Development of *Phaseolus vulgaris* Root Nodules

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We report developmental studies on the determinate type of root nodules in *Phaseolus vulgaris* induced by *Rhizobium leguminosarum* biovar *phaseoli*. We show the first steps of nodule induction, leading to the formation of the nodule primordium from cells of the root outer cortex and describe the development of the central tissue, including invaded and uninvaded cells. Cytological observations, such as cell size and the pattern of starch accumulation, show that cells of the central tissue are a heterogeneous population. *In situ* hybridization shows a specific pattern of expression of the leghemoglobin and uricase-II genes. In mature nodules older invaded cells appear to be located in coincidence with a window generated in the endodermis by the penetration of the infection threads in the central tissue. This region seems to be the eccentric origin of a radial gradient of development. A model for the development of the common bean nodule is proposed.

Additional keywords: infection threads, nodulins, organogenesis, symbiosis.

The symbiotic interaction between soil bacteria of the genus *Rhizobium* and roots of leguminous plants results in the development of nitrogen-fixing nodules. The formation of these new plant organs, which fix atmospheric nitrogen, makes the leguminous plants independent of the nitrogen available in the soil (Newcomb 1981). The development and function of root nodules depends on the coordinated differentiation of the two symbionts, and it is the consequence of the expression of specific bacterial and plant genes (Verma 1992).

The morphological and physiological steps leading to the formation of indeterminate nodules, as occurs in alfalfa or pea root nodules, has been extensively investigated. The first response of the legume upon contact with *Rhizobium* is the deformation and curling of root hairs. In this way bacteria are entrapped in the curls and enter the root hair by invagination of the plasma membrane. Around the invaginated membrane the plant forms, by deposition of cell wall-like material, a tubelike structure, called infection thread, in which bacteria proliferate (Long 1989). Concomitantly, cells of the inner root cortex dedifferentiate and start dividing to form the nodule primordium. Few distal cell layers of the primordium become small and rich in cytoplasm to form the apical, persistent nodule meristem from which the different tissues of the nodule develop. While the meristematic cells are pushed outwards, the infection threads reverse their growth direction by 180° to follow the nodule meristem. The release of bacteria from the infection threads takes place in the most proximal cell layer of the meristem. In this way new invaded cells are continuously added to the central tissue. Bacteria contained in the plant cytoplasm differentiate into nitrogen-fixing bacteroids (Nap and Bisseling 1990). Using cytological criteria it has been possible to divide the central tissue of mature nodules in zones: the apical, bacteria-free meristematic zone I; the invasion zone II, in which the infection threads penetrate the newly dividing cells; the nitrogen-fixing zone III, containing invaded cells filled with bacteroids and the senescent zone IV. Recently, an interzone II–III, localized between the invasion and the nitrogen-fixing zone, has been defined. The interzone II–III is characterized by starch deposition in amyloplasts of the invaded cells and by a major switch in gene expression (Vasse *et al.* 1990).

During nodule development the synthesis of nodule-specific plant proteins, the nodulins, occurs (for a review see Franssen *et al.* 1992). According to the timing of expression they can be divided into early and late nodulins. Early nodulins are involved in nodule organogenesis, while the expression of late nodulins is associated with bacteroid development and metabolic exchange between both symbionts. Studies on nodulin expression confirm the existence, in indeterminate-type nodules, of the above described developmental zones (Scheres *et al.* 1990).

Root nodules of soybean do not show a persistent meristem and, for this reason, are called determinate nodules. They have been described as globose structures where all cells of the central tissue are progressing through the same stage of development (Newcomb *et al.* 1979). In this type of nodule, mitotic activity occurs throughout the central zone, persists only over a period of a few weeks, and cell expansion—rather than cell division—is responsible for the increase in nodule size. The developmental zones of the central tissue, as defined for mature indeterminate nodules, are not found (Calvert *et al.* 1984; Selker 1988). The nodules found on common bean roots are also of the determinate type (Dart 1975; Vandenbosch *et al.* 1985), but scant observations are reported about them.

We show here developmental aspects of nodule formation induced by *Rhizobium leguminosarum* biovar *phaseoli* on *Phaseolus vulgaris* roots. We analyze these nodules at various stages of development and find a heterogeneity in size of the invaded cells. We also describe the pattern of amyloplasts accumulation and of nodulins (leghemoglobin and uricase-II) expression. Our results suggest a model for nodule development.

RESULTS

Formation of nodule primordium.

Using the clearing method described by Truchet *et al.* (1989), the first microscopically visible response of the *P.*

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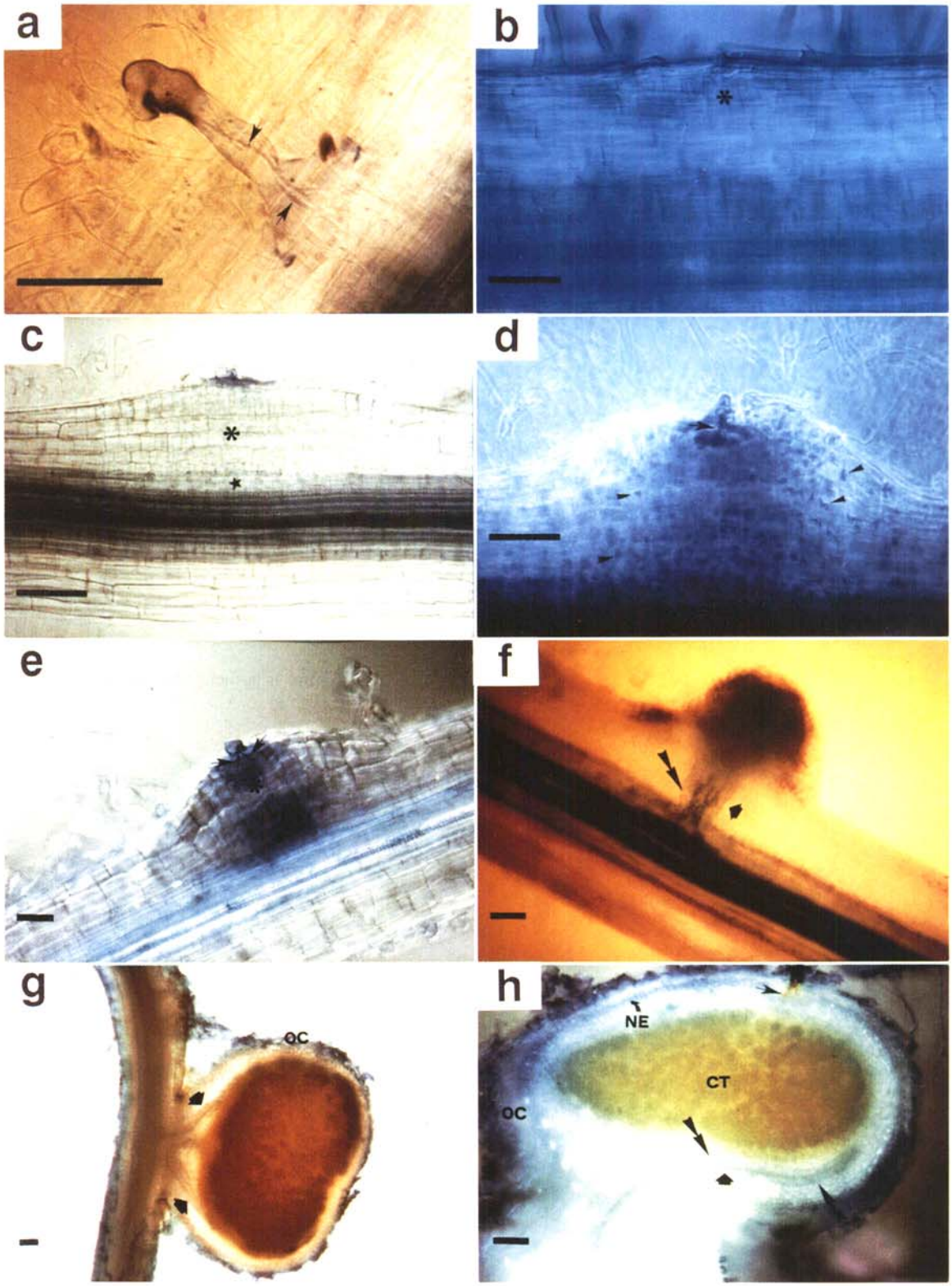

Fig. 1. Developmental stages of *Phaseolus vulgaris* nodules induced by *R. l.* bv. *phaseoli* strain CE3. Whole roots were fixed with paraformaldehyde, cleared with sodium hypochlorite, stained with methylene-blue and uncut material (A–F) or hand-free sections (G and H) were then viewed by bright-field microscopy. The figure shows: infection threads (thin arrows) in a root hair (A), in the root cortex (D and E) and in the mature nodule (H); mitotic activity of the root cortex cells in the vicinity of infection threads (asterisks in B, C, and E) and of the root central cylinder (stars in C and E); condensed nuclei of the nodule meristem (arrowheads in D); nodule vascular bundles (large arrows in F–H) and vascular endodermis (double arrowheads in F and H). In G and H hand-free sections of mature bean nodules with nodule outer cortex (OC), nodule endodermis (NE), and central tissue (CT) are shown. Bar equals 100 µm.

vulgaris root upon contact with *R. l.* bv. *phaseoli* is deformation and curling of root hairs (Fig. 1A,D,E). Curling leads to the entrapment of bacteria (not shown). The root hair reacts by depositing new cell wall material in the form of an inwardly growing tube, the infection thread (Fig. 1A,D,E,H). While infection is proceeding in the root hairs, several cells of the outer layers of the root cortex are reactivated and start to divide. The first cell divisions in the outer cortex take place near the infected root hair (Fig. 1B). Later on, cell division activity is also evident in the pericycle and in the inner cortex from which the nodule vascular bundles originate (Fig. 1C). The newly divided cells in the cortex show condensed nuclei in the cell center (Fig. 1D). Meanwhile, infection threads grow, ramify, and penetrate in the central part of the nodule primordium (Fig. 1E). Figure 1F clearly shows that the bean nodule primordium originates from the hypodermal and cortical cells of the root outer cortex. The endodermis of the root central cylinder continues with the endodermis of the forming nodule vasculature.

Fully developed root nodules (1–2 mm in diameter) are approximately spherical in shape, and their longitudinal diameter is usually shorter than their transverse one (Fig. 1G,H). Two major tissue types can be recognized: the peripheral tissue and the central tissue (Fig. 1G,H). The central tissue is surrounded by the peripheral tissue which includes, from the outside to the inside, the outer cortex, the nodule endodermis and the inner cortex (nodule parenchyma), which contains the vascular bundles. Figure 1G shows that the vascular bundles branch around the circumference of the nodule. The nodule endodermis completely surrounds the central tissue with the exception of a window generated by the penetration of infection threads (Fig. 1H).

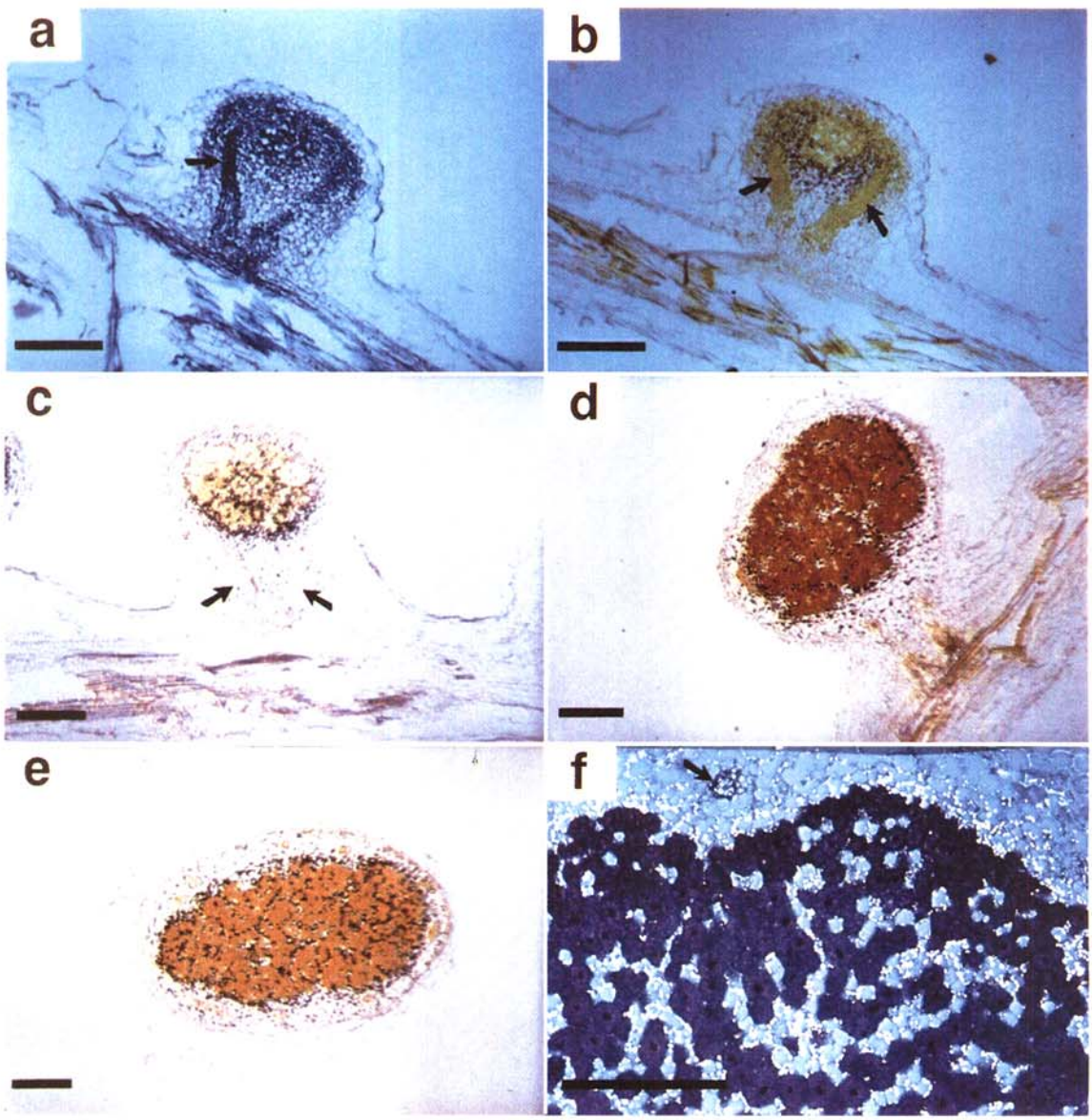

Fig. 2. Sections (7 μm) of nodules at different stages of development were stained with toluidine blue (A,F) or aqueous I_2-KI (B–E) and observed by light microscopy. Starch granules are stained in black with I_2-KI. Vascular bundles are indicated by arrows. A transversal section (F) of the mature nodule portion, indicated in the inset of Figure 3C, shows toluidine blue darkly stained invaded cells with nuclei in the central position and starch granules in vacuolated, uninvaded cells as white dots in phase-contrast microscopy. Bar equals 200 μm.

Starch accumulation.

In Figure 2 we show thin sections (7 μm) of nodules at different stages of development. The nodules were fixed, embedded in paraffin, stained with toluidine blue or I_2-KI and observed in bright-field microscopy.

In early emergent nodules (1 wk old) the central tissue is not yet organized, as shown in a longitudinal section stained with toluidine blue (Fig. 2A). A small cluster of cytoplasm rich cells is present in the central region; the vacuolated cells in this region are few; the vascular bundles and the nodule endodermis are forming. The early emergent nodule shows a wide base anchored to the plant root. A serial section of the same nodule (Fig. 2B) shows that small starch granules stained in black with I_2-KI are present, mainly in at least 10 uninvaded cell layers in the proximal part of the root.

In longitudinal sections of 2-wk-old nodules stained with I_2-KI (Fig. 2C) the central tissue is better organized and contains (not shown) invaded cells and smaller, uninvaded cells. Around it the inner cortex, the nodule endodermis, and the outer cortex are evident. Starch granules distribution shows a decreasing proximal-distal gradient: They are mainly present in at least three to four uninvaded cell layers and also appear evident in the interspersed, uninvaded cells of the central tissue. The base width decreases with respect to the length of the nodule as compared to the early emergent nodule of Figure 2B.

In longitudinal sections of mature nodules (3-wk-old; Fig. 2D), the base of anchorage is reduced and the vascular bundles connect the nodule to the root. Starch granules are present in the uninvaded cells interspersed among the invaded

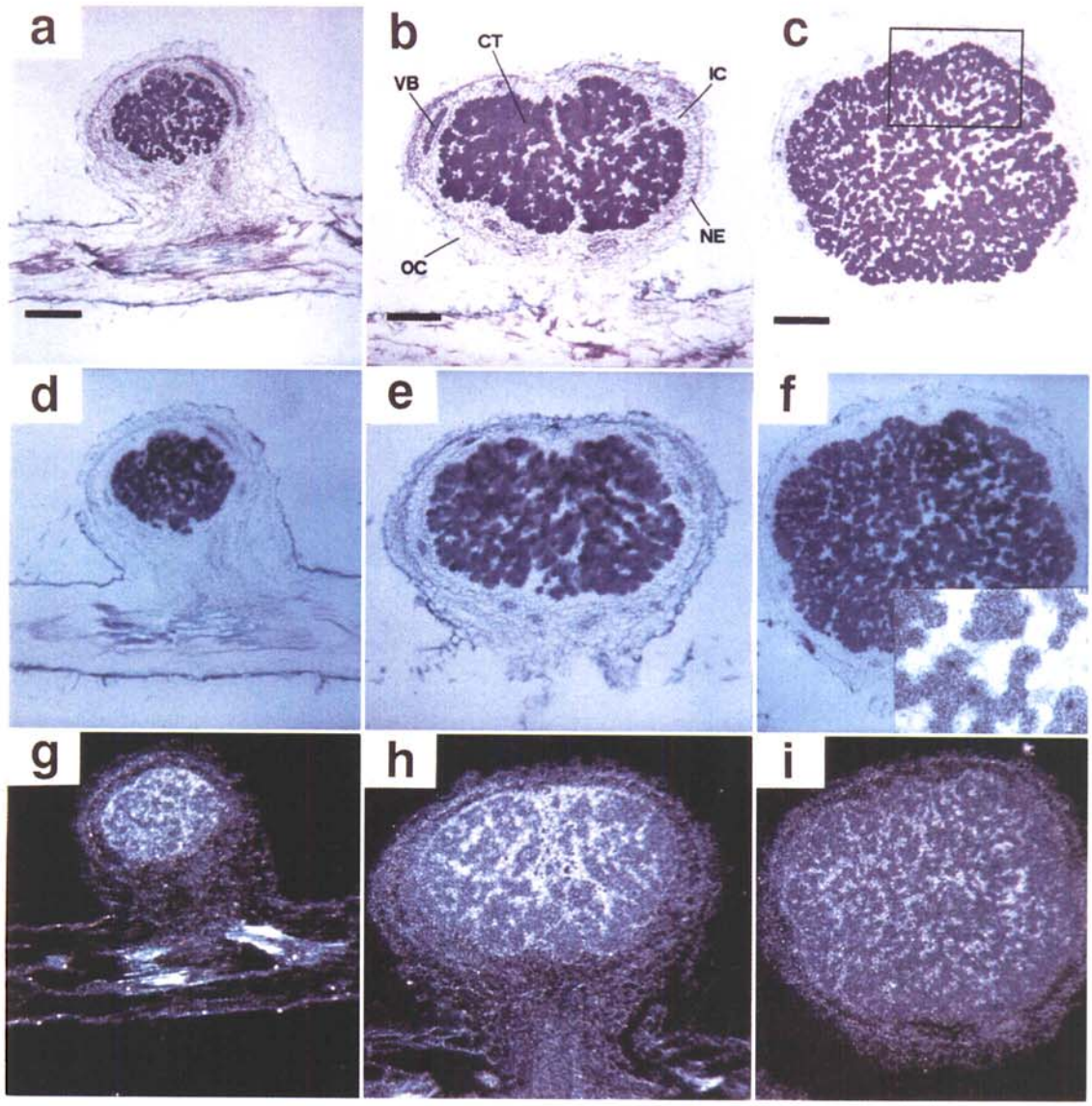

Fig. 3. Serial sections (7 μm) of young and mature nodules analyzed by staining with toluidine blue and *in situ* hybridization with leghemoglobin or uricase-II probes. Longitudinal sections of a young (A,D,G) or mature (B,E,H) nodule and transversal sections of a mature nodule (C,F,I). Bright-field micrographs of the sections stained with toluidine blue (A–C) show the central tissue (CT) completely surrounded by the peripheral tissues including the inner cortex (IC) which contains the vascular bundles (VB), the nodule endodermis (NE) and the outer cortex (OC). D–F are bright-field micrographs of the *in situ* localization of the leghemoglobin transcript. The hybridization signals are visible as dark dots in the invaded cells. G–I are dark field micrographs of the *in situ* localization of uricase-II transcript. Silver grains are visible as white dots in the uninvaded cells. Bar equals 200 μm.

ones and in few layers around the most peripheral invaded cells. They are almost absent in the distal part of the nodule. Transversal median sections (Fig. 2E) of mature nodules confirm the presence of starch granules accumulation in at least two uninvaded cell layers around the most peripheral invaded cells and a diffuse pattern of starch granules is also present in interspersed uninvaded cells among the invaded ones.

In Figure 2F we report a magnification of a portion of the transversal-median section of a mature nodule (shown below in Fig. 3C). The central tissue consists of large invaded cells and smaller, vacuolated uninvaded cells. The invaded cells are deeply stained with toluidine blue, their nuclei are in the cell center and the cytoplasm is densely packed with bacteroids (data not shown). Both types of cells, although not distributed according to a simple pattern, show extensive interconnections and the analysis of serial sections (data not shown) suggests that all the cells of one type may have connections with cells of the other type. The invaded cells at the periphery of the central tissue are smaller than those present inside, while all uninvaded cells are almost similar in shape. We also show the presence of starch granules visible as white granules in phase-contrast. These granules are accumulated in two to three layers of uninvaded cells around the most peripheral invaded cells.

Nodulins as markers of specific nodule tissues.

Thin sections (7 μm) of young and mature bean nodules stained with toluidine blue are shown in Figure 3A–C. In a young nodule (2-wk-old) the outer cortex, as well as the endodermis and a few layers of the inner cortex cells, are evident and the central tissue contains small invaded and uninvaded cells (Fig. 3A).

The peripheral tissue of the mature nodule (Fig. 3B,C) consists of three to four layers of large, vacuolated cortical cells (outer cortex), a layer of small sclerenchyma cells (nodule endodermis), and several layers of small, more densely packed, vacuolated parenchyma cells (inner cortex), which contain the vascular bundles. In longitudinal sections the invaded cells proximal to the original site of infection are larger than the others (Fig. 3B). The transversal-median section in Figure 3C shows that the most peripheral invaded cells are smaller than those present inside.

We used serial sections of the same nodules to analyze the expression of leghemoglobin (an oxygen-binding hemoprotein expressed in nitrogen-fixing nodules; Witty *et al.* 1986) and uricase-II (an enzyme involved in the assimilation of fixed nitrogen; Schubert 1986).

Using *in situ* hybridization we investigated the localization of the leghemoglobin transcript (Fig. 3D–F). A ^{35}S-labeled antisense-RNA probe transcribed from a homologous leghemoglobin cDNA fragment (Campos *et al.* 1987) was applied to 7-μm sections, and the site of hybridization was localized by autoradiography. The leghemoglobin transcript is synthesized exclusively in invaded cells of the central tissue, mainly those of the distal part, whilst it is not detected in uninvaded cells of the central tissue or in the peripheral tissues. In a transversal median section (Fig. 3F) we observe a strong hybridization signal in the central part of the nodule.

Figure 3G–I shows the expression, detected by *in situ* hybridization, of the uricase-II transcript (Sanchez *et al.* 1987). This gene is transcribed exclusively in uninvaded cells either interspersed or around the most peripheral invaded cells, mainly in the distal portion of the nodule (Fig. 3H). It is not detected in the invaded cells of the central tissue and in the vascular parenchyma. The transversal-median section in Figure 3I shows a strong expression of the uricase-II gene in the interspersed uninvaded cells between the invaded ones in the central part of the nodule. A control experiment (not reported) using the leghemoglobin and uricase-II sense probes shows no hybridization above background (see Materials and Methods).

DISCUSSION

We report studies on the development of *P. vulgaris* root nodules induced by *R. l.* bv. *phaseoli*. Common bean and soybean nodules are described as of the determinate type, but extensive experimental observations are reported only in the case of soybean (Newcomb *et al.* 1979; Calvert *et al.* 1984; Selker 1988).

We describe the first steps of *R. l.* bv. *phaseoli* invasion: curling of the root hair, formation of infection threads, and induction of mitotic activity in the root cortex. The root cortical cells dedifferentiate and start to divide, thus forming the nodule primordium. In temperate legumes, such as pea (Newcomb *et al.* 1979) and alfalfa (Truchet *et al.* 1989), the nodule primordium originates from cells located in the inner cortex. We observe that in bean the primordium originates from the outer cortex, similar to what happens in soybean (Calvert *et al.* 1984).

In fully developed bean nodules we recognize two major tissue types: the peripheral and the central tissues. The peripheral tissue consists of large, vacuolated, cortical cells (outer cortex), a layer of small schlerenchymatic cells (nodule endodermis) and several layers of small, densely packed, vacuolated cells (inner cortex) containing the nodule vascular bundles. The endodermis is located around the central tissue, with the exception of a small window generated by the penetration of infection threads. The central tissue consists of large invaded cells and smaller, vacuolated uninvaded cells. We observe that a gradient of progressively smaller invaded cells proceeds from the site of infection threads penetration towards the periphery. The distribution of uninvaded cells of the central tissue appears in bean analogous to those described in soybean nodules by Selker (1988) and Newcomb *et al.* (1979). Interconnected aggregates of uninvaded cells are arranged throughout the central region in such a way that they are in contact with virtually all invaded cells.

The pattern of starch accumulation in indeterminate nodules has been used to correlate morphological changes with the developmental stage. Amyloplasts first accumulate in the proximal half (15 cell layers) of the central tissue. Afterwards, this zone progressively decreases in size, and in mature nodules it is reduced to two layers of invaded cells of the distal part of the nodule, those corresponding to the interzone II–III (Vasse *et al.* 1990; Franssen *et al.* 1992). We observe in bean nodules starch deposition exclusively in uninvaded cells of the central tissue. In early emergent nodules small starch granules are present in at least 10 cell layers in the proximal part of the nodule. In mature nodules we observe amyloplasts in the uninvaded cells interspersed among the invaded ones and in few cell layers around the most peripheral invaded

cells. Starch granules show a proximal–distal decreasing gradient and are absent in the nodule region distal to the root. It is tempting to speculate that the differentiation program induced by *R. l.* bv. *phaseoli* causes the appearance of amyloplasts in uninvaded cells of the early emergent nodule and that later on starch is used up by nitrogen-fixing bacteroids present in the invaded cells. As a consequence, in mature nodules the pattern of starch accumulation may correlate to the developmental stage and nitrogen-fixing activity of the bacteroids. If so, the youngest invaded cells are present at the periphery of the central tissue and in the nodule region proximal to the root.

In pea nodules, leghemoglobin mRNA is detectable only in the cytosol of invaded plant cells of the early symbiotic zone, gradually reaching the maximum level of expression in the late symbiotic zone (Yang *et al.* 1991). In alfalfa nodules (de Billy *et al.* 1991) leghemoglobin mRNA expression is triggered in a single invaded cell layer rich in amyloplasts of the interzone II–III. We observe in bean nodules that the leghemoglobin gene is transcribed exclusively in invaded cells. In longitudinal sections, the leghemoglobin transcript is expressed according to a proximal-distal increasing gradient, while in transversal sections a central-peripheral decreasing gradient is present. Instead, in soybean nodule, antibodies raised against leghemoglobin decorate both invaded and uninvaded cells (Vandenbosch and Newcomb 1988). This discrepancy may be correlated to the difference in plants and/or level of gene expression analyzed.

Nodules of tropical legumes synthesize and transport fixed nitrogen in the form of ureides (Schubert 1986). In soybean nodules, the uricase-II gene product is found, by immunogold-labeling analysis, in uninvaded cells (Nguyen *et al.* 1985) and in cells of the vascular parenchyma (Vaughn and Stegink 1987). Moreover, marginal uricase-II activity was found in isolated cortical cells (Kohuchi *et al.* 1988). In bean nodules we observe that the uricase-II gene is transcribed in uninvaded cells either interspersed or around the most peripheral invaded cells and is more concentrated in the distal portion and in the center of the nodule. It is not detected in the invaded cells and in the vascular parenchyma. It appears, therefore, that uricase-II is mainly located in the uninvaded cells, making contacts with the larger invaded cells. Since uricase-II is involved in the conversion of fixed nitrogen into ureides, we speculate that the high level of expression of uricase-II in the uninvaded cells located closer to the infection starting point may correlate to the presence in this region of older invaded cells. These are devoid of starch and express heavily leghemoglobin mRNA. It is possible therefore that they are the most active nitrogen-fixing cells and that the expression of the uricase-II gene is induced by fixed nitrogen.

We propose (see Fig. 4) that globose nodules induced by *R. l.* bv. *phaseoli* result from two opposite centers of cell division: 1) Cells of the outer cortex multiply near the infected root hair, become infected, and give rise to further invaded cells of the central tissue, either by division or by sequential infection; 2) concomitantly, cells of the inner cortex start dividing and form an outer envelope that eventually surrounds the central tissue and forms the parenchyma and the vascular bundles. Therefore, the nodule results from the inward growth of invaded cells which are appropriately surrounded by specific plant tissues. In the distal part of a mature nodule and in coincidence with a window generated in the endodermis by the penetration of the infection threads, the bigger invaded cells are located. They show the strongest hybridization signal for the leghemoglobin transcript. In the uninvaded cells of this nodule region the uricase-II transcript is mainly present, whereas starch granules are almost absent. In contrast, in the most peripheral and proximal cells, starch granules are present and neither the uricase-II (in uninvaded cells) nor leghemoglobin (in invaded cells) transcript is observed. The data are in accordance with the eccentric origin of a radial gradient of nodule development and show that the nodule cells are not progressing through the same stage of development.

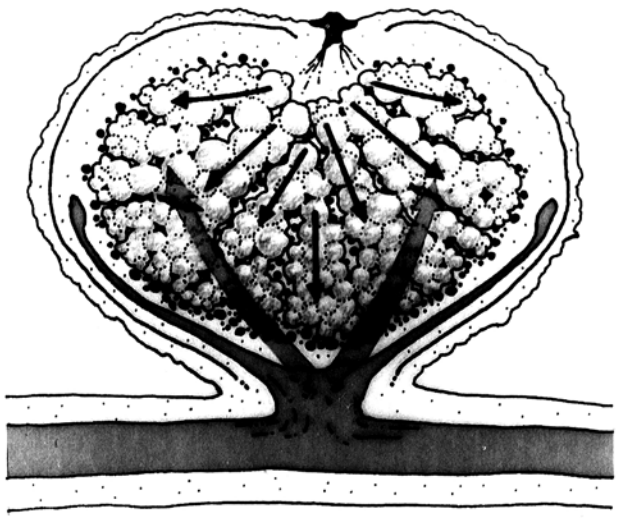

Fig. 4. Model for nodule development. The bean nodule results from two opposite centers of cell division: cells of the outer cortex multiply near the infected root hair, become infected and give rise to further invaded cells (thin arrows) which form the central tissue of the mature nodule. At the same time cells of the inner cortex start dividing and form (thick arrows) an outer envelope that eventually surrounds the central tissue and forms the parenchyma and the vascular bundles.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The wild-type strain of *R. l.* bv. *phaseoli* strain CE3 (Quinto *et al.* 1982) was grown on RMM minimal medium (Hooykaas *et al.* 1977) at 30° C. The nitrogen source used was KNO_3 at a concentration of 1 g/L.

Plant growth conditions.

Seeds of common bean (*P. vulgaris* L. 'negro Jamapa') were surface sterilized with 5% hydrogen peroxide for 30 min, washed extensively with sterile water, germinated for 72 hr in a petri dish in distilled water, and inoculated with *R. l.* bv. *phaseoli* diluted in Jensen medium (Vincent 1970) to a final OD_{600} of 0.1. The plants were grown at 23° C with an 8-hr daily light period.

Staining of whole fixed roots.

Whole plants were collected at different times after inoculation and fixed by immersion in 4% para-formaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2) for 1 hr under vacuum and 1 hr under atmospheric pressure. Fixed roots were cleared with sodium hypochlorite

as described by Truchet *et al.* (1989). After clearing, specimens were stained with methylene blue (0.01% in distilled water) and then observed by bright-field or dark-field microscopy. Staining or washing of the material was achieved by gently allowing the solution to flow between the slide and the coverslip.

Microscopy.

Individual nodules at different stages of development were excised from the plant and fixed immediately with 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2) for 4 hr at room temperature (Yang *et al.* 1991). After fixation, the nodules were rinsed in the same buffer, dehydrated by passing through a graded ethanol series, cleared with xylene, and embedded in paraffin (Paraplast Plus). Sections (7 μm thick) were cut with a Leitz microtome, adhered on TESPA-coated slides, and thereafter deparaffinized with graded xylene. The sections were rehydrated through decreasing ethanol concentrations and rinsed in 50 mM sodium phosphate buffer. The nodule organization was observed by staining with 0.25% toluidine blue in 50 mM sodium phosphate buffer (pH 7.2) and mounted with DePeX (Serva). To locate starch granules, the nodule sections were stained in a 0.1 M aqueous I_2-KI. The stained sections were photographed with a Nikon microscope in bright-field and epipolarization optics.

***In situ* hybridization.**

In situ hybridization was performed essentially as described by Cox and Goldberg (1988). Prior to hybridization the rehydrated nodule sections were incubated with 1 μg/ml proteinase K in 100 mM Tris/HCl, pH 7.5, 50 mM EDTA for 30 min at 37° C, rinsed three times in distilled water, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, at room temperature for 10 min. Subsequently the sections were dehydrated in a graded ethanol series and allowed to air dry. The hybridization with antisense or sense mRNA probes was performed for 16 hr at 55° C in a solution containing 50% formamide, 10% dextran sulphate, 0.5 mg/ml yeast tRNA, 1× Denhardt's solution, 300 mM NaCl, 20 mM Tris/HCl, pH 7.5, 5 mM EDTA. The hybridization solution contained approximately 3×10^4 cpm/ml. Following hybridization the nodule sections were washed in 50% formamide, 2× SSC, 10 mM DTT at 55° C, treated with 20 μg/ml of RNase A in 0.5 M NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA for 30 min at 37° C, rinsed three times in the same buffer (15 min, 37° C), washed in 0.2× SSC for 10 min at room temperature, dehydrated in graded ethanol, and air dried. Slides were coated with Kodak NTB2 nuclear emulsion and exposed for 2–10 days at 4° C. They were developed in Kodak D19 developer, fixed in Kodak Unifix and rinsed in distilled water. After washing slides were stained with 0.02% toluidine blue and observed by bright-field microscopy, dark-field microscopy, or phase-contrast microscopy using a Leitz microscope.

Preparation of antisense/sense RNA probes.

The 0.5-kb *Pst*I fragment from plasmid pNF-Lb01 containing the coding region of the *P. vulgaris* leghemoglobin gene (Campos *et al.* 1987) was recloned into the *Pst*I site of pGEM4 (Promega Inc.); the same resultant plasmid pRTLb-4 was linearized with either *Eco*RI or *Hin*dIII to prepare, respectively, the antisense- or sense-RNA used as a probe. The 0.6-kb *Hin*dIII fragment from plasmid pNF-UR07 (Sanchez *et al.* 1987) containing the coding region of the *P. vulgaris* uricase-II gene was recloned into the *Hin*dIII site of pGEM4 (Promega Inc.). The resultant plasmids pRTUR-4(+) and pRTUR-4(-) were linearized with *Bam*HI to prepare, respectively, the antisense- or sense-RNA used as a probe.

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