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Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant-signal, interacting with the *nodD* gene product

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We have identified a *nodD* gene from the wide host-range *Rhizobium* strain MPIK3030 (termed *nodD*1) which is essential for nodulation on *Macroptilium atropurpureum* (siratro). Experiments with *nodA–lacZ* gene fusions demonstrate that the MPIK3030 *nodD*1 regulates expression of the *nodABC* genes. Additionally, we used *nodC–lacZ* fusions of *Rhizobium meliloti* to show that the MPIK3030 *nodD*1 gene induces expression of these fusions by interacting with plant factors from siratro and from the non-host *Medicago sativa* (alfalfa). The *R. meliloti nodD* genes, however, only interact with alfalfa exudate. In line with these results, no complementation of MPIK3030 *nodD*1 mutants could be obtained on siratro with the *R. meliloti nodD* genes, while the MPIK3030 *nodD*1 can complement *nodD* mutants of *R. meliloti* on alfalfa. Furthermore, *R. meliloti* transconjugants harbouring the MPIK3030 *nodD*1 efficiently nodulate the illegitimate host siratro. When compared with other *nodD* sequences, the amino acid sequence of the MPIK3030 *nodD*1 shows a conserved amino-terminus, whereas the carboxy-terminus of the putative gene product diverges considerably. Studies on a chimeric MPIK3030/*R. meliloti nodD* gene indicates that the carboxy-terminal region is responsible for the interaction with plant factor(s) and may have evolved in different rhizobia specifically to interact with plant–host factors.

Key words: lacZ-fusions/gene regulation/host specificity/nodulation/*Rhizobium*

Introduction

The lack of naturally occurring nitrogen in most soils makes the symbiotic association of the Gram-negative bacterium *Rhizobium* with plants belonging to the Leguminosae ecologically as well as economically very important. For the development of a successful partnership between *Rhizobium* and legume, numerous genes on both sides interact in a multi-phase process culminating in the fixation of atmospheric nitrogen by the bacteroids in root nodules on the plant.

Among the genes found to be directly involved in symbiotic nitrogen fixation in fast-growing rhizobia (*fix, nif, nod* and *hsn*) (Johnston *et al.*, 1978; Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Hooykaas *et al.*, 1981), the common and host-specific nodulation genes are those which play an active part in the earliest stages of the infection process (Kondorosi *et al.*, 1984).

Nucleotide sequence data and interspecies complementation experiments of common *nod* genes from a series of *Rhizobium* species and their mutant derivatives, have not only revealed a high level of functional and structural conservation in the organization of the *nodABC* and *D* genes, but have also established that these genes are essential for nodulation irrespective of the plant host (Török *et al.*, 1984; Rossen *et al.*, 1984; Bachem *et al.*, 1985; Jacobs *et al.*, 1985; Egelhoff *et al.*, 1985; Putnoky and Kondorosi, 1986; Schofield and Watson, 1986; Scott, 1986).

In *R. meliloti* three *nodD* copies have been located on the Sym plasmid, two of which (designated *nodD*1 and *nodD*2) are necessary for efficient nodulation of alfalfa (*Medicago sativa*) (Göttfert *et al.*, 1986). The *nodD* genes from several *Rhizobium* species (*R. meliloti, R. leguminosarum* and *R. trifolii*) have been shown to regulate positively the expression of the nodulation genes in the presence of flavonoid compounds found in root exudates (Firmin *et al.*, 1986; Peters *et al.*, 1986; Redmond *et al.*, 1986). Although specific flavonoids seem to predominate in particular *Rhizobium*–plant interactions (e.g. luteolin in *R. meliloti*/alfalfa; Peters *et al.*, 1986) no host specificity of the plant signal has so far been reported.

In *R. meliloti* a cluster of host-specific nodulation genes has been located close to the common *nod* genes (*hsnABC* and the divergently transcribed *hsnD*) (Horvath *et al.*, 1986). These genes have otherwise been described as *nodFEG* and *H*, respectively (Rostas *et al.*, 1986; Debelle and Sharma, 1986). In *R. leguminosarum* and *R.trifolii* genes analogous to *hsnAB* have also been found (termed *nodFE*, respectively), and are believed to be involved in the modification of *Rhizobium* surface components (Shearman *et al.*, 1986; Schofield and Watson, 1986). Furthermore, a conserved sequence upstream of all putative transcriptional units containing nodulation genes from *R. meliloti* (*nod*-box) was shown to be essential for the expression of the

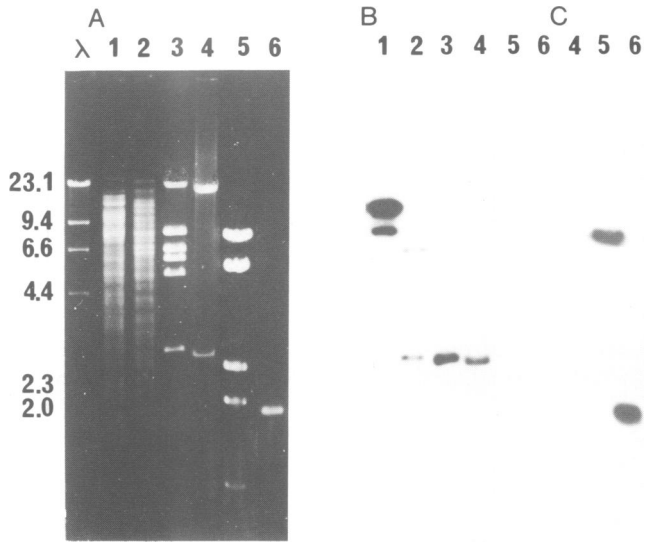

Fig. 1. Hybridization of ^{32}P-labelled *nodD*1 fragment from *R. meliloti* (**B**) *nod*-box (**C**) to the following *Eco*RI-digested DNA (**A**). **Lane 1** total DNA of AK631; **lane 2**, total DNA of MPIK3030; **lane 3**, pCB5001; **lane 4**, pBH264; **lane 5**, pCB1223, **lane 6**, isolated *Pst*I fragment of pCB1223 carrying the *nodA* gene of MPIK3030.

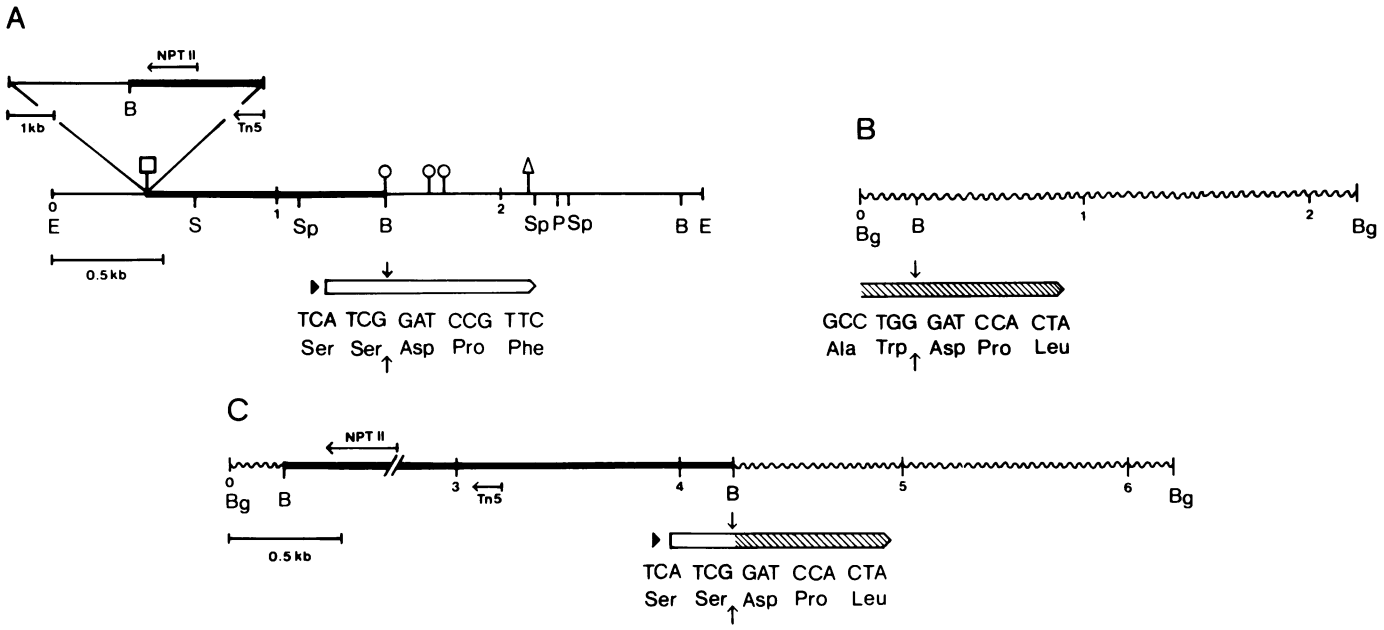

Fig. 2. Physical map of the 2.9-kb *Eco*RI fragment, carrying the MPIK3030 *nodD*1 gene (**A**), and construction of a chimeric *nodD* gene. The horizontal line (**A**) shows the 2.9-kb *Eco*RI fragment from MPIK3030. Nod phenotypes of Tn*5* insertion are indicated as: Nod$^-$ (⚲), delayed Nod$^-$ (⚲) and Nod$^+$(⚲). The region of DNA between the *Bam*HI site in the Nod$^+$ (⚲) Tn*5* insertion and the *Bam*HI site of the MPIK3030 *nodD*1 gene (heavy line) carrying the NPTII gene of Tn*5* and the coding region for 60 amino acids of the MPIK3030 *nodD*1 gene was cloned into the *Bam*HI site (**B**) present in the 2.2-kb *Bgl*II fragment (wavy line) carrying a promoterless *R. meliloti nodD*1 gene (pKSK5) without an initiation codon. The resulting 6.2-kb *Bgl*II fragment represented below (**C**) contains a gene fusion at the *Bam*HI site. The location of coding regions on both MPIK3030 and *R. meliloti* fragments is indicated by the large arrows (open and shaded arrows, respectively). The nucleotide and amino-acid sequence below the arrows show the precise location of the *Bam*HI site indicated by vertical arrows. Other symbols used are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P. *Pst*I; S, *Sal*I; Sp, *Sph*I; and ▶*nod*-box sequence.

Table I. Complementation and host-range extension of wild-type and *nodD* mutants of *R. meliloti* and MPIK3030 with plasmids carrying different *nodD* genes on siratro and alfalfa plants

	Plasmids					
	pBH264 (MPIK3030 *nodD*1)		pKSK5 (*R. meliloti nodD*1)		pBH256 (chimeric *nodD*)	
	Siratro	Alfalfa	Siratro	Alfalfa	Siratro	Alfalfa
AK631	+	+	−	+	−	+
PP659[a]	+[b]	+	−	+	−[b]	+
MPIK3030	+	−	+	−	+	−
BC166	+[b]	−	−	−	−[b]	−

[a]For nodulation kinetics see Figure 4A,B.
[b]Photographs of these phenotypes are shown in Figure 5.

downstream genes and is likely to be involved in the coordinate regulation of *nod* genes (Rostas *et al.*, 1986).

MPIK3030, a derivative of the wide host-range *Rhizobium* strain NGR234 (Trinick, 1980), is capable of nodulating siratro (*Macroptilium atropurpureum*), but not alfalfa. This strain contains Sym-plasmid-borne common *nodABC* genes (Bachem *et al.*, 1985; Djordjevic *et al.*, 1985) and two copies of *nodD* (Rodriguez-Quinones *et al.*, 1986). We have reported the isolation of a siratro-specific host-range region from the Sym-plasmid of MPIK3030, by assaying for nodulation on siratro, inoculated with an *R. meliloti* population containing a conjugally introduced pLAFR1 clone bank of MPIK3030 (Bachem *et al.*, 1986). A similar host-range region was also isolated from MPIK3030 by Broughton *et al.* (1976) and from NGR234 by Bassam *et al.* (1986). In MPIK3030 three separate regions involved in host-range extension were mapped on an 11.4-kb region of the plasmid pCB507, one of which was shown to be essential for host-range extension of *R. meliloti* to the non-host siratro.

In this paper we report on a *nodD* gene from MPIK3030 which is essential for nodulation on siratro and is able to suppress *nodD* mutations in *R. meliloti*. Furthermore, this gene is able additively to extend the host range of *R. meliloti* transconjugants to include siratro. We also provide evidence that, while the *R. meliloti nodD* genes cannot substitute the MPIK3030 *nodD* due to their inability to be activated by siratro plant-factor, the MPIK3030 *nodD* is not only able to interact with plant factors from its native host, siratro, but also with luteolin and alfalfa plant factor; our results therefore show that the *nodD* protein host-specifically mediates the regulation of nodulation genes.

Results

The MPIK3030 hsn region contains a nodD copy involved in siratro-specific nodulation

In preliminary complementation experiments we found that the plasmid pCB5001, containing around 34 kb of a host-specific nodulation region from MPIK3030, as well as the shorter 12.1-kb derivative pCB507, were able to complement *nodD*1 or *nodD*2 mutants of *R. meliloti*. In order to follow up these results, a 250-bp M13mp18 clone containing the 5′ section of the *R. meliloti nodD*1 gene (Göttfert *et al.*, 1986) was labelled with ^{32}P and used to probe *Eco*RI digests of the plasmid pCB5001 and total DNA from MPIK3030. As shown in Figure 1B, lane 2, two *Eco*RI fragments in MPIK3030 total DNA hybridized (6.5 and 2.9 kb) and the 2.9-kb *Eco*RI fragment also hybridized in pCB5001 (Figure 1B, lane 3). Since Tn*5* insertions in this region eliminate host-range extension in an *R. meliloti* background (Bachem *et al.*, 1986), we conclude that this copy of MPIK3030 *nodD*, referred to below as MPIK3030 *nodD*1, is involved in host-range determination.

To determine the effect of mutations in the host-range region

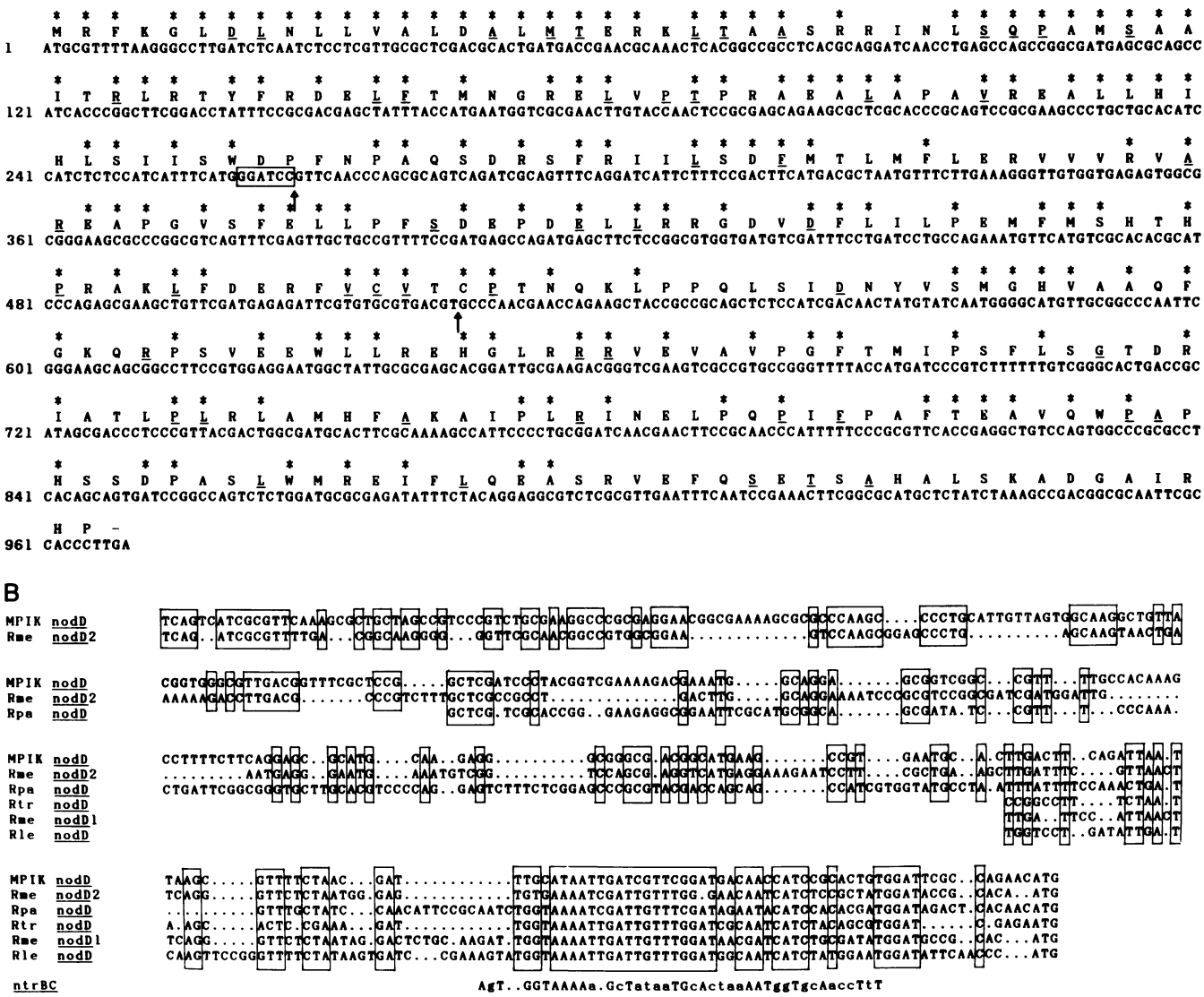

Fig. 3. Nucleotide and deduced amino-acid sequences of the MPIK3030 *nodD*1 gene and the comparison to other *nodD* genes (**A**). Amino acids marked with asterisks show conservation in all compared *nodD* sequences. Underlined amino acids indicate homology to the *lysR* gene from *E. coli*. The boxed *Bam*HI site was used in the construction of the chimeric *nodD* (Figure 2), and the arrows below the nucleotide sequence indicate positions of Tn*5* insertions in some mutants. Below (**B**) is shown a comparison of the 5′ non-coding region of the MPIK3030 *nodD*1 to the equivalent sequences of *nodD* genes from the following *Rhizobium* species: Rme, *R. meliloti* 41 (AK631); Rpa, *R. parasponia* Rtr, *R. trifolii*; Rle, *R. leguminosarum*. Boxed region indicates regions of more than 80% homology. The largest box indicates the *nod*-box core sequence, below which homology to the promoter sequence of *ntrBC* (Ferro-Luzzi Ames and Nikaido, 1985) is indicated by capital letters.

carrying *nodD*1 in an MPIK3030 background, Tn*5*-mutated plasmid derivatives of pCB507 (Bachem *et al.*, 1986) were mobilized into the wild-type MPIK3030 and Tn*5* mutations were introduced into the Sym plasmid by homologous recombination. The resulting homogenotes were tested for their symbiotic phenotype on siratro seedlings. Figure 2A shows the positions of selected Tn*5* insertion sites and the nodulation phenotype of the homogenotes. From this plant test it could be seen that the *nodD*1 gene of MPIK3030 is also essential for nodulation of siratro. Tn*5* mutations in the two left-hand regions of pCB507 showed a delayed nodulation phenotype in the homogenotes (data not shown).

Interspecies complementation with nodD genes from MPIK3030 and R. meliloti

To verify that the *nodD* function in MPIK3030, as well as the host-range extension ability in *R. meliloti* are properties of the same gene, the 2.9-kb *nodD*1 region from pCB507 hybridizing to the *R. meliloti nodD*1 probe was recloned into the transferable vector pPP375 and the resulting recombinant (pBH264) was mobilized into a series of *nodD* mutants of MPIK3030 and *R. meliloti* (see Table I). The transconjugants were tested for their nodulation phenotype on siratro and on alfalfa. The double *R. meliloti nodD*1/2 mutant PP659 (Göttfert *et al.*, 1986) carrying the MPIK3030 *nodD*1-region (pBH264), showed a clear restoration of nodulation on alfalfa (Figure 4A) and simultaneously extended the host range of the *R. meliloti* transconjugants to siratro (Table I, Figure 4B). As expected, the plasmid pBH264 is able also to suppress the Nod$^-$ phenotype of the Tn*5* mutations in the MPIK3030 *nodD*1 gene on siratro; however, the cloned *nodD*1 gene from *R. meliloti* could not complement the MPIK-3030 *nodD*1 mutants on siratro (Figure 4A,B and Table I).

Nucleotide and amino-acid sequences of the MPIK3030 nodD1 gene show conservation of the 5′ and divergence of the 3′ regions compared with other nodD genes

To identify differences in gene structure which could account for the host-range extension ability of the MPIK3030 *nodD*1,

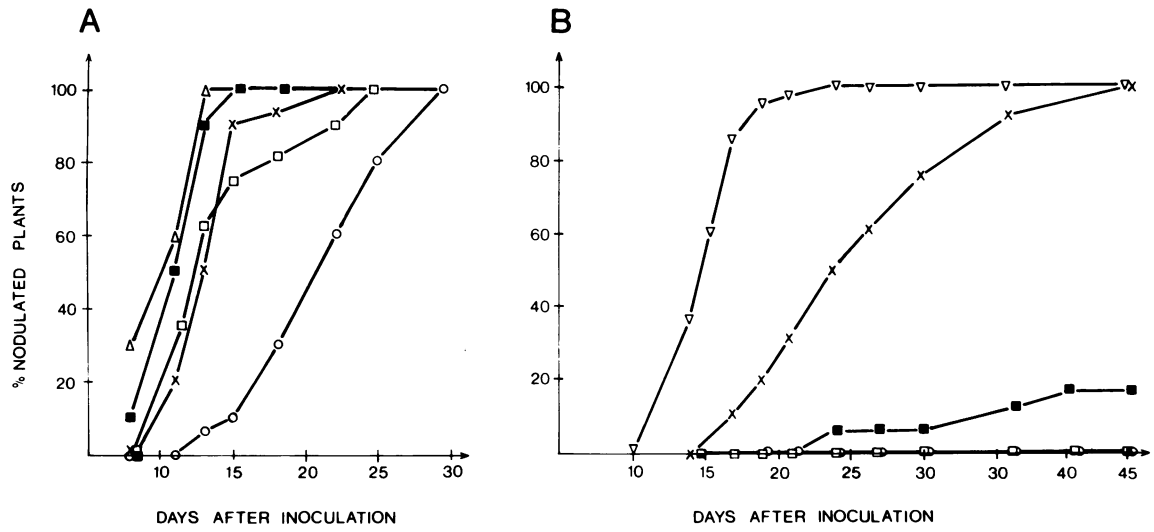

Fig. 4. Kinetics of nodule formation of wild-type MPIKL3030 (▽) on siratro (**B**) and wild-type *R. meliloti* (AK631, △) on alfalfa (**A**) and its derivative PP659 (○) complemented by different *nodD* genes: *nodD*1 of MPIK3030 (pBH264, ×), *nodD*1 of AK631 (pKSK5, □) and chimeric MPIK3030/*R. meliloti nodD* gene (pBH256, ■) on alfalfa (**A**) and on siratro (**B**).

we subcloned the 2.9-kb *Eco*RI fragment (Figure 1) in appropriate M13 vectors and determined the nucleotide sequence of the MPIK3030 *nodD*1 gene from MPIK3030. The restriction map of the sequenced region with the location of the MPIK3030 *nodD*1 ORF is shown in Figure 2A and the nucleotide and the derived amino-acid sequence of the structural gene is in Figure 3.

A computer-aided comparison to published *nodD* sequences [*R. meliloti nodD*1 and *nodD*2 (Göttfert *et al.*, 1986); *R. leguminosarum nodD* (Shearman *et al.*, 1986); *R. parasponia nodD* (Scott, 1986); *R. trifolii nodD* (Schofield and Watson, 1986)] indicated that the nucleotide sequence homology was ~50% in all cases (±2.5%). The distribution of the asterisks (Figure 3), indicating conserved amino acids in all compared sequences, clearly shows that the amino-terminal section of the *nodD* gene is the most highly conserved in different *Rhizobium* species. In total 48.4% of the amino-acid residues are conserved in all *nodD* copies. Chou–Fassman and hydrophobicity plots of the MPIK3030 *nodD*1 also reflects the similarity in the primary structure when compared with the other published sequences (data not shown).

A computer-aided gene-library search for homology, revealed that out of 25 of the best matches the *Escherichia coli lysR* gene (Stragier and Patte, 1983; Appelbaum *et al.*, 1985) exhibited significant homology to the MPIK3030 *nodD*1 gene (homologous amino acids shown underlined in Figure 3). The major homology was again found in the amino-terminal end of the MPIK3030 *nodD*1. It can also be seen that in most cases the homology to the regulatory *lysR* gene coincides with conserved amino acids between the various *nodD* proteins.

To verify the location of the Tn*5* insertions in the MPIK3030 *nodD*1, the insertion junctions of two pCB507::Tn*5* derivatives were sequenced by the method of McGraw (1984). The precise insertion positions are located within the MPIK3030 *nodD*1 coding region (marked with arrows below the sequence in Figure 3).

Although no hybridization to the radioactively labelled *nod*-box probe (Rostas *et al.*, 1986), could be found on the 2.9-kb fragment (Figure 1C, lane 4), sequence analysis revealed that the region upstream of MPIK3030 *nodD*1 contains a region with some homology to the *nod*-box. A computer-aided comparison of regions 5′ to published *nodD* genes (Figure 3B) showed a high level of conservation up to 90 bp upstream of the initiation codon, sections of which have >80% conservation among tested species (boxed sequences in Figure 3B). Interestingly, in those *nodD* upstream regions not associated with a *nodA* gene (*R. parasponia nodD, R. meliloti nodD*2 and MPIK3030 *nodD*1), the homology

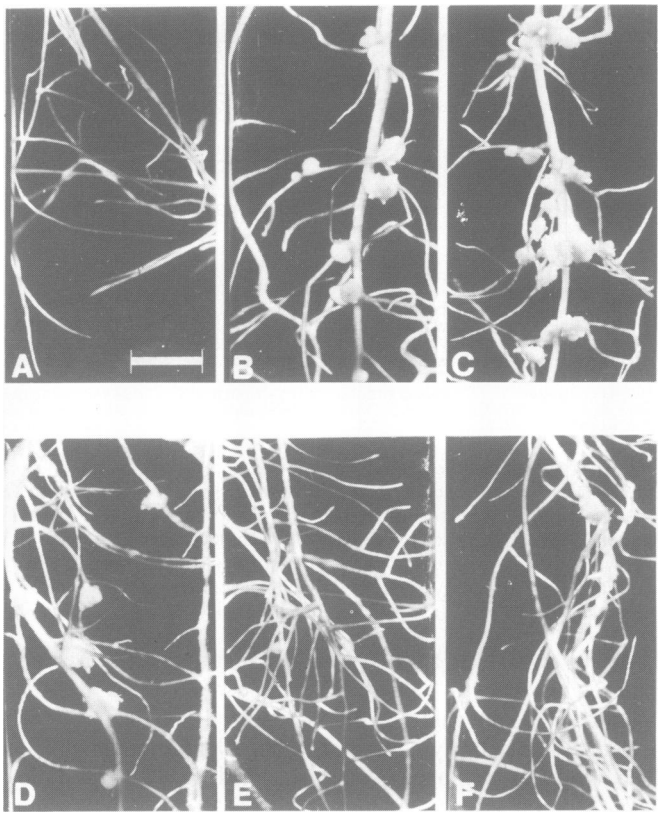

Fig. 5. Siratro roots and nodules induced by the following bacteria: uninoculated control (**A**), MPIK3030 (**B**), double *nodD* mutant of *R. meliloti* (PP659) and MPIK3030 *nodD*1 mutant strain (BC166) carrying the MPIK3030 *nodD*1 (pBH264) (**C**) and (**D**), respectively and PP659 (**E**) and BC166 (**F**) containing the chimeric *nodD* (pBH256).

Table II. The effect of various *nodD* genes and plant factors on the induction of *nodA−lacZ* (MPIK3030) and *nodC−lacZ* (*R. meliloti* 1021) fusions

Strain	Introduced plasmid (genes carried)	β-Galactosidase activity (u)			
		No Exudate	Luteolin	Alfalfa seed extract	Siratro seed extract
MPIK3030	–	1	2	2	2
	pBH291 (*nodA−lacZ* of MPIK3030)	2	92	50	132
	pRmM57 (*nodC−lacZ* of Rm 1021)	5	57	52	60
BC166	–	1	1	1	1
	pBH291 (*nodA−lacZ* of MPIK3030)	2	2	1	1
	pRmM57 (*nodC−lacZ* of Rm 1021)	1	3	2	1
JM57	–	3	4	4	4
	pKSK5 (*nodD*1 of Rm 41)	2	215	169	3
	pBH264 (*nodD*1 of MPIK3030)	8	356	256	215
	pBH256 (chimeric *nodD*)	5	33	60	7

extends considerably further (to ~340 bp upstream of the compared *nodD* initiation codons).

Significant homology was also detected between an *ntrBC* promoter in *Salmonella typhimurium* (Hanau *et al.*, 1983) and the *nod*-box region of the MPIK3030 *nodD*1 (Figure 3B). Ferro-Luzzi Ames and Nikaido (1985) found that this sequence conforms to a consensus *ntrC* protein-binding site from a series of nitrogen-regulated promoters of *S. typhimurium*.

A chimeric MPIK3030/R. meliloti nodD gene only complements nodD mutants of R. meliloti on alfalfa

To determine which section of the MPIK3030 *nodD*1 gene is responsible for the host-range extension, we constructed a chimeric *nodD* containing the 5′ region of the MPIK3030 *nodD*1 and the 3′ section of the *R. meliloti nodD*1 utilizing a common *Bam*HI site present in the structural genes of both *nodD*s (the cloning strategy of pBH256 is shown in Figure 2). The *R. meliloti* double *nodD*1/2 mutant PP659, carrying the chimeric *nodD* gene was able to nodulate alfalfa normally (Table I and Figure 4A). On siratro, however, this strain could not induce nodules, although the roots showed a clear thickening and browning response (Figure 5E). The MPIK3030 *nodD*1 mutant (BC166) containing the chimeric *nodD* gene, retained a Nod^- phenotype on siratro; however, small swellings occurring primarily on the larger roots are visible (Table I and Figure 5). From the sequence data it can be seen that both the sequence upstream of the initiation codon and the amino-terminal section of the putative gene product are more conserved than the carboxy-terminal region.

The MPIK3030 nodD1 activates nodABC promoters in conjunction with siratro and alfalfa plant-factors

From the results described above we have shown that a *nodD*1 gene of MPIK3030 can extend the host range of *R. meliloti* to siratro. As described for several *Rhizobium* species, the *nodD* gene is involved in the positive regulation of nodulation genes in conjunction with plant factors (Innes *et al.*, 1985; Mulligan and Long, 1985; Rossen *et al.*, 1985). To test whether this is also the case in MPIK3030, we constructed *nodA−lacZ* fusions from MPIK3030 and studied their induction with several plant exudates.

As a target for the mini-Mu::*lac* transposon, a 1.8-kb *Pst*I fragment, containing around 500 bp of the *nodA* gene and 1.3 kb of the upstream region, was subcloned from the MPIK3030 *nodABC* region (pBH277). Previous results from our laboratory showed that the 7.9-kb *Eco*RI common *nod* fragment of MPIK3030 carries sequence homology to the *nod*-box (Rostas *et al.*, 1986; and Figure 1C, lanes 5 and 6). To verify the presence of an intact *nod* promoter on the 1.8-kb *Pst*I fragment, a synthetic 25-bp oligonucleotide described by Rostas *et al.* (1986) was used to probe the isolated 1.8-kb *Pst*I fragment (Figure 1A, lane 6). The hybridization showed that the *nod*-box is indeed present on this fragment (Figure 1C, lane 6). In-frame *nodA−lacZ* fusions were obtained by assaying for β-galactosidase activity in MPIK3030 transconjugants on plates using X-gal as an indicator and screening for induction of β-galactosidase by siratro seed extract. One of these inducible fusions (designated pBH291) was shown to contain the MudII-*lac* transposon in the MPIK3030 *nodA* gene, and was used in further studies.

The *nodA−lacZ* fusion (pBH291) was subsequently checked for inducibility by siratro and alfalfa seed extracts and luteolin in β-galactosidase assays. As shown in Table II the *nodA−lacZ* fusion in MPIK3030 can be induced 66 times over background level by siratro seed extract. Moreover, luteolin and alfalfa seed extract also shows a significant induction in MPIK3030. In addition a *nodC−lacZ* fusion from *R. meliloti* 1021 (pRmM57) (kindly supplied by S.Long; Mulligan and Long, 1985) was introduced into MPIK3030 where induction was around 11 times over background with all plant factors. When pBH291 was tested in the MPIK3030 *nodD*1 mutant strain BC166, no significant induction could be measured irrespective of the plant factor used. A low level of induction of pRmM57 in a BC166 background was found however, which is probably due to a *nodD* gene on this construct (Table II).

Differential activation of nod gene expression using nodD genes from MPIK3030 and R. meliloti with different plant factors

To investigate the role of *nodD* in the recognition of different plant factors and the control of common *nod* gene expression, we mobilized the MPIK3030 *nodD*1 (pBH264) and the *R. meliloti* 41 *nodD*1 (pKSK5) (Kondorosi *et al.*, 1984) into the *R. meliloti* 1021 strain JM57, containing a Sym plasmid-integrated *nodC−lacZ* fusion (kindly supplied by S.Long; Mulligan and Long, 1985). The *nodC−lacZ* fusion in JM57 is inducible 1.5- to 2-fold; however, transconjugants containing pKSK5 can be induced 107-fold with luteolin. The induction by alfalfa seed extract is also high (85-fold). No induction, however, can be attained with siratro seed extract. In contrast, JM57 harbouring the MPIK3030 *nodD*1 gene (pBH264) is induced 27-fold over background by siratro seed extract and can also be induced by luteolin and alfalfa seed extracts (Table II).

Table III. Strains, plasmids and phages

Designation	Characteristics	Reference
Rhizobium meliloti		
AK631	Compact colony morphology variant *R. meliloti* 41	This laboratory
PP659	Double *nodD*1/2 mutant of AK631	Göttfert *et al.* (1986)
L5-30	*R. meliloti* wild-type strain	J.Denarie
1021	*R. meliloti* wild-type strain	F.Ausubel
JM57	1021 pSym *nodC*−*lacZ* fusion	Mulligan and Long (1985)
Rhizobium sp.		
MPIK3030	Str resistant derivative of NGR234	Pankhurst *et al.* (1983)
BC166	*nodD*1 mutant of MPIK3030	This work
Escherichia coli		
HB101	(*pro, leu, thi, lacY, endoI, recA, hsd*, Str^r)	Boyer and Roulland-Doussoix (1969)
JM101	(Δ(*lac, pro*) *sup*E, *thi str*A^r F′ (*tra*D36, *ro*AB, *lac*I^2 ZΔM15)	Messing (1983)
M8820	F^- *ara*D139, Δ (*ara, leu*) 7697, (*pro*AB, *arg, lac*POZYA)XIII, *str*A	Casadaban (1975)
MC4100	F^- *ara*D139, Δ (*lac*IPOZYA, *arg*F), U169, *rps*L, *thi*	Casadaban (1976)
Plasmid vectors		
pRK290	Wide host-range cloning vector (Inc Pl, Tc^r)	Ditta *et al.* (1980)
pPP375	pRK290 derivative carrying a poly-linker	Putnoky and Kondorosi (1986)
pRK2013	Helper plasmid for mobilization	Ditta *et al.* (1980)
pPH1JI	Inc Pl plasmid for homogenotization (Gen^r)	Beringer *et al.* (1978)
Recombinant plasmids		
pCB1223	pJB8 clone carrying the common *nod* region of MPIK3030	Bachem *et al.* (1985)
pCB5001	pLAFR1 clone carrying an *hsn* region of MPIK3030	Bachem *et al.* (1986)
pCB507	Shortened derivative of pCB5001 in pLAFR1	Bachem *et al.* (1986)
pKSK5	pRK290 clone carrying the common *nod* genes of AK631	Kondorosi *et al.* (1984)
pRmM57	pLAFR1 clone carrying a *nodC*−*lacZ* fusion	Mulligan and Long (1985)
pBH264	pPP375 clone carrying the *nodD* gene from MPIK3030	This work
pBH256	pRK290 clone carrying a *nodD* chimera	This work
pBH277	pPP375 clone carrying a 1.9-kb *Pst*I fragment of pCB1223	This work
pBH291	pBH277 clone carrying a *lacZ* fusion to the *nodA* gene	This work
Phages		
M13 mp18, 19	M13 cloning vectors used for sequencing	Norrander *et al.* (1983)
MudII PR13	MudII (*cat, 'lac*)	P.Ratet

When the chimeric *nodD* construct (pBH256) was tested in JM57, induction was observed only upon addition of alfalfa seed extract or luteolin (Table II). The lack of induction with siratro seed extract is in line with results obtained in the nodulation assays (Table I and II).

Discussion

In recent years substantial progress has been made in the understanding of the molecular basis of the nodulation process. Nodulation genes common to all rhizobia (common *nod*) and genes specific to particular plant−bacteria interactions (*hsn*) were shown to be coordinately regulated by the *nodD* gene, which was previously regarded as one of the common *nod* genes. In this paper we present evidence that host specificity is determined at regulational level by the *nodD* gene.

We show that one of the two *nodD* genes carried by MPIK3030 (*nodD*1) is essential for nodulation on siratro. With the aid of *lacZ* fusions in the *nodA* gene we also demonstrate that in conjunction with plant factors from its host, the MPIK3030 *nodD*1 gene positively regulates expression of nodulation genes in the same manner as has been described in several other systems (Innes *et al.*, 1985; Mulligan and Long, 1985; Rossen *et al.*, 1985). In addition to seed extract from siratro, the MPIK3030 *nodD*1 also interacts with alfalfa plant-factor in the form of luteolin or alfalfa seed extract to induce expression of the common *nod* genes. In contrast, siratro seed extract cannot induce *nodC*−*lacZ* fusions in *R. meliloti*. In line with this finding the MPIK3030 *nodD*1 gene restores nodulation ability to *R. meliloti nodD*1/2 mutants on alfalfa, although in the reciprocal experiment the *nodD*1 gene from *R. meliloti* cannot complement *nodD*1 mutants of MPIK3030. These results indicate that the *nodD*1 gene from the wide host-range strain MPIK3030 has a less stringent requirement for particular plant factors than the *nodD* from *R. meliloti* which specifically interacts with luteolin (Peters *et al.*, 1986). Alternatively, siratro produces factor(s) which block(s) the *R. meliloti nodD* genes, to which, however, the MPIK3030 *nodD*1 is insensitive. The presence of compounds antagonizing nodule induction has been recently reported by Firmin *et al.* (1986).

To delimit the region of the *nodD* gene giving rise to the specificity for various plant factors we compared the nucleotide and derived amino-acid sequences of *nodD* genes from several *Rhizobium* species. In the region upstream of the MPIK3030 *nodD*1 initiation codon a high level of conservation is found, con-

sisting of a *nod*-box which is also associated with all other copies of *nodD* genes and additionally, a long region of homology in *nodD* copies not linked to a *nodA* gene [*R. parasponia nodD* (Scott, 1986) and *R. meliloti nodD2* (Göttfert *et al.*, 1986)]. Although, in the comparison of the derived amino-acid sequences of different *nodD* genes, the amino-terminal region of the putative gene product is highly conserved, the carboxy-terminal region shows a striking divergence, leading to the speculation that this part of the *nodD* gene product is important for plant-factor interaction.

In experiments using a chimeric *nodD* gene (see Figure 2) to complement *nodD* mutants of *R. meliloti*, normal nodulation can be restored on alfalfa. However, nodulation on siratro is seriously impaired, although a reactive phenotype can still be observed. Confirmation of these results was also obtained from *nodC–lacZ* induction assays in *R. meliloti* where the chimera is able to respond to luteolin and alfalfa seed extracts (at a much lower level than either wild-type gene) and almost not at all to siratro seed extract. These results suggest that the fusion of the two *nodD* genes has changed the conformation of the hybrid protein such that it can no longer interact efficiently with siratro seed extract, but is still capable of accepting luteolin and alfalfa seed extract. Furthermore, the changed amino-acid sequence at the junction of the two fused *nodD* sections may account for the loss of efficient interaction with siratro seed extract and the lower level of specificity for luteolin in the resultant hybrid product (Table II).

The high conservation in the 5′ region of *nodD* genes irrespective of the plant factor necessary for induction of nodulation in a particular *Rhizobium*/legume system, suggests that this part of the gene is not directly responsible for interacting with specific plant factors. Moreover, one may speculate that divergence of the 3′ section during evolution allowed a selective specialization in the interaction of structurally varying inducing factors coming from different legume species. The multiple copies of *nodD* genes in various rhizobia (Rodriguez-Quinones *et al.*, 1986) may be responsible for the efficient interaction with one host, as seems to be the case for *R. meliloti* and alfalfa, or alternatively, for the specialized interaction with a series of different hosts.

When the wild-type *R. meliloti* and *nodD* mutant derivatives carrying the MPIK3030 *nodD*1 were tested for nodulation on siratro, a host not normally nodulated by *R. meliloti*, efficient host range extension is found. The fact that the *R. meliloti nodD* genes cannot extend the host range of MPIK3030 transconjugants to alfalfa indicates the importance of the *R. meliloti hsn* genes in this interaction (Horvath *et al.*, 1986). These genes can extend host range to alfalfa when introduced into MPIK3030 (Putnoky and Kondorosi, 1986).

The apparent lack of necessity for siratro-specific *hsn* genes in MPIK3030 indicates the possibility that the expression of the common *nod* genes is sufficient for siratro nodulation. However, the use of a plasmid which constitutively expresses the *nodABC* genes in *R. meliloti* does not cause this strain to induce nodulation on siratro (unpublished results). This result indicates that either more genes present in *R. meliloti*, as well as MPIK3030, are necessary for siratro nodulation, or that the expression of the common *nod* genes must be modulated during the early infection process.

In a paper by Bassam *et al.* (1986) the isolation of host specificity region from NGR234 (similar to the region isolated from MPIK3030) was described. This DNA section contained a region hybridizing to *nodD* from *R. trifolii* and could complement *R. trifolii nodD* mutants; however, the *hsn* region was mapped in two flanking sections of the *nodD*-like gene (Hsp loci I and II). Confirmation of the positions of Tn*5* insertions in the MPIK3030 *nodD*1 gene, which abolish host range extension in *R. meliloti* and result in a Nod$^-$ phenotype in MPIK3030, was obtained by sequencing the insertion junctions.

Until now the *nodD* gene was regarded as functioning in a common way in all *Rhizobium* species. Our results presented above show that the mode for action of the *nodD*1 from the wide host range strain MPIK3030 and those of *R. meliloti* are similar; however, the putative MPIK3030 *nodD*1 gene product allows a broader recognition of plant-host factors, and/or is less sensitive to putative inhibitory plant factors. Whether or not the second *nodD* copy of MPIK3030 is also involved in the wide host range of this strain is presently under investigation in our laboratory.

Materials and methods

Microbiological techniques

Bacterial strains, plasmids and phages are listed in Table III. All strains and mutants of *R. meliloti* were grown on YTB rich medium (Orosz *et al.*, 1973), while MPIK3030 and mutant derivatives were grown on TY complete medium (Beringer, 1974). LB (Luria Bertani-Broth) was used as a complete medium for *E. coli*. Minimal medium for all bacterial strains was GTS (Kiss *et al.*, 1979) supplemented with the appropriate antibiotics. Strains of *Rhizobium* and *E. coli* were grown at 30°C and 37°C, respectively. Triparental matings were performed as described previously (Kondorosi *et al.*, 1977a). Selection of transconjugants was done on GTS minimal medium with the appropriate antibiotics.

DNA Isolation and manipulation

Enzymes were purchased from Boehringer-Mannheim, FRG, and Bethesda Research Laboratories, Karlsruhe, FRG, and were used according to manufacturers' recommendations. Radioactively labelled reagents were obtained from the Isotope Institute (Budapest, Hungary). DNA cloning isolation and detection was carried out according to protocols described by Maniatis *et al.* (1982). DNA transfer to nitrocellulose membranes was carried out after the method of Southern (1975). The hybridization conditions with ^{32}P-labelled restriction fragments were as described earlier (Kondorosi *et al.*, 1982); however conditions for hybridization with the synthetic *nod*-box from *R. meliloti* were according to Rostas *et al.* (1986).

Sequence determination

DNA sequencing was performed using the dideoxy chain termination method in M13 vectors (Sanger *et al.*, 1977). The nucleotide sequences were determined on both strands using direct cloning of overlapping restriction fragments in complementary M13 vectors M13mp18 and M13mp19 (Norrander *et al.*, 1983).

Construction of lacZ fusions

Transcriptional fusions with the *lacZ* gene from *E. coli* were obtained with the aid of the mini-MudII-*lac* system (de Castilho *et al.*, 1984). Mu-mutagenesis was done in the strain MC4100 carrying a chromosomally integrated copy of a MudII-PR13 carrying a chloramphenicol-resistance marker kindly provided by Dr P.Ratet. Strain M8820 was used as recipient for the transduction of mutated and packaged plasmids. Cm, Tc-resistant transductants (around 3000 individual colonies) were used as donors in a mass conjugation into MPIK3030, where intact *nodA–lacZ* fusions were screened for, by replica plating on GTS (Tc) medium containing X-gal (50 μg/ml) with or without root exudate from siratro. Colonies, giving a blue colour on plates containing siratro root exudate while remaining colourless on GTS (Tc, X-gal), were then checked for plasmid content, and the position of the mini-Mu insertion was mapped with restriction enzymes.

Preparation of root exudates and induction of nod–lac-fusions

Plant exudates from alfalfa were prepared essentially according to the method described by Mulligan and Long (1985) but using 25–30 seeds/ml. Plant exudate from siratro was prepared as follows. After having been surface sterilized with mercuric chloride and rinsed thoroughly in water, the seeds were plated out on water agar and left to germinate for 3 days. Seedlings were then frozen in liquid nitrogen and homogenized in the cold, with pestle and mortar. The homogenate was subsequently extracted with 40% ethanol, and cleared by centrifugation. Excess ethanol was evaporated off overnight in an excicator under vacuum. After filter sterilization the exudate was tested for bacterial contamination on rich medium. Exudates (1/10 volume) and luteolin (10 μM final concentration) were given to rhizobia in logarithmic growth phase and incubated for a further 4 h, after which β-galactosidase assays were carried out according to Miller (1972).

Nodulation assays

Rhizobium strains were tested for their nodulation phenotype on siratro and alfalfa seedlings in test tubes as described previously (Kondorosi *et al.*, 1977b).

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The *nodD* sequence of MPIK3030 is almost identical to that of NGR234 as was kindly communicated to us by Nayudu and Rolfe prior to publication. Use of the *Pst*I fragment from pCB507 in complementation experiments showed that no sequences downstream of the MPIK3030 *nodD*1 are essential for host range extension.