A HISTOCHEMICAL STUDY OF ROOT NODULE DEVELOPMENT

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A HISTOCHEMICAL STUDY OF ROOT NODULE DEVELOPMENT

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ter verkrijging van de graad van
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The investigations described in this thesis were carried out at the department of Molecular Biology and the department of Plant Cytology and Morphology, Agricultural University, Wageningen, The Netherlands.

The front cover shows a dark field micrograph of a transection through a root of a six-day-old pea (*Pisum sativum* L.) root. Expression of the PsENOD12 gene in an early stage of root nodule formation is visualized by the presence of white silver grains.

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STELLINGEN

1 De integratie van moleculair-biologische en microscopische technieken leidt bij het onderzoek aan wortelknollen tot een verdieping van het inzicht in het ontwikkelingsproces.

Dit proefschrift

2 Nu immunolocalisatiewaarnemingen hebben laten zien dat het voorkomen van leghemoglobine beperkt is tot de laat-symbiontische zone van de erwtewortelknol, is de waarneming van Newcomb dat de "region of thread invasion" in een "pink zone" van de wortelknol ligt, uiterst onwaarschijnlijk geworden.

Newcomb (1976) Can. J. Bot. 54: 2163.
Dit proefschrift

3 Het strijdig zijn van de immunolocalisatie van xanthinedehydrogenase (XDH) in bepaalde organellen van de ongeïnfecteerde cellen van sojawortelknollen door Nguyen et al. met observaties uit voorgaande rapporten is te verklaren door aspecifieke hechting van hun antiserum tegen XDH aan zetmeelkorrels bevattende plastiden van de ongeïnfecteerde cellen.

Nguyen et al. (1986) Planta 167: 190.

4 Het maltraiteren van rijstwortels met celwand degraderende enzymen en polyethyleenglycol door Al-Mallah et al. mag dan wel leiden tot het terechtkomen van rhizobia in afgestorven cellen van een opgezwollen wortelcortex, maar heeft als zodanig weinig te maken met het aanzetten van rijst tot symbiontische wortelknolvorming.

Al-Mallah et al. (1989) J.Exp.Bot. 40: 473.

5 Het in een onderzoek combineren van de expertise van twee verschillende vakgroepen kan vergeleken worden met het tot stand komen van een *Rhizobium*/waardplant-symbiose: in beide gevallen is een uitgebalanceerde communicatiestructuur tussen de twee partners nodig, wil er geen voortijdige abortie plaatsvinden.

6 Gezien de aard van de beschikbare gegevens en de praktijk van de theorievorming is het paleontologisch onderzoek naar de afstammingsgeschiedenis van de mens veel interessanter vanuit een wetenschapssociologisch dan vanuit een evolutiebiologisch oogpunt.

R. Lewin (1987) Bones of contention, Simons & Schuster, Inc.
D.C. Johanson & M.A. Edey (1981) Lucy, The beginnings of humankind, Granada Publishing, Ltd.

7 De discussie over de verwerpelijkheid van het vogelsoortenjagen zoals gepraktizeerd door de "Dutch Birding Association" als te weinig nuttig voor natuurbescherming en/of -wetenschap getuigt van een misplaatst soort Hollands calvinisme, aangezien er uit de aard van de bezigheid, namelijk vrije-tijdsbesteding, geen enkele aanleiding is enig nut te vooronderstellen.

8 De discussie tussen jagers en verklaarde tegenstanders van de jacht heeft veel weg van een competentiestrijd over wie van beide partijen de meest adequate natuurkennis ten aanzien van het natuurbeheer bezit.

9 In al hun diversiteit komen de plannen voor een creatief natuurbeheer, zoals het uitzetten van bevers in de Biesbosch en het plan "Ooievaar", overeen in een voorspiegeling van een terugkeer naar een soort aards paradijs, waarvan het de vraag is of het echt bestaan heeft en, zo ja, of het ook in de gewenste vorm zal terugkeren.

10 De Nederlandse Spoorwegen N.V. zouden de door hen geproclameerde aansluiting bij het huidige milieubewustzijn het beste kunnen waarmaken door de eerste-klascoupés te vervangen door ruimte voor een efficiënt en betaalbaar vervoer van fietsen ten behoeve van de verplaatsing naar en van het station.

Stellingen bij het proefschrift "A histochemical study of root nodule development" door Clemens van de Wiel, te verdedigen op 11 juni 1991 te Wageningen.

Aan mijn ouders

CONTENTS

CHAPTER 1

Outline

In cooperation with soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* or *Azorhizobium*, many members of the legume family are able to form specialized organs on their roots, called root nodules. The bacteria, wrapped up inside a plant membrane, are accomodated in large parenchymatic cells located centrally in these root nodules. For this, they reward their host by converting atmospheric nitrogen into a form usable for the plant. The central infected tissue of the nodule is surrounded by a peripheral tissue provided with vascular bundles through which metabolites are exchanged with the other parts of the plant.

In the interaction with the bacteria, the host plant expresses specific genes that are not transcribed at a detectable level in other parts of the plant. The products of several of these genes are made during the formation of the nodule and are named early nodulins.

The present study aims at elucidating the role of these early nodulins in the formation and infection of the root nodules. For that purpose, we set out to combine the molecular approach of studying gene expression with the microscopical approach of studying the structural development of the nodule.

To provide a background to these studies, chapter II summarizes existing knowledge about nodule development from an anatomical/cytological point of view, supplemented with data on already described nodulins and with brief excursions into physiological phenomena relevant to the rest of our study.

In chapter III and IV, nodulin gene expression is analysed in common vetch (*Vicia sativa*) nodules elicited by a panel of bacterial strains with various defined genetic changes. Such nodules were blocked at different stages in the development of the central tissue depending on the bacterium involved; the precise stage at which the blockade occurred was determined by light- and electron-microscopical observations. In that way, insight could be gained in the diverse genetic information supplied by the bacterium for nodule development to proceed through the successive developmental stages and the induction of the appropriate nodulin genes going with it. Furthermore, the start of the expression of individual nodulin genes, for instance the early nodulin Nps-40', could be related to certain stages of central tissue development. In the case of the leghemoglobin genes, such a correlation between nodulin gene expression and specific developmental stages could be confirmed by the direct localization of the leghemoglobin proteins in pea (*Pisum sativum*) nodule sections comprising different consecutive developmental stages, by immunolabeling.

Such direct approach of studying nodulin gene expression in nodule sections was further pursued in the chapters V, VI, VII and VIII. In chapters V, VI and VII early nodulin gene transcripts for which sequenced cDNA clones had become available were localized by *in situ* hybridization: in chapter V, ENOD2 in soybean (*Glycine max*) and

pea nodules, respectively; in chapter VI, ENOD2 in alfalfa (*Medicago sativa*) nodules; and in chapter VII, PsENOD12 in pea. In chapter VIII an attempt to localize the Nps-40' protein by immunolabeling in pea nodules is described. By these *in situ* localization methods, different temporal and spatial patterns of gene expression for each early nodulin were determined. Speculations about the functions of the individual nodulins are made based upon the gene expression patterns and the amino acid sequences of the nodulins as deduced from the nucleotide sequence of the corresponding cDNA clones.

In addition, in chapter VI, *in situ* localization of MsENOD2 transcripts was performed on alfalfa nodules induced by certain engineered bacterial strains or by auxin transport inhibitors. Such nodules do not have bacteria in their central tissue and also differ in other structural details from effective nodules, but nevertheless were shown to exhibit a tissue-specific expression pattern of the MsENOD2 gene similar to effective nodules. In chapter VII the results of further experiments are reported pertaining to the influence of the bacterium on nodulin gene expression, particularly the involvement of bacterial factors and the bacterial *nod* genes in the induction of the expression of the PsENOD12 genes.

Finally, chapter IX summarizes the results of the *in situ* localization of early nodulin gene products. In the light of these results, the significance of our histochemical approach to elucidating the role of nodulins in root nodule development is discussed.

CHAPTER 2

General introduction

The Leguminosae are well known for the very intriguing property, found in the majority of its members, of developing root nodules in symbiosis with soil bacteria of the genera *Rhizobium, Bradyrhizobium* and/or *Azorhizobium*. The interaction between bacterium and plant is specific in terms of recognition between the partners, as mostly a distinct plant species establishes an effective symbiosis with a defined bacterial species. For instance, *Rhizobium meliloti* establishes an effective symbiosis only with *Medicago, Melilotus* and *Trigonella* species. However, there are also interactions with a less specific character, exemplified by the so-called broad host range strains that are accepted by a whole series of unrelated tropical legumes (Lim & Burton, 1982).

After inoculation with a host-specific bacterium, the plant forms a well organized and specialized outgrowth, the nodule. Cells in the root cortex are induced to resume meristematic activity and the newly formed cells develop into the nodule. The bacteria are eventually accomodated inside special cells in a central tissue of the nodule, after which they become large, often pleiomorphic-shaped bacteroids. They are separated from the plant cell cytoplasm by a membrane of host origin. The intracellular state enables the bacteria to fix atmospheric dinitrogen and to provide the host with the primary product of the fixation, ammonia, which is further assimilated by the host plant.

The development of root nodules and their functioning have been extensively studied from an anatomical and physiological point of view since the discovery of its nitrogen-fixing capability more than a century ago (Hellriegel, 1886). With the rise of molecular-biological techniques to study the structure and organization of genes and the regulation of their expression, the possibility was created to tackle also the molecular aspects of the infection of the root and the development of the root nodule.

On the bacterial side this led to the identification of genes involved in the induction of the nodulation process, the *nod* genes, and of genes involved in the nitrogen fixation process, the *nif/fix* genes. These genes are located on a large Sym(biotic) plasmid in *Rhizobium* strains, but on the chromosome in *Bradyrhizobium* strains. In addition, genes involved in the infection process, such as the *exo* genes that direct the synthesis of extracellular polysaccharides, and the *ndv* (nodule development) genes that are homologous to the chromosomal virulence genes, *chv*A and *chv*B, of the plant pathogen *Agrobacterium tumefaciens*, have similarly been found to be essential for establishment of the symbiosis (for review of the bacterial genes: Appelbaum, 1990).

On the plant side the efforts were directed towards the identification of genes that are only transcribed in root nodules. These genes were designated as nodulin genes (Van Kammen, 1984). Some of these genes are well characterized and their products have even been assigned clearly circumscribed functions, e.g. the leghemoglobins regulating the oxygen supply to the bacteroids (Appleby, 1984; Sheehy & Bergersen, 1986). The early

nodulin genes, which are expressed during development of the nodule well ahead of the onset of nitrogen fixation, have been implicated in processes of nodule development or infection (Nap & Bisseling, 1990). In order to establish their roles in these processes it is necessary to determine in which tissue of the nodule and at which moment during nodule development, these genes are expressed. To that end, *in situ* localization studies of nodulin gene products have been performed at both the mRNA and the protein level. These studies form the major theme of this thesis.

As a basis for the interpretation of our localization studies, a detailed description of the development of the root nodule from a morphological/anatomical point of view will be given in the following account. Where relevant, information about aspects of nodule physiology and the role of nodulins, as far as known, will be provided. Before this , the nodulin concept will first be briefly outlined.

NODULINS

By definition, nodulins are plant proteins that are only present in root nodules (Van Kammen, 1984). For practical reasons, nodulins are frequently identified by comparing gene expression in nodules and uninfected roots. Nodulins thus identified may then still prove to be present in other parts of the plant. In that case, it will be necessary to adapt the designation of these proteins as nodulins as new observations become available. One way of comparing gene expression in nodules and roots is the analysis of the proteins produced upon *in vitro* translation of RNA in a eukaryotic protein synthesis system by two-dimensional gel electrophoresis. The nodulins identified in that way are indicated by the letter N followed by the molecular weight in kD as determined by SDS/polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the initials of the plant species' scientific name are added in lower case to the "N" in order to be able to discriminate between nodulins of different plant species. For instance, Nvs-40 is a nodulin with an apparent molecular weight of 40 kD in common vetch (*Vicia sativa*). Should the nodulin be identified as a nodule-specific form of a protein with a known function occurring elsewhere in the plant, e.g. glutamine synthetase, the prefix "n" can be added to the name of the known protein if the nodulin is involved; thus, in our example, n-glutamine synthetase.

Another approach to identify nodulins has been the isolation of nodulin cDNA clones by differential screening of a cDNA library from nodule poly(A^+) RNA with

cDNA from nodule and uninfected root RNA, respectively. These nodulin cDNA clones are designated by the prefix NOD, preceded by the proper plant species initials. When the protein product encoded by the cDNA clone has been identified by hybrid-released translation, the nodulin can also be indicated by the letter N, followed by the molecular weight in kD as determined by SDS/PAGE of the hybrid-released translation product. Thus, the Ngm-75 nodulin corresponds with the GmENOD2 cDNA clone in soybean (*Glycine max*) (Franssen et al., 1987).

The nodulin genes have been divided into two classes on the basis of the time course of appearance of their products during nodule development. The early nodulin genes are expressed well in advance of the onset of nitrogen fixation. cDNA clones representing these genes are designated by ENOD after the plant species initials. This class of nodulin genes is thought to be involved in the formation of the nodule, including the infection process. The major part of the investigations described in this thesis is directed towards the determination of the spatial and temporal pattern of the expression of early nodulin genes. The majority of described nodulin genes are expressed around the onset of nitrogen fixation and are named late nodulin genes. They are exemplified by the well-studied leghemoglobin genes. The late nodulin genes are thought to be involved in establishing a proper environment for nitrogen fixation and metabolizing the fixed nitrogen for transport to the rest of the plant (Nap & Bisseling, 1990).

LEGUME ROOT NODULE DEVELOPMENT

Legume root nodule ontogeny and organization has been extensively reviewed, a.o. by Dart (1977), Goodchild (1977), Newcomb (1981) and Bergersen (1982). Basically, two nodule types are distinguished based on the presence or absence of a persistent meristem: an indeterminate and a determinate type, respectively. Examples of the determinate type are the nodules of soybean (*Glycine max*), bean (*Phaseolus vulgaris*) and birdsfoot trefoil (*Lotus* spec.). The indeterminate type is exemplified by nodules of pea (*Pisum sativum*), vetch (*Vicia* spec.), clover (*Trifolium* spec.) and alfalfa (*Medicago sativa*). In the following, the ontogeny of the two nodule types will be described separately, based mainly on the root nodules of soybean and pea, respectively. The latter two species have been the main subject of this thesis. The development of both types of nodules is represented schematically in Fig. 1; the end result is shown in Fig. 2. Light micrographs of nodule development are shown in chapter 5 for soybean and in chapters 3, 5 and 7 for pea.

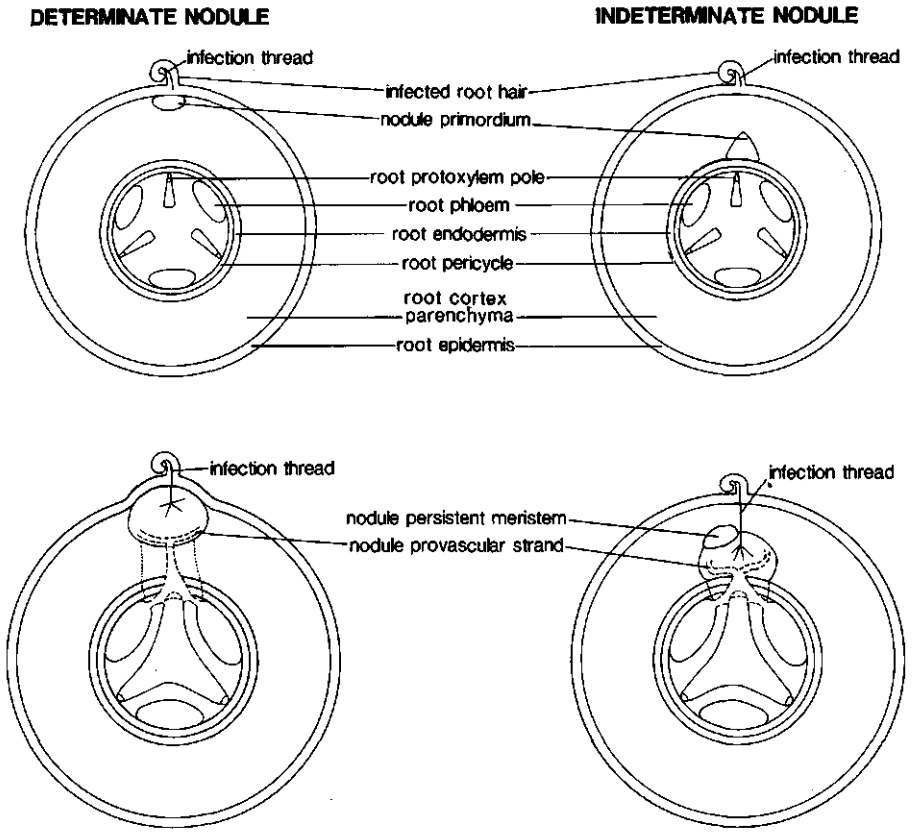

Fig. 1. Schematic representation of the ontogeny of the determinate (left part of the figure) and the indeterminate (right part of the figure) nodule. In the upper part of the figure infection thread formation in a root hair and concomitant initiation of a nodule primordium is shown. In the lower part of the figure a later stage is shown, in which the infection of the nodule primordium and the development of the nodule vasculature, including the linking up with the root vasculature, are taking place. In the indeterminate nodule the delimitation of the persistent meristem is indicated.

Indeterminate nodules

Specific tricyclic phenolic compounds, flavonoids, present in the root exudate of the host plant induce *Rhizobium* in the root vicinity to transcribe its *nod* genes. This is thought to occur via the interaction of these flavonoids with the product of the regulatory gene *nod*D. When activated by the proper -i.e. host-specific- flavonoid, this *nod*D product is thought to switch on the transcription of the other *nod* genes. Of these, the *nod*ABC genes, which form one operon, are absolutely necessary for the production of one or more signal(s) that induce(s) the deformation of root hairs and the initiation of nodule primordia in the plant's root cortex. Besides *nod*ABC, other *nod* genes are involved in certain aspects of nodule development, such as host recognition (Long et al., 1989). A rhizobial root hair deformation factor produced by *Rhizobium meliloti*, called NodRm-1, has recently been identified as a sulfated and acylated glucosamine tetrasaccharide by Lerouge et al. (1990). There is also evidence that perturbing the auxin/cytokinin balance in the root plays a role in eliciting nodule development (Hirsch et al., 1989).

Rhizobia are able to adhere to the root hair surface by a bacterial Ca^{2+}-dependent protein, the so-called rhicadhesin. Subsequently they can anchor themselves to the root hair surface by producing a network of cellulose fibrils (Smit et al., 1987). Lectins provided by the host plant have been implicated in the host-specific attachment of the bacteria, but their role is controversial and may depend on environmental conditions (Diaz et al., 1989a). The most compelling recent piece of evidence for the involvement of lectins in determining host-specificity is provided by Diaz et al. (1989b), who transformed white clover with a pea lectin gene. Transformation with this pea lectin gene enabled the white clover plants to become nodulated by the normally pea-specific bv. *viciae* of *Rhizobium leguminosarum*. The oligosaccharide character of the root hair-deforming factor NodRm-1 also suggests the involvement of lectins in the interaction with the rhizobia (Lerouge et al., 1990).

The curling of the root hairs, frequently described as the formation of "sheperd's crooks", leads to the entrapment of the bacteria. After entrapment, the bacteria are able to induce local lesions in the root hair cell wall. The host plant reacts by depositing new cell wall material around the lesion in the form of an inwardly growing tube (Callaham & Torrey, 1981). The tube is filled with proliferating bacteria surrounded by a matrix and becomes an infection thread. The infection thread grows towards the inner tangential wall of the root hair cell by a process of tip growth (Bakhuizen et al., 1988). During this growth, and later on, the infection thread remains at all times enveloped by the plant cell membrane. The growing tip is often accompanied by the host cell nucleus. In the majority

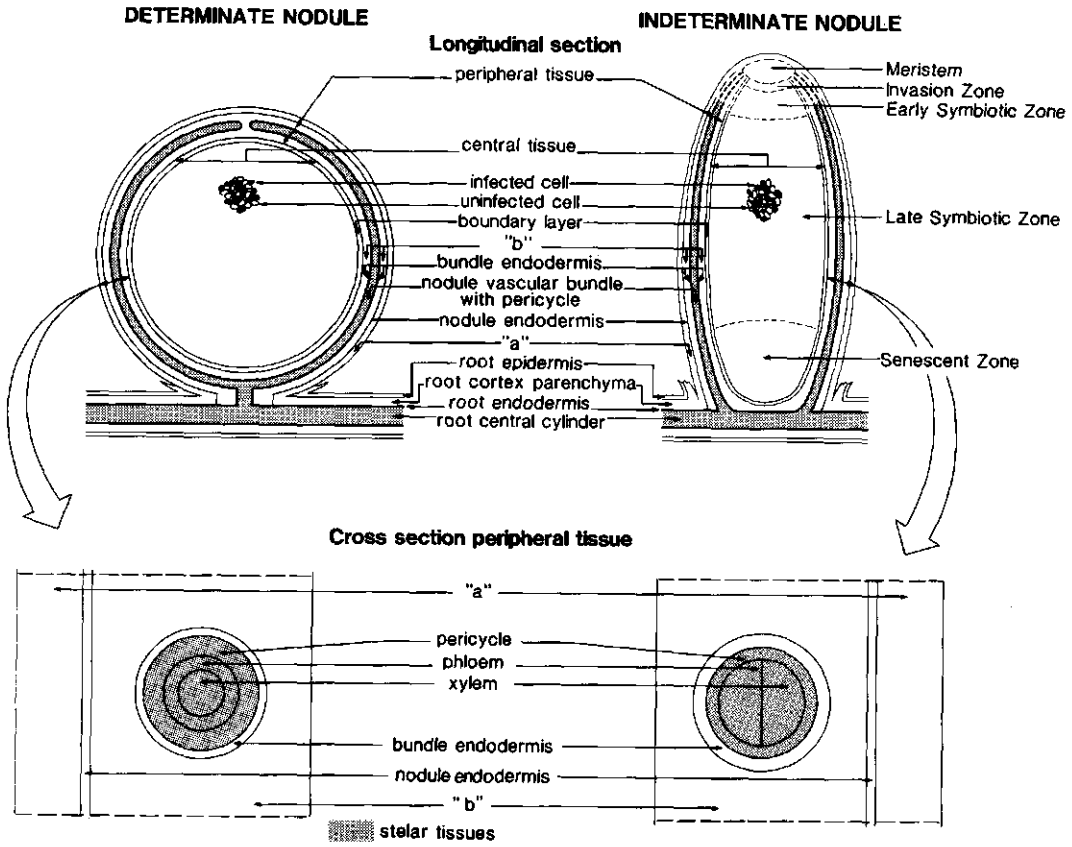

Fig. 2. Schematic representation of the determinate and the indeterminate nodule with all tissues and zonations mentioned in the text indicated. "a" Is the outer part of the peripheral tissue, usually called "outer cortex", but also "root cortical" or "host" tissue; "b" is the inner part of the peripheral tissue, usually called "inner cortex", but also "nodule cortex"; see the text for an explanation of this conflicting terminology.

of legumes in which the infection process has been studied, just emerging root hairs have been shown to be the main site of infection (Bauer, 1981; Bhuvaneswari et al., 1981). In some species, such as clover and alfalfa, also old hairs can be infected, after they have been induced to resume growth, manifested by branching or other signs of deformation (Bhuvaneswari & Solheim, 1985; Wood & Newcomb, 1989).

While infection is proceeding in the root hairs, several cells of the inner layers of the root cortex, mostly those adjacent to a protoxylem pole opposite the infection site (cf. Libbenga & Bogers, 1974), become activated (Fig. 1). The nuclei of these cells migrate to the middle of the large central vacuole via cytoplasmic strands. Next, the cells divide, at first in a transversal plane, later on also in radial and tangential planes (Libbenga & Harkes, 1973). This group of dividing cells forms the nodule primordium, also named nodule initial (Dart, 1977). As the cells continue with dividing, they become smaller and richer in cytoplasmic content and eventually the group takes on a hemispherical shape. Meanwhile, the infection thread grows through the cortex towards this developing nodule primordium. Infection thread growth from cell to cell proceeds via a mechanism of local dissolution of the host cell wall and formation of a new wall in the form of an inwardly growing tube, just as in the original infection of the root hair. Ahead of the growing infection thread, cells in the outer layers of the root cortex also become activated in preparation for infection. These cells deposit a thin additional wall layer and form cytoplasmic strands through their central vacuoles. The cytoplasmic strands subsequently serve as pathways for the growing infection thread (Bakhuizen et al., 1988).

The infection thread penetrates the nodule primordium most often at the middle of the distal end (Fig. 1). From there it ramifies into thinner threads that grow into cells in the central part of the primordium. Here, the release of rhizobia into the host cells takes place from swollen, unwalled tips of side branches of the infection thread, via an endocytotic process. The swollen tips can apparently also detach from the infection thread, before the rhizobia are released, and are then called "unwalled droplets" (Newcomb, 1976). After release into the plant cell, the rhizobia are called bacteroids (Bergersen, 1982). Within the host cytoplasm they remain surrounded by a membrane of host plant origin, called the peribacteroid membrane (Robertson et al., 1978). The bacteroids multiply, increase in size concomitantly with an increase of their DNA content (Bisseling et al., 1977), and finally almost completely fill the host cell. During bacterial proliferation, the host cell produces large amounts of peribacteroid membranes. Several late nodulins have been shown to be incorporated into the peribacteroid membrane, emphasizing the specific role of this membrane in the symbiosis (Verma et al., 1986). Especially in indeterminate nodules the bacteroids adopt a pleiomorphic shape (Sprent, 1980); they can, for instance, become club-shaped or branch dichotomously. The

nucleus, which has become multi-lobed, and a relatively small vacuole remain in a central position, but other organelles, like mitochondria and plastids, become confined to a peripheral position in the infected cells. The infected cell as a whole enlarges considerably.

Not all the cells of the central part of the primordium become infected cells. In a considerable number of cells, rhizobia are not released. These cells differentiate into uninfected cells, also named interstitial cells (Goodchild, 1977), characterized by a large central vacuole and plastids often containing prominent starch grains.

After the infection of the central part of the nodule primordium, the persistent meristem is established at the distal end of the nodule primordium next to the site where the infection thread originally penetrated the primordium (Fig. 1). This meristem enables the nodule to follow an indeterminate growth pattern. The persistent meristem is also called apical (e.g. Newcomb, 1981), distal (e.g. Hirsch et al., 1984) or nodule meristem (e.g. Bergersen, 1982). The meristem proximally adds new cells to the nodule, which leads to the emergence of the nodule through the outer layers of the root cortex. In the central tissue infection thread growth is now redirected distally and its branches penetrate a part of the cells that are formed by the meristem. These cells subsequently develop into infected cells next to the cells also arising from the meristem that remain uninfected. Thus, a zonation of consecutive stages of development arises in the central tissue (Newcomb, 1976; see Fig. 2). Going in a proximal direction from the apically localized persistent meristem, one first encounters an invasion zone in which infection threads penetrate plant cells and release rhizobia into the cells. Then an early symbiotic zone can be discerned in which the rhizobia proliferate untill the infected cells are almost completely filled with bacteroids, while the infected and uninfected cells themselves enlarge considerably. In the late symbiotic zone the rhizobia continue their differentiation culminating in the onset of nitrogen fixation.

In the late symbiotic zone several late nodulins, among which leghemoglobin accumulate in the cytoplasm of the infected cells (Robertson et al., 1984). Leghemoglobin transports oxygen to the bacteroids. Free oxygen diffuses only slowly into the central tissue due to a gas barrier posed by the restricted intercellular space in the inner part of the peripheral tissue, i.e. the peripheral tissue inside the nodule endodermis (see Fig. 2). A very low level of free oxygen in the infected cells is a prerequisite to protect the extremely oxygen-sensitive nitrogen-fixing enzyme nitrogenase against damage. At the same time, the bacteroids need large amounts of oxygen to generate the energy necessary for the nitrogen fixation process. The leghemoglobin apparently serves a dual role: it enables the infected cells to efficiently take up the oxygen, which so slowly diffuses through the inner part of the peripheral tissue, and to transport it to the bacteroids, while, at the same

time, keeping the concentration of free oxygen sufficiently low to prevent damage to nitrogenase (Witty et al., 1986).

In older nodules the late symbiotic zone is followed by a senescent zone at the base of the nodule, in which plant cells, together with the bacteroids, are degraded. The central tissue is delimited from the peripheral tissue by several layers of cells that morphologically resemble the uninfected cells of the rest of the central tissue (Goodchild, 1977), named boundary layer by Gresshoff and Delves (1986; see Fig. 2).

Returning to the nodule primordium, while the infection of the central part of the nodule primordium is going on, the peripheral tissue starts to be formed at the lateral sides of the primordium. Provascular strands are initiated by additional divisions, starting in the root pericycle opposite one of the protoxylem poles. From these pericyclic derivatives, division activity spreads out on the one hand into the residual procambial tissue between the xylem and the phloem of the root stele to bring about a connection at one or two sites with both the xylem strand itself and the phloem strands at either side of the xylem strand (Bond, 1948). On the other hand, division activity spreads out into the nodule peripheral tissue (Fig. 1). These provascular strands develop into dichotomously branching nodule vascular bundles. The nodule vascular bundles have a collateral organization, but, contrary to other plant organs, the phloem is located at the inner side, i.e. more closely to the central infected tissue than the xylem (Pate, 1976; see Fig. 2). The xylem contains tracheids, which first develop a ring/spiral and later on a scalariform secondary wall thickening pattern (Dart, 1977). Around the vascular tissue, a pericycle develops that in pea consists of a single layer of transfer cells, i.e. cells rich in cytoplasm with an extensive network of wall protuberances into the cell (Pate et al., 1969). Transfer cells also develop in the xylem parenchyma and pericycle of the root stele near the nodules (Newcomb & Peterson, 1979). Each of the vascular bundles will also be surrounded by a bundle endodermis exhibiting Casparian strips. This bundle endodermis is continuous with the root endodermis (Bond, 1948; see Fig. 2).

In addition, a nodule endodermis, also called common endodermis, is formed that divides the peripheral tissue into an inner part, containing the vascular tissue, and an outer part (Fig. 2). Frazer (1942) did not find Casparian strips in the nodule endodermis of pea and clover, among others. On the contrary, Casparian strips have been reported to develop in the nodule endodermis of pea by Bond (1948). At a later stage in development, the nodule endodermis cells deposit an additional suberin-containing wall layer all around the cell. The cells of the outer part of the peripheral tissue link up with the parenchymatic cells of the root cortex and also resemble the latter cells in morphology, in that they are relatively large, and loosely packed. No epidermal layer is discernable around the peripheral tissue. The parenchyma cells of the inner part of the peripheral

tissue differ from those of the outer part in that they usually are smaller and more densely packed, while their cell walls stain more heavily with toluidine blue (Goodchild, 1977). The limited intercellular space enables this tissue to restrict the rate of free oxygen diffusion into the central tissue, which helps to protect nitrogenase (see above). Several authors, e.g. Newcomb, 1981; Bergersen, 1982, have simply named the outer and inner parts of the peripheral tissue "outer" and "inner" nodule cortex, respectively. Furthermore, Newcomb (1981) stated that this nodule cortex, as a whole, is deposited by the apical (persistent) meristem. A different opinion is held by Bond (1948), which is supported by Libbenga and Harkes (1973). In their view, only the inner part of the peripheral tissue, together with the nodule endodermis, is deposited by the distal meristem and therefore belongs to the nodule proper. This inner part of the peripheral tissue then is named nodule cortex. The outer part of the peripheral tissue is regarded as a derivative of a few layers of root cortex cells around the nodule primordium, in which some divisions and considerable stretching must have occurred to accomodate the enlarging nodule. This outer part then is called root cortex (Bond, 1948), or host tissue (Libbenga and Harkes, 1973) surrounding the nodule. In this view, the nodule endodermis should be regarded as the outer boundary of the nodule proper. In chapter 5 of this thesis (≈ Van de Wiel et al., 1990), a new term has been proposed for the inner part of the peripheral tissue to avoid the potentially misleading term "cortex". The problems with terminology are further discussed in the general discussion (chapter 9).

The indeterminate nodule has a potential to branch dichotomously, apparently by a splitting of the persistent meristem. Thus, older nodules can develop a coralloid appearance. In perennial species the persistent meristem can resume activity each new growing season, leading to nodules with annular constrictions indicating the contributions from each growing season. In such species a periderm develops in the outer part of the peripheral tissue (Dart, 1977).

Determinate nodules

Many aspects of nodule development in determinate nodules are comparable to those of indeterminate nodules. The following section about determinate nodule development therefore forms an addition to the last section about indeterminate nodule development, with emphasis on the differences between the two types of nodules.

Like in the afore-described development of the indeterminate nodule, the primary infection in determinate nodule development occurs through adherence of the bacteria to root hairs, which are induced to curl and initiate infection threads (Turgeon & Bauer,

1982). In contrast, the initiation of nodule development occurs in a group of cells in the outer cell layers of the root cortex (Fig. 1). Here a nodule primordium is formed by the subdivision of highly vacuolate cortical cells. This subdivision leads to the formation of relatively small cells with a high cytoplasmic density, a few scattered vacuoles and large nuclei with prominent nucleoli. The primordium subsequently enlarges by the continuing division activity of these cells. From the primordium, cell division activity also radiates out towards the inner cell layers of the root cortex (Newcomb et al., 1979; Calvert et al., 1984). Here, between the nodule primordium and one of the protoxylem poles of the root stele, a provascular strand is formed that differentiates into a vascular bundle connecting the nodule vasculature to the root vascular system (Fig. 1). The connecting vascular bundle becomes surrounded by a bundle endodermis and a newly formed parenchymatic tissue that becomes continuous with the inner part of the peripheral tissue of the nodule. This newly formed parenchymatic tissue becomes surrounded by an endodermal layer, i.e. the part of the nodule endodermis that links up the part surrounding the nodule with the root endodermis (Fig. 2).

While the cell division activity of the nodule primordium continues, the infection thread grows out of the root hair cell and ramifies into the enlarging globular nodule primordium (Fig. 1). After a certain period, meristematic activity ceases, starting in the centre of the primordium (Vance et al., 1982). Here the infection threads begin to release rhizobia into the cytoplasm of the plant host cells, via an endocytotic process, just as described for indeterminate nodules. In soybean on the other hand, cells with released bacteria can still proceed with dividing for some time (Newcomb, 1981). The bacteroids proliferate, enlarge and come to fill the host cell cytoplasm. The infected cells as a whole increase in size enormously.

As in indeterminate nodules, the central tissue also contains uninfected cells. The uninfected cells constitute a more or less continuous network throughout the whole central tissue (Selker, 1988). In addition to a large central vacuole and amyloplasts these cells develop prominent peroxisomes. These peroxisomes contain large amounts of the late nodulin, n-uricase (Bergmann et al., 1983; Van den Bosch & Newcomb, 1986). Uricase catalyzes one of the final steps in the assimilation of the ammonia produced in nitrogen fixation, leading to the production of the ureides, allantoin and allantoic acid. These compounds are the main form in which nitrogen is transported to other parts of the plant in determinate nodules (Schubert, 1986).

Around the central tissue, a peripheral tissue develops. The peripheral tissue becomes divided into an inner and an outer part by an endodermal layer, the nodule endodermis (Fig. 2). No Casparian strips were found in the nodule endodermis of soybean by Frazer (1942). The vast majority of nodule endodermis cells develop into

sclereids in soybean (Bergersen, 1982). In the inner part of the peripheral tissue, dichotomously branching vascular bundles develop that meet at the base in the vascular bundle connecting the nodule to the root vascular system. It was originally thought that these vascular bundles form a closed system around the whole circumference of the nodule. However, recent work by Walsh et al. (1989) shows that these vascular bundles end blindly close to each other at the nodule apex. In the nodule vascular bundles, the phloem completely surrounds the xylem , i.e. the bundles show an amphicribral organization. There is a pericycle of several layers of cytoplasmic-rich cells, which do not exhibit the cell wall characteristics of transfer cells. Each vascular bundle is surrounded by a bundle endodermis with Casparian strips (Walsh et al., 1989) (Fig. 2).

The peripheral tissue becomes separated from the central tissue by a boundary layer of uninfected cells (Gresshoff & Delves, 1986; see Fig. 2). Also in the uninfected cells of the boundary layer, peroxisomes containing large amounts of n-uricase have been shown to be present (Newcomb et al., 1989). Later on, the outer part of the peripheral tissue gives rise to lenticels, which are positioned in line with the vascular bundles, giving the outside of the nodule a striated appearance (Corby, 1981).

Meristematic activity persists for the longest time in the apical periphery of the central tissue, but finally it comes to an end; thus, the primordium differentiates completely into a nitrogen-fixing nodule: a determinate growth pattern.

Several deviations from the above-described sequence of events in nodule development are known. These will be briefly reviewed in the next section.

Alternative modes of nodule formation and/or infection in legumes

In peanut (*Arachis hypogea)* (Chandler, 1978) and *Stylosanthes* sp. (Chandler et al., 1982) an alternative mode of infection has been described. Here the bradyrhizobia penetrate the main root intercellularly at the junction of epidermal cells and the large, septate root hairs that only occur at the sites where lateral roots have emerged. Further penetration of the root cortex also takes place intercellularly. Cell divisions are induced in the cortex of the lateral root, near to the attachment point to the main root, and these newly divided cells are infected from irregular wall ingrowths containing the rhizobia. After the release of the rhizobia, the infected cells divide repeatedly with each daughter cell receiving a part of the number of bacteroids. In this way a central nodular tissue is formed that does not contain uninfected cells. Sometimes stretches of non-infected cells are discerned, but these are probably partitions between separate infection zones

(Chandler, 1978). In their growth pattern the nodules resemble the determinate type. Although they are formed in the cortex of the lateral root, their vascular tissue becomes connected to the vascular system of the main root. An additional deviation from the soybean nodule described above is the absence of lenticels in the mature nodules (Corby, 1981).

Like in *Arachis*, the nodules of *Lupinus* spec. lack uninfected cells. Most of the central tissue apparently arises by repeated divisions of a few primarily infected cells, with the multiplying bacteria being distributed over the daughter cells (Dart, 1977). Infection of cells from intracellularly located infection threads has also been described for nodules of *Lupinus*, i.e. for *L. angustifolius*, by Robertson et al. (1978). In addition, *Lupinus* nodules have a peculiar growth pattern as persistent division activity occurs basi-laterally in the nodule. As a consequence, the nodules adopt a collar-shape around the root during their development. In some cases this peculiar shape might, however, also be due to the merging of several nodules lying close to each other (Dart, 1977).

In a survey of "primitive" legume trees, De Faria et al. (1987) described that all members of the subfamily Caesalpinioideae studied, and some of the Papilionoideae, had indeterminate nodules in which bacteria were apparently not released from the infection threads in the central tissue. Infection of the cells appeared to occur by intercellular spread rather than by infection threads. This phenomenon of so-called persistent infection threads is reminiscent of the situation in the *Bradyrhizobium*-induced nodules on the non-legume *Parasponia* (Smith et al., 1986) (see further below).

In *Sesbania rostrata*, root nodule formation does not only occur on the subterranean root, but also on the stem. Here so-called mamillae, which are in fact adventitious roots, are the targets for infection by *Azorhizobium sesbaniae* (Duhoux, 1984). Infection at first proceeds by the dissolution and subsequent penetration of epidermal and outer cortical cells of the adventitious root near its site of emergence from the stem. Infection pockets are formed, surrounded by radial rows of tangentially divided host cells. Several nodule primordia are formed in inner layers of the cortex of the adventitious root. Later on, these nodule primordia merge and form a single nodule between the already differentiated part of the stele at the base of the adventitious root and the root's apical meristem. From the primary infection pockets the bacteria penetrate intercellularly further into the cortex and subsequently into the nodule primordia (Tsien et al., 1983). Next, infection threads grow into cells of the nodule primordia and bacteria are released from these into the host cells. Around the infected tissue vascular strands develop that merge at the base with the vascular system of the adventitious root. This vascular system of the root in turn is connected to the ring of vascular tissue of the stem. A trace of the adventitious root apical meristem remains discernible at the distal end of the

mature nodule. The nodule itself shows a determinate growth pattern (Duhoux, 1984).

In another *Sesbania* species, *S. grandiflora*, nodule rootlets have been reported. These appear to arise from within the bundle endodermis of nodule vascular traces (Harris et al., 1949). On the nodules of other species, e.g. in the genus *Trifolium*, nodule rootlets can be induced by high temperature treatments. These also appear to be derived from the nodule vascular bundles (Nutman, 1956; Day & Dart, 1971).

On the whole, these deviations appear to be only minor variations of the afore-described modes of nodule development. Intercellular infection is often interpreted as intercellular infection thread formation (e.g. Duhoux, 1984), since the bacteria are often encased in a structure strongly resembling an infection thread as the bacteria are embedded in one or several rows in a matrix surrounded by a plant cell wall. As far as we know, it is not exactly described how this infection thread-like morphology is generated. Since the penetrating bacteria are not immediately surrounded by a plant membrane, the infection thread-like morphology will probably not arise by a process of tip growth as described for intracellular infection thread formation (see section "indeterminate nodules" and Bakhuizen et al., 1988). A more precise study of the mechanisms that cause the infection thread-like morphology and the role of the plant cell membrane in these processes is clearly required.

Even the "stem" nodules of *Sesbania rostrata* are actually formed in the cortex of a root, if an adventitious one, and the resulting nodule can be clearly recognized as belonging to the determinate type.

The development of rootlets on nodules, such as in *Sesbania grandiflora* and in *Trifolium* spec., is more reminiscent of the development of adventitious roots in an existing nodule than a fundamental change in the developmental program of a whole nodule, for example by a change in the pattern of differentiation of cells deposited by the persistent meristem. In nodules induced by the actinomycete *Frankia* on, e.g. *Alnus* spec., nodule rootlets can actually be formed by the nodule meristem (Becking, 1977) (see further below).

From the descriptions of nodule formation on the roots of Leguminosae, it will be apparent that the nodule can be considered as a true plant organ that shows a consistent anatomical organization and a well integrated physiological machinery specialized for symbiotic nitrogen fixation. The latter is exemplified by the tight regulation of the oxygen supply towards the bacteroids, in which the infected cells of the central tissue and the inner part of the nodule peripheral tissue cooperatively participate (Witty et al., 1986), and by the distribution of the nitrogen assimilation pathway over the infected and uninfected cells in the determinate nodule (cf. Dilworth & Glenn, 1984; Schubert, 1986).

In its anatomical organization, the leguminous root nodule system appears to

occupy a rather unique position among plant/microbe interactions. This can be illustrated by a comparison with other root-based nitrogen-fixing systems. In such systems, either no specialized root outgrowth is formed, as is the case for the intercellularly located *Azospirillum* spec. (Patriquin et al., 1983), or a structure is formed with the appearance of a modified lateral root, as is apparent in the nodules induced by the actinomycete *Frankia* spec. on species of several angiosperm families (Becking, 1977), and in the nodules induced by the cyanobacterium *Nostoc* spec. on cycads (Pate et al. 1988). Regarding these nodules as lateral roots can be justified by their root-pericyclic origin and their centrally located vascular tissue. Nevertheless, deviations from normal lateral root development occur: for instance, in *Comptonia peregrina* root cortical cell derivatives contribute to the formation of the *Frankia*-induced nodule, whereas the root cortex does not contribute to normal lateral root development (Callaham & Torrey, 1977). The *Frankia* symbiont is harboured in infected cells located in the central layers of the cortex (Becking, 1977). The *Nostoc* symbiont is located intercellularly in a specialized cell layer in the middle of the cortex (Pate et al., 1988). Nevertheless, examples of striking similarities between leguminous nodules and actinomycete-induced nodules can be found in the infection process, such as the initiation of infection threads in curled root hairs in many Leguminosae as well as in many host species of *Frankia* (Callaham et al., 1979), or in the physiology, like the regulation of the oxygen supply by hemoglobin in the infected cells and/or restrictions in the amount of intercellular space around the infected tissue (Tjepkema et al., 1988).

In contrast, it is hardly possible to consider the legume nodule as a modified lateral root. Its mode of origin (in the cortex instead of the pericycle), the central location of its infected tissue, and the peculiar organization of its peripheral tissue with the vascular tissue distributed over several bundles, each surrounded by a bundle endodermis, and the additional nodule endodermis dividing the peripheral tissue into two morphologically different parts, all argue against such a proposition.

This becomes the more striking, as one realizes that a same strain of *Bradyrhizobium* (e.g. NGR234) is able to elicit not only a leguminous type of root nodule on, for instance, the legume siratro (*Macroptilium atropurpureum*), but also a lateral root-like type of root nodule on the non-legume *Parasponia* (Ulmaceae) (Price et al., 1984; see further: Lancelle & Torrey, 1984a, b, and Scott & Bender, 1990). Since the *nod* genes of the *Bradyrhizobium* are involved in eliciting a nodule in *Parasponia* as well as in compatible legumes (Scott & Bender, 1990), apparently a comparable set of signals, produced under the direction of the *nod* genes, can lead to different responses, in terms of nodule structure, in legumes and in *Parasponia*. Indeed, within the legume family, the type of nodule is also largely determined by the host plant (Dart, 1977). On

the other hand, for example, a *R. meliloti* mutant with a Tn5 insertion 3 kb downstream from *nod*C elicits on alfalfa apparently bacteria-free nodules resembling lateral roots in that they have a centrally located vascular bundle. However, in contrast to normal lateral roots, this pseudonodule exhibits a nodule endodermis-like layer in its "cortex" (Dudley et al., 1987). It will be of considerable interest to determine the mode of origin of such aberrant nodules. From such studies it may become apparent whether the normal type of legume nodule and the lateral root-like type are in fact minor variations of the same developmental program. On the other hand, it might be the that small differences in a set of bacterial signals can result, with the same plant, in the initiation of two alternative, basically different, developmental pathways, i.e. a legume nodule or a lateral root, respectively.

In summary, the nodulin genes must play a specific and significant role in the development of symbiotically nitrogen-fixing root nodules. The elucidation of the functions of the nodulins and the regulation of the expression of the nodulin genes will contribute to the understanding of the molecular mechanisms resulting in the formation of the root nodule and in meeting the requirements for the functioning of this plant organ. Among the root nodules formed on different leguminous plant species there is rather a variation in anatomy and morphology. A comparative study of nodulin gene expression in different types of nodules might reveal to what extent similarities and differences in the various types of nodules are the result of variation in the basic mechanisms underlying the respective developmental programs. At the same time, such a study of legume nodule formation might offer opportunities to gain insight into the mechanism of plant organ development in general.

REFERENCES

Appelbaum, E., 1990, The *Rhizobium/Bradyrhizobium*-legume symbiosis, *in*: Molecular biology of symbiotic nitrogen fixation, P.M. Gresshoff, ed., CRC Press, Boca Raton, Florida.

Appleby, C.A., 1984, *Annu. Rev. Plant Physiol.*, 35, 443.

Bakhuizen, R., Van Spronsen, P.C., Díaz, C.L., and Kijne, J.W., 1988, The plant cytoskeleton in the *Rhizobium*-legume symbiosis, Ph.D. thesis, Leiden University, The Netherlands.

Bauer, W.D., 1981, *Ann. Rev. Plant Physiol.*, 32: 407.

Becking, J.H., 1977, Dinitrogen-fixing associations in higher plants other than legumes, *in*: A treatise on dinitrogen fixation, vol. III, R.W.F. Hardy and W.S. Silver, eds., Wiley, New York.

Bergersen, F.J., 1982, Root nodules of legumes: structure and functions, Research studies Press Wiley, Chichester.

Bergmann, H., Preddie, E., and Verma, D.P.S., 1983, *EMBO J.*, 2: 2333.

Bhuvaneswari, T.V., and Solheim, B., 1985, *Physiol. Plant.*, 63: 25.

Bhuvaneswari, T.V., Bhagwat, A.A., and Bauer, W.D., 1981, *Plant Physiol.*, 68: 1144.

Bisseling, T., Van den Bos, R.C., Van Kammen, A., Van den Ploeg, M., Van Duijm, P., and Houwers, A., 1977, *J. Gen. Microbiol.*, 101: 79.

Bond, L., 1948, *Bot. Gaz.*, 109: 411.

Callaham, D., and Torrey, J.G., 1977, *Can J. Bot.*, 55: 2306.

Callaham, D., Newcomb, W., Torrey, J.G., and Peterson, R.L., 1979, *Bot. Gaz. Suppl.*, 140: S1.

Callaham, D.A., and Torrey, J.G., 1981, *Can. J. Bot.*, 59: 1647.

Calvert, H.E., Pence, M.K., Pierce, M., Malik, N.S.A., and Bauer, W.D., 1984, *Can J. Bot.*, 62: 2375.

Chandler, M.R., 1978, *J. Exp. Bot.*, 29: 749.

Chandler, M.R., Date, R.E., and Roughly, R.J., 1982, *J. Exp. Bot.*, 33: 47.

Corby, H.D.L., 1981, The systematic value of leguminous root nodules, *in*: Advances in legume systematics, R.M. Polhill and P.H. Raven, eds., Royal Botanic Gardens, Kew.

Dart, P., 1977, Infection and development of leguminous nodules, *in*: A treatise on dinitrogen fixation, vol. III, R.W.F. Hardy and W.S. Silver, eds., Wiley, New York.

Day, J.M., and Dart, P.J., 1971, *Rep. Rothamsted Exp. Stat.*, 1970: 85.

De Faria, S.M., McInroy, S.G., and Sprent, J.I., 1987, *Can. J. Bot.*, 65: 553.

Diaz, C.L., Hengstmengel, M.E., Smit, G., Lugtenberg, B.J.J., and Kijne, J.W., 1989a, *in*: Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis, C.L. Diaz, Ph.D. thesis, Leiden University, The Netherlands.

Diaz, C.L., Melchers, L.S., Hooykaas, P.J.J., Lugtenberg, B.J.J., and Kijne, J.W., 1989b, *Nature*, 338: 579.

Dilworth, M., and Glenn, A., 1984, *TIBS*, 9: 519.

Dudley, M.E., Jacobs, T.W., and Long, S.R.,1987, *Planta* 171: 289.

Duhoux, E., 1984, *Can J. Bot.*, 62: 982.

Franssen, H.J., Nap, J.-P., Gloudemans, T., Stiekema, W., Van Dam, H., Govers, F., Louwerse, J., Van Kammen, A., and Bisseling, T., 1987, *Proc. Natl. Acad. Sci. USA*, 84: 4495.

Frazer, H.L., 1942, *Proc. Roy. Soc. (Edinburgh), Ser. B*, 61: 328.

Goodchild, D.J., 1977, Int. Rev. Cytol. Suppl., 6: 235.

Gresshoff, P.M., and Delves, A.C., 1986, Plant genetic approaches to symbiotic nodulation and nitrogen fixation in legumes, *in*: A genetic approach to plant biochemistry, A.D. Blonstein and P.J. King, eds., Springer, Heidelberg.

Harris, J.O., Allen, E.K., and Allen, O.N., 1949, *Am. J. Bot.*, 36: 651.

Hellriegel, H., 1886, Welche stickstoffquellen stehen der Pflanze zu Gebote?, *Z. Ver. Rübenzucker-Industrie Deutschen Reichs*, 36: 863.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T., 1989, *Proc. Natl. Acad. Sci. USA* 86: 1244.

Hirsch, A.M., Wilson, K.J., Jones, J.D.G., Bang, M., Walker, V.V., and Ausubel, F.M., 1984, *J. Bacteriol.*, 158: 1133.

Lancelle, S.A., and Torrey, J.G., 1984a, *Protoplasma*, 123: 26.

Lancelle, S.A., and Torrey, J.G., 1984b, *Can. J. Bot.*, 63: 25.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., and Dénarié, J., 1990, *Nature*, 344: 781.

Libbenga, K.R., and Bogers, R.J., 1974, Root-nodule morphogenesis, *in*: The biology of nitrogen fixation, A. Quispel, ed., North-Holland Publishing Company, Amsterdam.

Libbenga, K.R., and Harkes, P.A.A., 1973, *Planta*, 114:17.

Lim, G., and Burton, J.C., 1982, Nodulation status of the Leguminosae, *in*: Nitrogen fixation, Volume 2: *Rhizobium*, W.J. Broughton, ed., Clarendon Press, Oxford.

Long, S.R., (1989), *Cell*, 56: 203.

Nap, J.-P., and Bisseling, T., 1990, Nodulin function and nodulin gene regulation in root nodule development, *in*: Molecular biology of symbiotic nitrogen fixation, P.M. Gresshoff, ed., CRC Press, Boca Raton.

Nap, J.P., Van de Wiel, C., Spaink, H.P., Moerman, M., Van den Heuvel, M., Djordjevic, M.A., Van Lammeren, A.A.M., Van Kammen, A., and Bisseling, T., 1989, *Mol. Plant-Microbe Interac.*, 2: 53.

Newcomb, E.H., Kaneko, Y., and Van den Bosch, K.A., 1989, *Protoplasma*, 150: 150.

Newcomb, W., 1976, *Can. J. Bot.*, 54: 2163.

Newcomb, W., 1981, *Int. Rev. Cytol. Suppl.*, 13: 247.

Newcomb, W., and Peterson, R.L., 1979, *Can. J. Bot.*, 57: 2583.

Newcomb, W., Sippell, D., and Peterson, R.L., 1979, *Can. J. Bot.*, 57: 2603.

Nutman, P.S., 1956, *Biol Rev. Cambridge Phil. Soc.*, 31: 109.

Pate, J.S., 1976, Transport in symbiotic systems fixing nitrogen, *in*: Transport in plants, Encyclopedia of plant physiology, N.S., vol. 2, pt. B, Tissues and organs, U. Lüttge and M.G. Pitman, eds., Springer, Heidelberg.

Pate, J.S., Gunning, B.E.S., and Briarty, L.G., 1969, Planta, 85:11.

Pate, J.S., Lindblad, P., and Atkins, C.A., 1988, *Planta*, 176: 461.

Patriquin, D.G., Döbereiner, J. and Jain, D.K., 1983, *Can. J. Microbiol.*, 29: 900.

Price, G.D., Mohapatra, S.S., and Gesshoff, P.M., 1984, *Bot. Gaz.*, 145: 444.

Robertson, J.G., Lyttleton, P., Bullivant, S., and Grayston, G.F., 1978, *J. Cell Sci.*, 30: 129.

Robertson, J.G., Wells, B., Bisseling, T., Farnden, K.J.F., and Johnston, A.W.B., 1984, *Nature*, 311: 254.

Scheres, B., Van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., Zwartkruis, F., Wolters, A.-M., Gloudemans, T., Van Kammen, A., and Bisseling, T., 1990, *Cell*, 60, 281.

Schubert, K.R., 1986, *Ann. Rev. Plant Physiol.*, 37: 539.

Scott, K.F. and Bender, G.L., 1990, The *Parasponia-Bradyrhizobium* symbiosis, *in*: Molecular biology of symbiotic nitrogen fixation, P.M. Gresshoff, ed., CRC Press, Boca Raton.

Selker, J.M.L., 1988, *Protoplasma*, 147: 178.

Sheehy, J.E., and Bergersen, F.J., 1986, *Ann. Bot.*, 58, 121.

Smit, G., Kijne J.W., and Lugtenberg, B.J.J., 1987, *J. Bacteriol.*, 169: 4294.

Smith, C.A., Skvirsky, R.C., and Hirsch, A.M., 1986, *Can. J. Bot.*, 64: 1474.

Sprent, J.I., 1980, *Plant Cell Env.*, 3: 35.

Tjepkema, J.D., Pathirana, M.S., and Zeng, S., 1988, The gas diffusion pathway and hemoglobin content in actinorhizal nodules, *in*: Nitrogen fixation: hundred years after, H. Bothe, F.J. de Bruijn and W.E. Newton, eds., Gustav Fischer, Stuttgart, NY.

Tsien, H.C., Dreyfus, B.L., and Schmidt, E.L., 1983, *J. Bacteriol.*, 156: 888.

Turgeon, B.G., and Bauer, W.D., 1982, *Can. J. Bot.*, 60: 152.

Vance, C.P., Johnson, L.E.B., Stade, S, and Groat, R.G., 1982, *Can. J. Bot.*, 60: 505.

Van den Bosch, K.A., and Newcomb, E.H., 1986, *Planta*, 167: 425.

Van de Wiel, C., Nap, J.P., Van Lammeren, A.A.M., and Bisseling, T., 1988, *J. Plant Physiol.*, 132: 446.

Van de Wiel, C., Norris, J.H., Bochenek, B., Dickstein, R., Bisseling, T., and Hirsch, A.M., 1990a, *Plant Cell*, 2: 1009.

Van de Wiel, C., Scheres, B., Franssen, H., Van Lierop, M.-J., Van Lammeren, A., Van Kammen, A., and Bisseling, T., 1990b, *EMBO J.*, 9: 1.

Van Kammen, A., 1984, *Plant Mol. Biol. Rep.*, 2: 43.

Verma, D.P.S., Fortin, M.G., Stanley, J., Mauro, V.P., Purohit, S., and Morrison, M., 1986, *Plant Mol. Biol.*, 7: 51.

Walsh, K.B., McCully, M.E., and Canny, M.J., 1989, *Plant Cell Env.*, 12: 395.

Witty, J.F., Minchin, F.R., Skøt, L., and Sheehy, J.E., 1986, *Oxford Surv. Plant Mol. Cell Biol.*, 3: 275.

Wood, S.M., and Newcomb, W., 1989, *Can. J. Bot.*, 67: 3108.

CHAPTER 3

A defence response of the host plant might interfere with nodulin gene expression in *Vicia sativa* root nodules induced by an *Agrobacterium* transconjugant.

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SUMMARY

The *Agrobacterium* transconjugant LBA2712 harbours a complete *R. leguminosarum* sym plasmid in an *Agrobacterium* chromosomal background. On *Vicia sativa* (common vetch) this strain induces nodules in which a number of plant cells is filled with bacteria. Nevertheless the leghemoglobin (Lb) genes were not found to be expressed in these nodules, suggesting that the *Rhizobium* sym plasmid-borne genes are not sufficient for inducing Lb gene expression. Detailed structural analyses on the nodules induced by LBA2712 indicated that the bacteria are subject to degradation as soon as they are released from the infection thread. An immunocytological analysis of developing wild-type pea root nodules using a leghemoglobin antiserum showed that leghemoglobin is only detectable as from the third to fifth cell layer of the late symbiotic zone. Thus, the differentiation into infected and uninfected cells precedes leghemoglobin accumulation. In combination with the structural analysis of the nodules formed by the *Agrobacterium* transconjugant, it is concluded that the *Agrobacterium*-induced nodules do not reach the developmental stage in which the leghemoglobin genes normally are induced, despite the release of the bacteria from the infection thread. As the results presented indicate that apparently a host defence mechanism interferes with nodulin gene expression, it follows that an *Agrobacterium* transconjugant does not allow conclusions with respect to the involvement of sym plasmid-borne genes in the induction of the expression of late nodulin genes.

INTRODUCTION

During nodule formation on the roots of leguminous plants at least twenty nodulin genes are expressed (Legocki & Verma, 1980, Govers et al., 1985, Lang-Unnasch & Ausubel, 1985). We showed for pea (*Pisum sativum*) (Govers et al., 1985), soybean (*Glycine max*) (Gloudemans et al., 1987) and common vetch (*Vicia sativa*) (Moerman et al., 1987), that most nodulin genes are expressed shortly before or concomitantly with the onset of nitrogen fixation. This class of nodulin genes, of which the leghemoglobin (Lb) genes are the best studied so far, will be called late nodulin genes. In all three plant species a number of nodulin genes were expressed about a week before the onset of nitrogen fixation. This second class of nodulin genes is named early nodulin genes.

As an approach to identify the *Rhizobium* genes that are involved in the induction of nodulin genes, we analysed nodules induced by a strain harbouring the *Rhizobium* sym plasmid separated from the *Rhizobium* chromosome. The *Agrobacterium* transconjugant LBA2712 harbours the complete *R. leguminosarum* sym plasmid in an *Agrobacterium* chromosomal background (Hooykaas et al., 1982). On *V. sativa* this strain forms nodules in which some cells are filled with bacteria (Hooykaas et al., 1984, Moerman et al., 1987). Only early nodulin genes were found to be expressed and no expression of late nodulin genes could be detected in these nodules (Moerman et al., in press), suggesting that the *Agrobacterium* transconjugant lacks genetic information for inducing late nodulin gene expression. The apparent contradiction between the lack of late nodulin gene expression and the presence of infected cells prompted us to study the infection process in the nodules induced by the *Agrobacterium* transconjugant LBA2712 at the electron microscopical level. To obtain additional information about the correlation between the infection process and Lb gene expression we determined by an immunocytochemical method in which developmental zone of a wildtype pea nodule Lb was detectable.

RESULTS

Histology of nodules induced by the Agrobacterium transconjugant LBA2712

In nodules induced on *V. sativa* by an *Agrobacterium* cured from its Ti plasmid and carrying a complete sym plasmid from *R. leguminosarum* instead, designated strain LBA2712 (Hooykaas et al., 1982), two early nodulin genes are expressed, whereas no expression of late nodulin genes could be detected. Full details of these studies are presented in a separate paper (Moerman et al. in press). As an approach to understand this pattern of nodulin gene expression, we studied the histology of the nodules induced by LBA2712 at the electron microscopical level.

The overall morphology of the nodules induced by the *Agrobacterium* transconjugant is shown in transection in Fig. 1A. These nodules have many features in common with a wild-type nodule (Newcomb, 1981). That is, an apical meristem, an early symbiotic zone with penetrating infection threads, and a late symbiotic zone characterized by infected and uninfected cells, peripherically located vascular bundles and endodermis. In the early symbiotic zone bacteria are released from unwalled regions of the infection

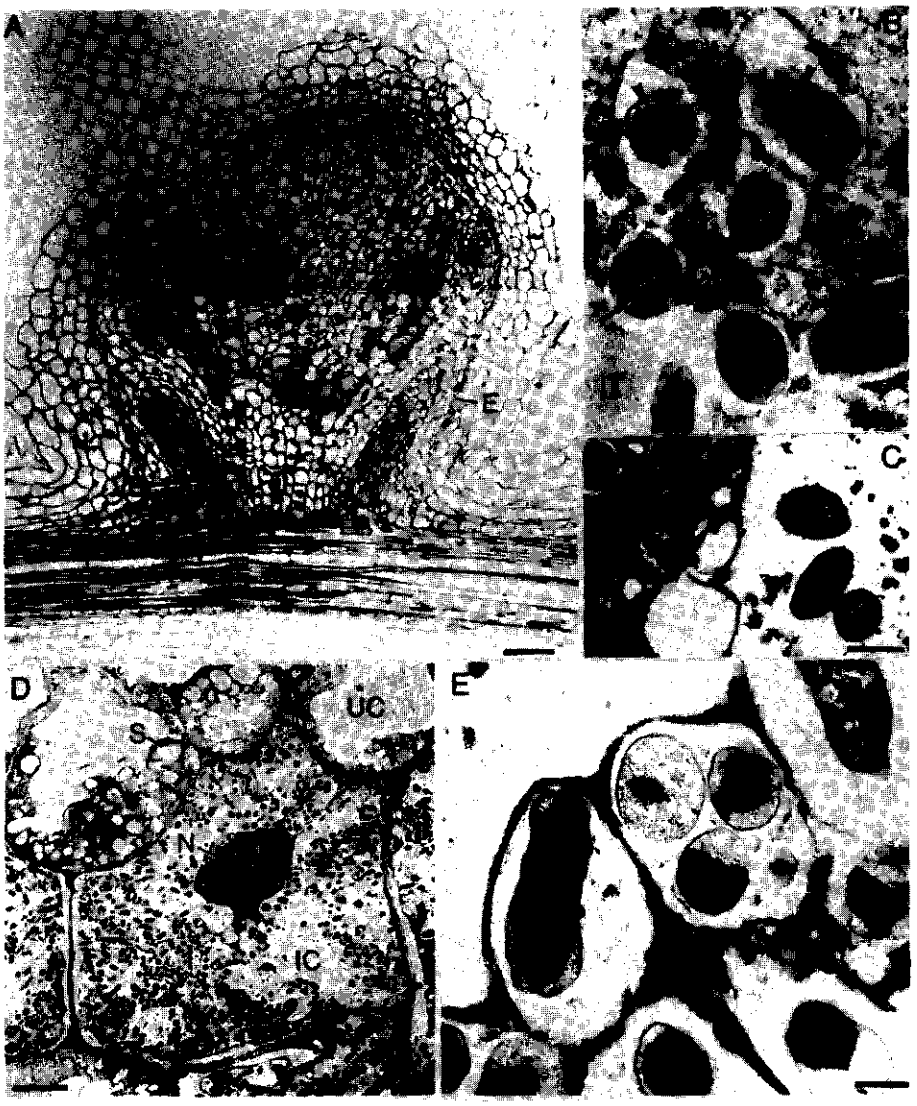


Fig. 1. Light (A) and electron (B - E) micrographs of a 16-day-old *Vicia* nodule induced by the *Agrobacterium* transconjugant LBA2712. A) Low magnification micrograph montage of a section showing the apical meristem (M), vascular bundles (VB) and endodermis (E). Bar = 100 µm. B) Detail of an unwalled part of an infection thread (IT) showing release of bacteria (arrow). The "empty spaces" in the outer region of the cytoplasm of the bacteria are indicated by arrowheads. Bar = 0.3 µm. C) Detail of an infected cell in the late symbiotic zone. Bacteria inside the remnants of an infection thread (left side of the micrograph) are degenerating, whereas the bacteria at the right side of the micrograph are apparently not within vesicles and look like vegetative bacteria. Bar = 1.0 µm. D) Detail of the late symbiotic zone. The contents of the infected cells (IC) are deteriorated. No organelles, except for a very dark staining nucleus (N), can be discerned. In contrast, the uninfected cells (UC) appear normal. They have a prominent central vacuole and large starch granules (S) inside plastids. Bar = 5 µm. E) Detail of an infected cell in the late symbiotic zone. The bacteria inside the vesicles in these cells are degenerating. Bar = 0.3 µm.

thread. By entering the cytoplasm of the host cells, the bacteria become surrounded by a host-derived membrane, the peribacteroid membrane (Fig. 1B).

However, immediately upon release from the infection thread the bacteria are characterized by "empty spaces" in the outer regions of their cytoplasm (Fig. 1B). In addition, aggregated electron dense material can be observed in the cytoplasm of bacteria within vesicles in the late symbiotic zone (Fig. 1E). These ultrastructural changes can be considered as features of bacterial degeneration (Kijne, 1975). In this late symbiotic zone, not only the bacteria that occur inside vesicles degenerate (Fig. 1E), but also bacteria within the remnants of infection threads (Fig. 1C) deteriorate, and the host cells (Fig. 1D) disintegrate. The nucleus and the cytoplasm of the host cell have become electron dense and all other cell organelles have disappeared completely (Fig. 1D). The overall pattern of breakdown bears resemblance to the premature senescence described for ineffective nodules from several plant species (Newcomb et al., 1977, Werner et al., 1980, Vance et al., 1980). However, some bacteria appear much less deteriorated than other bacteria observed in the infected cells of the late symbiotic zone (Fig. 1C). These bacteria are apparently not found within vesicles. They look like vegetative bacteria that possibly feed saprophytically on products of infected cell autolysis (Bergersen, 1974). In contrast to infected cells, the uninfected cells resemble the uninfected cells in wild-type nodules, as judged by cytological criteria. These cells have a prominent central vacuole, and in the cytoplasm plastids containing large starch granules are present. No indications of disintegration can be observed (Fig. 1D).

Localization of leghemoglobin in wild-type pea root nodules

To obtain further information about the correlation between late nodulin gene expression and root nodule organogenesis, we used Lb as a marker protein. By means of immunocytochemistry we have determined in which developmental zone of the pea root nodule Lb is present.

A toluidine blue stained section of a nodule from a 15-day-old pea plant is shown in Fig. 2. At 15 days after sowing and inoculation, the nodule has the distinct zonation of the indeterminate type of nodule (Newcomb, 1981). Details of the zones indicated by capitals in Fig. 2 are shown in the phase contrast micrographs in the right panel of Fig. 3. The details include the apical meristem (Fig. 3A), the early symbiotic zone in which cells become infected by *Rhizobium* (Fig. 3B) and progressively older cells of the late symbiotic zone in which the cells are fully differentiated into infected and uninfected cells. The cytoplasm of the infected cells is tightly packed with the characteristically Y-shaped

bacteroids (Fig. 3C-E).

The results of the indirect immunofluorescence method used to determine in which cells Lb was located are presented in the left panel of Fig. 3. The fluorescence micrographs correspond to the phase constrast micrographs in the right panel. Neither in the apical meristem (Fig. 3A), nor in the early symbiotic zone (Fig. 3B) fluorescence intensities could be discerned above the background of fluorescence in cortex or uninfected root tissue (not shown). In the youngest cell layers of the late symbiotic zone the nodule cells can be considered fully differentiated into infected and uninfected cells (Fig. 3C), because Y-shaped bacteroids fill the cells as in older cells of the same developmental zone (Fig. 3E). Despite this differentiation, fluorescence cannot be detected in these youngest cells of the late symbiotic zone (Fig. 3C). Fluorescence above background becomes detectable in the third to the fifth cell layer of the late symbiotic zone (Fig. 3E) and fluorescense intensities increase more and more towards the older part of the nodule (Fig. 3E). Fluorescence is found in the cytoplasm and nucleus of the infected cells, but is absent in uninfected cells, thus confirming earlier observations by Verma & Bal (1976) and Robertson et al. (1984).

DISCUSSION

Our study of nodulin gene expression in an *Agrobacterium* transconjugant aims at identifying the *Rhizobium* genes exclusively involved in inducing nodulin gene expression (Moerman et al., 1987). In the nodules formed by LBA2712 on *Vicia sativa* roots no Lb genes were found to be expressed (Moerman et al., 1987), despite the release of bacteria from the infection thread and differentiation into infected and uninfected cells. show that different plant species allow nodule development to proceed to different stages of infection, suggesting that plant species may vary in their "tolerance" towards deviant bacteria.

In a previous study we have shown that the same *Agrobacterium* transconjugant LBA2712, harbouring a complete *R. leguminosarum* sym plasmid instead of its Ti plasmid, forms nodules on the roots of pea plants that are totally devoid of intracellular bacteria, although infection thread-like structures are observed (Govers et al., 1986). The reason for this marked difference between pea and *Vicia* nodule development is not clear. In alfalfa, Wong et al. (1983) and Truchet et al. (1984) have shown that an *Agrobacterium* transconjugant carrying the *R. meliloti* sym plasmid gives rise to nodule-

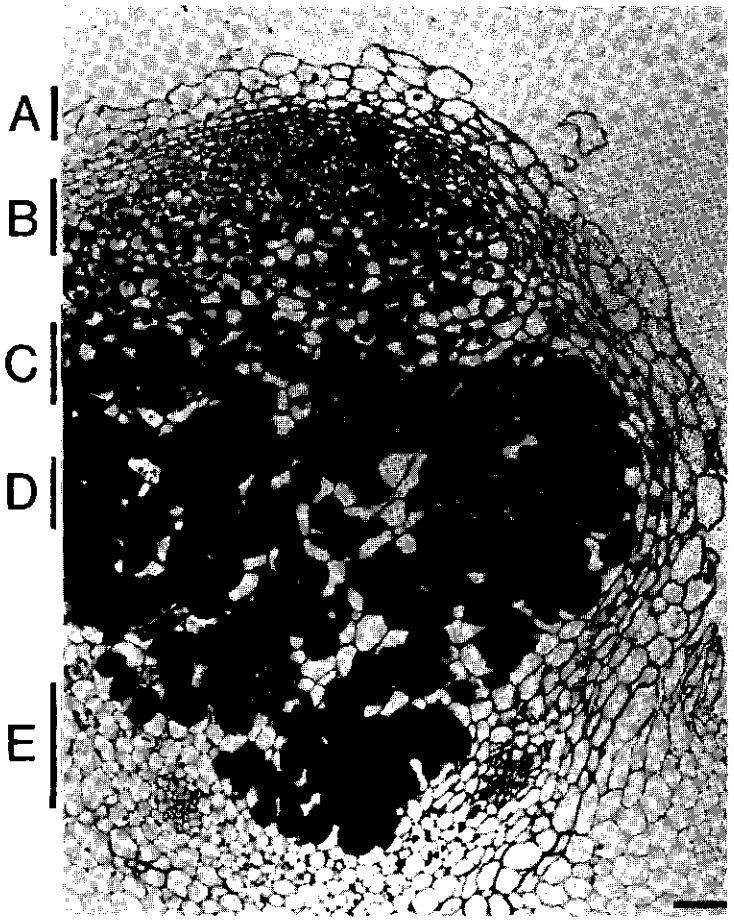
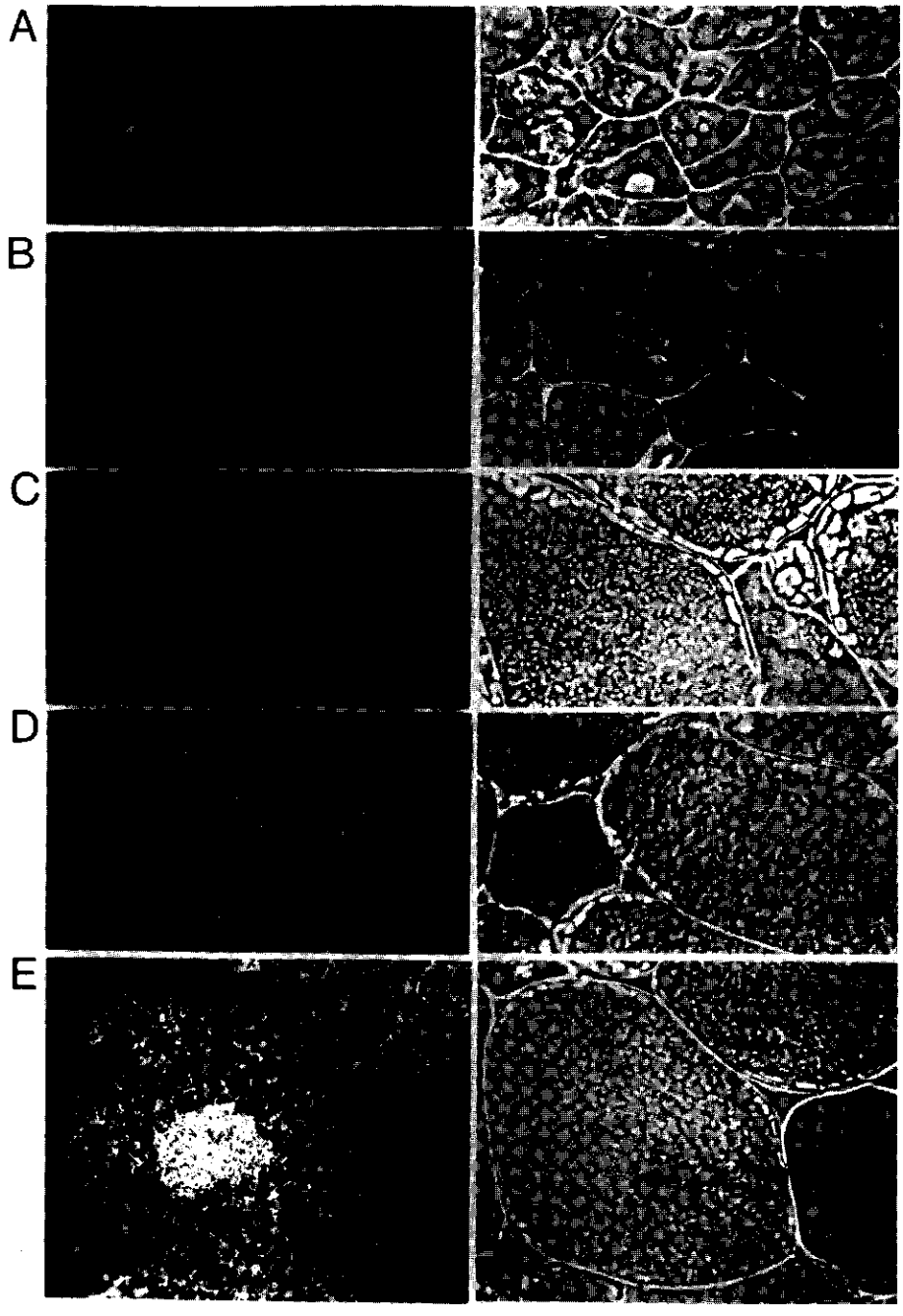


Fig. 2. Toluidine blue stained section of a 15-day-old pea nodule. Capitals indicate the parts of the characteristic developmental zones from the indeterminate nodule that are shown in Fig. 3. **A)** apical meristem. **B)** early symbiotic zone. **C - E)** progressively older parts of the late symbiotic zone. Bar = 100 µm.

Fig. 3. Localization of leghemoglobin in a 15-day-old pea nodule by indirect immunofluorescence. The left panel shows the fluorescence micrographs and the right panel the corresponding phase contrast micrographs of details of the developmental zones indicated by capitals in Fig. 2. In (B) an infection thread is indicated by an arrowhead. IC = infected cell, UC = uninfected cell. Bar = 10 µm.


A
B
C
D
E

like structures that are more disturbed in development than the nodules formed on pea by LBA2712, whereas in clover Hooykaas et al. (1981) have reported infected cells in nodules induced by a transconjugant carrying a *R. trifolii* sym plasmid. These results show that different plant species allow nodule development to proceed to different stages of infection, suggesting that plant species may vary in their "tolerance" towards deviant bacteria.

The absence of late nodulin gene expression in the nodules formed by LBA2712 suggests that *Rhizobium* genes outside the sym plasmid must be responsible for the induction of late nodulin gene expression. However, the strategy of introducing the sym plasmid in an *Agrobacterium* chromosomal background demands that the *Agrobacterium* chromosome itself does not interfere with the expression of nodulin genes. On the one hand the *Agrobacterium* chromosome should not harbour genes involved in the induction of nodulin gene expression, and on the other hand the *Agrobacterium* chromosome should not result in gene products that preclude nodulin gene expression at a particular stage of development. The first condition is met, because no nodulins were detectable in tumours formed on *Vicia* stems by wild-type *A. tumefaciens* (Moerman et al., 1987). But the histological data presented in this paper suggest that the second condition is not fulfilled.

Structural analyses on the electron microscopical level of the nodules formed on the roots of *V. sativa* by LBA2712 indicate that development of these nodules is similar to wild-type nodule development up to and including bacterial release from the infection thread and differentiation into infected and uninfected cells. However, soon after release from the infection thread, further development is severely disturbed and both bacterial and plant cells degenerate rapidly. Apparently, the release of the bacteria from the infection threads is correlated with a recognition step, in which is decided to allow or counteract the infection process.

Robertson et al. (1985) have pointed out that bacteria in the infection thread can be considered as being extracellular, while after release they can be regarded as being intracellular, although still separated from the plant cytoplasm by the peribacteroid membrane. Bradley et al. (1986) demonstrated the association between the bacteroid outer membrane and the peribacteroid membrane in pea root nodules. Bacterial lipopolysaccharides may also be deposited in the peribacteroid membrane (Bradley et al., 1986). It is conceivable that the *Agrobacterium* transconjugant LBA2712 fails in this first interaction between the membranes of plant and bacterium. As a consequence, the host plant recognizes the transconjugant as a pathogen. A pathogen that is subject to host defence. The importance of the *Rhizobium* outer cell surface in bypassing the host defence mechanism is furthermore demonstrated by the disturbed infection process

caused by mutations in *Rhizobium* genes that affect the outer surface of the bacterium. The reaction of the plant in this disturbed infection resembles a hypersensitive response (Rolfe et al., 1986).

To obtain additional information about the absence of Lb gene expression in the nodules formed by LBA2712, the correlation between Lb gene expression and nodule development was investigated by an immunocytochemical method. The immunocytological observations show that in wild-type pea root nodule development the complete morphological differentiation into infected and uninfected cells precedes the accumulation of Lb. Consequently, this differentiation process seems independent from the expression of the Lb genes. Assuming that Lb gene expression in pea and *Vicia* is regulated in a similar way, the *Vicia* Lb genes will be first expressed when the cells are completely filled with bacteria. As this developmental stage is obviously not reached in the nodules formed by the *Agrobacterium* transconjugant LBA2712, there will be no induction of Lb gene expression in these nodules. The absence of Lb gene expression as found previously (Moerman et al., 1987) is therefore in perfect agreement with the results of the immunocytological localization studies presented in this paper.

Since the *Agrobacterium* transconjugant is degraded directly upon release from the infection thread, the nodule does not reach the correct stage of development for the induction of the expression of Lb genes. Apparently, the second condition stated above for the use of an engineered *Agrobacterium* in the identification of *Rhizobium* genes involved in inducing nodulin gene expression is not fulfilled, because a counteraction of the host defence response precludes nodulin gene expression. As a consequence, the *Agrobacterium* transconjugant might have all the genetic information for the induction of the expression of the Lb genes. Therefore, definitive conclusions with respect to *Rhizobium* genes involved in inducing nodulin gene expression are not allowed for nodulin genes, which are not expressed in the nodules formed by such an engineered *Agrobacterium*. Chromosomal genes of *Rhizobium* may be involved in inducing late nodulin gene expression in an indirect manner by establishing a surface of the bacterium that prevents an interaction with the plant defence mechanism.

MATERIALS AND METHODS

Cultivation of plants and bacteria

Pisum sativum L. cv "Rondo" was cultured in gravel trays and inoculated as described by Bisseling et al. (1978). *Vicia sativa* subsp. *nigra* (L.)Ehrh. was germinated, cultured and inoculated as described by Moerman et al. (1987). The bacterial strains *Agrobacterium tumefaciens* LBA2712 and *Rhizobium leguminosarum* PRE (wild-type) were cultured as described (Bhuvaneswari et al., 1980).

Electron microscopy

Nodules from 16-day-old *Vicia* plants were fixed with 2.5% glutaraldehyde in 0.1 M sodiumphosphate buffer pH=7.2 for 1 hr at room temperature (RT). After washing in 0.1 M sodiumphosphate buffer pH=7.2 (1 hr, RT), the nodules were treated with 1% osmiumtetroxide in the same buffer for 1 hr (RT). After washing in 0.1 M sodiumphosphate buffer pH=7.2 (1 hr, RT), the nodules were dehydrated in a graded ethanol series and embedded in LR White resin according to the manufacturer's instructions (The London Resin Company Ltd., London). Sections were cut with glass knives on an LKB Ultrotome V. Semithin sections (0.5 - 2 µm) were stained with 1% toluidine blue 0 in 1% tetraborate in water. Ultrathin sections were stained at RT in an LKB Ultrostainer 2168 with uranyl acetate for 20 minutes and subsequently with lead citrate for 40 seconds. The sections were examined using a Philips EM301 transmission electron microscope operated at 60 kV.

Immunocytochemistry

Nodules from 15-day-old pea plants were fixed with 5% paraformaldehyde in 0.05 M sodiumphosphate buffer pH=7.2 for 1 hr at RT, washed in 0.05 M sodiumphosphate buffer pH=7.2 (1 hr, RT), dehydrated in a graded ethanol series on ice, and embedded in LR White resin as above. Semithin sections (0.5 µm) were cut on an LKB Ultrotome V and attached to poly-L-lysine coated slides. Sections were incubated for 1 hr at RT with an antiserum directed against pea leghemoglobin, diluted 1:500 in buffer A (Buffer A = 0.1 M Tris-HCl pH=7.4, 0.1 M NaCl, 1% Bovine Serum Albumine, 0.1% gelatin, 0.1% Tween-20, 0.1% Triton X-100, 0.05% sodium azide). In these experiments a recently made, monospecific, polyclonal anti-Lb serum was used that had a significantly higher titer than the antiserum used previously (by e.g. Govers et al., 1985). After washing for 1 hr in three changes of buffer A, the sections were incubated (1 hr, RT) with FITC-coupled goat-anti-rabbit (GAR) serum (Nordic, Tilburg),

diluted 1:25 in buffer A. After washing for 1 hr in three changes of buffer A, the sections were mounted with 20% (w/v) Mowiol 4-88 (Hoechst, Frankfurt am Main) in Citifluor (Citifluor Ltd., London) and examined under a Leitz Ortholux microscope with both phase contrast and epifluorescence (by a Xenon 150 lamp) illumination. In order to compare the fluorescence intensities from different parts of a nodule, details were photographed with the same exposure time. The appropriate exposure time was determined on the most intensely fluorescing part of the nodule. To obtain low magnification micrographs, consecutive sections from the same nodule were stained with 1% toluidine blue 0 in 1% tetraborate in water. Negative controls included the incubation of sections without adding the first antiserum, and the incubation of sections from uninfected root tissue.

REFERENCES

Bergersen, F.J., 1974, Formation and function of bacteroids, *in*: The Biology of Nitrogen Fixation, A. Quispel, ed., North Holland Publishing Company, Amsterdam.

Bhuvaneswari, T.V., Turgeon, B.G., and Bauer, W.D., 1980, *Plant Physiol.*, 66: 1027.

Bisseling, T., Van den Bos, R.C., and Van Kammen, A., 1978, Biochim. Biophys. Acta, 539: 1.

Bradley, D.J., Butcher, G.W., Galfre, G., Wood, E.A., and Brewin,N.J., 1986, *J. Cell Sci.*, 85: 47.

Gloudemans, T., De Vries, S., Bussink, H.-J , Malik, N.S.A., Franssen, H.J., Louwerse, J., and Bisseling,T., 1987, *Plant Mol. Biol.*, 8: 395.

Govers, F., Gloudemans, T., Moerman, M., Van Kammen, A., and Bisseling, T., 1985, *EMBO J.*, 4: 861.

Govers, F., Moerman, M., Downie, J.A., Hooykaas, P., Franssen, H.J., Louwerse, J., Van Kammen, A., and Bisseling,T., 1986, *Nature*, 323: 564.

Hooykaas, P.J.J., Van Brussel, A.A.N., Den Dulk-Ras, H., Van Slogteren, G.M.S., and Schilperoort, R.A., 1981, *Nature*, 291: 351.

Hooykaas, P.J.J., Snijdewint, F.G.M., and Schilperoort, R.A., 1982, *Plasmid*, 8: 73.

Hooykaas, P.J.J., Van Brussel, A.A.N., Van Veen, R.J.M., and Wijffelman, C.A., 1984, The expression of Sym plasmids and Ti plasmids in rhizobia and agrobacteria, *in*: Advances in Nitrogen Fixation Research, C. Veeger and W.E. Newton, eds., Nijhoff/Junk, The Hague.

Kijne, J., 1975, *Physiol. Plant Pathol.*, 7: 17.

Lang-Unnasch, N., and Ausubel, F.M., 1985, *Plant Physiol.*, 77: 833.

Legocki, R.P., and Verma, D.P.S., 1980, *Cell*, 20: 153.

Moerman, M., Nap, J.P., Govers, F., Schilperoort, R., Van Kammen, A., and Bisseling, T., 1987, *Plant Mol. Biol.*, 9: 171.

Newcomb, W., 1981, *Int. Rev. Cytol. Suppl.*, 13: 247.

Newcomb, W., Syono, K., and Torrey,J.G., 1977, *Can. J. Bot.*, 55: 1891.

Robertson, J.G., Wells, B., Bisseling, T., Farnden, K.J.F., and Johnston, A.W.B., 1984, *Nature*, 311: 254.

Robertson, J.G., Wells, B., Brewin, N.J., Wood, E., Knight, C.D., and Downie, J.A., 1985, *J. Cell Sci. Suppl.*, 2: 317.

Rolfe, B.G., Redmond, J.W., Batley, M., Chen, H., Djordjevic, S.P., Ridge, R.W., Bassam, B.J., Sargent, C.L., Dazzo, F.B., and Djordjevic, M.A., 1986, Intercellular communication and recognition in the *Rhizobium*-legume symbiosis, *in*: Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions, B. Lugtenberg, ed., Springer-Verlag, Berlin.

Truchet, G., Rosenberg, C., Vasse, J., Julliot, J.-S., Camut, S., and Dénarié, J., 1984, *J. Bacteriol.*, 157: 134.

Vance, C.P., Johnson, L.E.B., and Hardarson, G., 1980, *Physiol. Plant Pathol.*, 17: 167.

Verma, D.P.S., and Bal, A.K., 1976, *Proc. Natl. Acad. Sci. USA*, 73: 3843.

Werner, D., Mörschel, E., Stripf, R., and Winchenbach, B., 1980, *Planta*, 147: 320.

Wong, C.H., Pankhurst, E., Kondorosi, A., and Broughton, W.J., 1983, *J. Cell. Biol.*, 97: 787.

CHAPTER 4

The relationship between nodulin gene expression and the *Rhizobium nod* genes in *Vicia sativa* root nodule development.

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SUMMARY

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 upon 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. An Agrobacterium transconjugant 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.

INTRODUCTION

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 thirty 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 allows 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 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 *Rhizobium* genes in the induction of nodulin gene expression. Investigations using overlapping cosmid clones allowed to draw the conclusion that 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 in nodules induced by an *Agrobacterium* strain carrying the complete sym plasmid, the early but not the late nodulin genes were expressed (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 *Rhizobium* and *Agrobacterium* strains carrying exclusively the *nod* region from either the *R. leguminosarum* or the *R. trifolii* sym plasmid. We present further evidence that the *Rhizobium nod* genes 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 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.

RESULTS

Nod genes are the only sym plasmid DNA required for nodulin gene induction

We have used strain *R. leguminosarum* 248c(pMP104) which contains the cloned 11 kb *nod* region of the sym plasmid pRL1JI of *R. leguminosarum*. A physical and genetic map of the 11 kb nod region carrying the *nod*E, F, D, A, B, C, I, and J genes, inserted into a low-copy-number Inc P vector to yield plasmid pMP104 (Spaink et al, 1987), is given in fig 1. Strain 248c(pMP104) has the ability to form nodules on both vetch and pea. After introduction of pMP104 in the sym plasmid-cured *R. trifolii* strain ANU845, 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 248c(pMP104) is similar to that of the nodules induced by wild-type *R. leguminosarum* (Newcomb, 1981). These nodules have an apical meristem, peripherically located vascular bundles and a central tissue containing infected cells fully packed with bacteroids and uninfected cells. The bacteroids develop into pleiomorphic forms (fig. 2D), but fail to fix nitrogen due to the absence of *nif* and *fix* genes.

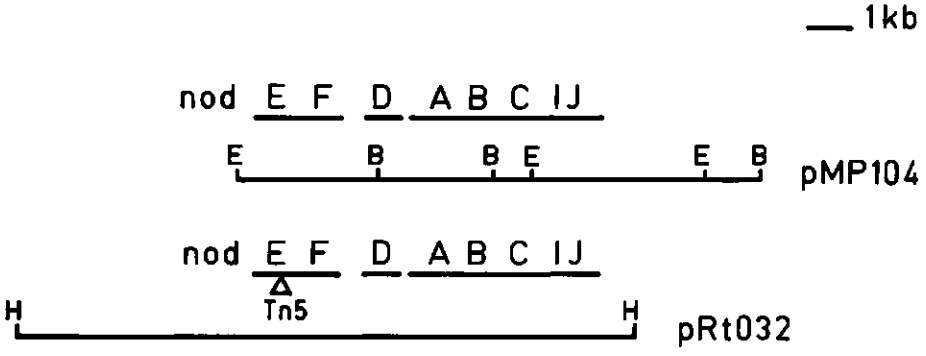

Fig. 1. Simplified physical and genetic maps of the *nod* region derived from the sym plasmid pRL1JI of *R. leguminosarum*, as present in pMP104, and the *nod* region derived from the sym plasmid pANU843 of *R. trifolii*, as present in pRt032. With the Tn5 in *nod*E at position K11, the latter plasmid becomes pRt032(*nod*E K11::Tn5). The maps are aligned to stress their similarities. In addition to the *nod* genes indicated, pRt032 contains the *nod* genes *nod*N, M and L, upstream from *nod*E, and pMP104 contains genes downstream of *nod*IJ. H, Hin dIII; E, Eco R1, B, Bam H1.

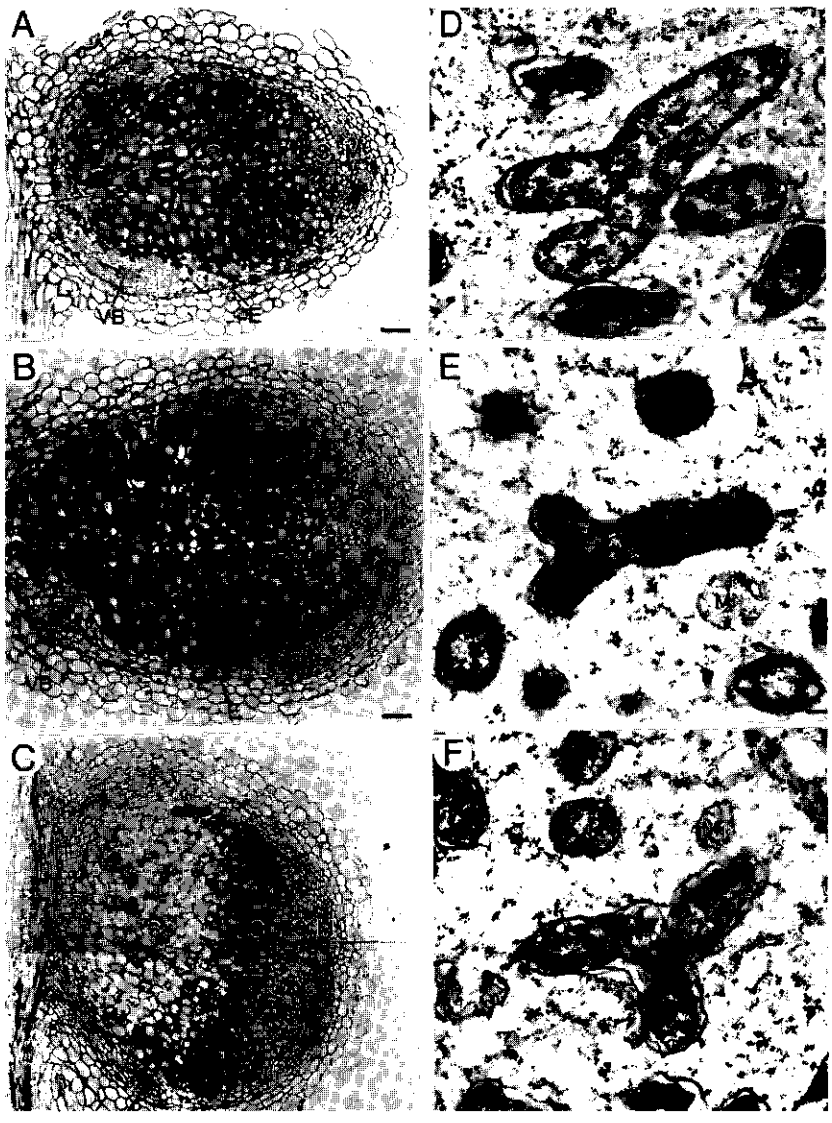

Fig. 2. Light micrograph montages (A - C) of nodules induced on vetch by (A) *R. trifolii* ANU845(pMP104), (B) *R.trifolii* ANU843(nodE K11::Tn5), and (C) *R.trifolii* ANU845(pRt032)(nodE K11::Tn5) respectively, and electron micrographs (D - F) of bacteroids of these strains. A) Three-week-old nodule induced by *R.trifolii* ANU845(pMP104). D) Bacteroid of *R.trifolii* ANU845(pMP104). B) Four-week-old nodule induced by *R.trifolii* ANU843(*nod*E K11::Tn5). E) Bacteroid of *R.trifolii* ANU843(nodE K11::Tn5). C) Three-week-old nodule induced by *R.trifolii* ANU845(pRt032)(*nod*E K11::Tn5). F) Bacteroid of *R.trifolii* ANU843(pRt032)(*nod*E K11::Tn5). 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)(*nod*E K11::Tn5)-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.

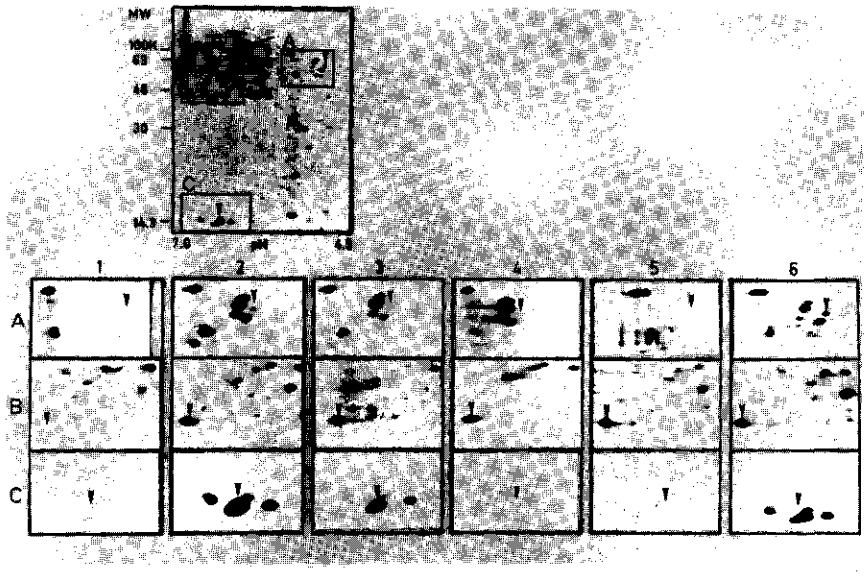

Fig. 3. Expression of three major nodulin genes induced in vetch by *R. trifolii* ANU845(pMP104), *R.trifolii* ANU843(*nod*E K11::Tn5), *R.trifolii* ANU845(pRt032) (*nod*E K11::Tn5), *A. tumefaciens* LBA4301(pMP104) and *R. leguminosarum* PRE, respectively. The upper part of the figure shows the fluorograph 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 panel 2: *R. trifolii* ANU845(pMP104), panel 3: *R.trifolii* ANU843(*nod*E K11::Tn5), panel 4: *R.trifolii* ANU845(pRt032)(*nod*E K11::Tn5), panel 5: *A.tumefaciens* LBA4301(pMP104) and 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) through (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.

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 fig. 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 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 the 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,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. 5D), just as uninfected cells in nodules induced by wild-type *Rhizobium*.

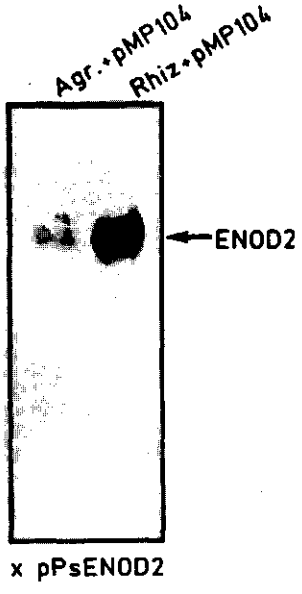

Fig. 4. Expression of the VsENOD2 gene in vetch root nodules. Autoradiograph of a Northern blot containing RNA isolated from four-week-old nodules induced on vetch by *A. tumefaciens* LBA4301(pMP104), indicated as Agr+pMP104, and in three-week-old nodules induced by *R. trifolii* ANU845(pMP104), indicated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

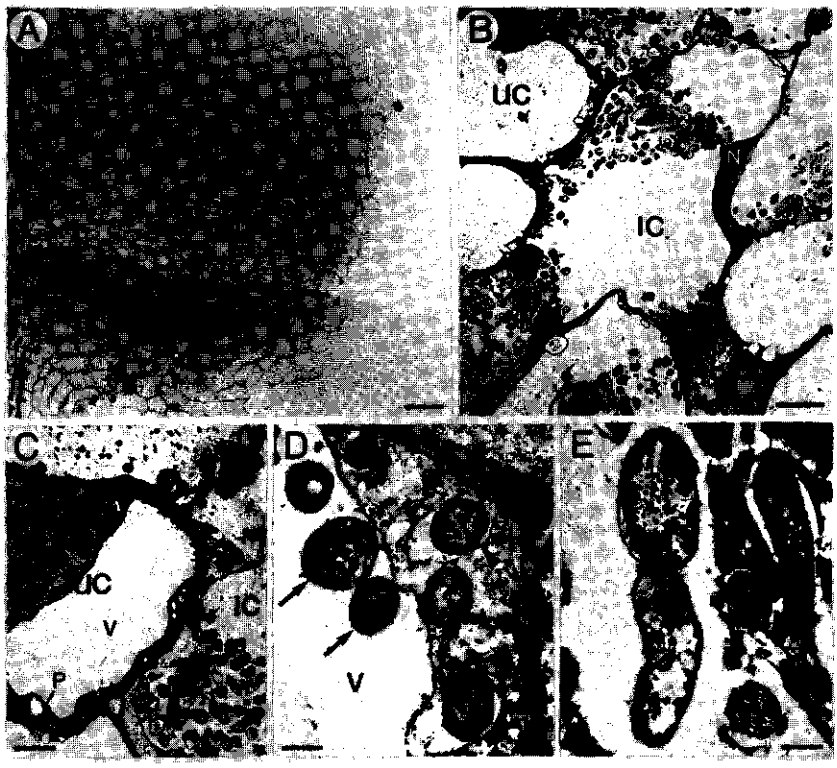

Fig. 5. Light micrograph montage (A) and electron micrographs (B - E) of a four-week-old nodule induced on vetch by the *Agrobacterium* transconjugant LBA4301(pMP104). **A)** Section showing the apical meristem (M), vascular bundles (VB) and endodermis (E). Bar= 100 µm. **B)** Detail of the late symbiotic 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.

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) respectively, 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 *Rhizobium* DNA that in combination with the *Agrobacterium* chromosome 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 on clover roots (Schofield et al, 1984). A physical and genetic map of the 14 kb region in plasmid pRt032, containing the *nod* genes *nod*N,M,L,F,E,D,A,B,C,I, and J, is shown in fig. 1. It has been found that a Tn5 insertion in *nod*E 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(*nod*E K11::Tn5), containing a Tn5 insertion in the *nod*E gene at position K11, in the sym plasmid-cured strain ANU845, the resulting strain ANU845(pRt032)(*nod*E K11::Tn5) 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)(*nod*E K11::Tn5) 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 development, senescence occurs soon afterwards. In a three-week-old nodule, only a few layers of fully packed cells and a large zone of senescence are observed (fig. 2C).

Analysis 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 the expression of other late nodulin genes, including the leghemoglobin genes (fig. 3, panel 4C), is not detectable in the nodules induced by ANU845(pRt032)(*nod*E K11::Tn5). These observations do not exclude that late nodulin genes are still expressed in the few fully infected cells observed in the ANU845(pRt032)(*nod*E K11::Tn5)-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)(*nod*E K11::Tn5)-induced nodules (figs. 6A and 6B). In contrast, high levels of immunogold silver labeling are found in the plant cytoplasm surrounding wild-type R. leguminosarum bacteroids (figs. 6C and 6D). These analyses of individual cells show that the leghemoglobin genes are not expressed in the fully infected cells of the ANU845(pRt032)(*nod*E K11::Tn5)-induced nodules at levels found in infected cells of nitrogen fixing nodules.

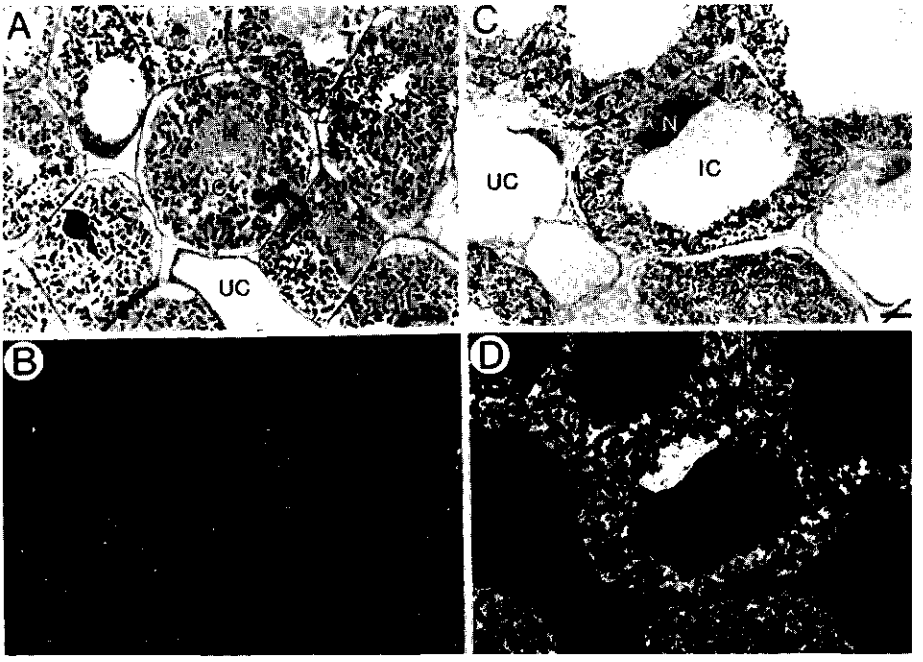

Fig. 6. Localization of leghemoglobin by immunogold silver labeling in a three-week-old nodule induced on vetch by *R.trifolii* ANU845(pRt032)(*nod*E K11::Tn5) (A, B) and in a three-week-old nodule induced on vetch by wild-type *R. leguminosarum* PRE (C, D). A and C are the bright field micrographs of 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)(*nod*E K11::Tn5) (B). IC, infected cell, UC, uninfected cell, N, nucleus. Bar = 10 μm.

Strain ANU845(pRt032)(*nod*E K11::Tn5) 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. Since the only difference between the two strains is the construct carrying the *nod* region, the *nod* region of *Rhizobium 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)(*nod*E K11::Tn5) 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 *R. trifolii* sym plasmid, we used strain ANU843(*nod*E K11::Tn5), which contains a complete *R. trifolii* sym plasmid with a Tn5 in *nod*E at position K11. Per vetch plant, strain ANU843(*nod*E K11::Tn5) formed on the average one nodule, which occurred primarily at lateral root emergences. Wild-type *R. trifolii* strain ANU843 very rarely induced a nodule on vetch, confirming the influence of the Tn5 mutation in *nod*E on host range (Djordjevic et al, 1985).

The histology of the nodules formed on vetch by ANU843(*nod*E K11::Tn5) was similar to the histology of nodules induced by wild-type *Rhizobium* (fig. 2B). The morphology of the ANU843(*nod*E K11::Tn5) 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(*nod*E K11::Tn5) revealed that all nodulin genes are expressed (fig. 3, panels 3A, 3B, and 3C, and fig. 4). This shows that the *R. trifolii* sym plasmid genes are equivalent to *R. leguminosarum* sym plasmid genes in establishing late nodulin gene expression. It indicates furthermore that the *nod*E mutation is not the cause for the failure of strain ANU845(pRt032)(*nod*E K11::Tn5) to induce late nodulin gene expression in vetch nodules.

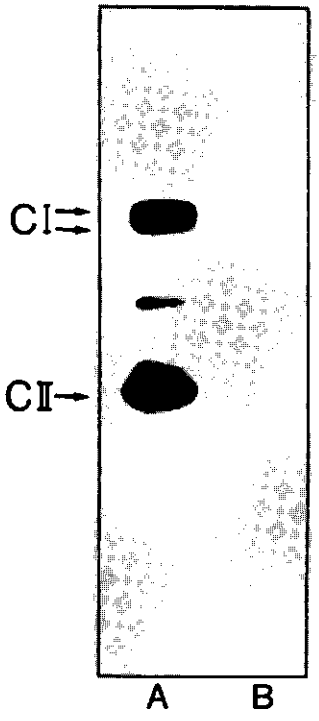

Fig. 7. Autoradiograph of a Western blot containing bacteroid proteins from wild-type *R.trifolii* bacteroids isolated from clover nodules (A) and ANU843(*nod*E K11::Tn5) bacteroids isolated from vetch nodules (B). The blot was incubated with antiserum raised against purified *R. leguminosarum* nitrogenase components CI and CII and ^{125}I-labeled protein A to detect the immune complexes.

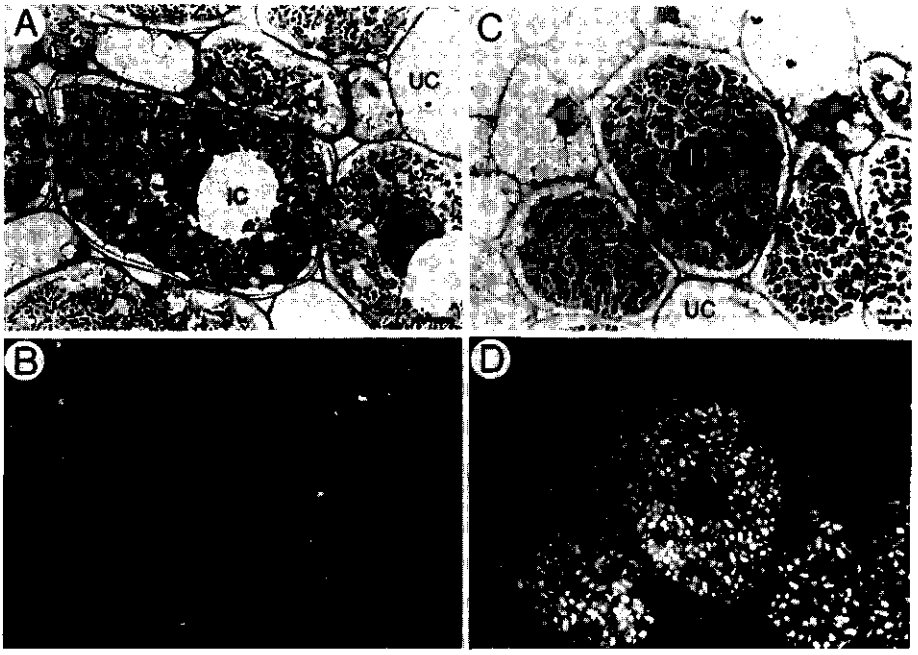

Fig. 8. Localization of nitrogenase by immunogold silver labeling in a four-week-old nodule induced on vetch by *R.trifolii* ANU845(*nod*E K11::Tn5) (A, B) and in a three-week-old nodule induced on white clover by wild-type *R. trifolii* ANU843 (C, D). A and C are the bright field micrographs of the 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 *R. trifolii* nitrogenase. No signal above background is observed in the infected cells of the nodule induced by ANU845(*nod*E K11::Tn5) (B). IC, infected cell, UC, uninfected cell, N, nucleus. Bar = 10 μm.

The nodules induced on vetch by ANU843(*nod*E K11::Tn5) 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 *R. trifolii* bacteroids, from clover nodules, and from ANU843(*nod*E K11::Tn5) bacteroids, from vetch nodules, were incubated with antisera against the components CI and CII of the *R. leguminosarum* nitrogenase complex. 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(*nod*E K11::Tn5) bacteroids, whereas the *R. trifolii* nitrogenase 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(*nod*E K11::Tn5) 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 *R. trifolii* bacteroids in clover nodule sections (figs. 8C and D) and wild-type *R. leguminosarum* bacteroids 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(*nod*E K11::Tn5)-induced nodules.

DISCUSSION

Strains of *Rhizobium* carrying only the *nod* region from a *R. leguminosarum* sym plasmid 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 *R. leguminosarum* sym plasmid is present in an *Agrobacterium* transconjugant, this *nod* region confers upon 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 an *Agrobacterium* transconjugant containing the cloned *nod* region of the *R. meliloti* sym plasmid (Dickstein et al, 1988).

Eight nod genes, *nod*E, 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 Tn5 mutants, it has been found that mutations in the *nod*D, A, B, and C genes abolish nodulation. These four genes are apparently indispensable for nodulation. Mutations in the *nod*E, 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 *nod*D gene encodes a regulatory protein required for the expression of all other *nod* genes (Rossen et al, 1987). The *nod*A, B, and C genes are essential for root hair curling, formation of the infection thread and the induction of cortical cell divisions (Downie et al, 1985; Debellé et al, 1986; Dudley et al, 1987). Therefore, the gene products of the *nod*A, 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 *nod*A, B, and C gene products accomplish these effects directly or by initiating a cascade of

reactions.

Transfer of a fragment carrying exclusively the *nod*D,A,B,C,E, and F region into a sym plasmid-cured *Rhizobium* does not confer upon the recipient strain the ability to induce nodules, although such a strain still causes root hair curling and infection thread formation (Knight et al, 1985, van Brussel et al, 1988). These observations suggest that the *nod*D,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 *nod*E, 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 *nod*E, 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, J, and I 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 *nod*E, F, J, or I gene is complemented by another sym plasmid gene, like *nod*N, M, or L, which are not present in pMP104. This question needs further clarification. The results of the phenotypical effects of mutations do not allow the decision whether the *nod*E, F, J, and I 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 *Agrobacterium* chromosome does not appear to contribute signals involved in 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 with 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 *Rhizobium* and *Agrobacterium* chromosome 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 towards 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 of the *nod* region being sufficient for the induction of early nodulin gene expression seems unequivocal, 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 *Agrobacterium* transconjugant LBA4301(pMP104) carrying the *nod* region, which seems to imply that the Agrobacterium chromosome lacks one or more genes that are

present on the *Rhizobium* chromosome 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 cytological data are suggestive of a plant defense response (see also van de Wiel et al, 1988). The outer membrane of the *Agrobacterium* transconjugant is likely to differ from the *Rhizobium* outer membrane, 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 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 *Rhizobium* strains containing the *nod* regions from *R. trifolii* and *R. leguminosarum*, respectively. Strains ANU845(pMP104) and ANU845(pRt032) (*nod*E K11::Tn5) 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)(*nod*E K11::Tn5) 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, i.e. 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 *nod*A and *nod*C genes are expressed in *R. meliloti* bacteroids, thus at a relatively advanced stage of nodule development. Although formal proof for the involvement of the *Rhizobium nod* genes in the induction of late nodulin gene expression cannot be obtained, these *nod* genes may very well be the only *Rhizobium* genes 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 *R.trifolii nod* region is unclear. There are differences in the extent of *nod* genes between the two constructs pMP104 and pRt1032(*nod*E K11::Tn5), but the differences upstream *nod*F (i.e. *nod*NML) and downstream *nod*IJ 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 *nod*E seems not responsible for the failure to induce the expression of late nodulin genes, because *R. trifolii* strain ANU843(*nod*E K11::Tn5), carrying the *nod*E 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. If a high-copy number plasmid carrying the *nod*A,B,C genes transcribed constitutively from a vector promoter, was introduced in a *R. leguminosarum* with a complete sym plasmid, nodulation ability was abolished completely (Knight et al, 1986). A two fold enhancement of *nod* gene expression was sufficient to result in a strain that induced on vetch only twenty procent of the number of nodules compared to wild-type strains and these nodules were ineffective (Hong et al, 1987). A slight difference in copy number between pMP104 and pRt032(*nod*E K11::Tn5) may therefore explain the observed differences in the nodulin gene expression pattern.

A remarkable finding is the ineffective nature of the nodules induced by *R. trifolii* ANU843(*nod*E K11::Tn5) 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(*nod*E K11::Tn5) 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 *Rhizobium* and *Agrobacterium* strains 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)(*nod*E K11::Tn5) contain only 2-4 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).

Table 1. Pattern of nodulin gene expression in nodules induced by the strains indicated

Bacterial strain	Early		Late	
	ENOD2	Nvs-40	Nvs-65	Lb
PRE	+	+	+	+
248^{c}(pMP104)	+	+	+	+
ANU843(*nod*E K11::Tn5)	+	+	+	+
ANU845(pMP104)	+	+	+	+
ANU845(pRt032)(*nod*E K11::Tn5)	+	+	+	−
LBA4301(pMP104)	+	+	−	−

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 nodules induced by ANU845(pRt032)(*nod*E K11::Tn5) 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)(*nod*E K11::Tn5). 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)(*nod*E K11::Tn5), 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.

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 2. Bacterial crosses were performed as described (Spaink et al, 1987) 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 tetracycline for pMP104 selection and 75 mg/L kanamycine for Tn5 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 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 label, and the signal was silver enhanced using the IntenSE^{TMII} silver enhancement kit (Janssen Pharmaceutica) according to the manufacturer's manual. After treatment, sections were stained with 0.1 % toluidine blue 0 for 1 minute, mounted in Euparal (Chroma) and examined under a Nikon microscope equipped with epipolarization optics (Philips 100 Watt halogene lamp).

Table 2. Bacterial strains and their relevant characteristics

Strain	Relevant characteristics*				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 & Wijffelman 1984
248^c (pMP104)		pR1nod+	nod+fix-	Tc^R	This study
Rhizobium trifolii					
ANU843 (wild type)	pSym+		nod-fix-		Schofield *et al.* 1983
ANU843 (*nod*E K11::Tn5)	pSym+		nod+fix-	Km^R	Djordjevic *et al* 1985
ANU845 (=cured ANU843)			nod-fix-		Schofield *et al.* 1983
ANU845 (pMP104)		pR1nod+	nod+fix-	Tc^R	This study
ANU845(pRt032)(*nodE* K11::Tn5)	pRtnod+		nod+fix-	$Km^R Cb^R$	Djordjevic *et al.* 1985
Agrobacterium tumefaciens					
LBA4301 (= cured Ach5)			nod-fix-		Hooykaas *et al.* 1982
LBA4301 (pMP104)		pR1nod+	nod+fix-	Tc^R	This study

*pSym, sym plasmid pRL1JI (*R.leguminosarum*) or pANU843 (*R.trifolii*); pR1nod, cloned *nod* region from the *R.leguminosarum* sym plasmid pRL1JI; pRtnod, cloned *nod* region from the *R.trifolii* sym plasmid pANU 843; nod, ability to nodulate vetch; fix, *in planta* nitrogen fixation on vetch; Tc, tetracycline; Km, kanamycin; Cb, carbenicillin.

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 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 32P-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 SDS/polyacrylamide gel 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 *R. leguminosarum* nitrogenase has been described before (Bisseling et al, 1980, Van de Wiel et al, 1988).

REFERENCES

Bisseling, T., Moen, L.L., Van den Bos, R.C., and Van Kammen, A., 1980, *J. Gen. Microbiol.*, 118: 377.

Bisseling, T., Been, C., Klugkist, J., Van Kammen, A., and Nadler, K., 1983, *EMBO J.*, 2: 961.

Bradley, D.J., Butcher, G.W., Galfre, G., Wood, E.A., and Brewin,N.J., 1986, *J. Cell Sci.*, 85: 47.

Debellé, F., Sharma, S.B., Rosenberg, C., Vasse, J., Maillet, F., Truchet, G., and Dénarié, J., 1986, Respective roles of common and specific *Rhizobium meliloti nod* genes in the control of lucerne infection, *in*: Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions, B. Lugtenberg, ed., Springer-Verlag, Berlin.

Dickstein, R., Bisseling, T., Reinhold, V.N., and Ausubel, F.M., 1988, *Genes and Development* 2: 677.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R., 1980, *Proc. Natl. Acad. Sci. USA*, 77: 7347.

Ditta, G., Virts, E., Palomares, A., and Kim, C.H., 1987, *J. Bacteriol.*, 169: 3217.

Djordjevic, M.A., P.R. Schofield, and B.G. Rolfe. 1985. Tn5 mutagenesis of *Rhizobium trifolii* host-specific nodulation genes result in mutants with altered host-range ability. Mol. Gen. Genet. 200: 463-471.

Downie, J.A., Knight, C.D., Johnston, A.W.B., and Rossen, L., 1985, *Mol. Gen. Genet.*, 189: 255.

Dudley, M.E., Jacobs, T.W., and Long, S.R.,1987, *Planta* 171: 289.

Fischer, H.M., and Hennecke, H., 1987, *Mol. Gen. Genet.*, 209: 621.

Franssen, H.J., Nap, J.-P., Gloudemans, T., Stiekema, W., Van Dam, H., Govers, F., Louwerse, J., Van Kammen, A., and Bisseling, T., 1987, *Proc. Natl. Acad. Sci. USA*, 84: 4495.

Gloudemans, T., De Vries, S., Bussink, H.-J , Malik, N.S.A., Franssen, H.J., Louwerse, J., and

Bisseling,T., 1987, *Plant Mol. Biol.*, 8: 395.

Govers, F., Gloudemans, T., Moerman, M., Van Kammen, A., and Bisseling, T., 1985, *EMBO J.*, 4: 861.

Govers, F., Moerman, M., Downie, J.A., Hooykaas, P., Franssen, H.J., Louwerse, J., Van Kammen, A., and Bisseling,T., 1986, *Nature*, 323: 564.

Hong, G.-F., Burn, J.L., and Johnston, A.W.B., 1987, Analysis of the mechanism of *nod* gene regulation in *Rhizobium leguminosarum*, *in*: Plant Molecular Biology, D. von Wettstein and N.-H. Chua, eds., Plenum Press, New York.

Hooykaas, P.J.J., Snijdewint, F.G.M., and Schilperoort, R.A., 1982, *Plasmid*, 8: 73.

Josey, D.P., Beynon, J.L., Johnston, A.W.B., and Beringer, J., 1979, *J. Appl. Microbiol.*, 46: 343.

Knight, C.D., Rossen, L., Robertson, J.G., Wells, B., and Downie, J.A., 1986, *J. Bacteriol.*, 166: 552.

Legocki, R.P., and Verma, D.P.S., 1980, *Cell*, 20: 153.

Lie, T.A., Soe-Agnie, I.E., Muller, G.J.L., and Gökdan, D., 1979, Environmental control of symbiotic nitrogen fixation: limitation to and flexibility of the legume-*Rhizobium* system, *in*: Proc. Symp. Soil Microbiol. Plants Nutrition 1976, W.J. Broughton, C.K. John, J.C. Rajaro and B. Lim, eds., University of Malaya, Kuala Lumpur.

Maniatis, T., Fritsch, E.F., and Sambrook, J., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, New York.

Moerman, M., Nap, J.P., Govers, F., Schilperoort, R., Van Kammen, A., and Bisseling, T., 1987, *Plant Mol. Biol.*, 9: 171.

Newcomb, W., 1981, *Int. Rev. Cytol. Suppl.*, 13: 247.

Priem, W.J.E., and Wijffelman, C.A., 1984, *FEMS Microbiol. Lett.*, 35: 247.

Rossen, L., Davis, E.O., and Johnston, A.W.B., 1987, *Trends Biochem. Sci.*, 12: 430.

Schmidt, J., John, M., Wieneke, U., Krössman, H.-D., and Schell, J., 1986, *Proc. Natl. Acad. Sci. USA*, 83: 9581.

Schofield, P.R., Djordjevic, M.A., Rolfe, B.G., Shine, J., and Watson, J.M., 1983, *Mol. Gen. Genet.*, 192: 459.

Schofield, P.R., Ridge, R.W., Rolfe, B.G., Shine, J, and Watson, J.M., 1984, *Plant Mol. Biol.*, 3: 3.

Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Pees, E., and Lugtenberg, B.J.J., 1987, *Plant Mol. Biol.*, 9: 27.

Van Brussel, A.A.N., Pees, E., Spaink, H.P., Tak, T., Wijffelman, C.A., Okker, R.J.H., Truchet, G., and Lugtenberg, B.J.J., 1988, Correlation between *Rhizobium leguminosarum nod* genes and nodulation phenotypes on *Vicia*, *in*: Nitrogen Fixation: Hundred Years After, H. Bothe, F.J. de Bruijn and W.E. Newton, eds., Gustav Fischer Verlag, Stuttgart.

Van Brussel, A.A.N., Tak, T., Wetselaar, A., Pees, E., and Wijffelman, 1982, *Plant Sci. Lett.*, 27: 317.

Van de Wiel, C., Nap, J.P., Van Lammeren, A., and Bisseling, T., 1988, *J. Plant Physiol.*, 132: 446.

Van Kammen, A., 1984, *Plant Mol. Biol. Rep.*, 2: 43.

Verma, D.P.S., and Brisson, N., eds., 1987, Molecular Genetics of Plant-Microbe Interactions, various reports, Nijhoff, Dordrecht.

Yelton, M.M., Mulligan, J.T., and Long, S.R., 1987, *J. Bacteriol.*, 169: 3094.

Zabel, P., Moerman, M., Van Straaten, F., Goldbach, R., and Van Kammen, A., 1982, *J. Virol.*, 41: 1083.

CHAPTER 5

The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules.

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SUMMARY

A pea cDNA clone homologous to the soybean early nodulin clone pGmENOD2 that most likely encodes a cell wall protein was isolated. The derived amino acid sequence of the pea ENOD2 protein shows that it contains the same repeating pentapeptides, ProProHisGluLys and ProProGluTyrGln, as the soybean ENOD2 protein. By *in situ* hybridization the expression of the ENOD2 gene was shown to occur only in the inner cortex of the indeterminate pea nodule. The transcription of the pea ENOD2 gene starts when the inner cortical cells develop from the nodule meristem. In the determinate soybean nodule the ENOD2 gene is expressed in the inner cortex as well as in cells surrounding the vascular bundle that connects the nodule with the root central cylinder. The name "nodule inner cortex" is misleading, as there is no direct homology with the root inner cortex. Therefore, we propose to consider this tissue as nodule parenchyma. A possible role of ENOD2 in a major function of the nodule parenchyma, namely creating an oxygen barrier for the central tissue with the *Rhizobium* containing cells, is discussed.

INTRODUCTION

Root nodules formed on the roots of leguminous plants are unique organs for symbiotic nitrogen fixation by *Rhizobium* bacteria. Root nodules are organized structures which develop from meristems newly formed in the cortex of the root as a result of the interaction with rhizobia. The mature root nodule is made up of a central tissue containing infected and uninfected cells, surrounded by a cortex. The nodule has a common endodermis which divides the cortex into an outer and an inner cortex. The inner cortex is traversed by vascular strands, each surrounded by a bundle endodermis. The strands are connected to the central cylinder of the root (for review see Newcomb, 1981; Bergersen, 1982).

By their morphology two main categories of leguminous nodules can be recognized, determinate and indeterminate nodules (for discussion see Sprent, 1980). Legumes such as *Pisum* (pea), *Trifolium* (clover) and *Medicago* (alfalfa) species develop indeterminate nodules, whereas determinate nodules are formed on the roots of for example *Glycine* (soybean) and *Phaseolus* (bean) species. Indeterminate root nodules

have a persistent meristem at the apex from which cells are continuously added to the cortical and central tissues. Consequently all tissues of these nodules are of graded age from the meristem to the root attachment point. The meristem of a determinate nodule ceases to divide two to three weeks after inoculation and it differentiates completely into nodular tissue (Newcomb, 1981).

The formation of root nodules involves the differential expression of a series of nodule-specific plant genes, the nodulin genes (Van Kammen, 1984). These genes have been divided into early and late nodulin genes. The early nodulin genes are already expressed at early stages of root nodule development, well before the onset of nitrogen fixation. The late nodulin genes are first expressed around the onset of nitrogen fixation, after a complete nodule structure has been formed. Several late nodulins e.g. leghemoglobin, n-uricase and nodulins present in the peribacteroid membrane, have been located in the central tissue of the nodule (Robertson *et al.*, 1984; Van den Bosch and Newcomb, 1986, 1988; Verma *et al.*, 1986). Involvement of nodulins in the function of the cortical tissues in the nodule has not been defined for so far.

Recently we have characterized the product of the early nodulin gene ENOD2 from soybean as a proline-rich protein built up of two repeating pentapeptides (Franssen *et al.*, 1987). In this paper we report the amino acid sequence of a homologous ENOD2 nodulin from pea. Moreover, we demonstrate that the ENOD2 gene is specifically expressed in the inner cortex of the determinate soybean nodule as well as in the inner cortex of the indeterminate pea nodule. In the discussion we suggest that the ENOD2 nodulin has a role in the characteristic morphology of the inner cortex and the function of this tissue as barrier for oxygen diffusion into the root nodules.

RESULTS

Sequence of the pea ENOD2 early nodulin

From a cDNA library prepared against polyA(+) RNA from 21-day-old pea root nodules several clones were selected that specifically hybridized with the insert from the soybean cDNA clone pGmENOD2 (Franssen *et al.*, 1987). The clone with the largest insert was named pPsENOD2. The insert of pPsENOD2, 558 bp in length, was sequenced and a partial amino acid sequence of the pea ENOD2 nodulin deduced from the cDNA sequence is shown in Figure 1. The sequence contains 336 nucleotides of an open

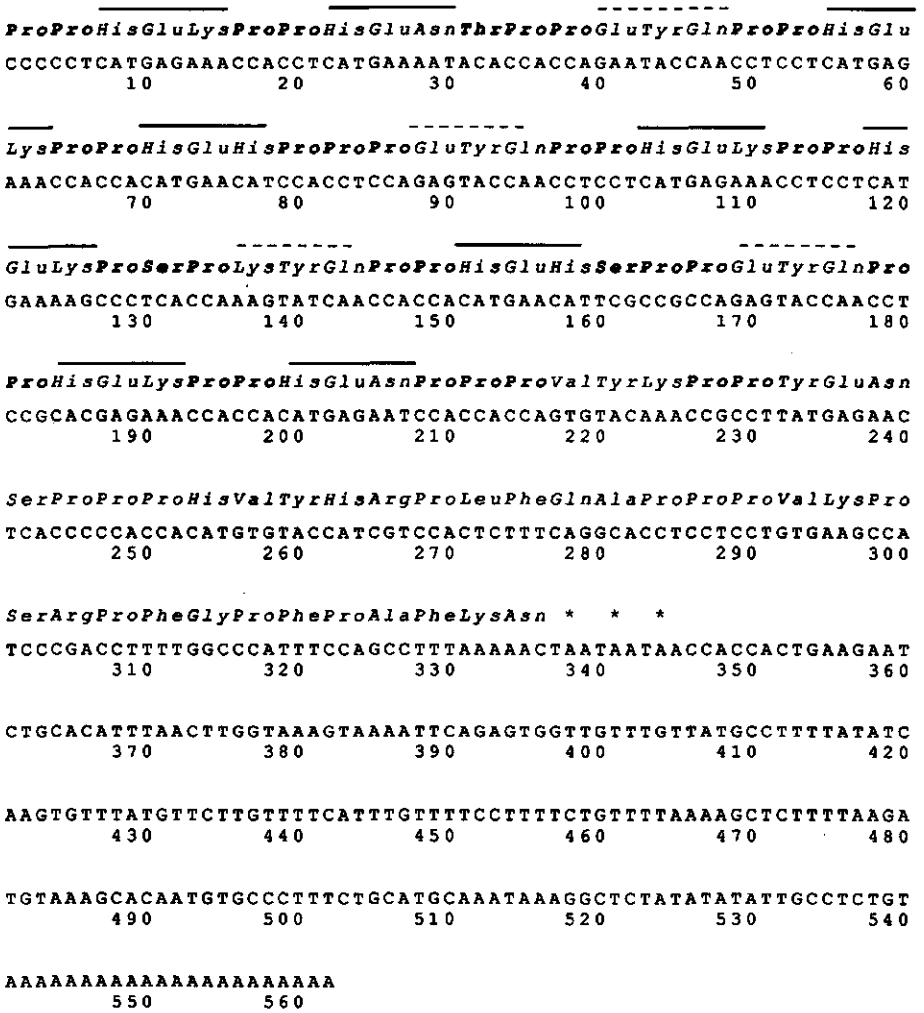


Fig. 1. cDNA and predicted amino acid sequence of the pPsENOD2 insert. Nucleotides 1-562 are determined from the pPsENOD2 insert. The sequence of nucleotides 34-562 is confirmed by analysis of an independently obtained ENOD2 cDNA clone. The amino acid sequence of the only long open reading frame is displayed over the nucleotide sequence. The amino acid triplets characteristic for the different types of pentapeptide repeats described in the text are overlined with unbroken and dashed bars, respectively. The three termination codons ending the reading frame are marked by asterixes. Nucleotides 511-517 encompass the polyadenylation signal.

reading frame (ORF) encoding 112 amino acids of the C-terminal end of the ENOD2 protein. The ORF ends with three successive termination codons and is followed by a 3' non-translated region of about 235 nucleotides in which a potential poly(A) addition signal is present and a short part of a poly(A) tail. The amino acid sequence reveals that the pea ENOD2 protein is very proline-rich and is mainly composed of two repeating pentapeptides, ProProHisGluLys and ProProGluTyrGln, respectively. Two ProProHisGluLys repeats alternate with one ProProGluTyrGln element. Southern blots containing pea genomic DNA digested with EcoRI or SphI and a dilution series of pPsENOD2 were hybridized with the insert of pPsENOD2. A 7.2 kb EcoRI fragment and a 4.6 kb SphI fragment hybridized to pPsENOD2. Moreover, comparison of the levels of hybridization of the pPsENOD2 dilution series and the pea genomic fragments, respectively, indicated that only one ENOD2 gene is present in the pea genome (data not shown).

Localization of the ENOD2 transcript in indeterminate pea nodules

We examined with the *in situ* hybridization technique in which nodular tissue the pea ENOD2 gene is expressed. Longitudinal sections of pea nodules from 20-day-old plants were hybridized with ^{35}S-labeled sense and antisense RNA transcribed from the insert of pPsENOD2. After autoradiography the anti-sense RNA probe appeared to hybridize with RNA present in the sections whereas the sense RNA probe did not (result not shown). The antisense RNA probe only hybridized with RNA present in the inner cortex of the nodule, suggesting that the pea ENOD2 gene is exclusively expressed in this nodular tissue (Figure 2A,B). The ENOD2 gene is expressed throughout the whole inner cortex; from the youngest cells directly adjacent to the meristem up to the oldest cells near the root attachment point. The vascular tissue traversing the nodule inner cortex does not contain detectable levels of the ENOD2 transcript.

The presence of the ENOD2 transcript in the inner cortical cells close to the nodule meristem indicated that expression of the ENOD2 gene is induced at a relatively early stage of development. To test this we also hybridized serial sections of nodule primordia of seven-, eight-, and ten-day-old roots to antisense RNA from pPsENOD2. The pea nodule primordia are initiated in the inner cell layers of the root cortex. At day seven no ENOD2 messenger was detectable in nodule primordia (data not shown). The ENOD2 transcript is first detectable in nodule primordia of an eight-day-old pea plant (Figure 3A,B). At this stage the infection thread, which transports the rhizobia from an infected root hair to the nodule primordium, has already reached the primordium and

branched off into different cells of the central part of the pimordium. Moreover the first differentiation of procambial strands (not shown in Figure 3A, but visible in consecutive sections of the same primordium) and the formation of an apical meristem have taken place (Figure 3A). The ENOD2 messenger is present in a few inner cortical cells at the base of the nodule primordium (Figure 3B). In nodules of ten-day-old pea plants, which is three days before the onset of nitrogen fixation, infected cells filled with bacteroids can be seen at the base of the nodule (Fig. 3C). The ENOD2 transcript is now present throughout the inner cortex as in the 20-day-old nodule (compare Figure 3C,D with Figure 2A,B).

Localization of the ENOD2 transcript in determinate soybean nodules

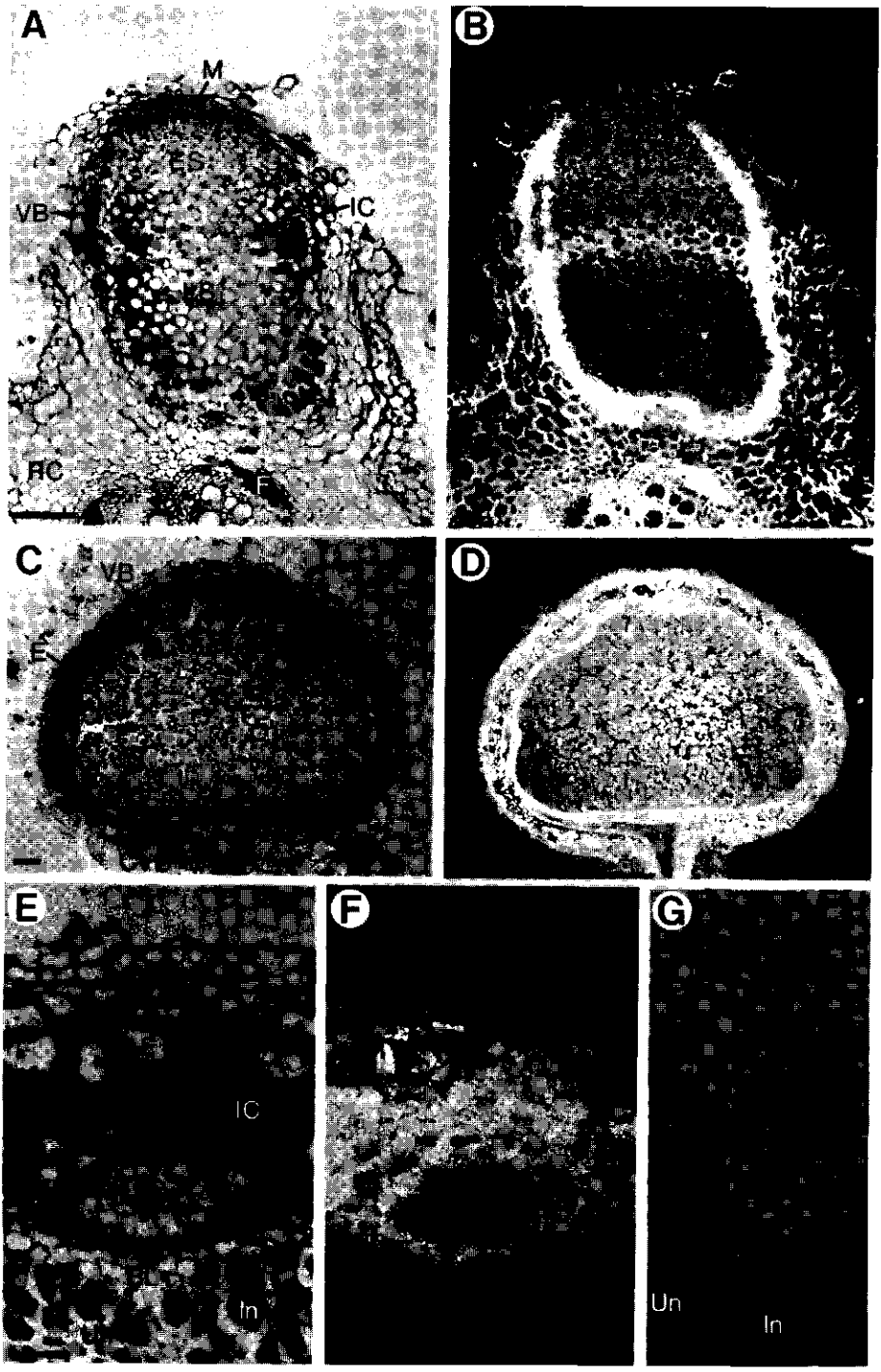
Sections of soybean nodules of 21-day-old plants were hybridized with ^{35}S-labeled antisense RNA made from the insert of pGmENOD2. Figures 2C and D show that as in pea nodules the soybean ENOD2 messenger is located in the nodule inner cortex and in the tissue surrounding the vascular bundle connecting the nodule to the central cylinder of the root. Since there is no persistent meristem in this type of nodule, the inner cortical tissue completely surrounds the central tissue of the mature nodule (compare Figure 2C,D with Figure 2A,B of pea). The distribution of the silver grains in the different nodule tissues is better shown in Figures 2E and F, which represent magnifications of a section through a 21-day-old soybean nodule hybridized with ENOD2 antisense RNA.

In order to obtain a good impression of the various tissues, a similar part of a section of a soybean nodule from a 21-day-old plant embedded in glycolmethacrylate resin, is shown in Figure 2G. Here the tissue morphology is better preserved than in paraffin. The inner cortical cells have fewer and smaller intercelullar spaces than the outer cortical cells. The endodermis that separates the inner and outer cortex mainly consists of large sclerenchymatic cells at this stage (Figure 2G). Figure 2E and F show that the vast majority of ENOD2 transcript is found in the inner cortex, but low levels of this messenger are also present in the endodermis and the outer cortical cell layer directly adjacent to it. The boundary cell layers of uninfected cells between the cortex and the central tissue, like the central tissue itself, appear to contain no ENOD2 transcript (Figures 2E and F).

Also in soybean we studied the appearance of ENOD2 transcript during nodule development. The earliest stage that we investigated was six days after sowing and inoculation. At this stage small bumps become just visible on the main root indicating the

presence of nodule primordia. The primordia of the determinate nodule type originate in the outer cell layers of the root cortex. At six days cell divisions have also been induced in the inner cell layers of the root cortex and the central part of these dividing cells is developing into vascular tissue that connects the root nodule with the central cylinder of the root (Figure 4A and C). At six days the soybean ENOD2 messenger is detectable in the newly formed tissue surrounding the procambial strand between the primordium and the root central cylinder and in inner cortical cells at the proximal and lateral sides of the nodule primordium (Figures 4B and D). In a ten-day-old plant the globular meristem has further developed into a central and a cortical tissue (Figure 4E). The ENOD2 gene is expressed in the nodule inner cortex as well as in the tissue surrounding the vascular strand that connects the nodule with the central cylinder (Figure 4E, F). At this stage the inner cortex at the distal part of the nodule already contains the ENOD2 messenger, albeit still at a lower level than in the proximal part of the nodule (Figure 4E, F). In nodules from 21-day-old plants similar amounts of the ENOD2 transcripts are present in all parts of the nodule inner cortex (cf. Figure 2C, D).

Fig. 2. Localization of ENOD2 transcripts by *in situ* hybridization in pea (A,B) and soybean (C-G) nodules. A) Bright field micrograph of a longitudinal section through a nodule from a 20-day-old pea plant. In the nodule from the top to the base, the apical meristem (M), and early (ES) and late (LS) symbiotic growth stages of the central tissue can be discerned. Over the nodule inner cortex (IC) an autoradiographic signal of black silver grains is present. No signal can be observed over the nodule outer cortex (OC) nor the vascular bundle (VB). The nodule endodermis can not be easily recognized in this section, since in pea the endodermis does not sclerify like in maturing soybean nodules (Fig. 2(C) and (E)). At the base of the nodule, part of the root is visible in transversal section. Here the cortex (RC), and a group of phloem fibers (F) and a xylem pole (X) of the central cylinder are indicated. Bar represents 200 µm. B) Dark field micrograph of the same section as in (A) showing the autoradiographic signal as white grains. C) Bright field micrograph of a longitudinal section through a nodule from a 21-day-old soybean plant. The central tissue (CT) is completely surrounded by an inner cortex (IC) over which an autoradiographic signal of black silver grains can be observed. This signal continues over the tissue surrounding the vascular bundle (CVB) that connects the nodule to the central cylinder of the root. E, endodermis, other abbreviations as in (A). Bar represents 200 µm. D) Dark field micrograph of the same section as in (C) showing the autoradiographic signal as white grains. E) Bright field micrograph of a detail of a section through the same nodule as in (C). From top to bottom the outer cortex (OC), the sclerified endodermis (E), the inner cortex (IC) with a vascular bundle (VB), the boundary layer (BL), and the infected (In) and uninfected (Un) cells of the central tissue can be discerned. Bar represents 50 µm. F) The same detail as shown in (E), photographed with a combination of bright field and epipolarization illumination. A strong autoradiographic signal of white grains is visible over the inner cortex. A lower signal is present over the endodermis and the adjacent layer of the outer cortex. G) Detail of a glycolmethacrylate section through a 21-day-old soybean nodule showing the same tissues at the same magnification as in (E) and (F). Abbreviations as in (E). The arrows indicate intercellular spaces and the arrowheads calcium oxalate crystals in the outer cortex (OC).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DISCUSSION

In this paper we have presented evidence that the early nodulin gene ENOD2 is specifically expressed during the formation of the tissue in determinate as well as in indeterminate root nodules that has so far been described as the inner cortex. Moreover, the occurrence of homologous ENOD2 genes encoding polypeptides with a conserved structure in different legume species (Franssen *et al.*, 1987; Dickstein *et al.*, 1988) strongly suggests that the ENOD2 protein has a role in the function of this root nodule tissue (Govers *et al.*, 1989).

In earlier studies we have demonstrated that in both soybean and pea the ENOD2 gene is expressed during early stages of nodule morphogenesis (Franssen *et al.*, 1987; Govers *et al.*, 1986). Besides, it has been shown that in soybean and alfalfa this early nodulin gene is expressed in so-called empty nodules that contain neither infection threads nor intracellular bacteria (Franssen *et al.*, 1987; Dickstein *et al.*, 1988). Such empty nodules are elicited on legume roots by certain *Rhizobium* and *Bradyrhizobium* strains and mutants (Finan *et al.*, 1985; Franssen *et al.*, 1987) and by *Agrobacterium* strains carrying the *Rhizobium meliloti nod* genes (Hirsch *et al.*, 1985; Truchet *et al.*, 1985). The expression of the ENOD2 gene in these empty nodules strongly suggested a role for the ENOD2 early nodulin in the formation of the nodule structure and not in the infection process. This conclusion has now been consolidated by our finding that the ENOD2 gene is specifically expressed upon differentiation of the nodule meristem into inner cortical cells.

Root nodules are organs with a histological organization that is markedly different from roots. Nevertheless since the one originates from the other these two organs might share homologous tissues. Thus, the names nodule inner cortex and root inner cortex suggest that these two tissues are closely related. However, by definition, the root cortex is inwardly delimited from the central cylinder by the endodermis. In nodules only what has hitherto been called the outer cortex has a similar position as the root cortex and is also connected with it at the base of the nodule. In contrast, what has been hitherto called the inner cortex has no positional relationship with the root cortex: it is located inside the nodule endodermis and around the vascular strands. In other plant parts, notably the stem, the cortex is also, by definition, always located outside the vascular system and, to our knowledge, never surrounding individual vascular strands. In addition, the morphology of the nodule inner cortical cells distinguishes this tissue from root cortical tissues. The nodule inner cortical cells have fewer and smaller intercellular spaces than most other cortical cells (figure 2G, see also Tjepkema and Yocum, 1974 and Witty *et*

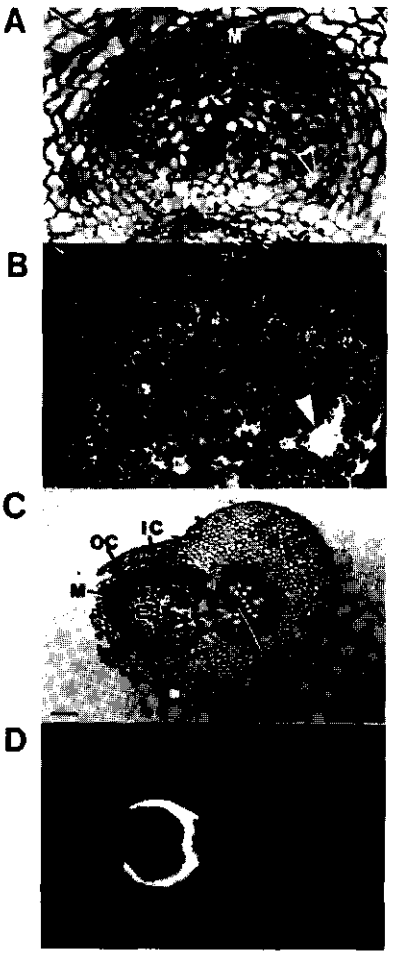


Fig. 3. Localization of ENOD2 transcripts by *in situ* hybridization during nodule development in pea. In the dark field micrographs (B) and (D), which correspond to the bright field micrographs (A) and (C), respectively, the autoradiographic signal is visible as white grains. A) Detail of a transection through an 8-day-old root showing a nodule primordium with an apical meristem (M). The arrow points to the part of the infection thread that has grown through the root cortex to the primordium. A few inner cortical cells containing an autoradiographic signal of black silver grains are indicated by the large arrowhead. CC, central cylinder of the root. Bar represents 50 µm. B) The autoradiographic signal over the inner cortical cells is indicated by the arrowhead. C) Transection through a root with a 10-day-old nodule. The autoradiographic signal of black silver grains is visible over the inner cortex (IC). CC, central cylinder of the root; RC, root cortex; M, apical meristem of the nodule; ES, early symbiotic growth zone of the nodule central tissue; OC, nodule outer cortex. Bar represents 250 µm.

al., 1986). Also at the molecular level the nodule inner cortex is different from the root cortex as we showed that at least one nodulin gene is specifically expressed during the formation of the nodule inner cortex. So both from an anatomical and a molecular point of view the name nodule inner cortex is misleading. Therefore we propose to consider this tissue as nodule parenchyma, while the nodule outer cortex can properly be described as nodule cortex. In determinate nodules the tissue that surrounds the vascular bundle connecting the nodule and the root central cylinder is morphologically very similar to the nodule parenchyma (see below). In addition, the ENOD2 gene is expressed in both tissues. Therefore we propose to consider also the tissue surrounding the connecting vascular bundle as nodule parenchyma.

The determination of the nucleotide sequence of the cloned pea ENOD2 cDNA, and the amino acid sequence derived from it, allow a comparison with the structures of

the soybean and alfalfa ENOD2 proteins that have been determined previously (Franssen *al.*, 1986). Also at the molecular level the nodule inner cortex is different from the root cortex as we showed that at least one nodulin gene is specifically expressed during the formation of the nodule inner cortex. So both from an anatomical and a molecular point of view the name nodule inner cortex is misleading. Therefore we propose to consider this tissue as nodule parenchyma, while the nodule outer cortex can properly be described as nodule cortex. In determinate nodules the tissue that surrounds the vascular

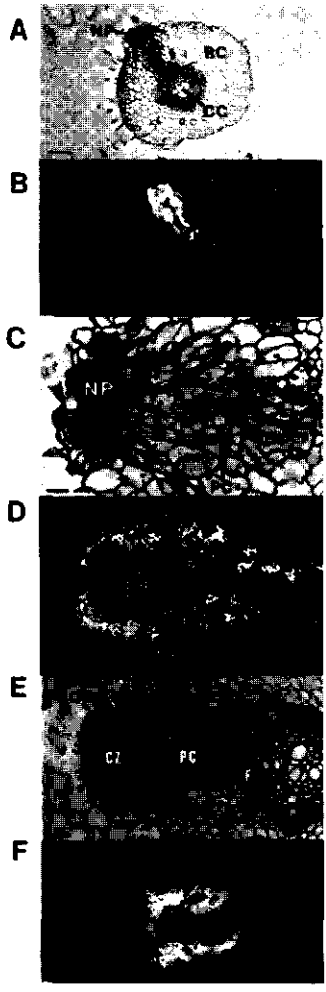


Fig. 4. Localization of ENOD2 transcripts by *in situ* hybridization during nodule development in soybean. In the dark field micrographs (B), (D) and (F), which correspond to the bright field micrographs (A), (C) and (E), respectively, the autoradiographic signal is visible as white grains. A) Transection through a six-day-old root with a nodule primordium (NP); CC, central vascular cylinder; RC, root cortex. Bar represents 250 µm. C) Detail of the root in (A) showing the nodule primordium (NP) and the procambial strand (PC) between the primordium and the central vascular cylinder of the root. The arrowhead indicates an infection thread in the basal part of a root hair cell. Bar represents 50 µm. D) shows the autoradiographic signal over the newly developed tissue surrounding the procambial strand and over the developing inner cortical cells in the lateral and basal parts of the nodule primordium. E) Detail of a transection through a 10-day-old root showing a nodule with the procambial strand (PC) connecting the nodule to the central vascular cylinder at one of the xylem poles. An autoradiographic signal of black silver grains is visible over the inner cortex (IC) and the tissue surrounding the procambial strand. CT, central tissue of the nodule; OC, nodule outer cortex; F, a group of phloem fibers in the central vascular cylinder of the root; RC, root cortex. Bar represents 100 µm.

bundle connecting the nodule and the root central cylinder is morphologically very similar to the nodule parenchyma (see below). In addition, the ENOD2 gene is expressed in both tissues. Therefore we propose to consider also the tissue surrounding the connecting vascular bundle as nodule parenchyma.

The determination of the nucleotide sequence of the cloned pea ENOD2 cDNA, and the amino acid sequence derived from it, allow a comparison with the structures of the soybean and alfalfa ENOD2 proteins that have been determined previously (Franssen *et al.*, 1987; Dickstein *et al.*, 1988, respectively). The pea ENOD2 protein appears to be composed of the same two repeating pentapeptides as the soybean ENOD2 protein or variants of these sequences with one amino acid replacement. However, whereas in the soybean ENOD2 protein the repeating elements occur alternately, in the pea ENOD2 protein two ProProHisGluLys repeats are alternated with the ProProGluTyrGln element. The latter organization also occurs in the alfalfa ENOD2 polypeptide, in which the same pentapeptides are present. This difference in structure between the soybean and pea/alfalfa ENOD2 proteins, respectively, suggests that the amino acid composition of the pentapeptides might be the main requirement for the function of the ENOD2 protein. A specific organization of the repeating elements seems less essential. The different distribution of the two pentapeptides in soybean and pea/alfalfa, respectively, might indicate that independent duplication events involving different basic polypeptide units gave rise to the different ENOD2 genes during the evolution of these legumes. However, more sequence data from a wider variety of legumes will be needed to substantiate this hypothesis.

The amino acid sequence of both the pea and soybean ENOD2 protein strongly resembles the recently described soybean protein 1A10 that occurs in cell walls of the axis tissue of germinating soybean seeds (Averyhart-Fullard *et al.*, 1988). This glycoprotein consists of at least 40 repeating ProProValTyrLys units and about 50% of the prolines are hydroxylated to hydroxyproline. Because of this similarity in structure it is very likely that also ENOD2 is a (hydroxy)proline-rich cell wall protein. Together with the carrot P33 protein, the1A10 and ENOD2 proteins seem to form a new class of cell wall proteins that are composed of pentapeptides containing two prolines. Their low Ser content forms a major difference with another important group of hydroxyproline-rich cell wal proteins, the extensins, which are characterized by (Hyp)4Ser-pentapeptide repeats (Cassab and Varner, 1988). Sequence analysis of two soybean ENOD2 genes revealing that a putative signal peptide is present at the N-terminus of the ENOD2 protein lends further support to the hypothesis that ENOD2 represents a cell wall protein (Franssen *et al.*, 1988).

The nodule parenchyma ("inner cortex") appears to be an important tissue in the

Rhizobium-legume symbiosis. The free oxygen concentration in a nodule shows a sharp decline across the nodule parenchyma to a very low value in the central tissue, which is a necessity to protect the extremely oxygen-sensitive nitrogen-fixing enzyme nitrogenase (Tjepkema and Yocum, 1974; Witty *et al.*, 1986). It was shown that this decline must be due to a high consumption rate of oxygen by the rhizobia in the infected cells of the central tissue combined with a diffusion barrier residing in the nodule parenchyma (Witty *et al.*, 1986). As oxygen diffusion through air is approximately 10^4 times faster than through water, it is very likely that in nodules oxygen diffusion occurs through the intercellular spaces. As mentioned above, the nodule parenchyma contains relatively few and small intercellular spaces. In contrast, in both ("outer") cortex and central tissue relatively wide intercellular spaces occur. By this specific morphology the nodule parenchyma will be able to form the oxygen diffusion barrier (Tjepkema and Yocum, 1974; Witty *et al.*, 1986). Since the differentiation of the cell wall will be a factor in determining tissue morphology, we propose that the putative cell wall protein ENOD2 is contributing to this special morphology of the nodule parenchyma.

In soybean the ENOD2 gene appears also to be expressed in the cells that surround the vascular strand connecting the nodule with the central cylinder of the root. In pea such a long connecting vascular bundle is lacking, since here the nodule originates more closely to the central cylinder. The cells surrounding the connecting vascular strand are morphologically similar to the nodule parenchyma of the nodule, i.e. they have relatively few and small intercellular spaces. This is consistent with the idea that the ENOD2 gene product can contribute to cell morphology. There are, however, no experimental data indicating that this tissue has a function similar to the nodule parenchyma that surrounds the central tissue.

MATERIALS AND METHODS

Growth conditions for plants

Soybean plants (*Glycine max* (L.) Merr. cv. Williams) and pea plants (*Pisum sativum* (L) cv. Rondo) were cultured as described before (Franssen *et al.*, 1987; Bisseling *et al.*, 1978). At the time of sowing the soybean seeds were inoculated with *Bradyrhizobium japonicum* USDA110 and the pea seeds were with *Rhizobium leguminosarum* biovar. *viciae* PRE.

Isolation and sequencing of pPsENOD2

A λgt11 cDNA library against RNA from root nodules of *Pisum sativum* (L.) cv. Sparkle was kindly provided by dr. G. Corruzi (Tigney *et al.*, 1987). Nitrocellulose replicas from plates containing 2000 plaques were made using standard procedures (Maniatis *et al.*, 1982). The plaques were screened with nick translated (Maniatis *et al.*, 1982) insert from the soybean cDNA clone pGmENOD2 (Franssen *et al.*, 1987). Phage DNA purification, insert isolation and cloning in pUC18 was according to standard procedures (Maniatis *et al.*, 1982). Both strands of the pPsENOD2 insert were sequenced using the chemical degradation method (Maxam and Gilbert, 1980).

In situ hybridization

The *in situ* hybridizations were performed essentially as described by Cox & Goldberg (1988). Nodules were fixed with 3% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 at room temperature (RT), dehydrated in graded ethanol and xylene series and embedded in paraplast. Sections, 7 μm thick, were attached to poly-L-lysine-coated slides. Sections were deparaffinized with xylene and rehydrated through a graded ethanol series. They were subsequently pretreated with 1 μg/ml proteinase K in 200 mM TrisHCl pH 7.5, 2 mM $CaCl_2$ at 37°C for 30 min and with 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0 at RT for 10 min, dehydrated in a graded ethanol series and air-dried. Sections were hybridized with (anti)sense RNA probes, which were made by transcribing pT7 clones (a kind gift of dr. S. Tabor) containing the inserts of pPsENOD2 and pGmENOD2.

The antisense RNA probes were radioactively labeled with ^{35}S-UTP (1000-1500 Ci/mmole, NEN). The probes were partially degraded to a length of 150 nucleotides by heating at 65°C in 0.2 M Na_2CO_3/0.2 M $NaHCO_3$. Sections were hybridized with RNA probes in 50% formamide, 0.3 M NaCl, 10 mM TrisHCl pH 7.5, 1 mM EDTA, 10% dextransulfate, 1 x Denhardt's, 70 mM DTT at 42°C for 16 hours. After washing three times in 4 x SSC, 5 mM DTT at RT slides were treated with 20 μg/ml RNase A in 0.5 M NaCl, 10 mM Tris/HCl pH 7.5, 5 mM EDTA at 37°C for 30 min and washed in the same buffer with 5 mM DTT at 37°C for 30 min. The final wash consisted of two times 2 x SSC, 1mM DTT at RT. Slides were dehydrated in graded ethanols (each with 300 mM ammoniumacetate) and 100% ethanol. After air- drying, slides were coated with Kodak NTB2 nuclear emulsion 1:1 diluted with 600 mM ammoniumacetate and exposed for one to three weeks at 4°C. They were developed in Kodak D19 developer for three minutes and fixed in Kodak Fix. Sections were stained with 0.025% toluidine blue 0 for 5 min and mounted with DPX.

For embedding in glycolmethacrylate resin nodules were fixed with 2.5% glutaraldehyde in 0.1 M sodiumphosphate buffer, pH 7.2 for three hours. After dehydration in a graded ethanol series the

nodules were embedded in Technovit resin according to the manufacturer's instructions (Kulzer, Friedrichsdorf, FRG). Section of 4 µm thickness were stained with 1% toluidine 0 blue for 1 min and mounted with Euparal.

Sections were photographed with a Nikon microscope equipped with dark field and epipolarization optics.

REFERENCES

Averyhart-Fullard, V., Datta, K., and Marcus, A., 1988, *Proc. Natl. Acad. Sci.USA*, 85: 1082.

Bergersen, F.J., 1982, Root Nodules of Legumes: Structure and Function. Research Studies Press, New York.

Bisseling, T., Van den Bos, R.C. and Van Kammen, A., 1978, *Biochim.Biophys. Acta*, 539: 1.

Cassab, G.J., and Varner, J.E., 1988, *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 39: 321.

Cox, K.H., and Goldberg, R.B., 1988, Analysis of plant gene expression, *in*: Plant Molecular Biology: A practical approach, C.H. Shaw, ed., IRL Press, Oxford.

Dickstein, R., Bisseling, T., Reinhold, V.N. and Ausubel, M., 1988, *Genes & Development*, 2: 677.

Finan, J.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C., and Signer, E., 1985, *Cell*, 40: 869.

Franssen, H.J., Nap, J.-P., Gloudemans, T., Stiekema, W., Van Dam, H., Govers, F., Louwerse, J., Van Kammen, A., and Bisseling, T., 1987, *Proc. Natl. Acad. Sci. USA*, 84: 4495.

Franssen, H.J., Scheres, B., Van de Wiel, C., and Bisseling, T., 1988, Characterization of soybean (hydroxy)proline-rich early nodulins, *in*: Molecular Genetics of Plant-Microbe Interactions, R. Palacios and D.P.S. Verma, eds., APS Press, St. Paul.

Govers, F., Franssen, H.J., Pieterse, C., Wilmer, J., and Bisseling, T., 1990, Function and regulation of the early nodulin gene ENOD2, *in*: Genetic Engineering of Crop Plants, P. Grierson and G. Lycett, eds., Butterworths Scientific, Guildford.

Govers, F., Moerman, M., Downie, J.A., Hooykaas, P., Franssen, H.J., Louwerse, J., Van Kammen, A., and Bisseling, T., 1986, *Nature*, 323: 564.

Hirsch, A.M., Drake, D., Jacobs, J.W., and Long, S.R., 1985, *J. Bacteriol.*,161: 223.

Maniatis, T., Fritsch, E.F., and Sambrook, J., 1982, Molecular cloning. A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Maxam, A.M., and Gilbert, W., 1980, *Methods Enzymol.*, 65: 499.

Newcomb, W., 1981, *Int. Rev. Cytol. Suppl.*, 13: 247.

Robertson, J.G., Wells, B., Bisseling, T., Farnden, K.J.F., and Johnston, A.W.B., 1984, *Nature*, 311:

254.

Sprent, J.I., 1980, *Plant Cell Env.*, 3: 35.

Tigney, S.V., Walker, E.L. and Coruzzi, G.M., 1987, *EMBO J.*, 6: 1.

Tjepkema, J.D., and Yocum, C.S., 1974, *Planta*, 119: 351.

Truchet, G., Debellé, F., Vasse, J., Terzaghi, B., Garnerone, A.-M., Rosenberg,C., Batut, J., Maillet, F., and Dénarié, J., 1985, *J. Bacteriol.*, 164: 1200.

Van den Bosch, K.A., and Newcomb, E.H., 1986, *Planta*, 167: 425.

Van den Bosch, K.A., and Newcomb, E.H., 1988, *Planta*, 175: 442.

Van Kammen, A., 1984, *Plant Mol. Biol. Rep.*, 2: 43.

Verma, D.P.S., Fortin, M.G., Stanley, J., Mauro, V.P., Purohit, S., and Morisson, N., 1986, *Plant Mol. Biol.*, 7: 51.

Witty, J.F., Minchin, F.R., Skøt, L., and Sheehy, J.E., 1986, *Oxford Surv. Plant Mol. Cell Biol.*, 3: 275.

CHAPTER 6

Nodulin gene expression and ENOD2 localization in effective, nitrogen-fixing and ineffective, bacteria-free nodules of alfalfa (*Medicago sativa*).

Clemens van de Wiel, Joanna Norris, Birgit Bochenek, Rebecca Dickstein,
Ton Bisseling, and Ann Hirsch.

SUMMARY

Alfalfa plants form bacteria-free root nodules in response to a number of agents, including *Rhizobium meliloti exo* mutants, *Agrobacterium tumefaciens* transconjugants carrying cloned *R. meliloti* nodulation genes, and compounds that function as auxin transport inhibitors, e.g. N-(1-naphthyl)phtalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA). These bacteria-free nodules contain transcripts for the early nodulin MsENOD2. In situ hybridization studies demonstrate that ENOD2 transcripts are localized in parenchyma cells at the base and along the periphery of wild-type *R. meliloti*-induced, nitrogen-fixing alfalfa nodules. The ENOD2 gene is also expressed in a comparable tissue-specific manner in nodules elicited by NPA and TIBA. In bacteria-free nodules induced by *R. meliloti exo* mutants and *A. tumefaciens* transconjugants carrying either one or both *R. meliloti* symbiotic plasmids, ENOD2 transcripts are usually restricted in their localization to parenchyma cells at the base of the nodule. On the basis of the spatial pattern of ENOD2 gene expression, we conclude that the developmental pathway of bacteria-free nodules, whether bacterially or chemically induced, is comparable to that of wild-type *R. meliloti*-induced nodules, and furthermore, that the auxin transport inhibitors in their action mimic some factor(s) that trigger nodule development.

INTRODUCTION

Legume root nodule development represents an excellent model system for investigating the question of how differentiated plant cells, in this case root cortical cells, can be induced to change their developmental destiny to initiate a new structure, in this case the nodule. Root nodule development in alfalfa (*Medicago sativa*) begins following specific recognition between the host plant and *Rhizobium meliloti*. Cells of the inner cortical layers of the root divide anticlinally, thus initiating a nodule primordium. This nodule primordium is soon invaded by an infection thread that originated in an infected root hair. Subsequently, rhizobia are released from branches of the infection thread into cells of the central part of the nodule primordium. Shortly thereafter, peripheral cells of the nodule primordium start to differentiate into the peripheral tissues of the nodule, including the vascular strands connecting the nodule to the root vascular system. At the

same time, the persistent nodule meristem, consisting of small, densely cytoplasmic, actively dividing cells, is organized at the apical (distal) end of the nodule primordium. This meristem provides the nodule with new cells that differentiate in the afore-mentioned nodule tissues, thus enabling the nodule to follow an indeterminate growth pattern.

Alfalfa plants form bacteria-free root nodules in response to auxin transport inhibitors (ATI's), such as N-(1-naphtyl)phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) (Hirsch *et al.*, 1989). These nodules show a histological organization resembling that of nodules induced by *Rhizobium meliloti*. In the ATI-induced nodules a peripheral tissue and a central tissue, the latter consisting of cells rich in plastids with prominent starch grains, can be distinguished. A narrow zone of meristematic cells is located at the distal end of the nodule. At the proximal end, vascular tissue connects the nodule to the root stele. The ATI-induced nodules contain transcripts for the nodulins Nms-30 and MsENOD2. However, transcripts for late nodulins, such as leghemoglobin, are not found in the ATI-induced nodules (Hirsch *et al.*, 1989). Thus, in several respects, ATI-induced nodules resemble the bacteria-free ("empty") nodules elicited on alfalfa by certain bacterial strains, such as *R. meliloti* mutants defective in exopolysaccharide synthesis (*exo*) (Finan *et al.*, 1985; Leigh *et al.*, 1987; Dickstein *et al.*, Norris *et al.*, 1988), or *A. tumefaciens* transconjugants carrying cloned *R. meliloti nod* genes (Wong *et al.*, 1983; Hirsch *et al.*, 1984; Truchet *et al.*, 1984; Hirsch.*et al.*, 1985; Dickstein *et al.*, 1988).

Because (early) nodulin genes are expressed in ATI-induced structures as well as in "empty" nodules formed in response to *R. meliloti exo* mutants or *A. tumefaciens* transconjugants carrying *R. meliloti* nodulation sequences, it was concluded that the ATI-induced nodules truly represent nodules comparable to those induced by *R. meliloti*. It was also suggested that the ATI's mimic the activity of the rhizobial *nod* genes, perhaps by perturbing the root's endogenous auxin/cytokinin balance (Hirsch *et al.*, 1989).

Recently, *in situ* hybridization studies have shown that the ENOD2 gene is exclusively expressed in the nodule parenchyma (the inner part of the peripheral tissue, formerly called "inner cortex") of soybean as well as pea (*Pisum sativum*) nodules (Van de Wiel et al., 1990). The nodule parenchyma is located between the central tissue of infected and uninfected cells, and the nodule cortex (the outer part of the peripheral tissue, formerly called "outer cortex"). The nodule parenchyma consists of highly vacuolated cells that are more densely packed, with fewer intercellular space, than the nodule cortex.

In this report we have determined whether in ATI-induced nodules the ENOD2 gene is also expressed in a tissue-specific manner comparable to that in wild-type *R. meliloti*-induced nodules. Such a comparison is of significance, because the growth

pattern of the ATI-induced structures deviates from the "normal" growth pattern of root nodules in several ways. Their meristematic activity is frequently spread out over a relatively large part of the distal end of the nodule instead of being focused to a limited area of the apex as in wild-type nodules. Also, vascular traces are confined to the proximal part of the nodule and do not separate into distinct strands extending distally into the peripheral tissue of the nodule (Hirsch et al., 1989). However, even among bacterial-induced "empty" nodules there is some variation in growth pattern (cf. Wong et al., 1983; Hirsch et al., 1984, 1985; Finan et al., 1985). Therefore, we included "empty" nodules elicited by different bacterial strains in this study. Our goal was to determine the spatial pattern of ENOD2 gene expression in ATI-induced nodules as well as in bacterial-induced "empty" nodules to test the proposition that the ATI-induced nodules are comparable to "true" nodules.

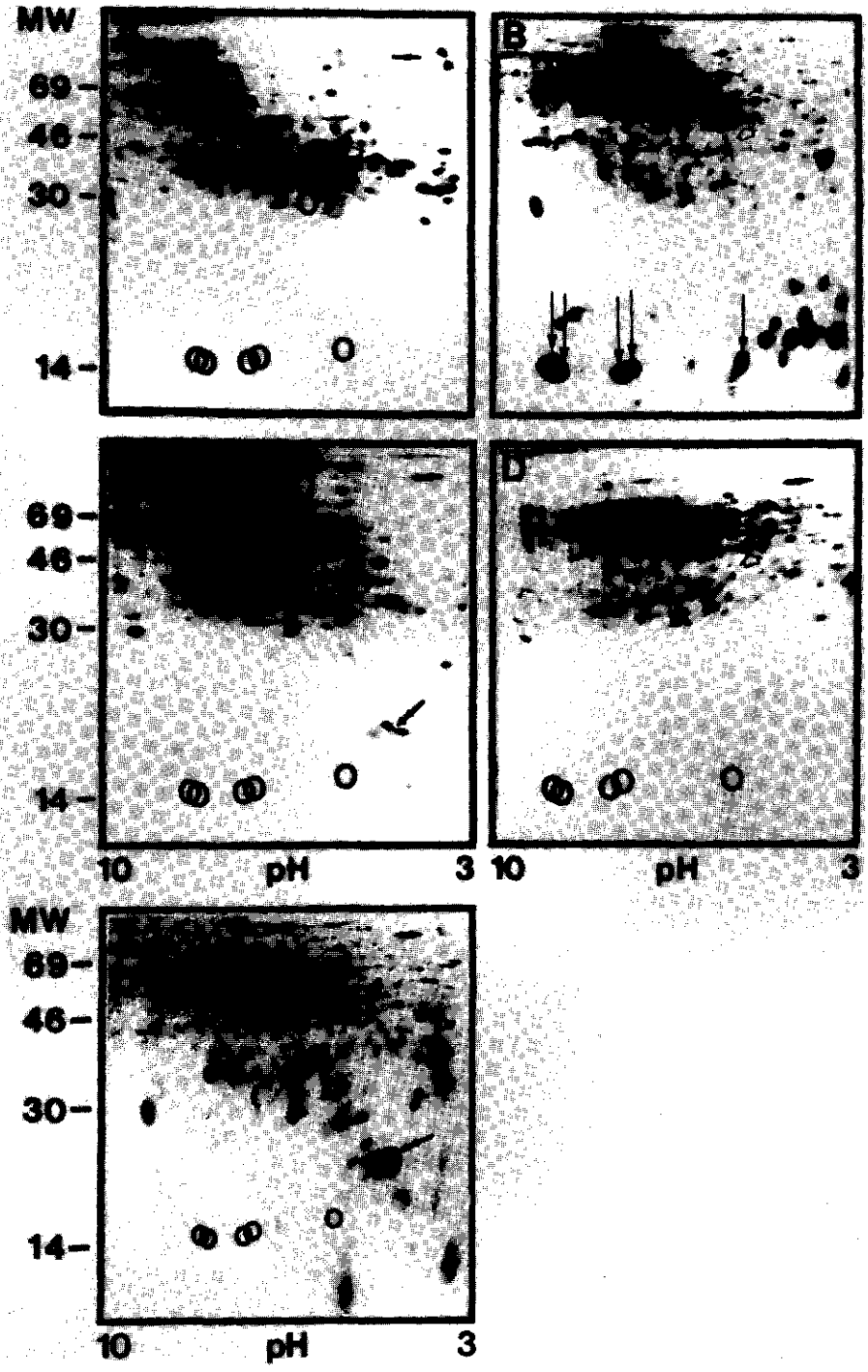
In situ hybridization analysis, utilizing an alfalfa ENOD2 probe, was used to determine where ENOD2 transcript is localized in ATI-induced nodules. We compared this localization with that in nodules formed following infection with wild type *R. meliloti* strains as well as several *R. meliloti exo* mutants and *A. tumefaciens* transconjugants carrying *R. meliloti* megaplasmids. Because some of the bacterial-induced nodules had not been analysed before, we also checked nodulin gene expression on two-dimensional gels of *in vitro* translated nodule RNA and examined RNA transfer blots hybridized to an alfalfa ENOD2 insert.

RESULTS

Nodule development and nodulin gene expression

The present study compares ATI-induced nodules with "empty" nodules formed after inoculation with *R. meliloti exo*A, *exo*B or *exo*F mutants or *A. tumefaciens* transconjugants carrying *R. meliloti* megaplasmids. As the nodules elicited by most of the strains used in the present study have not been described before, they will be compared here with the "empty" nodules already published.

Previous analyses of *in vitro* translated RNA on two-dimensional gels have shown that about 20 different nodulin translation products can be identified in wild-type *R. meliloti*-induced alfalfa nodules (Dickstein et al., 1988; Norris et al., 1988; cf. Figures 1A and B). In nodules formed in response to *R. meliloti exo* mutants or *A. tumefaciens*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transconjugants, only the nodulin translation product Nms30 can be detected (Dickstein et al., 1988; Norris et al., 1988; the latter describe our *exo*B mutant). In addition, a nodulin of variable isoforms, Nms25, that appears to be unique to *exo* mutant-induced nodules, has been described by Leigh et al. (1987). Figures 1C and F illustrate that the "empty" nodules used in the present study conform to this pattern of gene expression. Nms30 as well as Nms25 translation products are detectable in nodules elicited by *exo*A and *exo*F mutants. On the other hand, nodules formed in response to A128 inoculation appear to contain only the Nms30 transcript (Figure 1D).

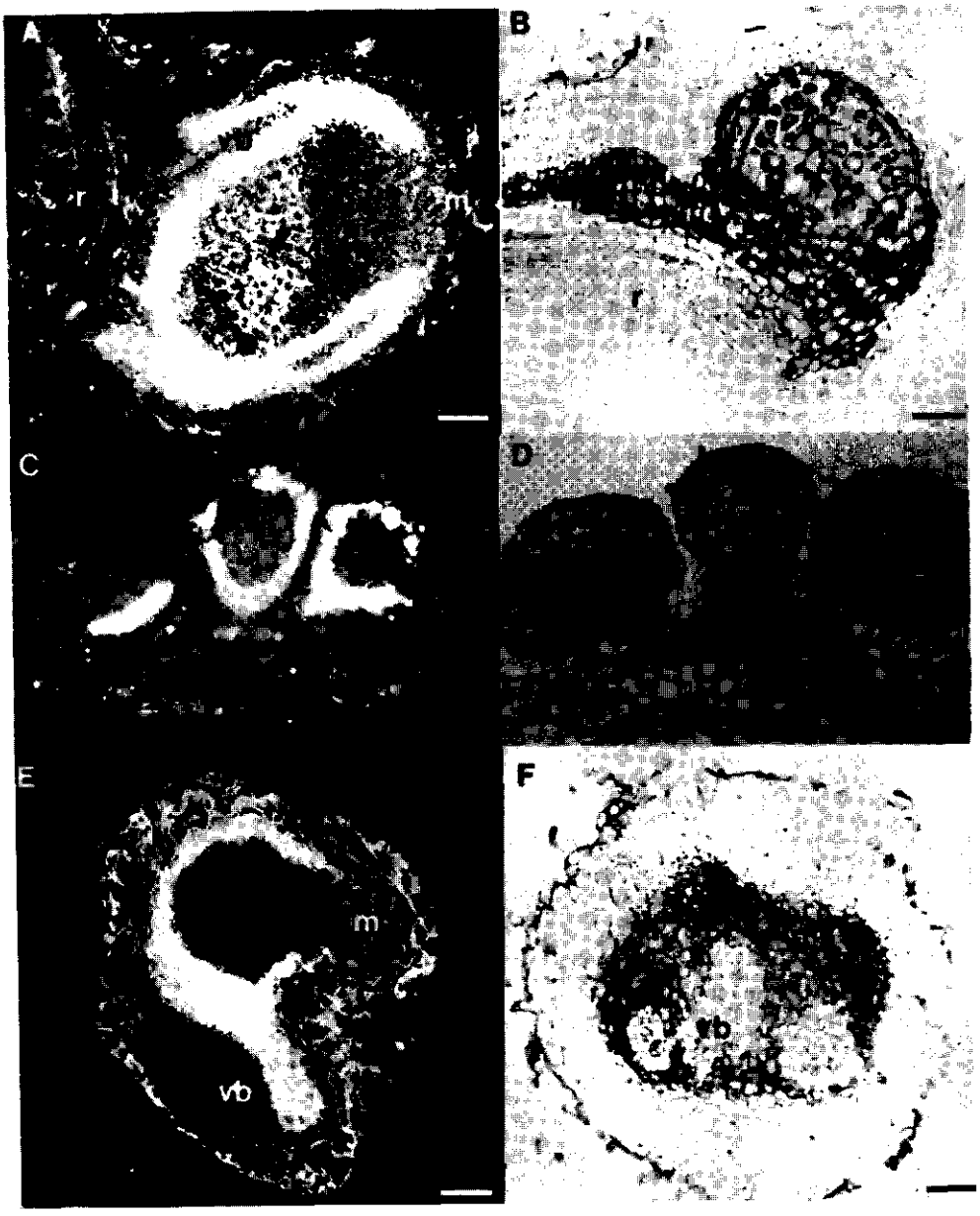
Thus, the pattern of nodulin gene expression is remarkably similar in all "empty" nodules, except for the nodulin Nms25, which apparently occurs only in *R. meliloti exo* mutant-induced nodules.

Localization of ENOD2 transcripts in nodules

Sections of the various alfalfa nodules were hybridized to antisense RNA transcribed from pA2ENOD2, which contains a 0.3 kb insert of alfalfa ENOD2 cDNA. The 4-week-old wild type Rm2011-induced nodule shows a hybridization pattern like the one described for pea (cf. Van de Wiel et al., 1990). The ENOD2 mRNA is present in parenchyma cells, constituting the nodule parenchyma, surrounding the central tissue of infected and uninfected cells. There is no ENOD2 transcript detectable at the top of the nodule, at the site of the persistent meristem. No transcript is found within the vascular strands traversing the nodule parenchyma (Figures 2A,B).

Four-week-old NPA- and TIBA-induced nodules are more or less similar to each other. Unlike a nodule elicited by wild type *R. meliloti*, the meristematic region commonly extends along the major part of the periphery of the nodule. A tissue

Fig. 1. Two-dimensional Polyacrylamide gel analysis of *in vitro* translations of total RNA isolated from alfalfa tissue. **A)** From root tissue. The open arrow (in all panels) points to glutamine synthetase (see Norris et al., 1988, for identification of this translation product as GS). The circles indicate prominent translation products that are evident following *in vitro* translations of RNA isolated from infected root nodules, but which are absent in root RNA, namely the leghemoglobins. **B)** From nitrogen-fixing nodules induced by wild type *R. meliloti* strain Rm1021. The boxed-in translation product is a nodule-specific/enhanced glutamine synthetase. The dark arrowhead points to Nms-30, and the arrows indicate leghemoglobins. **C)** From bacteria-free nodules induced by Rm7055(*exo*F::Tn5). Nms-30 is present (arrowhead) as well as Nms-25 (tandem arrows), but leghemoglobin translation products (circles) are absent. **D)** From bacteria-free nodules induced by A128 (*Agrobacterium tumefaciens* carrying Rm1021 pSyma). Nms-30 is present (arrowhead), but leghemoglobin translation products (circles) are not. **E)** From bacteria-free nodules induced by Rm7061(*exo*A::Tn5). The open arrow points to glutamine synthetase. Nms-30 is present (arrowhead) as well as Nms-25 (tandem arrows), but leghemoglobin translation products (circles) are not.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vb

consisting of vacuolated cells containing amyloplasts can be delimited in the center of the nodule. Around this central tissue a relatively narrow zone consisting of highly vacuolated, densely packed cells, reminiscent of the nodule parenchyma, contains transcripts that hybridize to the ENOD2 antisense RNA. Outside this zone a less densely packed ("outer") cortex where no ENOD2 transcript can be detected, is present (Figures 2C,D).

Figure 2E shows an example of a four-week-old *exo*B mutant-induced nodule. As in wild-type *R. meliloti*-induced nodules, it is possible to distinguish an apically localized persistent meristem, a peripheral zone and a central tissue, which, however, is free of intracellular bacteria. ENOD2 transcript is found in the densely packed, highly vacuolated parenchymatous tissue that constitutes the inner part of the peripheral tissue (the nodule paremchyma) (Figure 2E). This parenchymatous tissue is traversed by a vascular strand that is free of ENOD2 transcript (data not shown). The outer part of the peripheral tissue, which is more loosely packed, is also free of ENOD2 transcript (Figures 2E).

The majority of the nodules induced by the *exo*A, *exo*B and *exo*F mutants are not of the elongate, wild type-like sort of nodule. They are small and broad and meristematic activity, which is spread out along a relatively broad region of the subtending root, leads to the deposition of a limited amount of tissue in a plane perpendicular to the root axis. Consequently, little peripheral and central tissue is formed distally in the nodule (Figures 3A,B). ENOD2 transcripts are present in a zone of densely packed, highly vacuolated cells extending along the base of the nodule (Figures 3A,B). Also located basally in the nodule, several vascular traces, connected to the root stele, are present that do not contain

Fig. 2. Detection of MsENOD2 transcript in alfalfa nodules by *in situ* hybridization. Bar = 100 µm. **A)** Dark field photograph of a nitrogen-fixing nodule. MsENOD2 transcripts as visualized by the brightly reflecting silver grains (see Methods) are localized in parenchyma cells along the periphery of the nodule surrounding the vascular bundle (vb) and also along the base of the nodule. The persistent meristem (m) is devoid of MsENOD2 transcript. Median longitudinal section. **B)** Bright field photograph of a nitrogen-fixing nodule cut obliquely. The persistent meristem is out of the plane of section. MsENOD2 transcripts were localized on paraffin sections using the Boehringer-Mannheim RNA Labeling (Genius) kit (see Methods). A dark stain is present in parenchyma cells along the edge of the central tissue, including at the base of the nodule. The stain that appears to be in the root (r) is actually at the base of a second lobe of a two-lobed nodule. The second lobe is not observed in this section. **C)** Dark field photograph and **D)** bright field photograph of a cluster of nodules elicited two weeks after addition of NPA. MsENOD2 transcripts (brightly reflecting silver grains in C) are detected along the periphery of the nodule, but not in the root (r). Slightly oblique section. **E)** Dark field photograph of a five-week-old, bacteria-free nodule elicited by Rm5078(*exo*B::Tn5). The meristem (m) is devoid of MsENOD2 mRNA as is the outer part of the peripheral tissue. MsENOD2 transcripts extend along the periphery of the nodule and are also localized in parenchyma cells at the base of the nodule. The vascular bundle of the root stele (vb, observed in transversal section) is also devoid of MsENOD2 transcript. Non- median longitudinal section. **F)** Bright field photograph of a bacteria-free nodule induced by Rm7061(*exo*A::Tn5). The nodule has been sectioned transversely (see Fig. 3A for approximate location of the section). Several vascular bundles (vb) are evident; these are surrounded by parenchyma cells which express MsENOD2. The outer part of the peripheral tissue is devoid of MsENOD2 mRNA.

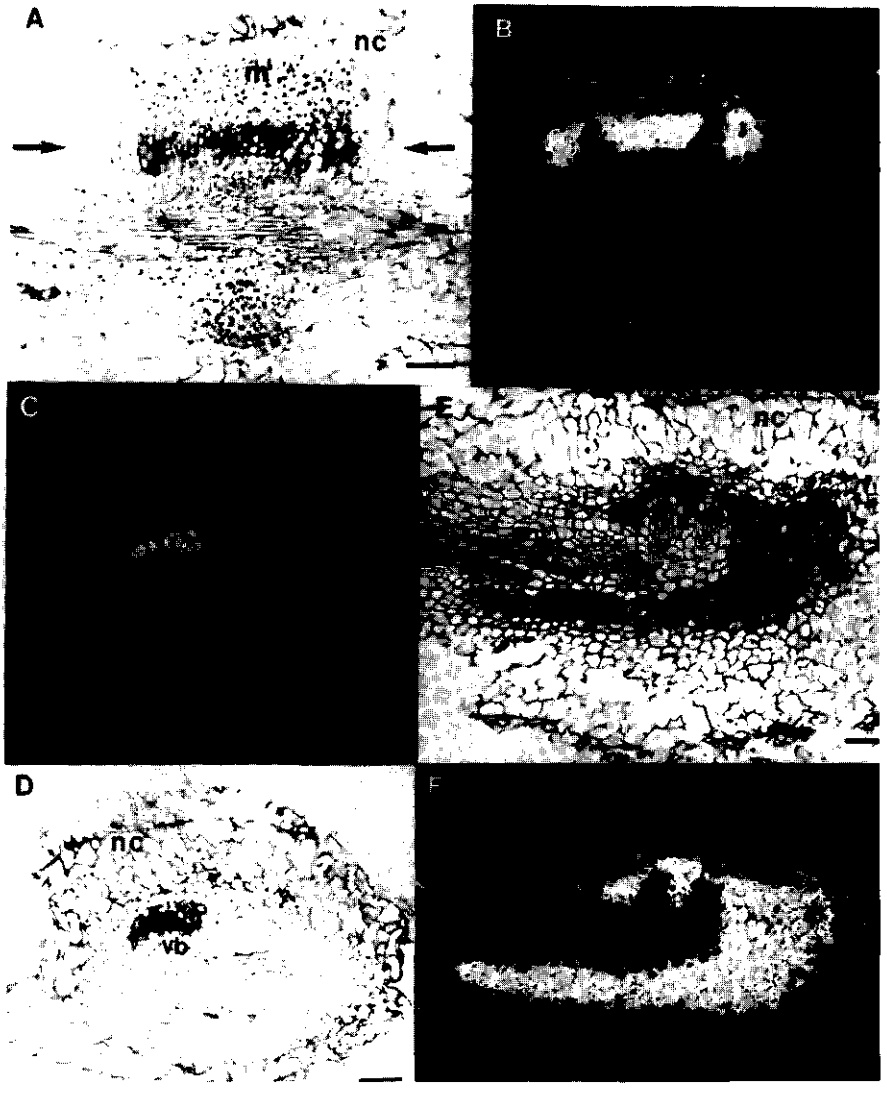

Fig. 3. Detection of MsENOD2 transcript in alfalfa nodules by *in situ* hybridization. Bar = 100 µm. (**A**) Bright field photograph and (**B**) dark field photograph of a three-week-old, bacteria-free nodule induced by Rm5078(*exo*B::Tn5). The presumed meristematic region (m) is very broad and lacks MsENOD2 mRNA. MsENOD2 transcripts are detected basally in the nodule, in parenchyma tissue that surrounds the vascular bundles (vb). The arrows denote the approximate location of the transverse section illustrated in Fig. 2F. Near median longitudinal section; nc: outer peripheral tissue of nodule. (**C**) Dark field photograph and (**D**) bright field photograph of a bacteria-free nodule induced by A135. MsENOD2 transcripts are localized in parenchyma cells around a vascular bundle (vb) of the nodule. A nodule outer peripheral tissue (nc) is observed, but cells typical of a meristematic region are not identifiable in this nodule. (**E**) Bright field photograph and (**F**) dark field photograph of a bacteria-free nodule induced by A128. The nodule is sectioned obliquely. MsENOD2 mRNA is detected basally in the nodule surrounding the vascular bundles (vb). Dividing cells typical of a meristematic region are not observed in this nodule, but the loosely packed outer peripheral tissue (nc) is present.

ENOD2 transcript. A more distally located tissue of the nodule, consisting of more cytoplasmic-rich cells containing amyloplasts, is also free of ENOD2 transcript (Figures 3A,B).

The nodules elicited by the *A. tumefaciens* transconjugants A128 (Figures 3E,F) and A135 (Figures 3C,D) are quite similar to the small and broad *exo* mutant-induced nodules described above. There is a limited amount of meristematic activity over a broad zone of the subtending root. Proximally, several vascular traces are connected to the root stele (Figure 3E,F). ENOD2 transcripts are clearly detectable in a zone of densely packed, vacuolated parenchymatous tissue, containing amyloplasts, located basally in these nodules and/or around the vascular traces. The distally located tissue of these nodules, which is highly vacuolated and free of amyloplasts, contains no ENOD2 transcript (Figures 3C,D and E,F).

DISCUSSION

Our *in situ* hybridization experiments clearly show that the ENOD2 gene is expressed in the nodule parenchyma of nitrogen-fixing alfalfa nodules, following a pattern in agreement with the recent observations of pea nodules (Van de Wiel et al., 1990). Moreover, we find that the ENOD2 gene is expressed in a tissue-specific manner in nodules elicited by the ATIs, NPA and TIBA. ENOD2 transcripts are present in a zone of densely packed, highly vacuolated parenchyma cells between a central tissue of cells, rich in amyloplasts, and an outer layer of more loosely packed cortical tissue. No ENOD2 mRNA was detected in the distal end of the nodule, the site of meristematic activity. The position and morphology of the ENOD2 gene-expressing zone correspond to those of the nodule parenchyma of wild-type *R. meliloti*-induced nodules. Hence, by using the criterion of tissue-specific ENOD2 gene expression, one may conclude that the "empty" ATI-elicited nodules are indeed comparable to nodules induced by wild type *R. meliloti*.

The *R. meliloti exo* mutants and *A. tumefaciens* transconjugants induce a heterogeneous population of nodules (cf. Wong et al., 1983; Hirsch et al., 1984, 1985; Finan et al., 1985). Relatively few are of the elongate type that most resembles the wild-type *R. meliloti*-induced nodule. In such nodules, the presence of ENOD2 transcripts was restricted to a tissue that, both positionally and morphologically, is comparable to the nodule parenchyma of wild-type nodules (cf. Figures 2E and Figures 2A,B). The vast majority of nodules, however, are of a small and broad type in which little peripheral and

central tissue is deposited perpendicularly to the long axis of the root. Nevertheless, ENOD2 gene expression is restricted to a zone of densely packed parenchymatous tissue extending along the base of the nodule and around the vasular traces (cf. Figure 3). The location of this zone is the same as where ENOD2 gene-expressing nodule parenchyma is present in wild-type *R. meliloti*-induced nodules. Thus, in spite of the anomalies related to the diffuse and limited meristematic activity of these "empty" nodules, the pattern of ENOD2 gene expression does not differ fundamentally from that observed in wild type *R. meliloti*-elicited nodules.

From the above-described examples it becomes apparent that localization studies of mRNAs that mark specific nodule tissues may provide insight into the nature of aberrant root nodules. Although distinct tissues, like nodule parenchyma, should be recognizable by the combination of positional and cytological criteria, such distinctions in tissue types are relatively difficult to assess by microscopical studies alone in "empty" nodules of the small and broad type described above. In the cases where a clearly differentiated nodule endodermis (cf. Truchet et al.,1984; Finan et al., 1985) is detectable, it is relatively easy to delimit nodule parenchyma cells from the more loosely packed ("outer") cortex. However, using the amount of intercellular space as a marker, delimitation from a bacteria-free central tissue is frequently more difficult, because these possible differences in the extent of intercellular space between the two tissues are not easy to discern. Furthermore, additional morphological criteria are hard to evaluate; the nodule parenchyma cells are often rich in amyloplasts, like the central tissue itself. Also, the extent of vacuolation in the central tissue is variable in the nodules of the small and broad type. In these cases, localization studies of ENOD2 messenger are helpful. Such analysis may also be important for understanding the developmental organization of even more peculiar types of nodules, such as those elicited on clover by *A. tumefaciens* or *R. leguminosarum* bv. *trifolii* transconjugants carrying *R. meliloti* nodulation sequences (Hirsch et al., 1985; Truchet et al., 1985), or the ones elicited on alfalfa by a *R. meliloti* with a Tn5 insertion 3 kb downstream from *nodC* (Dudley et al., 1987). Unlike other "empty" nodules, these pseudonodules more closely resemble lateral roots in that they have centrally located vascular tissue. In contrast to normal lateral roots, however, one such type of nodule has a nodule endodermis-like layer in its "cortex" (Dudley et al., 1987). Localization of ENOD2 transcripts might indicate whether a tissue comparable to the nodule parenchyma is present in these pseudonodules and, if so, where it is located. It then may be deduced how far these pseudonodules are developmentally comparable to *bona fide* root nodules.

Thus, based on the observation that the ATIs induce a normal pattern of ENOD2 gene expression, we conclude that in their action the ATIs mimic some bacterial factors

responsible for nodule development. The ATIs are known to cause alterations in the plant's endogenous hormone balance. Cell divisions, followed by nodule formation with concomitant tissue-specific expression of the ENOD2 gene, thus may be related to the hormonal status of the root. Recently, LeRouge et al. (1990) have identified a *R. meliloti*-produced molecule, NodRm-1, which elicits alfalfa root hair deformation -one of the earliest steps in nodule formation-, as a ß-sulfated tetraglucosamine with acetyl and acyl substitutions. The ATIs, which are often substituted benzoic acids, are structurally different from NodRm-1, an oligosaccharide. Thus, it is unlikely that both act at the same site. It is, however, conceivable that the ATIs interact with a consecutive site somewhere in the cascade of events normally triggered by NodRm-1, resulting in nodule development.

METHODS

Plant and bacterial material

Alfalfa (*Medicago sativa* L. cv. Iroquois) plants were grown as described by Norris et al. (1988). At the time of sowing, individual bins containing alfalfa seeds were left uninoculated or inoculated with either *Rhizobium meliloti* wild type strains Rm2011 or Rm1021, *R. meliloti* mutant in *exo*B (Rm5078), *exo*A (Rm7061) or *exo*F (Rm7055), or *Agrobacterium tumefaciens* transconjugants, either strain A128, which harbors the *nod/nif* symbiotic megaplasmid (pSyma), or strain A135, which carries pSyma and the megaplasmid bearing *exo* genes (pSymb) (Leigh et al., 1985; Finan et al., 1986). Plants were treated with the ATIs as described previously (Hirsch et al., 1989).

RNA analysis

Tissue was harvested, frozen in liquid nitrogen, and stored at -80oC until use. Total RNA was isolated from nodule or root tissue, *in vitro* translations performed, and the translation products separated by two-dimensional electrophoresis as described by Norris et al. (1988).

In situ hybridization

Tissues were fixed either as described by Van de Wiel et al. (1990) or in 4 % glutaraldehyde, 1,5 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Following fixation, the tissues were rinsed twice in buffer and dehydrated in a graded ethanol series until 50 % ethanol. The tissue was transferred to the first tertiary butyl alcohol step, dehydrated in the tertiary butyl alcohol series (Sass, 1953), and embedded in Paraplast. Sections, 7 or 8 µm thick, were affixed to poly-L-lysine-coated slides. The *in situ* hybridizations using (35S)UTP-labeled antisense RNA probes were based on a protocol published by Cox and Goldberg (1988) and were performed essentially as described by Van de Wiel et al. (1990). Following development of the emulsion, sections were stained either with 1 % aqueous safranin or with 0.05% toluidine blue.

Alternatively, the RNA Labeling (Genius) kit using digoxigenin-labeled UTP (Boehringer Mannheim) was utilized. Sense and antisense probes were made according to the manufacturer's directions and added to deparaffinized sections, which had been pretreated as described for radioactive probes. Some nodules were fixed overnight in formaldehyde-acetic acid-alcohol (FAA) (Sass, 1953), rinsed overnight in 50 % ethanol, slowly rehydrated, and then frozen on a block in liquid nitrogen. The nodules were sectioned at 16 µm using a cryostat and the sections were placed on poly-L-lysine-coated slides. The sections were gradually warmed to room temperature and further treated as for radioactive probes. 10-100 ng of probe/ml hybridization buffer/slide was added to the cryostat-sectioned material. The manufacturer's protocol for pre-hybridization, hybridization and post-hybridization was followed for both sense and antisense probes; sections were hybridized at 37oC overnight. MsENOD2 transcripts were detected immunologically according to the manufacturer's directions. For paraffin-embedded material, 12-72 hours were required for maximal color development, while for cryostat-sectioned material, color development generally occurred within 60 minutes. The sections were not counterstained.

Following dehydration through an ethanol seriers, the sections werte mounted with DPX or Eukitt, and were photographed either with a Nikon or Zeiss Axiophot microscope equipped with bright and dark field optics.

REFERENCES

Cox, K.H., and Goldberg, R.B., 1988, Analysis of plant gene expression, *in*: Plant Molecular Biology: A practical approach, C.H. Shaw, ed., IRL Press, Oxford.

Dickstein, R., Bisseling, T., Reinhold, V.N., and Ausubel, F.M., 1988, *Genes and Development*, 2: 677.

Dudley, M.E., Jacobs, T.W., and Long, S.R., 1987, *Planta*, 171: 289.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C., and Signer, E.R. (1985) Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell 40, 869-877.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C., and Signer, E.R., 1985, *Cell* 40, 869-877.

Franssen, H.J., Nap, J.-P., Gloudemans, T., Stiekema, W., Van Dam, H., Govers, F., Louwerse, J., Van Kammen, A., and Bisseling, T., 1987, *Proc. Natl. Acad. Sci. USA*, 84: 4495.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T., 1989, *Proc. Natl. Acad. Sci. USA* 86: 1244.

Hirsch, A.M., Drake, D., Jacobs, T.W., and Long, S.R., 1985, *J. Bacteriol.*, 161: 223.

Hirsch, A.M., Wilson, K.J., Jones, J.D.G., Bang, M., Walker, V.V., and Ausubel, F.M., 1984, *J. Bacteriol*,. 158: 1133.

Leigh, J.A., Reed, J.W., Hanks, J.F., Hirsch, A.M., and Walker, G.C., 1987, *Cell*, 51: 579.

Leigh, J.A., Signer, E.R., and Walker, G.C., 1985, *Proc. Natl. Acad. Sci. USA*, 82: 6231.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., and Dénarié, J., 1990, *Nature*, 344: 781.

Norris, J.H., Macol, L.A., and Hirsch, A.M., 1988, *Plant Physiol.* 88, 321-328.

Sass, J.E., 1953, Botanical Microtechnique, 3rd ed., Iowa State University Press, Ames, IA.

Truchet, G., Debelle, F., Vasse, J., Terzaghi, B., Garnerone, A.-M., Rosenberg, C., Batut, J., Maillet, F., and Dénarié, J., 1985, *J. Bacteriol.*, 164: 1200.

Truchet, G., Rosenberg, C., Vasse, J., Julliot, J.-S., Camut, S., and Dénarié, J., 1984, *J. Bacteriol.*, 157: 134.

Van de Wiel, C., Scheres, B., Franssen, H., Van Lierop, M.-J., Van Lammeren, A., Van Kammen, A., and Bisseling, T., 1990b, *EMBO J.*, 9: 1.

Witty, J.F., Minchin, F.R., Skøt, L., and Sheehy, J.E., 1986, *Oxford Surv. Plant Mol. Cell Biol.*, 3: 275.

Wong, C.H., Pankhurst, C.E., Kondorosi, A., and Broughton, W.J., 1983, *J. Cell Biol.*, 97, 787.

CHAPTER 7

The ENOD12 gene product is involved in the infection process during the pea-*Rhizobium* interaction.

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SUMMARY

The pea cDNA clone pPsENOD12 represents a gene involved in the infection process during the *Pisum sativum* L. - *Rhizobium leguminosarum* bv. *viciae* symbiosis. The ENOD12 protein is composed of pentapeptides containing two (hydroxy)prolines. The expression of the ENOD12 gene is induced in cells through which the infection thread is migrating, but also in cells that do not yet contain an infection thread. Soluble compounds from *Rhizobium* are involved in eliciting ENOD12 gene expression. *Rhizobium* common and host-specific nodulation genes are essential for the production of these compounds. Two ENOD12 genes are expressed in nodules and in stem tissue of uninoculated plants. The gene represented by the cloned ENOD12 mRNA is also expressed in flowers, but a different transcription start might be used.

INTRODUCTION

The symbiosis between *Rhizobium* bacteria and legumes results in the formation of highly organized structures, namely the infection threads and the root nodules. These nodules, in which nitrogen fixation takes place, consist of different tissues, organized in a specific way (Newcomb,1980).

The infection process starts with the deformation and curling of root hairs (Bauer, 1981). Curling is thought to achieve enclosure of attached bacteria, permitting them to locally modify and hydrolyse the cell wall of the root hairs (Callaham & Torrey, 1981; Turgeon & Bauer, 1985). At this site cell wall material is deposited by the plant and it forms tubular structures, the infection threads. Infection threads, containing dividing bacteria, grow into the root cortex. In pea roots the infection thread proceeds toward the inner cortical cell layers, where the following events occur: prior to penetration of the infection thread into root cortex cells, the cells change remarkably, as microtubules rearrange, an additional cell wall layer is deposited and a cytoplasmic bridge is formed by which the infection thread will migrate. The infection thread passes through the prepared cells by a cell wall degradation/deposition mechanism just like in root hairs Bakhuizen et al, 1988a, 1988b). Concomitantly with infection thread formation rhizobia induce the formation of a premeristem, the nodule primordium, in the inner cortical cell layers. Infection threads grow into the nodule primordium, where bacteria are released from the

infection thread tips (Libbenga and Bogers, 1974). Simultaneously at the apical site of the nodule primordium the meristem is formed. The meristem cells are smaller in size and have smaller vacuoles than the primordium cells. The direction of infection thread growth is then reversed as it now follows the apical meristem that grows out of the root by adding cells which differentiate into the various nodule tissues. In this way there is a continuous infection process in the so-called invasion zone, immediately adjacent to the meristem. Upon release of bacteria in the plant cells, the bacteria are encapsulated by a membrane of plant origin, and differentiate into N_2-fixing bacteroids (Newcomb, 1976).

It has been shown that both bacterial and plant genes are involved in nodule formation. For example the bacterial common and host specific nodulation (*nod*) genes are involved in root hair deformation, infection thread formation, and induction of cortical cell division (for review see Long, 1989). They are also essential for the induction of expression of nodule-specific plant genes, the nodulin genes (Van Kammen, 1984; Govers et al, 1986). Nodulin genes can, according to the the timing of their expression during nodule development, be divided into early and late nodulin genes (Govers et al, 1987). Early nodulins are involved in root hair deformation, infection or nodule morphogenesis. The best studied early nodulin is ENOD2. It is a (hydroxy)proline rich protein which is most likely a cell wall component (Franssen et al, 1987), that is formed in nodule parenchyma ('inner cortex') cells (Van De Wiel et al, 1990). Late nodulins are detectable after the nodule has developed and bacterial release has taken place. Therefore, they are neither involved in infection nor in nodule morphogenesis. Well characterized late nodulins are the leghemoglobins (Brisson and Verma, 1982) and a nodule specific uricase (Bergmann et al, 1983), involved in oxygen transport and nitrogen metabolism, respectively. Several late nodulins are located in the peribacteroid membrane, but their function is yet unknown (Fortin et al, 1985, 1987; Jacobs et al, 1987; Sandal et al, 1987).

The early nodulins characterized so far, GmENOD2, GmENOD13, and GmENOD55, are related to nodule morphogenesis and not to the infection process (Franssen et al, 1987, 1988). Our aim was to isolate a cDNA clone encoding a nodulin involved in the infection process, in order to investigate the role of plant genes in this process and the regulation of their expression by *Rhizobium*. The infection process occurs abundantly in the invasion zone of young pea nodules, where many cells derived from the meristem are penetrated by an infection thread. Therefore we decided to isolate early nodulin cDNA clones from a pea nodule cDNA library. The infection process occurs in root hairs as well as in nodules, so we selected putative infection-related clones by testing whether the corresponding genes are expressed both in root hairs of inoculated plants and in nodules. With this approach we obtained a cDNA clone representing an

early nodulin gene, involved in the infection process. In the following we report on the characterization of the ENOD12 cDNA clone, the regulation of expression of the corresponding gene by Rhizobium and its possible function in the infection process. Furthermore we discuss the evolutionary origin of this nodulin gene, since transcripts homologous to this cDNA clone were detected in stem and flower tissues.

RESULTS

Isolation of an infection-related early nodulin cDNA clone

A pea nodule cDNA library was differentially screened with cDNA probes made of RNA from nodules from 10 day old plants and uninfected roots from 8 day old plants, respectively. One of the isolated nodulin clones, pPsENOD12, appeared to encode an early nodulin potentially involved in the infection process. RNA transfer blot analysis revealed that the ENOD12 mRNA has a transient pattern of appearance during nodule development (figure 1A). It is already detectable in root segments of 8 day old infected pea plants, which do not possess macroscopically visible nodules. The mRNA reaches its maximum concentration from day 10 to day 13 and decreases in concentration thereafter. In contrast to ENOD12 mRNA the transcript of the pea early nodulin ENOD2 is first detectable on similar blots at day 10 and reaches a maximum concentration between day

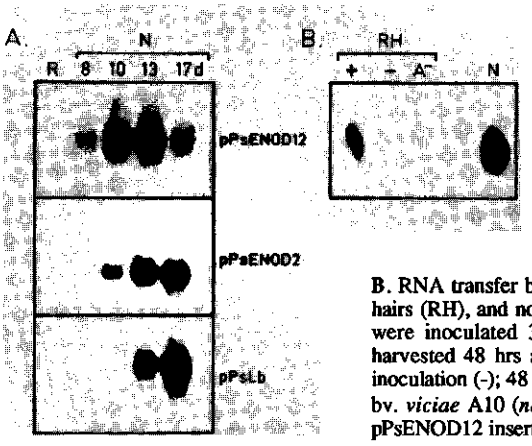


Fig. 1. RNA transfer blot analysis of RNA from roots, nodules and root hairs.
A. RNA transfer blots contain 10 µg of total RNA from uninoculated roots of 8 day old plants (R), and nodules (N), 8, 10, 13, and 17 days after sowing and inoculation. Blots were probed with pPsENOD12, pPsENOD2 and pPsLb inserts, respectively.
B. RNA transfer blot contains 20 µg of total RNA from root hairs (RH), and nodules 13 days after inoculation (N). Plants were inoculated 3 days after sowing and root hairs were harvested 48 hrs after inoculation (+); after 48 hrs without inoculation (-); 48 hrs after inoculation with *R.leguminosarum* bv. *viciae* A10 (*nodA::Tn5*).(A^-) The blot was probed with pPsENOD12 insert.

13 and 17, whereas the concentration remains constant thereafter. The mRNA of the late nodulin leghemoglobin is first detectable at day 13 and increases in concentration during the following days (figure 1A).

Next, we determined whether the ENOD12 gene was expressed in root hairs from inoculated plants using RNA transfer blot analysis. This study revealed that the ENOD12 transcript is present in root hairs 24 hours after inoculation, but not in the root hairs from uninoculated peas (figure 1B, RH + and - lanes). The presence of the ENOD12 transcript in root hairs of plants shortly after inoculation indicates that ENOD12 gene expression may correlate with the occurrence of the infection process.

Localization of ENOD12 mRNA in root cortex and nodule cells

The correlation between ENOD12 gene expression and the occurrence of infection threads was further examined by in situ hybridization. Roots of pea plants, inoculated with bacteria three days after sowing, were harvested 2 and 3 days after inoculation. They were embedded in paraffin and serial sections containing nodule primordia and infection threads were selected. These sections were hybridized to ^{35}S-labeled antisense ENOD12 RNA.

Two days after inoculation ENOD12 transcript appeared to be located in cortex cells containing the infection thread (figure 2A, figure 2B, small arrowhead + arrow). In root hairs where infection threads are present ENOD12 mRNA is also detected (figure 2A, figure 2B, arrow). By analysis of serial sections we determined the location of the tip of the infection thread (figure 2A, small arrowhead) and the ENOD12 transcript appeared not to be confined to the cells containing an infection thread. A track of cortical cells in front of the thread towards the central cylinder also contains ENOD12 mRNA (figure 2B). From cytological studies it is known that these cells undergo several morphological changes and become 'prepared' for infection thread passage (Bakhuizen et al, 1988a, 1988b). Moreover, ENOD12 transcript is also present in cells in the root inner cortex (figure 2A, figure 2B, large arrowhead), which will form the nodule primordium (Libbenga and Bogers, 1974).

Three days after inoculation a centre of mitotic activity, the nodule primordium (NP), is clearly visible in the inner cortical cell layers, containing dividing cells which possess enlarged nuclei and a higher content of cytoplasm then the root cortex cells (figure 2C). The infection thread (arrow) has not yet reached the primordium. At this stage ENOD12 mRNA can be detected in the cortex cells containing the infection thread, in the cells preparing for infection thread passage, and also in the cells that form the new

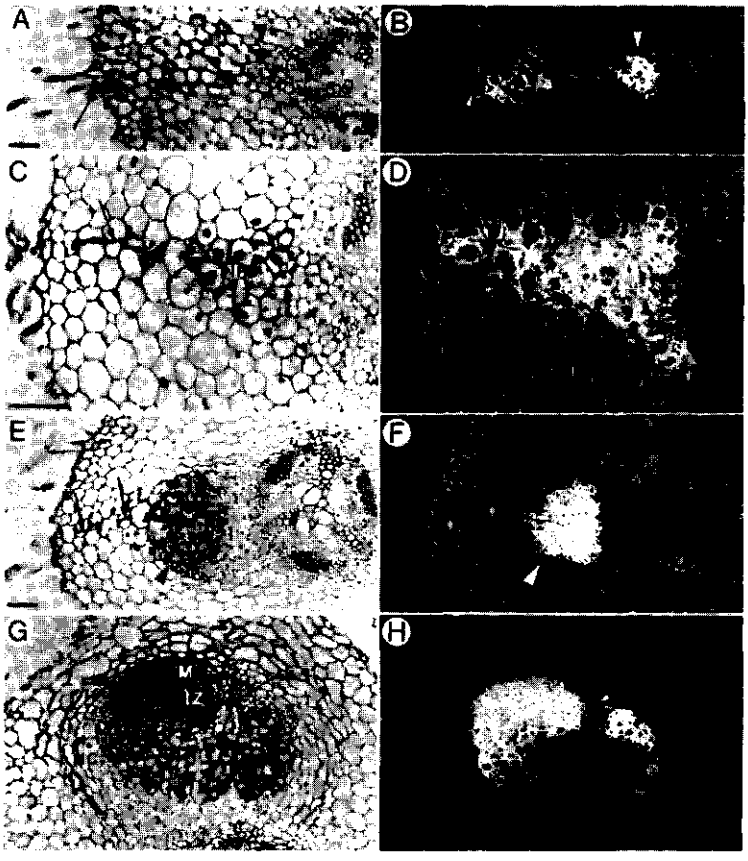


Fig. 2. Localization of ENOD12 transcripts in pea root segments at different stages of nodule development by in situ hybridization The right panel shows dark field micrographs corresponding with the bright field micrographs in the left panel. In the dark field micrographs, silver grains representing hybridization signal are visible as white spots. A/B, C/D, E/F, and G/H represent successive stages of nodule development. A: Transection of a five-day old root, two days after inoculation. An infection thread which is clearly visible at higher magnification can be observed in a root hair (arrow), the tip of which has reached the second cortical cell layer as deduced by analysis of a complete set of serial sections (small arrowhead). In the inner cortex the site of the future nodule primordium is marked (large arrowhead). C: Transection of a six day old root, three days after inoculation. Infection thread (arrow), infection thread tip located by analyzing a complete set of serial sections (arrowhead), and the nodule primordium (NP) in the root inner cortex are indicated. E: Transection of a seven day old root, four days after inoculation. The infection thread (arrows) has reached the nodule primordium (NP), and branches off into several thinner threads (small arrowheads), which grow into cells at the base of the primordium. A few cell layers at the periphery of the primordium, which will most likely develop into the cortex and, at the top, into the apical meristem of the nodule, do not contain hybridization signal (large arrowhead). G: Transection of a ten day old pea root, seven days after inoculation. The original site of entrance of the infection thread into the nodule primordium is indicated with an arrow. The apical meristem (M), invasion zone (IZ), early symbiotic zone (ES), and nodule cortex (NC) are indicated. Sections were hybridized with ^{35}S-labeled antisense ENOD12 RNA. Using sense RNA as a probe we did not observe hybridization signals (data not shown). Bar = 100 µm. Further abbreviations: X = xylem pole, F = phloem fibers of central cylinder.

centre of mitotic activity, the nodule primordium (figure 2C, figure 2D).

We also localized ENOD12 transcript in pea nodules at later stages of development. Sections from pea nodules of 7, 10 and 20 day old plants were hybridized with ^{35}S labeled antisense ENOD12 mRNA. In 7 day old plants infection threads are penetrating the nodule primordium (NP) (figure 2E). At this stage ENOD12 mRNA is located in all cells of the part of the nodule primordium where the infection thread branches (figure 2E, small arrowheads). Only a few small cells at the periphery of the primordium do not contain ENOD12 transcript (figure 2E, figure 2F, large arrowhead). These cells will form the apical meristem and the nodule cortex, while the other cells of the primordium are destined to become the first cells of the infected and the uninfected cell type (Libbenga & Bogers, 1974, C.v.d.W., unpubl. res.). In root cortex cells where the oldest part of the infection thread resides ENOD12 transcript is no longer detectable (figure 2E, figure 2F, arrows).

Nodules from 10 day old plants posses an apical meristem (M), containing small, actively dividing cells which are rich in cytoplasm (figure 2G). The different tissues are

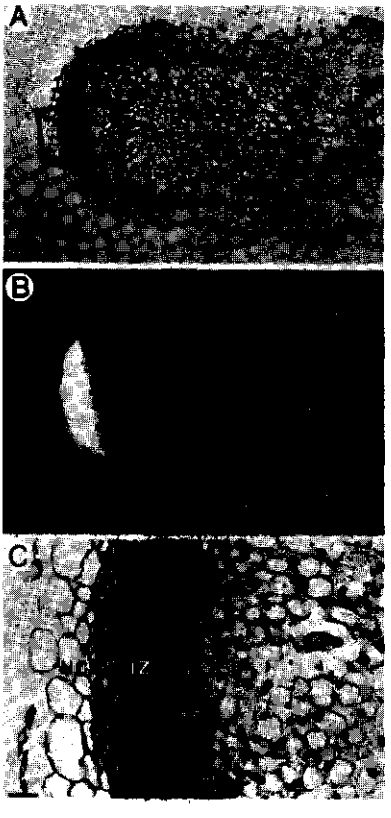


Figure 3. Localization of ENOD12 transcripts in nodules from 20 day old pea plants by in situ hybridization.A. Bright field micrograph of a longitudinal section of a 20 day old pea nodule. The arrow points to the remnant of the infection thread that originally penetrated the nodule primordium (cf. figure 2E,G). Late symbiotic zone (LS), vascular bundles (VB), and nodule cortex (NC), which developed from the apical meristem, are indicated. Part of the root to which the nodule is attached is visible in a transversal section. Here the root cortex (RC), a xylem pole (X) and phloem fibers (F) of the central cylinder are indicated. B: Dark field micrograph of the section shown in A. Silver grains representing hybridization signal are visible as white spots. C: Magnification of the bright field micrograph in A, as outlined in figure A. Silver grains representing hybridization signal are visble as black spots. The hybridization signal is strong over the invasion zone (IZ), and gradually diminishes over the early symbiotic zone (ES). Nodule cortex (NC) and meristem (M) are indicated. Sections were hybridized with ^{35}S-labeled antisense ENOD12 RNA. Bar in A: 300 µm. Bar in C: 50 µm.

graded in age from the apical meristem to the base of the nodule (Newcomb, 1976). Immediately adjacent to the meristem, in the invasion zone (IZ), cells are penetrated by infection threads, growing in reversed direction as they now follow the meristem. About half of the cells in the invasion zone are infected by bacteria released from infection threads. In the early symbiotic zone (ES) these cells differentiate into the infected cell type. The other cells which are not infected by rhizobia become the uninfected cell type. ENOD12 transcript is present in the invasion zone, adjacent to the meristem, but is not detectable in the meristematic cells (figure 2G, figure 2H). In the early symbiotic zone, where cells are elongating, the concentration of ENOD12 transcript is decreasing.

In nodules from 20 day old plants ENOD12 mRNA is detectable at the nodule apex (figure 3A, figure 3B). A magnification of figure 3A shows that the transcript is located in the zone where infection threads are growing (figure 3C). No transcript is detectable in the meristem, and the concentration of the transcript decreases in the early symbiotic zone. We conclude that, in 7 day old as well as in 10 and 20 day old nodules, ENOD12 mRNA is restricted to the region of the nodule where active infection thread growth occurs, and therefore it marks the invasion zone.

ENOD12 is a (hydroxy)proline rich protein

Further information on the nature of the ENOD12 early nodulin was obtained by determining the nucleotide sequence of the insert of pPsENOD12 (figure 4). The insert is 553 bp in length, excluding a short poly A stretch at the 3' end of the sequence. The mRNA measures approx. 600 bases, as determined on an RNA transfer blot, and primer extension analysis showed that less than 20 bases from the 5' end of the mRNA are missing in pPsENOD12 (figure 10). The sequence of the 5' end of the mRNA missing in the cDNA clone was determined by direct RNA sequencing (figure 4, small typeface). The cDNA sequence contains only one long open reading frame, starting with an ATG codon at position 7 which is the first and only methionine codon in the reading frame. From the derived amino acid sequence a molecular weight of 12.5 kD was calculated for the ENOD12 protein. A putative signal peptide containing a hydrophobic core sequence is present at the N-terminal part and the possible cleavage site of the signal peptide, marked with an arrow in figure 4, was determined by the rules of Von Heijne (1983). The major part of the following protein sequence consists of two pentapeptide repeating units. One of these units, Pro-Pro-Gln-Lys-Glu, indicated with solid lines, is well conserved throughout the protein sequence. The other unit is present as Pro-Pro-Val-Asn-Gly at the amino-terminal part and gradually every amino acid except the prolines is permuted to

give a Pro-Pro-His-Lys-Lys unit at the carboxy-terminal part of the polypeptide chain (dashed lines). At two positions a proline codon is changed into a threonine codon by a single base substitution. Further downstream to the carboxy terminus the proline repeat units are absent.

In vitro translation of hybrid-selected ENOD12 mRNA from nodules yielded one radioactive polypeptide of 12.5 kD when ^{35}S-methionine was used as radioactive amino acid. When the same selected mRNA was translated in the presence of ^{3}H-leucine two radioactive polypeptides of 12.5 and 14 kD were formed (figure 5). Since the smaller polypeptide is the one labeled with ^{35}S-methionine this cannot be a breakdown product of the larger polypeptide. Hence there appear to be two different ENOD12 mRNAs in nodules. The observation that there is no methionine encoded in the open reading frame

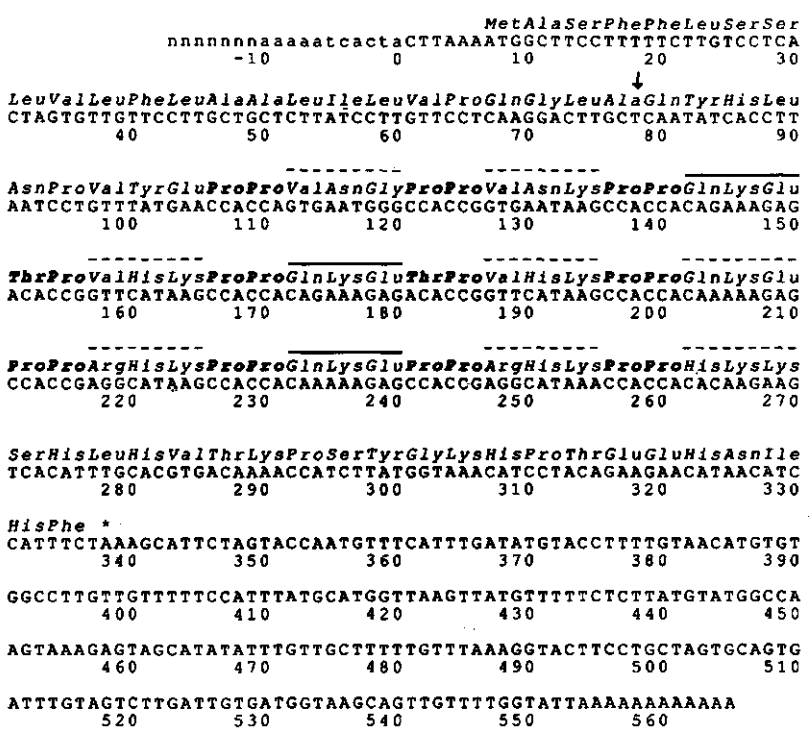


Fig. 4. cDNA and predicted amino acid sequence of the pPsENOD12 insert. Nucleotides 1-565 are determined from the pPsENOD12 insert. Nucleotides -18 (the 5' end of the mRNA) to 0 are determined using direct RNA sequencing (see materials and methods). The amino acid sequence of the only long open reading frame is depicted over the nucleotide sequence. The putative signal peptide cleavage site is marked with an arrow. Prolines in the repeat region are in bold typeface. The amino acid triplets characteristic of both types of pentapeptide repeats described in the text are overlined with unbroken and dashed bars, respectively. The termination codon ending the reading frame is marked by an asterix.

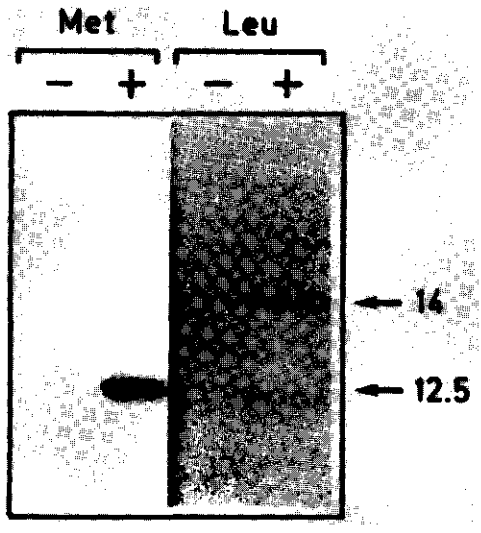


Fig. 5. In vitro translation products of hybrid-selected ENOD12 mRNA. ENOD12 mRNA was selected form total nodule RNA and translated in vitro in the presence of ^{35}S-methionine (Met) or ^{3}H-leucine (Leu) as radioactive amino acid. Filters used for hybrid selection contained pBR322 (-) or pPsENOD12 insert (+). The right two lanes are exposed 6-fold longer than the neighbouring two lanes to the left. The size (kD) of the in vitro translation products is indicated to the right side.

of pPsENOD12, except at the start, shows that the cDNA clone corresponds to the mRNA encoding the 14 kD polypeptide that can only be detected with ^{3}H-leucine. The observed discrepancy between the calculated and the apparent mol. wt. is rather common for proline-rich proteins (e.g. Franssen et al, 1987).

ENOD12 gene expression requires Rhizobium nod genes and is induced by excreted bacterial compounds

To determine which bacterial genes are essential for the induction of ENOD12 gene expression we analyzed the expression of this gene after inoculation with different Rhizobium mutants. The R.leguminosarum bv. viciae common nodulation genes *nod* ABC are required for initiation of cortical cell division, root hair curling, and infection thread formation (Wijffelman et al, 1985). In the *R.meliloti* - alfalfa interaction the host-specific *nod*EF genes are also important for infection thread formation (Horvath et al, 1986), and recently the involvement of *nod*EF in infection thread formation has also been demonstrated for the R.leguminosarum bv. viciae - Vicia hirsuta interaction (Van Brussel et al, 1988). Therefore we examined whether both common and host-specific *nod* genes are essential for eliciting ENOD12 gene expression in root hairs. Pea plants were inoculated with various mutant R.leguminosarum bv. viciae strains. As a control wild-type *R. leguminosarum* bv. *viciae* 248 was used. To obtain maximum sensitivity in detecting ENOD12 gene expression in root hairs we amplified cDNA specifically made from total root hair RNA by the polymerase chain reaction (PCR, see Saiki et al, 1985;

Mullis and Faloona, 1987). The amplified cDNA was visualized by DNA transfer blotting using pPsENOD12 cDNA insert as a probe. In this way the presence of ENOD12 transcript in 1 μg total root hair RNA, inoculated with wild-type *R. leguminosarum* bv. *viciae* could be visualized within several hours after exposure of a hybridized DNA transfer blot (figure 6, WT). Upon longer exposure a weak signal could also be observed in uninoculated root hair RNA (not shown). We do not know whether this signal is caused by low levels of ENOD12 mRNA or by residual chromosomal DNA present in the RNA preparations.

The *R. leguminosarum* bv. *viciae* A10 strain carries a Tn5 mutation in *nod*A which blocks the formation of the *nod*A, *nod*B, and *nod*C products (Wijffelman et al, 1985). In our first experiments we demonstrated by RNA transfer blot analysis that ENOD12 transcript is found in root hairs from plants inoculated with wild type *R.leguminosarum* bv. *viciae*, but not in root hairs from plants inoculated with *R. leguminosarum* bv. *viciae* A10 (figure 1B). Using the more sensitive PCR method it was confirmed that a mutation in *nod*A abolished the ability of bacteria to induce ENOD12 gene expression in root hairs (figure 6, lanes 2 and 3).

The Sym-plasmid cured *R. leguminosarum* bv. *viciae* 248^{c} (pMP104) strain, carrying the *nod*EFDABCIJ genes on the low-copy plasmid pMP104, is capable of nodulating Vicia (Spaink et al, 1987) and pea (H.P. Spaink, personal communitcation). This strain was able to induce ENOD12 gene expression (figure 6, lane 4). On the other hand the R. leguminosarum bv. viciae 248^{c} (pMP104 *nod*E::Tn5) strain, carrying a mutation in *nodE*, which forms no infection threads on Vicia (Van Brussel et al, 1988), showed no induction of ENOD12 gene expression (figure 6, lane 5). We concluded that both common and host-specific *nod* genes of Rhizobium are essential for eliciting

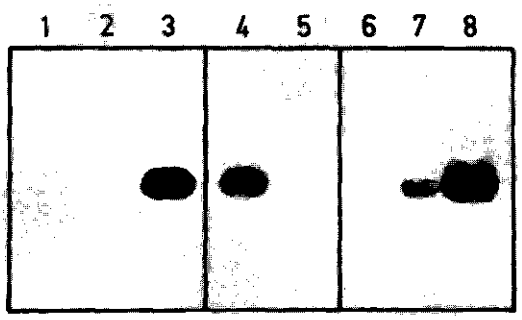


Fig. 6. PCR analysis of ENOD12 gene expression in root hair RNA. ENOD12 sequences from 1 μg total root hair RNA were amplified using 12 PCR cycles, and after electrophoresis a DNA transfer blot was probed with ^{32}P-labeled pPsENOD12 insert. The different lanes contain amplified root hair ENOD12 sequences from uninoculated plants (1 and 6); from plants inoculated with *R.leguminosarum* bv. *viciae* A10 (2), wild-type *R.leguminosarum* bv. *viciae* 248 (3 and 8), *R.leguminosarum* bv. *viciae* 248^{c} (pMP104) (4); *R.leguminosarum* bv. *viciae* 248^{c} (pMP104 *nod*E::Tn5) (5); and from plants inoculated with the cell-free supernatant of wild-type *R.leguminosarum* bv. *viciae* 248 induced with naringenin (7).

ENOD12 gene expression in pea plants.

It has been shown that, upon induction of the nod genes with a flavonoid, rhizobia excrete compounds that cause deformation of root hairs (Zaat et al, 1987). Therefore we studied whether the bacteria also excrete compounds capable of inducing the ENOD12 gene. Rhizobia were grown in the presence or absence of naringenin, a flavonoid inducing the *nod* genes. After removal of the bacteria (see experimental procedures) the Rhizobium-free culture medium was applied to 3 day old pea seedlings. Two days after inoculation root hairs were harvested and the presence of ENOD12 mRNA in root hair RNA was studied. The ENOD12 transcript was detectable in root hair RNA from plants treated with the medium of bacteria cultured in the presence of naringenin (figure 6, lane 7). On the other hand the culture media of bacteria grown in the absence of naringenin could not establish an increase in the amount of transcript (data not shown). We concluded that excreted compounds, formed after induction of the Rhizobium *nod* genes, are able to elicit ENOD12 gene expression in root hairs.

ENOD12 gene expression is not a defense reaction

The proline repeat units in the ENOD12 protein are quite similar to those in the amino acid sequence of hydroxyproline-rich glycoproteins (HRGPs), accumulating in plant tissue after wounding or upon interactions with pathogens (Chen and Varner, 1985; Corbin et al, 1987). Furthermore, infection thread formation has been viewed as a modified plant defense response (Vance, 1983; Djordjevic et al, 1987). This prompted us to investigate whether the ENOD12 gene or similar genes are induced as part of a defense response in pea. For these experiments we used the pathogenic fungus *Fusarium oxysporum* f. sp. *pisi*. While accumulation of HRGP transcripts was observed upon RNA transfer blot analysis of total RNA from pea roots inoculated with the fungus, no ENOD12 mRNA was detectable in these RNA preparations (data not shown). We can, therefore, conclude that the expression of the ENOD12 gene(s) during the infection process cannot be attributed to a general defense response following *Rhizobium* infection.

ENOD12 gene expression in stem and flower

We studied whether the ENOD12 gene or genes resembling ENOD12 are expressed in other parts of the plant since this might give some clues on the evolutionary

origin of the ENOD12 early nodulin. By RNA transfer blot analysis we were unable to detect ENOD12 transcripts in root tip, root elongation zone, mature root, hypocotyl, epicotyl, plumule, and leaf (figure 7). On the other hand, hybridizing RNA similar in size to ENOD12 transcript, but less abundant, was found in stem and flower RNA. (figure 7). In both tissues this RNA was also detectable with an ENOD12 probe, pPsENOD12-3', containing only the 3' region downstream from the BglI site at position 239 in the cDNA sequence (figure 4) (data not shown).

Using the in situ hybridization technique we localized ENOD12 mRNA in stem internode sections. The transcript appeared to be located in a zone of cortical cells surrounding the central ring of vascular bundles and the interfascicular cambium cells (figure 8). In flowers we were not able to localize the ENOD12 transcript unambiguously.

In vitro translation of hybrid-selected ENOD12 nodule mRNA resulted in two distinct polypeptides, as shown in figure 5. Therefore the existence of two or more ENOD12 genes in the pea genome seemed plausible. In order to obtain information on the number of ENOD12 genes we performed Southern blot analyses of restricted pea genomic DNA using the cDNA clones pPsENOD12 and pPsENOD12-3' as probes. Two EcoRI fragments of 4.5 and 5.5 kb hybridized to pPsENOD12. Both fragments also hybridized to the 3' region probe. Restriction with HindIII again yielded two fragments, 1.8 and 7 kb in size, hybridizing as well to pPsENOD12 as to the 3' region probe (data not shown). Since the two HindIII and the two EcoRI fragments hybridized to the same level with both pPsENOD12 and pPsENOD12-3' it appears very likely that there are two ENOD12 genes in the pea genome.

The presence of more than one ENOD12 gene raises the question of which genes are transcribed in the different tissues. To answer this question we could not use in vitro translation of hybrid-selected ENOD12 mRNA from stem and flower since in our hands the sensitivity of hybrid-released translation was insufficient with low abundant mRNA

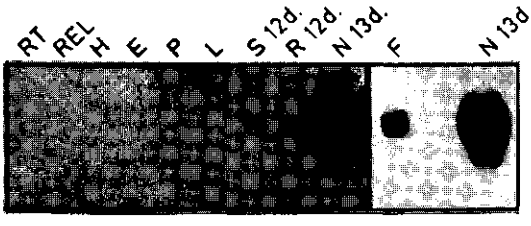


Fig. 7. RNA transfer blot analysis of various uninoculated pea tissues. RNA transfer blots contain 20 µg of total RNA from the following tissues; from 5 day old pea plants: root tip (RT), root elongation zone (REL), hypocotyl (H), epicotyl (E), plumule (P), leaf (L); from 12 day old pea plants: stem (S), root (R); nodules 13 days after inoculation (N), and from plants of varying age: flower (F). Blots were probed with pPsENOD12 insert; in lanes P to N of the left panel a faint band migrating with lower mobility than ENOD12 mRNA can be observed which represents aspecific binding of probe to the small ribosomal RNA.

and ^{3}H-leucine as the labeled amino acid. Specific probes for different transcripts could neither be used since all isolated ENOD12 cDNA clones corresponded to the same ENOD12 mRNA. Therefore we adapted the RNase mapping assay (Melton et al, 1984) to discriminate between different mRNAs by virtue of their complete or incomplete protection to RNase digestion. As a probe for RNase mapping we used the 3' region of the pPsENOD12 insert as the 5' region contains sequence duplications which will prevent accurate mapping. ^{32}P-labeled antisense ENOD12 mRNA was transcribed from the 3' region of the insert of pPsENOD12 as indicated in figure 9C1,2. This antisense RNA was hybridized to total RNA from roots and nodules, or to root RNA which was mixed with 1 ng unlabeled ENOD12 sense RNA transcribed from the 3' region of the insert of pPsENOD12, cloned in the opposite orientation towards the T7 promoter, as indicated in figure 9C3. After hybridization single-stranded RNA was digested using increasing amounts of RNase T1 and subsequently the RNA was separated by polyacrylamide gel electrophoresis. The RNase mapping experiment showed that a 216 bp sense-antisense ENOD12 RNA hybrid remained fully protected using increasing amounts of RNase T1

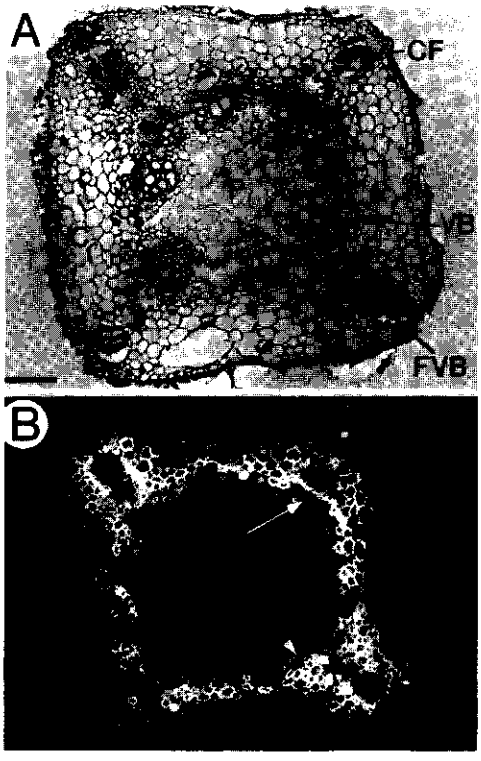
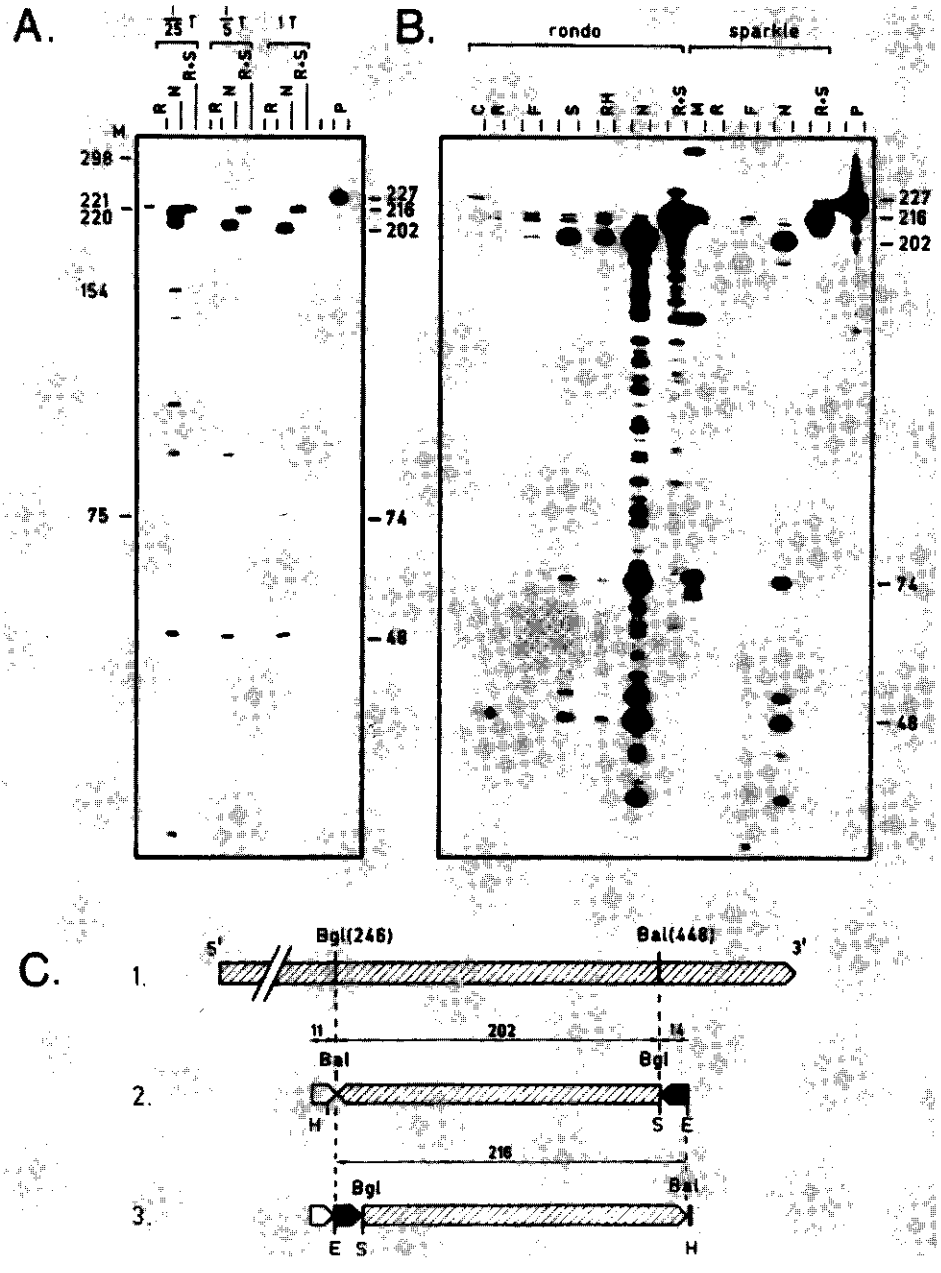


Fig. 8. Localization of ENOD12 transcripts in stem tissue by in situ hybridization. **A.** Bright field micrograph of a transection of the fourth internode of a 24-day-old pea plant, showing a central ring of vascular bundles (VB) with two fibrovascular bundles (FVB) and two cortical fiber bundles (CF) traversing the cortex (C). P: pith; arrow: fascicular cambium; arrowhead: interfascicular cambium. **B.** Dark field micrograph of the same section as in A. Silver grains representing hybridization signal are visible as white spots. The section was hybridized with ^{35}S-labeled ENOD12 antisense RNA. Bar = 300 μm.

(figure 9A, R+S lanes). This 216 bp band was absent when root RNA was hybridized to antisense ENOD12 RNA (figure 9A, R lanes). Hybridization of the antisense probe to nodule RNA and subsequent digestion resulted in a fully protected hybrid of 202 bp (fig. 9A, N lanes 1/5T and 1T) which was the expected size, as indicated in figure 9C. Using low concentrations of RNase T1 the trimming of the fully protected hybrid was still incomplete, resulting in products ranging in size between 202 and 220 bp (fig.9A, N lane 1/25T). In addition to the fully protected 202 bp hybrid, partially protected hybrids were formed (figure 9A, N lanes). Since these hybrids were not formed when the ENOD12 sense RNA was hybridized to the antisense probe (figure 9A, R+S lanes), we conclude that they originate from the second ENOD12 mRNA that occurs in nodules. Using increasing amounts of RNase many of these heterologous hybrids were degraded to smaller molecules, which cannot be analyzed on sequence gel as their size corresponds to the size of the fragments generated by digestion of the excess antisense RNA probes. However, the 74 and 48 bp fragments (figure 9A, N lanes) are examples of fragments which are not further degraded by increasing RNase concentrations. In conclusion, at fixed RNase concentration identical digestion patterns of the heterologous ENOD12 hybrid were obtained in different, independent experiments using nodule RNA from two different pea cultivars (figure 9A and 9B, N lanes). This indicates that with the RNase mapping method partially protected hybrids formed by hybridization of the antisense probe to a non-homologous mRNA are reproducibly detected as specific fragments on a

Fig. 9. RNase mapping of ENOD12 transcripts in nodule, stem, root hair and flower tissues. **A.** 20 µg total RNA from 8 day old roots (R), nodules 13 days after inoculation (N), and roots mixed with 1 ng 'sense' ENOD12 RNA transcribed from a T7 RNA polymerase vector (fig 9C,3) (R+S), was hybridized to a 227 nt 'antisense' ENOD12 RNA probe (fig 9C,2), followed by digestion with varying amounts of RNase T1 (1/25 T: 228 U/ml, 1/5 T: 1140 U/ml, 1 T: 5760 U/ml). Protected RNA molecules were separated by electrophoresis on a 6% polyacrylamide/urea gel and sizes were compared to pBR322 x HinfI size markers (M) and the input ENOD12 RNA probe (P). Vertical bars over the figure represent the borders of the different lanes. **B.** 20 µg total RNA form 8 day old roots (R), flowers (F), 4th internode stem sections from 35 day old plants (S), root hairs 48 hrs after inoculation (RH), nodules 13 days after inoculation (N), and root + 1 ng 'sense' ENOD12 RNA (R+S), was hybridized to 'antisense' ENOD12 RNA probe (P) as in A., followed by digestion with 5760 U/ml RNase T1 and electrophoresis on a 6% polyacrylamide/urea gel. Root, flower and nodule RNA was taken from two different cultivars, cv. 'Rondo' and cv. 'Sparkle'. A small portion of root RNA immediately after hybridization with probe without RNase digestion (C), and pBR322 x HinfI size markers (M), were also subjected to electrophoresis. Vertical bars over the figure represent borders of the different lanes. **C.** 1: Schematic representation of the cloned ENOD12 mRNA. The position of the restriction sites in the corresponding cDNA clone that were used to subclone the fragment used for RNase mapping is indicated; 2 and 3: Sequences present in the ENOD12 'antisense' (2), and 'sense' (3) T7 RNA transcripts. 202 nucleotides between the Bgl I site at position 246 and the Bal I site at position 448 in the pPsENOD12 cDNA (figure 9C1) (▨); 11 nt T7 RNA polymerase promoter region (▭); 14 nt pT7 polylinker (■). Complementary sequences are indicated by identically shaded inversed arrows. Dashed vertical lines depict the size of the hybridizing fragments upon hybridization of the antisense RNA probe to the homologous ENOD12 mRNA (202 nt.) and to the sense RNA transcript (216 nt.), respectively. Restriction sites in the corresponding DNA fragments: Bal = BalI, Bgl = BglI, E = EcoRI, H = HindIII, S = SmaI.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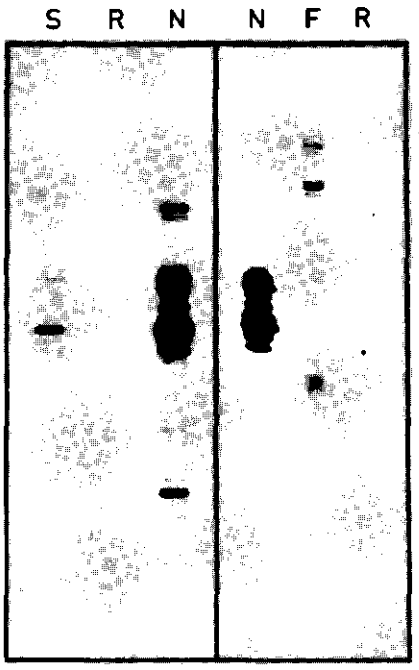

Fig. 10. Primer extension analysis of ENOD12 mRNA from nodule, stem and flower. An end-labeled oligonucleotide complementary to nt 70-90 of the cDNA sequence was annealed to total RNA from: 4th internode stem sections from 35 day old plants (S), 8 day old roots (R), nodules 13 days after inoculation (N), and flowers (F), extended with reverse transcriptase and subjected to electrophoresis on 6% polyacrylamide/urea gels. 20 µg total RNA was used for each extension, except in the extensions shown in the right panel where 80 µg total RNA from flowers and roots was used. The size of the largest reproducible extension fragment in each of the lanes is indicated to the right side (nt) to indicate the size differences between the two groups of extension products.

polyacrylamide gel. We concluded that RNase T1 mapping is useful for distinguishing between different mRNAs.

Using this RNase mapping method we analyzed RNA from stem and flower to determine which ENOD12 mRNAs occur in these tissues. We also analysed root hair RNA to investigate whether both ENOD12 mRNAs detected in nodule are already present at the early stages of infection thread formation. The 202 bp full-size protected hybrid as well as the 75 and 48 bp bands, specific for the partially degraded hybrid, are present in the RNase protection pattern of stem and root hair RNA as shown in figure 9B (S and RH lanes), indicating that in these parts of the plant the same ENOD12 mRNAs are present as in nodules. Using flower RNA we could visualize the full-size protected hybrid, indicating that mRNA corresponding to the isolated pPsENOD12 clone is present in flower tissue. We could not demonstrate in this experiment, nor in experiments using more flower RNA and prolonged exposures, the presence of both the 75 and 48 bp bands originating from the protected heterologous ENOD12 mRNA.

Long exposures like in figure 9B always revealed the presence of 216 and 212 bp fragments in all lanes including the root lane. The concentration of these fragments was dependent on the amount of probe input and not on the amount of hybridizing mRNA in the total RNA preparations. Therefore they are not due to the formation of a hybrid between probe and ENOD12 mRNA.

Since we could not detect differences between the ENOD12 mRNAs present in nodule, stem and flower using RNase mapping with the 3' region of pPsENOD12, we investigated whether differences were detectable at the 5' end of the ENOD12 mRNA in the different tissues. Therefore we extended a synthetic primer complementary to nucleotides 70-90 of the pPsENOD12 insert. After hybridizing with nodule, stem and flower RNA we compared the size of the extension products, as shown in figure 10 (lanes S and N). The sizes of the extension products with stem and nodule RNA appeared to be identical. Hence no difference in size of the 5' ends can be detected with this primer. In contrast, the extension products with flower RNA differed in size (figure 10, lane F). The largest extension product with flower RNA measured 16 extra nucleotides compared to the largest extension product with nodule RNA. This suggests that different transcriptional start sites are used on the gene corresponding to pPsENOD12 in flower and nodule, or alternatively the RNA transcript is differentially spliced at the 5' end in these tissues.

DISCUSSION

The ENOD12 gene product is involved in the infection process

The data presented in this paper demonstrate that the cDNA clone pPsENOD12 represents a gene encoding a (hydroxy-)proline-rich early nodulin, involved in the infection process which is part of the pea-*Rhizobium* interaction. pPsENOD12 is the first clone that represents a nodulin gene involved in a process occurring in root hairs. The expression of the ENOD12 gene in root hairs requires the presence of functional *nod* genes in Rhizobium. Therefore pPsENOD12 can be an important help for analyzing the mechanism by which the bacterial *nod* genes initiate the infection process.

Previously three cloned soybean early nodulins, GmENOD2, GmENOD13 and GmENOD55, have been shown to be involved in steps in nodule morphogenesis but not in the infection process (Franssen et al, 1987, 1988). Likewise the alfalfa and pea ENOD2 early nodulins are involved in nodule formation and not in the infection process (Dickstein et al, 1988; Van De Wiel et al, 1990). These early nodulins from soybean, alfalfa, and pea are all (hydroxy)proline-rich proteins. Strikingly, the infection related ENOD12 early nodulin is also proline-rich. As in the ENOD2 early nodulins from different species and in GmENOD13, the major part of the ENOD12 protein is composed

of repeating elements containing three amino acids interspersed with two or three prolines. The structure of these three early nodulins is very similar to the soybean cell wall protein 1A10 (Averyhart-Fullard et al, 1988). Because of the homology between 1A10 and ENOD12 we assume that ENOD12 is also a cell wall protein, involved in the infection process. The occurrence of a putative signal peptide, which might function in excreting the protein, is consistent with this hypothesis.

All root and nodule cells in which we have found ENOD12 transcript are sites of new cell wall synthesis and therefore possible sites of incorporation of the ENOD12 protein. In the root cortex cells containing the infection thread, the infection thread wall is formed. The root cortical cells preparing for infection thread passage, which contain ENOD12 mRNA, form an additional cell wall layer (Bakhuizen et al, 1988b). The dividing cells in the nodule primordium also form new cell walls. In the invasion zone of the developing nodule again the infection thread tips are sites of cell wall synthesis. Our present knowledge about ENOD12 does not allow predictions on a biochemical function of the protein in cell walls yet, but the absence of ENOD12 transcripts in pea roots infected with *F. oxysporum* indicates that the protein is not functional in a defense response.

Rhizobium nod genes are essential for the induction of ENOD12 gene expression.

Our observation that soluble compounds in a *Rhizobium*-free culture medium can induce ENOD12 gene expression, shows that physical contact between plant and bacterium is not a necessary prerequisite for ENOD12 gene expression. Therefore the direct role of bacterial genes in producing compounds involved in ENOD12 expression can be studied. The presence of ENOD12 transcript in root hairs of plants inoculated with the *R. leguminosarum* bv. *viciae* 248^{c} (pMP104) strain, carrying only cloned *nod*EFDABCIJ genes, shows that these Sym-plasmid genes are sufficient to induce ENOD12 gene expression. The absence of ENOD12 RNA in root hairs from plants inoculated with *R.leguminosarum* bv. *viciae* carrying a Tn5 mutation in *nod*A indicates that ENOD12 gene expression requires expression of at least one of the common *nod* genes. This is consistent with the fact that these genes are essential for the induction of the infection process. Furthermore the host-specific *nod*E gene, and/or the *nod*F gene present on the same operon, is also essential. Hence both common and host-specific *nod* genes appears to be involved in producing the factor(s) that elicit ENOD12 gene expression. The *R. meliloti nod*A and *nod*B genes have been shown to be involved in generating small soluble compounds that stimulate mitosis of plant protoplasts (Schmidt

et al, 1988). Faucher et al (1989) reported that *R. meliloti* common *nod* genes are essential for the production of root hair deformation factor, and that the *nod*H gene is involved in determining the host specificity of this factor. These authors hypothesize that the common *nod* genes produce a compound that can be modified to different factors, e.g. to root hair deformation factor by the *nod*H gene product. Whether the compound(s) inducing ENOD12 gene expression is also the result of a modification of a *nod*ABC dependent factor by the *nod*E product, or whether *nod*ABC and *nod*E enable production of different factors which are both necessary for induction of ENOD12 gene expression, cannot yet be decided.

Which molecular mechanisms lead to ENOD12 gene expression? We have shown that soluble compounds from *Rhizobium* which are excreted upon induction of the *nod* genes are the trigger in inducing expression of the early nodulin gene. Induction occurs in front of the growing infection thread and in the nodule primordium. This induction at significant distance from the bacteria indicates the involvement of factors which are capable to diffuse through several cell layers. In the invasion zone of the nodule ENOD12 mRNA is present in infected as well as in uninfected cells, as far as can be judged from our *in situ* hybridizations using ^{35}S-labeled probes. This observation is compatible with the notion of diffusible inducing compounds. Whether these are the bacterial compounds made under influence of the *nod* genes, or plant substances influenced by these compounds, is presently unknown. Clues to the mechanism involved in ENOD12 gene expression come from the observation that ENOD12 genes are expressed in the cells preparing for infection thread growth as well as in the mitotically reactivated cells of the initiating nodule primordium. A plant compound from the root vascular tissue, most likely present in the xylem, has been found to act in concert with plant hormones for the induction of primordia in the root inner cortex, similar to the nodule primordium (Libbenga et al, 1973). The root cortex cells preparing for infection thread passage show many structural analogies to cells in the nodule primordium and it has been postulated that a similar compound from the xylem and phytohormones are also involved in the preparation of these cells (Bakhuizen et al, 1988b). The analogy between these cells is supported by our observation that in both cell types ENOD12 genes are expressed. Hence we take into account that the postulated xylem factor and plant hormones are involved in the induction of ENOD12 gene expression. The necessary changes in phytohormone balance might be induced by the excreted bacterial compounds we have shown to be involved in induction of ENOD12 gene expression. The involvement of a xylem factor can explain the distribution of ENOD12 mRNA from the infection thread toward the nodule primordium near a xylem pole, at early stages of the infection process.

ENOD12 gene expression in stem and flower tissue

An important question concerning the evolutionary origin of the ENOD12 genes active in the Rhizobium infection process is whether the ENOD12 mRNAs in stem and flower are transcribed from the same genes. From Southern analysis and hybrid-released translation experiments we conclude that two genes are present in the pea genome, and they are both transcribed in nodules. Since the occurrence of these two mRNAs in stem and flower tissue could not be analyzed by standard means we successfully modified an RNase mapping procedure to distinguish between different ENOD12 transcripts. In general this method might be a useful tool to analyse differential transcription of gene families, since extensive cDNA cloning is not required. In summary, the conclusions from our RNase mapping and primer extension experiments are that both ENOD12 genes are expressed in nodule and stem tissue, whereas in flower tissue the expression of only one gene, corresponding to pPsENOD12, can be detected. The 5' end of this mRNA differs from that of its homologous counterpart in nodule. We assume that the difference in nodule and flower is due to a different start of transcription on the same gene, or by alternative splicing of an intron near the 5' end.

Nodulin genes are by definition genes exclusively expressed during root nodule formation and not in any other part of the plant (Van Kammen, 1984). Our finding that ENOD12 genes are expressed at a low level in flower and stem tissue shows that the ENOD12 genes are not true nodulin genes. However, in most other studies on nodulin genes the analyses have been restricted to root and nodule tissues. One can therefore expect that several genes considered to represent true nodulins are also used in other developmental programs in the plant. Recently this was demonstrated for the nodule specific glutamine synthetase gene. More detailed analyses showed that this gene is expressed at low levels in e.g. the stem of Phaseolus plants (Bennett et al, 1989). Also the expression of a globin gene in both roots and nodules from Parasponia suggests that leghemoglobin, the 'archetype' of the nodulins, might be expressed in non-symbiotic tissues (Bogusz et al, 1988). In conclusion, nodule formation involves not only genes that are specifically evolved for the benefit of the symbiosis, but also genes that are normally used in other parts of the plant, as examplified by the ENOD12 genes in pea and the "nodule specific" glutamine synthetase gene in bean. These genes are not expressed in uninoculated roots, and therefore their expression must be directly or indirectly induced by *Rhizobium* factors. It becomes an intriguing question whether *Rhizobium* is exploiting the regulatory mechanisms used in other parts of the plant, or whether new symbiotic regulatory mechanisms have evolved.

MATERIALS AND METHODS

Plant materials

Pea (*Pisum sativum* L. cv. rondo or sparkle) plants were cultured and inoculated with *R. leguminosarum* bv. *viciae* 248 as described by Bisseling et al (1978). Nodules were excised from root tissue, except in the case of pea plants 8 days after inoculation, where 2.5 cm sections of the main root, where nodules would appear, were harvested. Uninfected pea plants were cultured in the same way, and pieces of the main root were collected 8 days after sowing. Pea root hairs were brushed from the main root of seedlings, 48 hrs after inoculation of 3 day old seedlings, as described by Gloudemans et al. (1989).

Fusarium oxysporum mycelium was inoculated in Czapek-dox medium and grown for 2 days at 30°C. Pea plants were inoculated with this suspension three days after sowing, and cultured as above. Root tissue was harvested after various incubation times. All plant tissues were frozen in liquid nitrogen immediately after harvesting and stored at -70°C.

Preparation of Rhizobium-free culture medium

Bacterial free culture medium for the inoculation of plants was prepared as follows: *R.leguminosarum* bv. *viciae* 248 was grown in YMB medium to late log phase, diluted to $OD_{600} = 0.01$ in minimal medium and grown to late log phase, and diluted again 1:100 in plant medium containing 2μM naringenin. Bacteria were then grown to $OD_{600} = 0.3$. The culture was centrifiuged, and the supernatant was treated with chloroform and inoculated on 3 day old pea seedlings.

cDNA cloning

A λgt11 cDNA library, prepared from Pisum sativum cv. sparkle nodule RNA of 21 day old plants, was kindly provided by dr. G. Coruzzi (Tigney et al, 1987). Nitrocellulose replicas were made, containing phage DNA of approx. 3000 plaques, using standard procedures (Maniatis et al, 1982). ^{32}P-labeled cDNA probes were prepared from poly(A)+RNA of nodules from 10 day old plants, and of 8 day old, uninoculated roots. Replica filters were hybridized to either root or nodule cDNA as described by Franssen et al (1987). Plaques, giving a nodule-specific signal, were purified, phage DNA was isolated, and cDNA inserts were subcloned into pUC vectors using standard procedures (Maniatis et al, 1982).

RNA expression analyses

Total RNA from nodules and other tissues was isolated by phenol extraction and LiCl precipitation according to De Vries et al. (1982). Total RNA concentrations were measured spectrophotometrically. Equal amounts of total RNA, as indicated in the figure legends, were subjected to gel electrophoresis. RNA transfer blotting was performed as described by Franssen et al (1987), using GeneScreen membranes as support. Blots were hybridized to nick-translated cDNA inserts.

Genomic DNA isolation and blotting

Genomic DNA from pea leaves was isolated using the CTAB method, described by Rogers and Bendich (1988). Restriction enzyme digestions were performed under standard conditions. Digested DNA was electrophoresed on a 1% agarose gel and transferred to GeneScreen plus membranes (NEN) using ammonium acetate transfer (Rigaud et al, 1987). The blot was hybridized to nick-translated cDNA insert in 1M NaCl, 1%SDS, 10% dextran sulphate and 100 µg/ml denatured salmon sperm DNA at 65°C during 24 hr. Subsequently blots were washed, 2x10' in 2xSSC and 2x20' in 2xSSC/1% SDS at 65°C.

Hybrid-released translation

Selection of ENOD12 mRNA was done with the insert of PsENOD12 bound on DPT paper (BioRad) as described by Maniatis (1982). 50 µg denatured DNA was spotted on DPT discs of 0.5 cm^2. Hybridization to 1 mg total nodule RNA from 12 day old plants was done in 300 µl containing 50%(v/v) formamide; 0,1%SDS; 0.6 M NaCl; 4mM EDTA; 80 mM Tris-HCl (pH 7.8). Hybridization was initiated at 50°C and temperature was decreased to 37°C over a 6h period. Hybrid selected mRNA was translated in a wheat germ extract (BRL) using ^{35}S-met or ^{3}H-leu (NEN) as radioactive amino acid. Proteins were separated on SDS containing 25% acrylamide gels. Gels were fluorographed to Kodak XAR-5 films.

DNA sequencing

pPsENOD12 insert was sequenced using the double stranded dideoxy chain termination procedure (Korneluk et al, 1985). Both strands were sequenced entirely. Additionally, the insert of a second, independently obtained ENOD12 clone was sequenced, spanning nt 16-565 of the sequence in fig.3. Sequence data were stored and analyzed using programs written by R.Staden on VAX/VMS and

microVAX/VMS computers.

In situ hybridization

In situ hybridization was performed essentially as described by Cox & Goldberg (1988). The insert of pPsENOD12 was cloned in pT7-6 (kindly provided by dr. S.Tabor) and antisense RNA probes were transcribed using T7 RNA polymerase (New England Biolabs) and ^{35}S-UTP (NEN, 1000-1500 Ci/mmol) as the radioactive nucleotide. unlabeled UTP was not added. The probes were partially degraded to an average length of 150 nucleotides by heating for 90 min. at 60°C in 0.2 M Na_2CO_3/0.2 M $NaHCO_3$.

Plant tissues were fixed with 3% paraformaldehyde, 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4. Dehydration was performed in graded ethanol and xylol series and tissues were embedded in paraplast (Brunswick). 7 µm thick sections were attached to poly-L-lysine-coated slides. Sections were deparaffinized with xylol and rehydrated through a graded ethanol series. They were pretreated with 1 µg/ml proteinase K in 100 mM Tris/HCl, pH 7.5, 50 mM EDTA at 37°C for 30 min. and 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0 at room temperature for 10 min. Subsequently they were dehydrated in a graded ethanol series and air dried. Sections were hybridized with RNA probes (10^6 cpm/ml) in 50% formamide, 0.3 M NaCl, 10 mM Tris/HCl pH 7.5, 1 mM EDTA, 10% dextran sulphate, 1x Denhardt's, 500 µg/ml poly-A, 150 µg/ml yeast tRNA and 70 mM DTT for 16 hrs at 42°C. Washing was performed in 4 x SSC, 5 mM DTT at room temperature. Next sections were treated with 20 µg/ml RNase A in 0.5 M NaCl, 5 mM EDTA, 10 mM Tris/HCl pH 7.5 at 37°C for 30 min. and washed in the same buffer with 5 mM DTT at 37°C for 30 min. The final wash was twice 2 x SSC, 1 mM DTT at room temperature. Sections were dehydrated in graded ethanol and air dried. Slides were coated with Kodak NTB2 nuclear emulsion diluted with an equal volume 600 mM ammoniumacetate and exposed for 1-4 weeks at 4°C. They were developed in Kodak D19 developer for 3 min. and fixed in Kodak fix. Sections were stained with 0.025% toluidine blue O for 5 min. and mounted with DPX.

Polymerase chain reactions (PCR)

1 µg of the synthetic oligomer 5'-CGTGCAAATGTGACTTCTTG-3', complementary to nt. 263-283 of the ENOD12 cDNA sequence, and 1 µg root hair total RNA were annealed by heating 3 min. at 85°C in 10 µl annealing buffer (250 mM KCl, 1 mM EDTA and 10 mM Tris/HCl, pH 8.3), incubating for 30 min. at 52°C, and gradually cooling to 42°C for 30 min. 15 µl cDNA buffer (10 mM $MgCl_2$, 8 mM DTT, 0.4 mM of all four dNTPs, and 25 mM Tris/HCl, pH 8.3) and 5 U AMV reverse

transcriptase (Life Science) were added, and ENOD12-specific cDNA was synthesized at 42°C for 60 min. Then 55 µl Taq polymerase buffer (30 mM $(NH_4)_2SO_4$, 9 mM $MgCl_2$, 10 mM b-mercaptoethanol and 100 mM Tris/HCl, pH 8.8), 20 µl 5 mM dNTPs, 10 µl DMSO, 1.25 µl 2 M KCl, 1 µg of the synthetic oligomer 5'-CTTGTCCTCACTAGTGTT-3' (nt. 21-41 of the ENOD12 cDNA sequence), and 2 U Taq polymerase (Cetus) were added. The mixture was heated for 3 min. at 92°C, annealed for 2 min. at 52°C, and 12-16 of the following amplification cycles were performed: 5 min. at 70°C; 1 min. at 92°C; and 1 min. at 52°C. Amplified cDNA was ethanol precipitated, the pellet was dissolved in TE buffer and nucleic acids were separated on a 2% agarose gel. Upon DNA transfer blotting the amplified fragment was visualized by hybridization to ^{32}P-labeled ENOD12 insert. As established by three initial experiments the concentration differences between amplified ENOD12 cDNA from the different root hair RNA preparations reflected differences in the initial mRNA concentrations: 1) Differences in ENOD12 mRNA concentrations as revealed by the PCR method matched the differences revealed by RNA transfer blot analysis of 20 µg root hair RNA (cf figure 6 and 1B, WT and nodA$^-$ lanes); 2) PCR experiments on a dilution series of total nodule RNA revealed that, with 12-16 amplification cycles, initial mRNA concentration differences were reflected in the differences of the amounts of amplified ENOD12 cDNA; 3) amplification rates in different total root hair RNA preparations were compared by taking samples after different numbers of cycles. It appeared that the ENOD12 cDNA concentration indeed increased with the same rate in different root hair RNA preparations.

Primer extension analysis and RNA sequencing

The synthetic oligomer 5'-AGGTGATATTGAGCAAGTCC-3', complementary to nucleotide 70-90 of the pPsENOD12 sequence, was ^{32}P-labeled using T4 polynucleotide kinase (Pharmacia). 1.10^6 cpm of this primer was coprecipitated with 20 µg total RNA. Nucleic acids were resuspended in 6.25 µl annealing buffer (50 mM Tris/HCl, pH 8.2, 60 mM NaCl, 10 mM DTT), put at 68°C, and allowed to cool down to 35°C. 2.25 µl RT buffer (250 mM Tris/HCl, pH 8.2, 30 mM MgCl2, 500 mM NaCl, 50 mM DTT) 2.5 µl dNTP mixture (2 mM) and 0.5 µl AMV reverse transcriptase (Life Science, 25 U/µl) were added and primer extension was performed at 45°C for 20 min. Subsequently, 1 µl RNase A was was added and incubation was prolonged for 15 min. The mixture was extracted once with phenol/chloroform (1:1) and ethanol precipitated using 2 µg/ml tRNA as a carrier. Upon resuspension in 1.5 µl H_2O loading buffer was added and after denaturation samples were analyzed on a 6% polyacrylamide/8 M urea sequencing gel.

For RNA sequencing 5.10^6 cpm primer was coprecipitated with 80 µg total RNA. The precipitate was resuspended in 12.5 µl annealing buffer and annealed as described above. 4.5 µl RT buffer, 5 µl dNTP mixture (2 mM) and 25 U AMV RT were added. 4 µl of this solution was added to four separate tubes, containing 1 µl of one of the four dideoxyNTP's (800 µM). Extension was performed for

20 min. at 45°C. Subsequently 1 ul of the dNTP mixture was added for a chase reaction for 15 min. at 45°C. Samples were extracted, precipitated, and subjected to gel analysis as described above.

RNase mapping

The region of pPsENOD12 from the BglI site at position 246 up to the BalI site at position 448 containing 202 nucleotides from the 3' end of the cDNA was cloned into pT7-6. Antisense RNA was transcribed from this plasmid, after linearization immediately behind the insert, using T7 RNA polymerase (New England Biolabs) and ^{32}-P UTP (NEN) as labeled nucleotide. 50 µM unlabeled UTP was added to ensure 95-100% full size transcription. After synthesis the reaction was stopped with DNaseI (Boehringer) extracted once with phenol/chloroform (1:1) and once with chloroform, and unincorporated nucleotides were removed by spin-column chromatography (Maniatis et al 1982).

For RNase mapping 1.10^5 cpm of probe was coprecipitated with 20 µg total RNA. Pellets were resuspended in 30 µl hybridization buffer, and following denaturation at 85°C for 5 min the mix was incubated 16 hr at 45°C (Melton et al, 1984). Digestion with 640 to 5760 U/ml RNase T1 (BRL) was performed at 45°C for 60 min, RNases were removed by an additional incubation for 15 min with proteinase K and SDS at 37°C, all as described by Melton et al (1984). The mixture was extracted with phenol/chloroform (1:1) and precipitated with carrier tRNA and ethanol. The pellet was resuspended in H_2O, loading buffer was added and upon denaturation samples were analyzed on 6% polyacrylamide/ 8M urea sequencing gels.

REFERENCES

Averyhart-Fullard, V., Datta, K., and Marcus, A. (1988). A hydroxyproline rich protein in the soybean cell wall. Proc. Natl. Acad. Sci. USA 85, 1082-1085.

Bakhuizen, R., van Spronsen, P.C., Diaz, C.L., and Kijne, J.W. (1988a). Rearrangement of cytoplasm and endoplasmic microtubules in cortical cells of Pisum sativum L. roots prior to infection by Rhizobium leguminosarum biovar viciae. In: The plant cytoskeleton in the Rhizobium-legume symbiosis, Ph.D Thesis Leiden University, The Netherlands, pp. 57-81.

Bakhuizen, R., van Spronsen, P.C., and Kijne, J.W. (1988b). Cell wall degradation and infection thread initiation in pea root cortical cells during infection by Rhizobium leguminosarum biovar viciae. In: The plant cytoskeleton in the Rhizobium-legume symbiosis, Ph.D Thesis Leiden University, The Netherlands, pp. 83-107.

Bauer,W.D. (1981). Infection of legumes by Rhizobia. Ann. Rev. Plant Physiol. 32, 407-449.

Bennett, M.J., Lightfoot, D.A., and Cullimore, J.V. (1989). cDNA sequence and differential expression of the gene encoding the glutamine synthetase polypeptide of Phaseolus vulgaris L. Plant Mol. Biol. 12, 553-565.

Bergmann, H., Preddie, E., and Verma, D.P.S. (1983). Nodulin-35: a subunit of specific uricase (uricase II) induced and localized in the uninfected cells of soybean nodules. EMBO J. 2, 2333-2339.

Bisseling, T., Van den Bos, R.C., and Van Kammen, A. (1978). The effect of ammonium nitrate on the synthesis of nitrogenase and the concentration of leghemoglobin in pea root nodules induced by Rhizobium leguminosarum. Biochim. Biophys. Acta 539, 1-11.

Bogusz, D., Appleby, C.A., Landsmann, J., Dennis, E.S., Trinick, M., and Peacock, W.J. (1988). Functioning haemolglobin in non-nodulating plants. Nature 331, 178-180.

Brisson. N., and Verma, D.P.S. (1982). Soybean leghemoglobin gene family: normal, pseudo, and truncated genes. Proc. Natl. Acad. Sci. USA 79, 4055-4059.

Callaham, D.A., and Torrey, J.G. (1981). The structural basis for infection of root hairs of Trifolium repens by Rhizobium. Can. J. Bot. 59, 1947-1964.

Chen, J., and Varner, J.E. (1985). Isolation and characterization of cDNA clones for carrot extensin and a proline-rich 33-kDa protein. Proc. Natl. Acad. Sci. USA 82, 4399-4403.

Corbin, D.R., Sauer, N. and Lamb, C.J. (1987). Differential regulation of a hydroxyproline-rich glycoprotein gene family in wounded and infected plants. Mol. and Cell. Biol. 7, 4337-4344.

Cox, K.H., DeLeon, D.V., Angerer, L.M., and Angerer, R.C. (1984). Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. Dev. Biol. 101, 485-502.

Darvill, A.G., and Albersheim, P. (1984). Phytoalexins and their elicitors - a defense against microbial infection in plants. Ann. Rev. Physiol. 35, 243-275.

De Vries, S.C., Springer, J., and Wessels, J.H.G. (1982). Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. Planta 156, 129-135.

Dickstein, R., Bisseling, T., Reinhold, V.N., and Ausubel, F.M. (1988). Expression of nodule-specific genes in alfalfa root nodules blocked at an early stage of development. Genes and Development 2, 677-687.

Djordjevic, M.A., Gabriel, D.W., and Rolfe, B.G. (1987). Rhizobium - the refined parasite of legumes. Ann. Rev. Phytopathol. 25, 145-168.

Faucher, C., Camut, S., Dénarié, J., and Truchet, G. (1989). The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as avirulence genes in *Rhizobium leguminosarum* bv. Viciae and determine changes in the production of plant-specific extracellular signals. Mol. Plant-Microbe Interac. 2, 291-300

Fortin, M.G., Zelechowska, M., and Verma, D.P.S. (1985). Specific targeting of membrane nodulins to the bacteroid-enclosing compartment in soybean nodules. EMBO J. 4, 3041-3046.

Fortin, M.G., Morrison, N.A., and Verma, D.P.S. (1987). Nodulin-26, a peribacteroid membrane

nodulin is expressed independently of the development of the peribacteroid compartment. Nucl. Acids Res. 15, 813-824.

Franssen, H.J., Nap, J.P., Gloudemans, T., Stiekema, W., Van Dam, H., Govers, F., Louwerse, J., Van Kammen, A., and Bisseling, T. (1987). Characterization of cDNA for nodulin-75 of soybean: a gene product involved in early stages of root nodule development. Proc. Natl. Acad. Sci. USA 84, 4495-4499.

Franssen, H.J., Scheres, B., Van De Wiel, C., and Bisseling, T. (1988). Characterization of soybean (hydroxy)proline-rich early nodulins. In: Molecular Genetics of Plant-Microbe interactions, R.Palacios and D.P.Verma, eds. (St. Paul: APS Press), pp 321-326.

Gloudemans, T., Bhuvaneswari, T.V., Moerman, M., Van Brussel, T., Van Kammen, A., and Bisseling, T. (1989). Involvement of Rhizobium leguminosarum nodulation genes in gene expression in pea root hairs. Plant Mol. Biol. 12, 157-167.

Govers, F., Gloudemans, T., Moerman, M., Van Kammen, A., and Bisseling, T. (1986). Rhizobium *nod* genes are involved in inducing an early nodulin gene. Nature 323, 564-566.

Govers, F., Nap, J.P., Van Kammen, A, and Bisseling, T. (1987) Nodulins in the developing root nodule. Plant Physiol. Biochem. 25, 309-322.

Horvath, B., Kondorosi, E., John, M., Schmidt, J., Török, J., Györgypal, Z., Barabas, I., Wieneke, U., Schell, J., and Kondorosi, A. (1986). Organization, structure and function of Rhizobium meliloti nodulation genes determining host specificity in alfalfa. Cell 46, 335-343.

Jacobs, F.A., Zhang, M., Fortin, M.G., and Verma, D.P.S. (1987). Several nodulins of soybean share structural domains but differ in their subcellular locations. Nucl. Acids Res. 15, 1271-1280.

Korneluk, R.G., Quan, F., and Gravel, R.A. (1985). Rapid and reliable dideoxy sequencing of double-stranded DNA. Gene 40, 317-323.

Libbenga, K.R., Van Iren, F., Bogers, R.J., Schraag-Lamers, M.F. (1973). The role of hormones and gradients in the initiation of cortex proliferation and nodule formation in Pisum sativum L. Planta 114, 29-39.

Libbenga, K.R., and Bogers, R.J. (1974). Root nodule morphogenesis. In The Biology of Nitrogen Fixation, A. Quispel, ed. (Amsterdam, North Holland Publishing Company), pp. 430-472.

Long, S.R. (1989). Rhizobium-legume nodulation: life together in the underground. Cell 56, 203-214.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular cloning: a laboratory manual. (Cold Spring Harbor, NY, Cold Spring Harbor Laboratory)

Melton, D.A., Krieg, P.A., Rebabliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984). Efficient 'in vitro' synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nuc. Acids Res. 12, 7035-7056.

Mullis, K.B., and Faloona, F.A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Meth. Enzymol. 155, 335-350.

Newcomb, W. (1976). A correlated light and electron microscopic study of symbiotic growth and

differentiation in Pisum sativum root nodules. Can. J. Bot. 54, 2163-2186.

Newcomb, W. (1980). Control of morphogenesis and differentiation of pea root nodules. In Nitrogen Fixation, vol II, W.E. Newton and W.H. Orme-Johnson, eds. (Baltimore: University Park Press), pp 87-102.

Rigaud, G., Grange, T., and Pictet, R. (1987). The use of NaOH as transfer solution onto nylon membrane decreases the hybridization efficiency. Nuc. Acids Res. 15, 857.

Rogers, S.O., and Bendich, A.J. (1988). Extraction of DNA from plant tissues. In Plant Molecular Biology Manual, S.B. Gelvin, and R.A. Schilperoort, eds. (Dordrecht, Kluwer Academic publishers), A6, pp. 1-10.

Saiki, S., Scharf, F., Faloona, K., Mullis, G., Horn, G., Erlich, H., and Arnheim, N. (1985). Enzymatic amplification of b-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230, 1350-1354.

Sandal, N.N., Bojsen, K., and Marcker, K.A. (1987). A small family of nodule specific genes from soybean. Nucl. Acids Res. 15, 1507-1519.

Schmidt, J., Wingender, R., John, M., Wieneke, U., and Schell, J. (1988). *Rhizobium meliloti nodA* and *nodB* genes are involved in generating compounds that stimulate mitosis of plant cells. Proc. Natl. Acad. Sci. USA 85, 8578-8582.

Showalter, A.M., Bell, J.N., Cramer, C.L., Bailey, J.A., Varner, J.E., and Lamb, C.J. (1985). Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection. Proc. Natl. Acad. Sci. USA 82, 6551-6555

Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Pees, E., and Lugtenberg, B.J.J. (1987). Promoters in the nodulation region of the Rhizobium leguminosarum sym plasmid pRL1JI. Plant Mol. Biol. 9, 29-37.

Tigney, S.V., Walker, E.L., and Coruzzi, G.M. (1987). Glutamine syntetase genes of pea encode distinct polypeptides which are differentially expressed in leaves, roots and nodules. EMBO J. 6, 1-9.

Turgeon, B.G., and Bauer, W.D. (1985). Ultrastructure of infection-thread development during the infection of soybean by Rhizobium japonicum. Planta 163, 328-349.

Van Brussel, A.A.N., Pees, E., Spaink, H.P., Tak, T., Wijffelman, C.A., Okker, R.J.H., Truchet, G., and Lugtenberg, B.J.J. (1988). Correlation between Rhizobium leguminosarum nod genes and nodulation phenotypes on vicia. In Nitrogen fixation, hundred years after, H. Bothe, F.J. de Bruijn, and W.E. Newton, eds. (Stuttgart-New York: Gustav Fisher), p. 483.

Vance, C.P. (1983). Rhizobium infection and nodulation: a beneficial plant disease? An. Rev. Microbiol. 37, 399-424.

Van De Wiel, C., Scheres, B., Franssen, H.J., van Lierop, M.J., van Lammeren, A., Van Kammen, A., and Bisseling, T. (1990). The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. EMBO J., in the press.

Van Kammen, A. (1984). Suggested nomenclature for plant genes involved in nodulation and symbiosis.

Plant Mol. Biol. Rep. 2, 43-45.

Von Heijne, G. (1983). Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. 133, 17-21.

Wijffelman, C.A., Pees, E., Van Brussel, A.A.N., Okker, R.J.H., and Lugtenberg, B.J.J. (1985). Genetic and functional analysis of the nodulation region of Rhizobium leguminosarum sym plasmid pRL1JI. Arch. Microbiol. 143, 225-232.

Zaat, S.A.J., Van Brussel, A.A.N., Tak, T., Pees, E., and Lugtenberg, B.J.J. (1987). Flavonoids induce Rhizobium leguminosarum to produce nodDABC gene-related factors that cause thick, short roots and root hair responses on common vetch. J. Bacteriol. 169, 3388-3391.

CHAPTER 8

A molecular marker for the development of the uninfected cells in the central tissue of pea (*Pisum sativum*) root nodules.

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SUMMARY

The pea early nodulin Nps-40' that has been identified as an *in vitro* translation product of nodule mRNA, is shown to comigrate with a 40 kD protein isolated from pea nodules. Like the Nps-40' *in vitro* translation product, the putative Nps-40' *in vivo* protein appears relatively early during nodule development, well ahead of the late nodulin leghemoglobin. An antiserum raised against the putative Nps-40' *in vivo* protein reacted with both the putative Nps-40' *in vivo* protein and the Nps-40' *in vitro* translation product, affirming that these must be identical proteins. The antiserum also reacted with some other 40 kD proteins that were identified, using an antiserum against glutamine synthetase (GS) of bean, as various forms of GS. The cross reaction of the anti-Nps-40'-serum with the other 40 kD proteins could be diminished by titration with GS from bean. Using this titrated antiserum, the early nodulin was shown by immunolabeling to be exclusively located in the cytosol of the uninfected cells in the early and late symbiotic zone of pea nodules. This implies that the uninfected cells in the indeterminate pea nodule have a nodule-specific role in the symbiosis with *Rhizobium* bacteria, just like in the nodules of the determinate growth type, such as those of soybean. Furthermore, the anti-Nps-40'-serum cross-reacted with the nodulins Nvs-40 and Nms-30 of the indeterminate nodules of common vetch and alfalfa, respectively. The possible function of Nps-40'and the regulation of the expression of the gene for this early nodulin are discussed in the light of earlier studies on its occurrence various indeterminate nodules blocked at different stages of development due to the use of engineered bacterial strains.

INTRODUCTION

Root nodules of legumes formed upon infection with bacteria from the genera *Rhizobium* or *Bradyrhizobium* contain a specialized tissue, the central tissue, in which nitrogen fixation occurs. Two cell types can be recognized in this central tissue: the infected cells, which harbour the nitrogen-fixing bacteroids, and the uninfected cells, which occur intermingled with the infected cells. The uninfected cells are characterized by the presence of a large central vacuole and plastids, which often contain prominent starch grains.

Legume root nodules can be divided into two types: the determinate and the

indeterminate growth type (for review: Sprent, 1980). During the formation of both types of nodules, nodule-specific proteins -nodulins- are made. Most of the nodulins localized so far, are found in the infected cell type of determinate and/or indeterminate nodules (Nap & Bisseling, 1990; Scheres et al., 1990).

In the determinate nodules of soybean (*Glycine max*) a single nodulin was shown to occur specifically in the uninfected cells (Bergmann et al. 1983). This nodulin is a nodule-specific form of uricase (n-uricase) occurring in the peroxisomes of the uninfected cells in soybean (Van den Bosch & Newcomb, 1986; Kaneko & Newcomb, 1987), and also in cowpea (*Vigna unguiculata*) nodules (Webb & Newcomb, 1987). Uricase catalyzes one of the final steps in the ammonium assimilation pathway, leading to the production of the ureides, allantoin and allantoic acid. These compounds are in these species the main form in which the fixed nitrogen is transported to other parts of the plant (cf. Schubert, 1986). In the determinate soybean nodule, the uninfected cells have been shown to constitute a coherent network throughout the whole central tissue, which will allow for an efficient transportation of the ureides to the peripherally located vascular bundles of the nodule (Selker, 1988).

The indeterminate pea (*Pisum sativum*) nodules export fixed nitrogen in the form of amino acids like glutamine and asparagine (Schubert, 1986). In these nodules, uricase is not formed and nodulins specific for the uninfected cells of this type of nodule have not yet been identified. Consequently, it is unclear whether the uninfected cells in the indeterminate nodules also have a specific role in the assimilation of the fixed nitrogen or some other function related to the symbiosis.

In the indeterminate nodule the cells are of graded age from the persistent distal meristem to the root attachment point, which enables the examination of the development of different cell types in a single longitudinal section of a nodule (Newcomb, 1981). In that way, it was found that the development of the central tissue in pea nodules is attended by the sequential expression of several nodulin genes (Scheres et al., 1990). The first nodulin genes expressed during development are the early nodulin genes ENOD12 and ENOD5. Transcripts of these genes are detectable in the invasion zone, i.e. the zone of cells immediately adjacent to the distal meristem of the nodule. In this zone infection threads penetrate cells just added by the meristem, and release rhizobia into them. Whereas ENOD12 messenger is present in all cells of the invasion zone, the ENOD5 gene is only expressed in cells that have been penetrated by an infection thread (Scheres et al., 1990).

So, as soon as a cytological recognition of infected cells is possible, the infected cells are marked by the presence of ENOD5 transcripts. At this stage, it is difficult to determine with certainty whether a cell is developing into the uninfected cell type. All

cells are developing a central vacuole and it is hard to exclude that a bacteria-free cell will after all become infected. As mentioned above, we are not aware of any report indicating that the uninfected cells of indeterminate nodules are also marked by the expression of specific genes. As a consequence, there are at present no molecular markers indicating whether a cell is determined to become an uninfected one, and neither it is feasible to define at which stage of development a cell becomes committed to development into the uninfected type. This hampers the identification of the mechanism underlying development of this cell type. While the differentiation into an infected cell might just be related to the release of bacteria from an infection thread, it is unclear which event triggers the development into an uninfected cell.

In this report we present evidence that the indeterminate pea nodule contains a nodule-specific protein that is found exclusively in the uninfected cells and probably corresponds to Nps-40', formerly identified as a pea early nodulin by Govers et al. (1985). With antibodies against Nps-40' we studied the time course of its appearance during pea nodule development and also its occurrence in other legume species. The results of our present study together with those of earlier studies on Nps-40' gene will be used to discuss how the differentiation into uninfected cells might be induced by *Rhizobium*.

RESULTS

Raising of an antiserum against the Nps-40' protein

The early nodulin Nps-40' was originally identified as an *in vitro* translation product of mRNA from pea root nodules by Govers et al. (1985). By comparing the proteins in extracts of pea nodules and roots, after separation on two-dimensional gels, a nodule-specific protein was detected, with a molecular weight and iso-electric point comparable to that of the Nps-40' *in vitro* translation product. This nodule-specific protein already occurred in extracts from root nodules at day 10 after sowing and inoculation; this is well ahead of the appearance of the leghemoglobins at day 13, in conformity to the time course of appearance of Nps-40' as detected by *in vitro* translation of nodule mRNA. We confirmed that the 40 kD nodule-specific protein comigrated with the Nps-40' *in vitro* translation product, by electrophoresis of a mixture of protein extracted from nodules and *in vitro* translation products of nodule RNA (results not

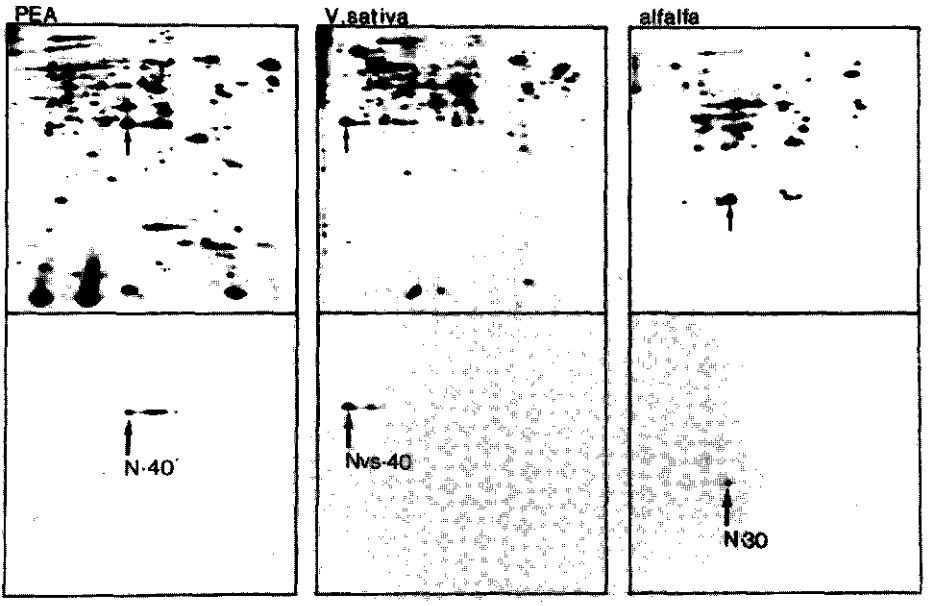


Fig. 1. Two-dimensional gel electrophoresis patterns of the *in vitro* translation products of nodule mRNA (upper panel) and of the *in vitro* translation products of nodule mRNA immunoprecipitated by an anti-Nps-40'-serum (lower panel) from pea, common vetch (V. sativa), and alfalfa, as indicated above the panels. In pea and vetch, the Nps-40' (N-40') and Nvs-40 *in vitro* translation products, respectively, are precipitated by the anti-Nps-40'-serum, together with some other 40 kD products; in alfalfa predominantly the Nms-30 *in vitro* translation product.

shown).

In order to raise an antiserum against Nps-40', we tried to purify the putative Nps-40' *in vivo* protein by ion exchange chromatography. However, for unknown reasons, we did not succeed in recovering the protein after chromatography on a DEAE column. Therefore, we separated proteins from ten-day-old nodules by preparative SDS/PAGE and used the mixture of proteins eluted from the 40 kD band to raise an antiserum. The specificity of this antiserum was tested by immuno-precipitation of *in vitro* translation products of nodule RNA. As shown in Fig. 1, the antiserum predominantly precipitated the Nps-40' protein. Hardly any precipitation of protein could be detected upon addition of the antiserum to the products obtained by *in vitro*-translation of root RNA (result not shown).

On a Western blot of proteins from pea nodules, separated by two-dimensional SDS/PAGE, the antiserum bound to several 40 kD proteins, one of them being Nps-40'. Most additional proteins, but not the putative Nps-40' protein, reacted with an antiserum

against glutamine synthetase from bean (*Phaseolus vulgaris*), suggesting that they are different isoforms of glutamine synthetase. Therefore, we decided to remove antibodies that might be directed against glutamine synthetase, by titration of the anti-Nps-40'-serum with purified glutamine synthetase from bean. This resulted indeed in a more specific antiserum, as on Western blots of nodule proteins, the purified antiserum then predominantly bound to the Nps-40' protein. A slight cross reaction with some other 40 kD proteins of different pI was still visible (results not shown). These proteins might represent posttranslationally modified forms of Nps-40', but a remaining cross-reaction with some forms of glutamine synthetase, or even other 40 kD proteins can not be excluded. The titrated antiserum was used to localize the Nps-40' protein on sections of pea nodules.

Besides we examined whether a protein similar to Nps-40' occurred in other leguminous species. For that purpose, we performed with the crude anti-Nps-40'-serum an immuno-precipitation experiment on proteins produced by *in vitro* translation of nodule RNA from vetch (*Vicia sativa*) and alfalfa (*Medicago sativa*). As shown in Fig. 1, the anti-Nps-40'-serum precipitated translation products previously identified as the Nvs-40 (Moerman et al., 1987) and Nms-30 nodulins (Lang-Unnasch & Ausubel, 1985), respectively. This suggests that early nodulins of the Nps-40' type are likewise important in other legumes of the indeterminate growth type. No proteins were observed to precipitate from *in vitro* translation products of mRNA from the determinate nodules of soybean with the anti-Nps-40'-serum.

Localization of Nps-40' in pea nodules

Sections of pea nodules were incubated with the titrated antiserum against Nps-40' and binding of the serum was visualized by silver-enhanced protein A-gold labelling. As shown in Fig. 2A, the titrated anti-Nps-40'-serum binds predominantly to uninfected cells, including the uninfected cells that form the boundary layer delimiting the central tissue from the peripheral tissue of the nodule. Moreover, a reaction with the anti- Nps-40'-serum is already clearly detectable in the early symbiotic zone of the nodule, which is consistent with the early appearance of Nps-40' during nodule development, as described above. The amount of anti-Nps-40'-serum binding diminishes in older parts of the late symbiotic zone (results not shown).

Since the possibility of cross-reaction of the anti-Nps-40'-serum with glutamine synthetase remained, sections of pea nodules were also incubated with the untitrated anti-Nps-40'-serum (Fig. 2B) and with a serum against glutamine synthetase of bean (Fig.

2C), respectively. By far the largest amounts of glutamine synthetase were detected in infected cells of the late symbiotic zone (Fig. 2C). This preponderance of glutamine synthetase in infected cells had already been shown for soybean (Verma et al., 1986). Contrary to the titrated anti-Nps-40'-serum, the untitrated antiserum bound not only to the uninfected cells, but also to the infected cells (Fig. 2B). These observations taken together imply that the binding of the anti-Nps-40'-antiserum to uninfected cells can not be attributed to a possible reaction with glutamine synthetase.

The subcellular location of the reaction with the anti-Nps-40'-serum was studied with the aid of electron microscopy, in which antibody binding was visualized by 10 nm gold particles coupled to protein A. In this way, it was shown that anti-Nps-40'-serum binding was predominantly to the cytoplasm of the uninfected cells (Fig. 3). Thus, very little antiserum binding was detected in the nucleus (Fig. 3A). Hardly any antiserum binding could be detected in mitochondria (Fig. 3B), plastids or the vacuole. Thus, the anti-Nps-40'-serum binding appears to be restricted to the cytosol. A possible binding to proteins in the endoplasmic reticulum can not be ruled out, since the latter cell organelle was not preserved well enough in our material to recognize it unequivocally.

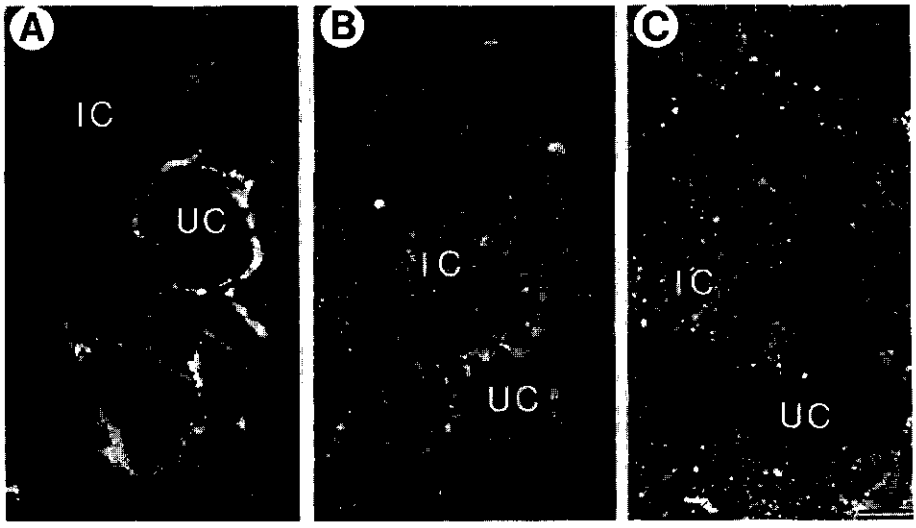


Fig. 2. Immunogold-silver labeling of semithin cryosections from a two-week-old pea nodule elicited by *R. leguminosarum* bv. *viciae*. The epipolarization micrographs show details of the central tissue in the late symbiotic zone, incubated with anti-Nps-40'-serum titrated with GS from bean (A), with untitrated anti-Nps-40'-serum (B), and with anti-bean GS-serum (C), respectively. In A, a prominent labeling signal in the form of white silver grains is visible over the parietally localized cytoplasm of the uninfected cells (UC); in B, silver grains are visible over both the infected (IC) and the uninfected cells; and in C, silver grains are predominantly present over the infected cells. Bar = 50 µm.

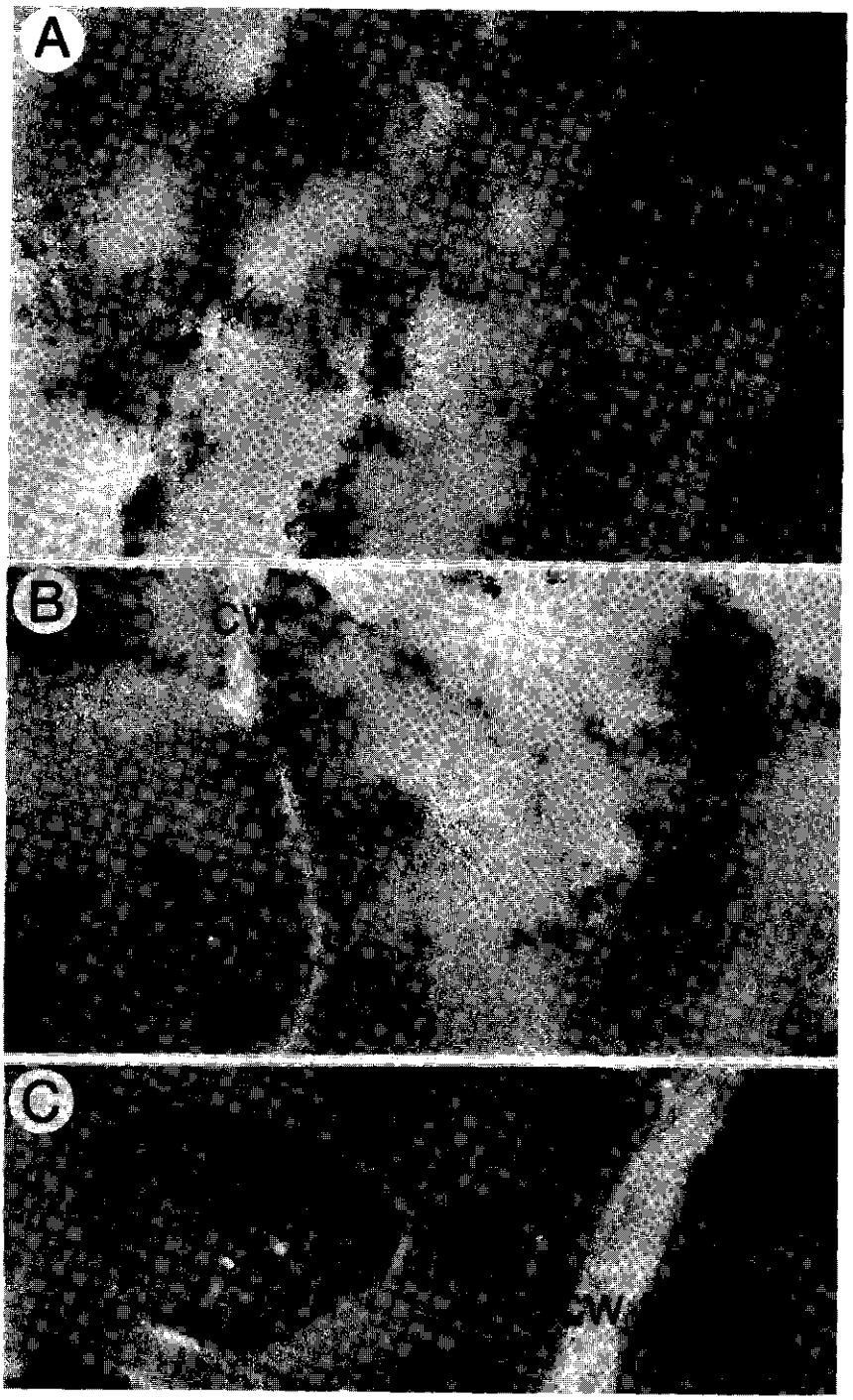
DISCUSSION

The results show the specific occurrence of a protein in the uninfected cells of the central tissue of pea nodules. This protein most likely corresponds to the early nodulin Nps-40', previously identified by two-dimensional SDS/PAGE of *in vitro* translated pea nodule mRNA by Govers et al. (1985).

For Nps-40' the corresponding cDNA is still lacking. In a set of pea early nodulin cDNA clones (Scheres et al., 1990), none could be identified as coding for Nps-40'. Therefore, in order to obtain a probe for the localization of the expression of the Nps-40' gene, an antiserum was raised, even though we did not succeed in purifying from nodule extracts the protein that exactly comigrates with the *in vitro* translated Nps-40' from other 40 kD proteins. These other 40 kD proteins most probably were isoforms of GS, as indicated by their reaction with an antiserum against purified GS from bean. Therefore, the specificity of our antiserum could be improved by titration with GS from bean. The antiserum reacted with the *in vitro* translated Nps-40' in immunoprecipitation experiments and with the putative *in vivo* protein on Western blots, affirming that these must be identical proteins. The titrated antiserum still gave a weak reaction on Western blots with 40 kD proteins other than Nps-40'. Anyhow, the titrated antiserum appeared to be very useful in demonstrating by immunolabeling the specific occurrence of Nps-40' in the uninfected cells, and gave virtually no reaction with proteins in the infected cells. Since GS was demonstrated to be prominent in the infected cells and relatively scarce in the uninfected cells, the weak cross reaction of the titrated antiserum with other 40 kD proteins on Western blots may be attributable to post-translationally modified forms of Nps-40', rather than to residual antibodies against GS. Also, there remains the remote possibility that the reaction of the titrated antiserum with uninfected cells is at least partly due to the presence of protein(s) other than GS or Nps-40', as the identity of the other 40 kD proteins has not yet been confirmed by independent methods.

In accordance with Nps-40' being an early nodulin, the protein specific for the uninfected cells is detectable histologically at a relatively early stage of nodule tissue differentiation, i.e. in the early symbiotic zone. The protein can, therefore, be regarded as a marker for the differentiation of the uninfected cell type.

Homologous early nodulins are present in vetch (Nvs-40, Moerman et al., 1987) and alfalfa (Nms-30, Lang-Unnasch & Ausubel, 1985), as indicated by their cross reaction with the anti-Nps-40'-serum. This suggests that N-40' might serve a role in the uninfected cells in several legumes of the indeterminate nodule growth type. Moreover, the exclusive presence of a nodule-specific protein in the uninfected cells of both the early


A
B
C

and late symbiotic zones indicates that, like in the determinate nodules, this cell type might play a specific role in the symbiosis in indeterminate nodules as well.

It is not apparent from our results whether the Nps-40' protein has a role in assimilating fixed nitrogen into a form suitable for transportation to the rest of the plant, like e.g. uricase in soybean (Schubert, 1986). Such a role for Nps-40' is not very likely for several reasons: 1) Nps-40' does not seem to be a form of glutamine synthetase, as it does not bind anti-glutamine synthetase-serum; 2) in pea, the fixed nitrogen is mainly exported out of the nodule in the form of glutamine and asparagine; these amino acids are formed in the infected cells (Schubert, 1986). Therefore, there is no *a priori* need for an extra specific cell type for the production of these amino acids can be reasoned, like for the ureides in soybean; 3) the relatively early appearance during nodule development argues against a role for N-40' in nitrogen assimilation.

With regard to the regulation of Nps-40' gene expression and the possible role of Nps-40' in the symbiosis, the following observations in nodules at some point blocked in their development are relevant. It was found that an *Agrobacterium tumefaciens* transconjugant, harbouring a Sym plasmid from *Rhizobium leguminosarum* bv. *viciae*, elicits nodules on pea that contain infection threads but no intracellular bacteria in the central tissue. In contrast, the same transconjugant induces nodules on vetch in which the release of bacteria from the infection thread and the differentiation of infected and uninfected cells take place. In the vetch nodules, Nvs-40 could be clearly detected (Moerman et al., 1987; cf. Nap et al., 1989), whereas in the pea nodules Nps-40' was not found (Govers et al., 1986) (see Table 1). However, in alfalfa, the Nms-30 transcript is always detected, even in nodules lacking not only intracellular bacteria but also infection threads in the central tissue (Dickstein et al., 1988; Norris et al., 1988) (Table 1). These so-called "empty" nodules are elicited not only by a variety of bacteria, among which *A. tumefaciens* transconjugants carrying nodulation sequences from the *R. meliloti* Sym plasmid, but also by treatment with auxin transport inhibitors, such as TIBA or NPA (Hirsch et al., 1989).

Fig. 3. Immunogold labeling of ultrathin resin sections from a two-week-old pea nodule elicited by *R. leguminosarum* bv. *viciae*. The electron micrographs show details of cells from the central tissue in the late symbiotic zone, incubated with anti-Nps-40'-serum. A) Detail of an uninfected cell in the boundary layer, showing labeling of black gold particles over the cytoplasm; hardly any label is present over the nucleus (N). B) Detail of two contiguous uninfected cells in the boundary layer; again gold particles are present over the cytoplasm, but not over the mitochondrion (Mt). CW = cell wall. C) Detail of an infected cell contiguous with an uninfected cell; gold particles are present over the cytoplasm of the uninfected cell (right), but lack over the infected cell (left), in which bacteroids (B) can be observed. Bar = 0.3 μm.

Table 1. N-40' gene expression in nodules induced by *A. tumefaciens* transconjugants carrying rhizobial *nod* genes

Host plant	Bacterium	IT in CT	BaR	N-40' gene expression	Reference
Pea	Atum(pSym)	+	–	– (Nps-40'	Govers et al, 1986
Vetch	Atum(pSym)	+	+	+ (Nvs-40	Moerman et al.,1987
Alfalfa	Atum(nod)	–	–	+ (Nms-30)	Dickstein et al., 1988

Abbreviations: Atum(pSym) = *A. tumefaciens* (LBA2712) carrying the symbiotic plasmid of *R. leguminosarum* bv. *viciae*; Atum(nod) = *A. tumefaciens* (1038/pMH36) carrying *nod* genes of *R. meliloti*; IT = infection threads; CT = central tissue of nodule; BaR = bacterial release from infection thread in central tissue of nodule.

The presence of Nvs-40 in the *A. tumefaciens* transconjugant-elicited nodules on vetch, together with the observation that in these nodules infected and uninfected cells become differentiated, are consistent with the observation that the homologous Nps-40' gene is exclusively expressed in the uninfected cells of wild type *R. leguminosarum* bv. *viciae*-induced nodules on pea. No clear differentiation between infected and uninfected cells is recognizable in the *A.tumefaciens* transconjugant-induced nodules on pea, despite the presence of infection threads in the central tissue. Thus, the lack of detectable Nps-40' might indicate that indeed no differentiation of uninfected cells has occurred, which might, in turn, be attributed to the absence of bacterial release in the central tissue of these pea nodules. However, in the alfalfa "empty" nodules elicited by *A. tumefaciens* transconjugants there is at present no possible way of distinguishing infected and uninfected cells in the central tissue, not by using the presence of infection threads as criterion either. Yet, in such "empty" alfalfa nodules, Nms-30 has clearly been demonstrated. This suggests that, at least in alfalfa, induction of the expression of the Nps-40'-homologous gene happens - and that perhaps even uninfected cells can differentiate -, without prior infection of the central tissue. However, it is unknown whether Nms-30 indeed is a molecular marker for the uninfected cells in alfalfa. For the interpretation of the central tissue of "empty" nodules and the mode of induction of the expression of the Nms-30 gene, it will thus be very important to be able to localize the Nms-30 gene product in alfalfa.

In conclusion, a set of early nodulins of different legume species (Nps-40' of pea, Nvs-40 of vetch, and Nms-30 of alfalfa) have been shown to be immunologically related. In order to determine to what extent they are comparable in the regulation of their production and their role in symbiosis, a more direct and reliable probe than the above-

described antiserum has to be obtained for detecting the expression of the Nps-40' gene and its homologues in vetch and alfalfa. For that purpose, cDNA clones representing the Nps-40' early nodulin are now being isolated.

MATERIALS AND METHODS

Cultivation of plants and bacteria

Pisum sativum ssp. *sativum* L. cv "Rondo" and *Vicia sativa* ssp *nigra* L. (Ehrh.) were cultured in gravel trays and inoculated as described by Bisseling et al. (1978). *Medicago sativa* was cultured on agar slants and inoculated as described by Meade et al. (1982). The bacterial strains *Rhizobium leguminosarum* PRE (wild-type) and *Rhizobium meliloti* 7023 (*nodA*$^-$) were cultured as described (Bhuvaneswari et al., 1980, and Leigh et al., 1985, respectively).

Protein analysis and raising of the antiserum

In vitro translation products (made according to Gloudemans et al., 1987) and *in vivo* proteins (isolated according to Studer et al., 1987) of pea nodules were compared by separating a mixture of 5 µl of *in vitro* translation products from RNA of 17-day-old nodules, and 40 µg of *in vivo* proteins isolated from 20-day-old nodules, by two-dimensional gel electrophoresis according to De Vries et al. (1982); the resulting gel was silver-stained, dried and fluorographed to pre-flashed Kodak XAR-5 film.

For purification of the putative Nps-40' protein, anion-exchange chromatography was performed with an FPLC type Mono Q HR 5/5 (Pharmacia) column, volume 1.8 ml.: 5 mg of proteins in 500 µl from 17-day-old pea nodules were centrifuged for 30 min. at 30.000g, filtrated through ø 0.22 µm filters,and loaded. The column was washed with 5 ml of 10 mM KAc/50 mM Tris-HCL pH 7.5, and eluted with, successively, gradients of 50 to 200 mM KAc (10 mM/ml) and 200 to 500 mM KAc (35 mM/ml), and 1 M KAc (10 ml), all in 50 mM Tris-HCl pH 7.5. Fractions of 0.5 ml were sampled every minute and 30 µl of each fraction was analyzed by 12.5 % PAGE with silver-staining. Fractions containing 40 kD proteins (3 times concentrated with Sephadex G-25 according to Saul & Don, 1984) were analyzed by two-dimensional electrophoresis. The fraction most abundant in 40 kD proteins was labeled with ^{125}I according to Zabel et al. (1982) and used as a marker for preparative gel electrophoresis.

For preparative gel electrophoresis, 5 mg *in vivo* protein from 15-day-old nodules mixed with the ^{125}I-labeled fraction rich in 40 kD proteins from ion exchange chromatography, were separated on 10

% PA gels for 6 hrs. at 150 V, dried and fluorographed to Kodak XAR-5 films. Slices with labeled 40 kD proteins were cut from the gels and washed 3 times for 20 hrs. at 4^0C in 10 mM Tris-HCl pH 5; the eluents were pooled, freeze-dried and saved at -80^0C.

Antiserum against the electrophoretically purified 40 kD proteins was prepared by immunizing New Zealand white rabbits, essentially as described by Bisseling et al. (1979). Immunoprecipitation was performed according to Govers et al. (1985). The antiserum was titrated twice over a column of glutamine synthetase (from bean, a generous gift of Dr. J. Cullimore; cf. Cullimore & Miflin, 1984) coupled to activated CH-Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Western blotting was performed as described by Studer et al. (1987).

Immunocytochemistry

For light microscopy, 15day-old nodules were fixed in 4% formaldehyde (freshly prepared from parafomaldehyde) in 50 mM sodium phosphate buffer (pH 7.2) for 2 hrs. at room temperature (RT), and subsequently infiltrated with 0.1 M sucrose in buffer for 2 hrs., with 1 M sucrose overnight, and with 2.3 M sucrose for 4 hrs., before being frozen in liquid propane. Semithin sections (1 µm) were cut on a Reichert Ultracut E equipped with an FC4 cryokit at -80^0C, and transferred in drops of 2.3 M sucrose to polylysine-coated slides. Labeling with anti-bean GS-serum (a generous gift from Dr. J.V. Cullimore; cf. Cullimore & Miflin, 1984) anti-Nps-40'-antiserum and visualization of the signal with silver-enhanced proteinA-gold particles were as described (Nap et al., 1989).

For electron microscopy, 15-day-old nodules were fixed in 3% formaldehyde/0.25 % glutaraldehyde, embedded in LR White resin and sectioned as described (Van de Wiel et al., 1988). Sections were labelled with anti-Nps-40'-serum and the signal visualized with proteinA attached to 10 nm gold particles as for light microscopy, omitting the silver enhancement treatment. Sections were post-stained and observed under a Philips EM301 as described (Nap et al., 1989).

REFERENCES

Bergmann, H., Preddie, E., and Verma, D.P.S., 1983, Nodulin-35: a subunit of specific uricase (uricase II) induced and localized in the uninfected cells of soybean nodules, *EMBO J.*, 2: 2333.

Bisseling, T., Van de Bos, R.C., Weststrate, M.W., Hakkaart, M.J.J., and Van Kammen, A., 1979, *Biochim. Biophys. Acta*, 562: 515.

Cullimore, J.V., and Miflin, B.J., 1984, *J. Exp. Bot.*, 35: 581.

De Vries, S.C., Springer, J., and Wessels, J.G.H., 1982, *Planta*, 156: 129.

Dickstein, R., Bisseling, T., Reinhold, V.N., and Ausubel, F.M. (1988) Expression of nodule-specific genes in alfalfa root nodules blocked at an early stage of development. *Genes and Development* 2, 677-687.

Gloudemans, T., De Vries, S.C., Bussink, H.-J., Malik, N.S.A., Franssen, H.J., Louwerse, J., and Bisseling, T., 1987, *Plant Mol. Biol.*, 8: 395.

Govers, F., Gloudemans, T., Moerman, M., Van Kammen, A., and Bisseling, T., 1985, *EMBO J.*, 4: 861.

Govers, F., Moerman, M., Downie, J.A., Hooykaas, P., Franssen, H.J., Louwerse, J., Van Kammen, A., and Bisseling, T., 1986, *Nature*, 323: 564.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T., 1989, *Proc. Natl. Acad. Sci. USA* 86: 1244.

Kaneko, Y., and Newcomb, E.H., 1987, *Protoplasma*, 140: 1

Lang-Unnasch, N., and Ausubel, F.M., 1985, *Plant Physiol.*, 77: 833.

Leigh, J.A., Signer, E.R., and Walker, G.C., 1985, *Proc. Natl. Acad. Sci. USA*, 82: 6231.

Meade, H.M., Long, S.R., Ruvkun, G.B., Brown, S.E., and Ausubel, F.M., 1982, *J. Bacteriol.*, 149: 114.

Moerman, M., Nap, J.P., Govers, F., Schilperoort, R., Van Kammen, A., and Bisseling, T., 1987, *Plant Mol. Biol.*, 9: 171.

Nap, J.-P., and Bisseling, T., 1990, Nodulin function and nodulin gene regulation in root nodule development, *in*: Molecular biology of symbiotic nitrogen fixation, P.M. Gresshoff, ed., CRC Press, Boca Raton.

Nap, J.P., Van de Wiel, C., Spaink, H.P., Moerman, M., Van den Heuvel, M., Djordjevic, M.A., Van Lammeren, A.A.M., Van Kammen, A., and Bisseling, T., 1989, *Mol. Plant-Microbe Interac.*, 2: 53.

Newcomb, W., 1981, *Int. Rev. Cytol. Suppl.*, 13: 247.

Norris, J.H., Macol, L.A., and Hirsch, A.M., 1988, *Plant Physiol.* 88, 321-328.

Saul, A., and Don, M., 1984, *Anal. Biochem.*, 138: 451.

Scheres, B., Van Engelen, F., Van der Knaap, E., Van de Wiel, C., Van Kammen, A., and Bisseling, T., 1990b, *Plant Cell*, 2: 687.

Schubert, K.R., 1986, *Ann. Rev. Plant Physiol.*, 37: 539.

Selker, J.M.L., 1988, *Protoplasma*, 147: 178.

Sprent, J.I., 1980, *Plant Cell Env.*, 3: 35.

Studer, D., Gloudemans, T., Franssen, H.J., Fischer, H.-M., Bisseling, T., and Hennecke, H., 1987, *Eur. J. Cell Biol.*, 45: 177.

Van den Bosch, K.A., and Newcomb, E.H., 1986, *Planta*, 167: 425.

Van de Wiel, C., Nap, J.P., Van Lammeren, A.A.M., and Bisseling, T., 1988, *J. Plant Physiol.*, 132: 446.

Verma, D.P.S., Fortin, M.G., Stanley, J., Mauro, V.P., Purohit, S., and Morrison, M., 1986, *Plant Mol.*

Biol., 7: 51.

Webb, M.A., and Newcomb, E.H., 1987, *Planta*, 172: 162.

Zabel, P., Moerman, M., Van Straaten, F., Goldbach, R., and Van Kammen, A., 1982, *J. Virol.*, 41: 1083.

CHAPTER 9

General discussion

INTRODUCTION

One of the major goals of the studies described in this thesis has been the integration of molecular data on nodulin gene expression with microscopical observations in order to obtain more insight into legume root nodule development. In the first part of the thesis (chapters 3 = Van de Wiel et al., 1988 and 4 = Nap et al., 1989) nodulin gene expression in pea (*Pisum sativum*) and vetch (*Vicia sativa*) was determined by two-dimensional gel electrophoresis of *in vitro* translation products and by probing RNA transfer blots with nodulin cDNA both of nitrogen-fixing nodules and of nodules that were blocked in several stages of development by the use of engineered bacterial strains. To determine at which stage the development of these nodules was blocked, they were studied with the aid of light and electron microscopy. All these nodules exhibited the basic structure of the leguminous root nodule: a central tissue surrounded by a peripheral tissue, the latter divided by a nodule endodermis into an inner and an outer part. The inner part was traversed by the vascular bundles that link up with the root vascular system. Disturbances in the nodules, blocked in development, were apparent at several stages in the differentiation of the infected cells in the central tissue. Thus, the expression of individual nodulin genes could be correlated with specific stages of central tissue development. In summary, the results indicated that for the expression of the Nps-40'/Nvs-40 gene to occur the bacteria had to be released from the infection thread, but further development of the infected cells was not necessary; in nodules showing expression of the Nps-40'/Nvs-40 genes, the uninfected cells developed an apparently normal morphology. The expression of the Nvs-65 gene could only be detected when undisturbed development of the infected cells to become completely filled with bacteroids was observed, whereas the induction of the expression of the leghemoglobin genes needed even further development of the infected cells. On the other hand, transcripts of the ENOD2 gene could be detected in all the nodules studied, even when no release of bacteria was observed. These results have been reviewed by Nap & Bisseling (1990).

Moreover, in some cases, e.g. if the *Rhizobium meliloti exo* mutants or *Agrobacterium tumefaciens* transconjugants harbouring *R. meliloti nod* genes are used as inducing agents, bacteria-free pseudonodules are formed on alfalfa (*Medicago sativa*) that, although formed under the direction of the *nod* genes, differ in morphologic details from normal nodules (cf. Finan et al., 1985; Hirsch et al., 1985). Yet, these "empty" nodules express two nodulin genes: ENOD2 and Nms-30, respectively (Dickstein et al., 1988; Norris et al., 1988). The Nms-30 gene is most probably the alfalfa counterpart of

the Nps-40' gene (chapter 8).

The approach followed in the initial stage of this study provides only indirect information about the respective processes of nodule development in which the nodulins are involved. More precise information could be obtained by utilizing a more direct approach, as for example *in situ* localization of the nodulin gene products. The data on the appearance of leghemoglobin during development could indeed be confirmed and extended by such an approach, namely by immunolocalization of the protein in sections of pea and vetch nodules, in chapters 3 and 4, respectively.

In the second part of this thesis the localization of nodulin gene products was further pursued, by *in situ* hybridization for the detection of nodulin transcripts in the chapters 5 (= Van de Wiel et al., 1990b), 6 (= Van de Wiel et al., 1990a) and 7 (=Scheres et al., 1990a), and by immunolabeling for the detection of a nodulin protein in chapter 8. By these methods more information was obtained, not only about the production of the Nps-40' protein and the ENOD2 transcript, but also about a set of pea nodulin genes for which cDNA clones had become available in the meantime.

Relevant data of the nodulins involved have been summarized in Table 1. Localization data are provided by the Figures 1, 2 and 3. One gene, ENOD2, is expressed in the inner part of the peripheral tissue of both growth types of nodule, i.e. the indeterminate nodules (= nodules with the capability of basically indefinite growth by the possession of a persistent meristem at the tip of the nodule)of pea and alfalfa as well as the determinate nodules (= nodules lacking in persistent meristematic activity) of soybean (Fig. 1, see also chapters 5 and 6). The majority of nodulin genes, however, are expressed in the developing central tissue of the indeterminate nodule of pea (Fig. 2, see also chapters 7 and 8 and Scheres et al., 1990b): the PsENOD12 gene in all cells of the invasion zone; the PsENOD5 gene in the infected cells of both the invasion zone and the early symbiotic zone; the PsENOD3 and PsENOD14 genes in the infected cells of the late part of the early symbiotic zone and the early part of the late symbiotic zone; and the Nps-40' gene to all probability in the uninfected cells of the early and late symbiotic zones. The PsENOD5 and PsENOD12 genes are already expressed at a relatively early stage of nodule development (Fig. 3, see also chapter 7 and Scheres et al., 1990b): the PsENOD5 gene in all cells with actively growing infection thread tips, the PsENOD12 gene in the cells forming the nodule primordium and the cells through which the infection thread (will) grow. No comparable cDNA clones were available for the study of central tissue development of the determinate soybean nodule. In the following will be discussed what the approach of localization *in situ* has contributed to our understanding of the role of nodulins in root nodule development.

Table 1. Early nodulins for which a probe is available for *in situ* localization

Early nodulin	Characteristics	References
GmENOD2	HRGP-like (extracellular protein?)	Franssen et al., 1987 Franssen et al., 1989
MsENOD2	homologous to GmENOD2	Dickstein et al., 1988
PsENOD2	homologous to GmENOD2	Van de Wiel et al., 1990b (chapter 5)
PsENOD3	cluster of 4 cysteines (metal-binding protein?)	Scheres et al., 1990b
PsENOD5	rich in Pro, Ala, Ser, Gly (arabinogalactan protein?)	Scheres et al., 1990b
PsENOD12	HRGP-like (extracellular protein?)	Scheres et al., 1990a (chapter 7)
PsENOD14	homologous to PsENOD3	Scheres et al., 1990b
Nps-40'	sequence unknown, antiserum raised	Govers et al., 1985, chapter 8

GmENOD clones were isolated from a soybean (*Glycine max*) nodule cDNA library, MsENOD clones were isolated from an alfalfa (*Medicago sativa*) nodule cDNA library, and PsENOD clones from a pea (*Pisum sativum*) nodule cDNA library. For Nps-40' (nodulin 40' of pea) no clone is available. Abbreviation: HRGP, hydroxiproline-rich glycoprotein.

NODULINS AS MARKERS OF SPECIFIC NODULE TISSUES AND/OR OF SPECIFIC STAGES IN THE DEVELOPMENT OF THESE TISSUES

The expression of each of the nodulin genes has been shown to be restricted to specific cell types of the nodule. The expression of a specific nodulin gene could therefore be used as an marker for the possible presence of the respective cell types. This might be of help in the interpretation of nodules that differ in the usual organization of the relevant tissues, such as some of the already mentioned "empty" nodules on alfalfa. This will be more fully discussed in relation to the expression pattern of the ENOD2 gene below.

Moreover, several nodulin genes appear to be expressed only transiently during the development of their respective tissues. This opens the possibility to use them as

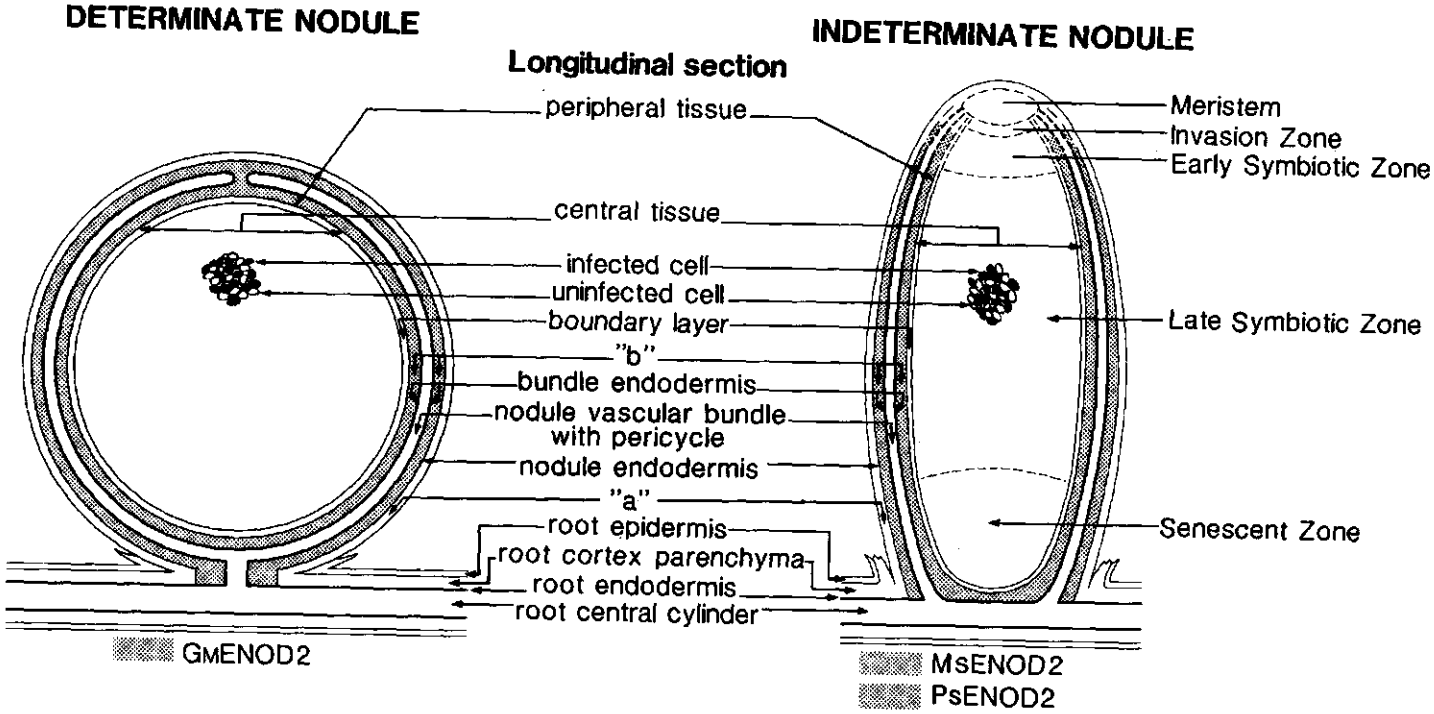


Fig. 1. Schematic representation of the *in situ* location of early nodulin ENOD2 transcripts in determinate (soybean) nodules and indeterminate (pea and alfalfa)nodules. "a" And "b" are the outer and inner part of the peripheral tissue, respectively; for a discussion of terminology for the peripheral tissue see the section on ENOD2 in the text.

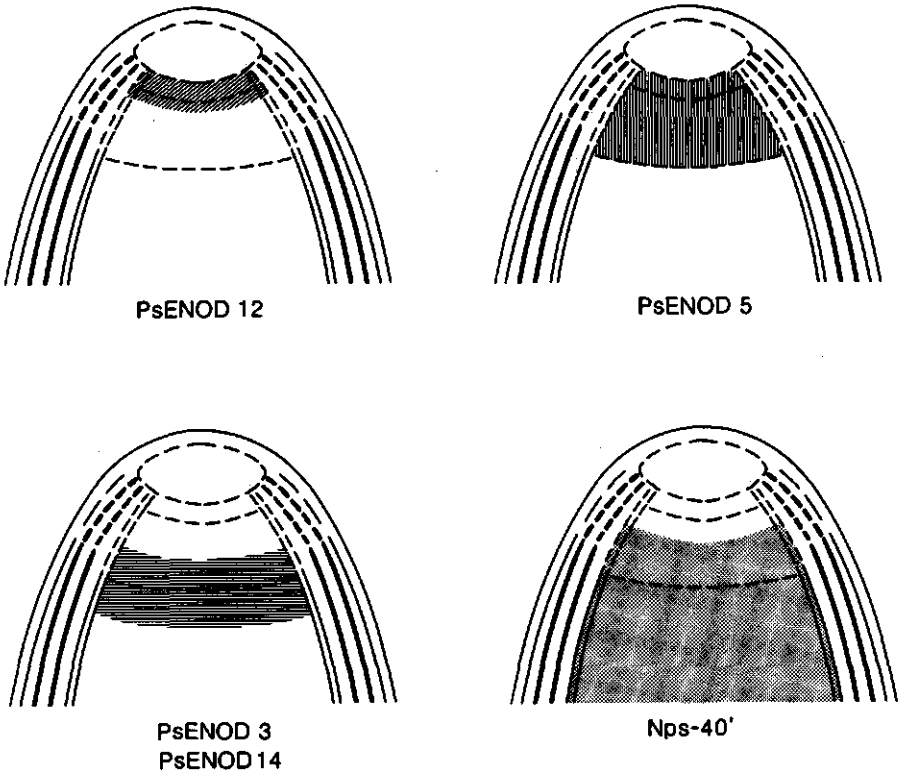


Fig. 2. Schematic representations of the tip of the indeterminate nodule depicted in Fig. 1, with the *in situ* locations of transcripts of the pea early nodulins PsENOD12, PsENOD3 and PsENOD14, and PsENOD5, respectively, and the most probable *in situ* location of the pea early nodulin Nps-40' protein. For the names of the depicted layers, see the corresponding part of Fig. 1.

markers for specific developmental stages of the relevant tissue. This will be illustrated with the nodulins present in the central tissue of the pea nodule.

In the indeterminate pea nodule, the sequential stages in the developmental process can be observed in a single longitudinal section, since the distal meristem continuously provides the nodule with new cells, which subsequently differentiate into the various nodular cell types. The relevant nodulin genes each are expressed at different developmental zones in the central tissue (Fig. 2, see also chapters 7 and 8 and Scheres et al., 1990b), as defined by Newcomb (1981). Transcripts of the PsENOD12 gene are detected in all cells of the invasion zone. Transcripts of the PsENOD5 gene are found in the infected cells of the invasion zone and the early symbiotic zone. The expression of the PsENOD5 gene is stronger in the infected cells of the early symbiotic zone than in those

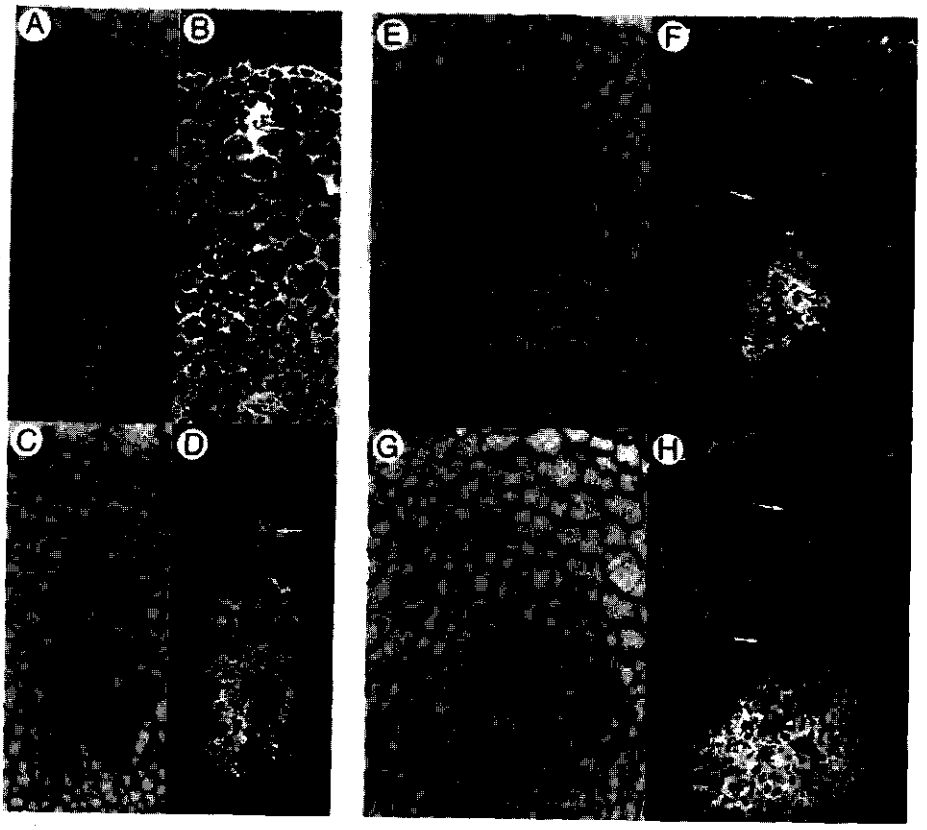


Fig. 3. Localization of ENOD5 and ENOD12 transcripts in root segments of 6 and 8 day old inoculated pea plants. The B, D, F, and H panels show dark field micrographs corresponding with bright field micrographs in the A, C, E, and G panels, respectively. In the dark field micrographs silver grains representing hybridization signal are visible as white spots. A and C: Root transections showing two infection events at similar developmental stages on 6 day old plants, 3 days after inoculation. The infection thread (arrow) has reached the third cortical cell layer, as determined by serial sectioning. In the inner cortex the nodule primordium (NP) is indicated. B: ENOD5 localization; D: ENOD12 localization. E and G: Similar stages of nodule development in roots of 8 day old plants, 5 days after inoculation. The infection thread (arrow) has reached the nodule primordium (NP), and branches off into several thinner threads (arrowheads), growing into the cells of the primordium where the first bacteria are released. F: ENOD5 localization; H: ENOD12 localization. Sections were hybridized with ^{35}S-labeled antisense ENOD5 and ENOD12 RNA. Bar = 50 µm. X = xylem pole of central cylinder.

of the invasion zone. The PsENOD3 and PsENOD14 genes are expressed in the infected cells of the late part of the early symbiotic zone and the early part of the late symbiotic zone. The Nps-40' protein seems restricted to the uninfected cells of the early and the late symbiotic zone.

Thus, some of the early nodulin transcripts are specifically present in zones

coinciding with the ones defined by Newcomb (1981), while others are not. The different developmental zones according to Newcomb (1981) have been defined on the basis of morphological/cytological criteria, mainly bearing upon growth stages of the bacteria. With the isolation of clones representing the above-mentioned early nodulin genes and the generation of antisera against early nodulin proteins, a set of molecular markers is available that enables to refine the description of the development of the two cell types in the central tissue. In particular, the transient character of the expression of some early nodulin genes improves the distinction of successive stages in the development of the infected cell type. In the description of the development of the infected cell type based on characters like the filling of the cells with bacteroids and the morphologic development of these bacteroids during the filling of the cells, the distinction of successive stages is more arbitrary, since these are processes following a more gradual course. An improved distinction of developmental stages, in turn, will facilitate the unravelling of the successive steps in nodule development and the identification of signals, both from the plant and the bacteria, involved in effecting these steps. In addition, the availibility of markers for the early development of infected cells (PsENOD5) as well as uninfected cells (Nps-40'), together with a marker for both cell types (PsENOD12), may open up possibilities to study the regulatory mechanisms underlying the differentiation of cells into the infected and the uninfected type, respectively.

The description of developmental stages will undoubtedly gain from knowledge of the function of the early nodulin gene products. This can be illustrated by recent studies on the location of the transcripts of a bacterial gene with a known function in the nitrogen fixation process, namely the *nif*H gene, which encodes component II of nitrogenase. It was shown that in the first two cell layers of the late symbiotic zone *nif*H transcripts are not yet detectable, while in the subsequent cell layer *nif*H transcripts are present at a maximal level (Yang et al., submitted). So, in the first few cell layers of the late symbiotic zone, as defined by the cytological criteria of Newcomb (1981), nitrogen fixation will not be possible. These results correlate well with those in alfalfa of Vasse et al.(1990), who extended the description of the central tissue zonation in the indeterminate alfalfa nodule by the cytological distinction of five successive growth stages in the development of the bacteroids. They showed in the first layers of the late symbiotic zone the presence of bacteroids of a growth stage that was correlated with the lack still of nitrogen fixation. The latter correlation was confirmed by studying bacteroids of strains defective in nitrogen fixation. Therefore, Vasse et al. (1990) suggested to name these first layers of the late symbiotic zone "interzone II-III" (in their terminology II = invasion zone + early symbiotic zone, and III = late symbiotic zone), and to name the part of the

late symbiotic zone with actively nitrogen-fixing bacteroids "nitrogen-fixing zone". The host cells themselves of this interzone II-III were characterized by an accumulation of starch. Such a starch accumulation is also observable in the infected cells at a corresponding position in the late symbiotic zone in pea root nodules where leghemoglobin is not yet detected (chapter 3). The absence of leghemoglobin in the so-called "interzone II-III" also correlates well with the absence of nitrogen fixation, in view of its function in regulating the oxygen supply to provide the bacteria with oxygen while keeping the concentration low so as to protect nitrogenase. These results taken together illustrate well the virtue of the combination of cytological and molecular approaches with the application of genetically engineered bacterial strains (see further the section on early nodulin gene expression in "empty" nodules below).

The potential of using molecular markers for the defining of developmental stages in the developing bacteroids has also been indicated with a slightly different approach by Sharma & Signer (1990). These authors monitored the expression of *R. meliloti* symbiotic genes in alfalfa nodule development by using bacteria that contained fusions of the promoters of the symbiotic genes with the *Escherichia coli gus*A reporter gene. The induction of the symbiotic gene promoters in the different symbiotic zones of the alfalfa nodule could thus be followed by the histochemical localization of the *gus*A gene product, ß-glucuronidase.

When the functions of the nodulins are unknown, they might be inferred from the temporal and spatial pattern of their production, combined with sequence data. This can be ventured, if the properties of the nodulin deduced from the amino acid sequence are consistent with a role in the specific function(s) of the tissue in which the nodulin is exclusively produced. The most clear-cut example of this is provided by the ENOD2 gene, of which the expression is restricted to all cells of a part of the peripheral tissue that has been shown to have a specialized function in regulating the oxygen influx into the nodule (chapter 5; cf. Witty et al.,1986). This is discussed more extensively in the section on ENOD2 gene expression below.

When inferring functions along these lines is more difficult, nodulin gene expression might still provide clues as to how the cytologically distinguished stages of nodule development relate to each other and how they are regulated by the interaction of bacterial- and host-derived signals. For example, the PsENOD12 genes are expressed in both the meristematic cells constituting the nodule primordium in the inner layers of the root cortex and, at a later stage, in the non-meristematic cells of the invasion zone of the nodule (Figs. 2 and 3, see also chapter 7). These observations may point to an interesting functional similarity between these different tissues. Moreover, the PsENOD12 gene expression pattern may be related to the same host and/or bacterial-derived regulatory

factors operating in both tissues. This will be discussed more fully in the following sections on the expression pattern of the PsENOD12 and PsENOD5 genes during nodule development.

The PsENOD12 gene

In the development of indeterminate nodules, two successive stages in meristematic activity can be distinguished: first, the meristematic activity at the very onset of nodule formation in the inner cell layers of the root cortex that constitutes a nodule primordium, and secondly, the meristematic activity organized in a persistent meristem that establishes itself in a subsequent stage at the distal end of the primordium, enabling the nodule to sustain indeterminate growth (Libbenga & Harkes, 1973). The meristematic cells in these two successive stages characteristically differ in the expression of the PsENOD12 genes (Figs. 2 and 3, see also chapter 7). No expression of these genes is detected in the persistent meristem of the second stage. On the other hand, the meristematic cells of the first stage do express the PsENOD12 genes. At the second stage of meristematic activity, the expression of the PsENOD12 genes becomes restricted to the invasion zone, i.e. the zone immediately proximal to the distal meristem where infection threads penetrate cells produced by the meristem.

When the root cortical cells that will form the nodule primordium are induced to express their PsENOD12 genes, the infection thread, containing the bacteria, is still in the outer cell layers of the root cortex. Hence, the nodule primordial cells must be amenable to react to a rhizobial signal carried over several cell layers. In the invasion zone of the nodule all the cells contain PsENOD12 transcripts, whereas only a part of the cells is penetrated by a branch of the infection thread (chapter 7; Scheres et al., 1990b). Therefore, it seems that also in the nodule tip, a rhizobial signal is operating which, although it might be a different one from that produced in the root cortex at this stage of nodule development, nevertheless is effective outside the cells actually containing bacteria and should therefore be able to reach the persistent distal meristem as well. Still, the PsENOD12 genes are not expressed in the persistent distal meristem, whereas the level of PsENOD12 transcripts is already maximal in the invasion zone cell layer directly adjacent to it, indicating that the persistent distal meristem is not susceptible to such a signal. These observations suggest that the persistent distal meristem cells must be different from the meristematic nodule primordial cells, not only in the pattern in which they deposit new cells, but also at the molecular level, as shown by the expression of the PsENOD12 genes. Nevertheless, it can not be excluded that at this developmental stage

the bacteria give off signals differing from those in the root cortex so that none of the meristematic cells is responsive to them. On the other hand, it may be hypothesized, that the activated cells of the inner cortex not simply "dedifferentiate" to form new cells for the future nodule, but also already specialize for a specific function in nodule formation, as indicated by the specific expression of the PsENOD12 genes in these cells.

Besides in the already mentioned sites, namely the nodule primordial cells and the nodule invasion zone, PsENOD12 transcripts are also detected in the root cortical cells through which the infection thread grows on its way to the nodule primordium (Fig. 3, chapter 7). Interestingly, both the cells of the outer root cortical layers and the cells of the inner root cortical layers that form the nodule primordium show a similar reaction to the stimulation by the rhizobia. In both cell types cytoplasmic strands start to intersect the large central vacuole of the original root cortical cells. In the inner cortical cells these cytoplasmic strands serve their usual role in positioning the nucleus in the centre of the cell prior to cell division; in the outer root cortical cells, however, these strands attain a new function, namely providing a sort of bridge for guiding the infection thread through the space normally occupied by the central vacuole of the cell. In this connection, Bakhuizen et al. (1988) suggested that the root outer cortical cells through which the infection thread will migrate start a cell division cycle in which they become arrested at the G2 phase. In the nodule invasion zone, also a vacuolation process is taking place, although reverse to that in the development of the nodule primordium: the cells of the invasion zone, which just have been deposited by the persistent meristem with scattered vacuoles, develop a single central vacuole not intersected by cytoplasmic strands. From these observations, it is still hard to tell if PsENOD12 might have a role in vacuolation phenomena (see also below).

Despite the apparent differences between the nodule primordium and the nodule invasion zone, it is tempting to hypothesize that they both express the PsENOD12 genes due to a comparable position in a developmental gradient of interacting signals. At the primordium stage, such a gradient might be brought about by an interaction between the rhizobia, coming in the infection thread from the outside, and (a) factor(s) derived from the protoxylem poles of the root. The nodule primordia mostly arise near these protoxylem poles and evidence for such a factor from the xylem comes from the experiments of Libbenga et al. (1973). In addition, PsENOD12 transcript could only be detected in infected root hairs, if a primordium could also be observed (Van de Wiel, unpublished preliminary observations), indicating that penetration of an infection thread *per se* is not sufficient for inducing PsENOD12 gene expression, but that an interaction with the initiation of nodule primordia is necessary for PsENOD12 gene expression to occur in infected root hairs. At a later stage, the invasion zone might be hypothesized to

be a zone where an interaction occurs between, again, the rhizobia in the incoming infection threads and a factor, now coming from the persistent meristem. Such a situation is conceivable, since during nodule development the growing infection thread is first directed towards the nodule primordium, but after establishment of the persistent meristem the growing infection thread is redirected over 180 degrees to infect the cells deposited by the persistent meristem. It would be very interesting to compare the postulated factors from the root xylem and the nodule persistent meristem, since the afore-mentioned observations that both should be involved in inducing PsENOD12 gene expression, suggest that they may be similar. Also, whether the bacteria utilize the same factor(s) in both situations, will have to be determined.

Further studies have shown that PsENOD12 transcripts can also be detected in other parts of the plant, namely in the flower and in older parts of the stem, though in smaller amounts than in the root nodule. Strictly speaking, PsENOD12 therefore no longer fulfils the requirements defined for a true nodulin. In the flower, no tissues have yet been found to express specifically the PsENOD12 gene(s). In the stem, however, PsENOD12 mRNA is only found in the inner cell layers of the cortex immediately adjacent to the ring of vascular bundles (chapter 7). These parenchymatic cells have relatively large central vacuoles and show neither signs of incipient meristematic activity nor the appearance of cytoplasmic strands through their vacuoles. Thus, no relationship with vacuolation phenomena could be implicated here.

These data provide no obvious clues as to the function of the PsENOD12 nodulin. Its amino acid sequence, as deduced from the cDNA sequence, indicates that it might be a cell wall protein (cf. Table 1), which is supported by its homology with the soybean 1A10 protein (Averyhart-Fullard et al., 1988). This is consistent with a role in nodule primordial meristematic activity as well as in infection thread growth, both being processes in which new cell walls are produced and with which PsENOD12 gene expression appears to be associated, as can be deduced from the PsENOD12 mRNA localization data discussed above. Also, the cells of the outer root cortical layers through which the infection thread makes its way to the nodule primordium, deposit an additional wall layer. In view of this, it would be worthwhile to check whether the cells of the inner layers of the stem cortex that express the PsENOD12 genes also deposit an additional cell wall layer and, if so, to compare the structure and the composition of this layer with those of the wall layers deposited in nodule development. Some cell wall-depositing activity might be suspected in the stem cortical cells, as cell stretching will be necessitated here as a result of an increase in thickness of the underlying stele.

Because the PsENOD12 genes are expressed in other parts of the plant than the nodule, it can be assumed that PsENOD12 has a role in normal plant developmental

processes not directly related to nodule formation, which it probably already had even before the development of the symbiosis in the course of evolution. Afterwards, PsENOD12 also proved useful for specific functions in nodule development during the evolution of the symbiosis (chapter 7).

If no specific clues to the function of PsENOD12 in the nodule have come from the studies of its presence in other parts of the plant than the nodule, the specific function of PsENOD12 in the nodule appears most likely to be related to the infection process. There is no consistent relationship of PsENOD12 gene expression with meristematic activity in the nodule, as is evident from its absence in the distal meristem. Neither are PsENOD12 transcripts detectable in root and shoot meristems. On the other hand, all sites in nodule development where PsENOD12 gene expression is found, sooner or later become involved in the infection process. Yet the function of PsENOD12 needs not necessarily be in formation of the infection thread, since not all cells of the nodule primordium expressing the PsENOD12 genes will eventually be penetrated by an infection thread. Rather, we would hypothesize that ENOD12, instead of having a function in infection thread growth itself, functions in preparing the walls of cells that *might* receive an infection thread, to enable these cells to sustain a controled infection process. In the outer cells of the root cortex this preparation involves depositing an additional cell wall layer, in the inner cells of the root cortex it is executed concomitantly with the formation of new cells, and in the cells of the nodule invasion zone it is attained during the differentiation of cells produced by the cell division activity of the nodule distal meristem.

The PsENOD5 gene

It is interesting at this point to compare the pattern of PsENOD5 gene expression with that of PsENOD12. The PsENOD5 gene is only expressed in cells with growing infection thread tips, including the outer cells of the root cortex in which no release of bacteria takes place. After the release of the bacteria from the infection threads, the amount of transcript increases concomitantly with the proliferation of bacteroids in the infected cells (Figs. 2 and 3, Scheres et al., 1990b). Thus, contrary to the situation with the PsENOD12 gene, PsENOD5 gene expression is apparently induced by a rhizobial factor that only operates at a distance of not more than a single cell. Moreover, there appears to be an induction of PsENOD5 gene expression proportional to the number of intracellular bacteria up till the point whereupon the bacteria cease to proliferate.

Regarding the possible function of the PsENOD5 protein, its amino acid

composition, as deduced from the cDNA sequence is reminiscent of that of arabinogalactan proteins. Nodule-specific forms of arabinogalactan proteins have been shown to accumulate in developing soybean nodules by Cassab (1986). In addition, there are some hydrophobic domains in the amino acid sequence of PsENOD5 indicating that the PsENOD5 protein might be inserted in a membrane. Arabinogalactan proteins can be inserted in the plasmamembrane (Knox et al., 1989). Therefore, it might be inferred that the PsENOD5 protein is part of both the infection thread membrane and the peribacteroid membrane, where it could serve a role in the interaction between the host plant and the bacterium. In this connection, Bradley et al. (1986) showed an intimate physical association between the plant peribacteroid membrane and the bacteroid outer membrane by immunolabeling with monoclonal antibodies against a plant membrane glycoprotein and bacteroid lipopolysaccharide, respectively.

The ENOD2 gene

One early nodulin gene, ENOD2, is exclusively expressed in the inner part of the peripheral tissue, usually called "inner cortex", in both the determinate nodules of soybean (*Glycine max*) (chapter 5) and the indeterminate nodules of pea (chapter 5) and alfalfa (*Medicago sativa*) (chapter 6) (Fig. 1). Since the ENOD2 gene apparently encodes a nodule-specific protein, the specific presence of the ENOD2 transcript in the inner part of the peripheral tissue indicates that not only the central tissue, but also a part of the peripheral tissue has a nodule-specific character.

The peripheral tissue has usually been called the nodule cortex (cf. Dart, 1977;Newcomb, 1981; Bergersen, 1982). The arrangement of tissues in this "cortex", however, is different from that of the cortex of the subtending root (cf. Fig. 1). A nodule endodermis forms the boundary between a tissue (the "outer cortex") that morphologically resembles the root cortex, and a different tissue (the "inner cortex"). Both the nodule "outer cortex" and the root cortex parenchyma join at the base of the nodule, separated from the rest of the nodule and the root stele by the nodule endodermis. The other part of the peripheral tissue, located inside the nodule endodermis (the "inner cortex"), consists of highly vacuolated, parenchymatous cells that are smaller and more densely packed, i.e. with fewer and smaller intercellular spaces (Witty et al., 1986), and that have walls staining more densely (Goodchild, 1977) than those of the peripheral tissue outside the endodermal layer (chapter 5). Moreover, it is traversed by vascular bundles each bounded by a second endodermal layer, named bundle endodermis, and in which no ENOD2 transcript was detected. Thus, the organization of

tissues inside of the nodule endodermis is unique for nodules, if compared to other plant organs. The unique character of the inner part of the peripheral tissue, based both on its position and on its morphology, is emphasized by the specific presence of the early nodulin ENOD2 transcript in this tissue in soybean, pea and alfalfa nodules.

These observations taken together prompted us to reconsider the terminology of the peripheral tissue. Indeed, one might simply distinguish a cortex and a medulla (the central tissue) in nodules. However, according to strict appliance of plant-anatomical terminology, the term "inner cortex" in the nodule is potentially misleading. By definition, the root endodermis delimits the root cortex from the stele with the vascular tissue (cf. Esau, 1977). The nodule endodermis, on the contrary, delimits only the nodule "outer cortex" from the rest of the nodule, including the "inner cortex". Thus, only this "outer cortex" has a position comparable to that of the root cortex parenchyma and might be called a cortex proper, in agreement with its morphology that is similar to that of the root cortex. Inside the root endodermis are the stelar tissues, which are organized in a single central cylinder. In the nodule, these stelar tissues are organized in several bundles that traverse the "inner cortex", bounded from the latter by the bundle endodermis. Hence, one must conclude that it is a tissue for which no counterpart can be found in the root. For that reason, we proposed to rename this tissue "nodule parenchyma", to avoid the potentially misleading term "inner cortex", which might be taken to imply a relationship with the root cortex that actually does not exist (Van de Wiel et al., 1990). This term, however, is still unsatisfactory, since it does not describe the specific position of the tissue in the nodule.

Next to the terminology of "inner" and "outer" cortex, there is also the terminology of Bond (1948), who named only the inner part of the peripheral tissue "nodule cortex". This is based on her conclusion from a developmental study of the pea nodule, that the outer part of the peripheral tissue must be regarded as a few layers of root cortical cells that must have undergone some cell divisions and a considerable amount of stretching to accomodate the growing nodule. In this view, only the inner part of the peripheral tissue is deposited by the persistent nodule meristem, with the inclusion of the nodule endodermis, which thus bounds the nodule proper from root cortical tissue. This view is consistent with our observation that transcripts for the nodulin ENOD2 are only present in the inner part of the peripheral tissue and that no nodulins have been observed in the outer part of the peripheral tissue. On the other hand, Newcomb states in his review of nodule morphogenesis and differentiation (1981), that all of the nodule peripheral tissue is derived from the distal face of the persistent meristem. Evidently, the question of the precise ontogeny of the various parts of the peripheral tissue must first be resolved, before a satisfactory terminology can be established.

The inner part of the peripheral tissue has been shown to function as a barrier towards the penetration of free oxygen into the central tissue (Witty et al., 1986). The flow of free oxygen into the central tissue needs to be regulated to protect the extremely oxygen-sensitive nitrogen-fixing enzyme nitrogenase. The restricted intercellular air-filled space, the location through which oxygen can diffuse most easily, might enable the nodule parenchyma to perform a function as oxygen barrier. From the cDNA nucleotide sequence it has been deduced that ENOD2 might be a cell wall protein (Table 1) and, therefore, we have suggested that ENOD2 contributes to a specific cell wall structure with limited intercellular space. Contrary to the transient appearance of most of the other early nodulins, the ENOD2 transcript remains present during the nitrogen-fixing stage. This is also consistent with a role in the regulation of the oxygen supply to the central tissue. That is, the oxygen barrier has been shown to be a variable one in connection with changes in the environment, such as nitrate stress (Witty et al., 1986). The ENOD2 protein could also contribute to, for instance, a flexible cell wall morphology, making it possible to change the extent of the intercellular space and thus the diffusion rate of oxygen.

Studying the location of ENOD2 gene expression proved also to be helpful in interpreting aberrant types of nodules, as will be discussed in the next section.

Early nodulin gene expression in "empty" nodules

In alfalfa, certain engineered bacterial strains (cf. Finan et al., 1985; Hirsch et al., 1985) as well as auxin transport inhibitors (ATIs; cf. Hirsch et al., 1989) elicit the formation of nodules which, in addition to being free of intracellular bacteria in their central tissue, may also differ in other details of their histology. Still, it could be shown that these nodules specifically express the MsENOD2 gene in cells that have a morphology and occupy a position in these nodules comparable to the cells expressing the MsENOD2 gene in nodules formed in response to wild type *R. meliloti* (chapter VI). These observations indicate that these nodules have a histological structure homologous to that of normal nitrogen-fixing nodules. This is particularly intriguing, because, in the case of the nodules induced by ATIs, it strongly suggests that the whole sequence of steps leading to the formation of a nodule, including the proper tissue-specific expression of the ENOD2 gene, can be triggered by a single exogenous signal. Such a proposition is consistent with the recent observation that the NodRm-1 molecule excreted by *R. meliloti* and identified by Lerouge et al. (1990), induces, in a host-specific way, nodule

formation on alfalfa (Roche et al., 1991). In this connection, it is of interest that certain vegetatively propagated clones of alfalfa form nodules in the absence of bacteria (Truchet et al. 1989). This implies that alfalfa has at its disposal the complete developmental program to form root nodules and only a trigger from the rhizobia is needed to set this program in motion. In the "spontaneously" nodulating alfalfa clones the need for this trigger apparently has been abolished by one or more mutations (Truchet et al., 1989). NodRm-1 is clearly different from the ATIs, but it is conceivable that both types of signal molecules interact at different levels of the same cascade of events contained in the nodule developmental program. Only if so, the action of plant hormones will be involved in the formation of root nodules.

In all the "empty" nodules discussed here, another early nodulin gene is expressed, namely the one encoding Nms-30, which is immunologically similar to the Nps-40' early nodulin of pea (chapter VIII). If Nms-30, like most probably Nps-40', turns out to be a marker of the uninfected cells, the question is raised whether indeed uninfected cells can differentiate without the presence of bacteria in the central tissue or that this lack of bacteria in the central tissue leads to disturbances in the normal specialization of cells in the developing central tissue. Further insight into the way uninfected cells differentiate or cell-specific gene expression is regulated in the central tissue may be gained by a combination of *in situ* localization studies and the use of engineered bacteria by which nodule development is blocked at specific stages. The application of engineered bacteria in elucidating the nature of bacterial signals involved in the induction of nodulin gene expression has already been shown to be useful in the first part of this thesis, although with one draw-back: If the expression of an individual nodulin gene fails, this needs not be simply due to the lack of a bacterial signal. Unfortunately, the possibility of the presence of bacterial signals eliciting a negative response, e.g. a defense response, in the host plant has to be considered, especially when transconjugants of the plant pathogen *Agrobacterium tumefaciens* are applied (see chapters III and IV, and also Nap & Bisseling, 1990). Except for this restriction, strains like the ones used in chapter III and IV, together with mutants, like for example *bar* (=bacterial release) mutants (De Maagd et al., 1989), will be useful in a further analysis of the differentiation of the central tissue, if combined with the above-described markers of diverse stages in central tissue development.

CONCLUDING REMARKS

During root nodule development, a range of tissues differentiates that can be distinguished by a combination of cytological and anatomical criteria. The development of several of these tissues is marked by the expression of nodule-specific genes. The majority of these genes are expressed in the developing central tissue, which comes as no surprise since this tissue will harbour the nitrogen-fixing bacteroids. However, also the inner part of the peripheral tissue turns out to express, in a persistent manner, at least one early nodulin gene, i.e. ENOD2 and this holds true for both the indeterminate pea and alfalfa nodules and the determinate soybean nodule. It has been shown that this pattern of gene expression can be related to functions of all these tissues that are specific for the symbiosis. Moreover, in pea nodules, the localization data of several nodulin and rhizobial transcripts have shown a transient expression pattern in the central tissue, which makes these nodulins useful in refining the characterization of successive developmental stages in the formation of the central tissue. This will be important in determining the type of signals by which the host plant and the bacteria interact to establish a properly functioning symbiosis.

In addition, localization of tissue-specific nodulin gene expression has appeared helpful in determining how far the pattern of development of the "empty' root nodules in alfalfa is comparable to that of "normal", nitrogen-fixing root nodules. It would be interesting to test whether this also contributes to the interpretation of the development of even more aberrant types of nodules, such as those appearing to be rather lateral roots than "true" leguminous root nodules (cf. Dudley et al., 1987).

Future research should be directed to the localization and further characterization of the corresponding nodulin proteins, in an attempt to understand their specific functions. For that purpose, antibodies will have to be obtained against these proteins. The study of the nodulin proteins might be of help in the further delimitation of specific nodule tissues and, furthermore, in the understanding of the specific functions of the various nodule tissues and perhaps even their organization into a coherently functioning symbiotic nodule. A combination of the localization of nodulin gene expression with the use of nodules, blocked at different stages of development, will help in the analysis of the regulation of gene expression effecting proper nodule development.

REFERENCES

Averyhart-Fullard, V., Datta, K. and Marcus, A. (1988) *Proc. Natl. Acad. Sci.USA*, 85, 1082-1085.

Bakhuizen, R., Van Spronsen, P.C., Díaz, C.L., and Kijne, J.W., 1988, The plant cytoskeleton in the *Rhizobium*-legume symbiosis, Ph.D. thesis, Leiden University, The Netherlands.

Bergersen, F.J., 1982, Root nodules of legumes: structure and functions, Research studies Press Wiley, Chichester.

Bond, L., 1948, *Bot. Gaz.*, 109: 411.

Bradley, D.J., Butcher, G.W., Wood, E.A., and Brewin, N.J., 1986, *J. Cell Sci.*, 85: 47.

Cassab, G.I., 1986, *Planta*, 168: 441.

Dart, P., 1977, Infection and development of leguminous nodules, *in*: A treatise on dinitrogen fixation, vol. III, R.W.F. Hardy and W.S. Silver, eds., Wiley, New York.

De Maagd, R.A., Rao, A.S., Mulders, I.H.M., Goossen-De Roo, L., Van Loosdrecht, M.C.M., Wijffelman, C.A., ande Lugtenberg, B.J.J., 1989, *J. Bacteriol.*, 171:1143.

Dickstein, R., Bisseling, T., Reinhold, V.N., and Ausubel, F.M.,1988, *Genes and Development* 2, 677-687.

Dudley, M.E., Jacobs, T.W., and Long, S.R., 1987, *Planta* 171, 289-301.

Esau, K., 1977, Anatomy of seed plants, Wiley, New York.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C., and Signer, E.R., 1985, *Cell* 40, 869-877.

Franssen, H.J., Nap, J.-P., Gloudemans, T., Stiekema, W., Van Dam, H., Govers, F., Louwerse, J., Van Kammen, A., and Bisseling, T., 1987, *Proc. Natl. Acad. Sci. USA*, 84: 4495.

Franssen, H.J., Thompson, D.V., Idler, K., Kormelink, R., Van Kammen, A., and Bisseling, T., 1989, *Plant Mol. Biol.*, 14: 103.

Govers, F., Gloudemans, T., Moerman, M., Van Kammen, A., and Bisseling, T., 1985, *EMBO J.*, 4: 861.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T., 1989, *Proc. Natl. Acad. Sci. USA* 86: 1244.

Hirsch, A.M., Drake, D., Jacobs, T.W., and Long, S.R., 1985, *J. Bacteriol.*, 161: 223.

Knox, J.P., Day, S., and Roberts, K., 1989, *Development*, 106: 47.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., and Dénarié, J., 1990, *Nature*, 344: 781.

Libbenga, K.R., and Harkes, P.A.A., 1973, *Planta*, 114:17.

Libbenga, K.R., Van Iren, F., Bogers, R.J., and Schraag-Lamers, M.F., 1973, *Planta*, 114: 29.

Nap, J.-P., and Bisseling, T., 1990, Nodulin function and nodulin gene regulation in root nodule development, *in*: Molecular biology of symbiotic nitrogen fixation, P.M. Gresshoff, ed., CRC

Press, Boca Raton.

Nap, J.P., Van de Wiel, C., Spaink, H.P., Moerman, M., Van den Heuvel, M., Djordjevic, M.A., Van Lammeren, A.A.M., Van Kammen, A., and Bisseling, T., 1989, *Mol. Plant-Microbe Interac.*, 2: 53.

Newcomb, W., 1981, *Int. Rev. Cytol. Suppl.*, 13: 247.

Norris, J.H., Macol, L.A., and Hirsch, A.M., 1988, *Plant Physiol.* 88, 321-328.

Roche, P., Lerouge, P., Prome, J.C., Faucher, C., Vasse, J., Maillet, F., Camut, S., De Billy, F., Dénarié, J., and Truchet, G., 1991, NodRm-1, a sulphated lipo-oligosaccharide signal of *Rhizobium meliloti* elicits hair deformation, cortical cell division and nodule organogenesis on alfalfa roots, *in*: Advances in molecular genetics of plant-microbe interactions, Vol. 1, H. Hennecke and D.P.S. Verma, eds., Kluwer Academic Publishers, Dordrecht.

Scheres, B., Van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., Zwartkruis, F., Wolters, A.-M., Gloudemans, T., Van Kammen, A., and Bisseling, T., 1990a, *Cell*, 60, 281.

Scheres, B., Van Engelen, F., Van der Knaap, E., Van de Wiel, C., Van Kammen, A., and Bisseling, T., 1990b, *Plant Cell*, 2: 687.

Sharma, S.B., and Signer, E.R., 1990, *Genes and Development*, 4: 344.

Truchet, G., Barker, D.G., Camut, S., De Billy, F., Vasse, J., and Huguet, T., 1989, Alfalfa nodulation in the absence of *Rhizobium*, *Mol. Gen. Genet.*, 219: 65.

Van de Wiel, C., Nap, J.P., Van Lammeren, A.A.M., and Bisseling, T., 1988, *J. Plant Physiol.*, 132: 446.

Van de Wiel, C., Norris, J.H., Bochenek, B., Dickstein, R., Bisseling, T., and Hirsch, A.M., 1990a, *Plant Cell*, 2: 1009.

Van de Wiel, C., Scheres, B., Franssen, H., Van Lierop, M.-J., Van Lammeren, A., Van Kammen, A., and Bisseling, T., 1990b, *EMBO J.*, 9: 1.

Vasse, J., De Billy, F., Camut, S., and Truchet, G., 1990, *J. Bacteriol.*, 172: 4295.

Witty, J.F., Minchin, F.R., Skøt, L., and Sheehy, J.E., 1986, *Oxford Surv. Plant Mol. Cell Biol.*, 3: 275.

Yang, W.C., Horvath, B., Hontelez, J., Van Kammen, A., and Bisseling, T., 1991, *submitted.*

Chapter 10

Samenvatting

Vlinderbloemige planten, zoals bijvoorbeeld erwt, wikke, luzerneklaver en soja, kunnen een samenwerkingsverband aangaan met bodembacteriën van de geslachten *Rhizobium* en *Bradyrhizobium*. Dit samenwerkingsverband heeft over het algemeen een uitermate specifiek karakter, d.w.z. de meeste bacteriesoorten kunnen slechts met een beperkt aantal gastheersoorten een effectieve interactie aangaan. Zo is *Rhizobium meliloti* alleen compatibel met soorten uit de geslachten *Medicago*, *Melilotus* en *Trigonella*. In interactie met de bacterie vormt de plant een gespecialiseerd orgaan, de wortelknol, waarin de bacterie wordt opgenomen. Als tegenprestatie voor deze gastvrijheid van de plant legt de bacterie stikstof uit de lucht vast in de vorm van ammonia en levert dit vervolgens aan de plant.

Gedurende de interactie komen zowel in de bacterie als in de plant specifieke genen tot expressie. Wat betreft de plant zijn een aantal genen geïdentificeerd die alleen in de wortelknol tot expressie komen. De producten van deze genen worden nodulines genoemd. Veel van deze genen -de late nodulinegenen- worden afgelezen kort voor en tijdens het plaatsvinden van de stikstoffixatie en zullen vermoedelijk ook voornamelijk een rol bij dit proces vervullen. Als voorbeeld hiervan kunnen de leghemoglobines gelden, die een functie in het zuurstoftransport naar de bacterie vervullen. Een andere groep van genen -de vroege nodulinegenen- komen aanmerkelijk eerder tot expressie, d.w.z. tijdens de infectie en de vorming van de wortelknol. Deze vroege nodulines vervullen een rol in de organogenese van de knol. Het verkrijgen van inzicht in deze rol en in de regulatie van de expressie van deze genen vormen het thema van dit proefschrift. Daartoe is vooral gekozen voor een aanpak waarbij de gegevens verkregen met moleculaire technieken voor het aantonen van genexpressie worden geïntegreerd met microscopische technieken om een goed beeld te krijgen van waar en wanneer gedurende de knolontwikkeling de nodulinegenen tot expressie komen.

Als achtergrond voor deze studies wordt in hoofdstuk 2 de ontwikkeling van de wortelknol beschreven. De bacteriën zetten de wortelharen aan zich te krommen en worden vervolgens ingevangen in deze kromming. Waar de bacteriën zich hebben gehecht aan de wortelhaar wordt de plantecelwand plaatselijk opgelost, waarop de wortelhaarcel nieuwe celwand afzet tegen de lesie in de oude celwand, in de vorm van een buis de cel in. De bacteriën komen in deze buis, de zogenaamde infectiedraad, terecht en worden via de topgroei van deze infectiedraad de wortel ingeleid. Tegelijkertijd worden locaal in de wortelcortex cellen geactiveerd om te gaan delen, wat leidt tot de vorming van een knolprimordium. De infectiedraad groeit naar het knolprimordium toe, waar hij zich vertakt en cellen in het centrale gedeeltevan het primordium binnendringt. Uit zijtakken van de infectiedraad komen de bacteriën vrij in de plantecellen. Ze blijven daarbij omgeven door een membraan van plantenoorsprong, de peribacteroidmembraan.

De bacteriën vullen de geïnfecteerde cellen en ontwikkelen zich tot stikstoffixerende bacteroiden. Daarnaast ontstaan ook ongeïnfecteerde cellen, die tesamen met de geïnfecteerde cellen het centrale weefsel van de knol vormen. Om een buitenste grenslaag van dit centrale weefsel, bestaande uit uitsluitend ongeïnfecteerde cellen, heen wordt een perifeer weefsel aangelegd. Dit perifere weefsel op zijn beurt wordt in een binnenst en een buitenst gedeelte opgedeeld door de vorming van een endodermisachtige laag, de knolendodermis genaamd. Het binnenste gedeelte van het perifere weefsel wordt doorsneden door vaatbundels die via de centrale cylinder van de wortel de knol aan het transportsysteem van de plant koppelen. Deze knolvaatbundels worden van binnen naar buiten omgeven door een pericykel en een bundelendodermis met bandjes van Caspari.

De knollen van leguminosen kunnen worden verdeeld in twee typen, uitgaande van het patroon van meristematische activiteit gedurende de knolontwikkeling. Bij het gedetermineerde type differentiëren alle cellen van het knolprimordium, dat ontstaat in de buitenste cellagen van de wortelcortex, in gespecialiseerd knolweefsel. Daarbij gaat alle delingsactiviteit verloren in de uitgegroeide, stikstoffixerende knol. Voorbeelden van dit type zijn de knollen van soja en boon. Bij het ongedetermineerde type wordt een persistent meristeem gevormd in de apex van het primordium, dat in de binnenste cellagen van de wortelcortex ontstaat. Dit persistente (apicale, distale) meristeem blijft gedurende een groot deel van het bestaan van de knol naar binnen toe cellen afzetten. De centraal afgezette cellen kunnen vervolgens weer vanuit het centrale weefsel geïnfecteerd worden. De infectiedraad, die eerst van buitenaf het knolprimordium ingroeide, draait daartoe zijn groeirichting 180^0 om teneinde het persistente meristeem te kunnen volgen. In dit knoltype kunnen dus in één longitudinale doorsnede de opeenvolgende stadia van knolontwikkeling bekeken worden. Voorbeelden van dit knoltype zijn de knollen van erwt, wikke, luzerneklaver en klaver. Tot slot worden nog enige varianten op de beschreven knolontwikkeling binnen de vlinderbloemigenfamilie besproken.

In de hoofdstukken 3 en 4 zijn knollen van smalbladige wikke bestudeerd die opgewekt zijn met verschillende genetisch gemodificeerde bacteriestammen. Dergelijke knollen bleken in diverse ontwikkelingsstadia gestoord te zijn, zoals kon worden vastgesteld m.b.v. licht- en electronenemicroscopische waarnemingen. Tevens kwamen de nodulinegenen in verschillende mate tot expressie, in samenhang met de mate waarin knolontwikkeling gestoord was. De gebruikte bacteriestammen waren transconjuganten van *Rhizobium leguminosarum* bv. *viciae* (specifieke symbiont van erwt en wikke) en bv. *trifolii* (specifieke symbiont van klaver), en *Agrobacterium tumefaciens* (pathogeen op een breed scala van dicotylen), waarin de normale plasmiden (in het geval van *A. tumefaciens* het Ti-plasmide dat verantwoordelijk is voor de tumorinductie op waardplanten) vervangen waren door een symplasmide of een deel daarvan met de

zogenaamde *nod* genen van *Rhizobium* erop. Deze *nod* genen zijn verantwoordelijk voor de knolvorming op de gastheer. Door het gebruik van verschillende transconjuganten kon worden nagegaan in welke mate de *nod* genen een rol spelen in het correct laten verlopen van de opeenvolgende ontwikkelingsstadia van de knol en in het tot expressie brengen van de verschillende nodulinegenen. Daarbij werd echter op één probleem gestuit: het uitblijven van de expressie van nodulinegenen kon niet zonder meer worden toegeschreven aan het ontbreken van genetische informatie in de transconjugant. Er werden aanwijzingen verkregen, dat de bacteriën een afweerreactie bij de plant opriepen. Dit zou kunnen betekenen dat bijv. de *A. tumefaciens*-transconjugant wel over de benodigde genetische informatie beschikt voor het induceren van nodulinegenexpressie, maar dat deze informatie gemaskeerd wordt door informatie van de transconjugant zelf met een negatief effect op de gastheer.

De waarnemingen aan de knollen die verstoord waren in hun ontwikkeling maakten het mogelijk de nodulinegenexpressie in correlatie te brengen met het bereiken van een bepaald ontwikkelingsstadium. Zo bleek het vroege noduline Nvs-40 al detecteerbaar als slechts het vrijkomen van de bacteriën uit de infectiedraad was opgetreden, tesamen met de differentiatie van ongeïnfecteerde cellen; het noduline Nvs-65 daarentegen kon slechts gedetecteerd worden, nadat de geïnfecteerde cellen geheel met bacteroiden gevuld waren. Transcripten voor het vroege noduline ENOD2 waren in alle gevallen detecteerbaar. Het optreden van leghemoglobine vereiste een nog verdere ontwikkeling van de geïnfecteerde cellen. Deze laatste waarneming kon bevestigd worden door de directe detectie van leghemoglobine m.b.v. immunolocalisatie in longitudinale doorsnedes van erwteknollen in hoofdstuk 3. Hieruit bleek dat leghemoglobine in de eerste cellagen van de laat-symbiontische zone, waarin de geïnfecteerde cellen al wel compleet met bacteroiden gevuld zijn, nog niet detecteerbaar was.

De directe benadering van *in-situ* -localisatie werd, nu uitgebreid met localisatie van transcripten middels *in-situ* -hybridisatie, voortgezet in de volgende hoofdstukken 5, 6, 7 en 8. In hoofdstuk 5 wordt de *in-situ*-localisatie van transcripten van het vroege noduline-gen ENOD2 in de gedetermineerde knollen van soja en de ongedetermineerde knollen van erwt beschreven. In beide knoltypen bleef de aanwezigheid van ENOD2 transcripten beperkt tot het binnenste gedeelte van het perifere weefsel, meestal aangeduid als de binnenste cortex. Dit benadrukt nog eens het knolspecifieke karakter van dit weefsel, hetgeen ook al gebleken was uit zijn specifieke morfologie en zijn specifieke positie, namelijk binnen een endodermis. Dit gaf ons aanleiding een alternatieve naam voor te stellen voor de "binnenste cortex", namelijk knolparenchym, teneinde verwarring te vermijden met de binnenste cortex van bijv. de wortel, die duidelijk niet vergelijkbaar is met dit zogenaamde knolparenchym. De van de cDNA afgeleide aminozuurvolgorde

laat een herhaling van twee verschillende pentapeptide-elementen, beide beginnend met twee prolines, zien, die voorafgegaan wordt door een signaalpeptide. Dit wijst erop, dat ENOD2 een extracellulair eiwit is. Daarom wordt gespeculeerd of ENOD2 bijdraagt tot de specifieke celwandordening van het binnenste gedeelte van het perifere weefsel met weinig intercellulaire ruimte, die op zijn beurt verantwoordelijk geacht wordt voor een specifieke functie van dit weefsel, namelijk het reguleren van de instroom van vrije zuurstof uit de lucht.

In hoofdstuk 6 zijn ENOD2-transcripten gelocaliseerd in knollen van luzerne-klaver. In dit geval werden niet alleen normale stikstoffixerende knollen bestudeerd, maar ook knollen die geïnduceerd waren door *R. meliloti exo*-mutanten of *A. tumefaciens* transconjuganten met één of twee symplasmides van *R. meliloti*, en zelfs knollen die ontstonden in reactie op behandeling van de wortels met auxinetransportremmers. Al deze knollen werden gekenmerkt door het ontbreken van bacteriën in het centrale weefsel, maar weken ook in andere details af van normale knollen. Niettemin werden in deze afwijkende knollen ENOD2-transcripten vastgesteld in een weefsel dat qua morfologie en positie overeenkwam met het binnenste gedeelte van het perifere weefsel van normale knollen. Dit leidt tot de opvatting dat deze knollen niet principieel afwijken van normale knollen. In het geval van de inductie door auxinetransportremmers impliceert dit, dat de basale wortelknolstructuur opgewekt kan worden door één betrekkelijk simpel signaalmolecuul.

Hoofdstuk 7 bespreekt het vroege noduline ENOD12 van de erwt. Boodschapper-RNA voor ENOD12 werd aangetroffen in de vroege cellen van het knolprimordium en de wortelcellen waardoor de infectiedraad naar het knolprimordium groeit; in de latere fasen van de knolvorming raakt het voorkomen van ENOD12-transcripten beperkt tot de invasiezone, waar een deel van de cellen die afgezet zijn door het persistente knolmeristeem gepenetreerd worden door infectiedraden. Net als bij ENOD2 het geval is, impliceert de uit de cDNA-sequentie afgeleide aminozuurvolgorde een eiwit met repeterende pentapeptide-elementen die telkens starten met twee prolines. Het repeterende gedeelte wordt voorafgegaan door een signaalpeptide, hetgeen wederom op een extracellulair eiwit wijst. Besproken wordt in hoeverre ENOD12 betrokken zou kunnen zijn bij het infectieproces gedurende de knolontwikkeling. Verder wordt aangetoond dat door de bacteriën uitgescheiden factoren ENOD12-genexpressie induceren in wortelharen. De bacteriële *nod*-genen blijken hierbij een belangrijke rol te spelen. Tenslotte blijken ENOD12-transcripten ook detecteerbaar in de binnenste cortexlagen van de oudere stengelgedeelten en in bloemweefsel. Klaarblijkelijk is het ENOD12-genproduct ook betrokken bij andere processen in de plant dan alleen de knolontwikkeling.

In hoofdstuk 8 wordt m.b.v. immunolocalisatie aannemelijk gemaakt dat het vroege noduline Nps-40' specifiek is voor de cytosol van de ongeïnfecteerde cellen van de erwt. Dit laat voor de eerste keer, voor zover ons bekend, zien, dat in de ongedetermineerde knol de ongeïnfecteerde cellen een specifieke rol moeten vervullen in de symbiose, zoals dat reeds is aangetoond bij de gedetermineerde knollen, bijv. die van soja. Immunoprecipitatie-experimenten geven aan dat vergelijkbare eiwitten voorkomen in smalbladige wikke en luzerneklaver, en wel de nodulines Nvs-40, respectievelijk Nms-30.
De regulatie van de genexpressie voor deze nodulines wordt besproken in het licht van vroegere waarnemingen aan knollen met verschillende ontwikkelingsstoornissen in het centrale weefsel.

Tot slot wordt in hoofdstuk 9 besproken, wat de localisatiestudies hebben bijgedragen aan inzicht in de rol van de nodulines in de ontwikkeling van de diverse weefsels van de wortelknol. Tevens wordt gespeculeerd over de regulatie van hun aanmaak gedurende knolontwikkeling.

ACCOUNT

The contents of the preceding chapters have been based on the following publications:

Nap, J.P., Van de Wiel, C., Spaink, H.P., Moerman, M., Van den Heuvel, M., Djordjevic, M.A., Van Lammeren, A.A.M., Van Kammen, A., and Bisseling, T., 1989, *Mol. Plant-Microbe Interac.*, 2: 53.

Scheres, B., Van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., Zwartkruis, F., Wolters, A.-M., Gloudemans, T., Van Kammen, A., and Bisseling, T., 1990a, *Cell*, 60, 281.

Scheres, B., Van Engelen, F., Van der Knaap, E., Van de Wiel, C., Van Kammen, A., and Bisseling, T., 1990b, *Plant Cell*, 2: 687.

Van de Wiel, C., and Bisseling, T., 1990, Tissue-specific expression of early nodulin genes, *in*: Plant molecular biology, R.G. Herrmann and B. Larkins, eds., Plenum, London, in press.

Van de Wiel, C., Nap, J.P., Van Lammeren, A.A.M., and Bisseling, T., 1988, *J. Plant Physiol.*, 132: 446.

Van de Wiel, C., Norris, J.H., Bochenek, B., Dickstein, R., Bisseling, T., and Hirsch, A.M., 1990a, *Plant Cell*, 2: 1009.

Van de Wiel, C., Scheres, B., Franssen, H., Van Lierop, M.-J., Van Lammeren, A., Van Kammen, A., and Bisseling, T., 1990b, *EMBO J.*, 9: 1.

NAWOORD

Het in dit boekje gepresenteerde onderzoek is tot stand gekomen binnen een samenwerkingsverband van twee Wageningse vakgroepen, te weten Moleculaire Biologie en Plantencytologie en -morfologie. Aan de inhoud van dit boekje hebben zodoende velen vanuit soms heel verschillende invalshoeken een belangrijke bijdrage geleverd. Op deze plaats wil ik er graag een aantal met name vermelden.

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CURRICULUM VITAE

Clemens Caspar Maria van de Wiel werd op 9 augustus 1959 geboren in Amsterdam. Aldaar bezocht hij het St.-Nicolaaslyceum, waar hij het eindexamen VWO behaalde in 1977. In dat zelfde jaar ving hij aan met de studie Biologie aan de Universiteit van Amsterdam. In 1985 behaalde hij daar het doctoraalexamen met als hoofdvak Bijzondere Plantkunde en als bijvakken Plantenfysiologie en Microbiologie. Vanaf februari 1986 werkte hij bij de vakgroepen Moleculaire biologie en Plantencytologie en -morfologie van de Landbouwuniversiteit Wageningen aan het onderzoek dat in dit proefschrift beschreven staat. Van juli 1990 tot en met januari 1991 was hij werkzaam bij de vakgroep Moleculaire Celbiologie van de Universiteit van Amsterdam aan een project dat door de Stichting voor Biologisch Onderzoek (BION) en de Stichting voor Ruimte-Onderzoek (SRON) gezamenlijk gefinancierd werd. Op 1 februari 1991 is hij in dienst getreden van het Centrum voor Rassenonderzoek en Zaadtechnologie (CRZ-DLO) te Wageningen.