NodW Is Essential for Full Expression of the Common Nodulation Genes in *Bradyrhizobium japonicum*

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The *Bradyrhizobium japonicum nodW* gene is required for nodulation of siratro, mung bean, and cowpea roots, but much less so for soybean nodulation. We found that a *nodW* mutant was unable to induce expression of the common *nodD1* and *nodYABCSUIJ* gene operons in the presence of either of the isoflavones genistein and daidzein. Under these conditions, the *nodW* mutant did not produce detectable amounts of the lipooligosaccharide nodulation factors. However, the mutant produced a detectable amount of the wild-type nodulation signal in the presence of soybean seed extracts. Furthermore, the host-specific nodulation phenotype of this mutant could be complemented with a plasmid carrying the *nodYABCS* genes that were expressed under the control of the *tac* promoter, indicating that the nodulation defect in the *nodW* mutant is due to a lack of expression of the common *nod* genes. A *B. japonicum nodD1-nodW* double mutant was virtually Nod⁻ on all plant hosts tested, including soybean. Likewise, *nod* gene expression and nod factor production by this mutant were severely impaired. Our data indicate that in *B. japonicum*, expression of the common nodulation genes in response to plant isoflavones and nodulation of legume hosts are mediated by both *nodD1* and *nodW*.

Additional keyword: symbiosis.

Bacteria of the genera *Rhizobium, Bradyrhizobium,* and *Azorhizobium* are able to interact with legume plant roots, leading to the formation of a new organ, the nitrogen-fixing root nodule. The nodulation (*nod, nol*) genes enable bacteria to induce nodule formation in a host-specific manner (Long 1989; Kondorosi *et al.* 1991a). Nodulation genes can be classified as either "common," such as *nodABC*, which are essential for nodulation, or "host-specific," which are strain- or species-specific and determine the bacterial host range. It has been shown that nodulation genes are required for the synthesis and export of lipooligosaccharide signal compounds that induce diverse responses such as root hair curling and meristem formation on legume roots (Lerouge *et al.* 1990; Spaink *et al.* 1991; Truchet *et al.* 1991; Sanjuan *et al.* 1992; Price *et al.* 1992; Schultze *et al.* 1992; Spaink 1992; Mergaert *et al.* 1993; Carlson *et al.* 1993). Expression of the nodulation genes occurs only in the presence of the plant host and requires 1) a NodD protein, a positive transcriptional regulator; 2) a *nod* box, a conserved DNA sequence that binds NodD and is located upstream of the inducible *nod* operons; and 3) an inducer, usually a flavonoid or an isoflavonoid excreted by the plant root (Györgypal *et al.* 1991; Fisher and Long 1992; Schlaman *et al.* 1992).

Some species of rhizobia possess a single *nodD* gene, whereas others may have as many as three *nodD* homologues. In rhizobial species that have only one *nodD* gene, such as *R. leguminosarum* bv. *viciae* and *A. caulinodans, nodD* mutants are Nod⁻ (Downie *et al.* 1985; Innes *et al.* 1985; Goethals *et al.* 1990), whereas mutations in only one of the three *nodD* genes in *R. meliloti* affect nodulation efficiency in a host-dependent manner (Göttfert *et al.* 1986; Honma and Ausubel 1987; Györgypal *et al.* 1988).

In *B. japonicum* strain USDA110, two copies of *nodD* have been characterized upstream of the common *nod* gene operon *nodYABCSUIJ* (Nieuwkoop *et al.* 1987; Göttfert *et al.* 1992) (Fig. 1). Unlike other rhizobial *nodD* genes, *B. japonicum nodD1* is inducible by isoflavones, such as genistein, and can be specifically induced by glycosyl derivatives of genistein and daidzein (Göttfert *et al.* 1987; Kosslak *et al.* 1988; Banfalvi *et al.* 1988; Smit *et al.* 1992). Mutations within *nodD1* drastically reduce the induction of *nod-lacZ* fusions by isoflavones (Banfalvi *et al.* 1988). Paradoxically, however, *nodD1* mutants still nodulate soybean and other legume hosts with only a small delay (Nieuwkoop *et al.* 1987; Göttfert *et al.* 1992). It was initially proposed that the presence of an active *nodD2* gene in *nodD1* mutants sustains expression of the nodulation genes, but Göttfert *et al.* (1992) recently reported that the *B. japonicum nodD2* gene has only a marginal effect on induction of the *nodYABCSUIJ* operon. Furthermore, a mutant strain deleted for both *nodD* copies still showed nodulation activity, albeit at a much lower rate than the wild-type strain. Efficient nodulation by *nodD1-nodD2* deletion mutants was restored only by genetic complementation with *nodD1,* but not with *nodD2,* which suggests that *nodD2* does not play a significant role in nodulation (Göttfert *et al.* 1992). Therefore, the apparent paradox remained unanswered as to why *nodD1* is critical for *nod* gene induction but not for efficient nodulation, especially since no further copies of *nodD* appear to be present in the *B. japonicum* genome (Göttfert *et al.* 1992).

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The *B. japonicum nodV* and *nodW* genes have been reported to be essential for nodulation of mung bean (*Vigna radiata*), cowpea (*V. unguiculata*), and siratro (*Macroptilium atropurpureum*) but contribute only marginally to nodulation of soybean (*Glycine max*) (Göttfert *et al.* 1990a). The predicted amino acid sequences of NodV and NodW suggest that they are members of a family of two-component regulatory proteins involved in signal transduction in prokaryotes, with NodV showing homology to the sensor subclass of proteins and NodW belonging to the transcriptional activator subclass.

In this work, we report that in concert with *nodD1, nodW* is essential for maximal induction of the common *nodYABCSUIJ* and *nodD1* operons in the presence of either of the isoflavones daidzein and genistein. Furthermore, the *nodD1* and *nodW* genes together are essential for nodulation, since *B. japonicum* strains in which *nodD1* and *nodW* were mutated virtually lost their ability to nodulate all of the plant hosts tested.

RESULTS

NodW is essential for induction of the common *nod* genes in response to isoflavones.

The rhizobial common *nod* genes have been reported to be involved in the synthesis of lipooligosaccharide signal compounds which induce root hair curling and cortical cell division (reviewed by Spaink [1992]). Variations of a basic, chitin-like structure seem to involve the action of host-specific *nod* gene products. Since the *nodV* and *nodW* genes have been reported to be determinants of host specificity in *B. japonicum* (Göttfert *et al.* 1990a), we investigated the possible role of these genes in Nod factor biosynthesis. Butanol extracts of ^{14}C-labeled cultures of the *nodW* mutant strain Bj613 grown in the presence or absence of isoflavones were analyzed by reverse-phase thin-layer chromatography (TLC). We found that the *nodW* mutant failed to produce detectable amounts of the Nod metabolite upon induction with either of the isoflavones genistein and daidzein (Fig. 2), but it produced a significant amount of the wild-type compound when induced with soybean seed extracts (Smit *et al.* 1992). These results suggest that, in the presence of genistein and daidzein, NodW is required for the expression of the proteins involved in the biosynthesis of the Nod metabolite. A *nodY'-'lacZ* or a *nodD1'-'lacZ* fusion was introduced into this strain and assayed for β-galactosidase activity in the presence of isoflavones. No induction of these fusions was observed in the presence of genistein or daidzein, which are strong inducers in the wild-type strain (Table 1). However, a sixfold increase in the β-galactosidase activity of the two *nod'-'lacZ* fusions was observed in the presence of soybean seed extracts (Table 1). The induction of *nod* genes in a *nodW* mutant strain was similar to that in a *nodD1* strain, BjΔ586.

In view of the requirement of the *nodW* gene for induction of the common *nod* genes, we speculated that constitutive expression of these genes should overcome the host-specific nodulation defect of strain Bj613. Plasmid pRJ570, which carries the *nodABCS* genes under the control of the *tac* promoter (see Fig. 1), was transferred into strain Bj613, and the resulting strain was tested for nodulation of cowpea, siratro, and mung bean roots. As shown in Table 2, plasmid pRJ570 was partially capable (on *V. radiata*) or fully capable (on *V. unguiculata* and *M. atropurpureum*) of complementing the

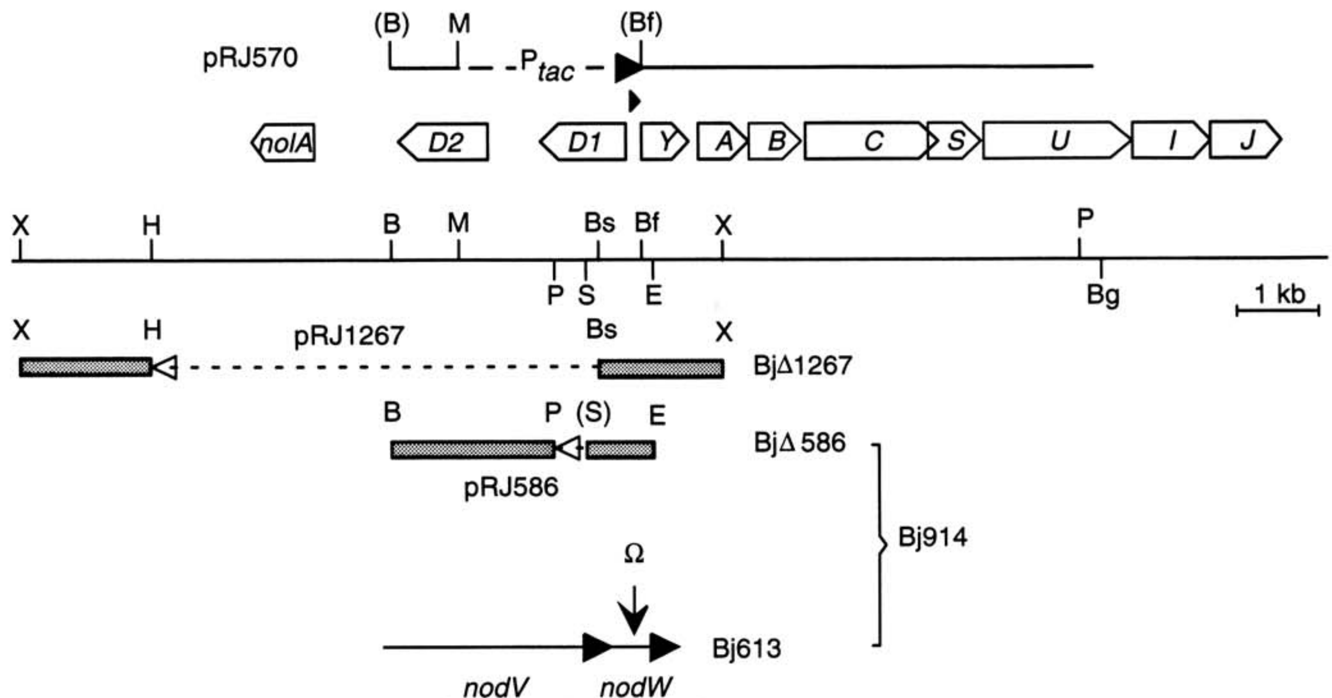

Fig. 1. Physical and genetic map of the *Bradyrhizobium japonicum* regions encoding *nod* gene regulators. The arrowhead between *nodD1* and *nodY* marks the orientation of the *nod* box. Restriction sites in parentheses were lost during the cloning procedure. Mutants are indicated by designations starting with Bj. The extent of deletions is marked by dashed lines. The presence and direction of an out-reading promoter is depicted by an arrowhead. The fragments used for the recombination event are indicated by shaded boxes. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; P, *Pst*I; X, *Xho*I; Bs, *Bsp*HI; Bf, *Bfr*I; M, *Mlu*I; Bg, *Bgl*II; Δ, deletion.

nodW mutant strain for nodulation of these plant hosts, indicating that the Nod⁻ phenotype of strain Bj613 is due to a lack of expression of the common *nod* genes.

To further investigate the role of *nodW* in *nod* gene expression and plant nodulation efficiency, a *nodD1-nodW* double mutant, strain Bj914, was generated (Fig. 1). As expected, this mutant was unable to induce a *nodY'*- or a *nodD1'-'lacZ* fusion in the presence of genistein or daidzein. However, it still showed a two- to threefold induction in the presence of soybean seed extracts (Table 1). Consistent with this result was the production of minimum amounts of Nod metabolites by this mutant in the presence of soybean seed extracts, which could only be observed after overexposure of the TLC plates (Fig. 2).

NodD1 and NodW are required for nodulation of legume hosts by *B. japonicum*.

With the exception of the *nodA, nodB,* and *nodC* genes, no other *B. japonicum* gene has been found to be absolutely essential for soybean nodulation. In some *Rhizobium* species *nodD* genes are essential for nodulation and *nod* gene expression, but in *B. japonicum nodD* genes are not absolutely required for either of these processes. Moreover, genes like *nodW,* which is essential for nodulation of alternate hosts such as siratro, cowpea, and mung bean, seem to play only a minor role in soybean nodulation (Göttfert *et al.* 1990a). The presence of overlapping regulatory circuits (i.e., NodD and NodW) could explain the dispensable nature of NodD or NodW for nodulation of specific hosts. This hypothesis would predict that strain Bj914 (*nodD1-nodW*) should be Nod⁻ on all plant hosts. In fact, strain Bj914 was unable to nodulate cowpea, mung bean, or siratro roots, as it lacks a functional *nodW* gene (Table 2) (Göttfert *et al.* 1990a). The ability of strain Bj914 (*nodD1-nodW*) to nodulate soybean was severely affected. Three weeks after inoculation, 80% of the plants inoculated with this strain had developed an average of only two nodules, compared with 26 nodules per plant induced by Bj613 (*nodW*) and 25 by the wild-type strain (Table 2). These results are in agreement with the *nod* gene expression data and also with the results concerning the production of Nod metabolites by strain Bj914 (Fig. 2 and Table 2).

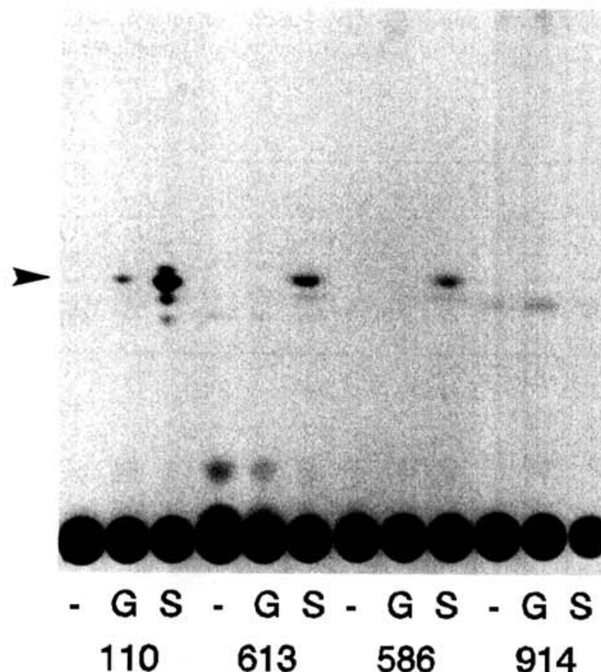

Fig. 2. Autoradiogram of ^{14}C-labeled Nod metabolites produced by *Bradyrhizobium japonicum* wild-type and mutant strains, in the absence (–) or presence of the isoflavone genistein (G) or soybean seed extracts (S). The samples were chromatographed on a reverse-phase thin-layer chromatography plate as described in Materials and Methods. Similar production of Nod factors is observed using daidzein instead of genistein (not shown). The arrowhead indicates the Nod metabolite produced by the wild-type strain (see text), which is missing in some of the mutants. 110, Wild-type strain; 613, Bj613 (*nodW*); 586, BjΔ586 (*nodD1*); 914, Bj914 (*nodW-nodD1*).

DISCUSSION

Current information concerning the regulation of *nod* gene expression in *Rhizobium* and *Bradyrhizobium* specifies that expression of the nodulation genes is induced by the presence of host-produced flavonoids and requires NodD and its corresponding DNA binding site, the *nod* box (Györgypal *et al.* 1991; Fisher and Long 1992; Schlaman *et al.* 1992). In this study, we report that, in addition to NodD1, NodW is required for induction of the *B. japonicum* nodulation genes in response to isoflavones. A *nodW* mutant was unable to induce a *nodY'-'lacZ* or a *nodD1'-'lacZ* fusion in the presence of either of the isoflavones genistein and daidzein, strong inducers of *nod* gene expression in wild-type *B. japonicum* (Göttfert *et al.* 1987; Kosslak *et al.* 1988; Banfalvi *et al.* 1988). Under the same conditions, this mutant produced no detectable amounts of a lipooligosaccharide Nod metabolite previously characterized in the wild-type strain USDA110, whose synthesis requires the expression of the *nodYABCSUIJ* operon (Sanjuan *et al.* 1992). In the presence of soybean seed extracts (Smit *et al.* 1992), strain Bj613 (NodW⁻) showed a sixfold increase in *nod* gene expression and also produced fairly large amounts of Nod factor. Furthermore, the host-specific phenotype of strain Bj613 could be reversed upon transfer of a plasmid carrying the *nodABCS* genes expressed constitutively, demonstrating that the host-specific phenotype exhibited by this mutant is due to a lack of expression of the common *nod* genes. The fact that plasmid pRj570 was only

Table 1. Expression of *nod'-'lacZ* fusions in *Bradyrhizobium japonicum* wild-type and mutant strains

	Activity (β-galactosidase units)[a] induced by:			
	No inducer	Daidzein	Genistein	SSE[b]
nodY'-'lacZ				
USDA110 (wild type)	8	50	650	934
Bj613 (*nodW*)	8	11	12	46
BjΔ586 (*nodD1*)	5	12	14	37
Bj914 (*nodD1, nodW*)	9	10	12	25
nodD1'-'lacZ				
USDA110 (wild type)	14	14	25	50
Bj613 (*nodW*)	7	9	10	38
BjΔ586 (*nodD1*)	10	9	10	33
Bj914 (*nodD1, nodW*)	11	8	9	23

[a] Mean values of three independent determinations with a standard deviation of less than 15%.
[b] Soybean seed extracts.

partially capable of complementing strain Bj613 for nodulation of *V. radiata* indicates that efficient nodulation of this host by *B. japonicum* may require genes located downstream of *nodS* or perhaps unknown genes regulated by *nodW*. These results are consistent with the fact that *nodW* mutants are able to nodulate soybean with relatively high efficiency, although they have lost the ability to nodulate other plant hosts (Göttfert *et al.* 1990a). The question, however, is why a *nodW* mutant exhibits a Nod⁻ phenotype on hosts like mung bean, cowpea, and siratro, but only a $\text{Nod}^{\text{delayed}}$ phenotype on soybean. One explanation could be that plants like siratro, mung bean, and cowpea produce signal molecules that are not able to lead to high *nod* gene expression in the presence of NodD1 alone. Thus, in a *nodW* mutant the level of Nod factor may be too low to give rise to the formation of nodules in those hosts. Soybean, however, may supply inducers (daidzein, genistein, and other substances present in soybean seed extracts) that could cause sufficiently high Nod factor levels with NodD1 alone. Alternatively, nodulation of soybean may require a lower amount of Nod factor than infection of the other plant hosts.

Results obtained with the *nodD1-nodW* double mutant strain Bj914 demonstrate that, for the most part, *nodW* is responsible for the ability of strain BjΔ586 (*nodD1*) to nodulate legume hosts (Göttfert *et al.* 1992). Similar conclusions can be drawn for the role of *nodD1* in a *nodW* mutant background. The residual *nod* gene expression and nodulation detected in strain Bj914 could be explained by the presence of *nodD2* (Göttfert *et al.* 1992). These data clearly demonstrate that in *B. japonicum* USDA110, both *nodD1* and *nodW* genes account for most of the expression of the *nodYABCSUIJ* and *nodD1* operons in response to isoflavones and also for the nodulation activity on all of the compatible hosts. Our results also show that in *B. japonicum* there is no correlation between *nod* gene induction levels and nodulation efficiency on soybean roots. Although conclusive data are still missing, this discrepancy between nodulation efficiency on soybean and *nod* gene induction levels may indicate the existence of additional complexity in the regulation of *nod* gene expression in *B. japonicum*, i.e., negative regulation (T. Dockendorff and G. Stacey, unpublished results), like that reported for *R. meliloti* (Kondorosi *et al.* 1989, 1991b).

The *nodV* and *nodW* genes belong to the prokaryotic family of two-component regulatory systems involved in a variety of signal transduction pathways (Göttfert *et al.* 1990a). Mutations in either of these two genes result in the complete loss of nodulation activity in certain plant hosts, such as siratro, mung bean, and cowpea, but have little effect on soybean nodulation. The unusual involvement of the NodV and NodW proteins in nodulation by *B. japonicum* has led to the proposal of a unique mode of *nod* gene regulation that differs from the regulation of nodulation genes in other rhizobial species. It has been proposed that NodV may be a membrane-bound protein that senses unknown signals from the plant and transduces the signal to NodW, which in turn activates the transcription of genes involved in host specificity (Göttfert *et al.* 1990a). The current results further support the initially proposed mechanism of action for NodVW. In addition, our results demonstrate that among the genes directly or indirectly regulated by NodW are the common nodulation genes. The possibility that other, yet unknown, genes may also be under the control of NodW is not ruled out. Also, the possibility that NodW may be involved in modifying plant signals required for NodD activation of *nod* promoters should be considered. Although further biochemical work is needed to demonstrate interaction of NodW with *nod* promoters, it is attractive to speculate that NodW may recognize specific sequences associated with *nod* genes. The involvement of both *nodD1* and *nodW* in the activation of *nod* gene expression raises the question of how these two proteins may interact with *nod* promoters, and perhaps with each other, to promote *nod* transcription. *Rhizobium* NodD proteins have been shown to bind to the so-called *nod* box, upstream of the *nod* gene operons. Although direct supporting data are missing, it is believed that NodD interacts with plant flavonoids, resulting in a conformational change that promotes transcription of the *nod* operons (Györgypal *et al.* 1991; Fisher and Long 1992; Schlaman *et al.* 1992). This model may also be valid for *B. japonicum* NodD proteins, but further complexity is added by the role of NodW. Indeed, NodW accounts for most of the *nod* gene induction and the nodulation activity in NodD1⁻ strains, which demonstrates that in the wild-type strain both proteins are needed for high levels of *nod* gene expression and maximum nodulation efficiency. The proposed role of NodV as a sensor of plant signals adds another point of complexity of *nod* gene regulation in *B. japonicum.* It will be of interest to see in which way the signal molecules for NodV differ from those for NodD.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The wild-type strain *B. japonicum* USDA110 or its derivative 110*spc*4 and mutant derivatives (see Fig. 1) were grown in PSY medium (Regensburger and Hennecke 1983) or RDY medium (Nieuwkoop *et al.* 1987) at 30° C. *Escherichia coli*

Table 2. Nodulation behavior of *Bradyrhizobium japonicum* 110*spc*4 (wild-type strain) and mutant derivatives

		Average number of nodules per plant[a]			
Strain	**Relevant characteristics**	***Vigna unguiculata***	***Macroptilium atropurpureum***	***Vigna radiata***	***Glycine max***
110*spc*4	Wild type	19 ± 9	17 ± 6	16 ± 7	25 ± 4
Bj613	*nodW*	0	0	0	26 ± 9
613 (pRJ570)	*nodW*; P*tacnodABCS*	17 ± 8	12 ± 5	2 ± 2	NT
Bj914	*nodD1-nodW*	0[b]	NT	NT	2 ± 2[c]

[a] Mean values from at least 20 plants in at least two independent tests. *G. max* was tested 2 wk after inoculation; the other plants were tested 3 wk after inoculation. NT, not tested.
[b] Values from 10 plants.
[c] No nodules on 20% of the plants.

strains were grown in Luria-Bertani medium at 37° C.

Strain Bj613 carries an Ω insertion in *nodW* (Göttfert *et al.* 1990a). Strain BjΔ586 is a *nodD1* mutant in which the DNA between the *Sal*I and *Pst*I sites within *nodD1* has been removed (Göttfert *et al.* 1992) (Fig. 1). Strain Bj914 combines the mutations of strains Bj613 and BjΔ586; it was generated by introducing plasmid pRJ586 (Göttfert *et al.* 1992) (Fig. 1) into strain Bj613 and selecting for double-homologous recombination. All of the mutations were verified by Southern blot hybridization.

Plasmid pRJ570 (Fig. 1) is a derivative of pRJ300, which carries an 8-kb insert containing the *nod* region from the end of *nodD2* to a *Sau*3A1 restriction site between the *Pst*I and the *Bgl*II sites within *nodU* (Göttfert *et al.* 1990a,b). The DNA between the *Mlu*I site within *nodD2* and the *Bfr*I site located at the 5′ end of *nodY* (Fig. 1) was replaced by the *Mlu*I-*Bam*HI fragment of plasmid pJF118HE (Fürste *et al.* 1986), which contains the *tac* promoter. The ends were made compatible by linker addition. The *tac* promoter reads toward *nodABCS,* and hence these genes are constitutively expressed.

Plant infection tests.

Soybean seeds (*G. max* (L.) Merr. cv. Williams) were provided by Jacques Seed Company, Prescott, WI. Cowpea (*V. unguiculata* (L.) Walp. cv. Red Caloona) and mung bean (*V. radiata* (L.) R. Wilcz.) seeds were provided by Wright Stephenson & Co., Seven Hills, Australia. Seeds from *M. atropurpureum* (Moc. & Sessé ex DC.) Urb. cv. Siratro were kindly provided by W. D. Broughton (Université de Genève, Geneva, Switzerland). Infection tests were carried out essentially as described previously (Göttfert *et al.* 1990a,b).

β-Galactosidase assays.

A *nodY′-′lacZ* or a *nodD1′-′lacZ* fusion, carried by plasmids pZB32 and pZB22, respectively (Banfalvi *et al.* 1988), was introduced into the wild-type and mutant strains by conjugation in triparental matings, with pRK2013 used as helper plasmid (Ditta *et al.* 1980), or by electroporation as described previously (Hattermann and Stacey 1990). Assays for β-galactosidase activity in the absence or presence of the isoflavones genistein (at a final concentration of 2 μM) and daidzein (2 μM) or soybean seed extracts, obtained as described previously (Smit *et al.* 1992), were done essentially as described by Banfalvi *et al.* (1988).

Analysis of Nod factor production by TLC.

Detection of ^{14}C-labeled Nod metabolites by reverse-phase TLC was performed as described previously (Spaink *et al.* 1992; Sanjuan *et al.* 1992).

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LITERATURE CITED

Banfalvi, Z., Nieuwkoop, A., Schell, M. G., Besl, L., and Stacey, G. 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum.* Mol. Gen. Genet. 214:420-424.

Carlson, R. W., Sanjuan, J., Bhat, U. R., Glushka, J., Spaink, H. M., Wijfjes, A. H., van Brussel, A., Stokkermans, T., Peters, N. K., and Stacey, G. 1993. The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type-I and type-II strains of *Bradyrhizobium japonicum.* J. Biol. Chem. 268:8372-8381.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti.* Proc. Natl. Acad. Sci. USA 77: 7347-7351.

Downie, J. A., Knight, C. D., Johnston, A. W. B., and Rossen, L. 1985. Identification of genes and gene products involved in the nodulation of peas by *Rhizobium leguminosarum.* Mol. Gen. Genet. 198:255-262.

Fisher, R. F., and Long, S. R. 1992. *Rhizobium*-plant signal exchange. Nature (London) 357:655-660.

Fürste, J. P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M., and Lanka, E. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene 48:119-131.

Goethals, K., van den Eede, G., Van Montagu, M., and Holters, M. 1990. Identification and characterization of a functional *nodD* gene in *Azorhizobium caulinodans.* J. Bacteriol. 172:2658-2666.

Göttfert, M., Grob, P., and Hennecke, H. 1990a. Proposed regulatory pathway encoded by the *nodV* and *nodW* genes, determinants of host specificity in *Bradyrhizobium japonicum.* Proc. Natl. Acad. Sci. USA 87:2680-2684.

Göttfert, M., Hitz, S., and Hennecke, H. 1990b. Identification of *nodS* and *nodU,* two inducible genes inserted between the *Bradyrhizobium japonicum nodYABC* and *nodIJ* genes. Mol. Plant-Microbe Interact. 3: 308-316.

Göttfert, M., Holzhäuser, D., Bäni, D., and Hennecke, H. 1992. Structural and functional analysis of two different *nodD* genes in *Bradyrhizobium japonicum* USDA110. Mol. Plant-Microbe Interact. 5:257-265.

Göttfert, M., Horvath, B., Kondorosi, E., Putnoky, P., Rodriguez-Quinones, F., and Kondorosi, A. 1986. At least two *nodD* genes are necessary for efficient nodulation of alfalfa by *Rhizobium meliloti.* J. Mol. Biol. 191:411-420.

Göttfert, M., Weber, J., and Hennecke, H. 1987. Induction of a *nodA-lacZ* fusion in *Bradyrhizobium japonicum* by an isoflavone. J. Plant Physiol. 132:394-397.

Györgypal, Z., Iyer, N., and Kondorosi, A. 1988. Three regulatory *nodD* alleles of diverged flavonoid-specificity are involved in host-dependent nodulation by *Rhizobium meliloti.* Mol. Gen. Genet. 212: 85-92.

Györgypal, Z., Kiss, G. B., and Kondorosi, A. 1991. Transduction of plant signal molecules by the *Rhizobium* NodD proteins. Bioessays 13: 575-581.

Hattermann, D., and Stacey, G. 1990. Efficient DNA transformation of *Bradyrhizobium japonicum* by electroporation. Appl. Environ. Microbiol. 56:833-836.

Honma, M. A., and Ausubel, F. M. 1987. *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. Proc. Natl. Acad. Sci. USA 84:8558-8562.

Innes, R. W., Kuempel, P. L., Plazinski, J., Canter-Cremers, H., Rolfe, B. G., and Djordjevic, M. A. 1985. Plant factors induce expression of nodulation and host-range genes in *Rhizobium trifolii.* Mol. Gen. Genet. 201:426-432.

Kondorosi, E., Gyuris, J., Schmidt, J., John, M., Duda, E., Hoffman, B., Schell, J., and Kondorosi, A. 1989. Positive and negative control of *nod* gene expression in *Rhizobium meliloti* is required for optimal nodulation. EMBO J. 8:1331-1340.

Kondorosi, A., Kondorosi, E., John, M., Schmidt, J., and Schell, J. 1991a. The role of the nodulation genes in bacterium-plant communication. Genet. Eng. 13:115-136.

Kondorosi, E., Pierre, M., Cren, M., Haumann, U., Buire, M., Hoffman, B., Schell, J., and Kondorosi, A. 1991b. Identification of *nolR,* a negative transacting factor controlling the *nod* regulon in *Rhizobium meliloti.* J. Mol. Biol. 222:885-896.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E., and Applebaum, E. A. 1988. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max.* Proc. Natl. Acad. Sci. USA 84:7428-7432.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Denarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligo-

saccharide signal. Nature (London) 344:781-784.

Long, S. R. 1989. *Rhizobium*-legume nodulation: Life together in the underground. Cell 56:203-214.

Mergaert, P., Van Montagu, M., Promé, J. C., and Holsters, M. 1993. Three unusual modifications, a D-arabinosyl, an *N*-methyl, and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571. Proc. Natl. Acad. Sci. USA 90:1551-1555.

Nieuwkoop, A. J., Banfalvi, Z., Deshmane, N., Gerhold, D., Schell, M. G., Sirotkin, K. M., and Stacey, G. 1987. A locus encoding host-range is linked to the common nodulation genes of *Bradyrhizobium japonicum*. J. Bacteriol. 169:2631-2638.

Price, N. P. J., Relic, B., Talmont, F., Lewin, A., Promé, D., Pueppke, S. G., Maillet, F., Denarié, J., Promé, J. C., and Broughton, W. J. 1992. Broad-host-range *Rhizobium* species strain NGR234 secretes a family of carbamoylated, and fucosylated, nodulation signals that are *O*-acetylated or sulphated. Mol. Microbiol. 6:3575-3584.

Regensburger, B., and Hennecke, H. 1983. RNA polymerase from *Rhizobium japonicum*. Arch. Microbiol. 135:103-109.

Sanjuan, J., Carlson, R. W., Spaink, H. P., Bhat, U. R., Barbour, W. M., Glushka, J., and Stacey, G. 1992. A 2-*O*-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. Proc. Natl. Acad. Sci. USA 89:8789-8793.

Schlaman, H. R., Okker, R. J., and Lugtenberg, B. J. J. 1992. Regulation of nodulation gene expression by NodD in rhizobia. J. Bacteriol. 174: 5177-5182.

Schultze, M., Quiclet-Sire, B., Kondorosi, E., Virelizier, H., Glushka, J., Endre, G., Gero, S., and Kondorosi, A. 1992. *Rhizobium meliloti* produces a family of sulfated lipo-oligosaccharides exhibiting different degrees of plant host specificity. Proc. Natl. Acad. Sci. USA 89:192-196.

Smit, G., Puvanesarajah, V., Carlson, R. W, Barbour, W. M., and Stacey, G. 1992. *Bradyrhizobium japonicum nodD1* can be specifically induced by soybean flavonoids that do not induce the *nodYABCSUIJ* operon. J. Biol. Chem. 267:310-318.

Spaink, H. P. 1992. Rhizobial lipo-oligosaccharides: Answers and questions. Plant Mol. Biol. 20:977-986.

Spaink, H. P., Aarts, A., Stacey, G., Bloemberg, G. V., Lugtenberg, B. J. J., and Kennedy, E. P. 1992. Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. Mol. Plant-Microbe Interact. 5:72-80.

Spaink, H. P., Sheeley, D. M., van Brussel, A., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host-specificity of *Rhizobium*. Nature (London) 354:125-130.

Truchet, G., Rouche, P., Lerouge, P., Vasse, J., Camut, S., De Billy, F., Promé, J. C., and Denarié, J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature (London) 351:670-673.