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Sinorhizobium meliloti nfe (Nodulation Formation Efficiency) Genes Exhibit Temporal and Spatial Expression Patterns Similar to Those of Genes Involved in Symbiotic Nitrogen Fixation

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The *nfe* genes (*nfeA, nfeB,* and *nfeD*) are involved in the nodulation efficiency and competitiveness of the *Sinorhizobium meliloti* strain GR4 on alfalfa roots. The *nfeA* and *nfeB* genes are preceded by functional *nif* consensus sequences and NifA binding motifs. Here, we determined the temporal and spatial expression patterns of the *nfe* genes in symbiosis with alfalfa. Translational fusions of the *nfe* promoters with the *gusA* gene and reverse transcription-polymerase chain reaction analyses indicate that they are expressed and translated within mature nitrogen-fixing nodules and not during early steps of nodule development. Within the nodules the three *nfe* genes exhibit a spatial expression pattern similar to that of genes involved in symbiotic nitrogen fixation. We show that *nfeB* and *nfeD* genes are expressed not only from their own promoters but also from the upstream *nfe* promoter sequences. Furthermore, with the use of specific antibodies the NfeB and NfeD proteins were detected within the root nodule bacteroid fraction. Finally, NfeB was inmunolocalized in the bacteroid cell membrane whereas NfeD was detected in the bacteroid cytoplasm.

Members of the Rhizobiaceae family form symbiotic associations with leguminous plants, resulting in the formation of a root-specific structure, the nodule, where the symbiotic nitrogen fixation takes place. Formation of this new organ is a complex developmental process involving a series of differential gene activation steps in both the bacterial and plant symbionts. In *Sinorhizobium meliloti,* most of the essential genes required during the symbiotic process are located in the harbored megaplasmids, named pSyms (Banfalvi et al. 1981; Finan et al. 1986; Hynes et al. 1986; Kondorosi et al. 1982). In addition to pSyms, some strains harbor one or more large cryptic plasmids (non-pSym) that carry genetic information that is not essential for symbiosis but could enhance the symbiotic process (Mercado-Blanco and Toro 1996). The *S. meliloti* strain GR4 harbors two cryptic plasmids (Toro and Olivares 1986), pRmeGR4a and pRmeGR4b. The latter contains the nodulation formation efficiency (*nfe*) genes (Soto et al. 1994, 1993), which are involved in the nodulation efficiency and competitiveness of this strain on alfalfa roots (Sanjuan and Olivares 1989; Soto et al. 1994; Toro and Olivares 1986). Earlier reports suggested that the *nfe* region contains symbiotically functional genes that transcribe from a *nifH* promoterlike DNA sequence in an NifA-RpoN (σ^{54})-dependent manner (Sanjuan and Olivares 1989, 1991). DNA sequencing and primer extension analyses showed, within the *nfe* region, three loci named *nfeA, nfeB,* and *nfeD,* flanked by insertion sequences, and that the *nfeA* and *nfeB* genes are preceded by functional *nif* consensus sequences and NifA binding motifs (Soto et al. 1993, 1994). It was also shown that in *Escherichia coli* cells expression of *nfeA* could occur in an NifA-independent manner (Soto et al. 1993). In vitro couple transcription-translation analysis suggested that the *nfeD* gene carries its own functional promoter (Soto et al. 1994). However, the regulation of *nfeD* expression was uncertain. Furthermore, to date, the temporal and spatial expression patterns of *nfeA, nfeB,* and *nfeD* genes during the symbiotic process with alfalfa have not been addressed.

Here, we report on the *S. meliloti nfe* gene expression in symbiosis with alfalfa. Our results show that the *nfe* genes exhibit temporal and spatial expression patterns similar to those of genes involved in symbiotic nitrogen fixation. We show that in symbiosis the *nfeB* and *nfeD* genes appear to be transcribed not only from their own promoter sequences but also from the upstream *nfe* promoters. This complex pattern of expression appears to result in a differential accumulation of the *nfe*-encoded products within mature nitrogen-fixing nodules.

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RESULTS

Spatial expression pattern of *nfeA, nfeB,* and *nfeD* genes in mature nitrogen-fixing nodules.

Translational fusions between *nfeA, nfeB,* and *nfeD* promoters and the *gusA* gene were constructed for monitoring *nfe* gene (Fig. 1) expression in alfalfa root nodules. Longitudinal sections (80 μm and 5 μm thick) of 20-day-old nodules, elicited by the *S. meliloti* strains GRM8 and 2011 harboring the three different fusions were observed for β-glucuronidase ac-

tivity. This enzymatic activity was visualized by light microscopy observation of the pigment resulting from degradation of the substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc). For all the strains analyzed, no staining was observed in the bacteria-free nodule tissues (meristematic zone I, outer cortex, endodermis, vascular bundles, and nodule parenchyma) or in the plant cells where bacteria are liberated from infection threads (prefixing zone II). In nodules elicited by GRM8 and 2011 (Fig. 2A and B) carrying fusion pGUS3 (p*nfeD*::*gusA*), blue staining, corresponding to β-glucuronidase activity, starts in the cell layers that stain with potassium iodide solution (non-nitrogen-fixing interzone II-III). The intensity of the signal was greatest in the interzone II-III and distal cell layers of zone III (nitrogen-fixing zone), decreasing progressively toward the proximal end of the nodule. For nodules elicited by GRM8 carrying fusion pGUS2 (p*nfeB*::*gusA*), staining accumulation was clearly visible in the infected cells of interzone II-III and nitrogen-fixing zone III but, unlike fusion pGUS3, the intensity of the signal was homogenous along the stained tissue (Fig. 2C). Interestingly, in *S. meliloti* 2011 the expression of fusion pGUS2 starts in the nitrogen-fixing zone III instead of interzone II-III (Fig. 2D), which indicates that the host genetic background has some role in the pattern of expression of the *nfeB* gene. In nodules elicited by GRM8 and 2011 carrying fusion pGUS1 (p*nfeA*::*gusA*), the staining appears in the interzone II-III and is extended through the nitrogen-fixing zone III. The expression level of β-glucuronidase activity by fusion pGUS1 was lower than that exhibited by the other two *nfe* promoter fusions (Fig. 2F and G).

Temporal expression of *nfeA, nfeB,* and *nfeD* genes during the symbiotic process.

To determine the temporal expression of the *nfeA, nfeB,* and *nfeD* genes, spot inoculation on alfalfa roots, with the *S. meliloti* strain GR4 as inoculant, was carried out. Total RNA was isolated at different time intervals after inoculation (1 to 7 days) from the inoculated root fragments and from bacterial cells grown in defined media. The presence of mRNA from each *nfe* gene was determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis with specific *nfe*-derived primers (see Materials and Methods). mRNA from the *nfe* genes was not detected from cells grown under vegetative conditions (data not shown). However, *nfeB* and *nfeD* RT-PCR-derived products were obtained from mRNA isolated from alfalfa roots 7 days after inoculation (Fig. 3A). This temporal expression pattern was similar to that obtained with the *nifH* gene (data not shown). In the case of *nfeA*, nonspecific RT-PCR products were visualized in the agarose gel. However, DNA hybridization of the corresponding Southern blots with a specific *nfeA* DNA probe revealed that the higher level of *nfeA* mRNA appears at the same time as *nifH* and the other *nfe* transcripts during the infection process (Fig. 3B).

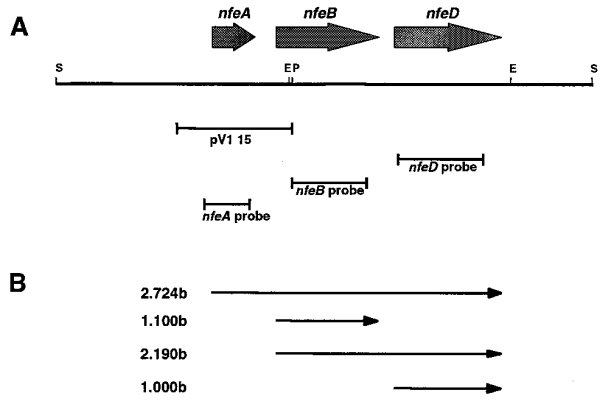

Fig. 1. *Sinorhizobium meliloti nfe* genes. **A,** Physical map of *nfe* genes on plasmid pRmeGR4b. Extension and location of DNA probes used in this work specific to *nfe* genes are indicated below map. Restriction sites indicated are *Eco*RI (E), *Pst*I (P), and *Sal*I (S). **B**, Pattern of transcription of *nfe* region based on analysis of Northern (RNA) blots (see Figure 4).

Functional character of *nfe* promoters in symbiosis.

The *nfeA, nfeB,* and *nfeD* genes are all preceded by promoter sequences, which suggests that each of these genes could be transcribed independently. However, transcriptional terminators are not detected at the DNA sequence level. Therefore, we tested whether the transcription of *nfeB* and *nfeD* genes could also be triggered from the other upstream *nfe* promoters. Northern (RNA) blot hybridizations with total RNA from 20-day-old nodules elicited by strains GR4, 2011 (pRmNT40), and 2011 were carried out. Approximately 20 μg of total RNA was loaded in a denaturing agarose gel, blotted, and hybridized against different DNA probes (Fig. 1A) specific to *nfeA, nfeB,* and *nfeD* genes.

The *nfeA* probe hybridizes with an mRNA transcript of 2.7 kb (Fig. 4A). As previously reported, *nfeA* shows in its 5′ end extensive sequence identity with *nifH* and with the upstream noncoding sequence of the *fixABCX* operon in its 3′ end (Soto et al. 1993). The fact that the detected signal was present in samples from strain 2011 (lacking *nfeA*) suggests that the probe is recognizing mRNA from the *nif* and/or *fix* region. On the other hand, a putative transcript containing the three *nfe* genes (2,724 bases) would be hidden by the detected transcript of 2.7 kb. This cross-hybridization could not be avoided by the use of nitrogen-fixing mutants, since it is well established that these mutants exhibit significant structural deviations from the wild-type nodules (Vasse et al. 1990), which could, in turn, modify the transcriptional pattern of other genes. Nevertheless, RT-PCR and Southern blot hybridization analyses confirmed the presence in the nodules elicited by *S. meliloti* strains carrying the *nfe* region of mRNAs containing *nfeA* (data not shown). Since the transcripts corresponding to *nfeA* alone (480 bases) or together with *nfeB* (1,634 bases) were not detected in the Northern blots, it is more likely that *nfeA* is contained within a transcript containing the three *nfe* genes. When the same blot was hybridized with an *nfeB* probe several bands with different intensity appeared (Fig. 4B). The strong signal of approximately 1.0 kb matches the size expected from monocistronic mRNA of *nfeB* transcribed from its own promoter. Another strong signal appears below the one of 1.0 kb that could correspond to degraded *nfeB* mRNA. Two weaker signals of approximately 2.0 and 2.5 kb were also detected that may correspond to a dicistronic mRNA (*nfeB* plus *nfeD*) transcribed from the *nfeB* promoter and a tricistronic mRNA transcribed from the *nfeA* promoter containing the three *nfe* genes, respectively. The same signals were detected when the hybridization was performed with the *nfeD* probe (Fig. 4C). With this probe, an additional 1.1-kb signal was

also detected, which corresponds to the length expected from monocistronic mRNA of *nfeD* transcribed from its own promoter.

The above results suggest that within the nodules there are at least four different mRNA transcribing from the *nfe* region (Fig. 1B). Thus, the *nfeB* and *nfeD* genes appear to be transcribed not only from their own promoter sequences but also from the upstream *nfe* promoters.

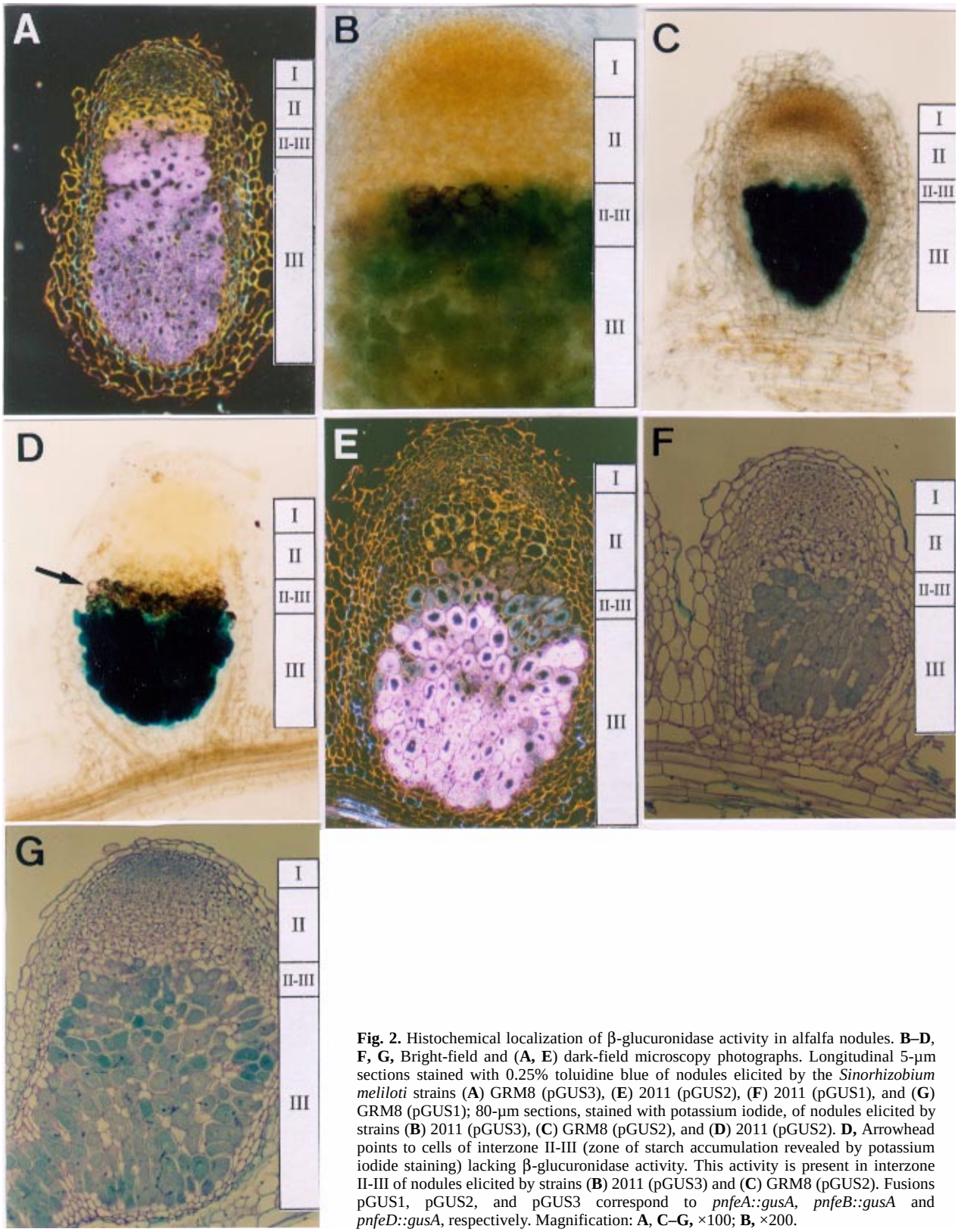

Fig. 2. Histochemical localization of β-glucuronidase activity in alfalfa nodules. **B–D**, **F, G,** Bright-field and (**A, E**) dark-field microscopy photographs. Longitudinal 5-µm sections stained with 0.25% toluidine blue of nodules elicited by the *Sinorhizobium meliloti* strains (**A**) GRM8 (pGUS3), (**E**) 2011 (pGUS2), (**F**) 2011 (pGUS1), and (**G**) GRM8 (pGUS1); 80-µm sections, stained with potassium iodide, of nodules elicited by strains (**B**) 2011 (pGUS3), (**C**) GRM8 (pGUS2), and (**D**) 2011 (pGUS2). **D,** Arrowhead points to cells of interzone II-III (zone of starch accumulation revealed by potassium iodide staining) lacking β-glucuronidase activity. This activity is present in interzone II-III of nodules elicited by strains (**B**) 2011 (pGUS3) and (**C**) GRM8 (pGUS2). Fusions pGUS1, pGUS2, and pGUS3 correspond to *pnfeA::gusA*, *pnfeB::gusA* and *pnfeD::gusA*, respectively. Magnification: **A**, **C–G,** ×100; **B,** ×200.

Detection of Nfe proteins in *S. meliloti* bacteroids.

Data above indicate that *S. meliloti nfe* genes are transcribed and translated in bacteroids within mature nitrogen-fixing nodules. To demonstrate that the *nfe*-encoded proteins were actually present in the bacteroids, *nfe*-specific antibodies were raised in rabbits and further purified by affinity in nitrocellulose. Proteins from bacteroid fraction of nodules elicited by *S. meliloti* strains with or without the *nfe* genes were separated on acrylamide gels, Western blotted (immunoblotted), and probed with the corresponding antibodies.

As shown in Figure 5A, antibodies raised against NfeA are able to detect this protein in *E. coli* BL21 (DE3) cells harboring plasmid pT7-55 (Fig. 5A, lane 3), which carries most of the *nfeA* gene under the T7 promoter. These antibodies recognize a polypeptide with the same apparent molecular mass as NfeA in GR4 bacteroid proteins (Fig. 5A, lane 1). However, this polypeptide was also present in bacteroids from *S. meliloti* strains lacking the *nfeA* gene (data not shown), suggesting a nonspecific protein recognition. With the purified antibodies (Fig. 5B), although they were still able to recognize the NfeA polypeptide from plasmid pT7-55, they did not recognize any protein from the GR4 bacteroid fraction. These results together with the low β-glucuronidase activity of fusion pGUS1 observed in mature nitrogen-fixing nodules suggest that the NfeA protein may be present in very small amounts in *S. meliloti* bacteroids expressing the *nfeA* gene.

As shown in Figure 6A, antibodies prepared against NfeB recognized a polypeptide of similar size in the bacteroid proteins from strains GR4 and 2011 (pRmNT40) that was absent in the bacteroid fraction of strain 2011 (pRK290). The antibodies also recognized the NfeB polypeptide resulting from

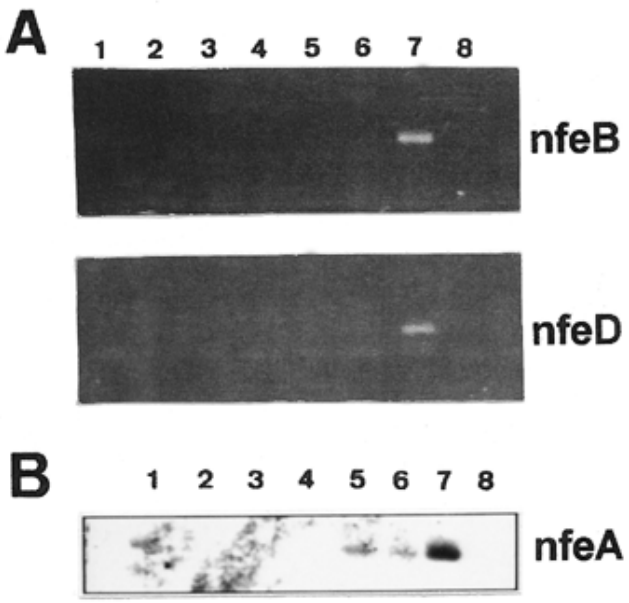

Fig. 3. Determination of temporal expression of *nfe* genes by reverse transcription-polymerase chain reaction (RT-PCR)**.** RNA was isolated from alfalfa roots, 1 (lane 1), 2 (lane 2), 3 (lane 3), 4 (lane 4), 5 (lane 5), 6 (lane 6), and 7 days (lane 7) after inoculation with the *Sinorhizobium meliloti* strain GR4. RT-PCR was carried out as described in Materials and Methods. Number of PCR amplification cycles was the same for all lanes in each sample. Controls without RT are in lane 8. **A,** RT-PCR-derived products for *nfeB* and *nfeD*. **B,** DNA hybridization of RT-PCR products derived from *nfeA* with a specific probe to *nfeA* (Fig. 1A). Major signal in lane 1 does not correspond to a hybridization band.

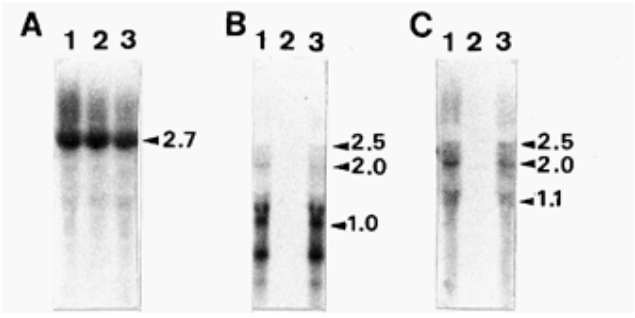

Fig. 4. Northern (RNA) blot on total RNA isolated from nodules elicited by the *Sinorhizobium meliloti* strains GR4 (lane 1), 2011 (lane 2), and 2011(pRmNT40) (lane 3). Total RNA was hybridized with DNA probes (Fig. 1) specific to (**A**) *nfeA*, (**B**) *nfeB*, and (**C**) *nfeD*. **A,** Arrowhead shows mRNA of *nifH*. Molecular weights indicated in kilobases.

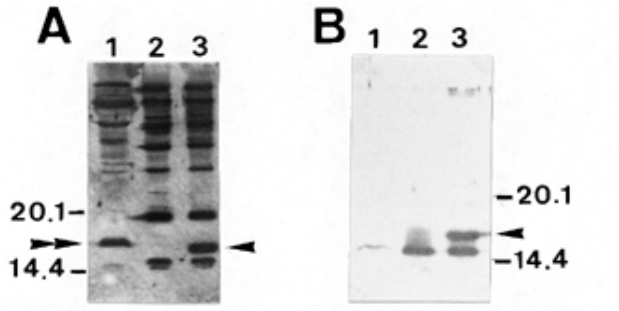

Fig. 5. Western blot (immunoblot) analysis of NfeA. Anti-NfeA antiserum (**A**) or purified anti-NfeA antibodies (**B**) were used to detect NfeA protein in bacteroid fraction of nodules elicited by the *Sinorhizobium meliloti* strain GR4 (lane 1), and extracts from induced *Escherichia coli* BL21 (DE3) cells carrying plasmids pT7-7 (lane 2) or pT7-55 (lane 3). **A (**lane 1), Double arrowhead shows nonspecific polypeptide recognized by NfeA antibodies in GR4 bacteroid proteins. **B (**lane 3), Arrowhead indicates NfeA polypeptide (expressed under T7 promoter in *E. coli* cells) recognized by purified anti-NfeA antibodies. Nature of fast migrating antigen band below NfeA polypeptide (**A** and **B**) is unknown.

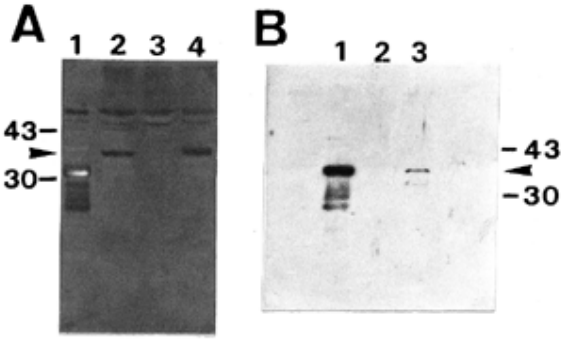

Fig. 6. Western blot (immunoblot) analysis of NfeB and NfeD. **A,** Anti-NfeB antibodies were used to detect NfeB protein in bacteroid fraction of nodules elicited by the *Sinorhizobium meliloti* strains GR4 (lane 2), 2011 (lane 3), and 2011 (pRmNT40) (lane 4), and in extracts from induced *Escherichia coli* BL21(DE3) cells carrying plasmid pT7-21.3 (lane 1). **B,** Purified anti-NfeD antibodies were used to detect NfeD protein in extracts from induced *E. coli* BL21(DE3) cells carrying plasmids pTE7-21 (lane 1) and pTEK7-21 (lane 2), and in the bacteroid fraction of nodules elicited by *S. meliloti* GR4 (lane 3).

expression of the *nfeB* gene from plasmid pT7-21.3 in *E. coli* cells. However, the specific polypeptide from the bacteroid fraction of strains GR4 and 2011 (pRmNT40) recognized by the antibodies exhibited a molecular mass 2.5 kDa higher than that expected for the native NfeB. This difference in the apparent molecular mass could be explained by posttranscriptional modifications of the NfeB protein in the bacteroid state.

Antibodies against NfeD recognized a polypeptide in the bacteroid fraction from strain GR4 that migrates in the polyacrylamide gel as the NfeD polypeptide resulting from expression of the *nfeD* gene from plasmid pTE7-21 in *E. coli* cells (Fig. 6B). This polypeptide, the size of which was expected from the deduced *nfeD* DNA sequence (Soto et al. 1994), was also present in the bacteroid fraction of strain 2011(pRmNT40) but not in strain 2011 (pRK290) (data not shown) nor in *E. coli* cells harboring plasmid pTEK7-21, which carries a mutated *nfeD* gene (Fig. 6B). These data indicate that the NfeD protein is present in the bacteroid fraction of *S. meliloti* strains expressing the *nfeD* gene.

Immunocytochemical localization of NfeB and NfeD within bacteroids.

The purified antibodies anti-NfeB and NfeD were used to immunolocalize the NfeB and NfeD proteins in mature nitrogen-fixing nodules. We performed immunolocalization on 700-Å sections taken from zone III of 20-day-old nodules elicited on alfalfa roots by *S. meliloti* GR4 (*nfe*$^+$) and its derivative strain GRM6 (*nfe* mutant), as a negative control. As shown in Figure 7A and B, labeling was observed in nodule sections elicited by strain GR4, with both anti-NfeB and anti-NfeD purified antibodies. In the case of the anti-NfeD (Fig. 7A) antibodies, gold particles were localized within bacteroids, which suggests that NfeD is a cytoplasmic protein. On the other hand, with anti-NfeB (Fig. 7B) antibodies, gold labeling was observed within bacteroids, both in cytoplasm and associated with external structures, which suggests that NfeB is a protein associated with the bacteroid cell membrane. Gold particles were not observed when antibodies were used on GRM6 sections (data not shown).

DISCUSSION

With the use of *gusA* translational fusions, we analyzed the expression of *S. meliloti nfe* genes in symbiosis with alfalfa. Within mature nitrogen-fixing nodules the three *nfe* genes *nfeA*, *nfeB*, and *nfeD*, when expressed in their host genetic background (GRM8), are switched on suddenly in the transition from prefixation zone II into interzone II-III. The interzone II-III is characterized by abundant starch deposits in the plastids of invaded host cells (Vasse et al. 1990), and appears to be a region where major changes take place during nodule development. Different reports have shown that in alfalfa nodules the *S. meliloti nif* and *fix* genes are switched on in the interzone II-III (Sharma and Signer 1990; Soupène et al. 1995). In pea nodules, Yang et al. (1991) described that *nifA*

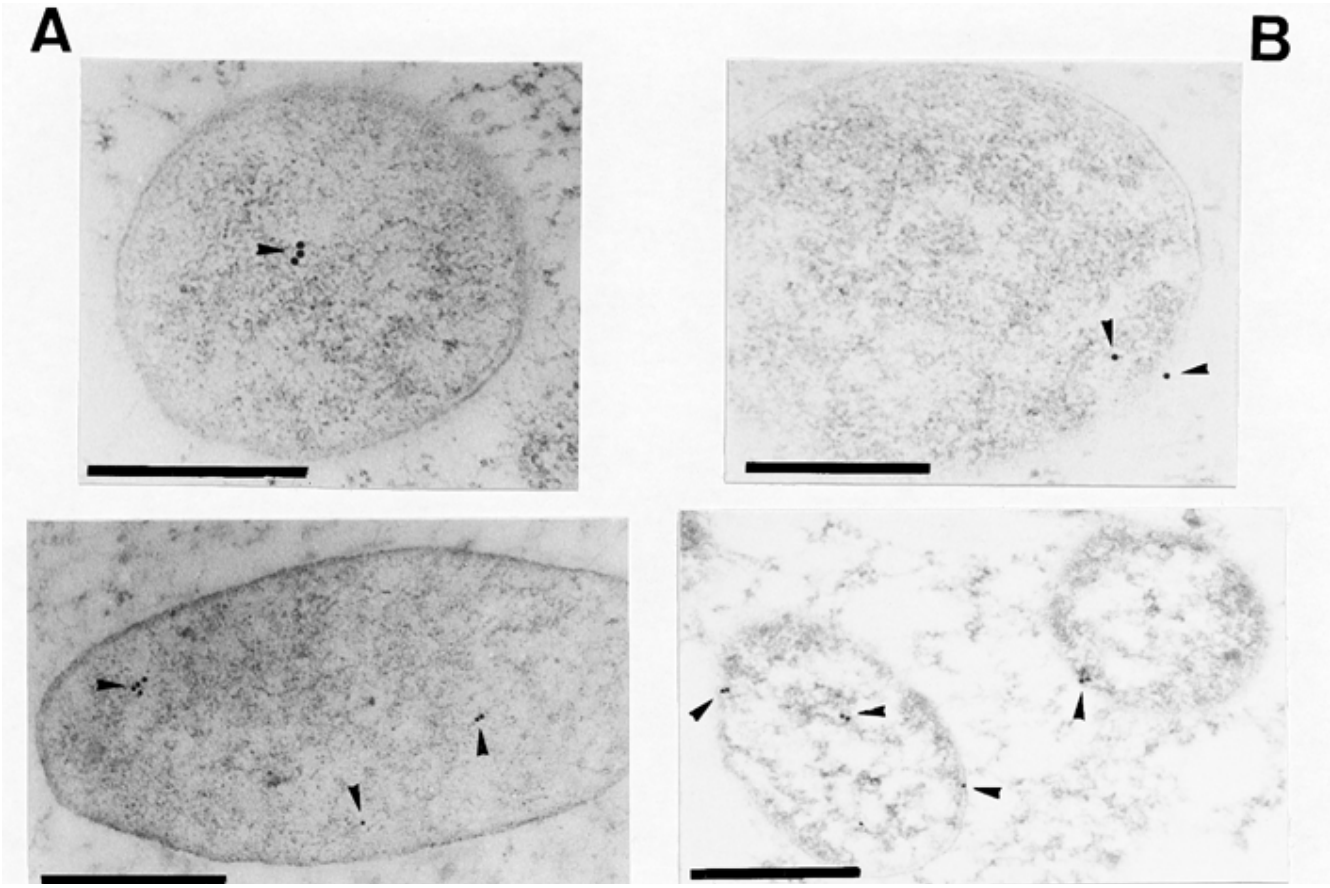

Fig. 7. Immunocitochemical localization of (**A**) NfeD and (**B**) NfeB. Arrowheads show colloidal gold. Bars, 1 µm.

expression was higher in the interzone II-III decreasing toward the proximal part of the nodule whereas *nifH* is expressed at the same levels in both interzone II-III and zone III. The spatial pattern of expression of *nfeD* in the nodule appears to be similar to that of *nifA* whereas the expression of *nfeA* and *nfeB* resembles that shown by *nifH*. In agreement with the spatial pattern of expression of the *nfe* genes within nitrogen-fixing nodules, spot inoculation assays did not reveal expression of these genes in the infection threads (data not shown). Furthermore, by RT-PCR analyses it was determined that during the symbiotic process the *S. meliloti nfe* genes are expressed at the same time as *nifH*, 7 days after inoculation, when the interzone II-III is already differentiated (Vasse et al. 1990). These results indicate a temporal and spatial coexpression of *nfe* and nitrogen fixation genes in alfalfa nodules and during the symbiotic process, suggesting that their induction may respond to the same nodule signals and transcriptional activators. Soto et al. (1993) found that *nfeA* and *nfeB* are preceded by functional *nif* consensus sequences and NifA binding motifs. Taken together, it is more likely that induction of *nifA* in the nodule would, in turn, activate the expression of *nfeA* and *nfeB* genes, as occurs with *nifH*. The fact that the expression pattern of *nfeB* depends on the host genetic background (see Results) suggests that in addition to *nifA* other host factor(s) could be involved. On the other hand, sequence analysis of the DNA upstream of *nfeD* (Soto et al. 1994) did not reveal either upstream activator sequences specific for *nifA* activation or a σ^{54} promoter. However, 218 bp upstream of the ATG start codon proposed for *nfeD*, within the coding sequence of *nfeB*, we found (N. Toro, *unpublished observation*) a DNA sequence with striking homology to the –54, –33 region of FixJ-dependent promoters (Galinier et al. 1994; Waelkens et al. 1992), including the motif ACC-AATA/TT with a single mismatch (G/C change at the third position). This region of the putative *nfeD* promoter lacks the –60 TAAG motif found in *fixK* promoters. Thus, it is possible that expression of *nfeD* in the nodule could be activated by the FixL/FixJ regulatory cascade in a manner similar to that of *nifA*, which would be in agreement with the spatial pattern of expression of *nfeD* in the nodule. However, we did not find that the level of expression of the *pnfeD*::*gusA* fusion could be increased by a plasmid (pDP3) expressing the *fixL* and *fixJ* under the Tac promoter (F. M. García-Rodríguez, *unpublished data*). Thus, further work is required to establish the mechanism of regulation of *nfeD* gene expression.

Although the *S. meliloti nfe* genes are all preceded by promoter sequences, transcription of this DNA region seems to follow a complex pattern. Northern blot analyses indicate the presence of mRNA transcripts containing *nfeB*, *nfeD*, and a transcript containing both genes. Although RT-PCR data indicate that mRNA containing *nfeA* is present in the infected roots (Fig. 3B) and in the nodules elicited by *S. meliloti* strains carrying the *nfe* genes (data not shown), we did not detect the *nfeA* transcript alone by Northern blot analysis. Furthermore, as shown in Figure 2, it was demonstrated that the promoter of the *nfeA* gene is functional within the nodules. Northern blot data suggest that there is a mRNAs transcript containing the three *nfe* genes. Taken together, the more plausible explanation for our data is that *nfeB* and *nfeD* genes are expressed not only from their own

Table 1. Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics	Source or reference
Escherichia coli		
HB101	*pro, leu, thi, lacY, endA, recA hsdR, hsdM,* Sm^r	Boyer and Roulland-Dussoix 1969
DH5α	*recA1, endA1, ϕ80d, lacZ, dm15*	Promega, Madison, WI
K38	HfrC(λ)	Russel and Model 1984
BL21(DE3)	F^-, *ompT [lon] hsdSB* (r^-_B m^-_B) DE3	Studier et al. 1990
Sinorhizobium meliloti		
GR4	Wild-type; Nod^+ Fix^+	Casadesús and Olivares 1979
GRM6	pRmeGR4b-cured derivative of GR4	Mercado-Blanco and Olivares 1993
GRM8	pRmeGR4a- and pRmeGR4b-cured derivative of GR4	Mercado-Blanco and Olivares 1993
2011	Wild-type; Nod^+ Fix^+	J. Denarié
Plasmids		
pRK2013	Helper plasmid for mobilization. Km^r	Figurski and Helinski 1979
pGP1-2	T7 polymerase vector; Km^r	Tabor and Richardson 1985
pT7-3/7-7	T7 promoter-expression vector; Ap^r	Tabor and Richardson 1985
pGEMEX™-1/-2	Expression vectors for fusion proteins with T7 gene 10; Km^r	Promega, Madison, WI
pGRF1	pGEMEX™-2 with *nfeA*	This study
pGRF2	pGEMEX™-1 with *nfeB*	This study
pGRF3	pGEMEX™-2 with *nfeD*	This study
pGUS1	Translational fusion between *pnfeA* (coordinates 1159–1678) and *gusA* in pBI101.3; Km^r	This study
pGUS2	Translational fusion between *pnfeB* (coordinates 1915–2361) and *gusA* in pBI101; Km^r	This study
pGUS3	Translational fusion between *pnfeD* (coordinates 2993–3345) and *gusA* in pBI101; Km^r	This study
pTE7-21	pT7-3 with an *Eco*RI fragment (coordinates 2352–4406) containing *nfeD* and 822 nucleotides of *nfeB*; Ap^r	This study
pTEK7-21	pTE7-21 with *nfeD* gene interrupted by Km^r cassette; Ap^r, Km^r	This study
pT7-21.3	pT7-7 with most part of *nfeB* (coordinates 2352–3345); Ap^r	This study
pT7-55	*nfeA* (coordinates 1678–2166) under T7 promoter in pT7-7; Ap^r	This study
pRmNT40	pRK290 with *Eco*RI insert of plasmid pRmeGR4b harboring *nfe* genes	Toro and Olivares 1986
pRK290	RK2 derivative; Tc^r	Ditta et al. 1980
pBI101/101.3	Derivatives of pBIN19 with a promoterless β-glucuronidase gene cassette	Clontech, Heidelberg, Germany
pV1 15	pUC18 with a insert of *nfeA* and *nfeB* (coordinates 1296–2361); Cm^r	Soto et al. 1993

promoters but also from the upstream *nfe* promoter sequences, and that the *nfeA* promoter induces transcription of the three *nfe* genes altogether.

The level of β-glucuronidase activity observed for each of the fusions suggests that the promoter upstream of *nfeB* functions as a stronger promoter than that of *nfeA*. The different strength of the promoter sequences and the pattern of transcription of the *nfe* genes also suggest a differential accumulation in the nodules of the corresponding *nfe*-encoded products. With purified antibodies, we detected in the bacteroid fraction the presence of NfeB and NfeD proteins, but not NfeA. In addition, the NfeB and NfeD proteins could be immunolocalized at the cellular level within the bacteroid. While NfeD seems to be a cytoplasmic protein, NfeB appears to be associated with the cell membrane. The *gusA* fusion results indicate that *nfeA* is translated in the nodule. Nevertheless, NfeA appears to be present at a very low level, which is in agreement with the low β-glucuronidase activity observed for the p*nfeA-gusA* fusion, and the weak signal detected for polycistronic mRNA containing the three *nfe* genes.

The *nfe* genes have a potential use for the construction of more competitive rhizobia for legume nodulation. Rhizopines are compounds synthesized in bacteroids within nodules and subsequently catabolized by the free-living rhizobial strains (Murphy et al. 1995). Synthesis of the rhizopine is invariably associated with the ability to catabolize it, providing a selective advantage for infection and therefore nodule occupancy to the rhizopine producing/catabolizing strain. The *nfeA* gene shows extensive homology to *nifH*, as occurs with the 5′ end of the first open reading frame (ORF1) of the rhizopine synthesis *mos* locus (Soto et al. 1993). In addition, the *mos* genes are transcriptionally regulated in a similar fashion to genes involved in symbiotic nitrogen fixation. Thus, it is possible that the infection advantage provided by the *nfe* genes results from the synthesis of an unknown yet rhizopine, which in turn can be catabolized by strain GR4.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

Bacterial strains and plasmids used and constructed in this work are listed in Table 1. *E. coli* was routinely grown at 37°C on Luria-Bertani (LB) medium (Sambrook et al. 1989) and rhizobial strains at 30°C on tryptone-yeast (TY) (Beringer 1974) or defined MM (Robertsen et al. 1981). Antibiotics were used as required at the following concentrations (in μg ml^{-1}): tetracycline, 10; ampicillin, 200; kanamycin, 50 for *E. coli* and 180 for *S. meliloti;* spectinomycin, 100; and streptomycin, 250 for *S. meliloti* and 50 for *E. coli*.

β-Glucuronidase expression in endosymbiotic conditions.

Seeds of *Medicago sativa* L. 'Aragón' were surface sterilized and germinated as described previously (Olivares et al. 1980) and the seedlings were grown aseptically in square petri plates on buffered nodulation medium agar (Cooper and Long 1994). Four-day-old alfalfa plants (roots 5 to 6 cm in length) were spot inoculated for root nodules formation as described by Dudley et al. (1987) with a bacterial suspension (10^9 cells ml^{-1}) of the *S. meliloti* strains 2011 and GRM8 carrying plasmids pGUS1, pGUS2, or pGUS3 (Table 1). Nodules and roots were fixed with 0.5% glutaraldehyde solution buffered with 10 mM sodium phosphate and 0.1 M sodium chloride (pH 7.0; 30 min) and then rinsed with 25 mM sodium phosphate buffer (5 × 5 min). The samples were transferred to the staining solution: sodium phosphate 100 mM pH 7.0 buffer; $K_4[Fe(CON)_6]$ 5 mM; $K_3[Fe(CON)_6]$ 5 mM and X-gluc 2 mM (15 min under vacuum followed by 12 to 24 h at atmospheric pressure and 37°C in the dark). Then the samples were rinsed with 10 mM pH 7.0 sodium phosphate buffer (5 × 5 min). The roots were cleared for 30 s with sodium hypochlorite and finally rinsed with distilled water (4 × 5 min) before being mounted in water and observed by bright-field microscopy. The nodules were fixed again with 4% paraformaldehyde and 0.25% glutaraldehyde in 10 mM sodium phosphate buffer (pH 6.8), NaCl 100 mM, and subjected to 1 h under vacuum followed by 3 h at atmospheric pressure. After this treatment, some nodules were embedded in 2% agarose, cut into 80-μm longitudinal slices, and stained with 0.1 M aqueous potassium iodide solution. Other nodules were dehydrated by being passed through a routine ethanol series, embedded in Technovit 8100, cut into 5-μm longitudinal sections, and stained with 0.25% toluidine blue. The sections were photographed with bright- or dark-field microscopy.

Inmunocytochemistry.

For embedding in acrylic resin, 20-day-old nodules were fixed in 2.5% (vol/vol) glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2 (2 × 10 min under vacuum followed by 2 h at atmospheric pressure) and then rinsed with the same buffer (3 × 1 h). The nodules were dehydrated by being passed through a routine ethanol series. Following infiltration for 4 h at 4°C, each time with a different mixture of absolute ethanol: LR White resin (London Resin Co., Woking, Surrey, UK) 2:1; 1:2; and with LR White resin for 24 h. Nodules were embedded in gelatin capsules and polymerized at 60°C for 24 h. Ultrathin sections were collected on nickel grids and used for immunolabeling. Nonspecific binding sites were blocked with 20 mg of BSA (bovine serum albumin) in TBS buffer (Tris-HCl 10 mM pH 7.4; NaCl 150 mM; 0.3% Tween 20 and N_3Na_2 0.2 mg/ml) for 1 h at 37°C. Sections were then transferred on drops of purified anti-NfeB and anti-NfeD antibodies diluted 1:2 in TBS buffer-BSA (20 mg/ml) 1 h at 37°C. After three washes in TBS buffer-BSA (2 mg/ml) for 5 min each, sections were placed on drops of goat anti-rabbit IgG conjugated to 5 nm of colloidal gold, diluted 1:40 with TBS buffer-BSA (2 mg/ml) 1 h at 37°C. Following three washes in TBS buffer-BSA (2 mg/ml) for 5 min each, samples were washed again with TBS buffer-BSA (2 mg/ml) plus 0.1% Triton X-100, and finally rinsed in fresh Milli Q water 3 × 5 min each. Samples were then counterstained with 2% uranyl acetate (10 min) and lead citrate (1 min) and observed on a Philips EM300 transmission electron microscope.

RNA isolation.

Total RNA isolation from alfalfa nodules elicited by *S. meliloti* was made by a single extraction with an acid guanidinium thiocyanate-phenol chloroform mixture as described (Chomczynski and Sacchi 1987). The amount and quality of the RNA samples was determined by spectrophotometry and agarose gel electrophoresis. Only RNA preparations having $A_{260}/A_{280} \geq 2$ were used for further studies.

Preparation of cDNA and PCR.

Total RNA (0.5 µg) was annealed with 100 ng of the corresponding annealing primer to the 3′ end of the mRNA, 5′-TGGTGCCGTCCTCTAAGTGC-3′ (coordinates 1809 to 1828) for *nfeA*, 5′-CGGAATTGACGACAGATCC-3′ (coordinates 3005 to 3024) for *nfeB*, and 5′-ATACTTGCTTGTGA CAGCCG-3′ (coordinates 3658 to 3677) for *nfeD*. Reactions were carried out in 5 µl of annealing buffer (250 mM KCl, 15 mM TrisHCl pH 8.3 and 1 mM EDTA) by boiling for 3 min and incubating at 60°C for 30 min. The RNA was reverse transcribed in 12 µl of 10 mM TrisHCl (pH 8.8), 50 mM KCl, 5mM $MgCl_2$, 1 mM dNTP, 20 U RNA guard (Boehringer Mannheim, Barcelona, Spain) and 7 U of AMV reverse transcriptase (Boehringer Mannheim). The reactions were incubated for 1 h at 42°C and heated for 5 min at 95°C. PCRs were carried out in a Robocycler 40 (Stratagene, Amsterdam). The cDNA samples were amplified in 25 µl of 10 mM TrisHCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 100 mM dNTP, 1 mM of the corresponding set of primers, and 1 U of *Taq* DNA polymerase. The 3′ end primers were the ones described above and the 5′ end primers were 5′-CCCGTCGA AAAACAAAATGC-3′ (coordinates 1713 to 1732) for *nfeA*, 5′-GTTCGACGGTGGTCATTACG-3′ (coordinates 2795 to 2814) for *nfeB*, and 5′-CACGATGCACTAATTCACG-3′ (coordinates 3453 to 3471) for *nfeD*. The amplification of synthesized cDNAs was carried out during 38 cycles for *nfeA* and *nfeB*, and 42 cycles for *nfeD* (95°C, 1 min; 60°C, 1 min; 72°C, 30 s) followed by an extension of 15 min at 72°C. PCR samples were analyzed by agarose gel electrophoresis. In the case of *nfeA*, Southern blotting and hybridization were carried out with an *nfeA*-derived DNA probe (pV115, see Table 1) labeled with digoxigenin with a DIG Luminescent Detection Kit (Boehringer-Mannheim).

Preparation of antibodies.

Fusion proteins between the T7 protein 10 and the *nfe* genes were produced by cloning *nfeA* and *nfeD* in pGEMEX-2 and *nfeB* in pGEMEX-1 (Promega, Madison, WI). For cloning, plasmid pGEMEX-2 was digested with *Apa*I and the 3′ ends removed with T4 DNA polymerase. *nfeA* was cloned as a *Bst*EII-*Xho*I fragment and *nfeD* as an *Spe*I-*Eco*RI fragment, after filling in the ends with Klenow DNA polymerase (Sambrook et al. 1989), resulting in plasmids pGRF1 and pGRF2, respectively (Table1). For cloning, pGEMEX-1 was previously digested with *Eco*RI and filled. *nfeB* was cloned as an *Eco*RI-*Spe*I fragment after filling in the 5′ overhangs, resulting in plasmid pGRF3 (Table 1). The fusion proteins were expressed in *E. coli* K38 (pGP1-2) as previously described (Tabor and Richardson 1985). The fusion proteins produced in this manner were purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), electroluted with a Bio-Rad 422 Electro-Eluter in the same buffer used for SDS-PAGE. Aliquot of 100 µg of protein were mixed 1:1 with Freund's complete adjuvant before being injected into rabbits (New Zealand White). Antibodies were purified by nitrocellulose immunoaffinity as previously described (Kelly et al. 1986; Sakakiraba et al. 1990).

For Western blotting, nodule bacteroid fractions were obtained as previously described (Caba 1987). The extracts were separated by SDS-PAGE with a Bio-Rad Mini-Protean 1 electrophoresis apparatus. Gels were blotted onto nitrocellulose membranes (Millipore Ibérica, Spain) with an LKB electroblotting transfer cell. Immunodetection was carried out with a chemiluminescence kit (Boehringer-Mannheim).

Northern blot hybridizations.

About 20 µg of total RNA from nodules, denatured with formaldehyde, was subjected to electrophoresis on 1.2% wt/vol agarose/MOPS (morpholinepropanesulfonic acid)/formaldehyde gels. Electrophoresis were carried out in 1× MOPS buffer and 80 V. Samples were mixed in 1:1 proportion with loading buffer (1.6 ml of formaldehyde, 5 ml of formamide, 0.5 ml of 20× MOPS, and ethidium bromide at 40 µg/ml), heated at 65°C for 10 min and chilled on ice for 5 min before being loaded in the gel. Fractionated RNA was transferred to nylon membranes (Boehringer Mannheim) in 20 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and baked 20 min at 120°C in vacuum. Filters were prehybridized for 2 h at 42°C in a buffer containing 6% vol/vol 20× SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 50% vol/vol formamide, 50 × Denhardt's reagent 1/10 diluted, 0.5% wt/ vol SDS, and salmon sperm DNA 100 µg/ml. Hybridization was carried out at 42°C overnight in the same solution without Denhardt's reagent and supplemented with the α^{32}P-dCTP-labeled DNA probe internal to *nfeA*, *nfeB*, or *nfeD* (Fig. 1). Probes were prepared with the Rediprime DNA Labelling System (Amersham Pharmacia Biotech, U.K.). Blots were washed in 2× SSC, 0.5% wt/vol SDS (2 × 5 min and 1 × 30 min) at room temperature and at 50°C in 1× SSC, 0.1% SDS 1 × 30 min and exposed overnight to X-OMAT films (Kodak, Rochester, NY) at –70ºC with an intensifying screen. For re-probing filters, blots were washed in 0.1% SDS at 68°C for 1 h.

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