EARLY NODULINS IN ROOT NODULE DEVELOPMENT

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op dinsdag 6 maart 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

ISN 268195

The investigations described in this thesis were carried out at the department of Molecular Biology, Agricultural University, Wageningen, The Netherlands, and were financially supported by a grant from the Netherlands Organization for Scientific Research (NWO), The Hague.

The front cover shows a redrawing from the calligraphy *shoshin* (beginner's mind) by Shunryu Suzuki-Roshi

NN08201, 1340

STELLINGEN

1. De argumenten, die Djordjevic et al aanvoeren voor hun visie dat de reactie van leguminosen op Rhizobium infectie een verfijnde pathogene respons is, zijn niet overtuigend.

M.A.Djordjevic et al (1987), Ann.Rev. Phytopathol. 25, 145-168.

2. In tegenstelling tot wat de titel van hun artikel aangeeft, leveren Suzuki en Verma geen bewijs voor het voorkomen van knolspecifieke proteine-kinases.

H.Suzuki en D.P.S Verma (1989), The Plant Cell 1, 373-379.

3. Het 'nodulatie'-fenotype dient te worden gestandaardiseerd om te voorkomen dat bacterien die een bepaald percentage planten met knollen veroorzaken door sommige auteurs als nod- en door anderen als nod+ wordt aangemerkt.

A.Hirsch et al (1985), J. Bact. 223-230.
J.P.Nap et al (1989), MPMI 2, 53-63.
H.P.Spaink et al (1989), EMBO J. 8, 2811-2818.

4. Het is onjuist om uit de zwakke homologie tussen homeobox-eiwitten en enkele algemene transcriptiefactoren te concluderen dat homeobox-bevattende eiwitten geen gedefinieerde functie in ontwikkelingsbiologische processen hebben.

M.Akam (1989), Cell 57, 347-349.

5. In een tijdperk waarin biologische vraagstukken multidisciplinair worden bestudeerd is het achterhaald om biochemici te overtuigen van het op zichzelf juiste argument dat reductionisme niet de enige benadering is voor het begrijpen van biologische systemen.

S.Rose (1988), Trends Bioch. Sci. 13, 160-162.

6. De titel "the egg came first, of course!" voor een manuscript over patroonvorming in Drosophila oogenese en embryogenese, wordt pas begrijpelijk bij het nagaan van de sexe van de auteurs.

L.J. Manseau en T.Schupbach (1989), Trends Genet. 5, 400-405.

7. Niet alleen militaire stellingen dienen, vanwege het aan hun bestaan ten grondslag liggende gebrek aan genuanceerd denken, tot een minimum aantal te worden beperkt.

Wageningen, 6 maart 1990 Ben Scheres

Aan mijn ouders

Aan Olga

CONTENTS

CHAPTER 1

Outline

The symbiotic interaction between bacteria of the genus *Rhizobium* and leguminous plants leads to the formation of root nodules, which are specific nitrogen-fixing organs on the roots of plants. Bacteria enter the root by infection threads, and concomitantly cell divisons are induced in the root cortex, which lead to the formation of a meristem. From this meristem the different tissues of the root nodule originate. In the nodule bacteria are released in plant cells and then differentiate into the endosymbiotic bacteroids. These bacteroids are capable of nitrogen fixation.

The formation of root nodules involves expression of both bacterial and plant genes. *Rhizobium* genes involved in nodule formation are the nodulation (*nod*) genes. Nodule-specific plant genes are termed nodulin genes. According to their timing of expression they can be divided into early and late nodulin genes. Early nodulin genes are expressed well before the onset of nitrogen fixation, at the time that the nodule tissue is formed and the roots become infected by bacteria, while expression of late nodulin genes starts shortly before the onset of nitrogen fixation, when the nodule structure has been formed. Therefore only early nodulins can be involved in the infection process and in nodule development. Early nodulin genes expressed during the pea (*Pisum sativum* L.) - *Rhizobium leguminosarum bv. viciae* interaction are the subject of this thesis. Several cDNA clones representing pea early nodulin genes have been isolated and they have been used to study root nodule development and the communication between bacteria and host plant.

In chapter 2 we review general aspects of plant development. Recent progresses in understanding the molecular mechanisms underlying animal development are listed, and the possible significance of such mechanisms for plant development is discussed. The features of the root nodule formation system that make it suitable to study particular questions on the molecular basis of plant development are put forward.

In chapter 3 the pea early nodulin cDNA clone pPsENOD2 is characterized. The nature of the encoded polypeptide is compared with that of the soybean early nodulin described before. ENOD2 transcripts are localized both in pea and soybean root nodules throughout successive stages of development by *in situ* hybridization. Data on the primary structure of the ENOD2 protein and localization data are then combined to hypothesise that the function of this early nodulin is to create an oxygen barrier in the root nodule.

In chapter 4 the early nodulin ENOD12 is described. The spatial distribution of the corresponding transcript throughout root nodule development is depicted to demonstrate the involvement of ENOD12 in the infection process. We describe the primary structure of the ENOD12 protein and we examine whether ENOD12 gene expression is related to a defense respons. Using a sensitive detection method based on the polymerase chain reaction (PCR) we demonstrate that ENOD12 gene expression is induced by excreted *Rhizobium* factors and that bacterial *nod* genes are involved. ENOD12 transcripts found in flower and stem tissue are compared to the ENOD12 mRNAs in nodules using, among other techniques, a novel adaptation of RNase mapping to determine whether the same genes are expressed in these different tissues or not.

In chapter 5 it is demonstrated that the accumulation pattern of the transcripts corresponding to the pPsENOD5, pPsENOD3 and pPsENOD14 cDNA clones differs from that of ENOD2 and ENOD12 mRNA. The distribution of the former three transcripts is compared with the distribution of ENOD12 mRNA and the late nodulin leghemoglobin transcript. It is shown that the different transcripts are present at successive stages of development of the infected cell type. The primary structure of the ENOD5, ENOD3 and ENOD14 early nodulins is determined and these data are combined with the localization data of the transcripts to speculate on functions of these proteins. The involvement of different factors to induce expression of different early and late nodulin genes is discussed.

In chapter 6 the results described in the previous three chapters are summarized and some additional data on early nodulins are presented. The significance of the availability of early nodulin gene probes to elucidate the mechanisms of communication between rhizobia and legumes, which underly the process of root nodule formation, is discussed. Finally, in chapter 7, the value of the obtained information on early nodulins for studying both specific and general aspects of root nodule development is discussed.

CHAPTER 2

Plant development and root nodule formation.

INTRODUCTION

Since the discovery that nitrogen fixation occurs in legume root nodules as the result of symbiosis with bacteria (Beyerinck, 1888), the interaction between (brady-)rhizobia and legumes has been studied extensively. Fixed nitrogen is of crucial importance to agriculture and this has been a major reason for studying the *Rhizobium*-legume symbiosis in detail. Also from a scientific point of view a symbiosis in which one of the partners is taken up into the cells of the other partner to act as an 'organelle' with a specific function (viz. nitrogen fixation) is intriguing. These two reasons explain the considerable amount of cytological and plant physiological research that has been devoted to root nodules for decades.

The rise of molecular biology has brought important new tools to the study of the *Rhizobium*-legume symbiosis. Our understanding has been deepened by analyses of both bacterial and plant genes involved in this process. This progress has been extensively reviewed elsewhere and will not be discussed here (e.g. Rolfe and Gresshoff, 1988; Morrison *et al*, 1988; Long, 1989). Rather I should like to substantiate that the *Rhizobium*-legume symbiosis, due to the achievements of molecular (genetic) research, becomes an attractive model system to study important aspects of plant development. An overview of the current knowledge on developmental processes in higher plants will be presented and compared with the principles of developmental programs in animals. Data on molecular mechanisms governing pattern formation and differentiation in animals will be listed, and the question whether similar mechanisms occur in plants will be adressed. Finally the importance of root nodule formation as a system for studying plant development will be discussed.

THE PROCESS OF DEVELOPMENT IN HIGHER PLANTS.

CONCEPTS IN DEVELOPMENT

Plants and animals are multicellular organisms that develop from a single cell, the zygote, which is the result of the fusion of male and female gametes. Upon division of the zygote morphologically and physiologically different cells are produced, a process referred to as differentiation. A body plan is established, and many different cell types are organized in

an orderly spatial distribution, referred to as morphogenesis. At the moment mechanisms of development have been studied in animals much more extensively than in plants. In animals several theoretical concepts have been defined that categorize events occurring during development. At the first stage of development, called commitment, the fate of a cell is set by its physical position. Commitment is mostly a theoretical concept, since experimental demonstration of commitment relies on the next stage in development, determination. Determination is defined as the progressive fixation of cell fates among a group of cells, and competence is the ability of a determined cell to respond to a signal to express the phenotype belonging to that specific fate. Determination is not necessarily the result of a single event but can be achieved in successive steps during development. Determination can occur early in development, prior to extensive cellular proliferation, as examplified in the 'mosaic type' animal embryos. On the other hand, determination can also occur late in development, as in 'regulative type' animal embryos. In regulative animal embryos a progressively refined network of positional cues appears to provide the information for determination, and it is difficult to unclutter determination and competence stages (For a detailed overview of concepts in developmental biology and of different ways of animal embryonic development see Walbot and Holder, 1987).

Examples exist where, upon determination, a single cell is able to exhibit the same developmental program in isolated form as *in situ*, and no further signals are involved. The term cell-autonomous development is used for these cases. The autonomy of blastomers specialized for muscle formation in the nematode *Caenorhabditis elegans* is an example of this type of development (Whittaker *et al*, 1977). In other cases a developmental pathway can only be continued when the cells acquire the competence to respond to a certain signal, as found in *Drosophila melanogaster* cell groups, which are determined to form progenitor cells for a particular type of imaginal disk at the cellular blastoderm stage. These cells can only follow the developmental pathway they are determined to after they have acquired the capacity to respond to the moulting hormone ecdysone at a later stage (Nöthinger, 1972).

SPECIFIC FEATURES OF PLANT DEVELOPMENT

It is by no means evident that plant and animal development follow the same principles. Features which clearly distinguish plant from animal development are 1) the establishment of only a rudimentary body plan during embryogenesis, 2) post-

embryogenic development from centres of mitotic activity, the meristems, and 3) the absence of cell mobility because of the presence of cell walls. In the following I shall briefly describe these three specific features, and try to establish which of the concepts like determination, competence, cell-autonomous development, and development according to positional information, will be useful to provide a theoretical framework in plant developmental biology.

Embryogenesis

While in most animals morphogenesis and differentiation occur largely during embryogenesis, in plants these processes span both the embryonic and the post-embryonic phase (Walbot, 1985). During plant embryogenesis a seed is produced which contains two different organ systems: the terminally developed cotyledon, which in general function as a food reserve, and a polarized axis with two cell clusters that become very different centres of mitotic activity after germination of the seed: the root and shoot meristems. Not much is understood about the mechanisms by which axis polarity, the meristems, and the body plan are established in the embryonic phase. In the brown alga *Fucus* the sperm cell entrance site polarizes the egg cell and marks the point of future rhizoid development (Jaffe, 1958). Redistribution of F-actin filaments has been shown to accompany polarization of the unicellular *Fucus* zygote (Kropf *et al*, 1989). In eggs of the amphibian *Xenopus laevis* polartity is likewise induced by the entrance of the sperm cell at a specific site, and followed by a redistribution of cytoplasm (Gerhart et al, 1981). Hence at least in non-vascular plants and amphibians polarization involves similar mechanisms. On the other hand, in *Drosophila* it is well established that maternally delivered cytoplasmic factors provide polarity to the egg cell, demonstrating that several ways to establish zygotic polarity exist in the animal kingdom (Ingham, 1988). It is not clear which mechanism provides polarity to the embryo in higher plants, but apparently the first cell division of the plant zygote, resulting in a terminally differentiated suspensor cell and the *embryo proper* cell which will give rise to the mature embryo, already provides the polarity that specifies the sites where root and shoot meristems are formed at later stages.

Meristem initiation has been studied in explanted tissues because the embryo, which is surrounded by carpel tissues, is inaccessible to manipulations. A classic experiment using cultured tobacco cells showed that the ratio of the plant hormones auxin and cytokinin determine the type of meristem that is formed (Skoog and Miller, 1957).

Experiments with cultured *Convolvulus arvensis* leaf segments confirmed the importance of the auxin to cytokinin balance, and demonstrated that commitment of new meristematic cells to develop into either root or shoot tissues was irreversible, and hence the result of two different and non-convertable developmental pathways (Christianson and Warnick, 1983). Here a clear analogy to the determination concept in animal systems exists, but on the other hand it is unknown whether differences in hormone balance result in determination of root and shoot meristems *in vivo*. The concept of competence might apply to the ability of the root and shoot mersitems to become active only at a certain time point, when the seed germinates.

Post-embryonic development

In plants post-embryonic differentiation starts when, upon germination of the seed, the root and shoot meristems exhibit cell divisions in directed geometric planes, and in that way lead to the formation of new organs and tissues. Initial cells in the meristem, which are morphologically different and have a longer cell cycle then the other meristematic cells, remain the source of new meristem cells, and resemble animal stem cells in function and characteristics (Barlow, 1978). In general, meristems can give rise to indeterminate growth patterns and can continuously generate vegetative and floral organs. The shoot meristem forms axillary meristems and thus enables the formation of lateral branches with a new apical meristem.

Root and shoot meristem cells are determined to root and shoot developmental programs, respectively. Nevertheless, these cells and their derivatives have a large developmental plasticity. The formation of stable tissue patterns in plants shows that cells become determined to a particular developmental fate, but this fate is very often reversible. The capacity of many (but not all) plant cells to respond to signals for the redirection of development throughout their life cycle, and the absence of irreversible developmental commitment even in a differentiated state is referred to as totipotency. This is in contrast with the general occurrence of irreversible determination in animal development, examplified by the cell autonomous development observed in lineage mutants from *Caenorhabditis elegans* (e.g. Horvitz and Sulston, 1980).

Many features during the post-embryonic growth phase demonstrate the plasticity of plant development, for example: 1) The shoot meristem of determinate plants can become re-directed as a whole by various stimuli during plant development. These stimuli can be of both internal and external nature, and they cause terminal differentiation of the

meristem into floral tissues, tendrils or thorns. 2) A subset of cells in the petiole becomes competent to respond to the hormone abscisic acid (ABA) and forms the leaf abscission layer. 3) Pericycle cells derived from the root meristem can be re-activated to form new meristems for lateral root formation. 4) Vascular cells derived from the procambium can become mitotically re-activated to establish secondary growth of the vascular cylinder. 5) A haploid germ line can be established from functionally differentiated diploid cells in pistil and stamens of the flower. 6) In monocots, intercalary meristems develop at the base of leafs to supply cells for leaf elongation. 7) Meristematic activity and new pattern formation can also be induced in plant tissues for wound healing after physical damage. This capacity of plant cells to retain totipotency after specialization, is also reflected by the relative ease with which many cell types can be used to regenerate complete plants *in vitro*. The regeneration potential of plant cells by far exceeds that of animal stem cells, which are always pre-specialized.

A major question in plant developmental biology is how the activity of meristems is programmed. This question has been addressed on an operational level mostly using the shoot meristem as a model, recently reviewed by Sussex (1989). The shoot meristem consists of three superimposed cell layers, of which the upper L1 layer forms exclusively epidermis tissue. All the other tissues are formed by a mixed population of derivatives from the other two L2 and L3 cell layers. L1 cells sometimes divide parallel to the surface and give rise to cells of the L2 layer, which then do not form epidermal cells anymore (Derman, 1953). This demonstrates that, already within the meristem, developmental fates are position dependent, and not lineage dependent, and that cells in the three layers are not irreversibly determined. In the fern *Osmunda cinnamomea* the upper five leaf primordia, after excision from the shoot, give rise to complete plants in sterile culture, but an increasingly higher number of explants from the next leaf primordia give rise to leaves instead of complete plants (Caponetti and Steeves, 1963). This indicates that the developmental fate of leaf primordia is acquired gradually. Additionally, scorable mutations induced in the shoot apical meristem of mature embryos of maize and sunflower, allowed clonal analysis of the progenitor cells. It appeared that different cells in the meristem formed distinct sectors in the plant, usually bounded by internodes. However, these sectors appeared to overlap when large numbers of plants were analysed, indicating that meristem cells were not precisely determined to form particular plant sectors (Coe and Neuffer, 1978; McDaniel and Poethig, 1988; Jegla and Sussex, 1988). A highly significant result of these experiments is that no evidence was obtained for pre-

specialization of meristematic cells to form different tissues, with the exception of the epidermis tissue mentioned above. All these observations indicate that determination of the developmental fate of single cells to form a particular tissue does not occur inside the shoot meristem during post-embryonic development.

Cell walls and development

In plants the presence of a cell wall fixes the relative positions of neighbouring cells and prevents cell mobility, an important means to achieve morphogenesis in animals. The post-embryogenic morphological pattern is formed by differences in meristematic division rates and division planes, and cell growth rates. Since developmental adjustments during morphogenesis cannot be made by re-grouping of cells this appears to be counteracted by the ability of cells in a fixed position to adjust their developmental fates to those of neighbouring cells. This implies the capacity of plant cells to receive information on the developmental stages of neighbouring cells, which can also be referred to as positional information. As stated before developmental fates are fixed late during post-embryonic development. Positional information is therefore likely to be of major importance in determination of cell fate.

Conclusions

The concept of determination appears applicable to the stable formation of root and shoot meristems during plant embryogenesis, and competence is possibly involved in determining the start of meristem activity upon germination. During the post-embryonic stage of development, which covers the most elaborate part of the cell differentiation and morphogenesis events in the plant life cycle, the developmental fate of cells derived from at least the shoot meristem is established late in development, when cells are fixed in position relative to their neighbours. Therefore developmental decisions rely heavily on positional cues. Since developmental fates of many cells remain flexible, the term determination can only be adapted to plant development if it is defined as a commitment to a particular developmental fate which is reversible.

MOLECULAR MECHANISMS INVOLVED IN DEVELOPMENT

ANIMAL DEVELOPMENT

Genes and animal development

Recently the combination of genetical, biochemical and molecular biological research has provided some insight into the molecular mechanisms governing animal development and the compounds involved, and before turning to plant development it is useful to list the emerging principles. The importance of genes in animal development has become clear from two kinds of observations: 1) single-gene mutations affect developmental decisions, e.g. the homeotic mutations in *Drosophila*; 2) differentiated cell types are marked by the expression of specific gene sets. The first set of observations show that animals contain in their genome a set of genes which affect developmental decisions during the formation of a multicellular organism from the zygote. The second set of observations demonstrate that cellular differentiation involves activation and repression of specific gene sets, leading to different cellular phenotypes. An important question regarding the genes which form developmental instructions is which molecular mechanisms allow these genes to affect developmental decisions. This question is now adressed in several experimental systems, and an overview will be provided below. The question of how specific gene sets are activated and repressed during cell differentiation, and how do these gene sets lead to the differentiated cell phenotype, is also being addressed, but only fragmentary knowledge about the modes of activation of large sets of specific genes has been obtained. Combinatorial use of different *cis* elements in promoter regions and *trans*-acting factors is thought to be the mechanism by which limited numbers of inductive compounds in different combinations can lead to a large diversity in gene expression patterns (Dickinson, 1988).

Molecular mechanisms of animal development

Animal development occurs mainly during embryogenesis and is characterized by an interplay of cell-autonomous differentiation programs and differentiation upon receival of positional cues. On the other hand the majority of plant development occurs after embryogenesis and positional cues appear most important. Therefore I shall below concentrate on those mechanisms which are related to positional information in animals.

In chicken wing buds, it was found by grafting experiments that cells receive positional information for specifying differentiation, and that differentiation is dependent on the distance to a tissue which is giving a positional signal. This resulted in the hypothesis that gradients of diffusible compounds of low molecular weight, so-called morphogens, provide positional information (Wolpert, 1969; Crick, 1970). In the original definition a morphogen is a positional signal that can produce multiple, concentration-dependent, developmental outcomes. Emerging details on the chemical nature of morphogen gradients that invoke differentiation in several animal systems will be discussed. Second, data concerning the molecular systems that lead from positional information provided by morphogens to cellular differentiation responses will be presented. Third, a group of molecules receiving positional information by cell-cell interactions, leading to all-or-none inductive events, is discussed. Fourth, the role of signal transduction in development will be regarded.

1) Positional signals

Several morphogens have been identified in animal systems. In the developing chicken wing bud retinoic acid appears to influence differentiation in a concentration-dependent manner. Furthermore, retinoic acid was shown to be present in developing chicken wing buds at different concentrations, consistent with the hypothesis that this compound is locally elicited and forms a concentration gradient (Thaller and Eichele, 1987).

During *Xenopus* embryogenesis, two peptide growth factor analogues induce ectoderm cells to differentiate into mesoderm derivates (Kimelman and Kirschner, 1987). The maternally deposited Vg1 mRNA, encoding the TGF-β homologue, has been shown to form a gradient in the developing embryo (Weeks and Melton, 1987). In explanted animal pole cells the two factors mentioned induce different mesodermal cell types when applied in different concentrations and they therefore seem to meet the morphogen definition (Smith, 1989).

Nurse cells distribute *bicoid* (*bcd*) mRNA into the anterior pole of the *Drosophila* oocyte. Upon fertilization this mRNA is translated and the resulting protein forms a concentration gradient in the zygote. The *bcd* protein contains a conserved 'homeobox' motif with DNA binding properties, and has been shown to induce transcription of at least the zygotic gap-class gene *hunchback* in a concentration-dependent manner (Struhl *et al.*, 1989). Hence the *bcd* protein can be regarded as a morphogen.

2) Receptors

Clearly several kinds of molecules conferring positional information during development exist in animals. Equally important in development are molecules by which cells or intracellular regions receive and subsequently respond to positional information. The presence of such systems marks the stage of competence to respond to developmental cues. A variety of molecular components of systems that appear to elaborate initial positional information both at uni- and multicellular stages have been reported on. Representative examples of the different classes of molecules involved are given below.

The retinoic acid receptor has homology to nuclear receptors for steroid/thyroid hormones with putative DNA-binding 'zinc finger' domains (Petkovitch *et al*, 1987). Steroid/thyroid hormones specifically direct differentiation processes, but not necessarily affect graded responses (for an overview see Walbot and Holder, 1987). This implies that the retinoic acid system is a member of a larger, related family of morphogens/hormones and receptors that govern particular stages of differentiation by direct transcriptional activation of target genes.

The transition of morphogenetic gradients in the unicellular zygote to increasingly complex patterns of differentiated cell states at blastula stage is becoming unravelled now in *Drosophila* embryogenesis. The emerging picture is that initial gradients, from which the *bcd* protein gradient is only one, cause differential expression of genes encoding transcriptional regulators. These transcriptional regulators bring about the expression of genes encoding different transcriptional regulators. In this way the products of several genes establish a hierarchic cascade of transcriptional regulation leading to a differential distribution of gene products. The differential distribution of the encoded proteins forms an increasingly complex spatial pattern which confers unique positional values to different regions in the embryo. In this way both segmentation and segment identity are established. Two classes of transcriptional regulators appear to be active with different putative DNA binding domains: the nucleic acid binding 'zinc-finger' and the homeobox domain, respectively (Ingham, 1988). The establishment of pattern by these transcriptional regulators requires interactions between them, as inferred from mathematical models that mimick pattern formation (Meinhardt, 1988; Nagorcka, 1988). Experimental evidence for activation and repression of genes by different homeobox proteins has been obtained (Krasnow *et al*, 1989), and synergism in activation and repression activities between different homeobox proteins has also been proven (Han *et al*, 1989).

3) Cell-cell interactions

Interestingly, while the first subdivision of the *Drosophila* embryo is accomplished mainly by a transcriptional regulation cascade, many cells at blastula stage are still pluripotent, and further differentiation requires intercellular communication. Examples of genes involved in developmental decisions by intercellular communication are the *notch*, the *dpp*^{Hind}, and the *sevenless* (*sev*) genes. During embryogenesis the *notch* gene is involved in the binary switch between neural and epidermal differentiation. The *dpp*^{Hind} gene is essential for the establishment of the dorsal cell type during embryogenesis. In the developing eye the *sev* gene determines the differentiation of the R7 photoreceptor cell. Both *notch*, *dpp*^{Hind} and *sev* gene products have protein domains homologous to peptide growth factors and transmembrane domains. Mosaic analysis of *sev* mutants indicates that the *sev* product is a receptor for a differentiation-inducing signal from neighbouring cells (Wharton *et al*, 1985; Hafen *et al*, 1988). Most likely also the *notch* and *dpp*^{Hind} gene products enable cells to receive a signal from an already differentiated neighbouring cell. This signal then specifies development of the receiving cell.

4) Signal transduction

It is also becoming clear that specific signal transduction pathways have an important role in animal development. The *Drosophila sev* receptor contains a protein tyrosine kinase domain, presumably capable of phosphorylation of specific target proteins (Hafen *et al*, 1988). Genetic evidence suggests interaction of the *notch* gene product with the *Enhancer of split* protein, a putative G-protein involved in signal transduction (D. Hartley *et al*, cited in Ingham, 1988). The *Drosophila* oncogene Abelson tyrosine kinase (*abl*) is involved in the development of axons in the embryonic central nerve system (Gertler *et al*, 1989). Finally, most likely also *Drosopila* oncogene c-*src* homologs, the tyrosine kinase D*src*28C proteins, are involved in segmentation as well as in neuronal differentiation (Vincent III *et al*, 1989).These examples show that specific signal transducers like G-proteins and protein kinases are involved in development.

Generality of molecular mechanisms in animal development

Drosophila embryonic development, from which most of the examples mentioned above were taken, exhibits many species-specific features. Still *Drosophila* appears to be useful to elucidate general molecular mechanisms that convert positional signals into actual

differentiation, because many homologous gene products are found in other animals: 1) At least three *Caenorhabditus elegans* genes that regulate differentiation have been shown to contain homeoboxes (see Levine and Hoey, 1988), and in mice the members of the *Hox* family contain homeoboxes. The spatial distribution of the different *Hox* transcripts during murine embryogenesis implies a role in development (Dressler and Gruss, 1988). 2) The mouse zinc-finger domain containing *mKr2* gene product is present throughout development of the central nervous system, and might be involved in development of this tissue (Dressler and Gruss, 1988). 3). The *Caenorhabditis elegans lin-12* gene product is one of the best studied receptors for an inductive molecule, and appears homologous in both structure and function to the *Drosophila notch*, *dpp*^{Hind}, and *sev* gene products. In several different cell lineages this gene product affects a binary switch between one cell fate and another (Greenwald *et al*, 1983). The *lin-12* protein has repeated peptide domains homologous to those in mammalian growth factors (Greenwald, 1985). At least in a gonad precursor cell depending on *lin-12* activity for differentiation, it was shown that the *lin-12* protein is the receptor for a positional signal from a neighbouring cell which specifies development (Seydoux and Greenwald, 1989). 4) The general importance for growth-factor like molecules and their receptors in mammalian development is well recognized.

Conclusions

Taken together, several main groups of gene products appear to be involved in regulating animal development: positional signals and their membrane-bound receptors, transcriptional regulators, and proteins which are part of specific signal transduction pathways. Important molecular mechanisms in animal development appear to be the generation of graded positional signals, which are then translated into concentration-dependent differential responses. Translation of signal into response occurs either by direct transcriptional activation or by signal transduction mechanisms leading to activation of specific subsets of proteins. Furthermore receptors which can recognize neighbouring cells exist, where the presence of the appropriate signal molecule on the neighbouring cell results in an all-or none developmental decision in the cell carrying the receptor.

The generalized picture of animal development is one of several cascades of spatial compartimentalization by different gradients of positional signals. The differential distribution of these signals causes certain cells to become determined. Specific signals elicited by or present on the surface of already determined cells can then further modulate

fine-grained development by cell-cell interactions (see also Ingham, 1988). Ultimately an elaboration of initial differences, obtained in a limited amount of cells, into large organized groups of cells with a specific pattern of gene expression that determines their phenotype has to be established to complete development.

PLANT DEVELOPMENT

The importance of genes in plant development

Indications for the importance of gene expression in plant development stem from the same arguments initially raised for animal systems. First, plant homeotic mutants in which one mutated gene affects specific developmental steps are known, and second, different plant tissues show specific gene expression patterns.

Single-gene mutations in *Arabidopsis* that alter flower development show the direct role of genes in specific developmental processes, and provide genetic evidence that development in plants indeed relies on positional information. A precise study on the phenotype of four recessive, chemically induced mutations revealed homeotic changes from stamens to petals in *agamous-1*; sepals to leaves and petals to staminoid petals in *apetala2-1*; petals to sepals and stamens to carpels in *apetala3-1*; and petals to sepals in *pistillata-1* (Bowman *et al*, 1989). Temperature-shift experiments on the temparature-sensitive *apetala* mutants revealed that the wild-type products act at the same time or after the primordia of the organs they affect are formed. Hence the wild-type products appear to allow primordial cells to determine their place in the developing flower and differentiate appropriately. Flower mutations in other plant species are described which are very similar, perhaps homologous (discussed by Bowman *et al*, 1989), indicating that the use of positional information in flower development is a universal mechanism in higher plants.

By studying hybridization kinetics Goldberg *et al* (1978), and Kamalay and Goldberg (1980) demonstrated that tobacco organ systems contain 24,000-27,000 polysomal RNA species, of which approx. 8,000 are shared in all organs, and at least 6,000 are unique to each organ system. Differences in nuclear mRNAs composition were also shown to exist between the different organ systems, but more overlap in nuclear mRNA composition then in polysomal mRNA composition between the different organs was observed, indicating that both transcriptional and post-transcriptional regulation occur to achieve differences in mRNA sets (Kamalay and Goldberg, 1984). The majority of the organ-

specific transcripts were shown to belong to the rare-class mRNAs (in total less then 0.01% of the mRNA mass). This mRNA complexity in plants is equivalent to the complexity in animals (Hastie and Bishop, 1976). Hence plants, albeit apparently more simple in structure and containing less morhpologically different cell types, show a complexity in gene regulation similar to that in animals. Stage-specific differences in rare-class mRNA composition of different mature organs were not observed during the later stages of soybean embryogenesis (Goldberg *et al.*, 1981). Therefore it is not clear whether regulation of rare class mRNA plays a role at that stages of embryo development. However, by molecular cloning numerous (medium-) abundant mRNAs have been shown to be regulated in a variety of developmental processes, emphasizing the importance of differential gene expression during development.

Compounds involved in plant development

At this moment research on the molecular biology of plant development is just starting. Plant physiologists and biochemists have however been putting effort in analyzing the effects of various compounds on development. Therefore a short description will be given of compounds shown to be involved in plant development. The existence of possible analogies to animal developmental mechanisms will be considered.

Plant growth regulators

Growth and development of plants is beyond doubt influenced by the plant growth regulators (phytohormones), five groups of small chemical compounds. Plant growth regulators have pleiotropic effects throughout the plant life cycle and the same set of growth regulators is involved in developmental processes (reviewed by Hall, 1984) as well as in coordination of overall plant activities (reviewed by Wareing, 1984). Space is too limited here to list all known effects of plant growth regulators, and to elaborate data on biosynthesis, active structures, and transport (for a recent overview see Roberts and Hooley, 1988). It is however relevant to call to mind briefly that growth regulators appear to induce meristem induction and subsequent differentiation of plant cells in a concentration dependent manner, which classifies them as positional signals resembling the animal morphogens: the ratio of auxin to cytokinin influences the decision to form root or shoot meristems in callus (Skoog and Miller, 1957). The formation of floem and xylem fibers in callus is dependent on the auxin concentration (Jeffs and Nothcote,

1967). In these examples plant growth regulators seem to establish determination of undifferentiated cells.

The nature of the molecular mechanisms underlying hormone-induced development is unknown. The general opinion is that, to account for the different responses of plant cells to one or more growth regulators, the existence of a multitude of receptors, capable for specifying responses, has to be proposed (Hall, 1984). The balance of certain hormones could then form a developmental field with analogies to morphogen gradients in animals. A specified developmental pathway could be triggered if appropriate receptors, reflecting the competence of the target cell, are present. Answers to basic questions on the action of plant growth regulators thus depend on description of receptors and growth regulator effects at the cellular level.

The auxins are the best studied plant growth regulators in terms of searching for putative receptors and analyzing effects at the cellular level. Evidence has been presented for the existence of both membrane-bound and soluble auxin receptors in tobacco cells (Libbenga *et al*, 1986). This points to the presence of an amount of target molecules for one single plant growth regulator that is unparallelled in animal systems, supporting the multiple receptor hypothesis to account for the many different responses of plant cells to auxins. For molecular cloning of a putative auxin-receptor, antibodies to an auxin-binding protein have been raised that block auxin-induced, ATPase-mediated proton import across tobacco protoplast membranes (Löbler and Klämbt, 1985; Barbier-Brygoo *et al*, 1989). The primary structure of the protein encoded by a cDNA clone isolated with the aid of these antibodies surprisingly provides evidence that not an integral membrane protein but a protein residing in the endoplasmatic reticulum is encoded (Tillmann *et al*, 1989). This indicates that the epitope on the membrane-bound protein reacting with the antibody is also present on a protein residing in the cytosol. It will have to be established whether the latter protein is an auxin receptor. The amino acid sequence derived from this putative auxin receptor protein is not homologous to previously described proteins, indicating that plant growth regulator receptors may be structurally unrelated to animal signal receptors. As to the subsequent activities of auxin upon binding to receptors, rapid induction of specific mRNA synthesis indicates that at least part of the auxin effects rely on transcriptional activation of target genes (Theologis, 1986). Whether plants are able to respond to other growth regulators by the means of various receptors is not yet conclusively established, although there are clues to the possible existence of

proteinaceous receptors for abscissic acid and gibberellins (Hornberg and Weiler, 1984; Stoddart, 1986).

The pleiotropic effect of plant growth regulators (phytohormones) on development has urged investigators to seek for more specific regulators of growth and differentiation in plants. Cell-wall derived oligosaccharins (oligosaccharides with regulatory activity) have been shown to influence morphogenetic pathways in tobacco thin-layer explants (Tran Thanh Van *et al*, 1985; Eberhard *et al*, 1989). These compounds can, at narrow concentration ranges comparable to those at which the classical plant growth regulators are effective, induce explants to form either vegetative buds, flowers, callus or roots. The authors conclude that plant growth regulators could affect the release of cell wall oligosaccharides as non-pleiotropic chemical messages *in vivo* which 'regulate a delineated set of biochemical processes that regulate morphogenesis'. Proof for this statement has to await further experimentation. Interesting with regard to this evidence for sugar residues as signal molecules, is the suggestion that sugar-binding glycoproteins termed lectins, which are widespread in the plant kingdom, may play a role as receptor in various cell-cell recognition events in plants (Knox and Clarke, 1984).

Phenylpropanoids, possibly derived from the plant cell wall, have been shown to replace cytokinin requirement for growth in cultured tobacco cells (Lynn *et al*, 1987). Hence not only sugars but also phenolic compounds from cell walls could be cues for growth and development in plants.

In conclusion, plant growth regulators, notably auxin, share certain characteristics with animal hormones: receptor-mediated activity, and hormone-induced selective gene expression. The growth regulator molecules themselves are however completely different from the morphogen/hormone molecules that provide positional information in animals. Neither gradient responses as with animal morphogens have been clearly demonstrated. Furthermore the question as to whether plant growth regulators are primary causative agents in *in vivo* differentiation events, or secondary compounds like oligosaccharins and phenylpropanoids play a decisive role, remains to be established.

Transcriptional regulators

The findings of Paz-Arez *et al* (1987) that the maize *C1* regulatory locus encodes a protein that is homologous to the transcriptional activator and proto-oncogene *c-myb* , and of Katagiri *et al* (1989) that two tobacco DNA binding proteins share homology with animal nuclear factors, can be seen as an argument for the conserved structure of

regulatory proteins in animal and plant kingdoms. Proteins with demonstrated transcriptional regulator activities, and involved in development, have to our knowledge not yet been identified in plants. However, the existence of a homeodomain in the yeast mating type proteins, which act as transcriptional activators invoking a developmental switch, proves that the use of this type of proteins is not restricted to the animal kingdom but might be used in all eukaryotes. This indicates that homologous proteins with analogous functions might be active in plants.

Signal transduction

Accumulating data on the relevance of signal transduction systems for plants have been recently reviewed by West *et al* (1989). In plants compounds have been found which could function in signal transduction systems like described for animals. Currently the existence of phosphatidylinositol pathways in plants is supported by more convincing data then the existence of cyclic nucleotide-mediated pathways. Phosphatidylinositol compounds and the membrane-bound phospholipase C enzyme that can generate them have been detected in plants (Boss, 1989). Especially interesting is the finding that auxin can induce cell division in arrested *Catharanthus roseus* cells, which is accompanied with a rapid generation of specific phoshatidylinositols (Ettlinger and Lehle, 1988). Several lines of evidence point to the importance of Ca^{2+} as second messenger in plants (Marmé, 1989). Ca^{2+}/calmodulin-dependent proteins kinases have been identified in plants (e.g. Blowers *et al*, 1985). Whether these compounds are functional in plant developmental processes remains to be established. The application of 'brute force' cloning of specific protein kinases and G-proteins in plants might be one way to establish the importance of signal transduction mechanisms in plant development (Palme *et al*, 1989).

Conclusions

A major effort of the research on the molecular basis of plant development is the identification of compounds possibly involved in pattern formation and differentiation. The emerging hypothesis is that phytohormones act as positional signals, whose pleiotropic effects could be mediated by different receptors, possible involving elements of signal transduction systems. The molecular structure of known developmental signals and putative receptors in plants does not resemble the structure of animal positional signals and receptors. On the other hand analogous transcriptional activators and signal transducing proteins exist in animals and plants. It can therefore be postulated that if the

mechanisms to convey positional information in plants and animals are different, the mechanisms to translate this information into differential gene expression may be rather similar. Models on molecular mechanisms leading from positional information to differentiation during embryogenesis and post-embryonic development are lacking so far. To establish these models questions regarding plant development will have to be asked in suitable experimental systems. Below I will discuss root nodule formation as such a system, capable of providing clues to the molecular mechanisms of post-embryonic development from meristems.

ROOT NODULE FORMATION AS A SYSTEM TO STUDY PLANT DEVELOPMENT

Root nodule formation involves development of an organ in which nitrogen fixation takes place. This particular case of organogenesis is induced by *Rhizobium* bacteria. In section A, I shall explain why the root nodule can be considered to be a unique plant organ, and I shall describe the series of events leading to nodule formation. In sections B and C, I shall discuss the intrinsic qualities that make root nodule formation suitable to study meristem formation and the subsequent differentiation leading to the different nodule cell types.

A). DESCRIPTION OF ROOT NODULE FORMATION.

Root nodules consist of several different tissues, schematically represented in figure 1: nodule cortex, endodermis, nodule parenchyma ('inner cortex') containing vascular strands, and a central tissue made up of cells infected with rhizobia and uninfected cells (Newcomb, 1976; chapter 3). The cell types of the central tissue are unique for root nodules as they have special functions bearing on symbiosis and assimilation of fixed nitrogen. The spatial organization of tissues like endodermis and vascular strands in root nodules is characteristic and is not found in other parts of the plant containing endodermis and vascular strands. Furthermore the origin of the nodule primordium in the root cortex is exceptional since organs do not normally arise from this tissue. For all these reasons the root nodule may be considered a unique organ.

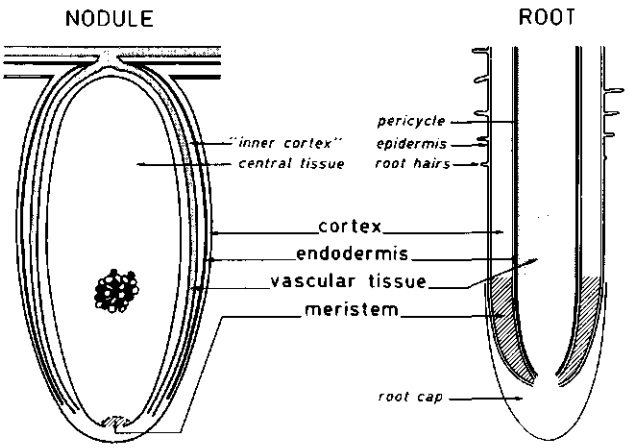

Figure 1. Anatomy of the root nodule compared to the root.

Two types of root nodules are distinguished, the indeterminate type found e.g. in pea and alfalfa infected with *Rhizobium* species, and the determinate type found in e.g. soybean infected with *Bradyrhizobium*. Indeterminate nodules contain a persistent meristem at the apex from which the different tissues of the nodule develop. In that respect the nodule meristem resembles the apical root and shoot meristems, which are also persistent and which are the source of the different root and shoot tissues. Since post-embryonic development in plants involves indeterminate meristems their formation and activity are of wide importance. In the following I shall therefore elaborate only on the formation of indeterminate root nodules. Determinate nodules which lack a true persistent meristem will not further be discussed.

Different stages of the formation of a pea root nodule are depicted in figure 2, copied from Libbenga and Harkes (1973). Bacteria first attach to root hairs, make them curl and enter the hairs after local hydrolysis of the cell wall. Upon entering the bacteria induce the plant cells to deposit cell wall material which forms a tubular structure, the infection thread. Through this thread the bacteria enter the plant. This type of infection process is a specific feature of the plant-*Rhizobium* interaction. In front of the growing infection thread tip (ti) a centre of mitotic activity is induced in the inner cortex which constitutes the nodule primordium, as shown in figure 2A and 2B. Upon reaching the primordium the infection thread branches (figure 2C, 2D). In the centre of the primordium the first bacteria are released into plant cells where they eventually differentiate into bacteroids (bt) capable of nitrogen fixation (figure 2E). When the bacteria are released from the infection thread, cells at the distal site of the primordium

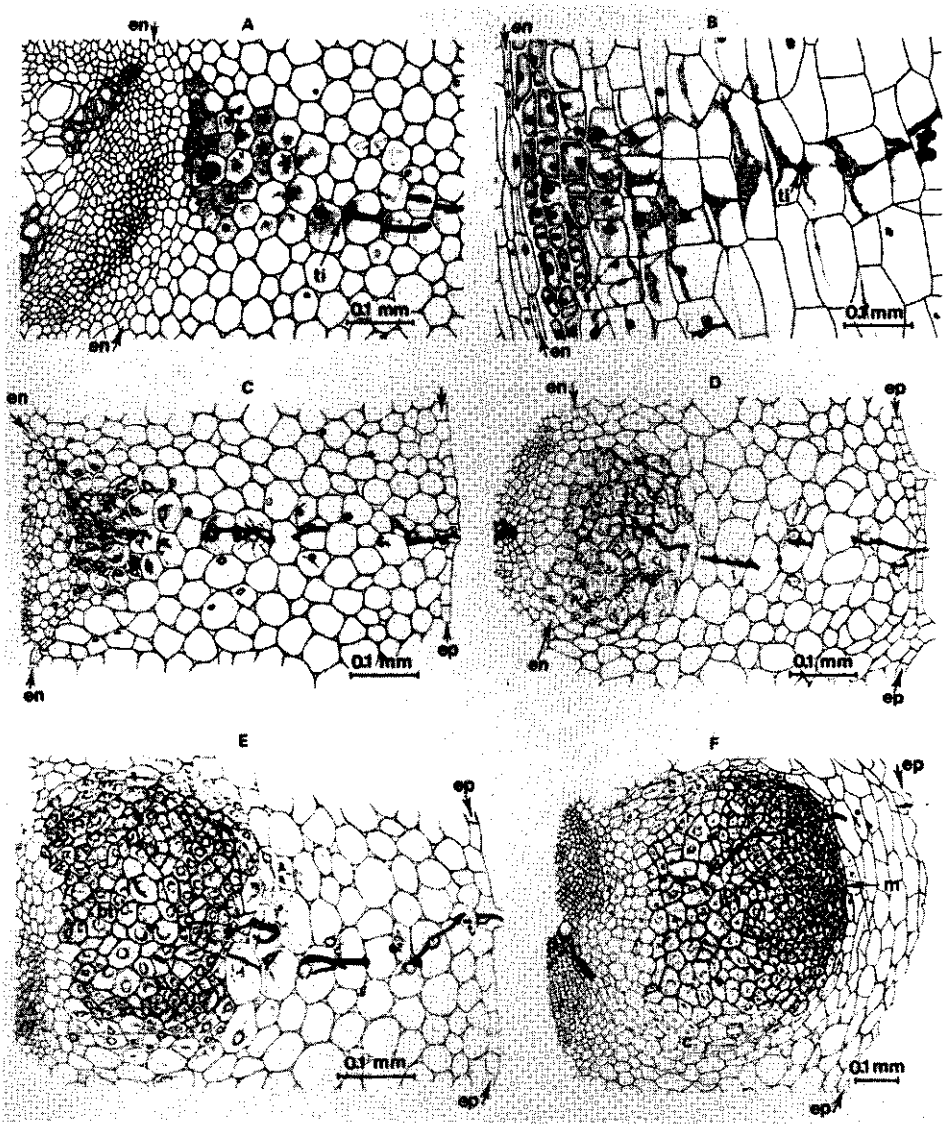

Figure 2. Pea root nodule development. A-F: successive stages of development from nodule primordium initiation to the formation of a root nodule containing an apical meristem. En: endodermis; ti: infection thread tip; ep: epidermis; bt: bacteroids; m: meristem. Reprinted from Libbenga and Harkes, 1973.

become small and rich in cytoplasm. These cells form the apical nodule meristem. The meristematic centre is pushed outward, while generating the different nodule tissues (figure 2F). The infection threads have now reversed their original growth direction by 180° and follow the meristem. In the zone immediately adjacent to the meristem the release of bacteria from the infection threads continues. In that way new infected cells are continuously added to the central tissue, that contains infected and uninfected cells in an appr. 1:1 ratio. Hence root nodule formation, induced by a symbiosis-specific infection process, includes such characteristic developmental events as the induction of meristematic activity and formation of a new plant organ.

B) ROOT NODULE FORMATION AND THE DEVELOPMENT OF APICAL MERISTEMS.

From preceeding paragraphs it will be clear that the question as to how meristems are initiated and how they are determined to generate specific tissues is crucial in the study of post-embryogenic development in plants. There are three arguments for considering root nodule formation a suitable system to study meristem formation: 1) the development of nodules, involving the formation of a nodule primordium and meristem, can be studied in a fixed spatial and temporal frame; 2) a limited, and therefore approachable, set of defined *Rhizobium* genes is involved in nodule primordium and meristem formation; 3) determination of the nodule meristem seems to be achieved by a limited number of compounds. These arguments will be elaborated below.

1. Controlled induction of primordium and nodule meristem formtion

The starting point of the induction of the primordium from which the nodule meristem is ultimately formed, is the first cell division in the root cortex. These cell divisions can be induced in a controlled way by spot-inoculation of *Rhizobium* on plants, which defines the time and location of primordium development (Calvert *et al*, 1984; Dudley *et al*, 1987). Similarly the time and site of appearance of the nodule meristem at the distal site of the primordium are defined. This enables the precise description of variations in primordium and meristem formation if mutant *Rhizobium* bacteria, rhizobial factors or mutant plants are used. Also the various stages at which specific plant genes are involved in this process can be studied by visualising the location of the corresponding transcripts at defined time points after inoculation by *in situ* hybridization (this thesis).

The site where the first mitotic activity is induced resides in the inner cortex, in almost all cases opposite the xylem poles. The specific localization of the responding cells raises the question whether this is a predetermined site, implying that inner cortex cells are the only ones competent to respond to a bacterial signal, or whether the initiation place is determined by gradients of positional signals produced by both the plant and *Rhizobium* bacteria. A clue for the latter mechanism can be inferred from the observation that *in vitro* a diffusible factor from the root vascular system can specify in a pea root explant system the cortical cells which become mitotically active at the proper auxin-cytokinin ratio (Libbenga *et al*, 1973). In the *Rhizobium*-legume symbiosis this ratio of plant hormones might be established by inductive signals from the bacterium (see below).

Although nodule meristem formation occurs at the time when the first bacteria are released in the primordium, bacterial release *per se* appears not to be the essential trigger for meristem induction as evidenced by *Rhizobium* exopolysaccharide-deficient mutants which do not infect the plant but are nevertheless able to elicit formation of a nodule meristem (Finan *et al*, 1985). At the other hand the observation that the nodule apical meristem only develops after a specific nodule primordium has been formed might be relevant. Traditionally the requirement of primordium formation prior to induction of meristematic activity has been explained by assuming that primordium formation is the necessary dedifferentiation event preceeding meristem formation. The presence of nodule-specific gene transcripts in the primordium which labels it as a specific tissue, and the observation that only a few distal cell layers of the nodule primordium form the meristem (Libbenga and Harkes, 1973; chapter 4), point to a more complicated function of primordium cells in induction, and perhaps determination, of the apical meristem.

In contrast with the root nodule meristem, the apical root and shoot meristems are not accessible to experimentation in a comparable way at the time when they are formed during embryogenic development. New foci of meristem activity that arise for example at the onset of lateral root development, are inaccessible to experimentation since they cannot be induced with external factors and for that reason their formation cannot be examined in fixed space and time frames. *In vitro* meristem induction with hormones on cultured cells (cf. Skoog and Miller, 1957) or with oligosaccharins on thin layer explants (Tran Thanh Van *et al*, 1985) can serve as an alternative to study *in vivo* meristem formation by the controlled external application of various growth regulators. However, if important clues on compounds possibly involved in meristem induction and specification have emerged from these studies, it remains difficult to establish whether

such compounds are also active in the *in vivo* situation. Because nodule formation is both externally inducable and occurring *in vivo*, the root nodule appears an attractive system to study general aspects of apical meristem formation.

2. Rhizobium genes and primordium/meristem formation

It has been established that a limited set of *Rhizobium* genes is involved in initiating the root nodule developmental program (cf. Long, 1989). This offers the unique possibility to interfere with a plant developmental process *in vivo* by manipulating the bacterial molecules involved in root nodule development using molecular genetic methods. Genetic manipulation of the production of endogenous plant compounds involved in development of, for example, the root and shoot meristems is not quite feasible for at least two reasons. First, plant genetics is technically more difficult than bacterial genetics because of the larger genome of plants. Second, even if the technical difficulties of applying plant genetics might be overcome, it is quite likely that mutations in meristem induction will be lethal or display pleiotropic effects.

The bacterial common nodulation (*nod*) genes have been shown to be essential for cortical cell division (Dudley *et al*, 1987), and most likely they produce a low molecular weight factor to achieve this (Schmidt *et al*, 1988). A striking observation has been that induction of cortical cell division, and subsequent meristem formation can be triggered by adding auxin transport inhibitors (Allen and Allen, 1940; Hirsch *et al*, 1989). Hence induction of nodule meristem initiation may involve plant growth regulators in an analogous way as postulated from *in vitro* meristem induction experiments (Skoog and Miller, 1957). This strongly suggests that also the bacterial factors that trigger cortical cell division and formation of a nodule meristem interfere with the plant hormone balance. If these compounds are comparable with those used in the formation of other meristems, mechanisms that influence the fine-grained distribution of plant growth regulators, possibly important for establishing positional information involved in meristem induction, can be investigated.

3. Determination of the nodule meristem

Apart from the possibility to study *in vivo* meristem formation in the root nodule system, it seems possible to study the mechanisms that lead to determination of the nodule apical meristem. An important observation in that respect is that the meristem induced by addition of auxin transport inhibitors to alfalfa roots gives rise to a pseudonodule

structure cytologically similar to bacterial root nodules, and in which at least one early nodulin gene transcript is present (Hirsch *et al*, 1989). The location of this transcript in pseudonodules corresponds to the location in bacterial nodules (Van De Wiel *et al*, in prep.). From these data it can be concluded that a single compound can establish *in vivo* induction of a meristem which is determined to form a tissue strongly resembling a bacterial root nodule. Therefore it is quite possible that compounds made by the *nod* gene products are also able to induce both meristem formation and determination. For example the factor identified by Schmidt *et al* (1988), might not only be involved in induction of cortical cell division but also in meristem specification. In addition, *nod* gene factors may cover other aspects of determination, required for the formation of a mature root nodule. The observation that *Agrobacterium* transconjugants carrying the cloned *Rhizobium nod* genes can induce formation of a cytologically normal nodule structure containing early nodulin transcripts is in agreement with the assumption that *nod* gene products govern both meristem induction and determination (Hirsch *et al*, 1984; Truchet *et al*, 1985; Dickstein *et al*, 1988; Nap *et al*, 1989). Characterization of the factors produced by the *nod* gene products can lead to an understanding of the mechanisms underlying meristem determination.

An intriguing question is how a limited number of bacterial compounds is capable of specifying a complex developmental pathway. This phenomenon might be explained if it is assumed that these compounds merely trigger and slightly modify an existing developmental pathway. It can be argued that the triggering and modification of an existing developmental pathway requires less information then the complete specification of a developmental process. In the following I will summarize some data that are in favor of considering root nodule development as a modified version of lateral root development.

Inoculation of the *R. meliloti* strain JT205, carrying a mutation in a Sym-plasmid gene, results in the formation of nodules that resemble lateral roots (Dudley *et al*, 1987). Root nodules formed by *Frankia* sp. on the non-legume *Alnus* and by *Rhizobium* species on the non-legume *Parasponia*, do resemble lateral roots in morphology (Drake *et al*, 1985; Lancelle and Torrey, 1984a,b); they contain an apical meristem and a central vascular tissue as in roots; the vascular tissue originates from the root pericycle, which is the site of origin of lateral roots. *Rhizobium nodABC* genes which bring about the formation of the lateral root-like nodules on *Parasponia* can also induce formation of nodules with normal morphology on legumes (Marvel *et al*, 1985). This indicates that

compounds initiating a developmental pathway containing elements of lateral root development can also, in a legume, induce developent of a meristem giving rise to the unique nodule structure. For that reason it is possible that root nodule development is a slightly modified version of lateral root development in *Parasponia*, and a more extensively modified version in legumes. If this is the case it is not only conceivable that a few bacterial compounds can trigger a developmental pathway, but it is also quite possible that (lateral) root development involves similar compounds and mechanisms as nodule development. Elucidation of the mechanisms by which bacterial compounds determine the nodule meristem can than gain access to mechanisms involved in the determination of other meristems.

CELLULAR DIFFERENTIATION IN THE DEVELOPING ROOT NODULE

Once the nodule meristem has been determined to form the different nodule-specific tissues, a further important question becomes how differentiation and the differential gene expression programs belonging to it are executed. During root nodule development cells derived from the apical meristem differentiate into the central tissue with infected and uninfected cells, while peripheral tissues like nodule cortex, endodermis, nodule parenchyma and vascular strands also originate from the meristem. During root nodule development the synthesis of nodule-specific plant proteins, the nodulins, occurs (Van Kammen, 1984). Whereas late nodulin gene expression is detectable in the mature nodule shortly before the onset of nitrogen fixation, early nodulin gene expression accompanies different stages of root nodule development (Nap and Bisseling, 1989; this thesis). Not only does early nodulin gene expression mark different cell types within the developing nodule (compare chapters 3 and 4), it also marks successive developmental stages of one cell type (chapter 5). The availability of early nodulin cDNA probes specific for particular cell types and/or particular developmental stages within one cell type, together with the possibility to apply *Rhizobium* mutants defective in nodulation can be used to study cellular differentiation. Mutants can be selected that cause developmental blocks at specific stages of root nodule development, like the *R. meliloti* JT205 strain which forms nodules that have only peripheral but no central tissues developed from the meristem (Dudley *et al*, 1987). The developmental stages reached in these nodules can be monitored by expression analysis of early nodulin genes. The molecular defect caused by the bacterial mutation can be identified, and this may provide insight in the bacterial

signals that give rise to particular stages of differentiation marked by early nodulin gene expression. As stated before, during development signal transduction mechanisms probably play a crucial role in translating extracellular signals into differentiation responses. The availability of both signal molecules produced by the *nod*- or other bacterial genes and different target genes (early and late nodulins) allows the investigation of signal transduction chains from both ends: first, putative receptor molecules for the different signals can be isolated and studied; second, the possibility to clone a set of sequentially induced nodulin genes (chapter 5) also allows studying the *cis* and *trans* elements involved in time- and tissue-dependent gene activation upon differentiation of cells from an apical meristem. In this way the combinatorial model for the induction of differential gene expression (Dickinson, 1988) can be tested in a plant system.

CONCLUDING REMARKS

Current information on compounds involved in plant development has not resulted in a firm basis for hypotheses about molecular mechanisms of plant development. In this chapter I have outlined the features specific for plant development compared with animal development and I have used current ideas about the molecular mechanisms underlying animal development to provide a framework for listing data on molecular mechanisms of plant development.

A major problem of plant development is the understanding of the formation and the post-embryonic development of apical meristems from which different specific tissues originate. I have pointed out that the development of a root nodule has features corresponding to apical meristems and that, for that reason, nodule development can serve as a model system to investigate important questions on plant development. These questions are: 1) which plant compounds carry positional information necessary for development *in vivo*?; 2) which gene products guide the determination of cells to generate specific tissues?; 3) how do the compounds regulating development establish differential gene expression and the differences among cell types belonging to it?

The first question, on the plant compounds carrying positional information, might be approached using the root nodule system because it is possible to identify bacterial compounds that direct plant developmental decisions. The second question, concerning the plant gene products involved in determining the fate of cells and the generation of specific tissues, may be adressed by analyzing the nature of the defects in the

differentiation of specific cell types produced by *Rhizobium* mutants. Unfortunately at present there is no set of plant mutants available in which genes involved in cell fate decisions during nodule formation are affected, which would greatly facilitate the identification of plant genes involved in nodule development. The third question, concerning the mechanism of differentiation, may be answered by analyzing signal molecules and their target genes in different cell types and at successive stages of development. The root nodule system offers with the early nodulin genes a good entry to identify the specific signal molecules that are involved in the regulation of the expression of plant genes related to successive stages of development. Ultimately this may lead to unravelling the route from developmental signals to a differentiated phenotype.

REFERENCES

Allen,O.N., and Allen,E.K. (1940). Proc. Acad. Sci. Bernice, P. Bishop museum spec. publ. 35, 15-16.

Barbier-Brygoo,H., Ephritikhine,G., Klämbt,D., Ghislain,M., and Guern,J. (1989). Functional evidence for an auxin receptor at the plasmalemma of tobacco mesophyll protoplasts. Proc. Natl. Acad. Sci. USA 86, 891-895.

Barlow,P.W. (1978). The concept of the stem cell in the context of plant growth and development. In: stem cells and tissue homeostasis, B.I. Lord, C.S. Potten and R.J. Cole, eds. Cambridge University Press, Cambridge, pp 87-113.

Beyerinck,M.W. (1888). Die bacterien der Papilionaccen knöllchen. Bot. Zeitiung 46-50, 725-804.

Blowers,D.P., Hetherington,A., and Trewavas,A. (1985). Isolation of plasma-membrane-bound calcium/calmodulin-regulated protein kinase from ea using western blotting. Planta 166, 208-215.

Boss,W.F. (1989). Phosphoinoside metabolism: its relation to signal transduction in plants. In: Second messengers in plants growth and development, W.F. Boss and D.J. Morré, eds. Alan R. Liss, New York, pp 227-255.

Bowman,J.C., Smyth,D.R., and Meyerowitz,E.M. (1989). Genes directing flower development in *Arabidopsis*. Plant Cell 1, 37-52.

Calvert,H.E., Pence,M.K., Pierce,M., Malik,N.S.A., and Bauer,W.D. (1984). Anatomical analysis of the development and the distribution of Rhizobium infections in soybean roots. Can. J. Bot. 62, 2357-2348.

Caponetti,J.D., and Steeves,T.A. (1963). Can. J. Bot. 41, 545.

Christianson,M.L, and Warnick,D.A. (1983). Competence and determination in the process of *in vitro* shoot regeneration. Dev. Biol. 95, 288-293.

Coe,Jr.,E.H., and Neuffer,M.G. (1978). Embryo cells and their destinies in the corn plant. In: The clonal basis of development, S.Subtelny and I.M.Sussex, eds. Academic Press, New York, pp 113-129.

Crick,F.H.C. (1970). Diffusion in embryogenesis. Nature 225, 420-422.

Derman,H. (1953). Periclinal cytochimeras and the origin of tissues in stem and leaf of peach. Am. J. Bot. 40, 154-168.

Dickinson,W.J. (1988). On the architecture of regulatory systems: evolutionary insights and implications. BioEsseays 8, 202-208.

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

Drake,D., Leonard,J.T., and Hirsch,A.M. (1985). Symbiotic genes in *Frankia*. In: Nitrogen fixation research progress, H.J. Evans, P.J. Bottomley and W.E. Newton, eds. Nijhoff Publishers, Dordrecht, p 147.

Dressler,G.R., and Gruss,P. (1988). Do multigene families regulate vertebrate developement? Trends Genet. 4, 214-219.

Dudley,M.E., Jacobs,T.W., and Long,S.R. (1987). Microscopic studies of cell divisions induced in alfalfa roots by Rhizobium meliloti. Planta 171, 289-301.

Eberhard,S. Duobrava,N., Marfa,V., Mohnen,D., Southwick,A., Darvill,A., and Albersheim,P. (1989). Pectic cell wall fragments regulate tobacco thin-cell-layer explant morphogenesis. Plant Cell 1, 747-755.

Ettlinger,C., and Lehle,L. (1988). Auxin induces rapid changes in phosphatidylinositol metabolites. Nature 331, 176-178.

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.

Gerhart,J., Ubbels,G., Black,S., Hara,K., and Kirschner,M. (1981). A reinvestigation of the role of the grey crescent in axis formation in *Xenopus laevis*. Nature 292, 511-516.

Gertler,F.B., Bennett,R.L, Clark,M.J., and Hoffmann,F.M. (1989). *Drosophila abl* tyrosine kinase in embryonic CNS axons: a role in axonogenesis is revealed through dosage-sensitive interactions with *disabled*. Cell 58, 103-113.

Goldberg,R.B., Hoschek,G., Ditta,G.S., Breidenbach,R.W. (1981). Abundance, diversity, and regulation of mRNA sequence sets in soybean embryogenesis. Dev. Biol. 83, 201-217.

Goldberg,R.B., Hoschek,G., Kamalay,J.C., and Timberlake,W.E. (1978). Sequence complexity of nuclear and polysomal RNA in leaves of the tobacco plant. Cell 14, 123-131.

Greenwald,I. (1985). *lin-12*, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. Cell 43, 583-590.

Greenwald,I.S, Sternberg,P.W., and Horvitz,H.R. (1983). The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. Cell 34, 435-444.

Hafen,E., Basler,K, Edstroem,J.E., and Rabin,G.M. (1988). Science 263, 55-63.

Hall,M.A. (1984). Hormones and plant development: cellular and molecular aspects. In: Developmental control in plants and animals, G.F. Graham and P.F. Wareing, eds. Blackwell Scientific Publications, Oxford, pp 313-330.

Han,K, Levine,M.S., and Manley,J.L. (1989). Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. Cell 56, 573-583.

Hastie,N.D., and Bishop, J.D. (1976). Cell 9, 761-774.

Hirsch,A.M., Wilson,K.J., Jones, J.D.G., Bang,M., Walker,V.V., and Ausubel,F.M. (1984). Rhizobium meliloti nodulation genes allow Agrobacterium tumefaciens and Escherichia coli to form pseudonodules on alfalfa. J. Bacteriol. 158, 1133-1143.

Hirsch,A.M., Bhuvaneswari,T.V., Torrey,J.G., and Bisseling,T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA 86, 1244-1248.

Hornberg,C., and Weiler,E.W. (1984). High affinity binding sites for abscisic acid on the plasmalemma of *Vicia faba* guard cells. Nature 319, 321-324.

Horvitz,H.R., and Sulston,J.E. (1980). Isolation and genetic characterization of cell-lineage mutants of the nematode C. elegans. Genetics 96, 435-454.

Ingham,P.W. (1988). The molecular genetics of embryonic pattern formation in *Drosophila*. Nature 335, 25-34.

Jaffé,L.F. (1968). Localization in the developing *Fucus* egg and the general role of localizing currents. Adv. Morphogen. 7, 492-499.

Jeffs,R.A., and Nothcote,D.M. (1967). The influence of indole-3-yl acetic acid and sugar on the pattern of induced differentiation in plant tissue culture. J. Cell. Sci. 2, 77-88.

Kamalay,J.C., and Goldberg,R.B. (1980). regulation of structural gene expression in tobacco. Cell 19, 935-946.

Kamalay,J.C., and Goldberg,R.B. (1984). Organ-sspecific nuclear RNAs in tobacco. Proc. Natl. Acad. Sci. USA 81, 2801-2805.

Katagiri,F., Lam,E., and Chua,N-H. (1989). Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature 340, 727-730.

Kimelman,D., and Kirschner,M. (1987). Synergistic induction of mesoderm by FGF and TGF-b and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. Cell 51, 869-877.

Knox,R.B. and Clarke,A.E. (1984). Cell recognition in plants. In: Developmental control in plants and animals, G.F. Graham and P.F. Wareing, eds. Blackwell Scientific Publications, Oxford, pp 191-217.

Krasnow,M.A., Saffman,E.E., Kornfeld,K., and Hogness,D.S. (1989). Transcriptional activation and repression by *Ultrabithorax* proteins in cultured *Drosophila* cells, Cell 57, 1031-1043.

Kropf,D.L., Berge,S.K., and Quatrano,R.S. (1989). Actin localization during *Fucus* embryogenesis. Plant Cell 1, 191-200.

Lancelle,S.A., and Torrey,J.G. (1984a). Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. I. Infection and early nodule initiation. Protoplasma 123, 26-37.

Lancelle,S.A., and Torrey,J.G. (1984b). Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. II. Nodule morphogenesis and symbiotic development. Can. J. Bot. 63, 25-35.

Levine,M., and Hoey,T. (1988). Homeobox proteins as sequence-specific transcription factors. Cell 55, 537-540.

Libbenga,K.R., and Harkes,P.A.A. (1973). Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum* L. Planta 114, 17-28.

Libbenga,K.R., Maan,A.C., Van Der Linde,P.C.G., and Mennes,A.M. (1986). Auxin receptors. In: Hormones, receptors and cellular interactions in plants, C.M. Chadwick and D.R. Garrod, eds. Cambridge University Press, Cambridge, pp 1-68.

Libbenga,K.R., Van Iren,F., Bogers,R.J., and 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.

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

Löbler,M., and Klämbt,D. (1985). J. Biol. Chem. 260, 9848.

Lynn,D.G., Chen,R.H., Menning,K.S., and Wood,H.N. (1987). The structural characterization of endogenous factors from *Vinca rosea* crown gall tumors that promote cell division of tobacco cells. Proc. Natl. Acad. Sci. USA 84, 615-619.

Marmé (1989). The role of calcium and calmodulin in signal transduction. In: Second messengers in plants growth and development, W.F. Boss and D.J. Morré, eds. Alan R. Liss, New York, pp 57-80.

Marvel,D.J., Kuldau,G., Hirsch,A., Richards,E., Torrey,J.G., and Ausubel,F.M. (1985). conservation of nodulation genes between Rhizobium meliloti and a slow-growing Rhizobium strain that nodulates a non-legume host. Proc. Natl. Acad. Sci. USA 82, 5841-5842.

McDaniel,C.N., and Poethig,R.S. (1988). Cell-lineage patterns in the shoot apical meristem of the germinating maize embryo. Planta 175, 13-22.

Meinhardt,H. (1988). Models for maternally supplied postional information and the activation of segmentation genes in *Drosophila* embryogenesis. Development 104 Supplement, 95-110.

Morrison,N.A., Bisseling,T., and Verma,D.P.S. (1988). Development and differentiation of the root nodule. Dev. Biol. 5, 405-425.

Nagorcka,B.N. (1988). A pattern formation mechanism to control spatial organization in the embryo of *Drosophila melanogaster*. J. Theor. Biol. 132, 277-306.

Nap,J.P., Van De Wiel,C., Spaink,H.P., Moerman,M., Van den Heuvel,M., Djordjevic,M.A., Van Lammeren,A., Van Kammen,A., and Bisseling,T. (1989). the relationship between nodulin gene expression and the Rhizobium nod genes in Vicia sativa root nodule development. Molecular Plant-Microbe Interac. 2, 53-63.

Nap,J.P., and Bisseling,T. (1989). Nodulin function and nodulin gene regulation in root nodule development. In: the molecular biology of symbiotic nitrogen fixation, P.M. Gresshoff, ed. CRC Press, Florida, pp. 181-229.

Newcomb,W. (1976). A correlated light and electron microscopic study of symbiotic growth and differentiation of pea root nodules. Can. J. Bot. 54, 2163-2186.

Nöthinger,R. (1972). The larval developement of imaginal disks. In: The Biology of Imaginal Disks. Results and Problems in Cell Differentiation, vol. 5, ed. H.Ursprung and R.Nöthinger, pp.1-34. Springer-Verlag, Berlin.

Palme,K., Diefenthal,T., Hesse,T., Nitschke,K., Campos,N., Feldwisch,J., Garbers,C., Hesse,F., Schwonke,S., and Schell,J. (1989). signalling elements in higher plants: identification and molecular analysis of an auxin-binding protein, GTP-binding regulatory proteins and calcium sensitive proteins. In: Molecular signals in microbe-plant symbiotic and pathogenic systems, B.J.J. Lugtenberg, ed. NATO-ASI series, in press.

Paz-Arez,J., Ghosal,D., Wienand,H., Peterson,P.A., and Saedler,H. (1987). The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. EMBO J. 6, 3553-3558.

Petkovitch,M. Brand,N.J., Krust,A., and Chambon,P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330, 444-450.

Roberts,J.A., and Hooley,R. (1986). Plant growth regulators. Chapman and Hall, New York.

Rolfe,B.G., and Gresshoff,P.M. (1988). Genetic analysis of legume nodule initiation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39, 297-319.

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.

Seydoux,G., and Greenwald,I. (1989). Cell autonomy of *lin-12* function in a cell fate decision in *C.elegans*. Cell 57, 1237-1245.

Skoog,F., and Miller,C.O. (1957). Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Symp. Soc. exp. Biol. 11, 118-131.

Smith,J. (1989). Positional signalling: where are we now? Trends Genet. 5, 165-166.

Stoddart,J.L. (1986). Gibberellin receptors. In: Hormones, receptors and cellular interactions in plants, C.M. Chadwick and D.R. Garrod, eds. Cambridge University Press, Cambridge, pp 91-114.

Struhl,G., Struhl,K. and Macdonald,P.M. (1989). The gradient morphogen *bicoid* is a concentration dependent transcriptional activator. Cell 57, 1259-1273.

Sussex,I.M. (1989). Developmental programming of the shoot meristem. Cell 56, 225-229.

Thaller,C., and Eichele,G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 327, 625-628.

Theologis,A. (1986). Rapid gene regulation by auxin. Ann. Rev. Plant Physiol. 37, 407-438.

Tillmann,U., Viola,G., Kayser,B., Siemeister,G., Hesse,T., Palme,K., Löbler,M., and Klämbt,D. (1989). cDNA clones of the auxin-binding protein from corn coleoptiles (Zea mays L.): isolation and characterization by immunological methods. EMBO J. 8, 2463-2467.

Tran Thanh Van,K., Toubart,P., Cousson,A., Darvill,A.G., Gollin,D.J., Chelf,P., and Albersheim,P. (1985). Manipulations of the morhphogenetic pathway of tobacco explants by oligosaccharins. Nature 314, 615-617.

Truchet,G., DeBelle,F., Vasse,J., Terzaghi,A.-M., Garnerone,C., Rosenberg,C, Batut,J., Maillet,F., and Denarié, J. (1985). Identification of a Rhizobium meliloti pSym2011 region controlling the host specificity of root hair curling and nodulation. J. Bacteriol 164, 1200-1210.

Van Kammen,A. (1984). Suggested nomenclature for plant genes involved in nodulation and symbiosis. Plant Mol. Biol. Rep. 2, 43-45.

Vincent,W.S.III, Gregory,R.J., and Wadsworth,S.C. (1989). Embryonic expression of a Drosophila src gene: alternate forms of the protien are expressed in segmental stripes and in the nervous system. Genes & Developm. 3, 334-347.

Walbot,V. (1985). On the life strategies of plants and animals. Treds Genet. 1, 165-169.

Walbot,V., and Holder,N. (1987). Developmental Biology, Random House, New York.

Wareing,P.F. (1984). Hormones and plant development: coordination and control in the whole plant and analogies with insect development. In: Developmental control in plants and animals, G.F. Graham and P.F. Wareing, eds. Blackwell Scientific Publications, Oxford, pp 331-348.

Wareing,P.F., Hamney,C.E.A., and Digby,J. (1964). The role of endogenous hormones in cambial activity and xylem differentiation. In: the formation of wood in forest trees, M.H. Zimmerman, ed. Academic Press, New York, pp 324-344.

Weeks,D.L., and Melton,D.A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for an growth factor related to TGF-b. Cell 51, 861-867.

West,C.A., Bruce,R., and Ren,Y-Y. (1989). Second messengers and their possible relevance for plants. In: Molecular signals in microbe-plant symbiotic and pathogenic systems, B.J.J. Lugtenberg, ed. NATO-ASI series, in press.

Wharton,K.A., Johansen,K.M., Xu,T., and Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus *Notch* implies a gene product that shares homology with proteins containing EGF-like repeats. Cell 43, 567-581.

Whittaker,J.R., Ortolani,G., and Farinella-Ferruza,A. (1977). Autonomy of acetylcholinesterase differentiation in muscle lineage cells of ascidian embryos. Dev. Biol. 55, 196-200.

Wolpert,L. (1969). Positional information and the spatial pattern of cellular differentiation. J.Theor. Biol. 25, 1-47.

CHAPTER 3

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

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EMBO Journal, in press.

ABSTRACT

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

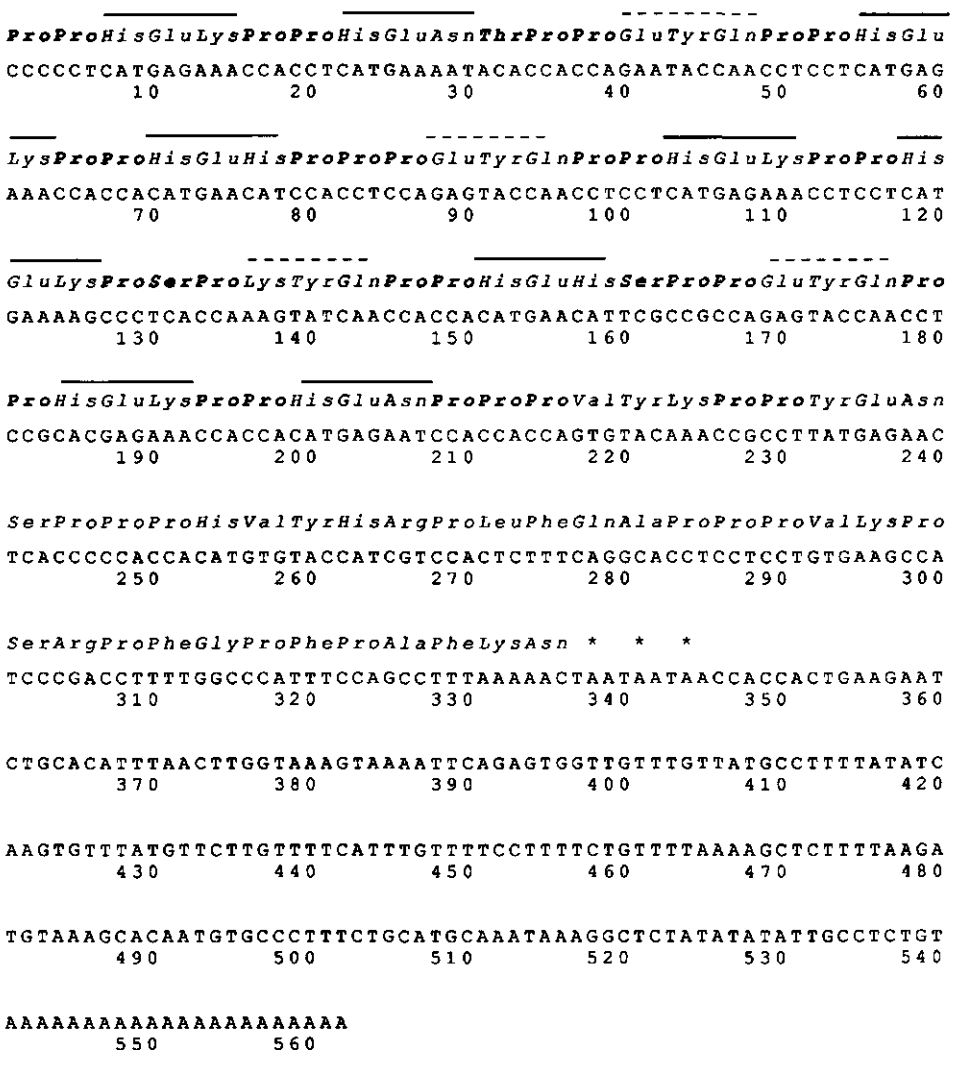

Fig. 1. cDNA and predicted amino acid sequence of the pPsENOD2 insert. Nucleotides 1-562 are determined from the pPsENOD2 insert. The sequence of nucleotides 34-562 is confirmed by analysis of an independently obtained ENOD2 cDNA clone. The amino acid sequence of the only long open reading frame is displayed 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.

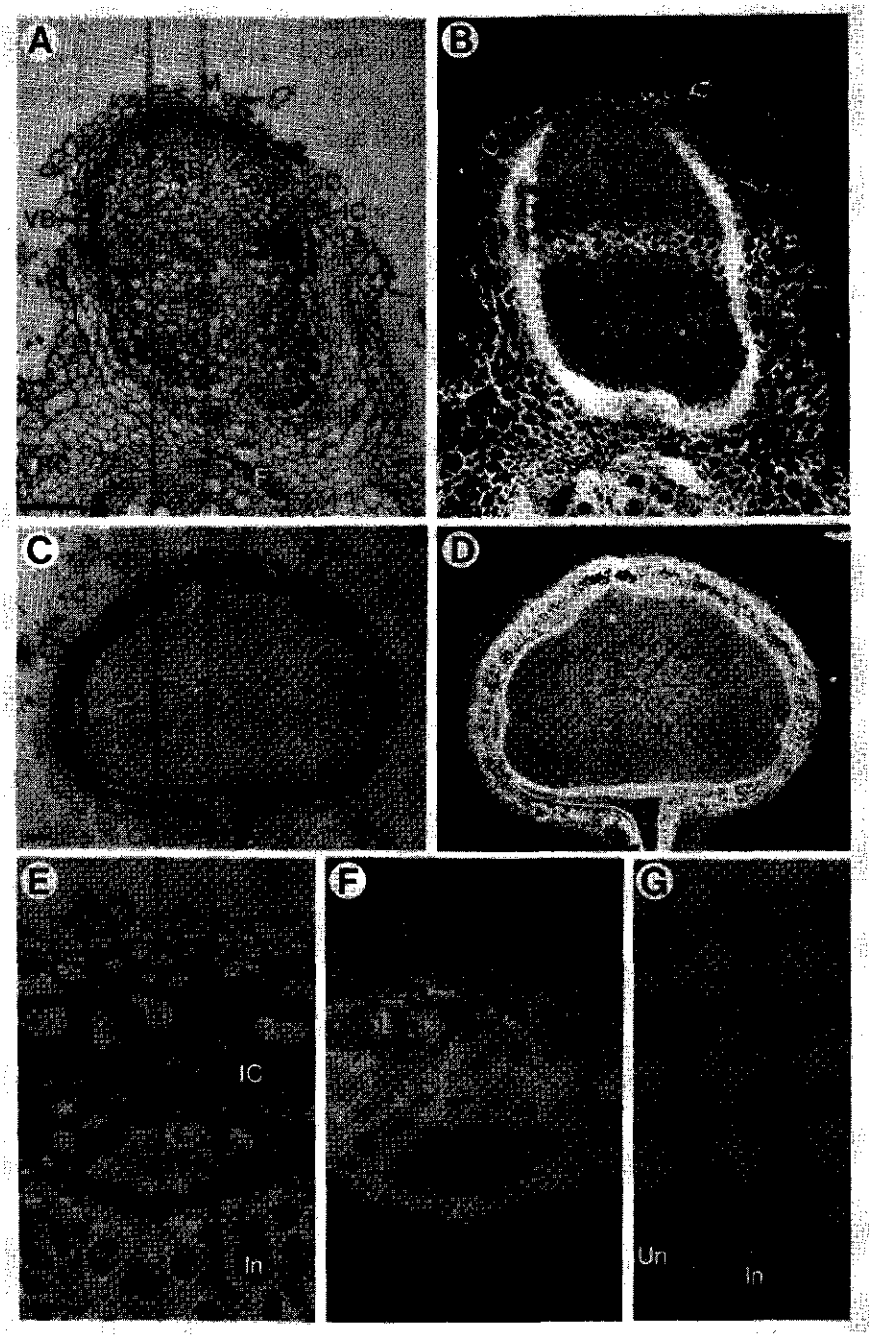

Figure 2

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).

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 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

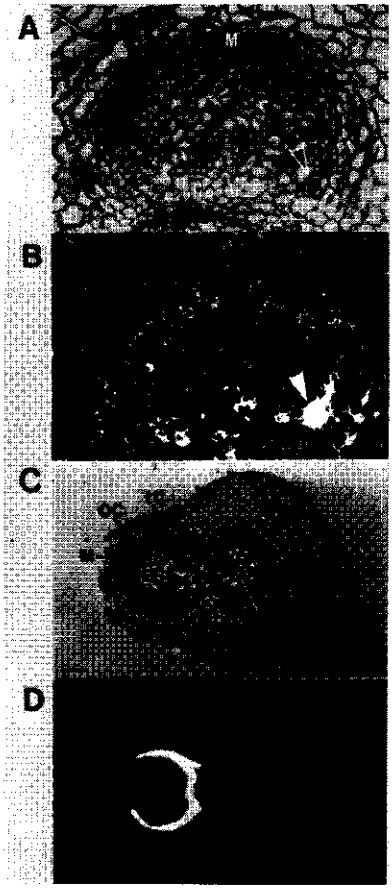

Fig. 3. Localization of ENOD2 transcripts by *in situ* hybridization during nodule development in pea.
In the dark field micrographs (B) and (D), which correspond to the bright field micrographs (A) and (C), respectively, the autoradiographic signal is visible as white grains. (A) Detail of a transection 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 represend 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.

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 ar pical 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

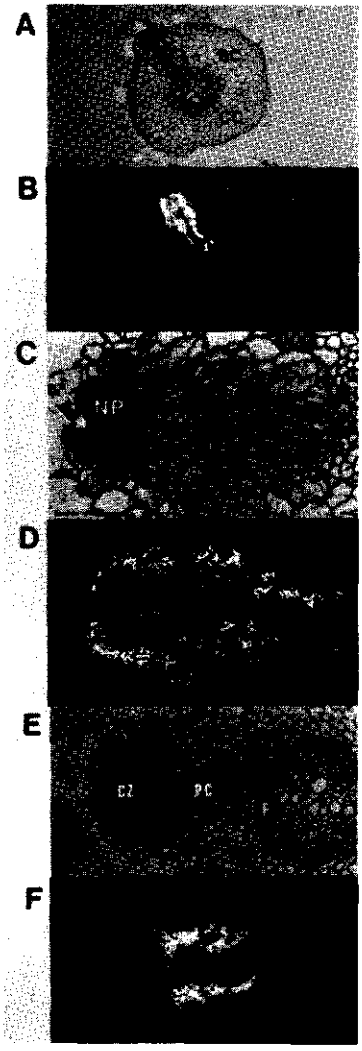

Fig. 4. Localization of ENOD2 transcripts by *in situ* hybridization during nodule development in soybean. In the dark field micrographs (B), (D) and (F), which correspond to the bright field micrographs (A), (C) and (E), respectively, the autoradiographic signal is visible as white grains.

(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.

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).

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 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 *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 *et al.* (1984), following a protocol kindly provided by Dr. M. van Montagu and Dr. G. Engler. 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.

ACKNOWLEDGEMENTS

We thank Dr. G. Truchet for helpful discussions and suggesting the term "nodule parenchyma", Prof. Dr. M.T.M. Willemse for constructive criticism of the manuscript, Mr. Sybout Massalt for photographic assistance, and Mrs. Gré Heitkönig and Mrs. Voline van Teeseling for preparing the manuscript.

REFERENCES

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

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, 1-11.

Cassab, G.J. and Varner, J.E. (1988) *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 39, 321-353.

Cox, K.H., DeLeon, D.V., Angerer, L.M. and Angerer, R.C. (1984) *Dev. Biol.*,101, 485-502.

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

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. 869-877.

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, 4495-4499.

Franssen, H.J., Scheres, B., Van de Wiel, C. and Bisseling, T. (1988) In: Palacios, R. and Verma, D.P.S. (eds.), *Molecular Genetics of Plant-Microbe Interactions.* APS Press, St. Paul, pp. 321-326.

Govers, F., Franssen, H.J., Pieterse, C., Wilmer, J. and Bisseling, T. (1989) In: Grierson, P. and Lycett, G. (eds.) *Genetic Engineering of Crop Plants.* Butterworths Scientific, Guildford, in press.

Govers, F., Moerman, M., Downie, J.A., Hooykaas, P., Franssen, H.J., Louwerse, J., Van Kammen, A. and Bisseling, T. (1986) *Nature*, 323, 564-566.

Hirsch, A.M., Drake, D., Jacobs, J.W. and Long, S.R. (1985) *J. Bacteriol.*,161, 223-230.

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, 499-560.

Newcomb, W. (1981) *Int. Rev. Cytol. Suppl.*, 13, 247-298.

Robertson, J.G., Wells, B., Bisseling, T., Farnden, K.J.F. and Johnston, A.W.B. (1984) *Nature*, 311, 254-256.

Sprent, J.I. (1980) *Plant Cell Env.*, 3, 35-43.

Tigney, S.V., Walker, E.L. and Coruzzi, G.M. (1987) *EMBO J.*, 6, 1-9.

Tjepkema, J.D. and Yocum, C.S. (1974) *Planta*, 119, 351-360.

Truchet, G., Debellé, F., Vasse, J., Terzaghi, B., Garnerone, A.-M., Rosenberg,C., Batut, J., Maillet, F. and Dénarié, J. (1985) *J. Bacteriol.*, 164, 1200-1210.

Van den Bosch, K.A. and Newcomb, E.H. (1986) *Planta*, 167, 425-436.

Van den Bosch, K.A. and Newcomb, E.H. (1988) *Planta*, 175, 442-451.

Van Kammen, A. (1984) *Plant Mol. Biol. Rep.*, 2, 43-45.

Verma, D.P.S., Fortin, M.G., Stanley, J., Mauro, V.P., Purohit, S. and Morisson, N. (1986) *Plant Mol. Biol.*, 7, 51-61.

Witty, J.F., Minchin, F.R., Skøt, L. and Sheehy, J.E. (1986) *Oxford Surv. Plant Mol. Cell Biol.*, 3, 275-314.

CHAPTER 4

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

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Cell, in press.

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

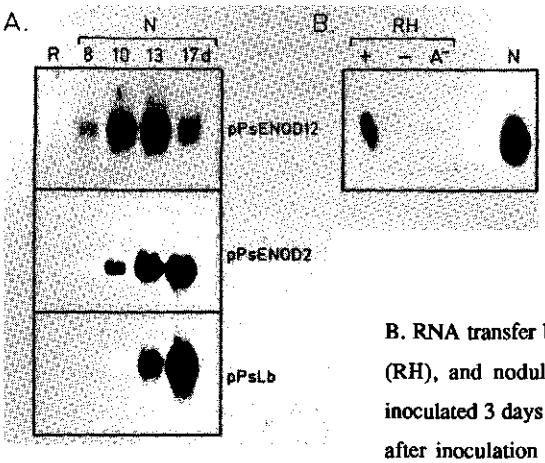


Figure 1. RNA transfer blot analysis of RNA from roots, nodules and root hairs.

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.

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 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.

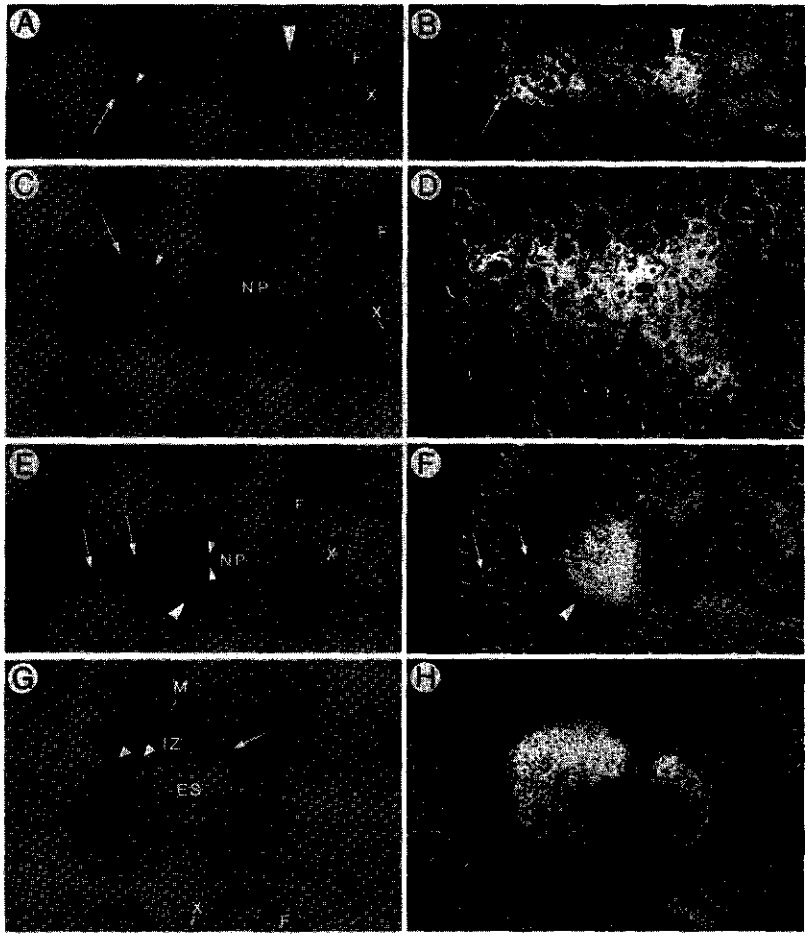
Localization of ENOD12 mRNA in root cortex and nodule cells

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,

Figure 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.

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 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 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.

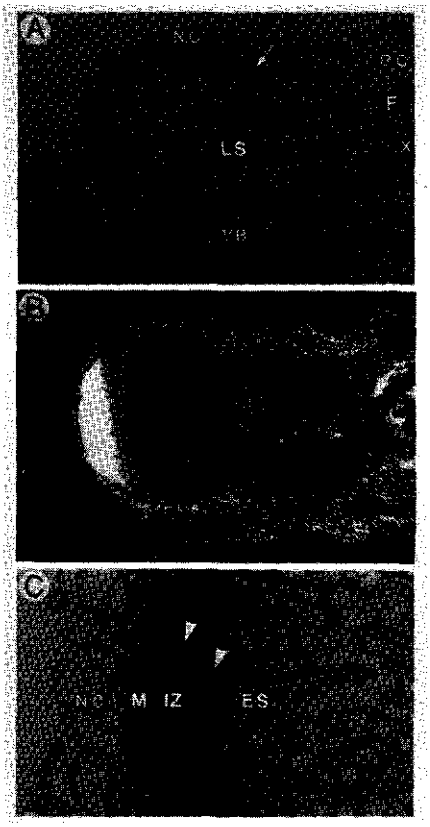


Figure 3. Localization of ENOD12 transcripts in nodules from 20 day old pea plants by in situ hybridization.
A. Bright field micrograph of a longitudinal section of a 20 day old pea nodule. The arrow points to the remnant of the infection thread that originally penetrated the nodule primordium (cf. figure 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.

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.

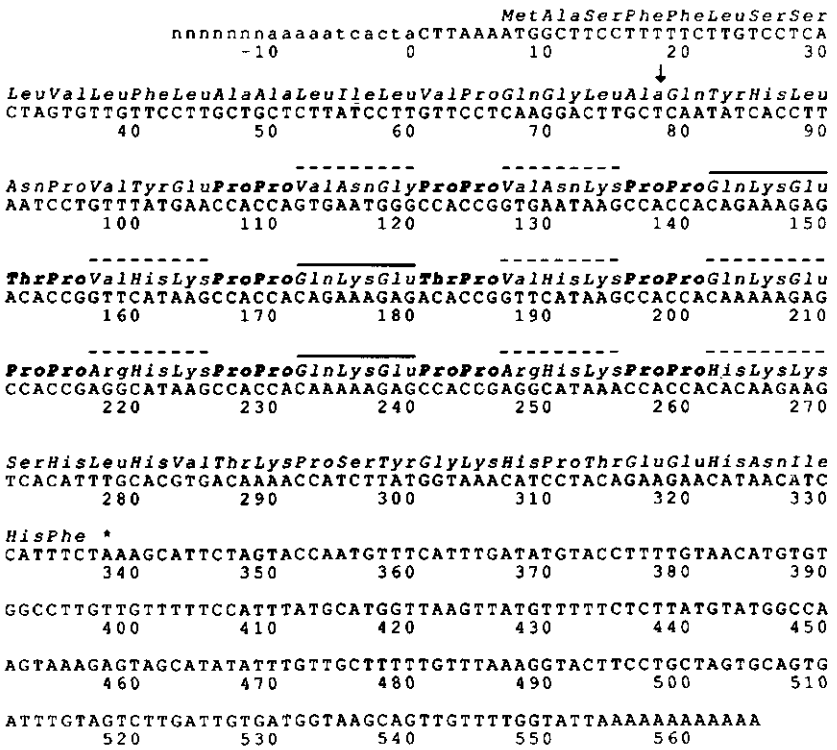


Figure 4. cDNA and predicted amino acid sequence of the pPsENOD12 insert.

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.

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

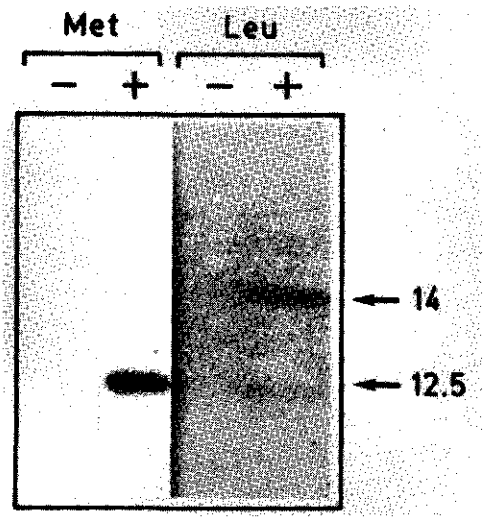


Figure 5. In vitro translation products of hybrid-selected ENOD12 mRNA.
ENOD12 mRNA was selected form total nodule RNA and translated in vitro in the presence of ^{35}S-methionine (Met) or ^{3}H-leucine (Leu) as radioactive amino acid. Filters used for hybrid selection contained pBR322 (-) or pPsENOD12 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.

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 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 *nodEF* genes are also important for infection thread formation (Horvath et al, 1986), and recently the involvement of *nodEF* 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 *nodA* which blocks the formation of the *nodA, nodB,* and *nodC* products (Wijffelman et al, 1985). In our first experiments we demonstrated by RNA transfer blot analysis that ENOD12 transcript is found

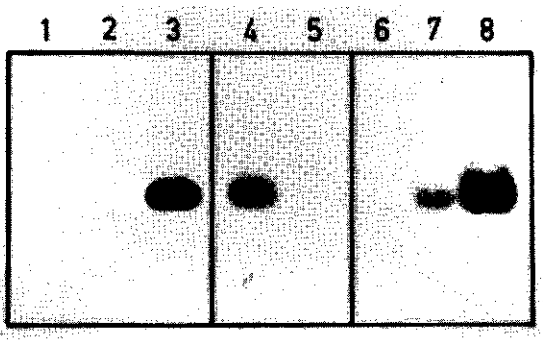


Figure 6. PCR analysis of ENOD12 gene expression in root hair RNA.
ENOD12 sequences from 1 μg total root hair RNA were amplified using 12 PCR cycles, and after electrophoresis a DNA transfer blot was probed with ^{32}P-labeled pPsENOD12 insert. The different lanes contain amplified root hair ENOD12 sequences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

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 *nodA* 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 *nodEFDABCIJ* 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 *nodE::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 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.

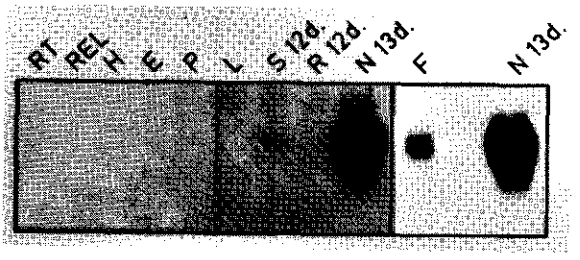


Figure 7. RNA transfer blot analysis of various uninoculated pea tissues.

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.

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.

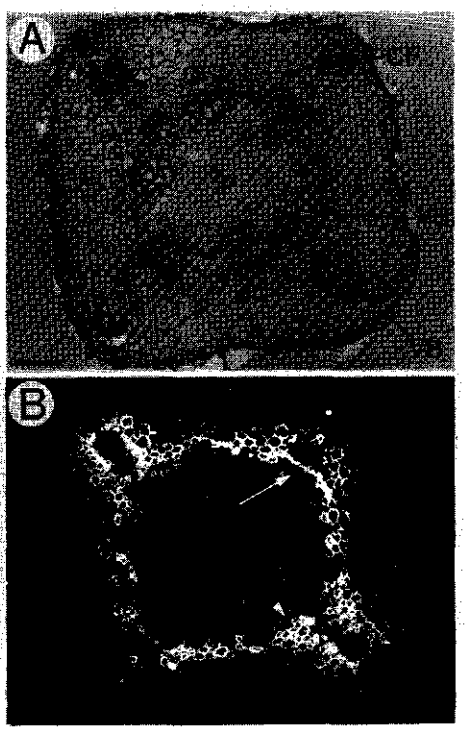


Figure 8. Localization of ENOD12 transcripts in stem tissue by in situ hybridization.

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.

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 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 (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 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

Figure 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

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.

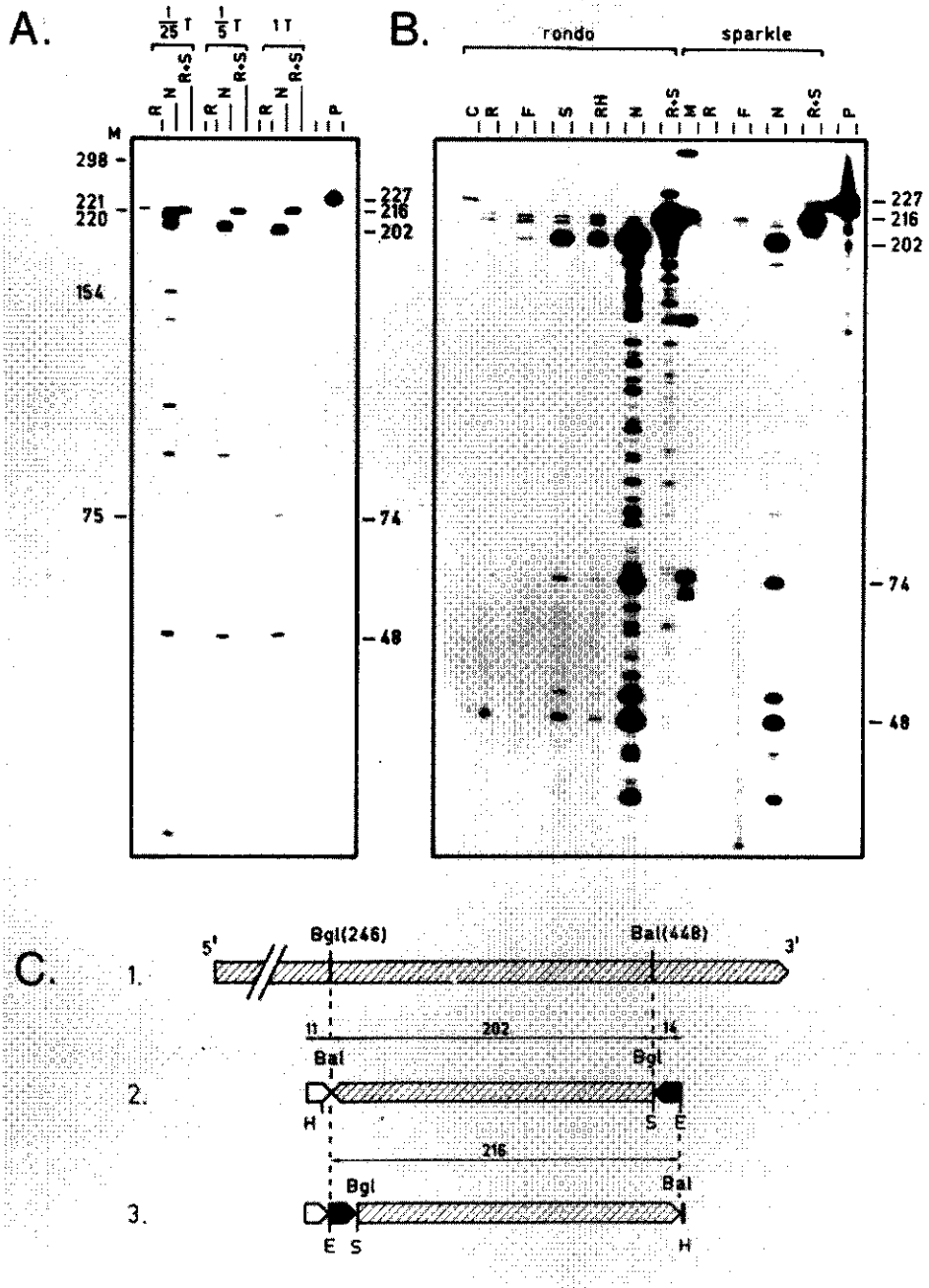

A.
1/25 T
1/5 T
1 T
R
N
R+S
P
M
298
221
220
154
75
227
216
202
74
48
B.
rondo
sparkle
C
R
F
S
RH
N
R+S
P
227
216
202
74
48
C.
1.
5'
3'
Bgl(246)
Bal(448)
11
202
14
2.
Bal
Bgl
H
S
E
216
3.
Bgl
Bal
E
S
H

Figure 9

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.

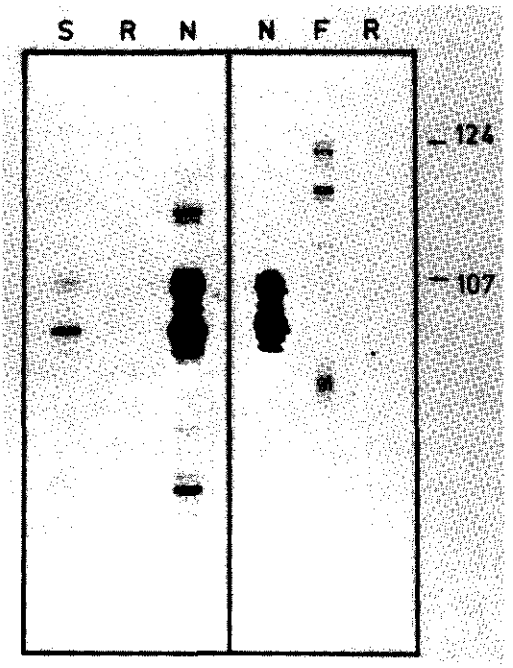


Figure 10. Primer extension analysis of ENOD12 mRNA from nodule, stem and flower. An end-labeled oligonucleotide complementary to nt 70-90 of the cDNA sequence was annealed to total RNA from: 4th internode stem sections from 35 day old plants (S), 8 day old roots (R), nodules 13 days after inoculation (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.

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 *nodEFDABCIJ* 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 *nodA* 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 *nodE* gene, and/or the *nodF* 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 *nodA* and *nodB* 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 *nodH* 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 *nodH* gene product. Whether the compound(s) inducing ENOD12 gene expression is also the result of a modification of a *nodABC* dependent factor by the *nodE* product, or whether *nodABC* and *nodE* 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.

EXPERIMENTAL PROCEDURES

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^{o}C during 24 hr. Subsequently blots were washed, 2x10' in 2xSSC and 2x20' in 2xSSC/1% SDS at 65^{o}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^{o}C and temperature was decreased to 37^{o}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 et al (1984), following a protocol kindly provided by Dr. M. van Montagu and Dr. G. Engler.

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.

ACKNOWLEDGEMENTS

We wish to thank dr. G. Coruzzi and dr. S. Tigney for providing us with a pea nodule cDNA library; dr. E. Pees for making available the R.leguminosarum bv. viciae A10 strain; dr. T.V. Bhuvaneswari for critical reading of the manuscript; dr. C. Díaz and dr. B. Bakhuizen for usefull suggestions; dr. A. van Lammeren for support; P. Madern, P. van Druten, S. Massalt and dr. S. de Vries for artwork; and P. de Kam for growing

plants. B.S. was supported by the Dutch Organisation for Scientific Research (N.W.O.). B.H. was supported by an EMBO fellowship.

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 5

Sequential induction of nodulin gene expression in the developing pea nodule.

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Submitted.

ABSTRACT

A set of cDNA clones have been characterized which represent early nodulin mRNAs from pea root nodules. By RNA transfer blot analyses the different early nodulin mRNAs were found to vary in time course of appearance during the development of the indeterminate pea root nodule. *In situ* hybridization studies demonstrated that the transcripts are located in different zones, representing subsequent steps in development of the central tissue of the root nodule. ENOD12 transcripts are present in every cell of the invasion zone, whereas ENOD5, ENOD3 and ENOD14 transcripts are restricted to the infected cells in successive but partially overlapping zones of the central tissue. We conclude that the corresponding nodulin genes are expressed at subsequent developmental stages. The nature of the nodulins that emerges from the amino acid sequence derived from the nucleotide sequences of the cDNAs, in combination with the localization data, are used to hypothesize about the possible functions of the early nodulins and the regulation of the expression of the corresponding genes by compounds produced by *Rhizobium*.

INTRODUCTION

The formation of nodules on roots of leguminous plants induced by *Rhizobium* bacteria proceeds according to Vincent (1980) as a "multistage sequence of interdependent steps". Based on cytological examinations of nodule development and the observation that this development can be blocked at discrete stages using plant or *Rhizobium* mutants, root nodule formation has been divided into three main stages that each can be subdivided into several steps (Vincent, 1980). First, in the so-called preinfection stage (stage 1), rhizobia attach to the root hairs and root hair deformation occurs. Next, in the infection and nodule formation stage (stage 2), infection threads containing rhizobia penetrate into roots, a nodule structure is formed, and bacteria are then released in plant cells and develop into bacteroids. Finally, in the nodule function stage (stage 3), the root nodule becomes a functional, nitrogen fixing, organ.

As has been shown by genetic studies plant genes are involved in each defined step of root nodule formation (for an overview see Vincent, 1980). However, none of the genes concerned have been cloned and as a consequence the characteristics of these genes and the encoded proteins remain unknown. On the other hand nodule-specific plant proteins, nodulins, have been identified (Van Kammen, 1984), and are studied extensively. They have

been divided into early and late nodulins. Late nodulin genes are first expressed during stage 3 of nodule development, when infection of the roots by bacteria has taken place and a nodule structure has been formed. Hence the expression of these genes is not related to the first two stages of nodule development. On the other hand, early nodulin genes are expressed during stage 1 and 2 of root nodule formation (Govers *et al.*, 1985; Gloudemans *et al.*, 1987). Late nodulin mRNAs have been cloned from several legumes, including pea (Govers *et al.*, 1987). cDNA clones representing early nodulins have been obtained from soybean (Franssen *et al.*, 1987; Franssen *et al.*, 1988), alfalfa (Dickstein *et al.*, 1988), and pea (Van De Wiel *et al.*, 1990; Scheres *et al.*, 1990). The pea early nodulin cDNA clone pPsENOD2 is homologous to the soybean pGmENOD2 and the product it codes for has been shown to be involved in the formation of the nodule parenchyma ('inner cortex') surrounding the central nodule tissue (Van De Wiel *et al.*, 1990). The pea ENOD12 early nodulin is involved in the infection process (Scheres *et al.*, 1990). Both ENOD2 and ENOD12 are proline-rich proteins composed of repeating pentapeptide units containing two (hydroxy-)prolines and are thought to be cell wall proteins.

The aim of the present study has been to obtain a set of pea early nodulin cDNA clones representing genes involved in the second stage of nodule development, when infection and nodule development take place. Both processes occur in pea nodules of 10 day old pea plants, when late nodulin mRNAs are not yet present (Govers *et al.*, 1985). Therefore we screened a nodule cDNA library with cDNA probes of RNA from nodules of 10 day old plants and uninfected roots, respectively. In this paper we describe the characterization of three early nodulin cDNA clones pPsENOD5, pPsENOD3, and pPsENOD14, and compare the spatial distribution of the corresponding mRNAs in the developing pea nodule with that of the pPsENOD2 and pPsENOD12 mRNAs by *in situ* hybridization. The results show that we have now available a series of cDNA clones corresponding to nodulin genes that are expressed in a sequential order and are related to different steps in the second stage of root nodule formation.

RESULTS

Isolation and characterization of early nodulin cDNA clones.

We differentially screened a nodule cDNA library with cDNA probes prepared from mRNA of roots of 8 day old uninoculated pea plants and nodules of 10 day old plants. In this way we

isolated three early nodulin cDNA clones, pPsENOD5, pPsENOD3, and pPsENOD14. The characteristics of these cDNA clones were determined and compared with those of the previously studied cDNA clones pPsENOD2 (Van De Wiel *et al.*, 1990) and pPsENOD12 (Scheres *et al.*, 1990).

The time course of appearance of the various early nodulin mRNAs during nodule development was studied by RNA transfer blot analyses. Identical blots containing total RNA from roots of 8 day old uninfected plants, root segments of 8 day old inoculated plants, and nodules of 10, 13, and 17 day old plants, were hybridized to the inserts of the three newly isolated early nodulin clones and the inserts of pPsENOD2 and pPsENOD12 (Figure 1). ENOD12 mRNA is already detectable in root segments of 8 day old inoculated plants, then accumulates to maximum amounts from day 10 to 13 and, after that, decreases in concentration. ENOD5 mRNA is first detectable at day 10, reaches a maximum level around day 13, and subsequently decreases in concentration. The ENOD2 mRNA is first detectable at day 10 on these RNA blots and increases in concentration during the next seven days. The accumulation patterns of both ENOD3 and ENOD14 mRNAs during nodule development are similar. Both transcripts (only hybridization to ENOD14 is shown) are first detectable at day 10. The transcripts reach their maximum concentration around day 13 and maintain this

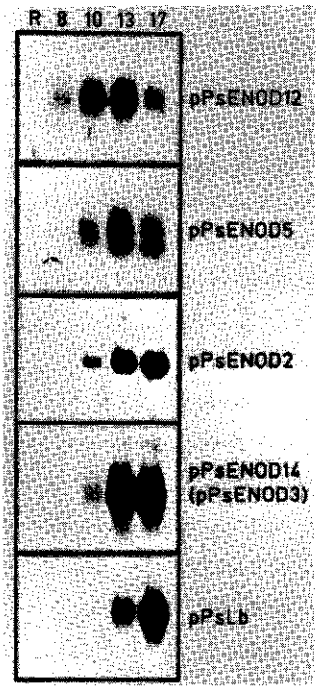

Fig. 1. RNA transfer blot analysis of RNA from roots and nodules.
RNA transfer blots contain 10 µg of total RNA from uninoculated roots of 8 day old plants (R), and nodules 8, 10, 13 and 17 days after sowing and inoculation, as indicated. Similar blots were probed with pPsENOD12, pPsENOD5, pPsENOD2, pPsENOD14 and pPsLb inserts, respectively.

concentration thereafter. Leghemoglobin (Lb) mRNA has been included in the experiment as a typical example of a late nodulin transcript. Lb mRNA is first detectable at day 13 and the concentration of the transcript increases thereupon. None of the (early) nodulin mRNAs are detectable in uninoculated pea roots (Figure 1), neither in hypocotyl, epicotyl, plumule, stem, leaf, and flower tissue, respectively (data not shown), with the exception of ENOD12 mRNA. As was reported previously (Scheres *et al.*, 1990) ENOD12 transcripts are detectable at low levels in stem and flower tissue.

To examine whether the early nodulin genes are specifically expressed during the symbiotic interaction of *Rhizobium* and legumes, but not during pathogenic interactions of microorganisms and pea roots, we studied whether early nodulin genes are expressed during infection of pea roots with the pathogenic fungus *Fusarium oxysporum f.sp. pisi*. Earlier we established that, during this pathogenic interaction, the ENOD12 early nodulin gene, which is involved in the bacterial infection process, is not expressed (Scheres *et al.*, 1990). Similar experiments showed that ENOD5, ENOD3, and ENOD14 transcripts are equally not detectable in pea roots upon inoculation with *F. oxysporum* (data not shown). Therefore the accumulation of these early nodulin transcripts during nodule formation cannot be attributed to a general defense response.

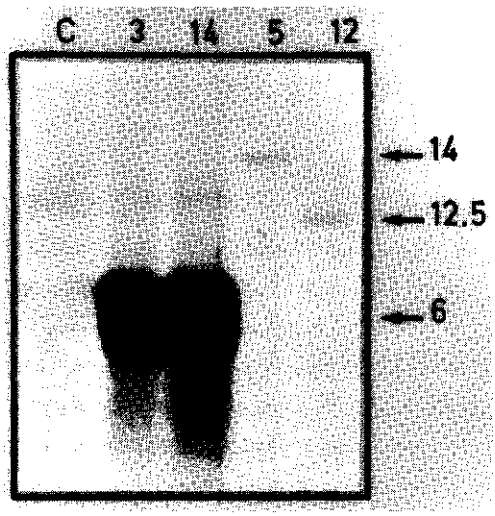

Fig. 2. *In vitro* translation products of hybrid selected early nodulin mRNAs.
Early nodulin mRNAs were selected from total nodule RNA and translated *in vitro* in the presence of ^{35}S-methionine as radioactive amino acid. Filters used for hybrid selection contained pBR322 (C), the inserts of pPsENOD3 (3), pPsENOD14 (14), pPsENOD5 (5), and pPsENOD12 (12). C, 3, 14: total RNA from nodules of 20 day old plants was used; 5, 12: total RNA from nodules of 13 day old plants was used. The size (kd) of the *in vitro* translation products as determined by comparison with size markers is indicated to the right side.

Hybrid-released translation of mRNA, selected with the inserts of pPsENOD5, pPsENOD3, and pPsENOD14 was performed to determine the size of the corresponding primary translation products. The insert of pPsENOD5 selected an mRNA that upon translation produced a 14 kd polypeptide, while the ENOD3 and ENOD14 mRNAs both encoded a 6 kd polypeptide (Figure 2). On a Southern blot containing pea genomic DNA digested with EcoR1, pPsENOD5 hybridized to a single 6.5 kb fragment, pPsENOD3 to a single 6.0 kb fragment, and pPsENOD14 to fragments of 15 kb, 6.7 kb, and also, with lower intensity, to fragments of 7.5 kb and 2.5 kb. None of the cloned cDNAs hybridized to *Rhizobium* DNA (data not shown). In conclusion, pPsENOD5 and pPsENOD3 appear to represent single or low copy plant genes while pPsENOD14 might represent a member of a small gene family.

Sequence analysis of pPsENOD5, pPsENOD3, and pPsENOD14

Further information about the nature of the early nodulins encoded by the different cDNA clones was obtained by determining the nucleotide sequence of the inserts of pPsENOD5, pPsENOD3, and pPsENOD14. The cDNA insert of pPsENOD5 is 553 bp in length and has a polyA stretch at the 3' end of the sequence while the corresponding mRNA has a size of 700 nt, as determined on an RNA transfer blot. By direct RNA sequencing the sequence of 20 nucleotides from the 5' end of the mRNA, missing in the cDNA clone, was determined (Figure 3., small typeface). The full sequence contained one large open reading frame that presumably starts with the first in-frame ATG codon, at position -5, as the sequences surrounding this ATG meet the requirements for the start codon consensus in plants (Lütcke *et al.*, 1987). The encoded protein of 135 amino acids has a mol. wt. of 14 kd, which matches exactly the value of the apparent mol.weight of the polypeptide produced upon hybrid released translation. The protein sequence is characterized by hydrophobic domains at the amino- as well as at the carboxy terminus (overlined). The N-terminal hydrophobic domain can be the core of a signal peptide of which the putative cleavage site (indicated by an arrow) can be predicted if the rules of Von Heijne (1983) are applied. Between amino acids 88 and 108 the polypeptide has a high proline content and in that part of the sequence prolines are alternated by either serines or alanines. Computer search in the NBRF database revealed no significant homology to other proteins. The high percentage of pro, ser, ala, and gly residues in the ENOD5 protein is reminiscent of the amino acid composition of arabinogalactan proteins (Van Holst *et al.*,

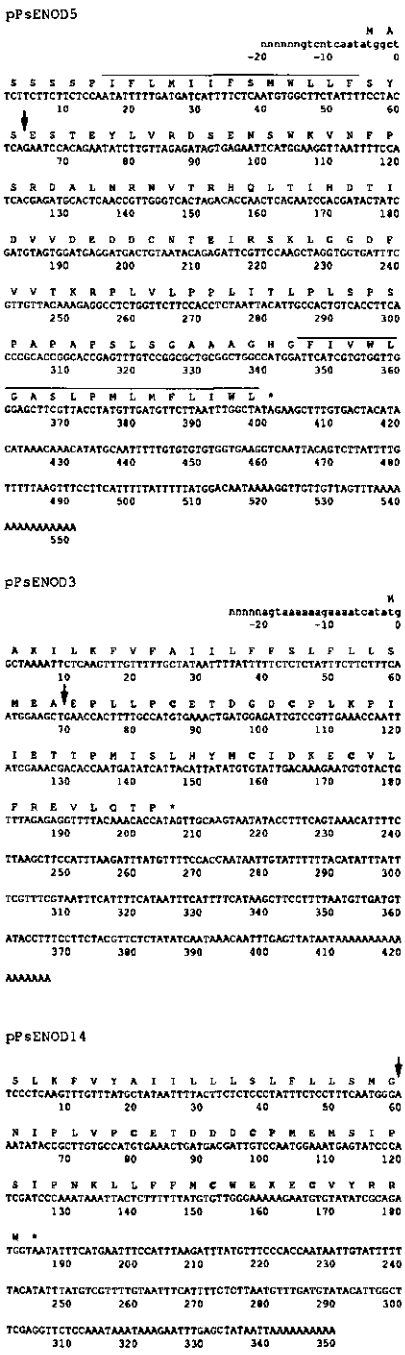

Fig. 3. Nucleotide sequences and the deduced amino acid sequences of the inserts of pPsENOD5, pPsENOD3, and pPsENOD14.
The amino acid sequences of the only long open reading frames present in the nucleotide sequences of the inserts of pPsENOD5, pPsENOD3, and pPsENOD14 are depicted over the nucleotide sequences. In pPsENOD5 and pPsENOD3 the sequence from the 5' end of the mRNA to the first base present in the cDNA clone (numbered 1) is indicated in small typeface. Putative signal peptide cleavage sites are marked with arrows. In the pPsENOD5 sequence hydrophobic regions are overlined. Termination codons ending the reading frames are marked with an asterix.

1981), of which however no sequences have been published so far.

The cDNA insert of pPsENOD3 is 430 bp in length, and has at the 3' end of its sequence a poly (A) stretch. The mRNA sizes 550 nucleotides on an RNA transfer blot. The sequence of 25 nucleotides from the 5' end of the mRNA, missing in the cDNA clone, was again determined by direct RNA sequencing (Figure 3, small typeface). The final cDNA sequence reveals a single large open reading frame starting with the ATG codon at position -2 (Figure 3), that conforms to the rules for initiation codons in plants (Lütcke *et al.*, 1987). The calculated mol. wt. of the encoded protein is 8 kd, slightly larger than the size determined for the polypeptide produced upon hybrid released translation. The protein has a putative signal peptide at the N-terminus for which the predicted cleavage site in the amino acid sequence (Von Heijne, 1983) is marked with an arrow. A remarkable feature of the protein sequence is the presence of four cysteines (bold typeface), arranged in two pairs, in such a way that they might be capable of binding a metal ion (Berg, 1986).

pPsENOD14 has an insert of 350 bp with at the 3' end of its sequence a poly (A) stretch While the mRNA was estimated to measure 500 nt. on an RNA transfer blot primer extension analysis showed that 29 nt. from the 5' end of the mRNA are missing in the cloned cDNA (data not shown). The only long open reading frame in the cDNA sequence starts at the 5' end and runs up to nucleotide 181, encoding an amino acid sequence of 61 amino acids. The ENOD14 and the ENOD3 cDNA sequences are 65% homologous. The homology is found in the coding as well as in the non-coding regions (Figure 4A). The amino acid sequences of ENOD3 and ENOD14 are 55% homologous (Figure 4B), which is lower than the 65% homology between ENOD3 and ENOD14 on the DNA sequence level. This discrepancy is due to a large number of single base substitutions leading to amino acid substitutions. The N-termini of ENOD3 and ENOD14 are 70% homologous. Therefore we assume that, like ENOD3, ENOD14 will also have a signal peptide. The predicted signal peptide cleavage site (Von Heijne, 1983), assuming that the hydrophobic regions in the ENOD3 and ENOD14 N-termini have a similar size, is indicated with an arrow in Figure 3. Just like the ENOD3 sequence, the amino acid sequence of the ENOD14 protein contains two cysteine pairs (bold typeface) and the distance between them is the same in both proteins. While the amino acids surrounding the cysteines are well conserved, the region between the two cysteine pairs shows substantial variation among the ENOD3 and ENOD14 proteins (Figure 4B). Neither ENOD3 nor ENOD14 are homologous to late nodulins in soybean that contain cysteines with a similar spacing (Jacobs *et al.*, 1987; Sandal *et al.*, 1987). They neither have the

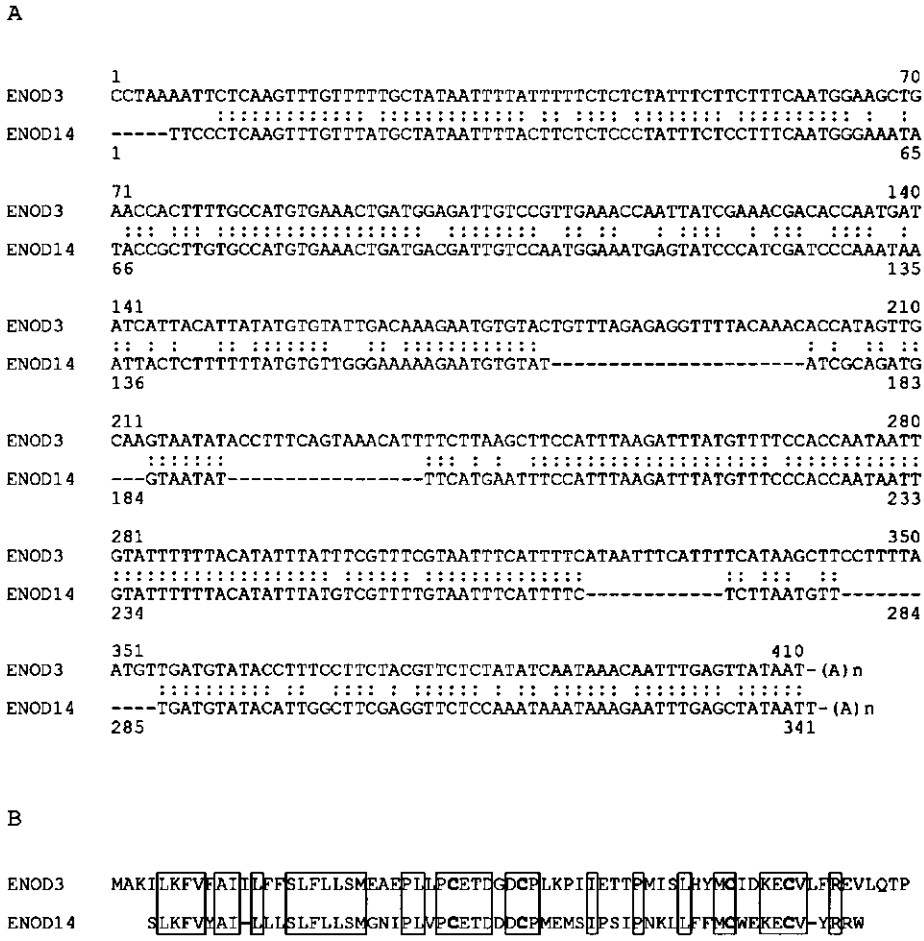

Fig. 4. Alignment of ENOD3 and ENOD14 nucleotide and amino acid sequences.

A: Alignment of the nucleotide sequences of the pPsENOD3 and pPsENOD14 cDNA inserts. Dots mark homologous bases. B: alignment of the ENOD3 and ENOD14 amino acid sequences. Homologous amino acids are boxed.

characteristics of DNA binding proteins with zinc-fingers, containing similarly spaced cysteines (Berg, 1986). Furthermore, searching the NBRF database did not reveal any other proteins with significant amino acid sequence homology to ENOD3 and ENOD14.

Spatial distribution of early nodulin transcripts in pea root nodules.

The distribution of the different early nodulin transcripts over the nodule tissue was studied in serial sections of nodules from 10 and 16 day old pea plants. Since pea nodules have a persistent meristem, the nodule tissues are of graded age from the apical meristem up to the root attachment point. A single longitudinal nodule section thus comprises various successive developmental stages of each particular cell type. The left panels of figure 5 and 6 show longitudinal sections of a nodule from 10 and 16 day old plants, respectively. The central part of the nodule can be divided into several zones (Newcomb, 1976). The meristem (M) is present at the apex. Adjacent to the meristem is the invasion zone (IZ), where infection thread growth and release of rhizobia occur. This zone is followed by the early symbiotic zone (ES) where the infected cells, containing bacteria, and the uninfected cells can first be distinguished and bacterial proliferation and cell enlargement occurs. The zone with fully elongated cells containing bacteroids, which are able to fix nitrogen, is the late symbiotic zone (S). This tissue is still lacking in nodules from 10 day old plants but clearly visible in sections of nodules from 16 day old plants (compare Figure 5 and Figure 6). Several other tissues surrounding the central part of the nodule are also derived from the apical meristem: the cortex (previously 'outer cortex', Van De Wiel *et al.*, 1990), the endodermis, the nodule parenchyma (previously 'inner cortex', Van De Wiel *et al.*, 1990) and the vascular tissue. Sections were analyzed by *in situ* hybridization using ^{35}S labeled antisense and sense RNA probes transcribed from the inserts of pPsENOD12, pPsENOD5, pPsENOD3, pPsENOD14, and pPsLb101 (Govers *et al.*, 1985), the latter representing the late nodulin leghemoglobin. Whereas none of the sense RNA probes hybridized to the sections at detectable levels (data not shown), the antisense probes hybridized with RNA present in different regions of the central tissue of the nodule, with exception of the ENOD2 probe. ENOD2 mRNA has been detected in the nodule parenchyma as described in a recent paper by Van De Wiel *et al.* (1990). *In situ* hybridizations with the ENOD12 transcript are included in these studies to allow direct comparison of the zones in the central tissue in which the various early nodulin genes are expressed.

In nodules from 10 day old plants ENOD12 transcript is present in the invasion zone (Figure 5). The cells of the invasion zone directly adjacent to the meristem contain the largest

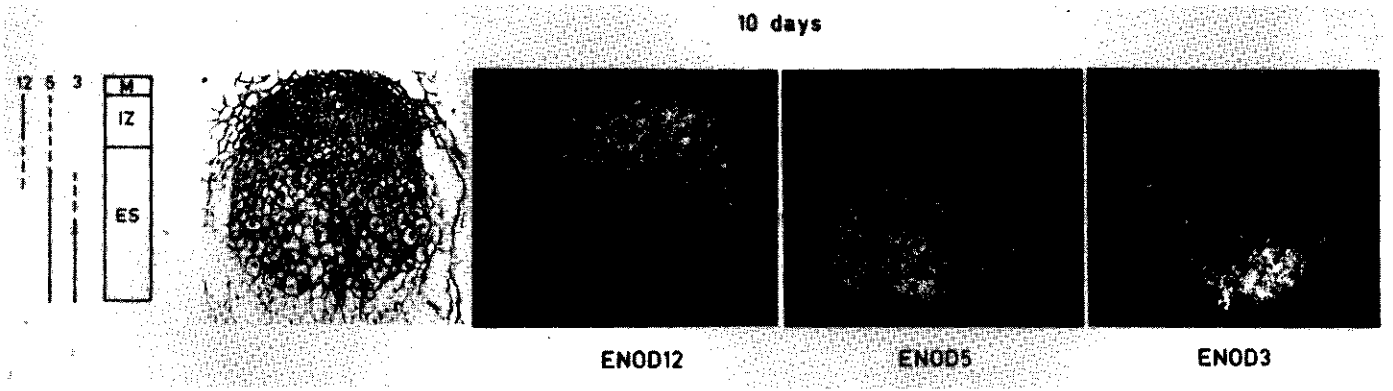

Fig. 5. Localization of ENOD12, ENOD5, and ENOD3 transcripts in nodules from 10 day old pea plants.

Left panel: bright field micrograph of a longitudinal section of a nodule from a 10 day old pea plant. The position of different zones in the central part of the nodule from top to basis is depicted to the left: meristem (M), invasion zone (IZ), and early symbiotic zone (ES). Dark panels: aligned epipolarization micrograph montages from longitudinal serial sections of the same 10 day nodule shown in the left panel. Silver grains representing hybridization signal are visible as white spots. Sections were hybridized with ^{35}S-labeled antisense RNA probes transcribed from the inserts of pPsENOD12, pPsENOD5, and pPsENOD3, respectively. The zones where the different transcripts are located in the centre of the nodule are aligned to the left of the figure: 12=ENOD12; 5=ENOD5; 3=ENOD3. Unbroken lines: high amount of transcript; dashed lines: low amount of transcript.

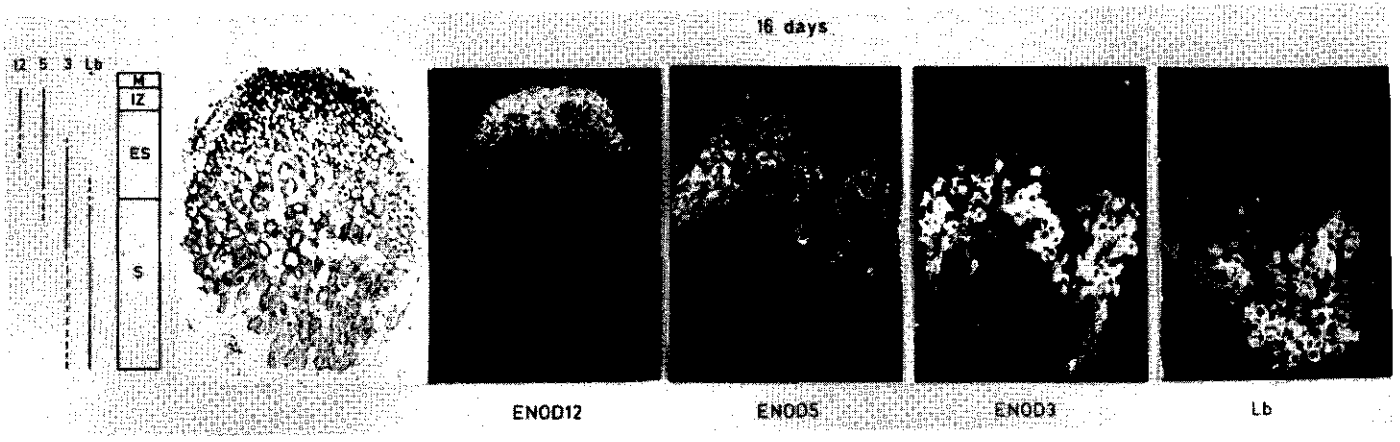

Fig.6. Localization of ENOD12, ENOD5, ENOD3, and leghemoglobin transcripts in root nodules from 16 day old pea plants.

Left panel: bright field micrograph of a longitudinal section of a nodule from a 16 day old pea plant. The position of zones in the central part of the nodule is depicted to the left: meristem (M), invasion zone (IZ), early symbiotic zone (ES), and late symbiotic zone (S). Dark panels: aligned epipolarization micrograph montages from longitudinal serial sections of the 16 day nodule shown in the left panel. Silver grains representing hybridization signal are visible as white spots. Sections were hybridized with ^{35}S-labeled antisense RNA probes translated from the inserts of pPsENOD12, pPsENOD5, pPsENOD3, and pPsLb1, respectively. The zones where the different transcripts are located in the centre of the nodule are aligned to the left of the figure: 12=ENOD12; 5=ENOD5; 3=ENOD3, Lb=leghemoglobin.

proportion of the ENOD12 transcripts, while in the early symbiotic zone the amount of ENOD12 transcripts is falling off. This decrease of ENOD12 mRNA in the early symbiotic zone is also observed in sections from nodules of 16 day old plants (Figure 6). ENOD12 mRNA is absent in the older part of the early symbiotic zone and in the symbiotic zone. The occurence of ENOD12 transcript in the invasion zone containing growing infection threads, together with its presence in root hairs and root cortex cells through which infection threads pass (see also Figure 9), have led to the conclusion that this early nodulin is involved in the infection process (Scheres *et al*, 1990).

Low amounts of ENOD5 transcripts are detected in the youngest cells of the invasion zone directly adjacent to the apical meristem in nodules from 10 day old plants. The largest amount of ENOD5 mRNA is present in the early symbiotic zone, in which bacterial proliferation and enlargement of the infected cells occur (Newcomb, 1976) (Figure 5). In nodules from 16 day old plants it can be seen that after reaching its maximum concentration in the early symbiotic zone, the amount of ENOD5 transcript decreases rapidly in the late symbiotic zone where it remains at a constant low level (Figure 6).

The location of ENOD3 and ENOD14 transcripts was found to be the same and therefore only pictures on the localization of ENOD3 mRNA are shown. In nodules from 10 day old plants ENOD3 mRNA is present in the older part of the early symbiotic zone, where cells have already substantially enlarged, and in which ENOD12 transcript is no longer detected (Figure 5). The ENOD3 and ENOD14 transcripts are thus first detectable at a later stage of development than the ENOD12 and ENOD5 mRNAs. In nodules from 16 day old plants ENOD3 transcript appears to reach its maximum proportion in the symbiotic zone where the amount of ENOD5 mRNA is decreasing (Figure 6). In nodules from 16 day old plants the oldest cells of the symbiotic zone show a decrease in ENOD3 mRNA. In such cells, where the ENOD3 mRNA concentration diminishes, the bacterial *nifH* mRNA encoding nitrogenase is first detectable with *in situ* hybridization (T.B., unpubl. res.). Maximum amounts of ENOD3 transcript, and of ENOD14 mRNA as well, therefore are present in the symbiotic zone just before the stage at which the bacteria start to fix nitrogen.

In nodules from 16 day old plants Lb transcripts are first detectable in cells of the late symbiotic zone where the level of ENOD3 mRNA is maximal (Figure 6). So the Lb mRNA concentration reaches its maximum amount at a later stage than ENOD3 mRNA and it remains at a high level in older parts of the symbiotic zone.

The central tissue of the nodule contains both the infected and the uninfected cell type. The data obtained with ^{35}S-labeled probes clearly show that all different early nodulin transcripts

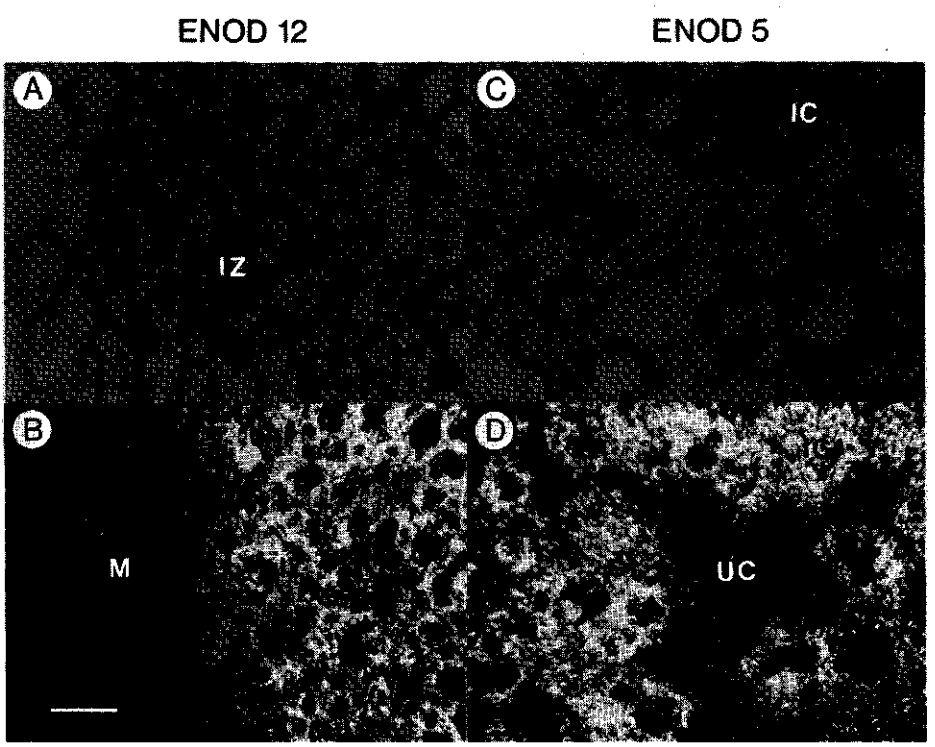

Fig.7. Localization of ENOD12 and ENOD5 transcripts using ^{3}H-labeled probes.

The lower panels show epipolarization micrographs corresponding with the bright field micrographs in the upper panels. A,B. Longitudinal section through the meristem (M) and invasion zone (IZ) of a nodule from a 16 day old pea plant. C,D. Longitudinal section through a nodule from a 16 day old pea plant showing a part of the symbiotic zone. Infected cells (IC) contain bacteria, and uninfected cells (UC) possess large vacuoles and cytoplasm with amyloplasts containing large starch grains which line the cell walls. Sections were hybridized with with ^{3}H-labeled antisense RNA probes translated from the inserts of pPsENOD12 (B) and pPsENOD5 (D)), respectively. Bar = 50 µm.

are present in the infected cell type. On the other hand it was not possible to exclude that transcripts were also present in the higly vacuolated, uninfected cells using ^{35}S-labeled probes. Therefore we also hybridized sections from nodules of 16 day old plants with ^{3}H-labelled probes, which allow more accurate localization. ENOD12 transcripts appeared to be present in all cells of the invasion zone, regardless whether they developed into infected- or uninfected cells (Figure 7A, 7B). The ENOD5, ENOD3, and Lb transcripts all appeared to be

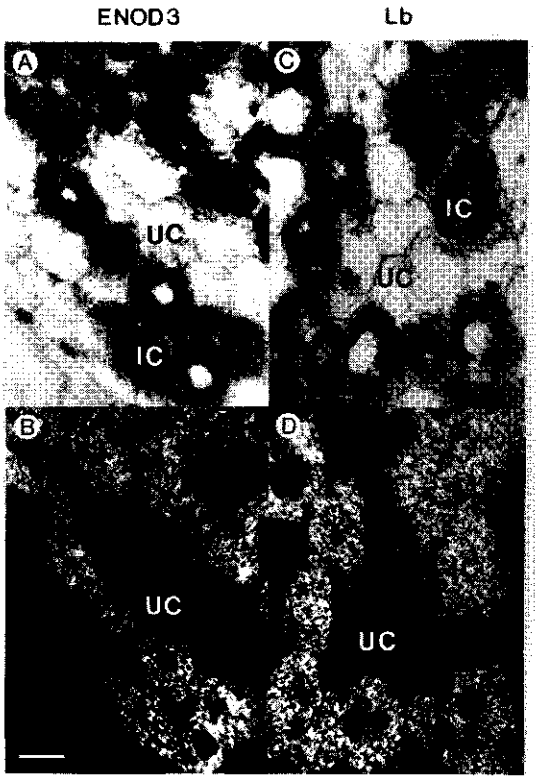

Fig.8. Localization of ENOD3 and leghemoglobin transcripts using ^{3}H-labeled probes.
The lower panels show epipolarization micrographs corresponding with the bright field micrographs in the upper panels. A,C: Longitudinal sections through a nodule from a 16 day old pea plant, showing a part of the symbiotic zone. Sections were hybridized with ^{3}H-labeled antisense RNA probes translated from the inserts of pPsENOD3 (B) and pPsLb101 (D), respectively. IC: infected cell; UC: uninfected cell. Bar = 50 µm.

detectable in the infected cells, but not in the thin layer of cytoplasm lining the cell wall of the higly vacuolated non-infected cells (Figure 7C, 7D; Figure 8).

Localization of ENOD5 and ENOD12 transcripts at early stages of root nodule development.

ENOD5 and ENOD12 transcripts are both detected in the youngest cells of the invasion zone, but while the ENOD12 transcript is present in these cells at maximum concentration, the ENOD5 mRNA is present at low concentration and this transcript reaches its maximum concentration later, in the early symbiotic zone. These data suggest that the ENOD5 gene is induced at a later stage of root nodule development than the ENOD12 gene. To test this assumption we hybridized ^{35}S-labeled ENOD5 and ENOD12 antisense RNA to sections of pea roots containing earlier stages of nodule development. In the root inner cortex of 6 day old plants, inoculated 3 days after sowing, the penetrating infection thread is visible in the outer

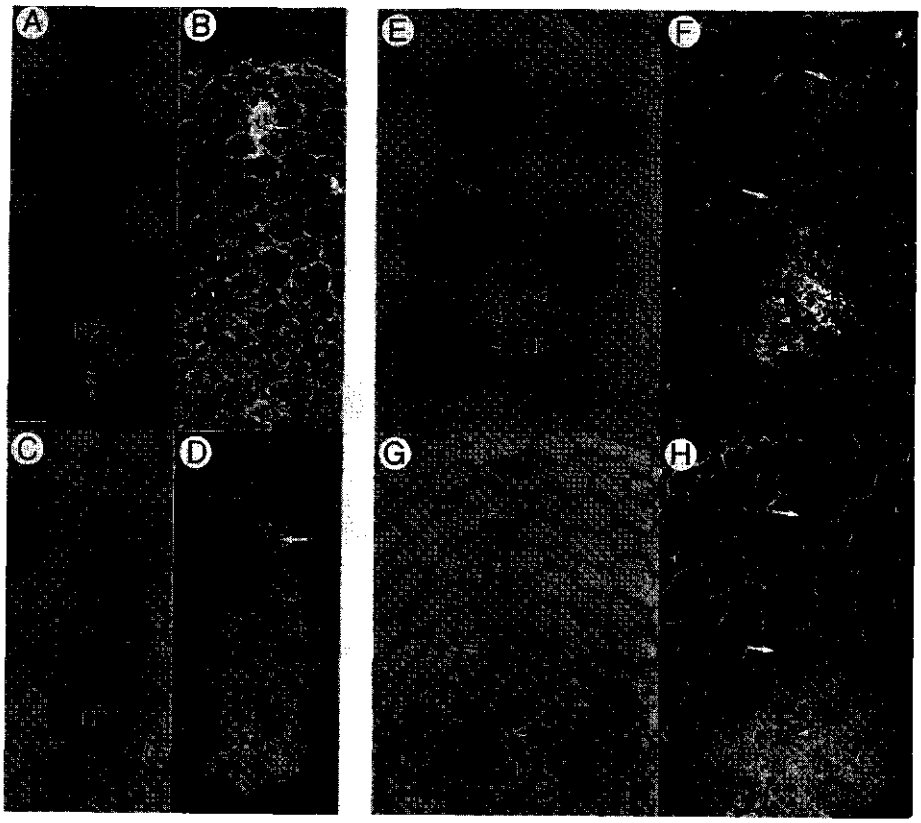

Fig.9. Localization of ENOD5 and ENOD12 transcripts in root segments of 6 and 8 day old inoculated pea plants.

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 of 6 day old plants, 3 days after inoculation, showing infection events at similar developmental stages. The infection thread (arrow) has reached the third/fourth 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: Cross sections through a pea root of 8 day old plants, 5 days after inoculation, showing similar stages of nodule development. 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.

root cortex (Figure 9A, 9C, arrow) while in the inner cortex the nodule primordium is developing. ENOD5 transcript is only detectable in root cortex cells containing the infection thread tip and in the cells through which the infection thread has just passed, and not in the nodule primordium (Figure 9B). On the other hand, ENOD12 mRNA is present in the cells carrying the invading infection thread, but also in the nodule primordium (Figure 9D). In 8 day old plants the infection thread has reached the nodule primordium (Figure 9E, 9G). At that stage ENOD5 transcript is detected in cells in the centre of the primordium (Figure 9F). Analyses at higher magnifications show that these cells contain branches of the infection thread (data not shown). The ENOD12 mRNA is present in all cells of the nodule primordium (Figure 9H). Hence ENOD5 and ENOD12 genes appear to be expressed at the same stage of the Rhizobium-pea interaction. There is however a major difference in accumulation of the transcripts during early stages of root nodule development: the ENOD5 transcript is restricted to the cells containing the actively growing infection thread, whereas ENOD12 mRNA is present well in advance of the growing thread.

DISCUSSION

Early nodulin transcripts mark successive stages of root nodule development.

Three early nodulin transcripts which are present in successive zones of the central tissue of the indeterminate pea nodule are characterized. Together with the previously described early nodulin transcript ENOD12 and the leghemoglobin mRNA they form a set of markers for successive developmental stages of the central tissue. While ENOD12 mRNA is present in both infected and uninfected cells of the invasion zone, the ENOD5, ENOD3, ENOD14, and Lb transcripts are restricted to the infected cell type. Therefore all five transcripts mentioned above mark different developmental stages of the infected cells, which are part of the actual symbiotic tissue. The location of the different mRNAs in the symbiotic tissue can be compared with the cytological zones according to Newcomb (1976), as indicated in figures 5 and 6. ENOD12 transcript is mainly present in the invasion zone; ENOD5 transcript in the invasion zone and the early symbiotic zone; and Lb mRNA in the late symbiotic zone. The location of these transcripts therefore roughly coincides with one or more zones described by cytological criteria. On the other hand the location of ENOD3 and ENOD14 mRNA does not mark a cytologically distinct zone. The possibility to describe root nodule development not only by

cytological criteria but also in molecular terms facilitates analysis of wild-type and mutant root nodule development.

Previously it has been recognized that nodulin genes are expressed at different time stages of nodule development (Govers *et al.*, 1985; Gloudemans *et al.*, 1987). This has led to the division between early and late nodulins. The accumulation of early nodulin transcripts in different zones of the pea nodule as shown in this paper implies that *Rhizobium* causes induction of nodulin gene expression at various time points. The division of nodulin genes into two classes according to their timing of expression should therefore not be regarded as reflecting the actual existence of two main time points at which *Rhizobium* induces plant gene expression. Rather it forms an arbitrary division within a set of genes induced by the bacterium in a sequential manner.

The accumulation patterns in time of different transcripts within the early nodulin class are already visualised on RNA transfer blots, as can be seen in figure 1, but are most clearly reflected by the *in situ* hybridization data. The apparent difference in time between onset of ENOD12 and ENOD5 gene expression as deduced from RNA transfer blot analyses appears not to be true. The *in situ* hybridization data show that a few cells express the ENOD5 gene at an early stage of bacterial infection, but the amount of transcript is not sufficient to be seen on transfer blots of RNA extracts at that time. Furthermore, from localization studies it is more evident that the ENOD5 gene is expressed at an earlier stage of development than the ENOD3/14 genes than from RNA transfer blots. Finally the transient accumulation patterns of ENOD12 and ENOD5 transcripts on an RNA transfer blot appear to reflect a decrease of the size of the zone in which the ENOD12 and ENOD5 mRNAs are present, relative to the total size of the nodule, rather than reflecting a decrease of the amount of transcript in a particular tissue zone.

Possible functions of early nodulins located in the central nodule tissue

In nodules the ENOD12 gene is expressed in the invasion zone which is the zone where active infection thread growth occurs (Newcomb, 1976). In addition ENOD12 gene expression is induced in root hairs, in root cortical cells through which the infection thread will migrate, and in the nodule primordium. It is likely that the (hydroxy-)proline-rich ENOD12 early nodulin is a cell wall component involved in preparing cells for the infection process, while the protein can also have a function in the formation of the nodule primordium (Scheres *et al.*, 1990).

The ENOD5 gene is expressed at early stages in cortex cells containing the infection thread tip so expression of this gene is induced during the same stage of development as the ENOD12 gene. The ENOD5 gene is expressed in the invasion zone and not in the meristem, which is consistent with a role of ENOD5 in the infection process. However, the highest levels of the ENOD5 transcript are present in infected cells of the early symbiotic zone. Therefore in the nodule the highest level of the ENOD5 transcript does not coincide with the zone where infection thread growth occurs. This might indicate that the protein is functional both during infection thread growth and bacterial proliferation. Cells containing the infection thread tip and cells containing released bacteria share as a common property the presence of rhizobia within the cell, only shielded from direct contact with the cytoplasm by the membrane surrounding the open infection thread tip or the peribacteroid membrane, respectively. The hydrophobic domains in the ENOD5 protein could point to a location of the protein within such membranes. On the other hand the resemblance in amino acid composition of a central region of the ENOD5 protein to that of arabinogalactan proteins (AGPs), might indicate that ENOD5 is an extracellular protein present in both the infection thread matrix and the peribacteroid space. The functions of AGPs are as yet unknown (Fincher *et al.*, 1983), but they are widely distributed and organ-specific forms occur (Knox *et al.*, 1989). In soybean nodules specific AGPs were detected, demonstrating that some nodulins can be AGPs (Cassab, 1986). We intend to establish whether ENOD5 is a nodule specific AGP or a membrane-associated protein by immunological analyses with the aid of antisera raised against ENOD5 peptides.

The inserts of pPsENOD3 and pPsENOD14 are 65% homologous. However, on Southern blots these inserts do not cross-hybridize under standard conditions (data not shown). Therefore we expect that also on nodule sections the ENOD3 and ENOD14 probes hybridize predominantly with their homologous RNAs. Since the ENOD3 and ENOD14 probes hybridize in the same zone of the nodule it is very likely that these two early nodulin genes are expressed in infected cells during the same stage of development, when the bacteria have proliferated to a certain extent. Remarkably, while the expression behaviour of the ENOD3 and ENOD14 genes is identical and the proteins have similar features, close comparison of the amino acid sequences reveals many amino acid substitutions indicating that the functions of the proteins might be similar but not identical. The ENOD3 and ENOD14 proteins consist of a signal peptide and a small polypeptide containing four cysteines which may bind a metal ion. Therefore we assume that these early nodulins have a metal ion transport function. Since the bacteroids require high amounts of molybdenum and iron for the synthesis of nitrogenase

(Shah and Brill, 1977), it is tempting to speculate that ENOD3 and ENOD14 have a role in the transport of these metals over the peribacteroid membrane to the bacteroid. Cysteine clusters and a putative signal peptide like in ENOD3 and ENOD14 are also present in a set of soybean nodulins, although there is no further sequence homology among these proteins (Jacobs *et al.*, 1987; Sandal *et al.*, 1987). One of these soybean proteins, N-23, is located in the peribacteroid membrane (Jacobs *et al.*, 1987), and hence like ENOD3 and ENOD14 present in infected cells. Nevertheless it seems too early to speculate that these soybean and pea nodulins have similar functions based on the correlations mentioned above.

Regulation of early nodulin gene expression by *Rhizobium.*

An intriguing question concerning the establishment of symbiosis is whether *Rhizobium* needs to elicit only a few or a multiplicity of signals to effect root nodule formation and the accompanying expression of nodulin genes. The availability of the set of cDNA clones described in this paper, representing genes expressed at different stages during nodule formation, adds to the possibility of exploring this problem.

In a previous paper we demonstrated that the ENOD12 gene is expressed in nodule primordia and root cortex cells that do not yet contain the infection thread so a diffusible compound from *Rhizobium*, involved in inducing this early nodulin gene, seems to act over a rather large distance (Scheres *et al.*, 1990). This is consistent with the finding reported in this paper that the ENOD12 gene is not only expressed in nodule cells containing bacteria, but in all cells of the invasion zone. The bacterial nodulation (*nod*) genes have been shown to be essential for ENOD12 gene expression, and soluble bacterial compounds are able to elicit ENOD12 gene expression in root hairs (Scheres *et al.*, 1990).

Our studies on the distribution of ENOD5 mRNA during nodule development indicate that bacterial compounds inducing ENOD5 gene expression appear to be active only in cells containing bacteria surrounded by either the infection thread tip membrane or the peribacteroid membrane. Apparently an intercellular barrier exists for the signal that induces ENOD5 gene expression. Hence there is a clear difference in the way *Rhizobium* induces ENOD5 and ENOD12 gene expression. While a factor acting over a long range is inducing ENOD12 gene expression most likely another factor acting over a short distance induces expression of the ENOD5 gene. The nature of the ENOD5 inducing signal and the bacterial genes involved in producing it is still unknown. However, the low level of ENOD5 mRNA in the infected cells

of the late symbiotic zone shows that the presence of rhizobia in a cell is not sufficient for the accumulation of the ENOD5 mRNA.

The observation that the amount of ENOD5 transcripts in the infected cells is already declining while the amount of ENOD3/14 transcript is maximal, indicates that different bacterial factors may be responsible for the induction of ENOD5 and ENOD3/14 gene expression. In a similar way different bacterial factors may induce ENOD3/14 and Lb gene expression, respectively, because the amount of ENOD3/14 transcript decreases in the zone where the amount of leghemoglobin transcript becomes maximal. Alternatively one bacterial signal, inducing a cascade of developmental events, may lead to differential expression of the ENOD5, ENOD3/14, and leghemoglobin nodulin genes. The argument for considering induction of ENOD12 and ENOD5 gene expression to be caused by different bacterial factors, namely the fact that one gene is induced at distance from infected cells and the other gene not, does not apply to the ENOD5, ENOD3/14, and Lb genes since these are all expressed in infected cells. Genetically defined bacterial mutants that do not produce the compounds necessary to invoke ENOD5, ENOD3/14, or Lb gene expression may provide clues to resolve whether one or more bacterial signals are involved in the induction of expression of these genes. Moreover these mutants may be usefull to elucidate the structure of different signal molecules. In this regard the bacterial release (*bar*) (De Maagd *et al.*, 1989) and bacteroid development (*bad*) mutants are promising since these mutants affect nodule development at the time that ENOD5 and ENOD3/14 genes are expressed.

MATERIALS AND METHODS

Plant materials

Pea (*Pisum sativum* cv. rondo or sparkle) plants were cultured and inoculated with *Rhizobium leguminosarum* bv. *viciae* 248 as described by Bisseling *et al.* (1978). Inoculation was performed directly upon sowing, unless stated otherwise. 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. *Fusarium oxysporum* mycelium was inoculated in Czapek-dox medium and grown for 2 days at 30°C. Pea plants were inoculated with this suspension 3 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.

cDNA cloning

A λgt11 cDNA library, prepared from *Pisum sativum* cv. sparkle lateral root nodule RNA, 21 days after infection, was kindly provided by dr. G. Coruzzi (Tingey *et al.*, 1987). Nitrocellulose replicas, containing phage DNA of appr. 3000 plaques, were made using standard procedures (Maniatis *et al.*, 1982). ^{32}P-labelled cDNA probes for differential screening were prepared from poly(A)$^+$RNA of nodules, 10 days after inoculation, and of 8 day old, uninfected 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 using another differential screening. Phage DNA was isolated and cDNA inserts were subcloned into pUC vectors using standard procedures (Maniatis *et al.*, 1982).

RNA transfer blot analysis

Total RNA from nodules and other tissues was isolated by phenol extraction and LiCl precipitation according to De Vries *et al.* (1982). RNA concentrations were measured spectrophotometrically and equal amounts of total RNA were used for gel blot analysis as stated in the legends. RNA transfer blotting was performed as described by Franssen *et al.* (1987), using GeneScreen membranes and nick-translated cDNA inserts or antisense RNA probes.

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 transferred to GeneScreen plus membranes using ammonium acetate transfer (Rigaud et al., 1987). The blot was hybridized to nick-translated cDNA insert probes 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 mRNA was done with inserts from the various cDNA clones, bound on DPT paper (BioRad) as described by Maniatis *et al.* (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 6 h period. Hybrid selected mRNA was translated in a wheat germ extract (BRL) using ^{35}S-methionine (NEN) as radioactive amino acid. Proteins were separated on SDS-containing 25% acrylamide gels. Gels were fluorographed to Kodak XAR-5 films.

DNA sequencing

cDNA inserts were sequenced using the double stranded dideoxy chain termination procedure (Korneluk *et al.*, 1985). Both strands were sequenced entirely. In all cases two independently obtained clones were sequenced to rule out the possibility of recombined cDNAs. Sequence data were stored and analyzed using programs written by R.Staden on VAX/VMS and microVAX/VMS computers.

In situ hybridization

All cDNA inserts were cloned in pT7-6 (a kind gift of 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) or ^{3}H-UTP (40 Ci/mmol) as the radioactive nucleotide. The probes were partially degraded to an average length of 150 nucleotides by heating at 60°C in 0.2 M Na_2CO_3/0.2 M $NaHCO_3$. Nodules, stems, and root sections were fixed with 3% paraformaldehyde and 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 70mM DTT for 16 hrs at 42°C. Washing was performed in 4 x SSC, 5 mM DTT at room temperature, followed by treatment 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 RT. Sections were dehydrated in 70% and 90% ethanol (each with 300 mM ammoniumacetate) and 100% ethanol, and air dried. Slides were coated with Kodak NBT2 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. Sections were photographed with a Nikon microscope equipped with dark field and epipolarization optics.

Primer extension analysis and RNA sequencing.

Synthetic oligomers complementary to nucleotide 118-138 of the pPsENOD5 insert, nucleotide 51-71 of the pPsENOD3 insert, and nucleotide 57-77 of the pPsENOD14 insert, were ^{32}P-labeled using T4 polynucleotide kinase (Pharmacia). 1.10^6 cpm of these primers 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 ddNTP's (800 µM). Extension was performed for 20 min. at 45°C. Subsequently 1 µl 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.

ACKNOWLEDGEMENTS

We thank dr. Scott Tigney and dr. Gloria Coruzzi for providing the nodule cDNA library, Peter van Druten for photography, Piet Madern for artwork, Piet de Kam for growing plants, and Sacco de Vries for formatting sequence data. B.S. was supported by a grant from the Dutch Organization of Science (N.W.O.).

REFERENCES

Berg,J.M. (1986) Science, 232, 485-487.

Bisseling,T., Van den Bos,R.C. and Van Kammen, A. (1978) Biochim. Biophys. Acta, 539, 1-11.

Cassab,G.J. (1986). Planta, 168, 441-446.

De Maagd,R.A., Rao,A.S., Mulders,I.H.M., Goossen-de Roo,L., van Loosdrecht,M.C.M., Wijffelman,C.A. and Lugtenberg,B.J.J. J. Bacteriol. In press.

De Vries,S.C., Springer,J. and Wessels,J.H.G. (1982) Planta, 156, 129-135.

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

Fincher,G.B., Stone,B.A. and Clarke, A.E. (1983) Ann. Rev. Plant Physiol., 34, 47-70.

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, 4495-4499.

Franssen,H.J., Scheres,B., Van De Wiel,C. and Bisseling,T. (1988) In Palacios,R. and Verma,D.P. (eds). Molecular Genetics of Plant-Microbe Interactions. APS Press, St. Paul, pp. 321-326.

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, 395-403.

Govers,F., Gloudemans,T., Moerman,M., Van Kammen,A. and Bisseling,T. (1985) EMBO J., 4, 861-867.

Govers,F., Nap,J.P., Van Kammen,A. and Bisseling,T. (1987) Plant Mol. Biol., 8, 425-435.

Jacobs,F.A., Zhang,M., Fortin,M.G. and Verma, D.P.S. (1987) Nucl. Acids Res., 15, 1271-1280.

Knox,J.P., Day,S. and Roberts,K. (1989) Development, 106, 47-56.

Korneluk,R.G., Quan,F. and Gravel,R.A. (1985) Gene, 40, 317-323.

Lütcke,H.A., Chow,K.C., Mickel,F.S., Moss,K.A., Kern,H.F. and Scheele,G.A. (1987) EMBO J., 6, 43-48.

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

Newcomb,W. (1976) Can. J. Bot., 54, 2163-2186.

Rigaud,G., Grange,T. and Pictet,R. (1987) Nuc. Acids Res., 15, 857.

Rogers,S.O. and Bendich,A.J. (1986) In Gelbin,S.B. and Schilperoort,R.A. (eds.) Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, pp. 1- 10.

Sandal,N.N., Bojsen,K. and Marcker,K.A. (1987) Nucl. Acids Res., 15, 1507-1519.

Scheres,B., Van De Wiel,C., Zalensky,A., Horvath,B., Spaink,H., Van Eck,H., Zwartkruis,F., Wolters,A., Gloudemans, T.,Van Kammen,A. and Bisseling,T. (1989) Cell, in press.

Shah,V.K., and Brill,W.J. (1977). Proc. Natl. Acad. Sci. USA, 3249-3253.

Tigney,S.V., Walker,E.L. and Coruzzi,G.M. (1987) EMBO J., 6, 1-9.

Van De Wiel,C., Scheres,B., Franssen,H.J., Van Lierop,M., Van Lammeren,A., Van Kammen,A. and Bisseling,T. (1990) EMBO J., in press.

Van Holst,G-J., Klis,F.M., De Wildt,P.J.M.,Hazenberg,C.A.M., Buijs,J. and Stegwee,D. (1981) Plant Physiol., 68, 910-913

Van Kammen,A. (1984) Plant Mol. Biol. Rep. 2, 43-45.

Vincent,J.M. (1980) In Newton,W.E. and Orme-Johnson,W.H. (eds.) Nitrogen Fixation II, University Park Press, Baltimore, pp. 103-109

Von Heijne,G. (1983) Eur. J. Biochem., 133, 17-21.

CHAPTER 6

Identification of *Rhizobium leguminosarum* genes and signal compounds involved in the induction of early nodulin gene expression.

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In: Molecular signals in microbe-plant symbiotic and pathogenic systems. B.J.J. Lugtenberg, (ed.), NATO ASI series, Springer Verlag, Heidelberg, pp. 367-377 (1990).

INTRODUCTION

The process of root nodule formation on legumes, induced by *Rhizobium*, can be looked upon as a sequence of several distinct steps. These steps have been defined by cytological studies on developing wild-type root nodules, and by analyses of nodules formed by either plant or bacterial mutants (Vincent 1980). Nowadays attachment of bacteria, root hair deformation and curling, induction of cortical cell division, infection thread formation, nodule development, bacterial release from infection threads, bacteroid development and effective nitrogen fixation are recognized as successive steps in root nodule formation (Vincent 1980). The multistep nature of root nodule formation has led to the hypothesis that at several stages in the *Rhizobium*-plant interaction signal molecules from either symbiotic partner are involved in inducing a process in the other partner. Identification of the different bacterial and plant signals and analysis of the mode of action of each separate compound would then significantly enlarge our knowledge about the establishment of symbiosis.

To date only two factors involved in the communication between the two symbionts have been identified. Using *nod-LacZ* reporter gene fusions, plant flavonoids have been identified as elicitors of the beginning of the communication. These flavonoids act in concert with the product of the constitutively expressed bacterial *nodD* gene to induce expression of the other *nod* genes (Mulligan and Long 1985). Upon induction of the *nod* genes the root hair deformation factor is formed by the bacteria. This factor has been identified using root hair deformation as qualitative bioassay (Bhuvaneswari & Solheim 1985). The relevant compound has however not been purified and the structure has not yet been determined due to the lacking of a quantitative assay. This demonstrates the importance of reporter genes for developing quantitative assays to be used for the purification of compounds, involved in different steps of root nodule formation.

We have set out to isolate nodule specific plant cDNA sequences representing genes of which the expression marks different stages of root nodule development. When we started, the already cloned plant nodulin sequences mainly represented genes expressed when nodule development, according to cytological criteria, is completed (reviewed in Govers *et al* 1987a). We have now isolated cDNA clones representing genes expressed earlier in nodule development and examined whether the expression of these genes correlates to distinct steps in root nodule formation. The information that may be obtained with such marker genes is twofold. First, characterization of the proteins encoded by these genes will provide information about the type of plant proteins essential for different developmental steps.

Second, if indeed the expression of a certain gene is characteristic for a step in root nodule development, the expression of such gene may be used to identify bacterial or plant signals necessary to invoke the developmental step in which the gene is involved. Here we shall briefly summarize the characteristics of a set of pea early nodulin cDNA clones. We will describe the correlation of the expression of different genes and different steps in root nodule formation. We further show how expression of these genes can be used to examine whether certain compounds can mimic the effect of signal molecules in the *Rhizobium*-plant interaction. We then focus on the question how one of the cloned cDNAs, pPsENOD12, may be used for developing an assay for purification of bacterial signal molecules.

EARLY NODULINS

Nodulin genes are differentially expressed during nodule development and therefore these genes have been divided into two groups, early and late nodulins (for review see Nap and Bisseling 1989). The late nodulin genes are first expressed shortly before or concomitantly with the onset of nitrogen fixation. The genes expressed at earlier stages of nodule development are named early nodulin genes.

pGmENOD2 was the first early nodulin for which a cDNA clone has been described (Franssen *et al* 1987). This cDNA clone was isolated from a soybean nodule cDNA library, and was found to encode a protein composed of 2 repeating penta- or hexapeptides, containing two or three prolines each. At the N-terminus a putative signal peptide is present. Later similar ENOD2 cDNA clones were isolated from alfalfa (Dickstein *et al* 1988), sesbania (F. de Bruijn, N-H.Chua, pers. comm.), and pea (our work). Besides pGmENOD2, two other soybean early nodulin clones have recently been described, pGmENOD13 and pGmENOD55 (Franssen *et al* 1988). ENOD13 has a structure similar to ENOD2. The 52 amino acid sequence of the polypeptide derived from the partial reading frame in the cloned sequence shows 70% homology to the C-terminal part of the ENOD2 protein. The structure of the ENOD55 protein differs from that of the ENOD2 protein, but ENOD55 is a proline rich protein as well. The proline rich region of the ENOD55 protein is confined to an internal domain of 32 amino acids and is composed of proline and serine residiues.

From *Pisum sativum*, the garden pea, we isolated five early nodulin clones pPsENOD2, pPsENOD12, pPsENOD5, pPsENOD3, and pPsENOD14, which could be involved in

different developmental steps, judged by the different time courses of accumulation of the corresponding mRNAs during nodule development. pPsENOD12 encodes a protein with a structure similar to that of the pea ENOD2 protein , since both are built up of a series of two repeating pentapeptides each containing two prolines. The three other amino acids in the pentapeptide unit of pPsENOD12 differ from those in ENOD2. In conclusion, the pea ENOD2 and ENOD12 early nodulins are, like the soybean early nodulins ENOD2 and ENOD13, (hydroxy-)proline rich proteins. It is thought that (hydroxy-)proline rich proteins are often associated with cell walls. Therefore we assume that at least part of these proline rich early nodulins are components of cell walls of the different cell types that are formed during symbiosis. The homology of ENOD2, ENOD13 and ENOD12 to the soybean cell wall protein 1A10 (Averyhart- Fullard *et al* 1988) supports this hypothesis. pPsENOD5 encodes a protein with hydrophobic regions at both the C- and the N-terminus, the latter possibly forming a signal peptide. This early nodulin also has a proline rich domain, which moreover has a high content of glycine, alanine, and serine. The amino acid composition of this domain of ENOD5 is reminiscent of that of arabinogalactan proteins (Van Holst *et al* 1981). To date no amino acid sequences of arabinogalactan proteins have been published, which prevents a more detailed comparison of ENOD5 and arabinogalactan proteins. Upon searching different databases no significant homology between ENOD5 and other previously described proteins was found. pPsENOD3 and pPsENOD14 both encode small nodulins, with a molecular weight of about 6 kD. These two early nodulins are 60% homologuous and contain as most striking characteristic a cluster of four cysteins, arranged in such a way that a metal can be bound. This suggests that the ENOD3 and ENOD14 proteins contain a metal ion. Similar motifs of four cysteines are found in the late nodulins Ngm-20, Ngm-23, Ngm26b, Ngm-27, and Ngm-44, forming a small gene family in soybean (Jacobs *et al* 1987). However, there is no significant sequence homology between these or any other proteins from which the sequence is stored in the different databases, and the ENOD3 and ENOD14 proteins.

NODULIN GENE EXPRESSION DURING NODULE DEVELOPMENT

Pea nodules belong to the indeterminate nodule type. These nodules have a persistent meristem. Hence all nodules contain cells at different stages of development: the youngest cells adjacent to the apical meristem and the oldest cells at the basal root attachment point. This type of nodules has the advantage that the presence of transcripts related to particular stages of root nodule development is less dependent on the age of the nodule. A further advantage is that it is possible to compare the expresssion patterns of the nodulin genes in different cell types during nodule development by comparing the spatial distribution of different nodulin transcripts in serial sections from one nodule using *in situ* hybridization.

The different pea early nodulin clones were hybridized with sections of nodules from plants of various ages. The plants start to fix nitrogen from day 13 after inoculation and sowing, and at this day the first late nodulin transcripts are detectable by RNA blot analysis (Govers *et al* 1987b). On sections of these nodules the leghemoglobin (Lb) cDNA clone pPsLb was used as a marker for the expression of late nodulin genes, and the *R.leguminosarum nifH* gene as a marker for expression of bacterial genes involved in nitrogen fixation.

The transcripts corresponding to four of the five selected early nodulin clones are present in the central tissue of the nodule. For ENOD2, on the other hand, the transcript was specifically located in the nodule inner cortex. The ENOD12, ENOD5, ENOD3, and ENOD14 transcripts are present in successive, but partially overlapping zones of the central tissue. The ENOD12 mRNA is present in a small zone directly adjacent to the meristem, the invasion zone. The ENOD5 transcript starts to accumulate in the cells where ENOD12 mRNA is still present, but the concentration reaches a maximum in the zone of the central tissue that contains enlarging cells. This zone has been named the early symbiotic zone as in this zone the first plant cells that contain bacteria are present (Newcomb 1981). The ENOD5 transcript is present at strongly reduced levels in the cells of the central tissue that have reached their maximum size. This is the late symbiotic zone. The ENOD3 and ENOD14 messengers start to accumulate in the cells where the ENOD5 transcript is present at a maximal level and the concentration of these transcripts reaches its maximum in the youngest 3-4 cell layers of the late symbiotic zone. In the older cells of the symbiotic zone the ENOD3 and ENOD14 mRNA concentration decreases. In these older cells the level of the transcript of the late nodulin Lb is at its maximum. The ENOD5, ENOD3, and ENOD14 mRNAs are only present in the cells containing rhizobia, while ENOD12 mRNA is present in all the cells of the invasion zone.

Therefore ENOD12 transcript is present in cells that will develop in either infected or uninfected cells. At the stage of development of the infected cells when the ENOD3 and 14 mRNA concentrations are decreasing, the nitrogenase mRNA can first be detected in the bacteroid . This proves that the infected cell becomes a functional nitrogen fixing cell at that stage. Therefore the decline of the ENOD3 and 14 mRNA concentrations marks the stage at which the infected cell is fully differentiated into a functional nodule cell.

In conclusion, the *in situ* hybridization studies show that the differentiation of meristematic cells into the infected cell type in the central zone of the nodule requires at least four successive steps of specific gene expression. These different steps are marked by the presence of ENOD12, ENOD5, ENOD3 and ENOD14, and Lb transcripts, respectively. Most likely several other late nodulin genes are expressed concomitantly with the Lb genes, but this has not yet been tested.

The availability of data on both the sequence and the localization of the early nodulin transcripts in some cases allows one to speculate about functions of the encoded proteins. As for the site of expression of the ENOD2 gene, it has been shown by Witty *et al* (1986) that the nodule inner cortex is the major barrier for oxygen diffusion within a root nodule. It is conceivable that ENOD2, being a putative cell wall protein, contributes to the absence of intercellular spaces in the inner cortex which causes this tissue to be the major oxygen barrier.

In situ hybridization studies have now revealed that ENOD12 genes are expressed not only in the nodule invasion zone but also in root hairs and in the root cortex during the infection process. ENOD12 transcripts are found in the cells that become prepared for infection thread growth (Bakhuizen *et al* 1989), and in cells containing the infection thread tip. Therefore it was concluded that ENOD12 has a role in the "preparation" of cells for infection thread growth and maybe also in the formation of the infection thread. Sequence analyses suggest that ENOD12 is a cell wall component. In the "prepared" root cortex cells an additional cell wall is formed, an infection thread contains a wall. Whether these walls indeed contain ENOD12 protein remains to be established by immunocytological studies.

Combination of structural data of ENOD5, ENOD3, and ENOD14 early nodulins, and the location of the corresponding transcripts, does not yet allow predictions about the functions of these early nodulins during development. Immunocytological localization of the proteins might give more clues. More direct evidence for the function of these early nodulins would require the succesfull application of reverse genetics, e.g. antisense RNA inhibition.

In view of the possible function of nodulins we have wondered if the expression of nodulin genes is related to defense responses to plant pathogens. The study of signals and transduction mechansisms for activation of plant defense genes is a well developed area of plant molecular biology (Lamb *et al* 1989). Also the *Rhizobium*-legume symbiosis has been viewed as a modified defense response (Djordjevic *et al* 1987). This hypothesis implies that during nodule development the expression of plant genes might be triggered as a result of bacterial signals and signal transduction pathways normally used as part of a response to pathogens. We tested whether pea early nodulin genes were expressed when pea roots were inoculated with the pathogen *Fusarum oxysporum* f. sp. *pisi*. While we found accumulation of defense related hydroxyproline rich glycoprotein mRNAs in these roots, no accumulation of early nodulin transcripts could be detected. We conclude that early nodulin gene expression is not related to a general plant defense respons and that the signals and transduction mechanisms that trigger early nodulin gene expression therefore differ from those occuring during a pathogenic interaction.

EARLY NODULIN GENES AS REPORTERS FOR BACTERIAL OR PLANT SIGNALS

The differential accumulation of nodulin transcripts during the differentiation of the infected cell type points to the occurence of successive steps in nodulin gene expression. Successive induction of gene expression can be caused by different bacterial signals, by different kinetics of gene expression in response to the same bacterial signal, or by different second messengers that are formed in the plant as a result of a process induced by one bacterial signal. The mechanisms used by *Rhizobium* to establish the differential gene expression during nodule development can now be studied with the set of nodulin cDNA clones described here. We do not pretend to have cloned all early nodulin transcripts, and hence we cannot expect that the six genes studied here are markers for all steps of plant-bacterium communication and nodule development. Still the answers obtained on the expression of the available nodulin genes can substantially increase the insight into the way plant and bacterium communicate. Here we will demonstrate the use of the pea ENOD12 and ENOD2 genes as marker genes.

A. Nodulin genes as tools to study compounds that mimic *Rhizobium* signals.

More than forty years ago Allen and Allen (1940) showed that compounds that block the polar transport of auxin induce nodule-like outgrowths on the roots of several plants. Later it was shown that auxins and cytokinins induce cell division in the inner cortex of root explants, possibly in cooperation with a factor from the xylem (Libbenga *et al* 1973). In these studies processes were induced that resemble steps occurring in root nodule formation. Unfortunately these processes could only be studied on a cytological level at that time, and it remained undecided whether these compounds really mimiced part of the nodule formation process. Now probes for the expression of several early and late nodulin genes are available and the expression of specific marker genes can be used to mark different steps in the *Rhizobium*-legume interaction. It is now possible to reexamine the effect of such compounds and re-evaluate data that were already buried in the archaeology of science. The nodule structures formed by anti-auxins were recently studied at the molecular level by Hirsch *et al* (1989). In such nodules formed on alfalfa roots upon treatment with the anti-auxins 2,3,5-triiodobenzoic acid (TIBA) or N-(1-naphtyl)-phthalamic acid (NPA) the alfalfa early nodulin genes ENOD2 and Nms-30 appeared to be expressed. Moreover we demonstrated that the ENOD2 transcripts are located in a tissue at the periphery of these nodule structures. By position and gene expression this tissue is therefore very similar to the nodule inner cortex, which points to the existence of both cytological and molecular similarities between anti-auxin formed nodules and nodules formed by *Rhizobium*.

If anti-auxins can induce cortical cell division and development of a nodule structure similar to that induced by rhizobia, an obvious question is whether *Rhizobium* produces analogous compounds. The occurence of a *Rhizobium* compound specifically involved in induction of cell division has been demonstrated, but the compound has not yet been characterized (Schmidt *et al* 1988). Two lines of evidence demonstrate a crucial role of the *nod* genes for the production of this factor. First, mutations in the common *nod* genes abolish the ability of *Rhizobium* to induce cortical cell division (Dudley *et al* 1987). Second, 12 kb of the *R.leguminosarum* Sym plasmid, containing the *nod* genes, can confer to *Agrobacterium tumefaciens* the ability to form root nodules, expressing the ENOD2 gene (Moerman *et al* 1987).

Based on the similarities in nodule development induced by anti-auxins and *nod* gene dependent bacterial compounds it is plausible that *Rhizobium nod* gene products interfere with

the phytohormone distribution in the legume root. The subsequent change in hormone balance could then induce centres of mitotic activity at certain sites in the root cortex. The observation that *Rhizobium nod* gene mutants can be complemented with the *A.tumefaciens* zeatin gene, which is involved in cytokinin synthesis, is in accordance with this hypothesis (J.Cooper, pers.comm.).

Upon treatment of pea roots with anti-auxins, also the expression of ENOD12 is induced in root hairs already after 48 hrs. Moreover the ENOD12 transcript is detectable in nodules formed on pea plants by the anti-auxin NPA. During the pea-*Rhizobium* symbiosis expression of this gene marks root hair-, root cortex-, and nodule cells involved in, or preparing for, infection thread growth. Specific microtubule rearrangement and nuclear migration in these cells also occurs in cells which, upon *Rhizobium* infection, become part of the centre of mitotic activity, the nodule primordium (Bakhuizen *et al* 1989). We demonstrated that in these cells ENOD12 gene expression is also induced. This would imply that cells preparing for infection thread passage and nodule primordium cells are similar as far as ENOD12 expression is concerned. Apparently anti-auxins can at least induce expression of the ENOD12 gene as a marker gene for these cell types. Since anti-auxins are able to elicit both ENOD12 and ENOD2 gene expression, they appear to mimic two different processes. First, ENOD12 nodulin gene expression, related to cells involved in both infection thread growth and the establishment of a nodule primordium, is induced. Second, meristematic activity indeed leads to differentiation into at least one nodule tissue where the proper nodulin gene, ENOD2, is expressed. Conclusively, anti-auxins induce a cascade of events, mimicing parts of both nodule morphogenesis and the *Rhizobium* infection process. Therefore signal molecules produced by *Rhizobium*, under the direction of a small set of *nod* genes, may similarly establish the morphogenesis of certain cell types and parts of the infection process by interfering with the plant hormone balance.

B. ENOD12 gene expression in root hairs as an assay for *Rhizobium* signal compounds.

Genes which are expressed in root hairs upon inoculation with *Rhizobium* seem most suitable to study bacterial signal compounds. First, these genes are expressed in a pre-existing cell type, and not in cells modified by *Rhizobium*. Hence the chain of events leading from a bacterial signal to expression of these plant genes might be less complicated. Second, root

hairs directly can be treated with putative bacterial signal compounds, and then isolated and analyzed for gene expression.

By *in vitro* translation of root hair RNA, two root hair specific pea transcripts, RH-42 and RH-44, have been shown to accumulate upon inoculation with *R.leguminosarum* (Gloudemans *et al* 1989). The accumulation of both transcripts was shown to be dependent on the bacterial *nodC* gene. Furthermore, the accumulation of RH-44 mRNA could be induced with a cell-free preparation of deformation factor, obtained from *R.leguminosarum* cultured in the presence of the *nod* gene inducer apigenin. Therefore, the appearance of RH-44 transcripts might be used as a molecular marker for the activity of the bacterial compound causing root hair deformation. Unfortunately the *in vitro* translation of root hair RNA followed by two dimensional gelelectrophoresis to detect this transcript, which has not yet been cloned, is too elaborate to use in a quantitative routine assay. Thus the use of RH-44 as marker has to wait untill more simple detection methods are available for this mRNA.

Another gene of which the expression could function as a possible marker for the action of *Rhizobium* signal compounds is the ENOD12 early nodulin gene. Also this gene is already expressed in root hairs 48 hrs after inoculation with *R.leguminosarum*, and we demonstrated that the expression of the bacterial common and host-specific *nod* genes is essential for eliciting ENOD12 gene expression. The sequence data available from the pPsENOD12 were used in designing a specific assay for the presence of ENOD12 transcripts, using reverse transcription and polymerase chain reactions. This semi-quantitative detection method is now used to test the ability of different *R.leguminosarum* mutant strains and of soluble compounds excreted by the bacteria to induce ENOD12 gene expression. Using a variety of *R.leguminosarum* strains containing only small regions of the sym-plasmid or deletions spanning different *nod* genes (Spaink 1989) it was shown that both the common *nod* genes *nodABC* and the host specific *nodE* gene are essential for ENOD12 gene expression. This is consistent with the observation that both *R.leguminosarum nodABC* and *nodE* are necessary for infection thread formation (Spaink 1989), the process to which ENOD12 gene expression is correlated. Furthermore it was shown that ENOD12 gene expression is elicited by a soluble compound, excreted by *Rhizobium* upon induction of the *nod* genes with pea root exudate.

The common *nod* genes of *R.leguminosarum* are known to be sufficient for excretion of the soluble factor that establishes root hair deformation (Zaat *et al* 1987). We were able to partially purify this deformation factor using root hair deformation on *Vicia sativa* as a bio-assay. We are currently investigating whether the factor that elicits ENOD12 gene expression

can be purified with a similar purification scheme, indicating that the compound has similar molecular properties. In this way we hope to establish whether the *nodE* gene product modifies a deformation factor made by a *nodABC* product in such a way that it is able to elicit ENOD12 gene expression. Alternatively, root hair deformation, established by the *nodABC* dependent deformation factor, might be a prerequisite for the abilitiy of a structurally unrelated *nodE* dependent factor to elicit ENOD12 gene expression. In the *R.meliloti*-alfalfa symbiosis not only the host specific nodulation genes *nod EF* and *nodG* (Horvath *et al* 1986) appear to be essential for infection thread formation, but also exopolysaccharide genes (Finan *et al* 1985). We also intend to investigate the role of exopolysaccharides in inducing expression of the infection related ENOD12 gene, and the possible link to *nod* gene products.

Summarizing the data on ENOD12 gene expression, soluble factors dependent on both *nod ABC* and *nodE* are required for induction of ENOD12 gene expression in root hairs. As stated in the previous section of this paragraph, ENOD12 transcript also accumulates in root hairs upon treatment with anti-auxins. This leads us to the hypothesis that bacterial compounds, made either by the *nodE* product alone, or by the *nodABC* and *nodE* products together, alter the hormone balance in roots to allow bacterial infection and the development of a nodule structure.

CONCLUDING REMARKS

During the development of root nodules bacterial and plant signals play an important role in establishing an effective symbiosis. We have obtained a set of cDNA clones that can serve as probes for the expression of genes that mark different stages of root nodule development, demonstrating that progressive development is accompanied by differential plant gene expression. The induction of the expression of these specific genes might be due to the action of bacterial and/or plant signals effective during nodule development. We have demonstrated the use of ENOD2 and ENOD12 in showing that anti-auxins mimic several aspects of root nodule formation induced by *Rhizobium*. Using ENOD12, we have designed an assay for bacterial compounds produced by the bacterial *nodABC* and *nodE* genes, which are necessary to induce expression of the ENOD12 gene. The purification and characterization of these compounds are under way.

The use of the ENOD5, ENOD3, and ENOD14 genes to identify signals involved in later steps of root nodule development will require a search for *R.leguminosarum* mutants unable to elicit expression of these genes, and characterization of the nature of the mutations. Possible candidate mutant strains for such a study are bacterial release (*bar*$^-$) (De Maagd *et al* 1989) and bacteroid development (*bad*$^-$) mutants.

REFERENCES

Allen, O.N., and Allen, E.K. (1940). Proc. Acad. Sci. Bernice P. Bishop Museum Spec. Publ. 35, 15-16

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. (1989). Rearrangement of cytoplasm and endoplasmic microtubules in cortical cells of *Pisum sativum* L. roots prior to infection by *Rhizobium leguminosarum* biovar viceae. Submitted

Bhuvaneswari, T.V., and Solheim, B. (1985). Root hari deformation in the white clover/*Rhizobium trifolii* symbiosis. Physiol. Plant. 63, 25-34

De Maagd, R.A., Rao, A.S., Mulders, L.H.M, Govers-de Roo, L., Van Loosdrecht, M.C.M., Wijffelman, C.A., Lugtenberg, B.J.J. (1989). Isolation and characterization of mutants of *R.leguminosarum* bv. Viciea strain 248 with altered lipopolysaccharides: possible role of surface charge or hydrophobicity in bacterial release from the infection thread. J. Bacteriol. 171, 1143-1150

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

Dudley, M.E., Jacobs, T.W., and Long. S.R. (1987). Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti*. Planta 171, 289-301

Finan, T.M., Hirsch, A.M., Leigh, J.A., Hohansen, E., Kuldau, G.A., Deegan, S., Walker, G.C., and Singer, E.R. (1985). Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. 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). 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), 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., Nap, J.P., Van Kammen, A., and Bisseling, T. (1987a). Nodulins in the developing root nodule. Plant Physiol. Biochem. 25, 309-322

Govers, F., Nap, J.P., Moerman, M., Franssen, H.J., Van Kammen, A., and Bisseling, T. (1987b). cDNA cloning and developmental expression of pea nodulin genes. Plant Mol. Biol. 8, 425-435

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA 86, 1244-1248

Horvath, B., Kondorosi, E., John, M., Schmidt, J., Torok, J., Gyorgypal, 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

Lamb, C.J., Lawton, M.A., Dron, M, and Dixon, R.A. (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56, 215-224

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

Moerman, M., Nap, J.P., Govers, F., Schilperoort, R., Van Kammen, A., and Bisseling, T. (1987). *Rhizobium nod* genes are involved in the induction of two early nodulin genes in *Vicia sativa* root nodules. Plant Mol. Biol. 9, 171-179.

Mulligan, J.T., and Long, S.R. (1985). Induction of *Rhizobium meliloti* nod C by plant exudate requires nod D. Proc. Natl. Acad. Sci. USA 82, 6609-6613

Nap, J.P., and Bisseling, T. (1989). Nodulin function and nodulin gene regulation in root nodule development. in: The Molecular Biology of Symbiotic Nitrogen Fixation, Gresshoff, P.M., Ed., CRC press, Florida, in press

Newcomb, W. (1981). Nodule morphogenesis and differentiation. in: Biology of of the Rhizobiaceae, Giles, K.L., and Atherly, A.G., Eds., Int. Rev. Cytol. Suppl 13, Academic Press, New York, 247-297

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

Spaink, H. (1989). The *Rhizobium*-plant symbiosis: bacterial gene regulation and host specificity. Thesis, Leiden.

Van Holst, G-J., Klis, F.M., De Wildt, P.J.M., Hazenberg, C.A.M., Buijs, J., and Stegwee, D. (1981). Arabinogalactan protein from a crude cell organelle fraction of *Phaseolus vulgaris* L. Plant Physiol. 68, 910-913

Vincent, J.M. (1980). Factors controlling the legume-*Rhizobium* symbiosis. in: Nitrogen Fixation II, Newton, W.E., and Orme-Johnson, W.H.,Eds., University Park Press, Baltimore, 103-109

Witty, J.F., Minchin, F.R., Skot, L., and Sheehy, J.E. (1986). Nitrogen fixation and oxygen in legume root nodules. Oxford surveys of plant and cellular biology 3, 275-315

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 7

Concluding remarks

Early nodulin gene expression during root nodule development

In this thesis it is demonstrated that pea early nodulin genes are expressed at different stages during root nodule development, and in different cell types. Cells which prepare for, or are involved in, infection thread formation, and cells which start to divide in the inner cortex to form the nodule primordium express the ENOD12 gene (chapter 4). The cells of the nodule parenchyma express the ENOD2 gene as soon as this cell layer is formed by the activity of the nodule meristem or primordium (Chapter 3). ENOD5 gene expression is switched on in cortex cells containing infection thread tips and reaches its highest level in elongating infected cells containing proliferating bacteria; ENOD3 and ENOD14 gene expression starts in infected cells when ENOD5 transcripts are already present in those cells, but before late nodulin transcripts are detectable. This illustrates that ENOD12, ENOD5, ENOD3 and ENOD14, and the late nodulin transcripts, mark succesive developmental stages of the infected cell type in the central tissue of the mature nodule (chapter 5). Nodule formation can now be described in molecular terms, which provides a substantial refinement of cytological descriptions.

Early nodulin function

Remarkably both the ENOD2 early nodulin, marking the nodule parenchyma, and the ENOD12 early nodulin, present in cells preparing for the infection process, are (hydroxy)-proline-rich proteins, and therefore most likely cell wall proteins (chapters 3 and 4). The occurrence of several specific cell wall proteins in nodule tissue, with different functions points to an important role of the plant cell wall in tissue differentiation. Developmental regulation of genes encoding (hydroxy-)proline-rich cell wall proteins has recently also been demonstrated in soybean (Hong *et al*, 1989), in agreement with the suggestion that (hydroxy)-proline-rich proteins can play in general a role in differentiation. Precise functions of the early nodulins are unknown, although the ENOD2 early nodulin might give the cell wall properties that enable it to function as an oxygen barrier. For further experiments it will be important to localize both ENOD2 and ENOD12 proteins in the cells and to prove whether they are indeed cell wall proteins and if so, in which parts of the wall they reside. The structure of the pentapeptide repeat units in ENOD2 and ENOD12, consisting of blocks of three amino acids flanked by two prolines, indicates that the blocks of three amino acids have a role in determining the specific function of the proteins. An analysis of the capacity of these amino acids to bind other cell wall components and determination of the conformation of the ENOD2

and ENOD12 early nodulins are essential to provide insights in the relationship between structure and function of these proteins.

The function of the ENOD5 protein is unknown, but the primary structure derived from the cDNA sequence allows some speculation as to the nature of the protein. The amino acid sequence of the proline-rich region in the ENOD5 polypeptide chain has some analogy to arabinogalactans, as stated in chapter 5. On the other hand hydrophobic regions in the amino acid sequence point to the possibility of ENOD5 being a membrane protein. It seems worthwhile to pursue a precise cellular localization of the ENOD5 protein to gain ideas about the possible function. Further insight about the role of ENOD5 in the root nodule might be obtained from transgenic plants expressing antisense ENOD5 RNA, if at least the blocking of the expression of ENOD5 results in a detectable phenotypic change.

The most striking feature of the ENOD3 and ENOD14 early nodulins are the cysteine clusters which indicate that these early nodulins might be capable of binding a metal ion. There is not sufficient amino acid sequence homology in the sequence between and around the cysteine clusters to allow ENOD3 and ENOD14 to be put in a particular class of proteins with cysteine clusters like for example the zinc-finger proteins, which are in several cases shown to be DNA binding proteins. We have hypothesized in chapter 5 that ENOD3 and ENOD14 might function in the transport of metal ions towards the bacteroids. If this is the case the proteins are expected to reside in the peribacteroid space and, again, localization of the protein in the cell using specific antibodies and immunogold labeling might provide a stronger basis for this hypothesis. After cleavage of the signal peptide the small ENOD3 and ENOD14 proteins are hydrophilic and presumably easily soluble and therefore their purification, allowing biochemical assays on metal binding properties, seems quite possible.

Early nodulins as tools to study development

Apart from the data obtained on the nature of the early nodulin proteins and their localizations the cloned early nodulin cDNAs provide several entries for the analysis of plant developmental processes. First, some genes that are expressed in root nodule formation upon *Rhizobium* infection, were found to be expressed in other plant tissues as well. If the mechanisms of regulation of these genes are analyzed and compared with regulatory mechanisms causing expression of the same genes in tissues in other parts of the plant, some insight in the regulation of the expression of these genes during differentiation of other tissues may be gained. Second, *Rhizobium* signal compounds invoking specific plant development can now

be isolated and examined for their function by assaying their capacity to induce early nodulin gene expression. Both possibilities are briefly elaborated below.

1). In our opinion the observation that ENOD12 gene expression is not restricted to nodules but also found in flower and stem tissues, is very interesting. Preliminary data of F. Govers *et al.* (1989, unpublished results) suggest that the ENOD2 is expressed in other parts of the plants as well. Hence some genes that are activated by *Rhizobium* at early stages of root nodule development are strictly speaking not nodulin genes. In bean it has been demonstrated that the gene of the late nodulin glutamine synthetase (GS) is expressed also not only in nodules but, at low levels, in stem tissue as well (Bennett *et al.*, 1989). Therefore throughout the development and functioning of root nodules, expression of plant genes is induced some of which are true nodulin genes according to the defenition, while others are not (Van Kammen, 1984). Whereas the function of GS in nitrogen metabolism is clear and appears to be the same in nodule and non-nodule tissue it is an intriguing, as yet unanswered question what the function of ENOD12 protein might be in stem and flower tissues and whether the function in these tissues is the same as in the root nodule. The ENOD12 gene is a typical examples of a gene of which the analysis of the induction of expression by *Rhizobium* in root nodules can be of help to gain better understanding of the specific expression of this gene in flower and stem tissue. As discussed in chapter 4 at least the bacterial compounds involved in ENOD12 gene expression can be identified using ENOD12 gene expression as an assay and in principle then the putative receptors for these compounds can be isolated and characterized. In addition the *cis* and *trans* regulatory factors essential for expression of ENOD12 can be studied. In this way the characterization of the compounds involved in the specific gene expression in root nodules may be of use to get some idea of the possible compounds involved in differentiation in stem and flower.

2). Evidence that probably a limited number of compounds is required to induce and specify a nodule meristem comes from two kinds of observations: first, if the small cluster of *nod* genes of *Rhizobium* is transferred to a avirulent *Agrobacterium tumefaciens* strain, that strain acquires the ability to induce formation of root nodules (e.g. Hirsch *et al.* 1984). Second, an auxin transport inhibitor can elicit formation of nodules cytologically and molecularly resembling *Rhizobium*-induced root nodules (Hirsch *et al.* 1989).

As discussed in chapter 4 it is feasible to identify the bacterial factors needed for ENOD12 gene expression, due to the possibility of rapid detection of ENOD12 transcripts in

root hairs upon addition of *Rhizobium* culture supernatants. Considering the different accumulation patterns of the mRNA of other early nodulins related to different steps in root nodule development, transcription of other early nodulin genes is probably induced by different bacterial factors (chapter 5). The expression of these early nodulin genes (ENOD5, 3, 14, 2) may be used as reporters to identify bacterial signal molecules active in inducing the corresponding steps in nodule development. As auxin transport inhibitors can form nodules, it seems plausible that the compounds produced by *Rhizobium* trigger root nodule formation by interfering with the plant hormone balance, which might be a mechanism of wide importance in plant development, as was discussed in chapter 2. Characterization of the compounds of which the production appears to be directed by *nod* genes, may therefore help to identify the type of endogenous plant compounds involved in meristem formation and specification in general.

References

Bennett, M.J., Lightfoot, D.A., and Cullimore, J.V. (1989). Plant Mol. Biol. 12, 553-565.

Hirsch, A.M., Wilson, K.J., Jones, J.D.G., Banof, M., Walker, V.V., and Ausubel, F.M. (1984). J. Bacteriol. 158, 1133-1143.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T. (1989). Proc. Natl. Acad. Sci. USA 86, 1244-1248.

Hong, J.C., Nagao, R.T., and Key, J.L. (1989). The Plant Cell 1, 937-943.

CHAPTER 8

Samenvatting

Bacteriën van het geslacht *Rhizobium* kunnen wortels van vlinderbloemige planten infecteren en induceren de vorming van wortelknollen. Dit zijn organen met een specifieke structuur waarin de bacterie in staat is om atmosferische stikstof (N_2) om te zetten in het voor de plant assimileerbare ammonia. Dit proces wordt symbiontische stikstoffixatie genoemd.

De vorming van wortelknollen kan worden verdeeld in vier stadia: 1) preïnfectie, 2) infectie en vorming van het knolweefsel; 3) knolfunctie, 4) knolveroudering. Het eerste stadium, de preïnfectie, begint met aanhechting van bacteriën aan wortelharen, en inductie van bacteriële nodulatie (*nod*) genen door flavonoiden, die door het wortelstelsel van de plant worden uitgescheiden. Bacteriële producten die onder invloed van de *nod* genen worden uitgescheiden doen wortelharen deformeren. Uiteindelijk resulteert dit in gekrulde wortelharen. In het tweede ontwikkelingsstadium induceren bacteriën die ingesloten zijn in gekrulde wortelharen de vorming van een draadvormig structuur, de infectiedraad. Door deze draad infecteren de bacterien de wortel. Tegelijkertijd worden celdelingen geïnduceerd in de cortex van de wortel. Zowel bij het infectieproces als bij de inductie van corticale celdelingen zijn producten van de bacteriele *nod* genen betrokken. De corticale celdelingen leiden tot de vorming van het knolprimordium. Uit de cellen van het knolprimordium ontstaat uiteindelijk de wortelknol. In het centrale deel van de wortelknol komen bacteriën vrij uit de infectiedraad. Ongeveer de helft van de cellen in het centrale deel van de wortelknol zijn gevuld met bacterien die zich ontwikkelen tot bacteroïden. In het derde stadium vindt stikstoffixatie plaats door de bacteroïden. De plant levert op zijn beurt voedingsstoffen aan de bacteroïd en er is dus sprake van een symbiose. Deze symbiose duurt een aantal weken totdat in de vierde fase, de verouderingsfase, de wortelknol afsterft.

In dit proefschrift staat de ontwikkeling van de wortelknol als gespecialiseerd orgaan voor symbiose centraal. In hoofdstuk 2 worden daarom algemene aspecten van de ontwikkelingsbiologie van planten kort samengevat. Een overzicht van de groeiende kennis omtrent moleculaire mechanismen die ten grondslag liggen aan de ontwikkeling van dieren wordt als referentiekader gebruikt om de fragmentarische kennis omtrent moleculaire mechanismen die betrokken zijn bij ontwikkeling van planten te rangschikken. De bruikbaarheid van wortelknolvorming als modelsysteem voor het bestuderen van specifieke vragen omtrent dergelijke moleculaire mechanismen wordt bediscussieerd.

In het experimentele werk beschreven in hoofdstukken 3, 4 en 5 staan genen centraal die specifiek in wortelknollen van de erwt (*Pisum sativum* L.) tot expressie komen tijdens het

tweede stadium van wortelknolvorming. De door deze genen gecodeerde eiwitten, nodulines genaamd, zullen dus betrokken zijn bij de vorming van de wortelknol of bij het infectieproces. Omdat de betreffende genen tot expressie komen ruim voordat stikstoffixatie detecteerbaar is worden ze 'vroege' noduline-genen genoemd. Vijf vroege noduline-genen, ENOD2, ENOD12, ENOD5, ENOD3, en ENOD14, zijn bestudeerd door het analyseren van cDNA klonen gemaakt tegen de corresponderende boodschapper RNAs die tijdens het tweede stadium van wortelknolvorming in wortelknollen detecteerbaar zijn. De accumulatie van deze boodschapper RNAs tijdens wortelknolvorming en de eventuele aanwezigheid van de transcripten in niet-knolweefsel is bestudeerd met RNA blots. De primaire structuur van de gecodeeerde eiwitten is vastgesteld via DNA sequentieanalyse aan de cDNA klonen en in een aantal gevallen via directe sequentiebepalingen in totaal knol RNA met oligonucleotiden als primers specifiek voor de verschillende transcripten. De transcripten en hun corresponderende genen zijn verder gekarakteriseerd met behulp van 'hybrid released' translaties en restrictie-analyses van hybridiserende genomische DNA fragmenten. De verschillende transcripten zijn gelokaliseerd in de zich ontwikkelende wortelknol met behulp van *in situ* hybridisatie.

In hoofdstuk 3 wordt beschreven dat het erwte- maar ook het soya-ENOD2 boodschapper RNA specifiek gelokaliseerd is in de 'inner cortex' van de wortelknol. Hiermee wordt aangetoond dat de op grond van de oude benaming gesuggereerde overeenkomst tussen 'inner cortex' en cortexcellen uit de wortel niet aanwezig is op moleculair niveau en de nieuwe benaming knolparenchym wordt geintroduceerd. De van de cDNA sequentie afgeleide aminozuurvolgorde leert dat het ENOD2 eiwit (hydroxy-)proline rijk is en bestaat uit repeterende pentapeptide eenheden met twee prolines. Uit de sequentiehomologie met hydroxyprolinerijke celwandeiwitten en uit de lokalisatiegegevens wordt afgeleid dat ENOD2 een celwandeiwit is dat mogelijk betrokken is bij de vorming van de zuurstofbarrière die gelegen is in het knolparenchym.

In hoofdstuk 4 wordt met behulp van uitgebreide lokalisatie-studies van het ENOD12 boodschapper RNA aangetoond dat het ENOD12 genproduct betrokken is bij het infectieproces en bij het ontstaan van het knolprimordium. Op grond van de uit de cDNA sequentie afgeleide aminozuurvolgorde blijkt ook hier sprake te zijn van een (hydroxy)proline rijk eiwit met repeterende pentapeptide eenheden. Op grond hiervan wordt gepostuleerd dat het ENOD12 vroege noduline functioneel is in de celwanden die worden aangelegd tijdens het infectieproces en de vorming van het knolprimordium. Met behulp van RNA blots is

aangetoond dat ENOD12 genen niet worden aangeschakeld bij infectie van erwtewortels met de pathogene schimmel *Fusarium oxysporum*. ENOD12 genexpressie wordt geïnduceerd in wortelharen door excretieproducten van *Rhizobium*. De betrokkenheid van zowel gemeenschappelijke ('common') als gastheerspecifieke bacteriele *nod* genen bij inductie van ENOD12 genexpressie wordt aangetoond met een gevoelige detectiemethode gebaseerd op de polymerase kettingreactie (PCR). Verder blijkt ENOD12 boodschapper RNA voor te komen in stengel- en bloemweefsel. Met gebruikmaking van RNase mapping en primer extensie experimenten wordt aangetoond dat de transcripten in stengel identiek zijn aan die in wortelknollen, hetgeen betekent dat *Rhizobium* expressie van genen kan induceren die normaal bij andere processen in de plant zijn betrokken.

In hoofstuk 5 wordt de karakterisering beschreven van de ENOD5, ENOD3 en ENOD14 boodschapper RNAs. Deze blijken gedurende de ontwikkeling van wortelknollen op een andere wijze te accumuleren dan de ENOD2 en ENOD12 boodschapper RNAs. De afgeleide aminozuurvolgorde van het ENOD5 genprodukt vertoont hydrofobe gebieden die het mogelijk tot een membraaneiwit bestempelen, maar een bepaald deel van het eiwit vertoont ook overeenkomsten in aminozuursamenstelling met de oplosbare arabinogalactan glycoproteinen. Mogelijk is dus ook ENOD5 een arabinogalactan (precursor-)eiwit. De ENOD3 en ENOD14 vroege nodulines zijn kleine eiwitten met een cysteïnecluster en mogelijk een signaalpeptide. Om die redenen en vanwege de waarneming dat ENOD3 en ENOD14 transcripten in geïnfecteerde cellen voorkomen wordt gepostuleerd dat ze betrokken zijn bij het transport van metaalionen naar de bacteroïd. De lokalisatie van ENOD12, ENOD5, ENOD3, ENOD14 en leghemoglobine transcripten in de wortelknol wordt uitgebreid onderling vergeleken met behulp van *in situ* hybridisatie. Deze transcripten blijken voor te komen in verschillende, maar gedeeltelijk overlappende zones van het centrale knolweefsel. Wortelknollen van de erwt bezitten een persistent meristeem, hetgeen impliceert dat verschillende zones in het centrale knolweefsel verschillende ontwikkelingsfasen representeren van geïnfecteerde en ongeïnfecteerde cellen. De jongste ontwikkelingsstadia grenzen direct aan het apicale meristeem en de oudste stadia bevinden zich het dichtst bij het aanhechtingspunt van de wortelknol aan de wortel. ENOD12 boodschapper RNA, dat voorkomt in zowel geïnfecteerde als ongeïnfecteerde cellen, en de andere vier transcripten die alleen in geïnfecteerde cellen van het centrale knolweefsel detecteerbaar zijn, markeren dus verschillende, overlappende fasen in de ontwikkeling van het geïnfecteerde celtype.

In hoofdstuk 6 worden de in hoofstuk 3-5 vermelde resultaten samengevat en aangevuld met andere gegevens over vroege nodulines. De bruikbaarheid van vroege noduline cDNA klonen als probes voor het identificeren van bacteriele genen en factoren die betrokken zijn bij wortelknolvorming wordt bediscussieerd.

In hoofstuk 7 wordt tenslotte aangegeven hoe het onderzoek aan vroege nodulines beschreven in dit proefschrift een aanzet kan vormen om zowel specifieke aspecten van wortelknolvorming als ook de relatie van dit proces met andere ontwikkelingsprocessen in de plant te bestuderen.

NAWOORD

Moleculair biologisch onderzoek gedijt het best in een plezierig samenwerkingsverband. Daarom is het vermelden in dit proefschrift van degenen die daartoe hebben bijgedragen geen formele aangelegenheid maar een zeer wezenlijk onderdeel van het totaal.

Ab van Kammen, mijn promotor, de menselijke betrokkenheid in de gewone omgang én de zakelijke afstand waarmee je teksten van passende kritiek voorzag vormden voor mij twee van de meest plezierige facetten in onze samenwerking.

Ton Bisseling, "chef", jouw wetenschappelijke gedrevenheid, je sterke realiteitszin ten opzichte van de waarde van proeven, én je gevoel voor vormgeving, zowel in als buiten het werk, zijn voor mij een voortdurende bron van inspiratie geweest.

Clemens Van De Wiel, jouw werk spreekt voor zich in dit proefschrift. Voor de zonder uitzondering prettige samenwerking en voor de volharding waarmee je de mooiste prentjes hebt verzameld ben ik je dankbaar.

De andere (ex-)"knollen" mensen, Ton Gloudemans, Francine Govers, Marja Moerman, Jan-Peter Nap: bedankt voor al jullie hulp en bijdragen tot de sfeer. Speciaal moet hier worden genoemd Henk Franssen: jij was vanaf het eerste begin niet alleen een goede collega maar een speciale vriend.

Fred van Engelen, Fried Zwartkruis, Marie-José van Lierop, Rafel Israels, Herman van Eck, Anne-Marie Wolters en Esther van der Knaap: jullie als student geleverde bijdrage aan dit proefschrift en/of aan experimenten die buiten het bestek van dit proefschrift vallen, is een erg belangrijke. Zonder die extra hersens en handen was het heel wat minder levendig geweest op lab 1.

Sacco de Vries, ik ben je erg dankbaar dat je voor Apples en niet voor Peren hebt gekozen.

Peter van Druten, Gré Heitkönig, Piet de Kam, Piet Madern, en Marie-José van Neerven: zonder jullie zou onze vakgroep, en dus ook ik, niet kunnen functioneren. Bedankt voor jullie bereidheid om ten allen tijde te fotograferen/afdrukken, typen, planten in te zetten, tekenen, en teksten op te maken.

Alle permanente en niet-permanente niet nader genoemde molbiollers, bedankt voor jullie bijdrage tot de unieke sfeer van onze vakgroep, voor jullie adviezen, buffers, gelplaten, enz. enz. Waarbij natuurlijk Peter Roelvink en Jan Verver met name moeten worden genoemd!

Bob Bakhuizen, Clara Díaz en Herman Spaink: bedankt voor jullie inbreng vanuit de "Leidsche hoek".

Michael "Crocodile" Djordjevic, Jeremy "Go" Weinman, Ann "PA" Hirsch, "TV" Bhuvana, "Takis" Katinakis and Andrei "PCR" Zalensky: it was good working and living together with you; thanks for all friendship and efforts!

Tenslotte Lobke, bedankt dat je me al die jaren trouw hebt uitgelaten, en natuurlijk Olga: dat je me daarna altijd hebt binnengelaten en het mij door je vriendschap, relativerend vermogen, lunchtrommeltjes en uiteraard ook kookkunst heel gemakkelijk hebt gemaakt om te promoveren.

CURRICULUM VITAE

Ben (Bernardus Johannes Godefridus) Scheres werd op 10 juli 1960 geboren te Echt. In 1978 behaalde hij het eindexamen Gymnasium β aan het Bisschoppelijk College St.-Jozef te Sittard. Aansluitend begon hij met de studierichting Planteziektenkunde aan de toenmalige Landbouwhogeschool. Het kandidaatsgetuigschrift werd behaald in 1982 (met lof). De ingenieursstudie omvatte de hoofdvakken Virologie (Prof. Dr. Ir. J.P. Van Der Want) en Moleculaire Biologie (Prof. Dr. A. Van Kammen), en de bijvakken Celbiologie (Prof. Dr. W.B. Van Muiswinkel) en Nematologie (Prof. Dr. Ir. A.F. Van Der Wal). Als stage voor het hoofdvak Virologie was hij in 1983 gedurende 6 maanden werkzaam in het Department of Plant Pathology van de University of California, Davis (Prof. Dr. R.J. Shepherd). In 1985 werd het ingenieursdiploma behaald (met lof). Van oktober 1985 tot mei 1989 werd het onderzoek beschreven in dit proefschrift uitgevoerd bij de vakgroep Moleculaire Biologie van de Landbouwuniversiteit, waarvan 3 jaar werden gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO). Vanaf 1 oktober 1989 is hij in opdracht van de vakgroep Moleculaire Biologie van de Landbouwuniversiteit werkzaam aan het Laboratorium voor Genetika te Gent.