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The Relationship between Nodulin Gene Expression and the *Rhizobium nod* Genes in *Vicia sativa* Root Nodule Development

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The role of the *Rhizobium nod* genes in the induction of nodulin gene expression was examined by analyzing nodules formed on vetch roots by bacterial strains containing only the *nod* region. Introduction of an 11-kb cloned *nod* region of the *R. leguminosarum* sym plasmid pRL1JI into sym plasmid-cured rhizobia conferred on the recipient strains the ability to induce nodules in which all nodulin genes were expressed. This proves that from the sym plasmid only the *nod* region is involved in the induction of nodulin gene expression. A transconjugant of *Agrobacterium* carrying the same *nod* region induces nodules in which only early nodulin gene expression is detected. Thus, the *nod* region is essential for the induction of early nodulin gene expression. In this case, nodule cytology may indicate that a defense response of the plant interferes with the induction of late nodulin gene expression. Indirect evidence is presented that indeed the *Rhizobium nod* genes are also in some way involved in the induction of the expression of late nodulin genes. The combination between histological data and pattern of nodulin gene expression furthermore reveals a correlation between nodule structure and nodulin gene expression. This correlation may aid in speculations about the functions of nodulins.

Additional keywords: leghemoglobin, nitrogenase, *R. trifolii*.

Leguminous plants are distinguished from other plant families by their ability to form nitrogen-fixing root nodules in close cooperation with bacteria from the genera *Rhizobium* or *Bradyrhizobium*. In several leguminous species, approximately 30 plant genes, so-called nodulin genes (Van Kammen 1984), have been identified that are exclusively expressed in the root nodule (Legocki and Verma 1980; Govers *et al.* 1985; Verma and Brisson 1987). In recent years it has been found that root nodule formation is attended by a differential expression of nodulin genes. This has resulted in a division of nodulin genes into two distinct classes, early and late nodulin genes.

Early nodulin genes are expressed well before the onset of nitrogen fixation, and early nodulins are most likely involved in the formation of the nodule structure and the infection process (Govers *et al.* 1986; Gloudemans *et al.* 1987). The best-studied example so far is the soybean early nodulin Ngm-75, which probably is a cell-wall constituent (Franssen *et al.* 1987). Late nodulin gene expression starts around the onset of nitrogen fixation, and late nodulins probably function in establishing and maintaining the proper conditions within the nodule that allow nitrogen fixation and ammonia assimilation to occur. Type members of the class of late nodulin genes are the leghemoglobin genes.

By analyzing the *in vitro* translation products of nodule and root RNA on two-dimensional (2-D) gels, we have previously identified one early (Nvs-40) and 15 late nodulin genes (Moerman *et al.* 1987) in vetch (*Vicia sativa* subsp. *nigra*). Vetch was used as test plant, because this small, leguminous plant responds rapidly to inoculation with engineered bacteria (van Brussel *et al.* 1982). Late nodulin genes included the leghemoglobins and Nvs-65. A second early nodulin, VsENOD2, was identified by northern blot analysis by using the soybean early nodulin cDNA clone pGmENOD2 (Franssen *et al.* 1987) as a probe.

Insight in the mechanisms of regulation of these nodulin genes may be gained by studying the involvement of genes of *Rhizobium* in the induction of nodulin gene expression. Investigations that used overlapping cosmid clones allowed us to draw the conclusion that the 10-kb *nod* region of the sym plasmid is sufficient for the induction of early and late nodulin gene expression, if present in a rhizobial chromosomal background. Because the early but not the late nodulin genes were expressed in nodules induced by a strain of *Agrobacterium* carrying the complete sym plasmid, (Moerman *et al.* 1987), we tentatively concluded that the 10-kb *nod* region on the sym plasmid carries at least the information for the induction of early nodulin gene expression.

In the present study, we have analyzed nodulin gene expression in nodules induced by engineered strains of *Rhizobium* and *Agrobacterium* carrying exclusively the *nod* region from the sym plasmid of either *R. leguminosarum* or *R. trifolii*. We present further evidence that the *nod* genes of *Rhizobium* are involved in the induction of the expression of early nodulin genes. Our results indicate that the *nod* genes are in some way involved in the induction of the expression of late nodulin genes. Analysis of nodulin gene

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expression in nodules increasingly disturbed in development shows that the process of root nodule development can be dissected into successive steps, each characterized by the start of expression of defined nodulin genes.

MATERIALS AND METHODS

Plants and bacteria. Vetch seeds were sterilized, germinated, inoculated, and cultured as described (Moerman *et al.* 1987). Bacterial strains and their relevant characteristics are listed in Table 1. Bacterial crosses were performed as described (Spaink *et al.* 1987) by using pRK2013 (Ditta *et al.* 1980) as helper plasmid. Bacteria were grown in YEM medium as described (Gloudemans *et al.* 1987) with 2.5 mg/L of tetracycline for pMP104 selection and 75 mg/L of kanamycin for Tn*5* selection. Nodules were excised from the roots with a scalpel. Root tips from uninfected plants were isolated 8 days after sowing. All tissues were immediately frozen in liquid nitrogen and stored at −80° C until use.

Microscopy and immunocytochemistry. Nodules were fixed with 2.5% glutaraldehyde and 1% osmiumtetroxide and embedded in LR White resin as described previously (Van de Wiel *et al.* 1988). Sections were cut with glass knives on an LKB Ultrotome V. Semithin sections (0.5–2.0 μm) were stained with 1% toluidine blue 0. Ultrathin sections were stained at room temperature in an LKB Ultrostainer 2168 with uranyl acetate for 20 min and then with lead citrate for 40 sec. Sections were examined by using a Philips EM 301 transmission electron microscope operated at 60 kV.

For immunocytochemistry, nodules were fixed in 4% paraformaldehyde, embedded in LR White resin, and attached to slides as described (Van de Wiel *et al.* 1988). Semithin sections (0.5–2.0 μm) were incubated with antiserum, followed by incubation with 10-nm gold particles coupled to protein A (Janssen Pharmaceutica) as secondary antiserum, and the signal was silver enhanced by using the IntenSE II silver enhancement kit (Janssen Pharmaceutica) according to manufacturer's manual. After treatment, sections were stained with 0.1% toluidine blue 0 for 1 min, mounted in Euparal (Chroma), and examined under a Nikon microscope equipped with epipolarization optics (Philips 100-watt halogene lamp).

RNA isolation, *in vitro* translation, and 2-D gel electrophoresis. Total RNA from plant tissue was isolated according to Govers *et al.* (1985). Approximately 2 μg of total RNA was translated *in vitro* in a rabbit reticulocyte lysate in a 6-μl reaction mixture as described (Moerman *et al.* 1987). Translation products were separated by 2-D gel electrophoresis, followed by fluorography of the dried gel to preflashed Kodak XAR5 film (Govers *et al.* 1985).

Northern blot analysis. Total RNA was denatured in dimethyl sulfoxide/glyoxal, electrophoresed in 0.8% agarose gels (Maniatis *et al.* 1982), and transferred to GeneScreen (New England Nuclear) membranes as described (Govers *et al.* 1985). The membranes were hybridized with ^{32}P-labeled probes (Maniatis *et al.* 1982) under the conditions previously described (Franssen *et al.* 1987).

Protein isolation and western blot analysis. Bacteroid proteins were isolated and separated by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis as previously described (Bisseling *et al.* 1983). Proteins were transferred to nitrocellulose by electroblotting (Zabel *et al.* 1982) and after incubation with antiserum visualized with ^{125}I-protein A according to Bisseling *et al.* (1983). Preparation of the antisera against leghemoglobin and the components CI and CII of the nitrogenase of *R. leguminosarum* has been described before (Bisseling *et al.* 1980; Van de Wiel *et al.* 1988).

RESULTS

***Nod* genes are the only sym plasmid DNA required for nodulin gene induction.** We have used strain *R. leguminosarum* 248^c(pMP104), which contains the cloned 11-kb *nod* region of the sym plasmid pRL1J1 of *R. leguminosarum*. A physical and genetic map of the 11-kb *nod* region, carrying the *nodE, F, D, A, B, C, I*, and *J* genes, inserted into a low copy number *IncP* vector to yield plasmid pMP104 (Spaink *et al.* 1987) is given in Figure 1. Strain 248^c(pMP104) has the ability to form nodule structures on both vetch and pea. After introduction of pMP104 in the sym plasmid-cured strain ANU845 of *R. trifolii*, the resulting strain ANU845(pMP104) also obtained the ability to induce nodules on vetch and pea. The histology of the nodules induced on vetch by ANU845(pMP104) (Fig. 2A) and by 248^c(pMP104) is similar to that of the nodules induced by wild-type *R. leguminosarum* (Newcomb 1981). These nodules have an apical meristem, peripherally located

Table 1. Bacterial strains and their relevant characteristics

Strain	Relevant characteristics[a]			Reference or source
Rhizobium leguminosarum				
PRE (wild type)	pSym+	nod+ fix+		Lie *et al.* 1979
248 (wild type)	pSym+	nod+ fix+		Josey *et al.* 1979
248^c (= cured 248)		nod− fix−		Priem and Wijffelman 1984
248^c (pMP104)	pRlnod+	nod+ fix−	Tc^R	This study
Rhizobium trifolii				
ANU843 (wild type)	pSym+	nod− fix−		Schofield *et al.* 1983
ANU843(*nodE* K11::Tn*5*)	pSym+	nod+ fix−	Km^R	Djordjevic *et al.* 1985
ANU845 (= cured ANU843)		nod− fix−		Schofield *et al.* 1983
ANU845(pMP104)	pRlnod+	nod+ fix−	Tc^R	This study
ANU845(pRt032)(*nodE* K11::Tn*5*)	pRtnod+	nod+ fix−	Km^R Cb^R	Djordjevic *et al.* 1985
Agrobacterium tumefaciens				
LBA4301 (= cured Ach5)		nod− fix−		Hooykaas *et al.* 1982
LBA4301(pMP104)	pRlnod+	nod+ fix−	Tc^R	This study

[a] pSym, sym plasmid pRL1J1 (*R. leguminosarum*) or pANU843 (*R. trifolii*); pRlnod, cloned *nod* region from the *R. leguminosarum* sym plasmid pRL1JI; pRtnod, cloned *nod* region from the *R. trifolii* sym plasmid pANU843; nod, ability to nodulate vetch; fix, *in planta* nitrogen fixation on vetch; Tc, tetracycline; Km, kanamycin; Cb, carbenicillin.

vascular bundles, and a central tissue containing uninfected cells and infected cells fully packed with bacteroids. The bacteroids develop into pleiomorphic forms (Fig. 2D), but fail to fix nitrogen due to the absence of *nif* and *fix* genes.

Irrespective of the *Rhizobium* chromosomal background, both early and late nodulin genes are expressed in the nodules induced on vetch by each of the strains containing pMP104. In Figure 3, the expression of three major nodulin genes, Nvs-40, Nvs-65, and vetch Lb, is shown for the ANU845(pMP104)-induced nodules in panels 2A, 2B, and 2C, respectively. All other identified late nodulin mRNAs are also present (data not shown). The presence of the early nodulin VsENOD2 is demonstrated by northern blot analysis by using pGmENOD2 (Franssen *et al.* 1987) as probe (Fig. 4). These results prove that the *nod* region is the only part of the sym plasmid that is essential for the induction of early and late nodulin gene expression.

The role of the *Rhizobium* chromosome in nodulin gene induction. To examine any role of the *Rhizobium* chromosome in nodulin gene induction, we constructed *Agrobacterium tumefaciens* LBA4301(pMP104). This Ti plasmid-cured *Agrobacterium*, containing plasmid pMP104 with the 11-kb *nod* region, efficiently induces nodules on vetch. These nodules have an apical meristem and vascular bundles at the periphery (Fig. 5A). Thus, such nodules are organized like wild-type nodules. In the early symbiotic zone, bacteria are released from the infection threads into the cytoplasm of the host cells and become surrounded by a peribacteroid membrane (Fig. 5D). The cytological data further indicate that after release from the infection threads, development of the infected cells is severely disturbed. Unlike wild-type nodule development, some bacteria are observed within the central vacuole (Fig. 5D). Bacteria never develop into Y-shaped structures and their cytoplasm becomes condensed (Fig. 5E), which suggests that the bacteria are subject to degradation. In addition, the nucleus and cytoplasm of the infected plant cells have become electron dense, and cell organelles have completely disappeared (compare Figs. 5B and E with Figs. 2D, E, F). These phenomena suggest a general collapse of the infected plant cells. This collapse may be due to the lack of a rhizobial signal, but it is also reminiscent of a plant-defense response. In contrast, the uninfected cells have a prominent central vacuole and they contain cell organelles, like plastids with prominent starch granules (Fig. 5C), just as uninfected cells in nodules induced by wild-type *Rhizobium*.

Analysis of the RNA isolated from these LBA4301-(pMP104)-induced nodules shows that the two early nodulin mRNAs, VsENOD2 (Fig. 4) and Nvs-40 (Fig. 3, panel 5B) are present, but late nodulin mRNAs are not detectable (Fig. 3, panels 5A and 5C). This result shows that the *nod* region is the only DNA of *Rhizobium* that in combination with the chromosome of *Agrobacterium* is necessary for the induction of early nodulin gene expression.

***Nod* genes relate to the induction of late nodulin gene expression.** Analogous to the 11-kb *nod* region of *R. leguminosarum*, a 14-kb *nod* region of *R. trifolii* contains all essential functions for the induction of a nodule structure on clover roots (Schofield *et al.* 1984). A physical and genetic map of the 14-kb region in plasmid pRt032, containing the *nod* genes *nodN, M, L, F, E, D, A, B, C, I,* and *J*, is shown in Figure 1. It has been found that a Tn*5* insertion in *nodE* of *R. trifolii* extends the host range of the recipient mutant strain to the pea/vetch cross-inoculation group (Djordjevic *et al.* 1985). After introduction of plasmid pRt032(*nodE* K11::Tn*5*), containing a Tn*5* insertion in the *nodE* gene at position K11, in the sym plasmid-cured strain ANU845, the resulting strain ANU845(pRt032)(*nodE* K11::Tn*5*) differs from ANU845(pMP104) essentially in the *R. trifolii* origin of its *nod* region. Thus, the capacities of two different *nod* regions in the induction of nodulin gene expression can be analyzed in nodules induced by each of the strains on the same host plant species. Strain ANU845(pRt032)(*nodE* K11::Tn*5*) induces nodules on vetch with a frequency of only about one nodule per five plants. The few nodules formed develop without delay like wild-type nodules (Fig. 2C) up to the stage in which infected cells become fully packed with rhizobia. Bacteria develop into pleiomorphic forms (Fig. 2F), but in contrast with wild-type nodule

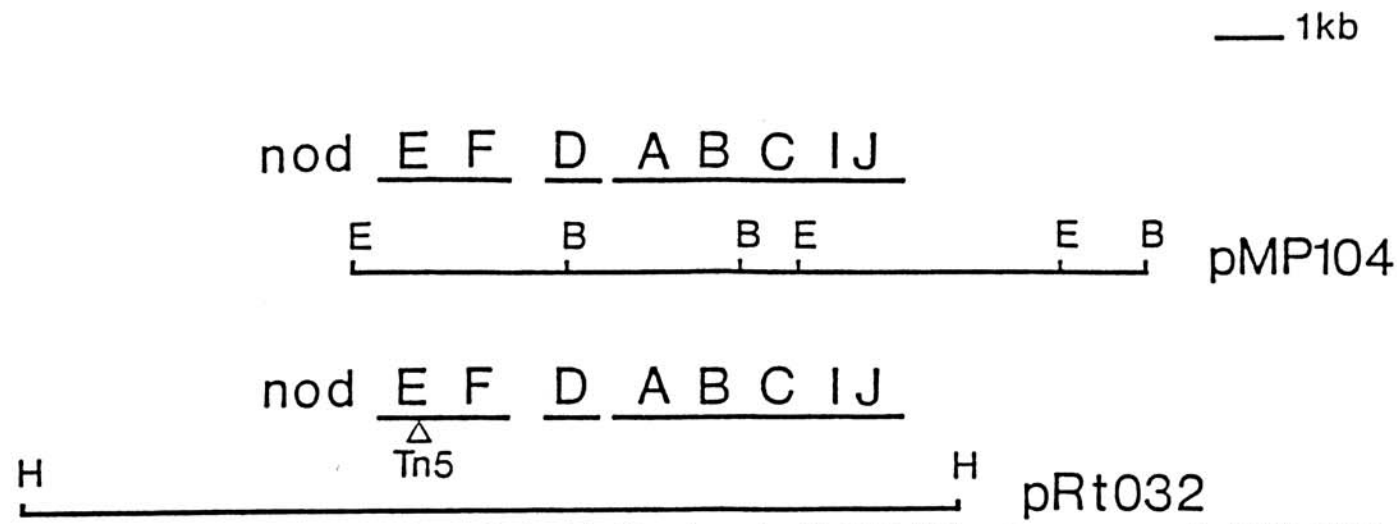

Fig. 1. Simplified physical and genetic maps of the *nod* region derived from the sym plasmid pRL1JI of *R. leguminosarum*, as present in pMP104, and the *nod* region derived from the sym plasmid pANU843 of *R. trifolii*, as present in pRt032. With the Tn*5* in *nod* at position K11, the latter plasmid becomes pRt032(*nod* K11::Tn*5*). The maps are aligned to stress their similarities. In addition to the *nod* genes indicated, pRt032 contains the *nod* genes *nodN, M*, and *L*, upstream from *nodE*, and pMP104 contains genes downstream of *nodIJ*. H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI.

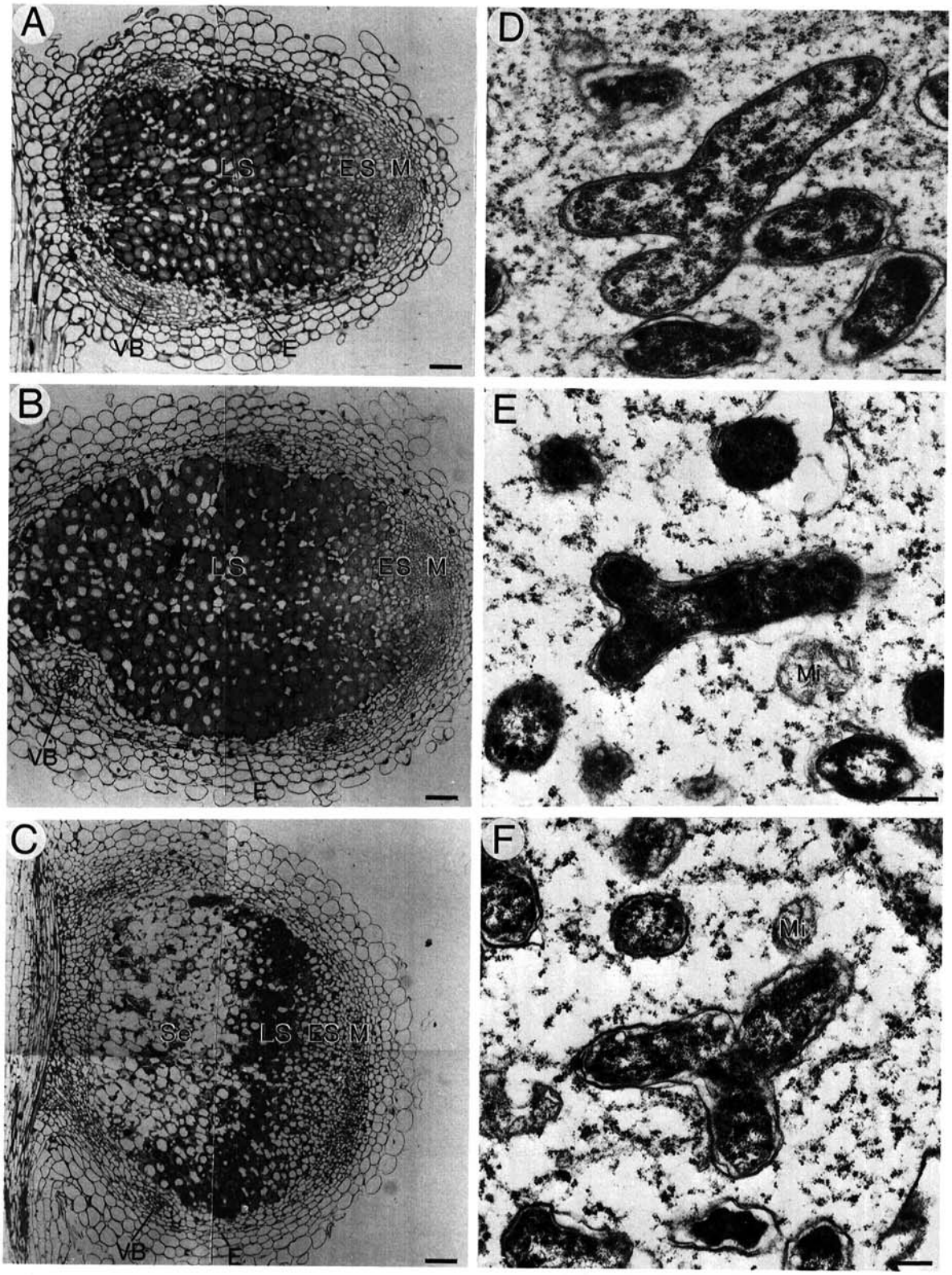

Fig. 2. Light micrograph montages (**A–C**) of nodules induced on vetch by (**A**) *R. trifolii* ANU845(pMP104), (**B**) *R. trifolii* ANU843(*nodE* K11::Tn*5*), and (**C**) *R. trifolii* ANU845(pRt032) (*nodE* K11::Tn*5*), and electron micrographs (**D–F**) of bacteroids of these strains. **A**, Three-wk-old nodule induced by *R. trifolii* ANU845(pMP104). **D**, Bacteroid of *R. trifolii* ANU845(pMP104). **B**, Four-wk-old nodule induced by *R. trifolii* ANU843(*nodE* K11::Tn*5*). **E**, Bacteroid of *R. trifolii* ANU843(*nodE* K11::Tn*5*). **C**, Three-wk-old nodule induced by *R. trifolii* ANU845(pRt032) (*nodE* K11::Tn*5*). **F**, Bacteroid of *R. trifolii* ANU845(pRt032) (*nodE* K11::Tn*5*). All three nodules (**A–C**) exhibit the characteristics of an indeterminate nodule: an apical meristem (M), peripheral vascular bundles (VB), and an endodermis (E). In addition, the ANU845(pRt032)(*nodE* K11::Tn*5*)-induced nodule (**C**) shows a large senescent zone (Se). Bar = 100 μm. The electron micrographs (**D–F**) show that in all cases, the bacteroids have differentiated into the characteristic Y-shaped form. Mi, mitochondrion. Bar = 0.3 μm.

development, senescence occurs soon afterwards. In a 3-wk-old nodule, only a few layers of fully packed cells and a large zone of senescence are observed (Fig. 2C).

Analyses of RNA isolated from these nodules show that both the Nvs-40 (Fig. 3, panel 4B) and the VsENOD2 (Fig. 4) gene are expressed. Also, the late nodulin gene Nvs-65 is expressed (Fig. 3, panel 4A), but expression of other late nodulin genes, including the leghemoglobin genes (Fig. 3, panel 4C), is not detectable in the nodules induced by ANU845(pRt032)(*nodE* K11::Tn*5*). These observations do not exclude that late nodulin genes are still expressed in the few fully infected cells observed in the ANU845(pRt032)-(*nodE* K11::Tn*5*)-induced nodules (Fig. 2C), because their expression might not be detectable in a total RNA preparation. Therefore, we examined the presence of leghemoglobin by means of immunocytochemistry. Sections incubated with antiserum directed against pea leghemoglobin, followed by immunogold silver labeling of the bound antibodies, showed only a low background labeling in the infected cells of the ANU845(pRt032)(*nodE* K11::Tn*5*)-induced nodules (Figs. 6A and B). In contrast, high levels of immunogold silver labeling are found in the plant cytoplasm surrounding wild-type bacteroids of *R. leguminosarum* (Figs. 6C and D). These analyses of individual cells show that the leghemoglobin genes are not expressed in the fully infected cells of the ANU845(pRt032)-(*nodE* K11::Tn*5*)-induced nodules at levels found in infected cells of nitrogen-fixing nodules.

Strain ANU845(pRt032)(*nodE* K11::Tn*5*) induced the expression of early nodulin genes and a single late nodulin gene, Nvs-65. As discussed in the previous section, strain ANU845(pMP104) induced nodules in which all early and late nodulin genes examined, including the leghemoglobin genes, are expressed. Because the only difference between the two strains is the construct carrying the *nod* region, the *nod* region of *R. leguminosarum* present in pMP104 appears in some way to be involved in the induction of the expression of late nodulin genes.

***R. trifolii* can induce early and late nodulin genes in vetch nodules.** The absence of most late nodulin gene transcripts in the vetch nodules formed by ANU845(pRt032)(*nodE* K11::Tn*5*) indicates that the genetic information on this mutated *nod* region is deficient in inducing the expression of late nodulin genes in vetch. To investigate further the genetic potentials of the sym plasmid of *R. trifolii*, we used strain ANU843(*nodE* K11::Tn*5*), which contains a complete sym plasmid of *R. trifolii* with a Tn*5* in *nodE* at position K11. Strain ANU843(*nodE* K11::Tn*5*) formed on the average one

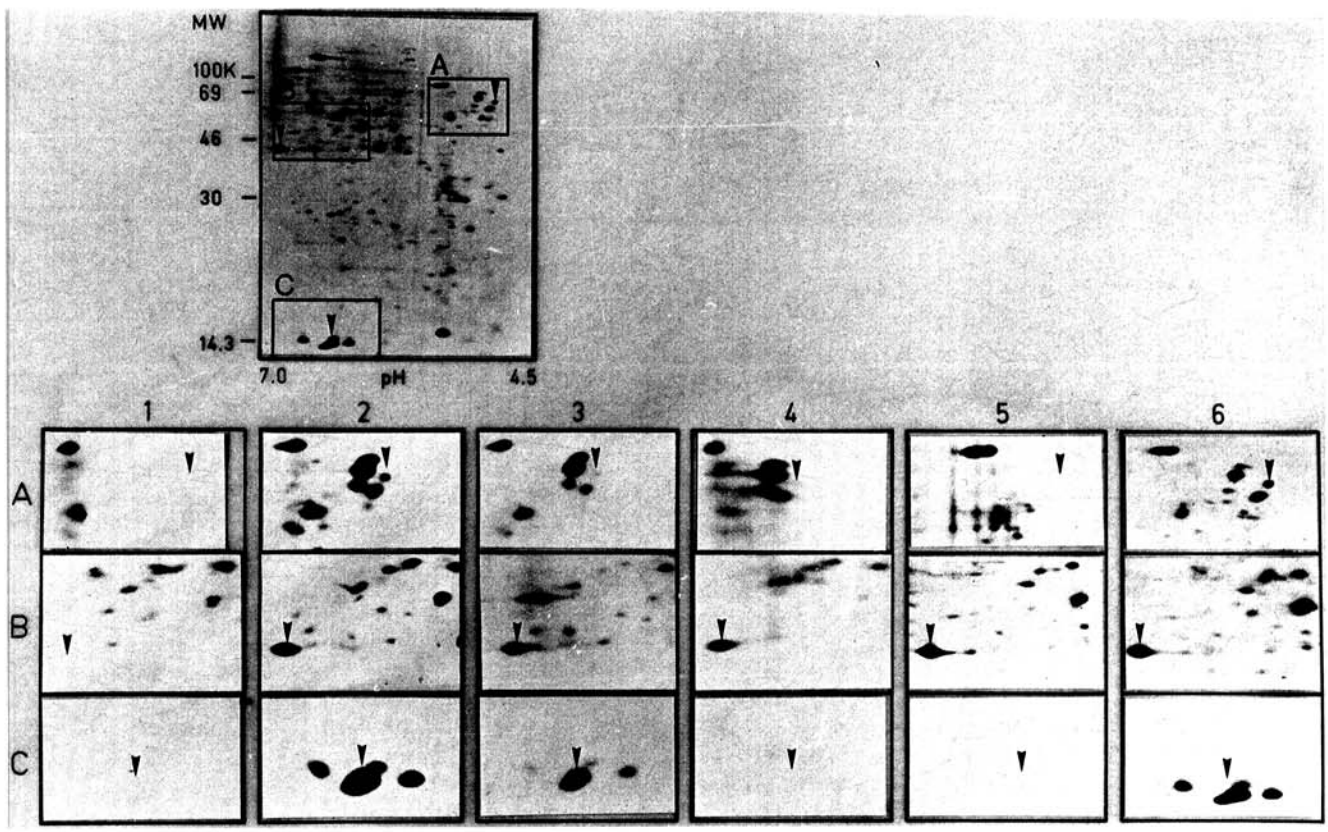

Fig. 3. Expression of three major nodulin genes induced in vetch by *R. trifolii* ANU845(pMP104), *R. trifolii* ANU843(*nodE* K11::Tn*5*), *R.trifolii* ANU845(pRt032)(*nodE* K11::Tn*5*), *A. tumefaciens* LBA4301(pMP104), and *R. leguminosarum* PRE. The upper part of the figure shows the fluorograph of a 2-D gel of *in vitro* translation products obtained with total RNA that was isolated from nitrogen-fixing vetch root nodules 15 days after sowing and inoculation with *R. leguminosarum* PRE. The major nodulin spots Nvs-65, Nvs-40, and vetch leghemoglobin (VsLb) are indicated by arrowheads. In the lower part of the figure, fluorographs are shown of *in vitro* translation products obtained from total RNA isolated from vetch root tips (panel 1) and from nodules induced on vetch by *R. trifolii* ANU845(pMP104)(panel 2); panel 3, *R.trifolii* ANU843(*nodE* K11::Tn*5*); panel 4, *R.trifolii* ANU845(pRt032)(*nodE* K11::Tn*5*); panel 5, *A.tumefaciens* LBA4301(pMP104); panel 6, *R. leguminosarum* PRE. Only the parts of the 2-D gels within the squares in the upper part are shown, as these contain the major nodulin *in vitro* translation products. The comparison is made in (**A**) for Nvs-65, in (**B**) for Nvs-40, and in (**C**) for VsLb. The gels used to obtain 1–4 were run in a different series than the gels used to obtain 5 and 6, which explains the minor differences that can be observed in the pattern of translation products between these fluorographs.

nodule per vetch plant, which occurred primarily at lateral root emergences. Wild-type strain ANU843 of *R. trifolii* very rarely induced a nodule on vetch, confirming the influence of the Tn*5* mutation in *nodE* on host range (Djordjevic *et al.* 1985).

The histology of the nodules formed on vetch by ANU843(*nodE* K11::Tn*5*) was similar to the histology of nodules induced by wild-type *Rhizobium* (Fig. 2B). The morphology of the ANU845(*nodE* K11::Tn*5*) bacteroids (Fig. 2E) was also similar to that of the ANU845(pMP104) bacteroids (Fig. 2D).

Analysis of RNA from the vetch nodules induced by ANU843(*nodE* K11::Tn*5*) revealed that all nodulin genes are expressed (Fig. 3, panels 3A, 3B, and 3C, and Fig. 4). This shows that the sym plasmid genes of *R. trifolii* are equivalent to sym plasmid genes of *R. leguminosarum* in establishing late nodulin gene expression. Furthermore, it indicates that the *nodE* mutation is not the cause for the failure of strain ANU845(pRt032)(*nodE* K11::Tn*5*) to induce late nodulin gene expression in vetch nodules.

The nodules induced on vetch by ANU843(*nodE* K11::Tn*5*) do not fix nitrogen. Because all early and late nodulin genes, as far as identified, are expressed in these vetch nodules, it appears unlikely that the ineffective nature of the nodules is due to the absence of certain nodulins. For a better understanding of the Fix$^-$ phenotype of these nodules, we examined whether the enzyme nitrogenase was produced. Western blots of total protein isolated from wild-type bacteroids of *R. trifolii*, from clover nodules, and from ANU843(*nodE* K11::Tn*5*) bacteroids, from vetch nodules, were incubated with antisera against the components CI and CII of the nitrogenase complex of *R. leguminosarum*. The bound antibodies were visualized with ^{125}I-protein A (Fig. 7). The fluorograph shows that no detectable levels of CI and CII were present in the ANU843(*nodE* K11::Tn*5*) bacteroids, whereas the nitrogenase of *R. trifolii* was easily detected in clover nodules by the antisera used. The lack of nitrogenase was confirmed in immunocytological studies. No immunogold silver labeling above background was detectable in the ANU843(*nodE* K11::Tn*5*) bacteroids after incubation of nodule sections with CI antiserum (Figs. 8A and B). In contrast, after the same treatment, a high level of immunogold silver labeling is observed in wild-type bacteroids of *R. trifolii* in clover nodule sections (Figs. 8C and D) and wild-type bacteroids of *R. leguminosarum* in vetch nodule sections (not shown). These observations indicate that despite the expression of all early and late nodulin genes, nitrogenase protein is not present in ANU843(*nodE* K11::Tn*5*)-induced nodules.

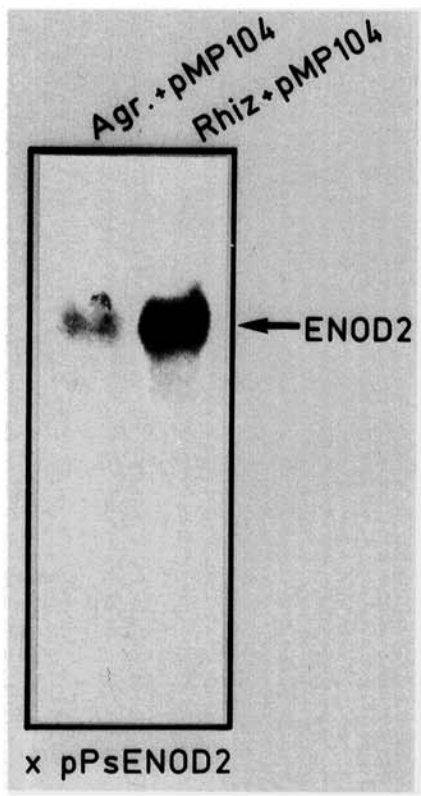

Fig. 4. Expression of the VsENOD2 gene in vetch root nodules. Autoradiograph of a northern blot containing RNA isolated from 4-wk-old nodules induced on vetch by *A. tumefaciens* LBA4301(pMP104), indicated as Agr+pMP104, and in 3-wk-old nodules induced by *R. trifolii* ANU845(pMP104), indicated as Rhiz+pMP104. The blot was hybridized with nick translated pGmENOD2.

DISCUSSION

Strains of *Rhizobium* carrying only the *nod* region from a sym plasmid of *R. leguminosarum* are able to induce nodules on the roots of vetch. In such nodules, both early and late nodulin genes are expressed. Furthermore, we have shown that if the *nod* region from a sym plasmid of *R. leguminosarum* is present in a transconjugant of *Agrobacterium*, this *nod* region confers on the *Agrobacterium* the ability to form a nodule on vetch. In these nodules, only early and no late nodulin gene expression is found. These results confirm and extend our previous findings (Moerman *et al.* 1987). Similar results have recently been found in nodules induced on alfalfa (*Medicago sativa*) by a transconjugant of *Agrobacterium* containing the cloned *nod* region of the sym plasmid of *R. meliloti* (Dickstein *et al.* 1988).

Eight *nod* genes, *nodE, F, D, A, B, C, I*, and *J*, have been identified in the *nod* region of *R. leguminosarum* present in pMP104. Analyzing the phenotypes of *nod* gene Tn*5* mutants, it has been found that mutations in the *nodA, B, C*, and *D* genes abolish nodulation. These four genes are apparently indispensable for nodulation. Mutations in the *nodE, F, I*, and *J* genes do not cause a complete inability to induce a nitrogen-fixing root nodule, but result in a delayed nodulation and a smaller number of nodules (Downie *et al.* 1985). The *nodD* gene encodes a regulatory protein required for the expression of all other *nod* genes (Rossen *et al.* 1987). The *nodA, B*, and *C* genes are essential for root hair curling, formation of the infection thread, and induction of cortical cell divisions (Downie *et al.* 1985; Debellé *et al.* 1986; Dudley *et al.* 1987). Therefore, the gene products of the *nodA, B*, and *C* genes are likely to be responsible for the generation of one or more signals that result in these three phenomena, followed by induction of early nodulin gene expression and formation of a nodule. It is unclear whether the *nodA, B*, and *C* gene products accomplish these effects directly or by initiating a cascade of reactions.

Transfer of a fragment carrying exclusively the *nodD, A, B, C, E*, and *F* region into a sym plasmid-cured *Rhizobium* does not confer on the recipient strain the ability to induce nodules, although such a strain still causes root hair curling

and infection thread formation (Knight *et al.* 1986; Van Brussel *et al.* 1988). These observations suggest that the *nodD, A, B,* and *C* genes are not sufficient by themselves for the induction of early nodulin gene expression and the formation of root nodules, but additional information encoded by the *nod* region is required. Indeed, mutations in the *nodE, F, I,* and *J* genes located on plasmid pMP104, if present in a sym plasmid-cured *Rhizobium*, result in a Nod$^-$ phenotype (Van Brussel *et al.* 1988). The gene products of the *nodE F, I,* and *J* genes thus appear indispensable for nodulation. On the other hand, if these genes are part of the complete sym plasmid, mutations in the *nodE, F, I,* and *J* genes result only in a delayed nodulation and a reduction of the number of nodules (Downie *et al.* 1985). Possibly the function of the mutated *nodE, F, I,* or *J* gene is complemented by another sym plasmid gene, such as *nodN, M,* or *L,* which is not present in pMP104. This question needs further clarification. The results of the phenotypical effects of mutations do not allow the decision as to whether the *nodE, F, I,* and *J* gene products have any role in the induction of early nodulin gene expression.

As reported previously, early nodulin gene expression is not detectable in tumors, and the chromosome of *Agrobacterium* does not appear to contribute signals involved in

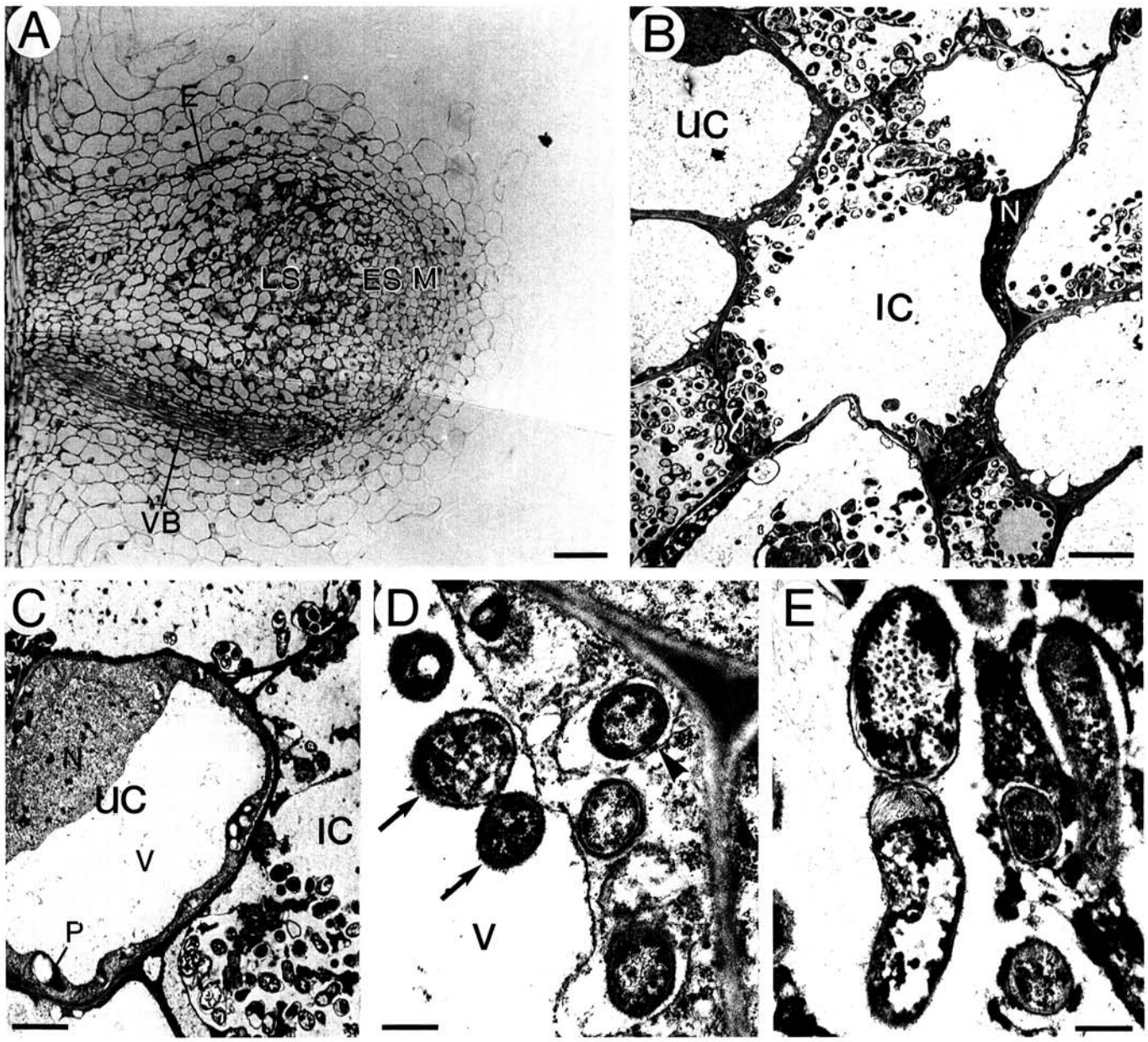

Fig. 5. Light micrograph montage (**A**) and electron micrographs (**B–E**) of a 4-wk-old nodule induced on vetch by the *Agrobacterium* transconjugant LBA4301(pMP104). **A**, Section showing the apical meristem (M), vascular bundles (VB), and endodermis (E). Bar = 100 μm. **B**, Detail of the late symbiotic zone. The contents of the infected cell (IC) have deteriorated. No organelles can be discerned, except for a very dark staining nucleus (N). The uninfected cell (UC) appears normal. Bar = 5 μm. **C**, Detail of an uninfected cell in the late symbiotic zone. The contents of the uninfected cell (UC) appear unaffected, in contrast to the contents of the infected cells (IC). The uninfected cell shows a prominent central vacuole (V), a nucleus (N), and plastids with starch granules (P). Bar = 3 μm. **D**, Detail of an infected cell in the early symbiotic zone. In addition to bacteria surrounded by a peribacteroid membrane (arrowhead) in the cytoplasm, several bacteria (arrows) are found within the central vacuole (V). Bar = 0.4 μm. **E**, Detail of an infected cell showing bacteroids in the late symbiotic zone. The bacteroids exhibit a condensed cytoplasm, and the plant cytoplasm is staining dark, which is a sign of severe degradation. Bar = 0.4 μm.

the induction of early nodulin genes (Moerman *et al.* 1987). Recently, it was shown that the *nod* genes from *R. meliloti* are inducible to levels comparable to the level found in wild-type *R. meliloti* if these genes are present in *A. tumefaciens*, but not if they are present in other Gram-negative bacteria such as *Escherichia coli* or *Pseudomonas savastanoi* (Yelton *et al.* 1987). If the chromosomes of *Rhizobium* and *Agrobacterium* have common characteristics that allow the induction of the *nod* genes, the common chromosomal genes will be essential for root nodule formation. It seems unlikely that these common genes have a role in generating signals toward the plant for the induction of early nodulin gene expression. These chromosomal genes will rather support the basic physiology of the bacterium, which in turn will be important for creating the conditions allowing the interactions between bacterium and host plant.

Whereas the evidence seems unequivocal of the *nod* region being sufficient for the induction of early nodulin gene expression, it is not clear whether the *nod* region alone, or in cooperation with non-sym plasmid genes, also regulates the induction of late nodulin gene expression. None of the late nodulin genes is expressed in nodules induced by the transconjugant LBA4301(pMP104) of *Agrobacterium* carrying the *nod* region, which seems to imply that the chromosome of *Agrobacterium* lacks one or more genes that are present on the chromosome of *Rhizobium* and that are involved in the induction of late nodulin gene expression. However, our data indicate that the agrobacteria start to degenerate after they are released from the infection threads. The cytologial data are suggestive of a plant defense response (see Van de Wiel *et al.* 1988). The outer membrane of the transconjugant of *Agrobacterium* is likely to differ from the outer membrane of *Rhizobium*, and bacterial membrane components become part of the peribacteroid membrane (Bradley *et al.* 1986). Upon release of bacteria from the infection threads, the plant may thus detect an aberrant bacterial surface and react with a defense response. Such an active role of the plant in rejecting the invading bacteria remains to be proven; more passive phenomena may also cause the observed degeneration of bacteria and plant tissue. However, irrespective of the precise mechanism of the interference of nodule development, late nodulin gene expression may have been prevented or aborted. Therefore, the lack of late nodulin gene expression in the nodules induced by LBA4301(pMP104) neither excludes nor proves that additional genes besides the *nod* region are required for the induction of late nodulin

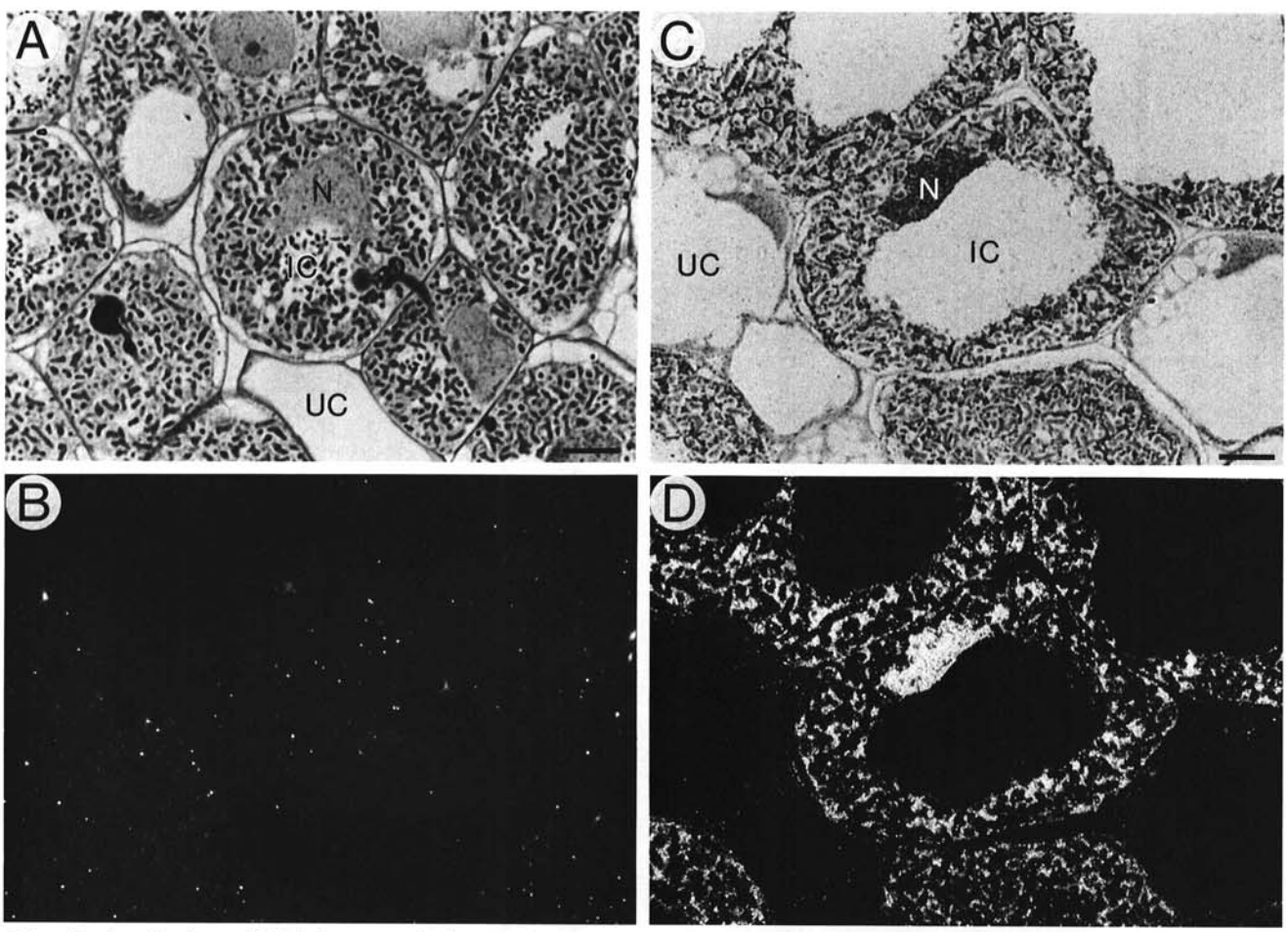

Fig. 6. Localization of leghemoglobin by immunogold silver labeling in a 3-wk-old nodule induced on vetch by *R.trifolii* ANU845(pRt032)(*nodE*K11::Tn*5*) (**A, B**) and in a 3-wk-old nodule induced on vetch by wild-type *R. leguminosarum* PRE (**C, D**). **A** and **C** are the bright field micrographs of the epipolarization micrographs shown in **B** and **D**, respectively. The strong signal observed in the infected cells in the nodule induced by wild-type *R. leguminosarum* PRE (**D**) is present as a dark silver label in the bright field micrograph **C**. No signal above background is observed in the cells of the nodule induced by ANU845(pRt032)(*nodE* K11::Tn*5*) (**B**). IC, infected cell; UC, uninfected cell; N, nucleus. Bar = 10 μm.

gene expression.

Indications that the *nod* genes are indeed involved in the induction of late nodulin gene expression come from our studies of nodules induced by strains of *Rhizobium* containing the *nod* regions from *R. trifolii* and *R. leguminosarum*, respectively. Strains ANU845(pMP104) and ANU845(pRt032) (*nodE* K11::Tn*5*) differ only in the *nod* region construct they contain, and both strains are able to nodulate vetch. In the nodules induced on vetch by ANU845(pMP104), all early and late nodulin genes are expressed, whereas in the nodules induced by ANU845-(pRt032)(*nodE* K11::Tn*5*), the majority of the late nodulin genes is not expressed. This difference in the pattern of late nodulin gene expression should be attributed to the only difference between the two strains, that is, to the *nod* region construct. Hence, the *nod* genes have a role in the induction of late nodulin gene expression. This conclusion is supported by the observation of Schmidt *et al.* (1986) that the *nodA* and *nodC* genes are expressed in bacteroids of *R. meliloti*, thus at a relatively advanced stage of nodule development. Although formal proof for the involvement of the *nod* genes of *Rhizobium* in the induction of late nodulin gene expression cannot be obtained, these *nod* genes may very well be the only genes of *Rhizobium* essential for the induction of the expression of all nodulin genes.

The reason for the absence of the transcripts from most late nodulin genes in nodules induced by the strain with the *nod* region of *R.trifolii* is unclear. There are differences in the extent of *nod* genes between the two constructs pMP104 and pRt1032(*nodE* K11::Tn*5*), but the differences upstream

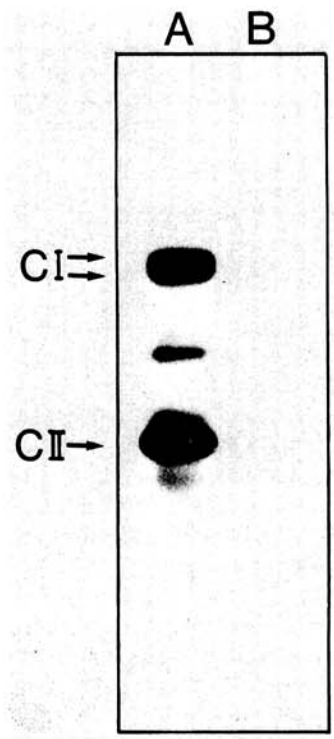

Fig. 7. Autoradiograph of a western blot containing bacteroid proteins from wild-type bacteroids of *R.trifolii* isolated from clover nodules (**A**) and ANU843(*nodE* K11::Tn*5*) bacteroids isolated from vetch nodules (**B**). The blot was incubated with antiserum raised against purified nitrogenase components CI and CII of *R. leguminosarum* and ^{125}I-labeled protein A to detect the immune complexes.

nodF (i.e., *nodNML*) and downstream *nodIJ* appear not to be essential for nodule formation in view of the phenotypical analysis of mutations of these genes when part of the total sym plasmid. In addition, it is hard to imagine how the addition of the *nod* genes *NML* would disturb the action of the other *nod* genes. The mutation in *nodE* seems to be not responsible for the failure to induce the expression of late nodulin genes, because strain ANU843(*nodE* K11::Tn*5*) of *R. trifolii*, carrying the *nodE* K11 mutation in the complete sym plasmid, induces nodules on vetch in which all late nodulin genes are expressed. It has been found that the amount of *nod* gene products appears critical for the proper development of nodules. When a high-copy number plasmid carrying the *nodA, B, C* genes, transcribed constitutively from a vector promoter, was introduced in *R. leguminosarum* with a complete sym plasmid, nodulation ability was abolished completely (Knight *et al.* 1986). A twofold enhancement of *nod* gene expression was sufficient to result in a strain that induced on vetch only 20% of the number of nodules compared with wild-type strains, and these nodules were ineffective (Hong *et al.* 1987). A slight difference in copy number between pMP104 and pRt032(*nodE* K11::Tn*5*) may therefore explain the observed differences in the nodulin gene expression pattern.

A remarkable finding is the ineffective nature of the nodules induced by ANU843(*nodE* K11::Tn*5*) of *R. trifolii* due to the lack of nitrogenase. The *nif* and *fix* genes were present in this strain, and both early and late nodulin genes were expressed in the nodules formed, so all prerequisites for nitrogen fixation on vetch seem fulfilled. The same strain induces nitrogen-fixing nodules on subterranean clover (*Trifolium subterraneum*; Djordjevic *et al.* 1985), showing that ANU843(*nodE* K11::Tn*5*) has all genetic potentials for nitrogen fixation. We do not know whether the nitrogenase protein has been broken down, or *nif* gene expression is disturbed. In the latter case, vetch nodules possibly lack a factor that is present in subterranean clover nodules and that is involved in the induction of nitrogenase gene expression. This presumably plant-specific factor may thus be a host-specific regulating factor in the induction of bacterial nitrogenase gene expression, in addition to the recently suggested role of low oxygen concentrations (Ditta *et al.* 1987; Fischer and Hennecke 1987).

The strains of *Rhizobium* and *Agrobacterium* used in this study induce nodules in which development is increasingly disturbed. Combining the histological data of the various nodule types with the pattern of nodulin gene expression in these nodules (Table 2), a correlation is found between nodule structure and nodulin gene expression. The nodules formed on vetch by strain ANU845(pRt032)(*nodE* K11::Tn*5*) contain only two to four layers of fully packed infected cells. The absence of the expression of most late nodulin genes in these nodules suggests that these late nodulin genes are not expressed in the youngest cells that are fully packed with bacteroids. This conclusion is in agreement with our immunocytological localization studies of leghemoglobin in wild-type pea nodules (Van de Wiel *et al.* 1988).

In nodules induced by LBA4301(pMP104), bacteria were released from the infection threads, but late nodulin gene expression was not detectable. Apparently, release from the infection threads is not sufficient to induce the expression of late nodulin genes. Comparison of the histology of the

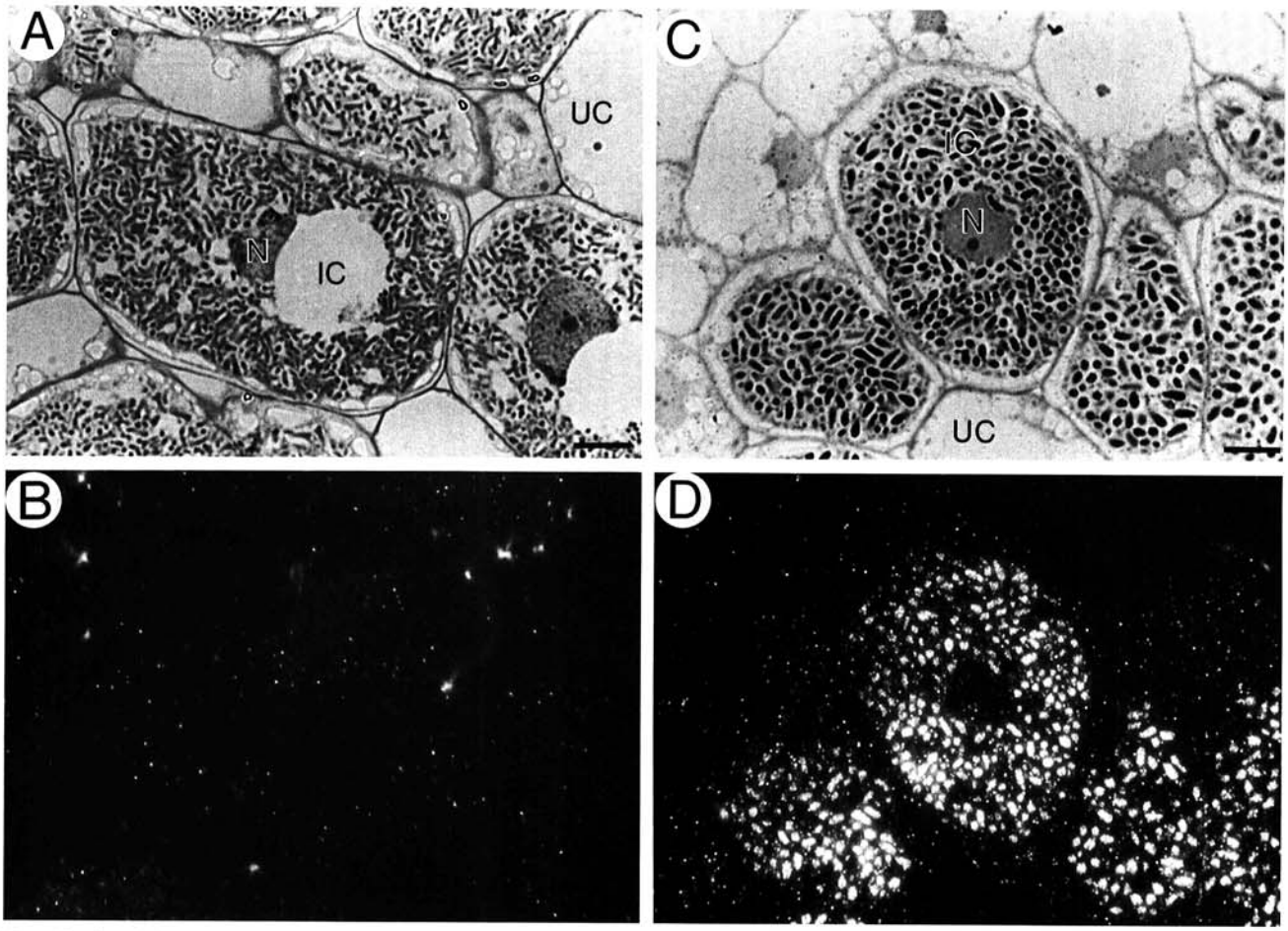

Fig. 8. Localization of nitrogenase by immunogold silver labeling in a 4-wk-old nodule induced on vetch by *R.trifolii* ANU843(*nodE* K11::Tn*5*) (**A, B**) and in a 3-wk-old nodule induced on white clover by wild-type *R. trifolii* ANU843 (**C, D**). **A** and **C** are the bright field micrographs of the epipolarization micrographs shown in **B** and **D**, respectively. The strong signal observed in the bacteroids in the nodule induced by wild-type *R. trifolii* ANU843 (**D**), present as a dark silver label in the bright field micrograph **C**, shows that the antiserum used is able to visualize the nitrogenase of *R. trifolii*. No signal above background is observed in the infected cells of the nodule induced by ANU843(*nodE* K11::Tn*5*) (**B**). IC, infected cell; UC, uninfected cell; N, nucleus. Bar = 10 μm.

Table 2. Pattern of nodulin gene expression in nodules induced by the strains indicated

	Early		Late	
Bacterial strain	**ENOD2**	**Nvs-40**	**Nvs-65**	**Lb**
PRE	+	+	+	+
248^c (pMP104)	+	+	+	+
ANU843(*nodE* K11::Tn*5*)	+	+	+	+
ANU845(pMP104)	+	+	+	+
ANU845(pRt032)(*nodE* K11::Tn*5*)	+	+	+	−
LBA4301(pMP104)	+	+	−	−

nodules induced by ANU845(pRt032)(*nodE* K11::Tn*5*) and LBA4301(pMP104) showed that in the LBA4301(pMP104)-induced nodules, fully infected cells were not found, whereas some fully infected cells were found in the nodules induced by strain ANU845(pRt032)(*nodE* K11::Tn*5*). The presence of fully infected cells correlates with the expression of the Nvs-65 gene, suggesting that the Nvs-65 gene is probably first expressed in the youngest cells that are completely filled with bacteria. In view of the time course of expression of the Nvs-65 gene, this gene is member of the class of late nodulin genes (Moerman *et al.* 1987). Because the Nvs-65 gene is expressed in the nodules induced by ANU845(pRt032)(*nodE* K11::Tn*5*), whereas leghemoglobin gene expression is not detectable, it appears likely that the Nvs-65 gene is regulated differently from the leghemoglobin genes. Consequently, late nodulin genes must be subdivided into two subclasses, the expression of which is regulated differently and correlates with a step in the developmental program of the root nodule.

Electron microscopical observations indicate that uninfected cells in the LBA4301(pMP104)-induced nodules develop like uninfected cells in wild-type nodules. The absence of expression of the identified late nodulin genes in nodules induced by LBA4301(pMP104) may indicate that these late nodulin genes are not expressed in uninfected cells. Correlations like these between the expression of a particular nodulin gene on the one hand, and nodule development up to a certain stage on the other, may be of use in determining the cell type in which a particular nodulin gene is expressed and must be borne in mind in speculations about the function of nodulins.

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