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Positive and negative control of *nod* gene expression in *Rhizobium meliloti* is required for optimal nodulation

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We show that expression of common nodulation genes in *Rhizobium meliloti* is under positive as well as negative control. A repressor protein was found to be involved in the negative control of *nod* gene expression. Whereas the activator NodD protein binds to the conserved *cis*-regulatory element (*nod*-box) required for coordinated regulation of *nod* genes, the repressor binds to the overlapping *nodD1* and *nodA* promoters, at the RNA polymerase binding site. A model depicting the possible interaction of the plant-derived *nod* gene inducer (luteolin), the NodD and the repressor with the *nod* promoter elements is presented. Mutants lacking the repressor exhibited delayed nodulation phenotype, indicating that fine tuning of *nod* gene expression is required for optimal nodulation of the plant host.

Key words: activator NodD/*cis*-regulatory elements/*nod* repressor/nodulation gene regulation/plant–*Rhizobium* interaction

Introduction

The induction of nitrogen-fixing root nodules on leguminous plants by rhizobial strains requires multiple interactions between the two partners. In different *Rhizobium* and *Bradyrhizobium* species, several gene sets (*nod* genes) have been identified which control the early steps of nodulation [for reviews see Kondorosi and Kondorosi (1986) and Rossen *et al.* (1987)]. The common nodulation genes (*nodABC*) (Kondorosi *et al.*, 1984) are essential and conserved in all rhizobia, both functionally and at the DNA sequence level. Other sets of genes determine the host-specificity of nodulation (*hsnABCD* genes) (Horvath *et al.*, 1986) [also called *nodFEGH* (Debelle and Sharma, 1986; Fisher *et al.*, 1987a) in *Rhizobium meliloti*; *nodFE* genes (Shearman *et al.*, 1986; Schofield and Watson, 1986) in *R.leguminosarum* biovars. *viciae* and *trifolii*]. Additional nodulation genes required for optimal and efficient nodulation have also been reported (Putnoky and Kondorosi, 1986; Renalier *et al.*, 1987; Surin and Downie, 1988).

The expression of these genes can be activated in the presence of the constitutively expressed *nodD* gene (Mulligan and Long, 1985; Rossen *et al.*, 1985; Horvath *et al.*, 1987) by flavonoid or isoflavonoid compounds (Firmin *et al.*, 1986; Peters *et al.*, 1986; Redmond *et al.*, 1986; Kosslak *et al.*, 1987; Zaat *et al.*, 1987) that are exuded from the roots of the host plants. The NodD proteins of different *Rhizobium* species interact with different flavonoids. The C-terminal part of NodD determines flavonoid- as well as host-specificity (Horvath *et al.*, 1987; Spaink *et al.*, 1987; Burn *et al.*, 1987), suggesting that NodD may directly interact with specific flavonoids. Some *Rhizobium* species contain more than one *nodD* gene (Rodriguez-Quinones *et al.*, 1987). For instance, three copies of *nodD (nodD1, nodD2* and *nodD3*) have been detected in *R.meliloti* and shown to contribute differentially to nodulation of distinct plant hosts (Göttfert *et al.*, 1986; Honma and Ausubel, 1987; Györgypal *et al.*, 1988).

Highly conserved DNA motifs (47-bp-long *nod*-boxes) (Rostas *et al.*, 1986) have been found upstream of the flavonoid-inducible transcriptional units. The *nod*-box is required for *nod* gene activation, suggesting its involvement in the coordinated regulation of the *nod* transcriptional units (Rostas *et al.*, 1986). The transcriptional initiation sites of *nodA*, *nodF* and *nodH* were mapped 26–28 bp downstream from the proximal end of the *nod*-box (Fisher *et al.*, 1987a,b, 1988). Recently, NodD-specific complex formation with *nod*-box fragments was demonstrated (Hong *et al.*, 1987; Fisher *et al.*, 1988).

The level of expression of the inducible *nod* genes varied in the different *Rhizobium* species. In *R.leguminosarum* and *R.trifolii* the induction was high (up to 100-fold) and the *nodD* was shown to repress its own transcription (Rossen *et al.*, 1985; Innes *et al.*, 1985). In *R.meliloti* the induction was low unless additional *nodD* copies were provided (Mulligan and Long, 1985) but negative autoregulation of *nodD* was not detected.

We investigated whether a negative regulatory factor exists in *R.meliloti*, and in this paper we report that expression of nodulation genes is controlled not only by the activator NodD but also by a negative *trans*-acting factor. Moreover, we found that this dual control is necessary for a more successful interaction between the bacterium and its host plant.

Results

Low level of nodC expression in R.meliloti strain AK631

We measured the level of expression of the common *nod* genes in two widely used laboratory strains of *R.meliloti*, AK631 and 1021. Induction of β-galactosidase activity of translational *nodABC–lacZ* fusions by *Medicago sativa* seed exudate or luteolin was low in both strains: using pEK901, containing a *nodC–lacZ* translational fusion, the induction was 1.4-fold in AK631 (Table I, line 4) and 1.6-fold in 1021 (Table I, line 3). When strain 1021 contained both *nodD1* and the *nodC–lacZ* fusion on plasmid pRmM57, β-galactosidase activity was induced to high level with luteolin (Mulligan and Long, 1985). Introducing pRmM57

Table I. Expression of *nod–lacZ* fusions in *R.meliloti* strains

Strains	*nod* genes on		β-Galactosidase units	
	p*Sym*[a]	Vector	−L[c]	+L
1. Rm1021	wt[b]	–	9	11
2. AK631	wt	–	6	7
3. Rm1021(pEK901)	wt	*nodC–lacZ*	34	54
4. AK631(pEK901)	wt	*nodC–lacZ*	32	44
5. Rm1021(pRmM57)	wt	*nodC–lacZ* + *nodD1*	41	1125
6. AK631(pRmM57)	wt	*nodC–lacZ* + *nodD1*	36	108
7. Rm1021(pEK902)	wt	*nodD1–lacZ*	684	692
8. AK631(pEK902)	wt	*nodD1–lacZ*	328	308
9. Rm1021(pRmM61)	wt	*nodD1–lacZ*	419	382
10. AK631(pRmM61)	wt	*nodD1–lacZ*	132	134
11. JM57	*nodC–lacZ*	–	8	39
12. JM57(pEK7059)	*nodC–lacZ*	Repressor	7	8
13. JM57(pLAFR1+4.4kb)	*nodC–lacZ*	Repressor	7	8
14. JM57(pEK7143)	*nodC–lacZ*	Repressor::Tn*5*	8	37
15. AK631*rep*$^-$(pRM57)	wt	*nodC–lacZ* + *nodD1*	36	779
16. AK631*rep*$^-$(pRmM61)	wt	*nodD1–lacZ*	316	322

[a]*Rhizobium meliloti* megaplasmid carrying the majority of the symbiotic genes.
[b]wt, wild type.
[c]L, luteolin.

(kindly provided by S.Long) into AK631 resulted in a level of β-galactosidase activity ~10-fold lower than in 1021 (Table I, lines 6 and 5).

To find an explanation for these differences in *nodC–lacZ* induction in these two strains, we measured the level of expression of the regulatory *nodD1* gene. Translational *nodD1–lacZ* fusions introduced on high and low copy number plasmids [pEK902 (see Materials and methods) and pRmM61 (Mulligan and Long, 1985)] expressed constitutively in both strains (Table I, lines 7–10) but the expression in AK631 was found to be lower: ~50% on the high and 30% on the low copy number plasmids. This may, at least in part, explain the differences in *nodC–lacZ* induction.

Low induction of the *nod* genes was not a unique feature of AK631, since in 80% of the *R.meliloti* strains we tested the induced level of *nodC–lacZ* expression was also low (data not shown). These observations suggested that in these strains *nod* gene regulation might be more complex and that a negative regulatory factor(s), a repressor-type element, could be involved.

Cloning of a DNA region from AK631 encoding the putative repressor

For cloning the repressor we used strain JM57, a derivative of 1021, which carries a *nodC–lacZ* fusion inserted into the symbiotic megaplasmid (Mulligan and Long, 1985). Upon addition of luteolin the β-galactosidase activity in JM57 is induced 4- to 5-fold (Table I, line 11). This induction of the *nodC–lacZ* fusion was easily detectable on agar plates containing *M.sativa* exudate and X-gal. Colonies of JM57 turned light blue after 3 days, while the parent strain 1021, as well as AK631, reached the same level of colour only after 7 days. A gene library of AK631, constructed in pLAFR1 (Putnoky and Kondorosi, 1986), was conjugally transferred into JM57, and colonies remaining white after 7 days were obtained at a frequency of ~0.1%. Plasmid DNA was isolated from these colonies, transformed into *Escherichia coli* HB101 and re-introduced into JM57. As exemplified in Table I, line 12, the addition of luteolin did not induce β-galactosidase activity in JM57 carrying one of these plasmids (pEK7059). The plasmid pEK7059, carrying the putative repressor locus, was selected for further studies.

The DNA region coding for the putative repressor was further delimited with partial *Eco*RI digestion and religation of pEK7059. The deletion derivatives were conjugally transferred into JM57. In these transconjugants inhibition of β-galactosidase induction correlated with the presence of a 4.4-kb *Eco*RI fragment (Table I, line 13), indicating that the gene(s) encoding the repressor (*rep*) was located on this fragment. Moreover, induction of the *nodC–lacZ* fusion was not repressed when JM57 carried pEK7059 with a Tn5 insertion in the 4.4-kb fragment (pEK7143), probably inactivating the repressor gene (Table I, line 14). Hybridizing the repressor-encoding region to Eckhardt gel (Banfalvi *et al.*, 1981) the gene was mapped on the chromosome in AK631 and was found to be present also in strain 1021 (data not shown), indicating that strain 1021 has probably an inactive homologue of the repressor.

In order to obtain isogenic repressor minus and repressor plus derivatives of strain AK631, Tn*5* mutants of pEK7059 carrying Tn*5* insertions in the repressor or outside of the gene were transferred to AK631 and the Tn*5*-carrying fragments were recombined into the genome by the marker-exchange technique (Ruvkun and Ausubel, 1981). Plasmids pRmM57 and pRmM61, carrying the *nodC–lacZ* and *nodD1–lacZ* fusions respectively, were then introduced in the *rep*$^-$ and rep$^+$ derivatives of AK631. Expression of *nod* genes in the *rep*$^+$ Tn*5*-derivative of AK631 was the same as in the wild type. In the *rep*$^-$ strain, however, expression of both *nodC–lacZ* and *nodD1–lacZ* fusions was comparable to that of Rm1021 carrying pRmM57 or pRmM61. Induction of *nodC–lacZ* fusion with luteolin was found to be 21-fold (Table I, line 15) and expression of the *nodD1–lacZ* fusion (both in the absence and presence of luteolin) also increased (line 16), indicating that the putative repressor gene is indeed involved in controlling *nod* gene expression.

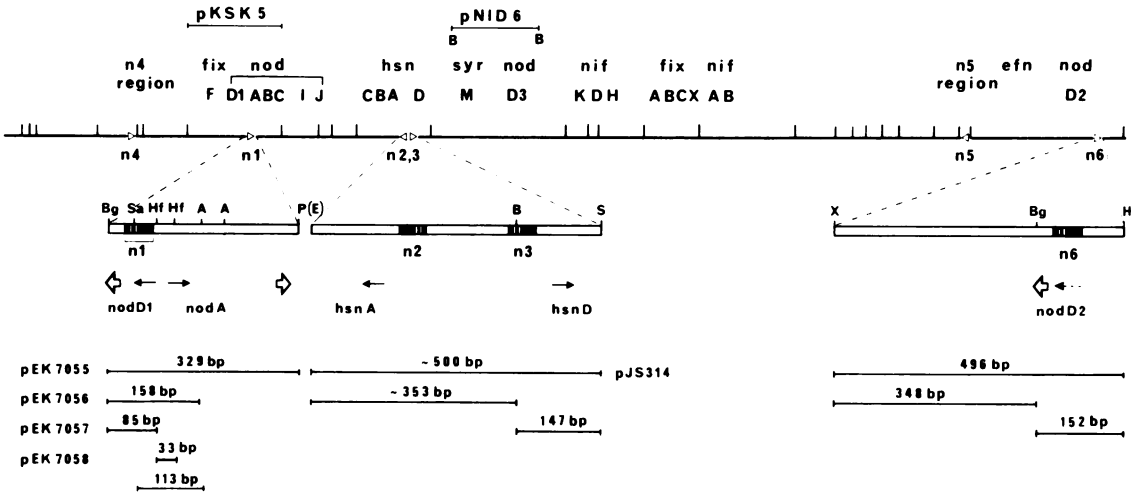

Fig. 1. Map of the *R.meliloti* AK631 *nod*−*nif* region. *Top*: *Eco*RI physical map (vertical bars) and location of symbiotic genes. *fix* and *nif* genes encode later steps of symbiosis. *syrM*: a symbiotic regulatory region (unpublished data), found also in strain 1021 and designated by S.Long (personal communication). Triangles show the *nod*-box sequences (n1−n6) in front of the flavone-inducible *nod* transcriptional units. pKSK5 carries the *nodD1,ABC* genes in pRK290, pNID6 contains a 7.1-kp fragment with *nodD3* and *syrM* in pRK290. **Middle**: detailed map of the enlarged n1, n2, n3 and n6 regions. Black arrows, transcriptional initiation sites (Fisher *et al.*, 1987a,b): white arrows, translational initiation sites. A, *Ava*II; B, *Bam*HI; H, *Hin*dIII; Hf, *Hin*fI; P, *Pvu*II, S, *Sph*I; Sa, *Sau*3A; X, *Xba*I; (E), *Eco*RI (this *Eco*RI sites does not exist on the megaplasmid, it was obtained by attachment of an *Eco*RI linker to a *Bal*31 generated end of the *hsn* fragment). **Bottom**: DNA fragments used for gel retardation experiments. Plasmids pEK7055−7058 carry fragments of the n1 region in pUC19, pJS314 contains the n2 and n3 regions in pACYC184.

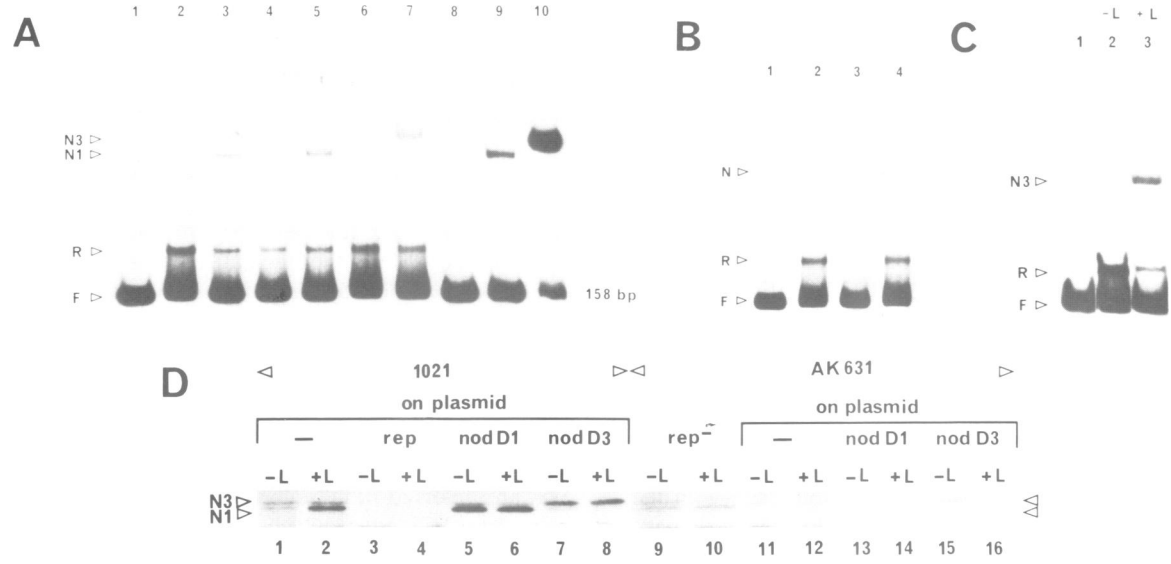

Fig. 2. Complex formation between the 158-bp fragment carrying n1 and the overlapping *nodD1* and *nodA* promoters, and protein extracts of *R.meliloti* strains. (**A**) Retardation of the 158-bp DNA with protein extracts prepared from luteolin-induced *R.meliloti* strains and with their derivatives carrying extra *nodD1* on pKSK5, or *nodD3* on pNID6. Source of extracts: none (**lane 1**), AK631 (**lane 2**), AK631(pKSK6) (**lane 3**), ZB138 (**lane 4**), ZB138 (pKSK5) (**lane 5**), ZB138 (pKSK5,*nodD1*::Tn*5*) (**lane 6**), AK631(pNID6) (**lane 7**), 1021 (**lane 8**), 1021(pKSK5) (**lane 9**), 1021(pNID6) (**lane 10**). (**B**) Complex formation with protein extracts from AK631 (**lane 2**), 1021 (**lane 3**) and 1021 carrying the putative repressor gene on pEK7059 (**lane 4**); **lane 1**, without extract. (**C**) Complex formation of luteolin-uninduced (**lane 2**) and luteolin-induced (**lane 3**) AK631(pNID6) protein extracts; **lane 1**, without extract. F, free DNA; R, repressor−DNA complex; N, NodD-specific complex; N1 and N3, NodD1- and NodD3-specific complexes. (**D**) Determination of the amount of NodD protein in uninduced (−L) and induced (+L) *R.meliloti* strains by immunoblotting and autoradiography. Extra *nodD1* and *nodD3* copies were provided on plasmids pKSK5 and pNID6 respectively, and the repressor region on pEK7059. The *rep*⁻ derivative of AK631 (**lanes 9** and **10**) was generated by Tn*5* mutagenesis.

Protein extracts from strains AK631 and 1021 interact differently with the 5′ upstream region of the nodABC transcriptional unit

Using gel retardation assays we investigated the interaction of possible positive and negative *trans*-acting factors with the DNA region containing the *nodABC* and *nodD1* promoters. Five different ^{32}P-labelled DNA fragments (329, 158, 85, 113 and 33 bp) from the n1 region (see Figure 1) were incubated with extracts (cleared cell lysates) prepared from strains AK631 and 1021, as well as from strains carrying cloned *nodD1* and *nodD3* genes of AK631. Extracts from the wild-type strain AK631 and from all its derivatives, including ZB138 where the entire *nod*−*nif* region was deleted, caused retardation of the 158-bp fragment (R in Figure 2A, lanes 2−7). This retardation was not observed with extracts from strain 1021 (lanes 8−10).

However, extracts from 1021 harbouring the putative repressor locus (either on plasmid pEK7059 or on its deletion derivatives containing the 4.4-kb fragment) yielded a retarded complex of the same mobility as the extract from AK631 (Figure 2B, lanes 2 and 4). This result indicates that the DNA region responsible for repression of the *nodC−lacZ* fusion determines the synthesis of a protein which binds to the *nod* promoter region.

With extracts from 1021 another, highly-retarded complex was formed (Figure 2A, lane 8), which increased upon introduction of *nodD1* on pKSK5 or *nodD3* on pNID6 (N1, N3; Figure 2A, lanes 9 and 10). The mobility of the retarded complexes was slightly different, with extracts containing NodD proteins encoded by different *nodD* genes. This highly retarded complex was not detectable with the AK631 extract (lane 2) but was weakly present when pKSK5 or pNID6 were introduced into strain AK631 (Figure 2A, lanes 3, 5 and 7). A Tn*5* insertion mutation in *nodD1* abolished this complex formation (Figure 2A, lane 6). The highly retarded complexes correlated with the presence and amount of NodD proteins and also with induction with the *nodC−lacZ* fusion in the particular strain, suggesting that the NodD protein is directly involved in the formation of this complex.

Effect of inducer on the nod promoter DNA−protein complex formation

Extracts from uninduced and luteolin-induced 1021 cultures did not differ significantly in their ability to form NodD-specific complexes (data not shown and Fisher *et al.*, 1988). In contrast, NodD-specific complex formation could be clearly demonstrated only with extracts from AK631 induced with luteolin (Figure 2A, lanes 3, 5 and 7). There was a marked difference between extracts from uninduced and induced cells of AK631 harbouring *nodD3* on pNID6 (Figure 2C). Addition of the uninduced extract to the 158-bp fragment resulted in very weak NodD3-specific and strong repressor binding (Figure 2C, lane 2). In contrast, with extracts from induced bacteria, the NodD3−DNA interaction was strong and the repressor binding was weaker (Figure 2C, lane 3). When increasing amounts of luteolin were added *in vitro* to the extract of uninduced AK631 carrying pNID6, the NodD3-specific complex formation increased ~2- to 3-fold but no detectable change in the binding of the repressor could be observed (data not shown).

Weak NodD-specific binding correlates with low NodD protein concentration in AK631

Western blot analysis of extracts from different *R.meliloti* strains (Figure 2D) showed that antibodies directed against the NodD1 protein (N1) are also reactive with the somewhat larger NodD3 protein (N3). In the wild-type strain 1021, which has no functional repressor the amount of both NodD1 and NodD3 was relatively high (Figure 2D, lane 1). Upon induction with luteolin (L) an increase in the level of NodD1 was observed (Figure 2D, lane 2). Our unpublished data indicate that the probable binding of luteolin to NodD1 leads to an increased accumulation of this protein in the bacterial membrane, which may both cause a higher stability of NodD1.

When the repressor gene from AK631 was introduced into 1021, both NodD proteins were synthesized in much lower amounts (Figure 2D, lanes 3 and 4). This low level of *nodD* gene expression was also observed with the wild-type strain AK631 carrying the repressor gene (Figure 2D, lanes 11 and 12). In the absence of repressor, the NodD proteins were synthesized in AK631 in detectable amounts (Figure 2D, lanes 9 and 10).

When extra *nodD1* and *nodD3* copies on plasmids pKSK5 and pNID6 were introduced into the strains 1021 and AK631, the expression of *nodD* was considerably higher in 1021 (Figure 2D, lanes 5−8) than in AK631 (lanes 13−16). Furthermore, induction with luteolin had no effect on the levels of NodD. These results suggest that the presence of the repressor is important for regulating the concentration of the activator NodD in the cell.

NodD-nod-box binding requires both halves of the nod-box

The retardation pattern of the 85-bp DNA fragment carrying the entire *nod*-box but lacking the overlapping region of the *nodD1* and *nodA* promoters, with extracts from AK631 and 1021 is shown in Figure 3A. Repressor binding, which is specific for the AK631 extracts, was not detected with the 85-bp fragment. The appearance of the highly retarded complexes (N1; Figure 3A, lanes 3, 5, 8 and 9; N3; lanes 7 and 10), however, correlated with the presence and amount of NodD1 and NodD3 proteins in the extracts.

To demonstrate direct binding of NodD protein to the *nod*-box, the NodD protein−DNA complexes were immunoprecipitated with anti-NodD antibodies. Plasmid pEK963, carrying the *nod*-box on a 600-bp fragment in vector pHC624 (Boros *et al.*, 1984) was digested with *Bgl*II and *Hin*fI, generating the *nod*-box on a 85-bp fragment. All DNA fragments were labelled, incubated with bacterial extracts and then with anti-NodD antibodies. The DNA from the immunocomplexes was reisolated and electrophoresed (Figure 3B). The only DNA fragment bound to NodD was the 85-bp *nod*-box fragment (Figure 3B, lane 3).

To investigate whether the entire *nod*-box is involved in binding of the NodD protein, the *nod*-box was cut into halves by digestion of the 158-bp fragment with *Sau*3A (see Figure 1), which generated a 113-bp fragment carrying the most conserved half of the *nod*-box and the overlapping region of the *nodD1* and *nodA* promoters. The 113-bp fragment exhibited drastically decreased NodD-specific binding (Figure 3C), indicating that both halves of the *nod*-box are involved in the interaction with NodD. Similarly, using *Sau*3A-digested and labelled pEK7056 DNA for NodD binding, no DNA was recovered from the immunocomplexes.

We have identified six *nod*-box sequences (n1−n6) in *R.meliloti* AK631 (Rostas *et al.*, 1986). Further gel retardation assays revealed that not only n1 (between *nodD1* and *nodA*) but also the other *nod*-box sequences tested (n2, n3 and n6) are able to bind NodD1, as well as NodD3 proteins. As an example, binding of NodD protein to a 496-bp fragment carrying n6 (Figure 1) is shown in Figure 3D.

The repressor protein binds to a 33-bp DNA fragment containing the transcriptional initiation site of nodA and nodD1

Complex formation between the 113-bp fragment and AK631 extracts (Figure 3C, lanes 2−4) indicated that the repressor binding site is located downstream of the *nod*-box. This site was found on a 33-bp fragment, overlapping the transcriptional initiation sites of the divergently transcribed *nodA* and nodD1 genes (Figure 1). This fragment was retarded

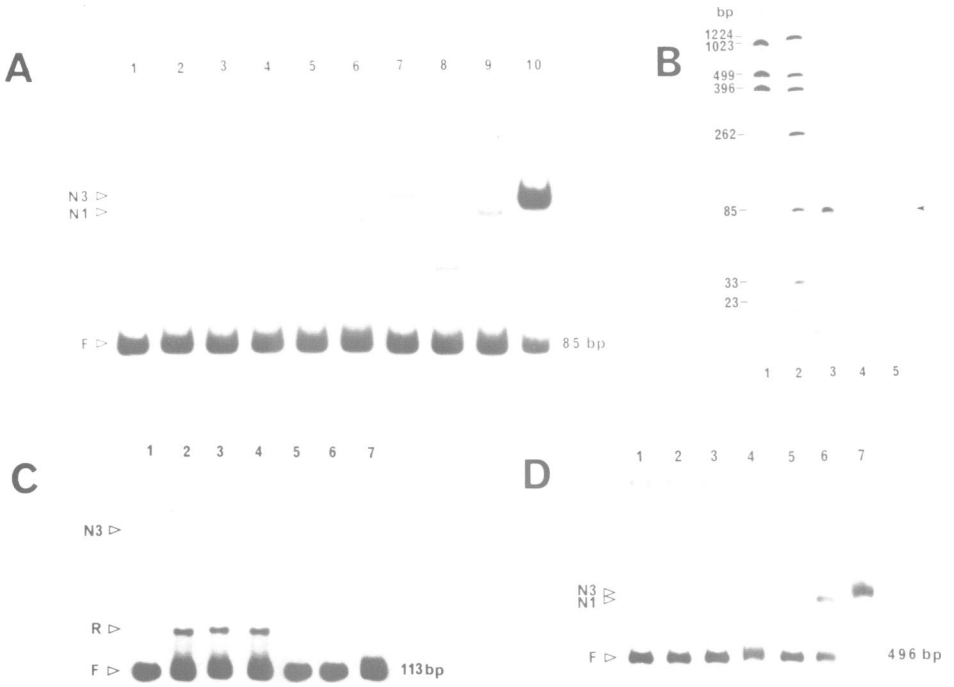

Fig. 3. Binding of the NodD protein to the *nod*-box. (**A**) Retardation of the *nod*-box containing the 85-bp fragment with extracts of luteolin-induced *R.meliloti* strains and with their derivatives carrying extra *nodD1* on pKSK5, or *nodD3* on pNID6. Source of extracts: none (**lane 1**), AK631 (**lane 2**), AK631(pKSK5) (**lane 3**), ZB138 (**lane 4**), ZB138(pKSK5) (**lane 5**), ZB138 (pKSK5,*nodD1*::Tn*5*) (**lane 6**), AK631(pNID6) (**lane 7**), 1021 (**lane 8**), 1021(pKSK6) (**lane 9**), 1021(pNID6) (**lane 10**). Another complex, migrating between the free DNA and the NodD-specific complexes (not correlating with the presence of NodD), was formed with extracts from both AK631 and 1021 strains. (**B**) Evidence for direct binding between NodD and the *nod*-box. *Bgl*II−*Hin*fI digested and labelled DNA fragments of the vector pHC624 (**lane** 1) and the *nod*-box containing recombinant plasmid pEK963 (**lane 2**) were used for complex formation. pEK963 DNA fragments were incubated with extracts of 1021(pKSK5) (**lane 3**) and ZB138 (**lane 4**) and pHC624 DNA fragments with extract of 1021(pKSK5) (**lane 5**). The NodD-bound DNA was immunoprecipitated with anti-NodD1 antibodies on the surface of protein-A-bearing *S.aureus* cells. Labelled DNA fragments reisolated from the immunocomplexes were electrophoresed on 5% polyacrylamide gel. Arrow points to the 85-bp *nod*-box fragment recovered from the immunocomplexes. No DNA fragment was recovered either when the vector DNA was incubated with NodD1-containing extract (**lane 4**) or when the *nod*-box-containing pEK963 DNA was treated with the extract of strain ZB138, lacking all three *nodD* copies (**lane 5**). (**C**) Retardation of the 113-bp fragment carrying half of the *nod*-box as well as the overlapping *nodD1*−*nodA* promoter region with extracts of luteolin-induced AK631 (**lane 2**), AK631(pKSK5) (**lane 3**), AK631(pNID6) (**lane 4**), 1021 (**lane 5**), 1021(pKSK5) (**lane 6**), 1021(pNID6) (**lane 7**); **lane 1**, without extract. (**D**) Retardation of the 496-bp fragment carrying the n6 region with extracts of luteolin-induced AK631 (**lane 2**), AK631(pKSK5) (**lane 3**), AK631(pNID6) (**lane 4**), 1021 (**lane 5**), 1021(pKSK5) (**lane 6**), 1021(pNID6) (**lane 7**); **lane 1**, without extract. F, free DNA; R, repressor−DNA complex; N1 and N3, NodD1- and NodD3-specific complexes.

by the extract from AK631 (Figure 4A, lane 2), but not by extract from 1021 (lane 5), with the exception of the 1021 transconjugant containing the cloned repressor gene in pEK7059 (lane 3). Formation of the repressor−DNA complex was abolished when the extracts were treated with proteinase K.

Binding of the repressor to the 152-bp fragment from the n6 region was also detected, but there was no binding to the 353-bp fragment from n2 or the 147-bp fragment from n3 (Figure 4A). Conservation of a 21-bp-long sequence in the n1 and n6 regions was found on the repressor-binding fragments but not in the n2 and n3 regions. Competition experiments with the synthesized 21-bp oligonucleotide (Figure 4A, lane 4) and with fragments of the *nod*-box regions also supported that the repressor binds specifically to this DNA sequence. Furthermore, the repressor was detected in 17 out of 21 additional *R.meliloti* strains of different geographical origin (Figure 4B).

Protection of the overlapping nodD1−nodA promoter sequences by trans-acting factors in DNase I footprinting assays

To further delimit the DNA sequences which bind the *trans*-acting factors, the extracts used in gel retardation assays were examined by footprinting experiments (Figure 5). NodD-specific protection with AK631 extracts was obtained when the extract was made from luteolin-induced AK631, carrying *nodD3* on pNID6. This extract protected the entire *nod*-box in two regions which were separated with DNase I hypersensitive sites on both strands. Extracts from uninduced and induced 1021(pNID6) cells protected the same nucleotide sequences as the extract from the induced AK631(pNID6), but the protection was stronger. Thus, the strength of protection correlated with the intensity of retardation.

Extracts from AK631 and its derivatives protected 27 nucleotides on the *nodD*-coding strand and 33 nucleotides on the *nodA*-coding strand in the overlapping region of the *nodD1* and *nodA* promoters (Figure 5), due to binding of the repressor. The protection was weaker when extracts were made of cells containing increased amounts of NodD protein (AK631 carrying pKSK5 or pNID6) (Figure 5, *nodD*-coding strand). This decrease was even more evident when extracts of induced bacteria were used. The protection was particularly weak with the extract from AK631(pNID6) where, upon induction, a strong protection of the *nod*-box was observed. These results also suggest that the inducer

may interact with the NodD protein, and in the repressor-containing strain this results in weakened binding of the repressor to the *nod* promoter.

Rhizobium meliloti synthesizing the nod repressor is more efficient in nodule induction than the repressor negative derivative

The nodulation ability of the AK631 strain and its isogenic *rep*$^+$ and *rep*$^-$ was compared on *M.sativa*. We found that the *rep*$^+$ strain is significantly more efficient in nodule initiation, as indicated by the percentage of nodulated plants between days 5 and 20 after infection (Figure 6A), as well as by the number of plants with higher nodule number than the average during the first days of nodulation (Figure 6B). Similar results were found when strain 1021 was compared with the 1021 transconjugant harbouring the repressor-containing plasmid (Figure 6A).

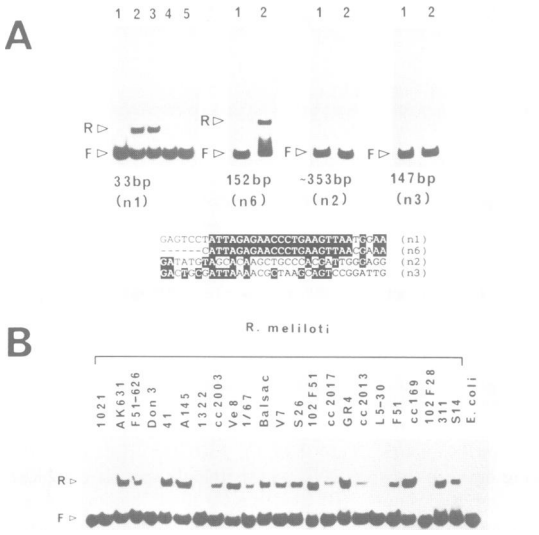

Fig. 4. Repressor binding to the n1 and n6 promoter regions from different *R.meliloti* extracts. (**A**) Retardation of DNA fragments from n1, n6, n2 and n3 regions with extracts of AK631 (**lane 2**), 1021 carrying the repressor region on pEK7059 (**lane 3**) and 1021 (**lane 5**). **Lane 4**, competition of repressor complex formation of AK631 extract with 100-fold excess of the synthesized 21-bp oligonucleotide conserved in the n1 and n6 regions; **lane 1**, without extract. **Lower part**: conservation of the 33-bp repressor binding sequence of the n1 region in sequences downstream from the n6, n2 and n3 *nod*-boxes. Nucleotides conserved are in black boxes. (**B**) Retardation of the 33-bp fragment with different *R.meliloti* extracts. R, Repressor complex; F, free DNA.

Discussion

Fine tuning of nod gene expression

We have shown that both positive and negative *trans*-acting factors (the NodD protein and a repressor-type protein) control the expression of the common *nod* genes in *R.meliloti*. This dual control provides a mechanism for fine tuning of the expression of nodulation genes, thus allowing a more successful interaction of the *Rhizobium* partner with the plant host.

We have presented evidence that most *R.meliloti* strains and field isolates produce the repressor and using isogenic strains the repressor-producing strain was more efficient in nodule induction than the strains lacking the repressor. This finding is in line with earlier reports showing that a low level of *nod* gene expression is sufficient for nodulation (Mulligan and Long, 1985) and overexpression of *nodABC* genes inhibits nodulation (Knight *et al.*, 1986). Considering that the common *nod* gene products are involved in the production of a plant-hormone-like compound (Schmidt *et al.*, 1988) and probably, as a consequence of nodule induction, the plant suppresses further nodule initiation (Caetano-Anolles and Bauer, 1988), it seems that nodule induction is finely modulated by both partners. This fine tuning is likely to be an important factor in determining the competitiveness of a *Rhizobium* strain in the soil.

A model for regulation of common nod genes

We propose the following model for the regulation of *nod* genes in *R.meliloti* strains AK631 and 1021 (Figure 7). In uninduced 1021 cells the *nodD* gene is expressed constitutively, the RNA polymerase binds to the *nodD* promoter and transcribes *nodD*, resulting in the production of NodD protein which binds to the *nod*-box. In AK631, the repressor competes with RNA polymerase for the binding

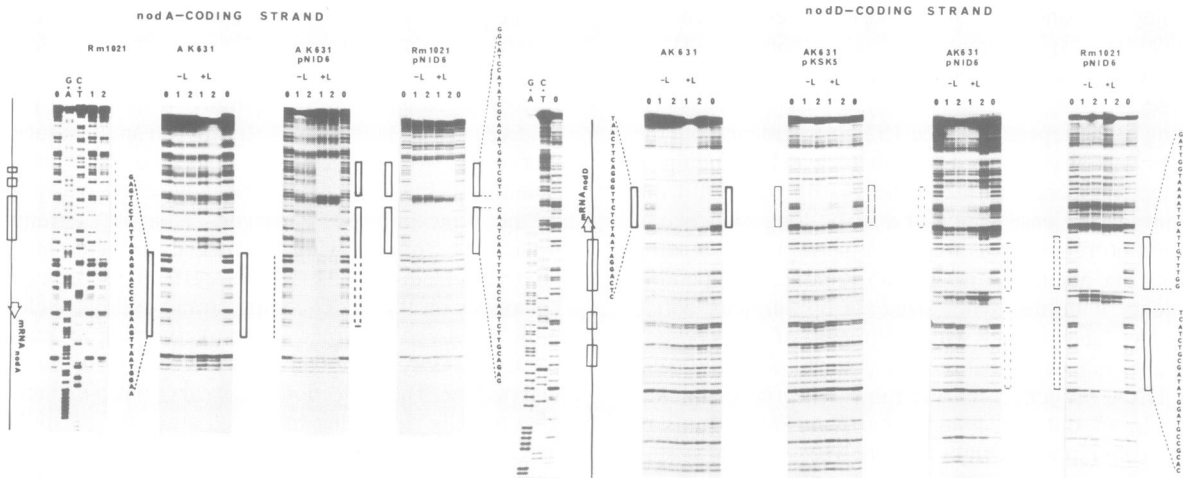

Fig. 5. DNase I protection of the n1 and the overlapping *nodD1*–*nodA* promoter region with *R.meliloti* protein extracts. −L, Uninduced; +L, luteolin-induced extracts. Protection with 100 μg (**lane 1**) and 200 μg protein (**lane 2**) and without protein (**lane 0**). Protected sequences are as indicated.

site. Therefore, binding of RNA polymerase in AK631 is occasional, resulting in the production of low amounts of the NodD protein. The low NodD concentration and a possible interfering effect of strong repressor binding (sterical effect, conformational change) cause weak binding of NodD to the *nod*-box. In luteolin-induced 1021 cells, the NodD protein interacts with the inducer which probably results in a conformational change of the NodD−DNA complex, leading to the transcription of *nodABC* genes. Expression of *nodABC* genes does not cause a drastic change in the expression of *nodD1*. The relatively low level of *nodABC* transcription still allows transcription to the opposite direction resulting only in a slight decrease of *nodD1* expression (Table I, line 9). In induced AK631 cells, the NodD−inducer complex binds to the *nod*-box with higher affinity and formation of the NodD−inducer−*nod* box complex promotes binding of RNA polymerase to the *nodA* promoter, as in the case of 1021. The RNA polymerase binding, in turn, competes with the repressor for the binding site and leads to the transcription of *nodABC*. Thus,

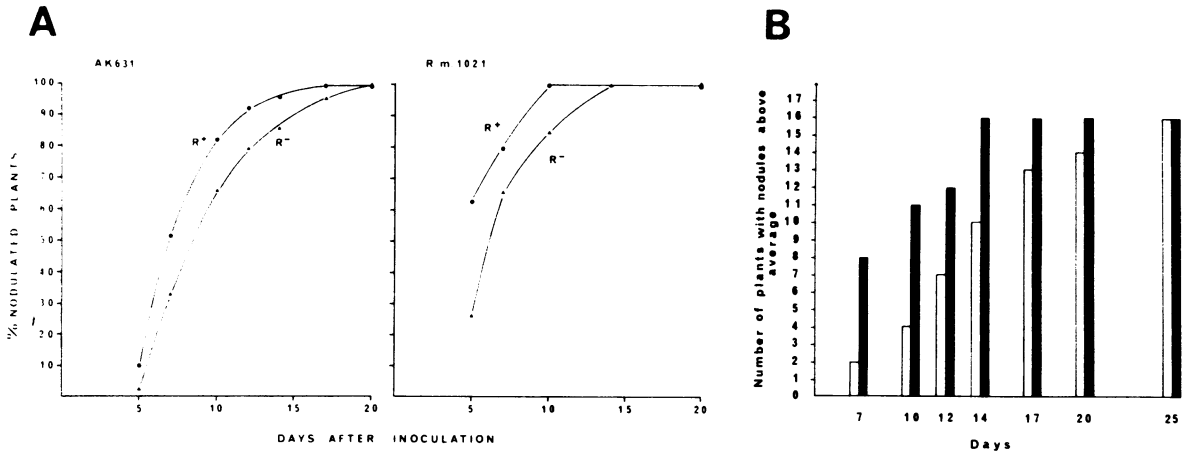

Fig. 6. Nodulation kinetics of *rep*$^+$ and *rep*$^-$ *R.meliloti* strains. (**A**) Percentage of nodulated *M.sativa* plants after inoculation with isogenic *rep*$^+$ (R^+) and *rep*$^-$ (R^-) derivatives of AK631 and with 1021 (R^-) and 1021 (pEK7059) (R^+). (**B**) Number of plants with higher nodule number than average. The average nodule number of nodulated plants at the given day was calculated and the number of plants with nodule number higher than average are presented. Thirty plants were inoculated with the *rep*$^+$ (black columns) or *rep*$^-$ (white columns) derivatives of AK631.

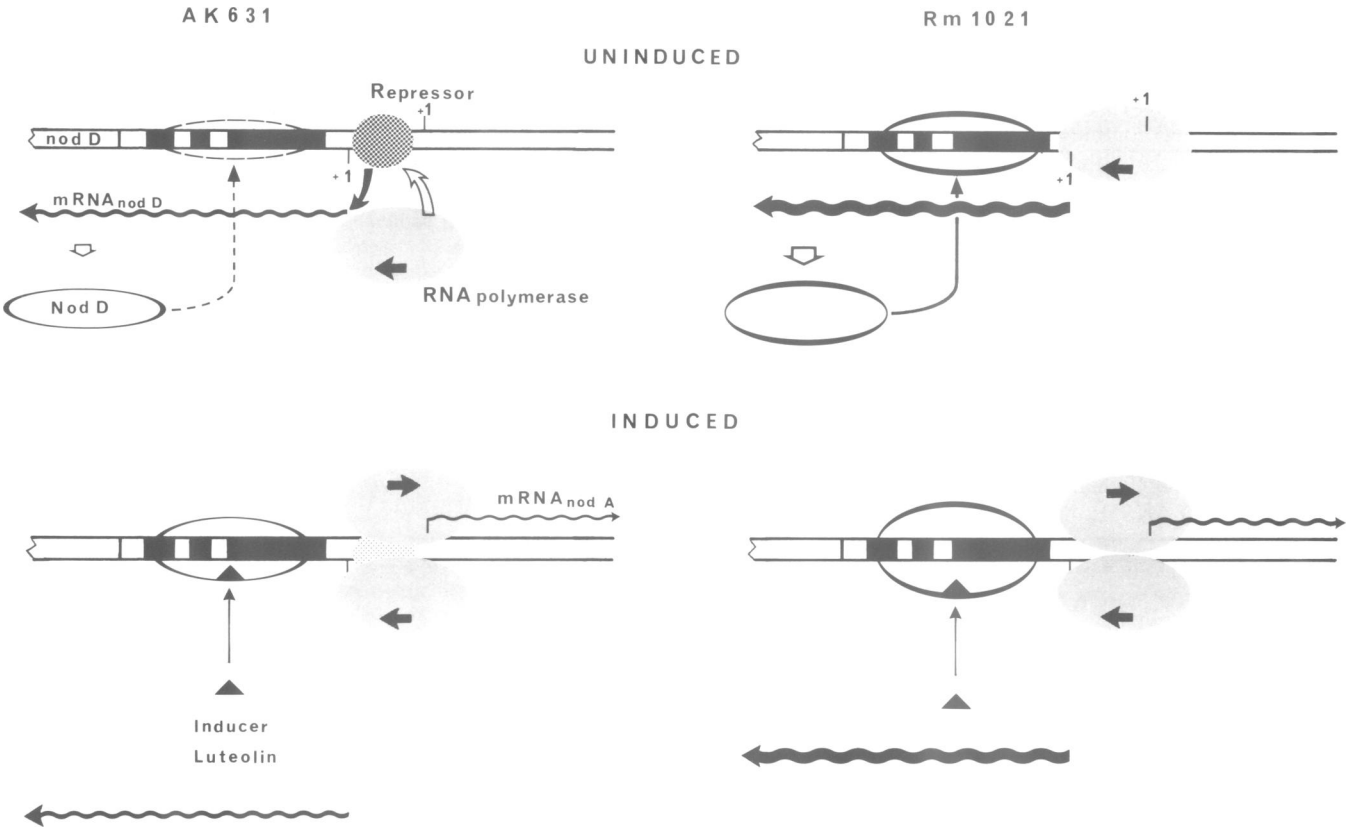

Fig. 7. A model for common *nod* gene regulation in *R.meliloti* strains AK631 and 1021.

according to this model, the flavonoid is required for high affinity binding of NodD to the *nod*-box, which will result in the transcription of the *nodABC* genes.

Specific aspects of the model

NodD–nod-box interaction. Significant binding of NodD protein to the *nod*-box was detected only with extracts of luteolin-induced AK631 strains, carrying either the cloned *nodD1* or the *nodD3* gene. Independently, using a somewhat different approach, Fisher *et al.* (1988) had demonstrated the complex formation of *nod* promoter fragments with proteins from *R.meliloti* extracts overproducing NodD, as well as with purified NodD. Our results support these observations and in addition, our DNase I footprinting and immunoprecipitation experiments indicate that both 'halves' of the *nod*-box are required for efficient NodD-binding. The 47-bp *nod*-box is a relatively large DNA region to interact only with one protein and the possibility that additional proteins bind to the *nod*-box and regulate *nod* gene expression cannot be excluded.

Role of repressor binding. In contrast to the positive activation of *nod* genes by NodD, the negative control of *nod* gene expression by the repressor protein described in this paper for AK631 has not been detected in all rhizobia. Different rhizobia may have different unique components of *nod* gene regulation. For instance, the *nodD* gene is autoregulated in *R.leguminosarum* (Rossen *et al.*, 1985), but not in *R.meliloti* (Mulligan and Long, 1985). The repressor-like protein was found not only in AK631 but also in >80% of other *R.meliloti* strains or field isolates. Strain 1021 was unable to produce the repressor, but it is likely to contain the inactive homologue of the repressor gene. In addition, *nod* gene induction is rather low in some other *Rhizobium* or *Bradyrhizobium* species (unpublished data; Banfalvi *et al.*, 1988). The approach described in this paper may be applicable to search for a repressor in these strains.

A 21-bp-long continuous stretch from the repressor-protected sequence of the *nodD1* promoter was found to be conserved in the *nodD2* promoter, downstream of the n6 *nod*-box (Rostas *et al.*, 1986), which binds also the repressor. The repressor does not interact with the *hsnA* and *hsnD* promoters where this sequence is more diverged. The binding of the repressor to the overlapping *nodD*-inducible *nod* promoters may sterically prevent the RNA polymerase from interacting with either of the two promoters.

Role of inducer. In contrast to other laboratories (Hong *et al.*, 1987; Fisher *et al.*, 1988), we found that under certain conditions, namely when extra copies of the *nodD3* region were present in AK631, the addition of luteolin considerably increased the strength of the NodD–*nod*-box interaction. This effect of luteolin was also detected *in vitro* upon addition of luteolin to the protein extract prepared from uninduced AK631 (pNID6) cells.

The molecular basis for the effect of the inducer on complex formation is not known. Earlier molecular genetic studies indicated that the inducer may interact directly with the NodD protein (Horvath *et al.*, 1987; Burn *et al.*, 1987), but a chemical demonstration is still lacking. Nevertheless, it is tempting to speculate that the inducer causes conformational change of the NodD protein which increases its binding affinity to the *nod*-box. This property of NodD would resemble that of the AraC protein which regulates the transcription of the L-arabinose operon. The AraC binds to the same promoter site with and without the inducer. In the absence of the inducer the AraC protein binding to the promoter site is not sufficient to stimulate RNA polymerase binding (Hendrickson and Schleif, 1984).

The requirement for extra *nodD* genes suggests that the inducer exerts its effect on the repressor-*nod* promoter complex formation via NodD. By analogy to other regulatory systems (Hahn *et al.*, 1986), it is possible that the flavonoid modifies the conformation of NodD in a way that its increased affinity to bind to the *nod*-box interferes with the binding of the repressor to the *nod* promoter.

Materials and methods

Bacterial strains and plasmids

AK631, a compact colony morphology variant of *R.meliloti* 41, ZB138, a megaplasmid deletion mutant of AK631, lacking all the three *nodD* copies and all *nod*-box sequences (Kondorosi *et al.*, 1984), *R.meliloti* 1021, and JM57, a derivative of *R.meliloti* 1021, carrying a *nodC–lacZ* fusion on the megaplasmid (Mulligan and Long, 1985) as well as 21 other *R.meliloti* strains, field isolates of different geographical origin (listed in Figure 4B and kindly provided by Ilona Barabas and Jean Denarie) were used. Media and growth conditions were as described previously (Kondorosi *et al.*, 1984). Plasmids pKSK5 (Kondorosi *et al.*, 1984) and pNID6 (Györgypal *et al.*, 1988) carry the common *nod* or the *nodD3* region respectively in vector pRK290. pEK901 and pEK902 plasmids were constructed by cloning the 2237-bp *Bam*HI–*Hin*dIII and 570-bp *Bam*HI–*Pvu*II fragments of the common *nod* region (Török *et al.*, 1984) into pNM481 and pNM480 vectors (Minton, 1984) respectively, resulting in translational fusions of *nodC* or *nodD1* gene with *lacZ*. The nod–*lacZ* fusions were isolated from these constructs on *Eco*RI–*Dra*I fragments and cloned into the *Eco*RI–*Sma*I site of the broad host range vector pPR33 (Ratet *et al.*, 1988), that was followed by insertion of the *E.coli rho*-independent *rrnB* T_1T_2 terminator sequence (kindly provided by I.Boros) into the *Eco*RI site upstream of the *nodA* or *nodD1* promoter region inhibiting transcriptional readthrough from the vector. Plasmids pEK7055–7058 carried restriction fragments of the overlapping *nodA–nodD1* promoter region (Figure 1), which were filled in with Klenow and ligated to the *Sma*I site of pUC19. pEK963 contains the n1 *nod*-box on the 570-bp *Bam*HI–*Pvu*II fragment of the common *nod* region in vector pHC624 (Boros *et al.*, 1984). pJS314 (Horvath *et al.*, 1986) contains n2 and n3 on a 500-bp fragment of the *hsn* region, generated by *Bal*31 digestion and attachment of *Eco*RI linker (Figure 1).

Tn5 mutagenesis and bacterial crosses

Tn*5* mutagenesis of pEK7059, mobilization of the pRK290 derivatives into *R.meliloti* (Ditta *et al.*, 1980) and gene replacement by homologous recombination (Ruvkun and Ausubel, 1981) were performed as described (Kondorosi *et al.*, 1984).

Hybridization

Southern hybridization and hybridization of the ^{32}P-labelled DNA fragments to Eckhardt gel (Eckhardt, 1978), which separated the endogenous *R.meliloti* plasmids from the chromosome, were performed according to Banfalvi *et al.* (1981).

Nodulation assay

This was carried out as described previously (Horvath *et al.*, 1986). Nodule number of *M.sativa* L. var. Nagyszenas induced by *R.meliloti* strains was counted every 2nd or 3rd day. To demonstrate significant differences in nodulation kinetics among *Rhizobium* strains, 90 seedlings were inoculated with every strain, using three separate inoculation batches. The nodulation data were evaluated and presented on two different ways (Figure 6), showing in both cases significant differences between rep^+ and rep^- strains.

β-Galactosidase activity of nod–lacZ fusions

Rhizobium meliloti strains indicated in Table I were diluted 100-fold from overnight cultures in YTB or in YTB containing the appropriate antibiotics for plasmid selection in the absence and presence of luteolin (5 μM). Bacteria were grown at 30°C for 16 h and β-galactosidase activity was determined from 3–5 independent measurements according to Miller (1972).

Gel electrophoresis DNA binding assay

Reduction in the electrophoretic mobility of the labelled DNA fragments was used to detect binding of proteins to DNA (Fried and Crothers, 1981). Restriction fragments indicated in Figure 1 were isolated and end-labelled with Klenow fragment and [α-^{32}P]dATP or [α-^{32}P]dCTP. Binding reaction was carried out at 23°C for 20 min in 20-μl aliquots containing 10 mM Tris−HCl, pH 7.5, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 5% glycerol, 1 μg sonicated ZB138 DNA (as non-specific competitor), ~1 ng radiolabelled DNA and 10 μg protein. Free and protein-bound DNA were separated on 4% polyacrylamide gels in TBE (Tris−borate−EDTA) buffer; gels were prerun for 2 h at 20 mA. Gels were electrophoresed at 15 mA at room temperature, then transferred to Whatman 3MM, dried and autoradiographed. The 21-bp oligonucleotide used in competition experiments was kindly synthesized by B.Gronenborn.

Production of anti-NodD antibodies and determination of the amount of NodD protein in Rhizobium cells

For production of the anti-NodD antibodies the NodD1 protein was over-expressed by gene fusion of the *cI* repressor sequence and *nodD1* gene of AK631. The strategy was the same as that reported for construction of *cI−nodA* gene fusion (Schmidt *et al.*, 1986), but using a 1.2-kb blunt-ended *nodD1* fragment (starting from the *Bgl*II site of *nodD1*), yielding the expression plasmid pJS4035, which encoded ~98% of the NodD1 protein (Göttfert *et al.*, 1986). The CI−NodD1 fusion protein was overexpressed in *E.coli* W3110 containing pJS4035 and purified from inclusion bodies as described for the CI−NodA fusion protein (Schmidt *et al.*, 1986). Polyclonal antibodies directed against the purified NodD fusion protein were raised in rabbits and were purified as described earlier (Schmidt *et al.*, 1986). The amount of NodD protein was determined from OD_{600} = 1.0 pellets of *R.meliloti* cells. The bacterial proteins were separated in 12% SDS−polyacrylamide gels and transferred to nitrocellulose which was incubated with anti-NodD antibodies and then with ^{125}I-labelled protein A (5 μCi, Amersham) as described previously (Schmidt *et al.*, 1986).

Immunoprecipitation of NodD protein−DNA complexes

The procedure of Benson and Pirotta (1987) was used with some modifications. Plasmid DNAs (pHC624 and pEK963) were cut with *Bgl*II and *Hin*fI and end-labelled with Klenow fragment and [α-^{32}P]dATP. The labelled fragments (~30 ng) were incubated on ice for 30 min with bacterial extracts (40−80 μg protein) in the presence of 3 μg competitor DNA (sonicated pUC18) in 25 μl binding buffer (20 mM Tris−HCl, pH 7.5, 150 mM NaCl, 0.25 mM EDTA, 3 mM $MgCl_2$, 1 mM DTT, 10% glycerol). Then anti-NodD1 antibodies (1 μg) were added to the mixtures and incubation continued on ice for a further 30 min. *Staphylococcus aureus* cells (20 μl of a 10% washed suspension, BRL) were then added and the mixtures were kept on ice for 30 min with occasional mixing. After dilution with 300 μl of binding buffer the immunocomplexes bound to protein A present on the surface of bacteria were collected by centrifugation, washed twice with binding buffer and were pelleted through a 10% sucrose cushion in binding buffer (1 ml). The pellet was suspended in 50 μl of TE (pH 7.6) containing 2 μg of competitor DNA and phenol extracted. The DNA in the aqueous phase was analysed by PAGE and autoradiography.

DNase I footprinting

DNase I protection experiments were performed on the 158-bp fragment containing the nl *nod*-box and the overlapping *nodA* and *nodD1* promoters. pEK7056 was cut either at the unique *Eco*RI or *Hin*dIII site, labelled by fill-in with Klenow polymerase and recut with either *Hin*dIII or *Eco*RI. Cleared cell lysates (100−200 μg) were preincubated on ice for 10 min with 1−3 μg sonicated ZB138 DNA in 20 μl binding buffer (10 mM Tris−HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 5% glycerol). For the naked DNA, the protein extracts were replaced by an equal amount of BSA. Following preincubation 1−2 × 10^4 c.p.m. of the labelled DNA was added and incubation was continued for 10 min at 20°C. The DNA was digested for 90 s at 20°C with 2 μl of 50 mg/ml freshly diluted DNase I (Sigma). The reaction was terminated by addition of 100 μl of stop solution containing 50 mM EDTA, 0.2% SDS, 200 μg tRNA, 10 mg/ml proteinase K and incubated first at 37°C for 30 min and then at 56°C for 30 min. The samples were deproteinized and purified by multiple extractions with phenol and phenol−chloroform and precipitated twice with ethanol. The digestion products were separated on 8% denaturing polyacrylamide gels. The sequence ladders were prepared according to the standard Maxam−Gilbert protocol.

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