasmid constructs are shown in the lower part. The extent of deletions in the mutants is marked by dashed lines. The presence and direction of an out-reading promoter from the inserted resistance gene are depicted by an arrowhead. The fragments used for the recombination event are indicated by shaded boxes. Hatched boxes depict *nodD* containing fragments used for the complementation of mutant Δ1267. The right *Bam*HI site of plasmids pRJ106/pRJ300 was created artificially. Detailed constructions are described in the text. Abbreviations: B, *Bam*HI; Bf, *Bfr*I; Bg, *Bgl*II; Bs, *Bsp*HI; Bt, *Bst*EII; E, *Eco*RI; H, *Hind*III; N, *Nhe*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*II; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I; Δ, deletion.

type throughout this paper, has been described previously (Regensburger and Hennecke 1983). Plasmid pSUPPOL1 (P. Grob, unpublished) is a derivative of pSUP202 and contains a multiple cloning site in the *Pst*I restriction site. The multiple cloning site was isolated from pBluescript II SK+ (Stratagene, La Jolla, CA) and reads *Dra*II-*Xho*I-*Sal*I/*Hinc*II/*Acc*I-*Cla*I-*Hind*III-*Eco*RV-*Eco*RI-*Pst*I-*Sma*I-*Bam*HI-*Spe*I-*Xba*I-*Not*I-*Xma*III-*Sac*II; the *Sac*II restriction site is proximal to the chloramphenicol resistance gene. A similar plasmid, pSUPPOL2, differs from pSUPPOL1 only by the orientation of the polylinker. Plasmid pSUP202X is also a derivative of pSUP202 and carries an *Xho*I linker within the *Eco*RI restriction site (C. Kündig, unpublished). Plasmid pRKPOL2 (P. Grob, unpublished) is a derivative of the broad host range vector pRK290 (Ditta *et al.* 1980) containing a multiple cloning site in the *Eco*RI restriction site. The multiple cloning site was isolated from pBluescript II SK+ and reads *Eco*RI-*Apa*I-*Dra*II-*Xho*I-*Sal*I/*Hinc*II/*Acc*I-*Cla*I-*Hind*III-*Eco*RV-*Eco*RI-*Pst*I-*Sma*I-*Bam*HI-*Spe*I-*Xba*I-*Not*I-*Xma*III-*Sac*II-*Eco*RI; the *Sac*II restriction site is proximal to the tetracycline resistance gene. The *lacZ* fusion vectors pNM480X and pNM481X (P. Grob, unpublished) are identical to pNM480 and pNM481 (Minton 1984), respectively, except that they contain an *Xba*I linker within the *Stu*I restriction site located near the 3' end of *lacA*.

Media and bacterial growth conditions. *E. coli* was grown in Luria-Bertani medium (Miller 1972) at 37° C. *B. japonicum* was grown in peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke 1983) at 30° C. For plasmid selection, antibiotics were used as described (Göttfert *et al.* 1989).

DNA manipulations. Recombinant DNA techniques were performed following established protocols as compiled by Maniatis *et al.* (1982). We labeled probes for hybridizations by using the nonradioactive labeling kit from Boehringer, Mannheim, Germany.

DNA sequence analysis. We established the DNA sequence on both strands by using the dideoxynucleotide chain termination method (Sanger *et al.* 1977) with the help of a DNA sequencer model 370A of Applied Biosystems (Foster City, CA). For computer-aided DNA and protein sequence analyses, the software package (Release 6.1) of the UWGCG (Genetics Computer Group of the University of Wisconsin, Madison, WI) was used. Percentages of identity values were determined by using the Gap program. Database searches were done by using the TFastA and Wordsearch programs in conjunction with the database GenEMBL (Release 62.0).

Construction of plasmids carrying *nodD1* or *nodD2* and of plasmids for mutagenesis. The *nodD1*- or *nodD2*-carrying fragments used in this study are depicted in Figure 1; they were cloned into the broad host range vector pRKPOL2 and yielded plasmids pRJ1271 (*nodD1*), pRJ1272 (*nodD2*), and pRJ1275 (*nodD2*). All *nodD*-containing fragments were isolated from plasmid pRJ106 (Lamb and Hennecke 1986).

To delete most of the *nod* gene cluster, we first constructed plasmid pRJ1240; it consists of the vector pSUPPOL1 and a 4.4-kb insert fragment. The insert contains a kanamycin resistance cassette (derived from pUC4KIXX;

Pharmacia, Uppsala, Sweden) flanked by two fragments of *B. japonicum* DNA as shown in Figure 1. The flanking regions provided the DNA needed for marker exchange by double homologous recombination. To delete both *nodD* genes and the *nolA* region located downstream, we constructed pRJ1267; this plasmid consists of the vector pSUP202X and a 9.4-kb insert fragment that contains a kanamycin resistance cassette (derived from pUC4KIXX) flanked by two *B. japonicum* DNA fragments as shown in Figure 1. These flanking regions provided the DNA for marker exchange. Plasmid pRJ1283 is similar to pRJ1267, except that it lacks the *nod*-box region (Fig. 1).

To create a mutation in *nodD1*, we generated two different constructs. Total DNA of the *nodD1* mutant strain 341 (Fig. 1; Göttfert *et al.* 1989) was cut with *Eco*RI and *Bam*HI, and the 5.4-kb fragment containing the mutated *nodD1* gene was cloned into pSUP202, yielding pRJ585. Then, pRJ585 was cut with *Pst*I and religated, thereby deleting 383 bp of *nodD1* internal DNA and yielding pRJ586 (Fig. 1).

Mobilization of pRJ1240, pRJ1267, pRJ1283, pRJ585, and pRJ586 into *B. japonicum* 110*spc*4 and selection for marker exchange events were done as described previously (Göttfert *et al.* 1989), and these procedures gave rise to mutant strains Δ1240, Δ1267, Δ1283, 585, and Δ586, respectively (Fig. 1). The correct genomic structure of the chromosomal mutations was verified by Southern blot hybridizations.

Construction of translational *nod'-'lacZ* fusions. To construct a translational *nodD2'-'lacZ* fusion, pRJ103 (Fig. 1; Göttfert *et al.* 1989) was partially digested with *Sma*I, and the resulting linear DNA was then digested with *Eco*RI. A 2.9-kb *Eco*RI-*Sma*I fragment containing *nodD2* was cloned into pNM480X and pNM481X and yielded pRJ1234 (out-of-frame *nodD2'-'lacZ* fusion) and pRJ1235 (in-frame *nodD2'-'lacZ* fusion), respectively (not shown). The correct fusion sites were confirmed by DNA sequence analysis. pRJ1234 and pRJ1235 were cut with *Eco*RI, and *Bgl*II linkers were added. Subsequently, the DNA was cut with *Xba*I, and the *nodD2'-'lacZ* fusion fragments were cloned into the broad host range vector pRKPOL2 and yielded plasmids pRJ1248 (in-frame fusion; Fig. 1) and pRJ1249 (out-of-frame fusion).

To construct translational *nodC'-'lacZ* fusion plasmids containing different *nodD* copies, we used pRJ458 as the starting material. This plasmid already carries the *nodC'-'lacZ* fusion and both *nodD* copies (Fig. 1; Göttfert *et al.* 1990). To remove both *nodD* genes, we linearized pRJ458 with *Sac*II and added an *Xba*I linker. The DNA was cut with *Bam*HI, and the *Xba*I-*Bam*HI fragment containing the *nodC'-'lacZ* fusion was cloned into pRKPOL2, which resulted in plasmid pRJ566 (Fig. 1). To clone a *nodC'-'lacZ* fusion plasmid carrying only *nodD1*, we cut pRJ458 with *Bst*EII and added an *Xba*I linker. This DNA was then cut with *Bam*HI, and the *Xba*I-*Bam*HI fragment carrying the *nodC'-'lacZ* fusion was cloned into pRKPOL2, which resulted in plasmid pRJ571 (Fig. 1). For the construction of a *nodC'-'lacZ* fusion plasmid carrying only the *nodD2* copy, we isolated a 1.8-kb *Bam*HI-*Xho*I fragment, encompassing *nodD2*, and attached *Spe*I linkers to both ends. The resulting *Spe*I fragment was cloned into pRJ566, which resulted in the pRJ582 plasmid construct as shown

in Figure 1. All *nodC'-'lacZ* fusion plasmids were finally transferred into *B. japonicum* by triparental mating as described by Ditta *et al.* (1980).

β-**Galactosidase assays.** Strains were grown as described previously (Göttfert *et al.* 1990). Measurement of *β*-galactosidase activity (in duplicate) was done by taking 100-*μ*l samples of cells grown to an optical density of A_{600} = 0.4–0.8. The cell samples were permeabilized by the addition of 10 *μ*l of chloroform and sodium dodecyl sulfate (SDS), which was already present in the Z-buffer (35 mg of SDS per 1 L of Z-buffer). Otherwise, the *β*-galactosidase assays and calculation of enzyme units were done as described (Miller 1972).

Sources of flavonoid compounds. Daidzein was from

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1    CTTAAGGAGAAACAACAACGCGCGCCCATGGTGCGCTCCCACGTCTGGGAGCTCTTCTCTTAGCTCACGTGAACGACCGGAAACCATCGTGGCGCGTCT

100  AATTGCTTTTTCCAAACTTCAGTTTGCTATCCAACCTCCCCCAGTTTGGTAAAATCGATTGTTTCGATAGAACACATCCACACGATGGATAGACTCATC
                                               A

199  ACATGCGGTTCAAGGGACTTGATCTAAATCTTCTCGTTGCGCTCGACGCCGTGATGACGGCGCGCAACCTCACAGCGGCGGCTCGCAAAATCAATCTGA
     M  R  F  K  G  L  D  L  N  L  L  V  A  L  D  A  V  M  T  A  R  N  L  T  A  A  A  R  K  I  N  L  S

298  GCCAGCCTGCTATGAGCGCTGCGATCGCACGGCTGCGCACCTATTTCCGCGATGAACTCTTTACTATGAGAGGTCGCGAACTCGTCCCGACACCTGGCG
     Q  P  A  M  S  A  A  I  A  R  L  R  T  Y  F  R  D  E  L  F  T  M  R  G  R  E  L  V  P  T  P  G  A

397  CGGAAGCGCTTGCAGGTCCGGTTCGCGAGGCCCTGCTGCACATCCAACTCTCAATCATATCGCGGGACGCGCTCGACCCTGCTCAATCGAGCCGACGCT
     E  A  L  A  G  P  V  R  E  A  L  L  H  I  Q  L  S  I  I  S  R  D  A  L  D  P  A  Q  S  S  R  R  F

496  TCAGGGTCATTCTCTCAGATTTCATGACGATCGTTTTTTTCCGCAGAATTGTGGACCGCATGGCGCAAGAAGCCCCCGCGGTGCGCTTCGAACTGCTGC
     R  V  I  L  S  D  F  M  T  I  V  F  F  R  R  I  V  D  R  M  A  Q  E  A  P  A  V  R  F  E  L  L  P

595  CATTTTCTGATGAACCGGATGACCTGCTGCGGCGGGGCGAGGTCGACTTTCTCATTCTGCCGGAACTTTTCATGTCGAGCGCGCACCCTAAGGCGACGC
     F  S  D  E  P  D  D  L  L  R  R  G  E  V  D  F  L  I  L  P  E  L  F  M  S  S  A  H  P  K  A  T  L

694  TGTTCGACGAGAGCCTCGTATGCGTCGGATGCCGCGCGAACAAGCAGCTATCCCGGCAGCTTACGTTCGAACAATACATCTCGATGGGGCACGTTACTG
     F  D  E  S  L  V  C  V  G  C  R  A  N  K  Q  L  S  R  Q  L  T  F  E  Q  Y  I  S  M  G  H  V  T  A

793  CCAAGTTCGGACGCGCACTGAGACCGAACCTCGAAGAATGGTTTTTGCTTGAGCACGGCCTGAGGAGACGAATTGAGGTCGTCGTGCAGGGCTTTAGCC
     K  F  G  R  A  L  R  P  N  L  E  E  W  F  L  L  E  H  G  L  R  R  R  I  E  V  V  V  Q  G  F  S  L

892  TGATTCCGCCCCTGTTGCTAGACACGAGCCGTATCGGCACGATGCCCTTACGACTGGCCAGGCACTTCGAAAAGCGGATGCCGTTGCGGATCATCCAAC
     I  P  P  L  L  L  D  T  S  R  I  G  T  M  P  L  R  L  A  R  H  F  E  K  R  M  P  L  R  I  I  Q  P

991  CGCCCCTCCCCCTGCCCACATTCACAGAGGCCCTGCAGTGGCCCTCATTCCACAATACCGACCCCGCGAGCATCTGGATGCGTCGGATATTGCTCGAGG
     P  L  P  L  P  T  F  T  E  A  L  Q  W  P  S  F  H  N  T  D  P  A  S  I  W  M  R  R  I  L  L  E  E

1090 AAGCATCCAACATGGCATCTGGGGACCAGGAGCCTCCAACTCGCAGGCGCTGTTAGGCTTACCTTAAGCTGCGCTCTGAACATCCCCGGCACACTGTAG
     A  S  N  M  A  S  G  D  Q  E  P  P  T  R  R  R  C  *

1189 AACCTTCGACGTGCCCCGTTTTCTTTCGTCTTTCCAGGGTATGGAGGTGAGAAGGAGCCACAGACGCGCAATGCGACGATCGGTCGCGAAGCCTTTTCC

1288 AGTTGTGCGCGTGGTGACCTCGAAGGTGCGGTTTGATGCGCTCACGACGCATTCAGGCTACGTCCGGCAACCATCGCAGAAGAATATGCCTGCCGTGCA

1387 AATGTTTGGCTCTATCACTAAAATGTCTTGAACCGCACATTGCGTCAGGTTGTAGGTTGCTCTCGTGACAAGCGCTGCACGACCCAGCTGTCGACACCG

1486 CGCTTTGGCACGCTGACGAACACTGGGCTGATCTGCCATCAGCGGACTGAACGACCCTGCTGCGTGGCCGGCCGGAACCGGCAAGACCAATTAGCTTGG

1585 AGCTCCCGACGCTTTCCCGCCGCTGCCGATCGGCTTTCCGCCTGCTCTCCATTGTTGCGGTCATCTCGCAAGCTTCCTTTCCCGGGCGCAGGAATGAGC

1684 CGATGTCCATTGGGGCGGGCCAAGACGATTGCTCCAGGCGCTGGCGCCACCGCACGCTATTGCGCAGCAACGACGTTTGTGCCGGATAGGAAGAGATGC
                                                                                                 M  R

1783 GTTTCAAAGGTCTGGATCTAAATCTTCTGGTCGCGCTGGATGCTCTAATAACTGAGCGCAACCTCTCGTCAGCCGCACGCAAGATCAATCTTAGTCAGC
     F  K  G  L  D  L  N  L  L  V  A  L  D  A  L  I  T  E  R  N  L  S  S  A  A  R  K  I  N  L  S  Q  P

1882 CGGCAATGAGCGCAGCCGTTGCCCGGCTGCGCAAGCATTTCCGTGATGAACTGTTTGGAATGAGGGGGCGCGAACTTGTCTTGAGCTCACGCGCGGAAG
     A  M  S  A  A  V  A  R  L  R  K  H  F  R  D  E  L  F  G  M  R  G  R  E  L  V  L  S  S  R  A  E  G

1981 GGCTCGCGGCTCCTGTGCGCGAGGCTCTGATGCACATTGAACTCTCGATTATGGCCCGACATCCGTTCGACCCAGCTCGATTGAACCGCCGATTCAGGA
     L  A  A  P  V  R  E  A  L  M  H  I  E  L  S  I  M  A  R  H  P  F  D  P  A  R  L  N  R  R  F  R  I

2080 TCGTCCTTTCTGACTTCGTAACAGTTGTGCTCTTCCGAAATGTCGTAGCACGCGTCACGCGAGAAGCCCCCGCCGTCAGCTTCGAATTGGCTGCGCCGA
     V  L  S  D  F  V  T  V  V  L  F  R  N  V  V  A  R  V  T  R  E  A  P  A  V  S  F  E  L  A  A  P  T

2179 CCGATGAACACGAGCTGCTCCTCCGCCGCGGTGAAGTCGATTTTGTTATCCGGCCAGATTTTTTCATGTCCAGCACGCACCCCAGAGCGGCCCTATTCG
     D  E  H  E  L  L  L  R  R  G  E  V  D  F  V  I  R  P  D  F  F  M  S  S  T  H  P  R  A  A  L  F  E

2278 AGGAGCGACTCGTCTGCGTAGGCTGCTGCACCAATAGGGAATTACAACCCCGGCTTACATTCGATCGATATATGTCGATGGGTCACGTTGCAGTTAAGC
     E  R  L  V  C  V  G  C  C  T  N  R  E  L  Q  P  R  L  T  F  D  R  Y  M  S  M  G  H  V  A  V  K  H

2377 ACGGAGGTGCGCCCCGGACGCCAGTTGAGCATTCCTTTTTGACTGATCTCGGACCCACGCGGCGCATCGACATCCTCGTGCAGAGCTTCAGCATGATCC
     G  G  A  P  R  T  P  V  E  H  S  F  L  T  D  L  G  P  T  R  R  I  D  I  L  V  Q  S  F  S  M  I  P

2476 CGCCTCTCATAGTTGGGACGAACCGCATAGGCACGATGCCATTAGGGCTCGTGAGGCATTTCCAAAGAACGATGCCCCTTCGGATCGTTGAGCTTCCGC
     P  L  I  V  G  T  N  R  I  G  T  M  P  L  G  L  V  R  H  F  Q  R  T  M  P  L  R  I  V  E  L  P  H

2575 ATCCATTCCCCGCCTTCACCGAGGCGGTCCAATGGCCCTCACTTCACAACAGCGACCCGGGAAGTCTGTGGATGAGGGACATTTTGTTTCAGGAGGCCA
     P  F  P  A  F  T  E  A  V  Q  W  P  S  L  H  N  S  D  P  G  S  L  W  M  R  D  I  L  F  Q  E  A  T

2674 CCCGCATGGCAACTACACAAGAGCTCCGTGTGACCAGCAGCCCGGAAGACGCGGAGCCCCCGGGACATTTCGTGCGATCCGTCTCACCGTTGCCGTAAG
     R  M  A  T  T  Q  E  L  R  V  T  S  S  P  E  D  A  E  P  P  G  H  F  V  R  S  V  S  P  L  P  *

2773 CACATTCGCGAAGTCTACGAACCTCACATTCCAACACTTGTCTCAACACACAATTCCTGCTCGCCTGGCTTGCTCTTATTGCCGGGGGAAAGGATCC 2869
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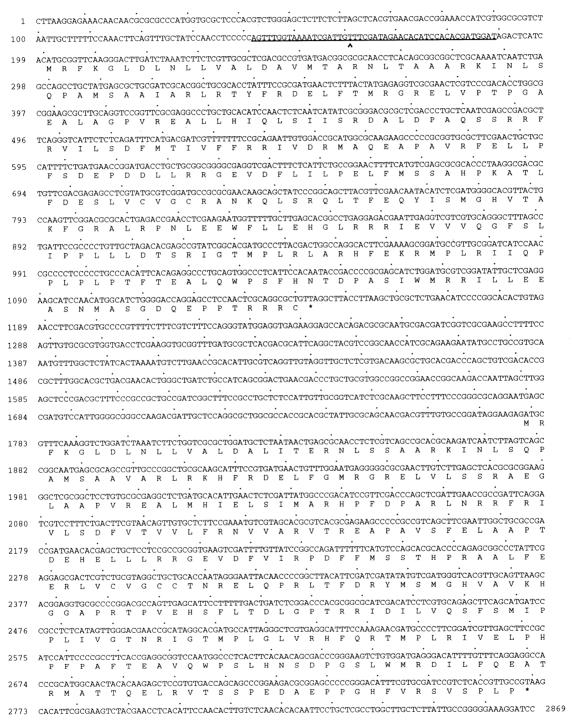
Fig. 2. Nucleotide sequence of a *Bfr*I-*Bam*HI fragment containing the *nodD1* and *nodD2* genes. *nodD1* extends from positions 201 to 1,142, and *nodD2* extends from positions 1,779 to 2,768. The predicted amino acid sequences are given below the nucleotide sequence. The transcriptional start of *nodD1* (Wang and Stacey 1991) is depicted by an arrowhead (position 161). The sequence corresponding to the *nod*-box that precedes *nodY* is underlined (positions 142–189; compare with Fig. 1). The sequence of the first 247 nucleotides shown here was also determined by Nieuwkoop *et al.* (1987).

ICN Biomedicals (Plainview, NY); genistein was from Carl Roth GmbH (Karlsruhe, Germany); and coumestrol was from Eastman Kodak (Rochester, NY). D. A. Phillips (University of California, Davis) provided 4,4'-dihydroxy-2'-methoxychalcone.

Plant infection tests. Soybean seeds (*Glycine max* (L.) Merr. 'Williams') were provided by Jaques Seed Co. (Prescott, WI). Seeds from mung bean (*Vigna radiata* (L.) R. Wilcz.) and *Macroptilium atropurpureum* (Moc. & Sessé ex DC) Urb. 'Siratro' were provided by W. D. Broughton (Université de Genève, Switzerland). The plant infection tests were carried out as described previously (Göttfert *et al.* 1990).

RESULTS

DNA sequence and expression of *nodD1* and *nodD2*. For a detailed molecular analysis of the *B. japonicum nodD* gene region, we determined the nucleotide sequence of a 2,869-bp *Bfr*I-*Bam*HI fragment (Figs. 1,2). The two open reading frames of *nodD1* (nucleotides 201–1,142) and *nodD2* (nucleotides 1,779–2,768) are separated by 633 nucleotides. The predicted molecular weights of the deduced gene products are 35,854 (NodD1) and 37,249 (NodD2). Wang and Stacey (1991) have mapped the transcription start site of *nodD1* 40 nucleotides upstream of the translational start codon and delimited the promoter region to 42 nucleotides further upstream. We compared the promoter region of *nodD1* to the *nodD2* upstream region, but could not identify any significant homology. No secondary structures potentially able to serve as rho-independent transcriptional stop signals were identified downstream of *nodD1* or *nodD2*. The *nodD1-nodD2* intervening sequence was used in a data bank search for homologies. Some similarity was found to DNA of *Bradyrhizobium* sp. *Parasponia*, also located downstream of *nodD* (Scott 1986), but not to DNA of any other organism.

From previous work, it is known that *nodD1* is weakly expressed under free living conditions and that the NodD1 protein activates its own expression about threefold (Banfalvi *et al.* 1988). To analyze if *nodD2* is expressed at all, we introduced plasmid pRJ1248 containing a translational *nodD2'-'lacZ* fusion (Fig. 1) into the *B. japonicum* wild type. However, the β-galactosidase activities obtained were never higher than background values. Therefore, the strain was streaked out on PSY X-Gal medium, which provides a more sensitive, though only qualitative, means of testing gene expression. After incubation at 30° C for about 2 wk, the colonies showed a faint blue color, indicating that *nodD2* was indeed expressed, albeit at a very low level. This expression level was not visibly changed by adding daidzein to the medium or by transferring the plasmid into strain Δ1240, a mutant deleted for both *nodD* copies (Fig. 1). In all these experiments, plasmid pRJ1249, which carries an out-of-frame *nodD2'-'lacZ* fusion, was used as a control; this plasmid did not lead to any detectable β-galactosidase activity.

Amino acid sequence comparisons of the deduced *nodD1* and *nodD2* gene products. Figure 3 shows an amino acid sequence alignment of the two predicted *B. japonicum* NodD proteins. Both NodD proteins share 62% identical

amino acid residues at corresponding positions and are very similar to NodD proteins of other *Azorhizobium*, *Rhizobium*, and *Bradyrhizobium* strains. The highest homology (91.7% identical amino acid residues) was found between NodD1 of *B. japonicum* and NodD of *Bradyrhizobium* sp. *Parasponia* (Scott 1986), and the lowest homology (47.9% identity) was between NodD2 of *B. japonicum* and NodD of *A. caulinodans* (Goethals *et al.* 1990).

Regulation of *nodC* expression by *nodD1* and *nodD2*. Previous work has shown that the *nodC* gene is part of a long *nod* gene operon including *nodY*, *nodA*, *nodB*, *nodC*, *nodS*, *nodU*, and probably *nodI* and *nodJ* as well (Fig. 1; Göttfert *et al.* 1990). Upstream of and divergent from this operon, the two *nodD* homologs were identified (Appelbaum *et al.* 1988a; Göttfert *et al.* 1989). Plasmid pRJ458 (Fig. 1) contains both *nodD* genes and a translational *nodC'-'lacZ* fusion, of which the latter is inducible by daidzein (Göttfert *et al.* 1990). Because we were interested in the contribution of both *nodD* copies to the transcriptional activation of the *nodYABCSUIJ* operon, we constructed derivatives of pRJ458 containing either *nodD1* (pRJ571) or *nodD2* (pRJ582), or neither of them (pRJ566). To avoid any interference of *nod* genes located on plasmids with those located on the chromosome, we transferred pRJ458 and its derivatives into the *B. japonicum* deletion strain Δ1240 (Fig. 1). Measurements of β-galactosidase activities revealed that the presence of *nodD1*, but not of *nodD2*, was responsible for expression of the *nodC* gene (Table 1). The *nodD1*-dependent expression could be further increased by a factor of two to four with the addition of the inducer daidzein. As with daidzein alone, *nodD2* also did not promote *nod* gene expression with a mixture of inducers, consisting of daidzein, genistein, coumestrol, and 4,4'-dihydroxy-2'-methoxychalcone; the latter substance is a potent inducer of *nod* genes in *R. meliloti* (Maxwell *et al.* 1989) and *B. japonicum* (not shown).

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NodD1    1 MRFKGLDLNLLVALDAVMTARNLTAAARKINLSQPAMSAAIARLRTYFRD
           |||||||||||||||||   | |||  ||||||||||||||| |||   |||
NodD2    1 MRFKGLDLNLLVALDALITERNLSSAARKINLSQPAMSAAVARLRKHFRD

NodD1   51 ELFTMRGRELVPTPGAEALAGPVREALLHIQLSIISRDALDPAQSSRRFR
           ||| ||||||||   || || |||||| || |||  |   ||| ||||
NodD2   51 ELFGMRGRELVLSSRAEGLAAPVREALMHIELSIMARHPFDPARLNRRFR

NodD1  101 VILSDFMTIVFFRRIVDRMAQEAPAVRFELLPFSDEPDDLLRRGEVDFLI
           |||| | |   || |  |    ||   ||  ||     || ||||||||| |
NodD2  101 IVLSDFVTVVLFRNVVARVTREAPAVSFELAAPTDEHELLLRRGEVDFVI

NodD1  151 LPELFMSSAHPKATLFDESLVCVGCRANKQLSRQLTFEQYISMGHVTAKF
           |  |||| || | || | |||||||  |  |   ||| | ||||| |
NodD2  151 RPDFFMSSTHPRAALFEERLVCVGCCTNRELQPRLTFDRYMSMGHVAVKH

NodD1  201 GRALRPNLEEWFLLEHGLRRRIEVVVQGFSLIPPLLLDTSRIGTMPLRLA
           | | |  |  ||  |  |||      ||  || || ||||  | |||||||| |
NodD2  201 GGAPRTPVEHSFLTDLGPTRRIDILVQSFSMIPPLIVGTNRIGTMPLGLV

NodD1  251 RHFEKRMPLRIIQPPLPLPTFTEALQWPSFHNTDPASIWMRRILLEEASN
           |||  |||||  |  | |   ||||   |||| |||| || || |||
NodD2  251 RHFQRTMPLRIVELPHPFPAFTEAVQWPSLHNSDPGSLWMRDILFQEATR

NodD1  301 MASGDQEPPTRRRC              314
           ||       |
NodD2  301 MATTQELRVTSSPEDAEPPGHFVRSVSPLP 330
```

Fig. 3. Amino acid sequence alignment of the NodD1 and NodD2 proteins of *Bradyrhizobium japonicum*. Identical amino acids are connected by vertical lines. A putative helix-turn-helix DNA binding motif (Henikoff *et al.* 1988) near the N-terminal end is underlined.

Difficulties encountered during construction of a *nodD1-nodD2* deletion mutant. It was shown previously that a *nodD1* insertion mutant was not drastically reduced in its nodulation ability on soybean (Nieuwkoop *et al.* 1987; Göttfert *et al.* 1989) or on mung bean and siratro (Göttfert *et al.* 1989), and it was proposed that the retained nodulation ability was due to the presence of the second copy of *nodD*. Because *nodD1* has a sandwich position between the *nodYABCSUIJ* cluster and *nodD2*, a disadvantage of insertion of a resistance marker into *nodD1* is that the out-reading promoter of the resistance gene could influence the expression of neighbouring genes and, hence, falsify the outcome of nodulation assays. Therefore, we decided to first construct a deletion spanning both *nodD* genes and to use a corresponding mutant later in complementation assays by providing either *nodD1* or *nodD2 in trans*. However, despite numerous attempts, we were unable to obtain *nodD1-nodD2* deletion mutants, even when more than 2 kb of DNA on both sides of the deleted *nodD* region were offered for marker exchange mutagenesis. An explanation for this failure was then given by our observation that broad host range plasmids, whenever they contained the DNA region downstream of *nodD2* (including *nolA*), showed a more than 100-fold reduced efficiency of conjugational transfer. The same observation was also made independently by Sadowsky *et al.* (1991). These problems made it necessary to delete the region downstream of *nodD2* as well. Because such a deletion removed *nolA*, which was shown to be involved in the genotype-specific nodulation of soybean (Sadowsky *et al.* 1991), we had to find out to what extent the *nolA* region itself contributed to nodulation efficiency. This was tested by complementing strain Δ1240 with plasmid pRJ300, which contained all essential *nod* genes but lacked the *nodUIJ* genes and the *nolA* region (Fig. 1). We have shown previously that the *nodUIJ* genes do not influence nodulation to a detectable level under our assay conditions (Göttfert *et al.* 1990). Strain Δ1240 (pRJ300) nodulated siratro and mung bean like the wild type (Fig. 4), indicating that the *nolA* region was not necessary for an efficient nodulation of these hosts. Whereas nodulation of soybean by Δ1240 (pRJ300) was delayed by about 3 days (not shown), the final nodule number, 21 days after infection, did not differ significantly from the wild type (Fig. 4). Having made sure that the *nolA* region was not essential for nodulation, we then constructed strain Δ1267 (Fig. 1), in which both *nodD* genes and the

nolA region were deleted. The construction of this mutant succeeded without problems. The absence of *nodD1* and *nodD2* in strain Δ1267 was obviously responsible for a strong reduction in nodule number on siratro and especially on mung bean (Fig. 4). Nodulation of soybean was delayed, but this reduced nodule number was compensated 3 wk after infection by numerous nodules on secondary roots.

Complementation of the *nodD1-nodD2* deletion mutant Δ1267 by either *nodD1* or *nodD2*. The strongly impaired nodulation ability of strain Δ1267, at least with siratro and mung bean as hosts, was a useful trait for studying the contribution of each of the two *nodD* copies to the nodulation phenotype in this mutant background. For this purpose, strain Δ1267 was complemented either with the *nodD1*-containing plasmid pRJ1271 or with the *nodD2*-containing plasmids pRJ1272 and pRJ1275 (Fig. 1). From the work of Wang and Stacey (1991), we deduced that pRJ1271 carried the *nodD1* promoter. Although pRJ1272 carried the entire *nodD1-nodD2* intergenic region, we did not know whether the *nodD2* promoter was located therein. In fact, it could not be ruled out a priori that *nodD2* was transcribed by read-through from the *nodD1* promoter. For this reason, plasmid pRJ1275 in which *nodD1* is destroyed by deletion was constructed, and transcription from the *nodD1* promoter into *nodD2* (if it occurs) should remain unaffected (Fig. 1). The results of all complementation assays are presented in Figure 4. The data clearly show that the *nodD1*-carrying plasmid (pRJ1271), but not the *nodD2*-carrying plasmids, could compensate for the nodulation defect of strain Δ1267. To test if the *nod*-box was involved in the residual nodulation ability of Δ1267, we extended the deletion up to the *Bfr*I restriction site located between the *nod*-box and the translational start of *nodY*, which yielded mutant Δ1283 (Fig. 1). However, Δ1283 was still able to nodulate (Fig. 4).

Nodulation phenotype of *nodD1* mutants. The finding that *nodD1*, but not *nodD2*, is essential for efficient nodulation somehow contradicts our previous finding (Göttfert *et al.* 1989) that the *nodD1* mutant strain 341 (Fig. 1) was not as strongly reduced in its nodulation ability. To rule out that this was caused by a potential second-site suppressor activity elsewhere on the genome, we constructed two additional *nodD1* mutant strains. First, the mutated *nodD1* fragment of strain 341 was isolated and used to mutagenize the wild type again (see Materials and Methods). This resulted in strain 585 (Fig. 1), which should

Table 1. Activation of a *nodC'-'lacZ* fusion in *Bradyrhizobium japonicum* by *nodD1* but not by *nodD2*

Plasmid[a]	Genes present on plasmid	Presence of daidzein[b]	β-Galactosidase activity[c] (U)
pRJ458	*nodD2-nodD1-nodYABC'-'lacZ*	+	260 ± 20
pRJ458	*nodD2-nodD1-nodYABC'-'lacZ*	−	82 ± 3.9
pRJ566	*nodYABC'-'lacZ*	+	9.2 ± 0.4
pRJ566	*nodYABC'-'lacZ*	−	8.8 ± 0.4
pRJ571	*nodD1-nodYABC'-'lacZ*	+	314 ± 22
pRJ571	*nodD1-nodYABC'-'lacZ*	−	150 ± 5.6
pRJ582	*nodD2-nodYABC'-'lacZ*	+	8.1 ± 0.7
pRJ582	*nodD2-nodYABC'-'lacZ*	−	7.9 ± 0.5

[a] Strain Δ1240 used.
[b] Daidzein was added to the medium at 5 μm final concentration (+) or omitted (−).
[c] Mean values of five independent cultures; each experiment was done in duplicate. The standard deviation is indicated. β-Galactosidase activity units (U) were calculated as given by Miller (1972).

be isogenic with strain 341, as far as the *nodD1* insertion mutation is concerned. Second, strain Δ586 was constructed, which differs from strain 585 in that it has a deletion of 383 bp of *nodD1* internal DNA immediately downstream of the kanamycin resistance marker (Fig. 1). The results of the nodulation assays with these mutants are depicted in Figure 4. Strain 585, like strain 341, was a little or not delayed on soybean and siratro and showed a delay of about 4 days on mung bean. By contrast, the nodulation of strain Δ586 was considerably reduced on siratro and mung bean and somewhat delayed on soybean.

DISCUSSION

In this study, we present a structural and functional analysis of the *B. japonicum nodD1* and *nodD2* genes. The two predicted NodD proteins, as derived from the nucleotide sequence of the respective genes, shared more than 60% amino acid sequence identity and had all the structural characteristics known for NodD proteins from other rhizobia: a highly conserved N-terminal part containing the helix-turn-helix motif for DNA binding and a less well conserved C-terminal part that was previously implicated in host-specific recognition of flavonoid inducer molecules (Horvath *et al.* 1987; Burn *et al.* 1989).

nodD1 turned out to be the most essential gene for activation at the *nodYABCSUIJ* operon, as documented by three lines of evidence. First, the expression of a plasmid-borne, translational *nodC'-'lacZ* fusion could be activated only when the *nodD1* gene was present on the same plasmid. Second, the *nodD1*-carrying plasmid pRJ1271, but not the *nodD2*-carrying plasmids pRJ1273 and pRJ1275, could compensate the nodulation defect of the *nodD1-nodD2* double mutant Δ1267 by genetic complementation. Third, nodulation activity of the *nodD1* mutant strain Δ586, which suffers an internal deletion of 383 bp, was strongly reduced, in particular with mung bean and siratro as the host plants. Obviously, the presence of an intact second *nodD* copy (*nodD2*) did not help to overcome the nodulation defect in strain Δ586. Similarly, the *nodD1*::Tn5 insertion mutant strain AN314 (Banfalvi *et al.* 1988; provided by G. Stacey) caused a severe nodulation-defective phenotype on mung bean and siratro (not shown).

The aforementioned results are in conflict with the mutant strain 341, which has a kanamycin resistance cassette inserted in *nodD1* and produces only a slightly reduced
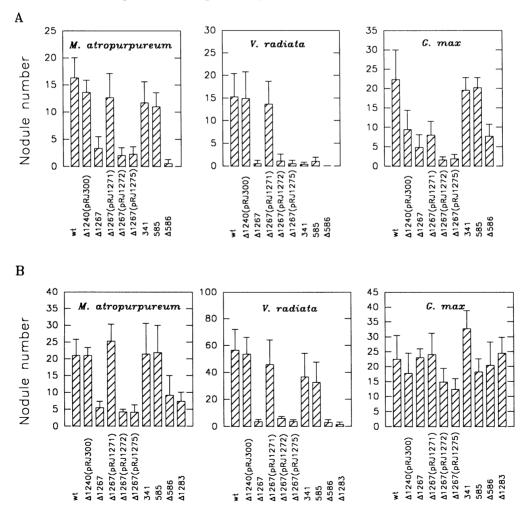
A

B

Fig. 4. Nodulation of three host plants by *Bradyrhizobium japonicum nodD* mutants or plasmid-carrying derivatives thereof. Each data column represents the mean of at least five plants tested and was confirmed by one repetition. A, Data obtained 12 days after inoculation. B, Data obtained 21 days after inoculation.

nodulation efficiency as compared with the wild type (Göttfert et al. 1989). We constructed the same type of mutant again (strain 585) and found that both strains 585 and 341 had the same, only marginally defective nodulation phenotype. At present, we cannot offer a plausible explanation why this is so. One speculation is that in strains 585 and 341, the C-terminal part of NodD1 is still produced (there is a potential GTG start codon within the Tn5 part) and associates with NodD2(?) to give a functional NodD protein. The fact that strain Δ586, which differs from strain 585 only by the deletion of the 3' end of nodD1, is strongly affected in its nodulation ability supports this speculation. We consider this idea as a basis for the design of further experiments, such as deleting nodD2 in strain 585.

An unexpected result was that the nodD1-nodD2 double mutant Δ1267 was still able to nodulate its host plants, albeit with reduced efficiency. Mutants of all rhizobial strains investigated so far, which lack all nodD copies, are completely Nod⁻ (Downie et al. 1985; Innes et al. 1985; Honma and Ausubel 1987; Goethals et al. 1990). This suggests that some residual transcription of the absolutely essential nodABC genes must still go on in strain Δ1267, for which either a third nodD copy or an unidentified promoter activity might be responsible. To test if the nod-box, the most likely target for a putative NodD3 protein, was involved in the residual nodulation ability of Δ1267, we extended the deletion up to the BfrI restriction site located between the nod-box and the translational start of nodY; mutant Δ1283 was yielded. However, even strain Δ1283 was still able to nodulate to some extent, which is evidence that a hypothetical third nodD copy is not responsible for the residual nodulation ability of Δ1267. The validity of this assumption is also supported by our finding that nodD1 or nodD2 gene probes did not give additional signals in hybridization experiments with B. japonicum total DNA. Therefore, we assume that an unidentified promoter activity is responsible for nodABC transcription in strain Δ1267. These experiments also shed some light on the difficulties frequently encountered in working with nod mutants of B. japonicum. It now appears to us as if only minute quantities of the nodA, nodB, and nodC gene products suffice to elicit a substantial nodulation response, in particular with soybean. The extraordinarily high tolerance of the soybean nodulation assay may often obscure subtle phenotypic differences when nod mutants other than nodA⁻ or nodC⁻ strains are tested.

This report shows that B. japonicum USDA110 has two different homologs of nodD. Although nodD1 is essential for maximal nod gene expression, no function could so far be attributed to nodD2. This situation may be typical for rhizobia harbouring two nodD copies, because the nodD2 genes of B. japonicum USDA123 (Appelbaum et al. 1988a), R. fredii USDA191 (Appelbaum et al. 1988b), and Rhizobium sp. NGR234 (Bassam et al. 1986; Cervantes et al. 1990) also do not appear to have a significant function in nod gene induction.

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