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Temporal and Spatial Co-expression of Hydrogenase and Nitrogenase Genes from *Rhizobium leguminosarum* bv. *viciae* in Pea (*Pisum sativum* L.) Root Nodules

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Expression of the *Rhizobium leguminosarum* bv. *viciae* hydrogen uptake (*hup*) genes in pea root nodules has been studied by in situ hybridization. Accumulation of transcripts specific for the hydrogenase structural genes (*hupSL*) abruptly started at the beginning of the interzone II-III and continued throughout the nitrogen-fixing zone III. This accumulation paralleled that of *nifA*- and *nifH*-specific mRNAs, and *hupSL* mRNAs were detected in the same cells as *nif* mRNAs. This suggests that the signals or regulators that trigger nitrogenase expression in nodules are also responsible for hydrogenase induction. Mutations in several hydrogenase accessory genes did not affect the pattern of *hupSL* expression, suggesting that these genes are not involved in hydrogenase regulation.

Additional keywords: *hup* genes, hydrogen oxidation, symbiotic gene expression.

Rhizobium leguminosarum bv. *viciae*, the N_2-fixing symbiont of peas, induces the synthesis of a nickel-containing hydrogenase capable of recycling the hydrogen gas evolved by the nitrogenase complex in pea root nodules. The genetic determinants for this H_2-uptake (*hup*) system have been isolated from *R. l. viciae* strain B10 (Seifert et al. 1984) and UPM791 (Leyva et al. 1987). The *hup* genes are clustered in a DNA region of about 15 kb of the symbiotic plasmid (Leyva et al. 1990). Sequence analysis of the *hup* region from *R. l.* bv. *viciae* strain UPM791 identified 17 genes, closely linked and transcribed in the same direction (Hidalgo et al. 1990; Hidalgo et al. 1992; Rey et al. 1992; Rey et al. 1993) (Fig. 1). A similar array of genes is present in strain B10 (Schneider et al. 1990; EMBL database Z36981 and Z36982). The first two genes in the *hup* cluster, *hupS,* and *hupL*, encode the hydrogenase structural polypeptides (Hidalgo et al. 1990; Schneider et al. 1990). The remaining genes include an operon (*hyp*) involved in nickel incorporation into the apoenzyme (Rey et al. 1993; Rey et al. 1994) and genes encoding hydrogenase accessory proteins whose precise molecular functions are not known.

Most studies on hydrogenase regulation in endosymbiotic bacteria have been carried out with vegetative cells of *Bradyrhizobium japonicum,* the microsymbiont of soybeans (Kim and Maier 1990; Kim et al. 1991; Kim et al. 1993). *B. japonicum* Hup$^+$ strains efficiently express hydrogenase activity in microaerobic, free-living conditions in the presence of nickel and hydrogen. Contrary to *B. japonicum, R. l. viciae* does not readily express hydrogenase in microaerobic, free-living conditions, and efficient expression has only been detected in the symbiotic state (Palacios et al. 1990). In this species, mutant complementation studies (Leyva et al. 1990) and expression analysis of *hup::lacZ* fusions (Palacios et al. 1990), indicated that *hup* genes are organized in several transcriptional units that are differently regulated. The region containing the *hyp* operon (Fig. 1) is expressed in microaerobically-grown cells, whereas transcription of the remaining genes, including the hydrogenase structural operon, has only been observed in symbiosis with peas. Transcription of the hydrogenase structural operon, which includes *hupS*, *hupL*, and four additional genes (*hupCDEF*), is initiated from a σ^{54}-type promoter located 56 bp upstream of *hupS* (Hidalgo et al. 1992). The study of regulation of hydrogenase expression in response to environmental stimuli (Ni, hydrogen, oxygen) is complicated by the symbiotic state. *In situ* hybridization analysis has previously been applied to locate *R. l. viciae* mRNAs, particularly *nifA* and *nifH*, in root nodules (Yang et al. 1991). In this work, we have used this technique to investigate *hup*-specific transcription in pea nodules.

Root nodules from peas (*Pisum sativum* L.) were harvested 16 days (cv. Rondo or Poneka) and 13 or 20 days (cv. Poneka) after inoculation with either *R. l. viciae* strain B10 (wild-type, Hup$^+$) or UPM791 (wild-type, Hup$^+$), or derivative Hup$^-$ mutants (Leyva et al. 1990; H. J. Schmitt et al. IV International Conference on the Molecular Biology of Hydrogenases. Noordwijkerhout, The Netherlands, August 14–19,

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1994). Preparation of nodule thin sections for hybridization with RNA probes, and visualization of the results were performed as previously described (Yang et al. 1991). Briefly, fixed nodules were dehydrated, embedded in paraffin, and sections (7 μm thick) cut with a microtome. Sections were hybridized with RNA probes as described by Van De Wiel et al. (1990), coated with Amersham LM-1 emulsion and exposed at 4°C. After exposure, sections were developed, fixed, counterstained with toluidine blue, and mounted. For observation and photography, bright field, dark field, and epipolarization optics were used. To generate a *hup*-specific RNA probe, a 429-bp *Eco*RI-*Sal*I restriction fragment internal to the *hupL* gene from plasmids pAL618 (containing the *hup* gene cluster from strain UPM791; Leyva et al. 1990) or pRlB505 (containing the *hup* gene cluster from strain B10, H. J. Schmitt et al., IV International Conference on the Molecular Biology of Hydrogenases. Noordwijkerhout, The Netherlands, August 14–19, 1994) was subcloned into pBluescript vectors (Short et al. 1988) and the antisense/sense RNAs were produced by in vitro transcription from the resulting plasmids by the T7 or T3 RNA polymerase systems in the presence of ^{35}S-labeled UTP, as previously described (Van De Wiel et al. 1990). For the in situ detection of *nifH* and *nifA* mRNAs in pea nodule thin sections, radioactively labeled antisense RNA probes were prepared as previously described (Yang et al. 1991).

Figure 2 (A–D and H) shows longitudinal sections of nodules from 16-day-old plants hybridized with ^{35}S-labeled antisense *hupL*, *nifH*, and *nifA* RNA probes. The *hupL* mRNA was clearly detectable in the infected cells of the interzone II-III and nitrogen-fixing zone III (as defined by Vasse et al. 1990; see also Franssen et al. 1992) of nodules from *R. l. viciae* wild-type strains UPM791 (Fig. 2A,B) and B10 (Fig. 3). The intense signal observed with the *hupL* probe (Fig. 2B) started in the same cell layer of interzone II-III as the signals obtained when antisense *nifH* and *nifA* RNAs were used as hybridization probes (Fig. 2C,D). No hybridization signal was detected with the *hupL* sense RNA probe in nodules from the wild-type strain or with the antisense *hupL* probe in nodules from *R. l. viciae* mutant strain AL51 (Fig. 2H), which carries a Tn*5* polar insertion into the *hupS* gene. This shows that the signal observed with antisense *hupL* probe in the wild-type strain was specific for *hupL* mRNA. By using adjacent sections of pea nodules, we were able to show that *hupL* mRNA appeared in the same cell layers of interzone II-III where *nifH* is first detectable (Fig. 2F,G). Like *nifH* mRNA (Yang et al. 1991), *hupL* mRNA is immediately present in these cells at maximal levels. In pea nodules from *R. l. viciae* strain PRE (wild-type, Hup$^-$), it has previously been shown that *nifH* and *nifA* mRNAs are first detectable in the interzone II-III (Yang et al. 1991; Franssen et al. 1992). Therefore, we conclude that *hupL* gene expression strictly parallels that of *nifA* and *nifH* genes. The transition of zone II into interzone II-III appears to be a region where major changes take place during nodule development. Within bacteroids, apart from the sudden onset of transcription of *nifA* and *nifH* (Yang et al. 1991; Franssen et al. 1992) and now *hupL* (this study), an opposite down-regulation for *ropA* has been demonstrated at this transition (de Maagd et al. 1994). The *ropA* gene encodes outer membrane protein IIIa, a major outer membrane protein in free-living cells that is depleted in pea bacteroids (de Maagd et al. 1989). On the plant side, the interzone II-III is characterized by the sudden accumulation of amyloplasts at the periphery of the infected cells (Vasse et al. 1990; Franssen et al. 1992), and the expression of several nodulins starts (PsNOD6, see de Maagd et al. 1994; alfalfa leghemoglobin, De Billy et al. 1991) or ends (PsENOD5, Franssen et al. 1992) in this region.

An obvious consequence of the observed temporal and spatial co-expression of *hupL* and *nif* genes in pea nodules is that their induction may respond to the same nodule signals and bacteroid activating factors. One possibility is that oxygen regulates *hupSL* gene expression through the *nifA* gene product. The microaerobic conditions in the nodules, created both by respiratory activity and by the presence of O_2 diffusion barriers in the nodule parenchyma (De Lorenzo et al. 1993; Ianetta et al. 1993; Van De Wiel et al. 1990), are supposed to activate *nifA* expression in symbiosis. The induction of the *nifA* gene would, in turn, result in a prompt activation of the *hupSL* genes. In order to test this possibility, the *hup* genes from *R. leguminosarum* UPM791, contained in cosmid pAL618, were introduced in *R. leguminosarum* PRE (wild-type, Hup$^-$; Lie et al. 1979) and its isogenic derivatives PRE2106 (*nifA*::Tn*5*; Schetgens et al. 1985), PRE2107 (*nifA*::Tn*5*; Schetgens et al. 1985), and PRE106 (*nifD*::Tn*5*; Schetgens et al. 1984). In situ hybridization experiments with antisense *hupL*, *nifA*, and *nifH* probes were carried out on Rondo pea nodules produced by these strains. No expression of *hupL* could be observed either in the NifA$^-$ or NifD$^-$ backgrounds (data not shown). In addition, expression of *nifA* and *nifH* was impaired in the NifD$^-$ background (data not shown), suggesting that nodule zonation in these nodules was

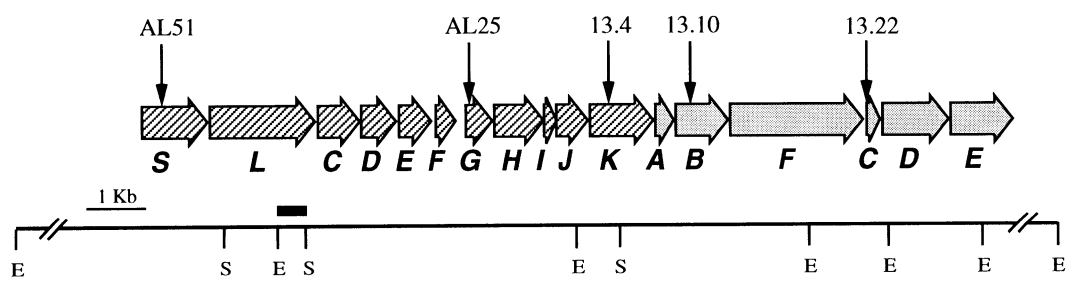


Fig. 1. Genetic organization of the *Rhizobium leguminosarum* bv. *viciae hup* cluster. Arrowed boxes above the restriction map represent the *hup* (striped) and *hyp* (solid gray) genes identified in the cluster. The location of the transposon (AL25, AL51) or cassette insertions (13.4, 13.10, 13.22) in H_2-oxidation defective *hup* and *hyp* mutants is shown by vertical arrows. The solid-black horizontal bar indicates the DNA fragment from which sense or antisense RNA probes were synthesized for in situ hybridization assays. Restriction sites: E, *Eco*RI; S, *Sal*I.

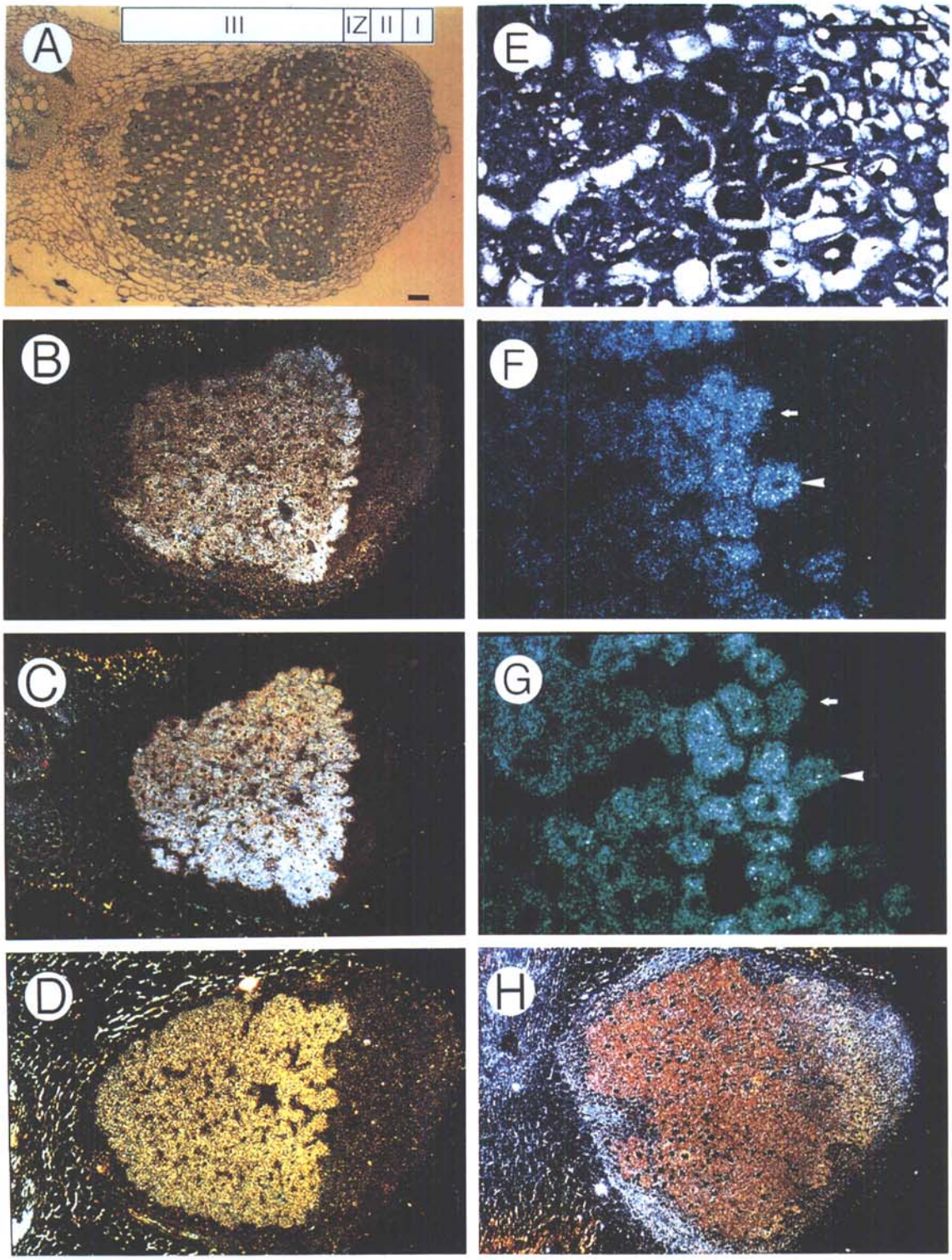
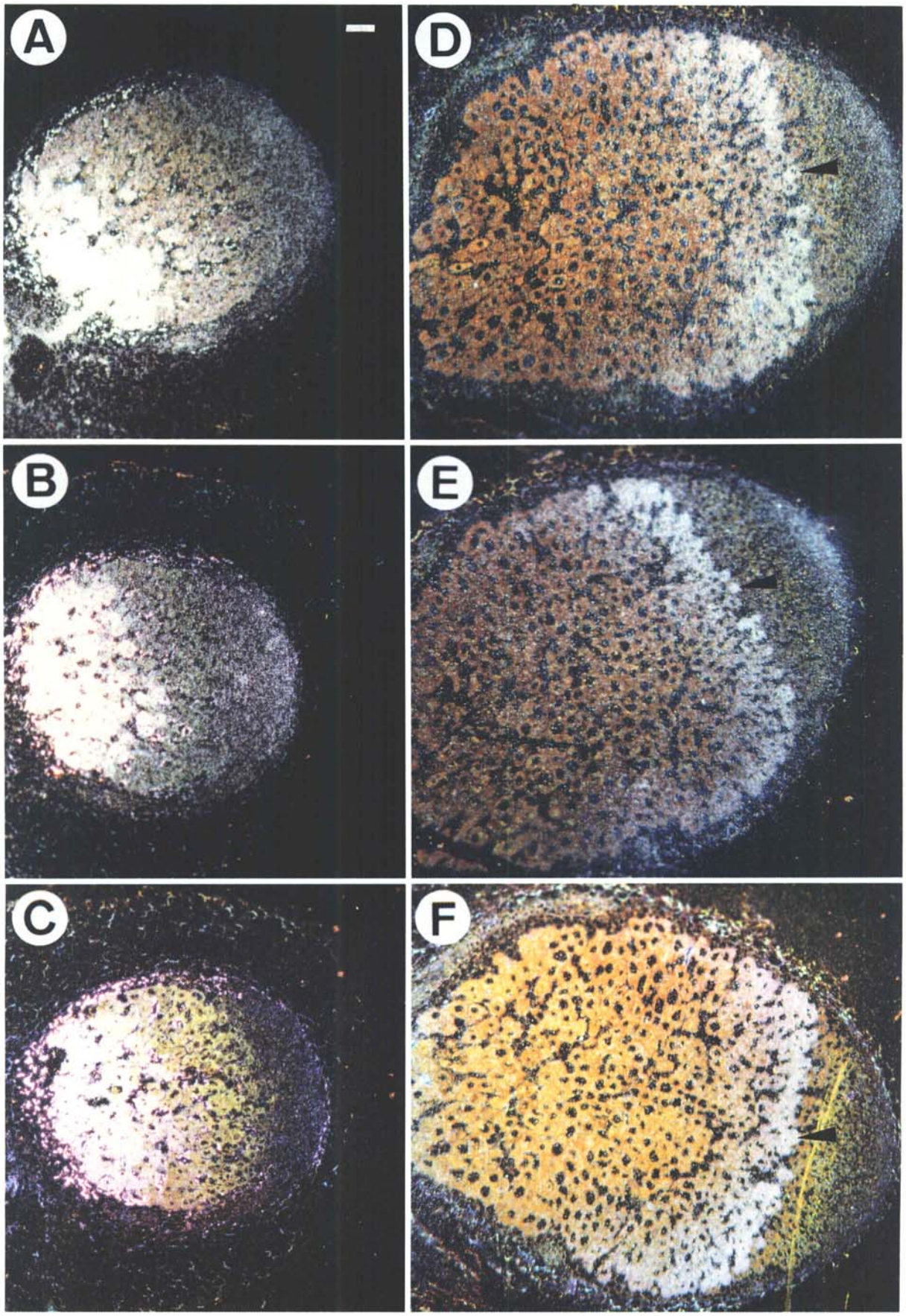


Fig. 2. Localization of *hup* and *nif* transcripts in longitudinal sections of 16-day-old pea (*Pisum sativum* cv. Rondo) nodules induced by *Rhizobium leguminosarum* bv. *viciae* strain UPM791 (**A–G**) or mutant AL51 (**H**). **A, E,** Bright-field micrographs; **B, C, D, H,** Dark-field micrographs in which silver grains are visible as white dots; **F, G,** Epipolarization micrographs. A, B, and C are adjacent sections. **A, B,** Sections hybridized with antisense *hupL* RNA probe; zone I (I), infection zone II (II), interzone II-III (IZ), and nitrogen-fixing zone III (III) are indicated. **C,** Section hybridized with antisense *nifH* RNA probe. **D,** Section hybridized with antisense *nifA* RNA probe. **E,** Detail of A. Arrows and arrowheads in E, F, and G indicate the same cells. Bar = 100 μm. **F,** Epipolarization micrograph of E. **G,** Epipolarization micrograph of a detail of C, showing the same area as E. **H,** Section hybridized with antisense *hupL* RNA probe. This section was exposed for 4 weeks. All other sections were exposed for 3 weeks. Bars = 100 μm.


A
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affected by their Fix$^-$ phenotype, even when experiments were performed with 13-day-old nodules to minimize nitrogen starvation effects. Thus, the altered pattern of bacteroid gene expression in Fix$^-$ nodules did not provide conclusive evidence of a direct implication of NifA on *hup* expression.

A second possibility to rationalize the co-expression of the *hupSL* operon and *nif* genes is that a signal resulting from nitrogenase activity is responsible for *hupSL* activation. The obvious candidate is H_2 produced from the N_2 reduction reaction. In *B. japonicum* vegetative cells, H_2 and nickel are required for hydrogenase induction in addition to microaerobiosis (Kim et al. 1993; Kim and Maier 1990; Kim et al. 1991). The combined requirement of H_2 and low O_2 for hydrogenase expression in pea nodules would result in initiation of *hupSL* transcription in a well-defined cell layer. However, this hypothesis requires the existence of an H_2-sensing protein and, so far, hydrogenase is the only protein known to respond to hydrogen. Besides, we have observed no induction of hydrogenase activity in free-living cells of *R. l. viciae* grown under the same conditions (low O_2, H_2, Ni) that derepress the hydrogenase activity in free-living cells of *B. japonicum* (Palacios et al. 1990).

We verified the spatial and temporal co-expression of genes *hupL*, *nifH*, and *nifA* during different stages of root nodule development. The rhizobial transcription in 13-day-old (Fig, 3A–C), 20-day-old (Fig. 3D–F), and 23-day-old (data not shown) nodules of pea plants infected with strain B10 was also analyzed via in situ hybridizations. As with 16-day-old nodules, transcription of all three genes started within the first cell layers of the interzone II-III. In 13-day-old nodules, similar levels of *hupL* transcripts were detected throughout the nitrogen-fixing zone III (Fig. 3A). In 20-day-old nodules (Fig. 3D), the concentration of *hupL*-specific mRNA in the cell layers following the first induced layers decreased to lower levels. This decrease in *hupL* expression in the older cell layers of the 20-day-old nodules paralleled a reduction in *nifA* (Fig. 3E) and *nifH* (Fig. 3F) expression.

In order to examine the effect of accessory genes on the symbiotic expression of *hupSL*, longitudinal thin sections of nodules from *R. l. viciae* Hup$^-$ mutant AL25, generated by Tn*5* insertion in *hupG* gene (Leyva et al. 1987), or from Hup$^-$ mutants 13-4, 13-10 and 13-22, generated by insertion of a *lacZ*/Km^R cassette into *hupK, hypB*, and *hypC*, respectively (H. J. Schmitt et al., IV International Conference on the Molecular Biology of Hydrogenases. Noordwijkerhout, The Netherlands, August 14–19, 1994) were hybridized with the antisense *hupL* RNA probe. In no case did the *hupL*-specific hybridization pattern differ from that obtained with nodules from the corresponding wild-type strain (data not shown). This result suggests that transcription of the *hupSL* operon is not dependent on the gene functions affected by the insertions.

In summary, in this paper we demonstrate by in situ hybridization experiments that hydrogenase structural *hupSL* genes are co-expressed with *nifA* and *nifH* genes in the same nodule cells. This suggests that nitrogenase and hydrogenase activities are co-regulated in the nodule. We were also able to show that the products of hydrogenase accessory genes *hupG*, *hupK*, *hypB*, and *hypC* are not necessary for *hupSL* expression in bacteroids. Further experiments are needed to identify the factors that specifically affect transcription of hydrogenase genes in pea nodules.

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Fig. 3. Localization of *hupL* transcripts in pea (*Pisum sativum* cv. Poneka) root nodules of different age induced by *Rhizobium leguminosarum* bv. *viciae* strain B10. All plates are dark-field micrographs. **A–C,** Adjacent sections of a 13-day-old nodule. **D–F,** Adjacent sections of a 20-day-old nodule. **A, D,** Sections hybridized with antisense *hupL* RNA probe. **B, E,** Sections hybridized with antisense *nifA* RNA probe. **C, F,** Sections hybridized with antisense *nifH* RNA probe. These sections were exposed for 2 weeks. All other sections were exposed for 3 weeks. Arroweads in D, E and F indicate the same cells. Bar = 100 μm.

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