Down-Regulation of Expression of the *Rhizobium leguminosarum* Outer Membrane Protein Gene *ropA* Occurs Abruptly in Interzone II-III of Pea Nodules and Can Be Uncoupled from *nif* Gene Activation

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The expression of the *Rhizobium leguminosarum* bv. *viciae* outer membrane protein gene *ropA* during nodule development was studied using immuno-electron microscopy and *in situ* hybridization. Using immunochemical detection in isolated cell envelopes it had been shown earlier that the RopA outer membrane antigen disappears during bacteroid development (R. A. de Maagd, R. de Rijk, I H. M. Mulders, and B. J. J. Lugtenberg, J. Bacteriol. 171:1136-1142, 1989). In the present study we used immuno-electron microscopy on vetch nodule sections to show that the decrease in RopA protein expression occurs in the nodule after release of the bacteria from the infection thread, during the transition in morphology from that of the free-living bacterium into that of the bacteroid. Detection of *ropA* mRNA in sections of pea nodules by *in situ* hybridization revealed a sudden decrease in messenger level at the transition from prefixation zone II to interzone II–III. This decrease coincided with a sudden increase in *nifH* mRNA levels. Although the decrease in *ropA* messenger and appearance of *nif* messenger are spatially correlated we could show that *ropA* down-regulation can be uncoupled from *nif* gene activation by using a strain that induces non-nitrogen fixing nodules on pea but does develop into bacteroids. The importance of the transition of prefixation zone II to interzone II-III as a developmental switch for bacteroid development is discussed.

Additional keywords: bacteroid differentiation, nitrogen fixation.

The development of an effective symbiosis between plants of the *Leguminosae* family and rhizobia involves a series of steps in which plant genes as well as bacterial genes play a role. For the bacterium this results in differentiation into a specialized nitrogen-fixing form, the bacteroid, which shows extensive morphological and molecular differences with free-living bacteria. Most notably, the mature bacteroid expresses the *nif* genes that are responsible for the fixation of atmospheric nitrogen. On the plant side, nodulin genes are expressed exclusively in the nodule. According to the timing of their appearance the nodulins can be divided into an early and a late subgroup (Nap and Bisseling 1990; Franssen *et al.* 1992).

Pea nodules are of the indeterminate type and, therefore, in mature nodules all developmental stages of the plant tissues as well as of the infecting bacterium can be observed, progressing from the distal meristematic zone to the proximal senescent zone. Vasse *et al.* (1990) proposed a nomenclature of zonation for alfalfa nodules, which is also applicable to pea nodules (Franssen *et al.* 1992).

Recently we have described the cloning and characterization of *ropA*, a surface protein gene of *Rhizobium leguminosarum* bv. *viciae*, encoding part of the surface antigen group III (de Maagd *et al.* 1992). *ropA* encodes one of the two proteins (OMPIIIa, M_r = 36 kDa) that together with their peptidoglycan residue-containing derivatives make up outer membrane protein antigen group III of free-living bacteria (de Maagd *et al.* 1989a, 1989b). Using Western blotting with monoclonal antibodies, it was shown that antigen group III is severely depleted in cell envelopes of pea nodule bacteroids, when compared to cell envelopes of free-living bacteria (de Maagd *et al.* 1989a). This depletion, as well as that of the antigen group II is a phenomenon that has been shown to occur in bacteroids of different host plant/*Rhizobium*-combinations, suggesting that this change is an essential part of the development of an effective symbiosis (H P. Roest, R. A. de Maagfd, I. H. Mulders, C. A.Wyffelman, and B. J. J., unpublished).

In this manuscript we demonstrate, by using immuno-electron microscopy, that the expression of antigen group III diminishes during bacteroid development after release from the infection thread. Furthermore, applying *in situ* hybridization we have shown that down-regulation of expression of *ropA* occurs at the messenger RNA level and very abruptly at the same developmental stage where *nif* gene expression is first detectable. Using a *Rhizobium* mutant that does not fix nitrogen while bacteroid development does occur, we have shown that the down-regulation of *ropA* messenger level is not dependent on *nif* gene expression.

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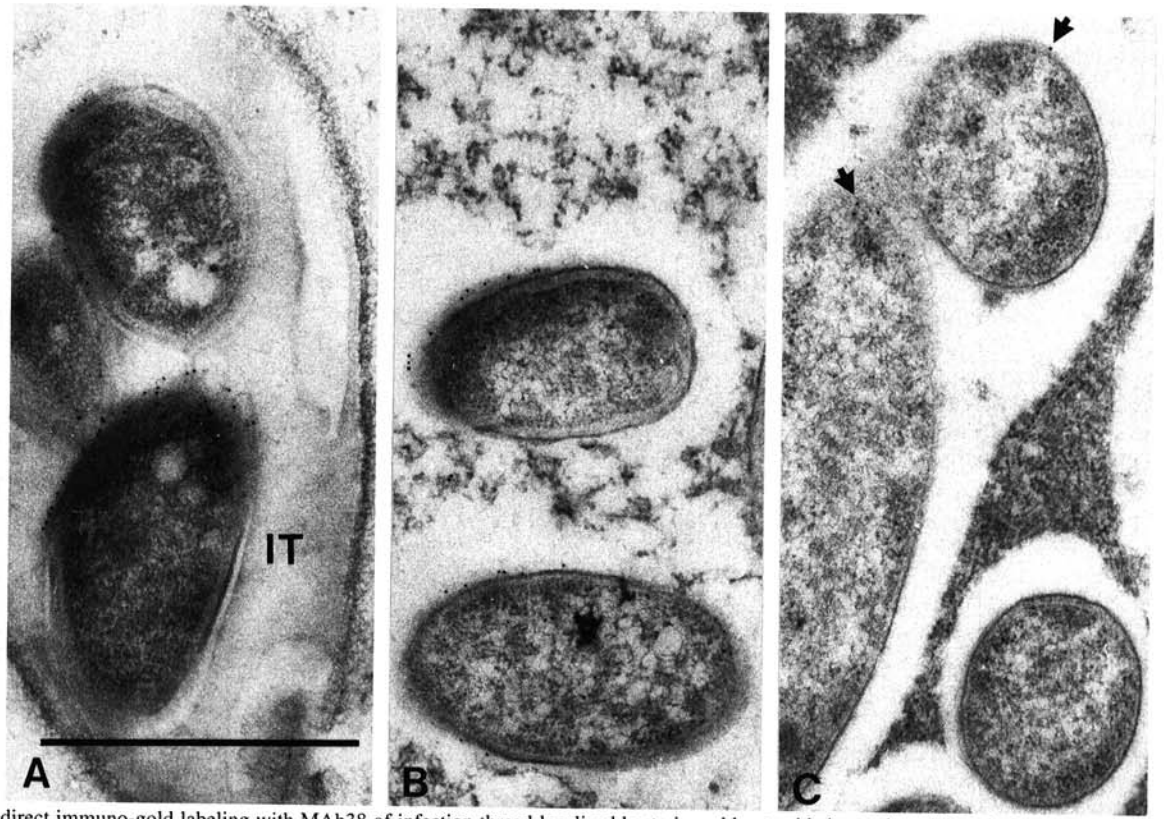

Fig. 1. Indirect immuno-gold labeling with MAb38 of infection thread-localized bacteria and bacteroids in vetch nodules. **A,** Bacteria in infection thread (IT). **B,** "Young" bacteroids in plant cytoplasm. **C,** Mature bacteroids (arrowheads indicate the rare gold particles). Bar = 1 μm.

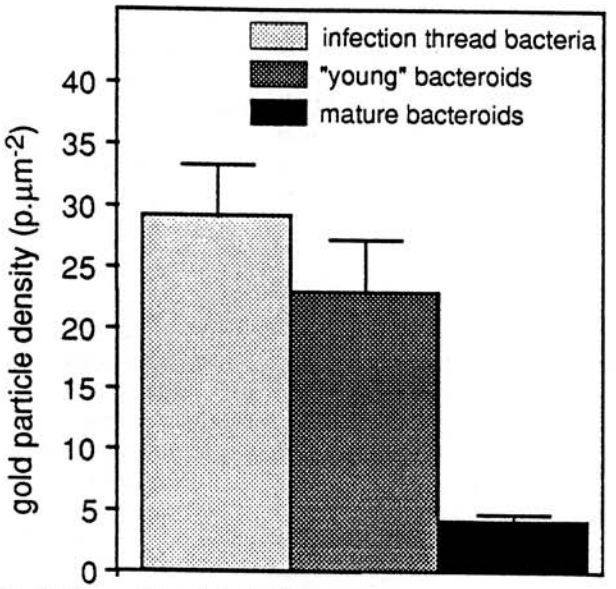

Fig. 2. Mean gold particle density (particles per square micrometer) in sections of immuno-gold labeled infection thread and infection droplet bacteria, "young" bacteroids, and mature bacteroids.

RESULTS

Immuno-electron microscopic detection of group III antigens in nodules.

Initially, the decrease in group III antigen during symbiosis was found by immunochemical comparisons of free-living bacteria with bacteroids from pea nodules (de Maagd *et al.* 1989a). To determine whether decrease of antigen group III expression takes place inside the nodule, immuno-electron microscopy with monoclonal antibody MAb38 (de Maagd *et al.* 1989a) was used to detect this antigen group in thin sections of vetch (*Vicia sativa*) nodules. MAb38 had been shown earlier to preferentially recognize the nondenatured antigen group III oligomers on Western blots as well as on intact cells of *R. l.* bv. *viciae* strain 248. The level of labeling of bacteria or bacteroids was quantified for three categories, broadly representing subsequent developmental stages. Bacteria and "young" bacteroids (categories 1 and 2, respectively) were from cells containing infection threads, infection droplets, and just released bacteria. These cells are not yet completely packed with rhizobia, and the bacteria still retain the free-living morphology. Mature bacteroids (category 3) were from cells adjacent to the cells described above. The cytoplasm of these cells is fully packed with rhizobia, and only a small layer of cytoplasm surrounds the bacteroids, with all large plant cell organelles at the periphery. A decrease in labeling in mature bacteroids (Fig. 1C) as compared to newly released bacteria (Fig. 1B) and infection thread bacteria (Fig. 1A) was observed. To quantify these data, the number of gold particles per square micrometer section of each category was averaged from a large number of photographs (for details, see Methods section). The results of this quantification are shown in Figure 2. Statistical comparison of each pair of two categories using the Student's *t* test showed that while the difference between categories 1 and 2 is not significant, the difference between categories 1 and 3, as well as that between categories 2 and 3,

are indeed significant ($P < 0.01$). It can be concluded from these results that decrease in antigen group III expression indeed occurs inside the nodule, when the released rhizobia change in morphology from the free-living to the bacteroid stage. Since in these vetch nodules the fully packed cells are spatially very close to the cells containing "young bacteroids" distinction of developmental zones as in alfalfa nodules (Vasse *et al.* 1990) and pea nodules (Franssen *et al.* 1992) is not possible. However, the results shown here suggest that the decrease in *ropA* protein levels occurs at a developmental stage that is comparable to that in interzone II–III of alfalfa and pea nodules.

In situ localization of *ropA* transcripts.

To determine whether the decreased detectability of antigen group III in developing bacteroids is the result of an actual decrease in expression rather than of degradation, for example by lytic enzymes in the peribacteroid space, we examined the level of *ropA* mRNA in pea nodules by *in situ* hybridization with a *ropA* probe. This technique would also allow us to determine in what particular developmental stage, if any, down-regulation takes place. Longitudinal sections of 16-day-old pea nodules induced by *R. l.* bv. *viciae* strain 248 were hybridized with a radioactive antisense-RNA-probe derived from the cloned *ropA* gene. Figure 3A shows a phase-contrast micrograph of a pea nodule section in which the different developmental zones can be identified. The three to four cell layers of interzone II–III can be easily identified here by their bright white appearance caused by amyloplast accumulation. Figure 3B shows an overview of a longitudinal section (adjoining that of Fig. 3A) of a nodule hybridized with a radioactively labeled *ropA* probe and with the different developmental zones indicated according to Franssen *et al.* (1992). Labeling at a low level is detectable in the youngest distal cells of the prefixation zone II. The density of silver particles increases towards the older, proximal part of zone II, probably as a result of bacterial proliferation. However, at the transition of the prefixation zone II to interzone II–III the intensity of the signal decreases abruptly. The same section viewed by dark-field microscopy (Fig. 3C) shows the same pattern. It can also be seen here that in the interzone II–III the hybridization signal decreased to a low, though still detectable level that remains constant throughout the rest of the proximal part of the infected tissue of the nodule. A higher magnification of the transition region (Fig. 3E) shows that this decrease occurs abruptly from one cell layer to the next layer, with almost no intermediary levels. This decrease in *ropA* mRNA level coincides with the appearance of amyloplasts in the infected cells, and so it exactly matches the transition from the prefixation II into the interzone II–III. Control sections hybridized with sense-*ropA* RNA probe showed no signal above background levels.

The pattern of hybridization of the *ropA* probe showed a striking complementarity with the patterns of hybridization observed earlier with *nifA* and *nifH* probes (Yang *et al.* 1991; Franssen *et al.* 1992). We therefore hybridized adjacent sections of the same nodule with a *nifH* probe. The overview of this section shows the abrupt start of expression of *nifH* at the beginning of the interzone II–III and the expression level remaining constant throughout zone III (Fig. 3D). A higher magnification, of the same region as was shown in Figure 3E (Fig. 3F), shows that expression of *nifH* starts abruptly and that the first cell layer in which *nifH* is expressed is the first cell layer in which *ropA* expression abruptly decreases (compare arrows in Fig. 3E and F). In conclusion, the switching off of *ropA* expression and the turning on of *nifH* gene expression both occur exactly at the transition from zone II into the interzone II–III, indicating that these processes may be regulated through a similar mechanism.

Down-regulation of the *ropA* mRNA level can be uncoupled from the activation of *nif* gene expression.

The exact coincidence of *ropA* down-regulation and the start of *nif* gene transcription prompted us to address the question whether *nif* gene transcription and *ropA* down-regulation might be regulated by the same mechanism. For this purpose we looked at pea nodules induced by strain K11.pMP258. This is a *nodE*::Tn*5* mutant of *R. l.* bv. *trifolii* strain ANU843 (Djordjevic *et al.* 1985) containing a cloned *nodE* gene of the *R. l.* bv. *viciae* strain 248 Sym plasmid pRL1JI, under control of the *nodA* promoter of the same plasmid (Spaink *et al.* 1989). This strain nodulates various host plants of biovar *viciae* strains such as vetch and pea, but forms ineffective nodules on these plants (Spaink *et al.* 1989; H. P. Spaink, unpublished results). Light microscopy of 7-μm sections of a pea nodule induced by this strain showed infected cells with bacteroids (Fig. 3G). This shows that, although the bacteroids are not able to fix nitrogen, release from the infection threads, subsequent proliferation, and to some degree normal bacteroid development do occur. The bacterial strain from which mutant K11 is derived, biovar *trifolii* strain ANU843, has a group of outer membrane proteins related to the group III antigens of strain 248. This relationship consists of immunological cross-reactivity (de Maagd 1989) as well as of the occurrence of two strongly cross-hybridizing DNA fragments in Southern blots of ANU843 DNA probed with *ropA* (H. P. Roest *et al.*, unpublished results). Moreover, immunochemical comparison of free-living bacteria and bacteroids from pea nodules of strain ANU843 containing biovar *viciae* Sym plasmid pRL1JI showed that, as in strain 248, this antigen group is severely reduced in bacteroids (H. P. Roest, unpublished). *In situ* hybridization of 7-μm sections of K11.pMP258-induced pea nodules with a *ropA*-antisense probe (Fig. 3G) revealed a pattern of mRNA distribution that is similar to that in pea nodules formed by strain 248 (Fig. 3B). The occurrence of a few hybridizing cells in the central tissue of the nodule (Fig. 3G), suggests that the down-regulation of *ropA* expression is slightly leaky in these nodules. However, in adjacent sections of the same nodule we were unable to detect *nifH* mRNA accumulation by *in situ* hybridization (Fig. 3H). These results show that the down-regulation of *ropA* mRNA level during bacteroid development can be uncoupled from the activation of *nif* gene transcription.

DISCUSSION

In this study we have followed the expression of the *ropA* outer membrane protein gene of *R. l.* bv. *viciae* during symbiosis. *ropA* expression appears to be regulated at the mRNA level, showing a sudden, sharp decrease from one cell layer to the next layer at the transition from zone II to

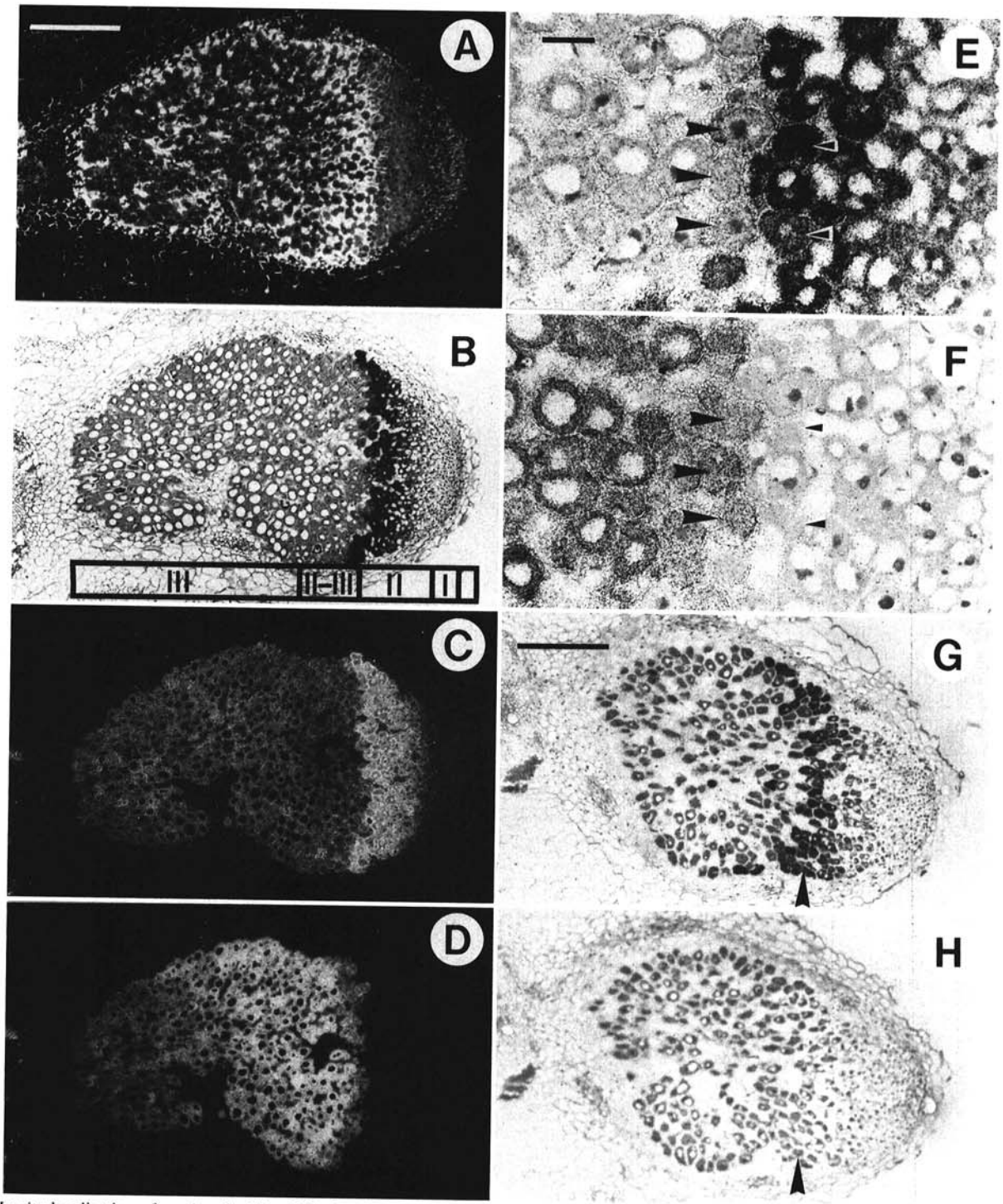

Fig. 3. *In situ* localization of *ropA* and *nifH* mRNA in adjacent longitudinal sections of 16-day-old pea nodules. Nodules were induced by *Rhizobium leguminosarum* bv. *viciae* strain 248 (A–F). **A,** Phase-contrast micrograph showing amyloplast accumulation. Bar = 50 μm. **B,** Bright-field micrograph of a nodule section hybridized with ^{35}S-labeled antisense *ropA* probe. Cells containing high concentrations of silver grains are black. The organization of pea nodule tissue is presented in the box. I. Meristem, II. Prefixation zone, II–III. Interzone, III. Nitrogen fixation zone. **C,** Dark-field micrograph of B. White dots are the signal. **D,** Dark field micrograph of an adjacent section of A hybridized with ^{35}S-labeled antisense *nifH* RNA probe. **E,** Higher magnification of B. White arrowheads indicate cells having the highest level of silver grains, black arrowheads indicate cells with significantly less silver grains. Bar = 5 μm. **F,** Higher magnification of a bright field micrograph of D. Arrowheads indicate the same cells as in E. **G,** Bright-field micrograph of a longitudinal section of a pea nodule induced by *R. leguminosarum* strain K11.pMP258, hybridized with an antisense *ropA* RNA probe. Bar = 50 μm. **H,** Bright-field micrograph of an adjacent section of G hybridized with an antisense *nifH* RNA probe. The lack of silver grains was confirmed by dark-field microscopy.

interzone II–III. Although this decrease occurs at exactly the same stage as where *nif* gene transcription is activated, these processes could be uncoupled.

We first addressed the question where the decrease of expression takes place by looking at occurrence of the MAb38-epitope of antigen group III. This showed that expression of the epitope decreases inside the nodule, between release from the infection thread and development into mature bacteroids. Subsequently, we localized the *ropA* messenger in sections of pea nodules. Down-regulation of the *ropA* messenger level occurred at the transition of zone II to interzone II–III, clearly showing that this transition is an important region in the nodule for bacteroid differentiation. The interzone II–III can be identified microscopically by the presence of amyloplasts (Vasse *et al.* 1990; Franssen *et al.* 1992). During nodule development it is initially a major region of the central tissue, but it decreases to only a few cell layers in mature nodules (Franssen *et al.* 1992). Whereas in zone II proliferation of the bacteria inside the infected plant cells appears to be the main feature, in interzone II–III the morphological and molecular changes start to take place that will eventually give rise to the mature, nitrogen-fixing bacteroid. Interzone II–III represents not only a crucial stage in bacteroid development: Expression of plant-derived nodulins also seems to change in this zone. The early nodulin gene PsENOD5 is highly expressed in zone II, but its expression suddenly drops at the transition to interzone II–III (Franssen *et al.* 1992). Furthermore the expression of the late nodulin gene PsNOD6 is induced at this transition (I. Kardailsky, C. van de Weil, W. C. Yang, and T. Bisseling, personal communication). Also, in alfalfa in interzone II–III expression of the late nodulin leghemoglobin first occurs (De Billy *et al.* 1991).

Some of the changes in surface structure occurring in bacteroid development can be mimicked *in vitro* by applying growth conditions reminiscent of conditions that are thought to occur in nodules, such as low oxygen pressure and availability of succinate as major carbon source (Sindhu *et al.* 1990). We have investigated the influence of a large number of *in vitro* growth conditions on the activity of the *ropA*-promoter, to identify possible factors that may cause down-regulation of transcription in the nodule. Only high calcium concentrations were found to repress *ropA* expression as measured with promoter/*lacZ*-fusions (H. P. Roest, I. H. M. Mulders, and R. A. de Maagd, unpublished results). Although a sharp increase in calcium concentration in the peribacteroid environment might be responsible for the drop in *ropA*-expression occurring in the interzone II–III, we find it unlikely that such a sharp change in calcium concentration could occur from one cell layer to the next. Clearly, at present not enough is known about the composition of the peribacteroid environment to answer these questions.

Although the down-regulation of *ropA* expression and the activation of *nif* gene transcription are occurring in exactly the same stage of bacteroid development, we were able to uncouple these processes using a fix$^-$ bacterial strain. This result complements those of Roest *et al.* (unpublished), where it was shown that in cell envelopes of bacteroids of *nifA* and *nifH* bacterial mutants that do not form mature, nitrogen-fixing bacteroids, group III antigen levels have nevertheless decreased. In contrast to these *nif* mutants, we used a strain that contains a full complement of *nif* and *fix* genes, that allow it to fix nitrogen in clover nodules. Nevertheless, in pea nodules *nifH* was not expressed in this strain, revealing another level of complexity of *nif* gene regulation. Our results show that activation of *nif* gene transcription is also not a prerequisite for *ropA* down-regulation. As for *ropA* regulation, the signal(s) responsible for *nif* gene transcription activation in nodules has not been identified. Low oxygen pressure is necessary, and in *in vitro* studies of *R. meliloti* it was sufficient for *nifA* transcription (Ditta *et al.* 1987). However, it may not be the sole signal *in vivo* since there is no evidence for a sudden drop in oxygen pressure occurring from one cell layer to the next at the transition of zone II into interzone II–III (Yang *et al.* 1991).

Taken together our results and those of others indicate that in indeterminate nodules the transition of zone II into interzone II–III is a region where major molecular changes during bacteroid and plant tissue development occur as a result of a possibly novel and probably complex exchange of signals between the bacterium and its host.

MATERIALS AND METHODS

Plant materials, bacterial strains, plasmids, and growth conditions.

Pea (*Pisum sativum* L. 'Finale') was grown on gravel, and vetch (*Vicia sativa* ssp. *nigra*) was grown on agar slants (Van Brussel *et al.* 1982). In all experiments, *R. l.* bv. *viciae* strain 248 (Josey *et al.* 1979) was used, unless mentioned otherwise. *R. leguminosarum* strain K11.pMP258 is described elsewhere (Spaink *et al.* 1989).

Electron microscopy.

Vetch nodules were harvested 21 days after inoculation and fixed overnight at 4° C in 1% glutaraldehyde-2% paraformaldehyde-0.1 M sodium cacodylate, pH 7.2. Fixed nodules were dehydrated in an ethanol series (30, 50, and 70% at 20° C, 96% and 100% at –35° C) and infiltrated with LR White acrylic resin (Agar Scientific Ltd., Stansted, U.K.) at –35° C. The resin was polymerized using 0.5% benzoinmethylether as a catalyst for 24 hr at –20° C and for 24 hr at room temperature under UV light. Ultrathin sections were collected on collodion-coated nickel grids and immunolabeled as described previously (Goosen-de Roo *et al.* 1991). Quantification of labeling based on gold particle density was done as described before (Goosen-de Roo *et al.* 1991). Number of cells for which gold particles were counted: 127, 50, and 70 for infection thread/droplet bacteria, "young" bacteroids, and mature bacteroids, respectively. Number of section areas counted: 19, 14, and 14, respectively. For the two-by-two comparison of different developmental stages a Student's *t* test was used to determine the statistical significance of observed differences.

In situ hybridization.

Pea nodules were harvested 16 days after inoculation and fixed immediately with 4% paraformaldehyde and 0.25% glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.2) supplemented with 100 mM sodium chloride for 4 hr. The nodules were dehydrated by passing through a routine ethanol series and were embedded in paraffin. Sections (7 μm thick) were hybridized according to a method derived from Cox and Gold-

berg (1988) as described before (Van de Wiel *et al.* 1990; Yang *et al.* 1991).

Labeling of antisense/sense RNAs.

For *ropA*, a 1.3-kilobase *Cla*I-*Bam*HI-fragment of pMP2202 (de Maagd *et al.* 1992) containing the full open reading frame as well as most of the untranslated leader, was cloned in the vector pBluescript KS (Stratagene, La Jolla, CA), resulting in plasmid pMP2242. For antisense RNA production, pMP2242 was cut with *Xho*I and *in vitro* transcribed by T7 polymerase. For sense RNA production, the plasmid was cut with *Bam*HI and transcribed by T3 polymerase. Radioactive labeling was performed as described by Van de Wiel *et al.* (1990). Preparation and labeling of the *nifH* probe was described before (Yang *et al.* 1991).

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