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Additional nodulation genes on the Sym plasmid of *Rhizobium leguminosarum* biovar *viciae*

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Abstract

A *Rhizobium leguminosarum* biovar *viciae* strain lacking a 40 kb DNA region of the Sym plasmid pRL1IJ to the left (3′ side) of gene *nodE* failed to nodulate *Vicia sativa* plants. Therefore this DNA region was investigated for the presence of additional nodulation genes.

Complementation experiments indicated that the DNA region to the left (3′ side) of *nodE* is functionally homologous between *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii*. In this DNA region, three nodulation genes were identified, *nodT*, *nodM* and *nodL*. Tn*phoA* insertions in the *nodT* gene, about 4.5 kb to the left of *nodE*, lead to a delay in nodulation on *Trifolium subterraneum*, but not on *V. sativa* plants. Tn*phoA* insertions in gene *nodM* have no detectable influence on nodulation.

Finally, Tn*phoA* insertions in the *nodL* gene affected nodulation so that only rarely nodules were induced on the inoculated plants. The nucleotide sequence of this gene is presented. On the basis of the sequence a membrane integrated protein is predicted with a molecular weight of 20.1 kDa. Microscopical analysis of the infection process by *nodL* mutants indicate a role for *nodL* in maintaining the stability of the infection thread. Experiments using transcriptional *lacZ* fusions suggest that *nodL* belongs to the same transcriptional unit as *nodF,E*. Very low expression of *nodL* seems to be sufficient for biological activity.

Introduction

In recent years many genes involved in nodulation have been identified on so-called Sym plasmids of various *Rhizobium* strains. The nodulation *(nod)* genes, *nodE,F,D,A,B,C,I,J*, on the pRL1JI Sym plasmid of *R. leguminosarum* biovar *viciae* can be subdivided into three transcriptional units. In front of the *nodE,F* and the *nodA,B,C,I,J* transcriptional unit an inducible promotor region was described, in which a conserved DNA sequence of 49 base pairs (bp), the so-called *nod* box is present [8, 15, 16, 19, 20, 21]. In *R. leguminosarum* bv. *viciae*, a third *nod* box followed by two open reading frames (designated *nodM* and *nodN*) located to the left of *nodE* has been reported [21, 22]. Finally, one open reading frame, presumably involved in nodulation has been located between *nodE* and *nodM* [4]. This open reading frame is defined as *nodL*.

Tn5 insertions in the various *nod* genes of the pRL1JI Sym plasmid have different effects on nodulation. Mutants of *R. leguminosarum* bv. *viciae* in which a Tn5 is inserted in *nodI* or *J*

nodulate all test plants belonging to the pea cross inoculation group slightly delayed [1, 3]. Tn5 insertions in all other *nod* genes lead either to a complete Nod$^-$ phenotype (*nodD,A,B,C*) or to a reduced nodulation phenotype (*nodE* or *nodF*) [3, 25].

An IncP vector containing the *nodE,F,D,A,-B,C,I,J* genes (pMP104) is not capable of conferring nodulation ability to the Sym plasmid cured *R. leguminosarum* bv. *trifolii* strain LPR5045 when inoculated on *V. sativa* plants [21]. This indicates that additional genes on the *R. leguminosarum* bv. *viciae* Sym plasmid pRL1JI might be involved in nodulation.

We therefore analyzed the Sym plasmid pRL1JI for the presence of additional loci. In this paper we show that there are at least three *nod* genes, *nodT*, *nodM* and *nodL*, located left of *nodE* which influence the nodulation on *V. sativa*, *V. hirsuta* or *T. subterraneum* plants by *R. leguminosarum* bv. *viciae*.

Materials and methods

Bacterial strains and plasmids

All bacterial strains and plasmids used are listed in Table 1. Bacterial strains were grown and maintained on standard media described by Wijffelman *et al.* [24]. If appropriate, antibiotics were added in concentrations described previ-

Table 1. Bacterial strains and plasmids.

Strain	Relevant characteristics	Sources
RBL5515	*R. leguminosarum* bv. *trifolii* RCR5, *rif str.*, cured for its Sym plasmid	[13]
RBL5523	RBL5515 pRL1JI : : Tn1831(3)	[13]
RBL5580	RBL5515 harboring pRL1JI with a 50 kb deletion from the left of *nodF* down to the *nifHDK* genes	[20]
RBL54	*R. leguminosarum* bv. *trifolii*	[1]
RBL56	*R. leguminosarum* bv. *trifolii*	Van Brussel, Leiden
LPR5020	*R. leguminosarum* bv. *trifolii* RCR5, *str*	[7]
ANU843	*R. leguminosarum* bv. *trifolii*	[14]
ANU251	ANU843, *nod* : : Tn5	[18]
Plasmids		
pRL1JI	Sym plasmid from *R. leguminosarum* bv. *viciae* wild-type strain 248	[9]
pANU251	Sym-plasmid from strain ANU251	[18]
pMP92	IncP vector, Tc^r	[21]
pMP100	*Eco* RI fragment cloned in pMP92	This paper
pMP104	*Eco* RI-*Bam* HI fragment cloned in pMP92	[21]
pMP168	*Bcl* I-*Sma* I fragment cloned in pMP190	[21]
pMP180	*Bam* HI-*Kpn* I *nod* DNA fragment in pMP92	This paper
pMP190	IncQ transcriptional indicator vector, Cm^r	[21]
pMP255	*Eco* RI-*Kpn* I fragment cloned in pMP92	This paper
pMP425	*Eco* RI-*Sal* I fragment cloned in pMP92	This paper
pMP428	*Bam* HI-*Pst* I fragment cloned in pMP190	This paper
pMP429	*Bam* HI-*Pst* I fragment cloned in pMP190	This paper
pMP430	*Acc* I-*Pst* I fragment cloned in pMP190	This paper
pMP431	*Acc* I-*Pst* I fragment cloned in pMP190	This paper
pMP501	*Bgl* II-*Sal* I fragment cloned in pMP92	This paper
pHC311	IncP vector, Sp^r	Van Veen, Leiden

Abbreviations: Cm^r, chloramphenicol; *rif*, rifampicin; Sp^r, spectinomycin; *str*, streptomycin, Tc^r, tetracyclin; Sym, Symbiosis.

ously [1]. Bacterial matings were performed according to standard microbiological techniques.

Recombinant DNA techniques

Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim and used according to the manufacturer's prescriptions. DNA restriction patterns were determined using a horizontal agarose mini-gel system. After enzymatic digestion, DNA fragments needed for cloning experiments were isolated from low melting agarose (0.7%) by electroelution as described by Maniatis *et al.* [10]. DNA sequencing was performed as described by Sanger *et al.* [17].

Transposon mutagenesis

For random mutagenesis of specific DNA regions Tn*phoA* described by Manoil and Beckwith [11] was used. To obtain Tn*phoA* inserts in plasmid pMP180, this plasmid was mated into *Escherichia coli* strain 1164 in which Tn*phoA* was inserted in the chromosome. Plasmid DNA was then isolated from these bacteria and transformed into *E. coli* 1164, followed by selection for Tc^r, Km^r colonies. The position of Tn*phoA* insertion in the plasmids isolated from these colonies was determined by restriction enzyme analysis.

Homogenotization

In order to homogenotize Tn*phoA* into the Sym plasmid, pMP180 was mated into *R. leguminosarum* bv. *viciae* strain 248. Then plasmid pHC311 (Sp^r) was introduced in this strain. After selection for Sp^r, Km^r, the colonies were screened for resistance against tetracycline. The Sym plasmid from Tc^s colonies was checked for the presence of Tn*phoA* in the correct gene by hybridization experiments.

Plant nodulation test

All plant seeds were sterilized by subsequent soakings in concentrated sulfuric acid and bleach as described by Van Brussel *et al.* [23]. After germination, seedlings with a rooth length of about 1 cm were transferred to test tubes containing Jensen agar slants [23]. After incubation of the seedlings for 24 h at 20 °C, suspensions of bacteria in sterile water were added to the seedlings in a concentration of 10^7 bacteria per seedling. The plantlets were then transferred to a climate chamber and checked regularly for nodulation. The conditions of the climate chamber were the following. The light intensity on the bench level is 28 000 lux, the daylight period 16 h. The relative air humidity is kept at $62 \pm 4\%$. The temperature of the air in the climate chamber is kept at 21 ± 1 °C. During daylight periods, the temperature inside the plant assay tubes is 23 ± 1 °C, during the 8-hour dark periods the temperature is 21 ± 1 °C.

As a standard practice bacteria were reisolated from root nodules and checked for their antibiotic resistance markers. Reisolated bacteria were once again tested for their nodulation behaviour. Seeds of *V. hirsuta* and *T. subterraneum* were kind gifts of Drs J. Engelman and B.G. Rolfe respectively. *T. pratense* and *T. repens* seeds were purchased from the CEBECO company, Rotterdam, Netherlands. Seeds from *V. sativa* subsp. nigra were harvested from plants grown in our own laboratory.

Plant root exudates

Plant root exudates were obtained by growing one seedling per three ml sterile Jensen medium for seven days as described by Zaat *et al.* [26]. Sterile exudates were maintained at 4 °C.

Induction assays

To test DNA fragments of the nodulation region of Sym plasmid pRL1JI for the presence of promoter activity, the fragments were cloned into

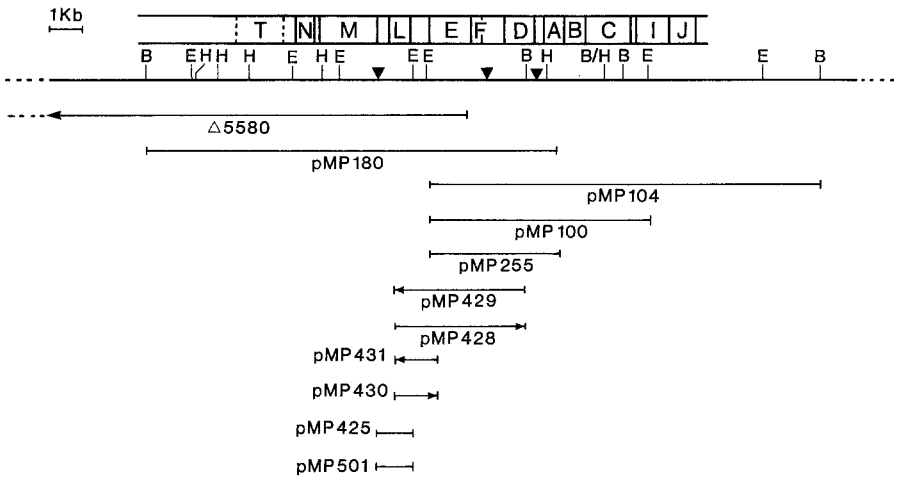

Fig. 1. Map of the nodulation region of the *R. leguminosarum* bv. *viceae* Sym plasmid pRL1JI. The deletion present in the pRL1JI Sym plasmid of *R. leguminosarum* bv. *viceae* strain RBL5580 is represented by a bar with an arrow pointing to *nifHDK*. Small arrows indicate the orientation of cloned DNA fragments towards *lacZ*, when cloned in the transcription indicator vector pMP190. Arrowheads indicate the position of *nod* boxes. The size and position of *nodM,N* was based on the sequence as determined by Surin and Downie [22]. The dotted lines indicate the presumptive borders of the *nodT* gene, which has not yet been sequenced.

lacZ transcription indicator plasmid pMP190, after which the resulting plasmids were transferred to strain RBL5523. The resulting strains were grown for 24 h at 28 °C in induction medium [20, 26]. Then inducing substances were added after which the incubation was continued for 16 h. The amount of β-galactosidase was then measured according to Miller *et al.* [12].

Results

An additional Nod *region is required for nodulation*

In order to determine which genes are involved in nodulation on *V. sativa* plants, we cloned several DNA fragments of the pRL1JI *nod* region into the vector pMP92. The resulting plasmids, pMP100 (*nodA,B,C,D,E,F*), pMP104 (*nodA,B,C,D,E,F, I,J*), pMP255 (*nodD,E,F*) and pMP180 (*nodD,E,F,L,M,N,T*) (Fig. 1) were conjugated into several *Rhizobium* strains.

When the Sym plasmid-cured *Rhizobium* strain RBL5515, containing any one of these plasmids, was inoculated on *V. sativa* plants, no nodules were observed within four weeks (Table 2). It thus appears that the genes *nodA,B,C,D,E,F,I,J* cloned on an IncP plasmid are not sufficient to confer the ability to noculate *V. sativa* plants to Sym plasmid-cured *Rhizobium* strains.

The same plasmids were also introduced in the deletion mutant strain RBL5580, with a deletion in its Sym plasmid which spans the whole DNA region to the left (3′ side) of *nodF* (see Fig. 1). Only strain RBL5580 harboring pMP180 induced nodules on *V. sativa* (Table 2). *Rhizobium* strain RBL5580 carrying one of the other plasmids

Table 2. Nodulation of *V. sativa* by *Rhizobium* strains harboring various clones of the nodulation region of the *R. leguminosarum* bv. *viceae* Sym plasmid pRL1JI.

Plasmid	*Rhizobium* strain		
	RBL5515	RBL5580	LPR5020[a]
pMP100	–	–	+
pMP104	–	–	+
pMP255	–	–	+
pMP180	–	+	+

[a] Comparable results were obtained with *R. leguminosarum* bv. *trifolii* strains RBL54, RBL56 and ANU843; –, no nodulation within four weeks after inoculation; +, nodules appeared within 10 days after inoculation.

failed to nodulate *V. sativa* plants. In conclusion, the *nod* region of the *R. leguminosarum* bv. *viciae* pRL1JI Sym plasmid to the left (3′ side) of *nodE* as present on pMP180 carries essential information for the nodulation of *V. sativa*.

The plasmids pMP100, pMP104, pMP255 and pMP180 were also introduced into several wild-type *R. leguminosarum* bv. *trifolii* strains. Strains LPR5020, RBL56, RBL54 and ANU843 carrying any one of these clones nodulated *V. sativa* plants just as well as *Rhizobium* strain RBL5580 pMP180 (Table 2). This suggests that the *nod* region to the left (3′ side) of *nodE* mentioned above is common between *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae*.

Additional genes nodT, nodM, *and* nodL

To identify which part(s) of the DNA region present in pMP180 is important to the nodulation process, we mutated pMP180 by transposon Tn*phoA* mutagenesis. Subsequently the Tn*phoA* insertions in the *nod* DNA fragment of 32 mutants, were mapped by restriction enzyme analysis (Fig. 2). These mutant plasmids were conjugated separately into *Rhizobium* strain RBL5580 and tested for nodulation on *V. sativa* plants.

Rhizobium strain RBL5580 harboring pMP180, *nodD*::*TnphoA* nodulates *V. sativa* plants as well as strain RBL5580 pMP180 (Fig. 2 and 3). This was expected since *Rhizobium* strain RBL5580 has an intact *nodD* gene. When strain RBL5580 carrying pMP180, *nodE*::Tn*phoA* was inoculated on *V. sativa*, no nodules were observed (Fig. 2). On *V. sativa*, this strain induces excessive root hair curling (Hac^{++}), but no infection threads (inf^{-}).

All three derivatives of *Rhizobium* strain RBL5580pMP180 which carry a Tn*phoA* insertion in *nodL*, only induce a few delayed nodules on less than ten percent of the inoculated *V. sativa* plants (Fig. 2). These *nodL* mutant strains induce excessive root hair curling and about three times as many infection threads as *Rhizobium* strain RBL5580 pMP180. However, most of these infection threads were abortive. One derivative of pMP180, pMP576, which carries a Tn*phoA* inserted in between *nodE* and *nodL*, nodulates all plants (Fig. 2).

R. leguminosarum bv. *viciae* strain RBL5580

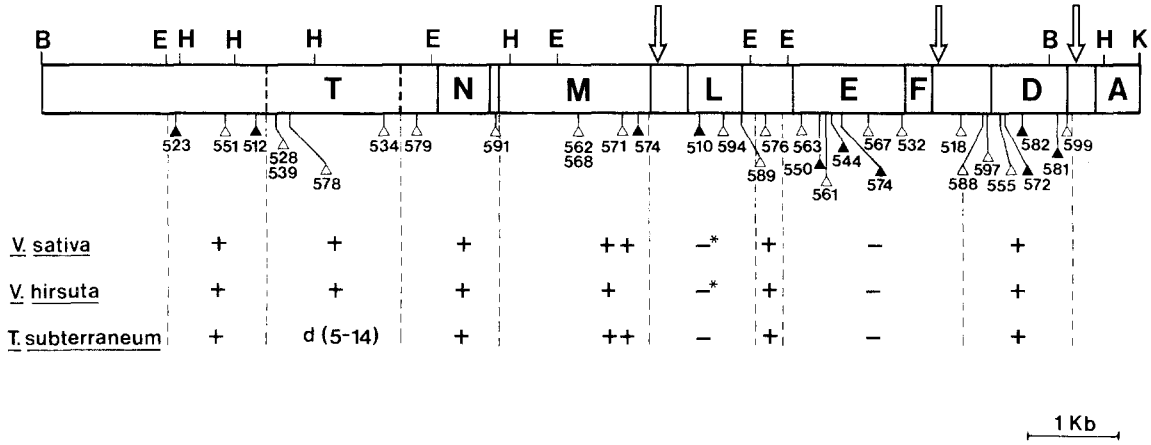

Fig. 2. Position of Tn*phoA* insertions in pMP180 and the influence of these mutations on nodulation when introduced into strain RBL5580. One orientation of Tn*phoA* is indicated by open arrowheads and the other by closed ones. Arrows indicate the position of *nod* boxes. The size and position of *nodM,N* was based on the sequence published by Surin and Downie [22]. The dotted lines around *nodT* indicate the presumptive borders of this gene. The *nodT* gene has not yet been sequenced. −, no nodules occurred within four weeks after inoculation; −*, delayed nodules were induced on a maximum of 10% of the inoculated plants; +, nodulation comparable to that induced by *Rhizobium* strain RBL5580pMP180; ++, nodules appeared about two days earlier in comparison to strain RBL5580pMP180; d, nodulation was delayed. The numbers in parenthesis indicate the number of days by which the nodulation was delayed in comparison to strain RBL5580pMP180.

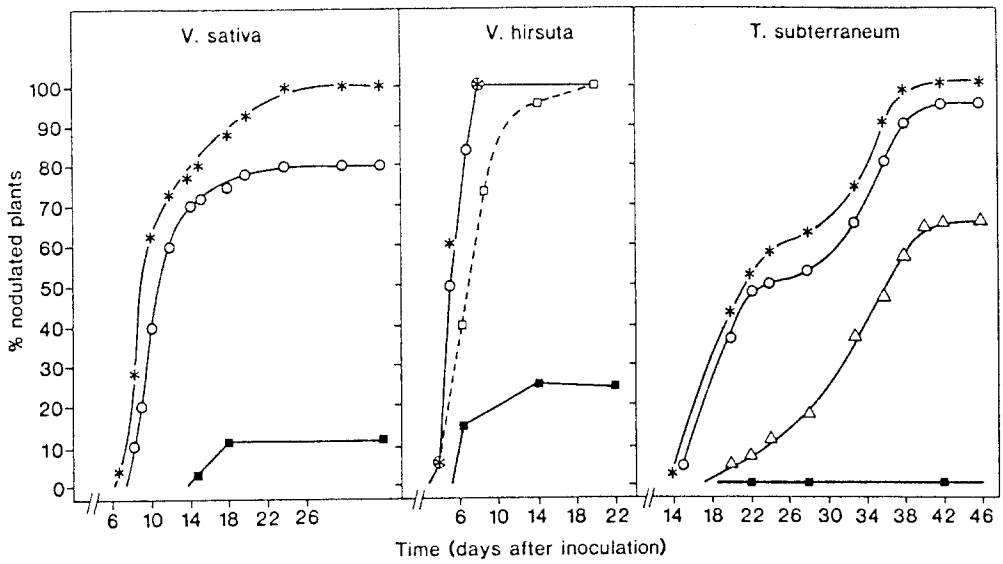

Fig. 3. Nodulation kinetics of *R. leguminosarum* bv. *viciae* strain RBL5580 harboring various Tn*phoA* derivatives of pMP180 when inoculated on (A) *V. sativa*, (B) *V. hirsuta* and (C) *T. subterraneum*. Indicated are the nodulation kinetics of the following strains: ○, RBL5580 pMP180; △, RBL5580 pMP180,*nodT*::Tn*phoA* (pMP534); ∗, RBL5580 pMP180, *nodM*::Tn*phoA* (pMP562); ■, RBL5580 pMP180, *nodL*::Tn*phoA* (pMP510); □, RBL5515 pRL1JI, *nodL510*::Tn*phoA*. The nodulation kinetics of *R. leguminosarum* bv. *viceae* wild-type strain RBL5523 are comparable to that of strain RBL5580pMP562.

harboring pMP180 with Tn*phoA* insertions in *nodM* nodulated *V. sativa* reproducibly better than strain RBL5580 pMP180 did (Fig. 3), whereas they also induce a slightly higher number of nodules. In fact the nodulation phenotype of strain RBL5580 pMP180, *nodM*::Tn*phoA* was comparable to that of wild-type strain RBL5523.

RBL5580 carrying plasmid pMP591 nodulated *V. sativa* as well as strain RBL5580 pMP180. We believe the Tn*phoA* of plasmid pMP591 to be located at the beginning of the *nodN* gene (Fig. 2), which indicates that mutation of *nodN* has no detectable influence on nodulation. However the method we used for determining the positions of the various Tn*phoA* insertions is not precise enough to distinghuish 100 bp differences. It is therefore possible that the Tn*phoA* insertion present in plasmid pMP591 is located at the extreme end of *nodM*. It is known that transposon insertions at the very end of genes can leave the protein products derived from these genes active.

Strain RBL5580 harbouring pMP180::Tn*phoA* derivatives in which the transposon was inserted to the left of *nodM* nodulated *V. sativa* plants equally well as strain RBL5580pMP180 itself (Fig. 2).

All 32 *Rhizobium* RBL5580pMP180::Tn*phoA* mutant strains were also inoculated on *V. hirsuta*. The results were comparable with those on *V. sativa*, except that when inoculated on the former plants, strain RBL5580 harboring pMP180 with Tn*phoA* insertions in gene *nodM* did not cause better nodulation than the parental strain RBL5580pMP180 or wild-type *Rhizobium* strain RBL5523 (Figs. 2 and 3).

In adition, the same 32 mutant strains were inoculated on *T. subterraneum*. The only difference with the results obtained with *V. sativa* was that four mutants, namely strain RBL5580 harboring pMP528, pMP534, pMP539 or pMP578, nodulated *T. subterraneum* plants with a delay of 5 to 14 days (Figs. 2 and 3). Apparently the gene defined by these mutants, herein called *nodT*, is involved in the nodulation of *T. subterraneum* plants, but not in the nodulation of *V. sativa* or *V. hirsuta* plants.

*Nodulation by pRL1JI : : Tn*phoA

Nodulation experiments in which *Rhizobium* strains harboring cloned *nod* genes are used, tend to be regarded as somewhat artificial. We therefore homogenotized the transposons present in the genes *nodL* (pMP510), *nodM* (pMP562), *nodN* (pMP591) and *nodT* (pMP534 and pMP528) of pMP180 back into the PRL1JI Sym plasmid (See Fig. 2). The mutant Sym plasmids were then introduced in *Rhizobium* strain RBL5515 and inoculated on *V. sativa*, *V. hirsuta* and *T. subterraneum*. The results of these nodulation experiments were completely comparable with those in which the corresponding mutants of strain RBL5580 pMP180 were used. Two exceptions however have to be made.

First, *Rhizobium* strain RBL5515 pRL1JI, *nodM562* : : Tn*phoA* nodulated *V. sativa* as well as parental strain RBL5523 and mutant strain RBL5580 pMP180, *nodM* : : Tn*phoA* (pMP562) (Fig. 3). This indicates that when *nodM* is present on a multicopy vector, as in strain RBL5580 pMP180, it causes a slight deregulation of the nodulation process, resulting in a decrease in the percentage of plants nodulated (Fig. 3).

Second, *Rhizobium* strain RBL5515 pRL1JI, *nodL510* : : Tn*phoA* nodulated all *V. hirsuta* plants (Fig. 3), which is in contrast with the results obtained with strain RBL5580 pMP180, *nodL* : : Tn*phoA* (Fig. 3). However the nodulation by strain RBL5515 PRL1JI,*nodL510* : : Tn*phoA* is slightly delayed (Fig. 3), whereas the average number of nodules induced on these plants was only half of that induced by RBL5523 (data not shown).

In conclusion, it seems that mutation of *nodL* on the Sym plasmid pRL1JI causes a decrease in nodulation efficiency. This decrease is much stronger on *V. sativa* and *T. subterraneum* than on *V. hirsuta* and therefore species-dependent.

Gene nodL *is common*

We described above that *R. leguminosarum* bv. *viciae* strain RBL5580 harboring pMP180 with a Tn*phoA* inserted in *nodL* nearly completely failed to nodulate *V. sativa* plants. To determine whether a mutation in *nodL* could be complemented by a non-homologous Sym plasmid, the mutant plasmid pMP510 (*nodL* : : Tn*phoA*) was introduced in *R. leguminosarum* bv *trifolii* strain ANU843. The resulting strain nodulated both *V. sativa and T. pratense* plants (Table 3), indicating that the mutation in *nodL* was indeed complemented. The same results were obtained when pMP510 was replaced by one of the other plasmids mutated in *nodL* (Table 3).

The *nod* regions of *R. leguminosarum* bv. *viciae* and bv. *trifolii* are very colinear [21]. We therefores chose a mutant of *R. leguminosarum* bv. *trifolii* strain ANU843 which had a Tn5 inserted in a region comparable to *nodL* of *R. leguminosarum* bv. *viciae*, namely ANU251. Strain ANU251 has a Nod$^-$ phenotype on *T. pratense*. The mutant plasmid pMP510 (pMP180,*nodL* : : Tn*phoA*) was transferred to strain ANU251. When inoculated on *T. pratense* or *V. sativa* plants, this strain hardly induced any nodules at all (Table 3). When in strain ANU251 pMP510 the mutant plasmid pMP510 was replaced by the parental plasmid pMP180, both plant species

Table 3. Complementation of *nodL*.

Strain	Nodulation on	
	V. sativa	*T. pratense*
ANU843 pMP180	+	+
ANU843 pMP180, *nodL* : : Tn*phoA* (pMP510)[a]	+	+
ANU251	–	–
ANU251 pMP180	+	+
ANU251 pMP180, *nodL* : : Tn*phoA* (pMP510)[a]	–	–
ANU251 pMP425	ND	+
ANU251 pMP501	ND	+

ND, not determined; –, delayed nodules were induced on a maximum of 10% of the inoculated plants; +, nodules were induced within 10 days on at least 90% of the inoculated plants.

[a] Comparable results were obtained when plasmid pMP510 was replaced by plasmids pMP589, pMP594 or pRL1JI, *nodL510* : : Tn*phoA*.

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1       ACGGAACCTGCCAAGTTGAATCCGCACATCTGATTGTGCCCTCCCCAAGCTGGTGAAACG

61      CAAGGTCGCTGGTGCCGTCAGCCCCTTGTGCAGGATTTGCCTCGCTTGAGCAGACGACTT

121     CAACCGGCCGCCAAAGGGGAAAGATTGCTGTTTACTCCTCTGTGAGCTCTGCCGTTATTA

181     GTTGAATTCCAGCGGCAATCACCGACCACCCCCACGAACAAGATCTGAGAAGATACGATG
                                                                  M

241     ACACGCAGCCAAAAAGAGAAGATGCTAGCAGGCGAAATGTACAACGCAGCGGACCCCGAG
        T  R  S  Q  K  E  K  M  L  A  G  E  M  Y  N  A  A  D  P  E

301     ATCCAAGCCGAGTTGCTCCTCACCGGGGCTTGGTTGAAGCGGTATAATGACACGCTGGGC
        I  Q  A  E  L  L  L  T  G  A  W  L  K  R  Y  N  D  T  L  G

361     GACTCCGCCGAGCGGTGGAATGGGCTCCTTTTAGAACGATTGGGAGCAGTCGGGCTTGGA
        D  S  A  E  R  W  N  G  L  L  L  E  R  L  G  A  V  G  L  G

421     GCGGTTATTCGTCCTCCGTTCCACTGCGATTACGGGTTCAACATCCGGATCGGAGCTTGG
        A  V  I  R  P  P  F  H  C  D  Y  G  F  N  I  R  I  G  A  W

481     GTTTACATCAATTACAACTGCGTCATTCTTGACGTGGCAGCGGTAACTATCGGTGACGGA
        V  Y  I  N  Y  N  C  V  I  L  D  V  A  A  V  T  I  G  D  G

541     ACCGCAATAGGCCCTGCTGTGCAGATTTACACCGCGGACCATCCACATGATCCAGAGCAG
        T  A  I  G  P  A  V  Q  I  Y  T  A  D  H  P  H  D  P  E  Q

601     CGCCAGGCTGGACTGCAGTTGGGACGACCTGTCAGCATTGGCAGGCACGCCTGGATTGGC
        R  Q  A  G  L  Q  L  G  R  P  V  S  I  G  R  H  A  W  I  G

661     GGTGGAGCAATCATTCTCCCGGGCGTGACAATTGGCGATCACGCTGTCATTGGCGCTGGT
        G  G  A  I  I  L  P  G  V  T  I  G  D  H  A  V  I  G  A  G

721     AGTGTGGTCACGCGAGATGTTCCTGCAGGAAGCACGGCCATGGGAAATCCAGCTCGGGTC
        S  V  V  T  R  D  V  P  A  G  S  T  A  M  G  N  P  A  R  V

781     AAGGCTGGTGGACGCTTGCCGAAATCATAAAAGCAGGCCTTGGGGTAAATCCGGCGGTAA
        K  A  G  G  R  L  P  K  S  *

841     AGGGCTGGTTTCTTTGCTTCTGCGAACGGACTTCTGGCCGCCATGGGCCTTTCGGTTCCA

901     ACTGTCGGCACTTTCACTCTGACGCCAACCTCTTTGCAAATGCGGCTGCGCAGTCCTGCT

961     GACCGATTCGTGATCATGTTTGCATGCAAAGGGGCAACTACGCCTTAGGGTCAGCCCGCA

1021    GACACATACCGCTTCACCTGCAGAAAAACGAATTGCCTTTCAGCCCGTGCGCCGATGAAA
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Fig. 4. Nucleotide sequence of the *nod* region DNA fragment containing the *nodL* gene. The predicted amino-acid sequence of the *nodL* gene product is indicated by the single-letter code.

were nodulated. Therefore, the Tn5 insertion of strain ANU251 is located in a locus functionally comparable to *nodL* of the pRL1JI Sym plasmid. Cross-hybridization studies confirmed the presence of a homologous locus on the expected place of the *R. leguminosarum* bv. *trifolii* strain ANU843 Sym plasmid (data not shown). These findings also imply that *nodL* is functionally common between *R. leguminosarum* biovar *viciae* and *R. leguminosarum* bv. *trifolii*.

Sequence of nodL

The DNA fragment of the pRL1JI Sym plasmid containing *nodL* was sequenced. The nucleotide sequence shows a large open reading frame of 572 basepairs (Fig. 4). The ATG transcriptional start triplet of *nodL* is located at 596 base pairs from the end of *nodE*. The direction of transcription is from right to left (Fig. 1). Based on the sequence a protein is predicted of 20.1 kDa containing 190 amino acids. Screening against the EMBL data bank of known proteins showed no homology. The predicted *nodL* protein was evaluated for its

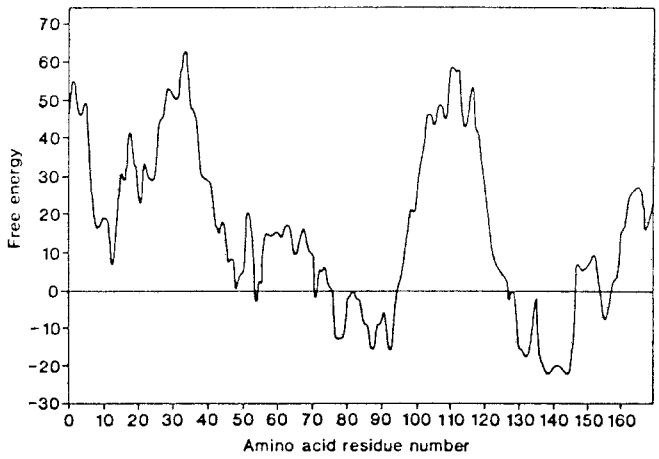

Fig. 5. Analysis of the predicted amino-acid sequence of *nodL* for the free energy of transfer from water to oil (kcal/mol) according to the method of Engelman *et al.* [6]. A window of 20 amino-acids was used in this analysis.

tendency to form transmembrane helices by the method of Engelman *et al.* [6]. This evaluation showed a hydrophobic region at the carboxy terminal end (Fig. 5). According to Engelman *et al.* [6] a protein which has a region which scores below – 20 kcal/mol on a scale of free energy of transfer from water to oil, while using a window of 20 amino acid residues, is very likely inserted in a membrane. This suggests strongly that the *nodL* gene product is anchored in the cell membrane.

Transcriptional control of nodL

It has already been reported that in front of *nodF* a promotor is located which harbours a *nod* box also found in the promotor region in front of *nodA* and *nodM* [4, 20]. Nevertheless a strain carrying a Tn*phoA* insertion of pMP180 in between *nodE* and *nodL* nodulated the test plants (Fig. 2). Although Tn5 insertions in *Rhizobium* are not always polar [5], this result made it questionable if *nodL* belonged to the same transcriptional unit as *nodF,E.* In order to investigate this, several DNA fragments (see Fig. 1) were cloned in both orientations in the expression vector pMP190, the essential part of which consists of a polylinker in front of the promoter-less *lacZ* gene of *E. coli* [21]. The resulting plasmids were transferred to *Rhizobium* strain RBL5523 and the activity of promoters present on the DNA inserts were measured after induction with 1.5 μM naringenin or undiluted *V. sativa* root exudate.

In this test system, plasmid pMP168, which contains the promoter in front of *nodF* and part of gene *nodF* itself (see Fig. 1), gave rise to about 400 units of β-galactosidase activity without induction and to approximately 6000 units after induction (Table 4). Thus, the expression of *nodF* increased

Table 4. Transcription of *nod* DNA fragments[a]

Plasmid	Promotor activity (units of β-galactosidase) after induction by		
	No inducer	Naringenin 1.5 μM	*V. sativa* root exudate
pMP168	400	6100	7825
pMP428	569	760	630
pMP429	475	1350	1100
pMP430	376	275	270
pMP431	432	355	535

[a] Fragments of the Sym plasmid pRL1JI *nod* region were cloned in pMP190 and the resulting plasmids were individually transferred to strain RBL5523. The latter strains were subsequently tested for the presence of promoter activity.

about 15-fold after induction of the *nodF* promoter.

Under similar conditions, the β-galactosidase activity of plasmid pMP429, which contains gene *nodF*, gene *nodE* and the major part of gene *nodL* (see Fig. 1), only increased two- to threefold after induction (Table 4). Plasmid pMP428 which contains the same DNA fragment as pMP429 but in the reversed orientation, showed no significant increase in activity after induction. This indicates that the expression of gene *nodL* increases only marginally after induction of the promoter in front of gene *nodF*.

In addition, plasmid pMP430, which contains only the intergenic region between gene *nodE* and *nodL* and part of the gene *nodL* itself (Fig. 1), did not cause an increase in β-galactosidase activity after induction (Table 4). Comparable results were obtained with plasmid pMP431, which contains the DNA fragment of pMP430 in the reversed orientation. When random clones were introduced in pMP190, the β-galactosidase activity varied between 150 and 700 units [21]. In conclusion, none of these results are indicative for a promoter in front of gene *nodL*. Therefore it seems likely that the transcription of *nodL* is regulated by the promoter in front of *nodF*. If so, the expression level of *nodL* seems to be exceptionally low.

To obtain more information about the transcription of *nodL* we also used a functional approach. An *Eco* RI-*Sal* I Sym plasmid pRL1JI *nod* region DNA fragment, containing about 50 bp of the intergenic region immediately in front of *nodL* and *nodL* itself, was ligated into the multiple cloning site of vector pMP92, thus forming pMP425 (Fig. 1). The vector pMP92 has no detectable promoter activity reading into the multiple clonings site [2]. The plasmid, pMP425, was introduced in *R. leguminosarum* bv. *trifolii* strain ANU251 (*nodL*::Tn5), and inoculated on *T. pratense* plants. The plants were nodulated by this strain (Table 3).

In addition, the *nod* DNA fragment inserted in pMP425 was deleted down to the restriction endonuclease *Bgl* II site, which is located 9 bp in front of the ATG site of *nodL*. The resulting Plasmid, pMP501 (Fig. 1), was also able to complement mutant strain UNA251 after inoculation on *T. pratense* plants, indicating that sufficient *nodL* gene product is made in this strain. In conclusion marginal expression of *nodL* is apparently already sufficient for its biological activity.

Discussion

In this paper we have shown that the DNA region to the left (3′ side) of *nodE* present on the *R. leguminosarum* bv. *viciae* Sym plasmid pRl1JI is involved in nodulation. This finding seems to contradict the conclusions of Downie *et al.* [2] that the overlapping region of cosmid clones pIJ1085 and pIJ1089, consisting of *nod* genes *nodE,F,D,A,B,C,I,J*, carries all the information necessary for nodulation. However they show that, when inoculated on peas, a cured *R. leguminosarum* strain harboring pIJ1085 induces about 4 times less nodules than the same strain harboring pIJ1089. Interestingly, pIJ1085 carries no *nod* DNA sequences to the left (3′ side) of *nodE*. When we inoculated a *R. leguminosarum* bv. *viciae* strain harboring only *nod* genes *nodE,F,D,A,B,C,I,J* (strain RBL5580 pMP104) delayed nodules were induced on peas (data not shown) but no nodules at all on *V. sativa* (Table 2). Thus it seems that the need for this region is species-dependent. On peas this DNA region is involved in the efficiency of nodulation, whereas on *V. sativa* this region is involved in the induction of nodules.

Our results show that this DNA region to the left (3′ side) of *nodE* carries at least three genes involved in nodulation. Gene *nodT* is located at the extreme left of pMP180 (Fig. 2). Mutations in *nodT* cause a delayed nodulation phenotype only when inoculated on *T. subterraneum* plants.

Under laboratory conditions, mutations in *nodM* (Fig. 2) have no influence on nodulation. It is possible, however, that *nodM* has a regulatory function. Our results show that when *nodM* is present on a multicopy vector as in strain RBL5580 pMP180 it decreases the efficiency of

nodulation. However since recently another gene, *nodN*, is identified in between *nodM* and *nodT* [22], it is also possible that this phenotype is due to the absence of expression of *nodN*. Strain RBL5580 harboring pMP180 with a Tn*phoA* inserted in the beginning of the *nodN* gene (pMP591) nodulates as well as RBL5580 pMP180. The noted deregulation of nodulation is therefore a result of the *nodM* gene.

The last and perhaps most interesting gene is *nodL* (Fig. 2), which gene is functionally homologous between *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii*. Based on the sequences (see also [22]), the reading frame of *nodL* is read down from the end of *nodE* in the direction of *nodM*. It is very likely that the predicted *nodL* protein is inserted in the membrane.

Since the expression of *nodL* was slightly increased after induction of the *nodF* promotor, it seems that *nodL* is located in the same transcriptional unit as *nodF* and *nodE*. One line of evidence against this notion is the location of the Tn*phoA* insertion on plasmid pMP576 (see Fig. 2). However, this apparent contradiction can easily be understood by the finding that the polarity of Tn5 insertions in *Rhizobium* strains is not always complete [5] and that the gene *nodL* deleted for all possible promoter regions was still able to complement a *R. leguminosarum* bv. *trifolii* strain mutated in *nodL*. Apparently, the level of expression at which *nodL* has biological activity is extremely low and lies beyond the sensitivity of our detection system.

Functionally, the *nodL* gene seems to play a role in the stabilization of infection threads, since those formed by *Rhizobium* strains mutated in *nodL* were mostly abortive. In addition, the *nodL* gene also seems to have some bearing on the regulation of root hair curling, as strains mutated in *nodL* cause excessive root hair curling. This phenomenon has also been described for mutants of *R. leguminosarum* bv. *viciae* mutated in genes *nodE* and *nodF* [25]. The protein predicted from the *nodL* nucleotide sequence showed no homologies after comparisons with the EMBL protein databank. This makes it very difficult to speculate on a mechanism by which the *nodL* gene product functions in the proposed stabilization of infection threads.

Surin and Downie [22] have recently reported that *nodL* mutants of Sym plasmid pRL1JI nodulate *V. sativa* and *V. hirsuta* to an extent almost comparable to that of a wild-type strain, which seems in contradiction with the findings reported here. However the background strains used in both experiments are not identical. When we introduce our mutant Sym plasmid pRL1JI,*nodL*::Tn*phoA* in the Sym plasmid-cured *R. leguminosarum* bv. *phaseoli* 8401 background used by Surin and Downie [22], we also find nodulation comparable to a wild-type *R. leguminosarum* bv. *viciae* strain on *V. sativa*. However, when the Sym plasmid pRL1JI, *nodL*::Tn*phoA* is introduced in the Sym plasmid cured derivative of *R. leguminosarum* bv. *trifolii* strain ANU845 or a cured derivative of *R. leguminosarum* bv. *viciae* strain 248, very poor nodultion on *V. sativa* is seen. This poor nodulation phenotype on *V. sativa* was completely comparable to the results obtained with *Rhizobium* strain RBL5515 pRL1JI,*nodL*::Tn*phoA* (Fig. 3). Apparently, in these latter strains a functional *nodL* gene is necessary for normal nodulation, whereas it is not required in strain 8401. In other words, the necessity for the presence of a functional *nodL* gene is not only dependent on the plant species, but also determined by the *Rhizobium* background used. This latter indicates that not only genes of the Sym plasmid are determinants for nodulation.

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