ROOT NODULE ORGANOGENESIS:

Molecular Characterization of the Zonation of the Central Tissue

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Statements

1. The sudden transition of gene expression and cell morphology from one cell layer to another is a key step in the formation of a symbiotic nitrogen fixing root nodule.

This Thesis

2. Characterisation of the function of ENOD40 will be of great importance for understanding root nodule development as well as plant development in general.

This Thesis

3. The activation of phenylpropanoid biosynthesis pathway by *Rhizobium* is more related to plant development than to a plant defense response.

This Thesis

4. The role of flavonoids in plant development is still underestimated.

5. The observations of De Billy *et al.* do not prove that leghemoglobin gene transcription is triggered in a single cell layer of the interzone in the indeterminate nitrogen-fixing root nodule of alfalfa.

De Billy, F., *et al.*, 1991, Plant J. 1,27-35

6. The studies of Savouré *et al.* do not prove that the cell cycle is activated in a suspension culture of *Medicago sativa* by Nod factors.

Savouré, A., *et al.*, 1994. EMBO J. 13,1093-1102

7. In their suggestion that a bacterial protein binds to the leghemoglobin promoter, Welters *et al.* overlooked that this protein has to pass three membranes.

Welters, P., *et al.*, 1993. Plant Physiol. 102,1095-1107

8. China should adopt the concept of democracy, irrespective of its cultural and traditional background.

9. It is hard to walk to the correct direction in the dark.

10. The admiration in the western world for recent Chinese films does not arise so much from the cinematographic quality of the films as from frustration with the lost "good old world".

11. When a Chinese says "yes", it does not mean that he or she agrees with you; it only means that he or she has listened to you.

Statements from the thesis entitled "Root Nodule Organogenesis: Molecular Characterisation of the Zonation of the Central Tissue". Wei-Cai Yang, Wageningen, 24 March 1994.

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CONTENTS

Chapter 1

Scope

Legume plants form root nodules by interacting with the soil bacterium, *Rhizobium*. In these nodules bacteria are able to convert atmospheric nitrogen into ammonia which is used by the host plants as nitrogen source. Therefore symbiotic nitrogen fixation in root nodules is of great importance for agriculture.

Root nodule formation involves several developmental stages, namely are: induction of cell divisions in the root cortex, formation of nodule primordium and meristem, and finally differentiation of the meristem into nodule tissues. A mature nodule is composed of a central tissue where bacteria are hosted and several peripheral tissues. The induction of nodule specific genes of the host plants as well as the bacteria in a temporally and spatially controlled manner regulates the development of root nodules. The aim of the research described in this thesis was to investigate mechanisms that control nodule development. For this purpose genes of interest have been isolated and their expression was studied by means of the *in situ* hybridization technique.

In chapter 2 a general introduction summarizing what we know about nodule development at present is given with an emphasis on gene expression and exchange of signals between the host plant and the rhizobia.

Early studies of Allen *et al.* (1953) and more recently Hirsch *et al.* (1989) on polar auxin transport inhibitors (ATIs) provided evidences that exogenously applied ATIs cause the formation of nodule-like structures on several legume plants. These studies showed that auxin plays a major role in nodule development. Since certain flavonoids, e.g. quercetin, are endogenous ATIs, we studied the expression of chalcone synthase (CHS) genes, which encode a key enzyme in flavonoid biosynthesis, *in situ* during nodule development. The results are presented in chapter 3.

To study gene expression during nodule development, two nodulin genes, ENOD40 and NOD6, were isolated and their expression during nodule development was studied by *in situ* hybridization. In chapter 4, a cDNA clone of the early nodulin gene ENOD40 was characterized. The pattern of expression of ENOD40 during soybean and pea nodule development suggested that it may play an important role in nodule formation. In chapter 5, the isolation of the late nodulin gene NOD6 was described and its expression pattern was compared with that of other nodulin genes.

In chapters 6 and 7, the expression patterns in pea nodules of several bacterial genes were studied. These genes are *nif*A and *nif*H, and *rop*A The expression pattern of *nif*A and *nif*H in nodules is described in chapter 6. The expression of the *rop*A gene, which encodes a bacterial outer membrane protein, is described in chapter 7. The expression pattern of *rop*A in nodules is compared with that of *nif*H. The *rop*A protein was localized at a ultrastructural level by immunocytochemistry.

In chapter 8, the results reported in this thesis are discussed with respect to the mechanisms that controls the induction of cortical cell divisions, meristem formation and formation of zones in the nodule central tissue.

Chapter 2

General introduction

INTRODUCTION

Soil bacteria of the genera *Rhizobium, Bradyrhizobium,* and *Azorhizobium* (here collectively called *Rhizobium*) have the ability to induce the formation of nodules on the roots of leguminous plants. In these nodules the bacteria are able to convert atmospheric nitrogen into ammonia, a process named symbiotic nitrogen fixation. This plant-microbe interaction has a symbiotic nature since the plant provides photosynthetic compounds to the bacteria and in return the bacteria supply fixed nitrogen for plant growth.

The formation of root nodules involves several consecutive steps. In short, bacteria multiply in the rhizosphere, chemotactically move to the root surface and colonize the root. Root hairs deform and curl, and subsequently the bacteria invade these hairs through tubular structures, called infection threads. These infection threads grow towards the base of the root hairs and then penetrate root cortical cells. Concommitantly with the infection process, cell divisions are induced in the root cortex, by which nodule primordia are formed. The infection threads grow towards these primordia and after penetrating the plant cells, bacteria are released from the threads by endocytosis. Then these nodule primordia differentiate into nitrogen fixing nodules (for reviews see Libbenga and Bogers, 1974; Vincent, 1980; Newcomb, 1981; Brewin, 1991).

A typical characteristic of the legume-*Rhizobium* symbiosis is its host specific nature. For example, *Rhizobium leguminosarum* bv. *viciae* can nodulate pea and vetch, but is unable to nodulate alfalfa. While *R. meliloti* can nodulate alfalfa but not pea and vetch (Table 1). This host specificity is determined by the two symbionts.

In this review we will discuss the successive developmental stages of nodule formation. Of each stage, we will discuss the cytological characteristics, the signal exchange between the two symbionts and the changes in gene expression with an emphasis on plant genes. We will start with a short description of the bacterial genes involved in nodule formation.

BACTERIAL GENES INVOLVED IN NODULE DEVELOPMENT

The genes in *Rhizobium* involved in infection and in nodule formation and functioning have been studied extensively (for reviews see Long, 1989; 1992; Martinez, *et al.*, 1990; Dénarié, *et al.*, 1992; Fischer and Long, 1992; Kondorosi 1992; Leigh and Coplin, 1992). In the following two groups of bacterial genes will be discussed in some detail; 1) the nodulation genes, *nod* and *nol* (the nodulation genes which represents *nod* genes after letter Z) and, 2) *Rhizobium* genes encoding surface polysaccharides. The latter group includes genes involved in the synthesis of extracellular polysaccharides (*exo*), lipopolysaccharides (*lps*) and ß-glucans (*ndv*). The rhizobial genes involved in nitrogen fixation (*nif* and *fix*) will not be discussed in this review.

Table 1. Rhizobium Host Specificity

Rhizobium	Host plant
Rhizobium leguminosarum	
biovar *viciae*	*Viciae, Pisum, Lathyrus, Lens*
biovar *phaseoli*	*Phaseolus*
biovar *trifolii*	*Trifolium*
Rhizobium fredii	*Glycine, Vigna*
Rhizobium loti	*Lotus, Anthyllis*
Rhizobium meliloti	*Melilotus, Medicago*
Rhizobium tropicii	*Phaseolus, Leucaena, Macroptilium*
Rhizobium sp. NGR234	Various tropical legumes, non-legume *Parasponia*
Bradyrhizobium japonicum	*Glycine, Vigna*
Azorhizobium caulinodans	*Sesbania rostrata*

The nodulation genes

The *nod* genes have been classified into three groups; the regulatory *nod*D genes, the common *nod* genes (*nod*ABCIJ) and the host-specific *nod* genes (also called *hsn*). The organization of the *nod* genes of *R. leguminosarum* bv. *viciae* and *R. meliloti* is presented in Figure 1.

The regulatory *nod*D genes are found in all *Rhizobium* species (Long, 1989; 1992). In general, the *nod*D genes are constitutively expressed, whereas the transcription of the other *nod* genes requires the NodD protein and specific host plant-secreted flavonoids or related phenolic compounds (for review see Peters and Verma, 1990). It has been postulated that NodD is a transcriptional activator since the NodD protein in the presence of flavonoids binds to a 50- to 60-bp long highly conserved nucleotide sequence, the so-called *nod* box, present in *nod* gene promoters (for review see Long, 1989; 1992; Dénarié, *et al.*, 1992).

*nod*D exists as a single gene in *R. leguminosarum* bv. *viciae* and bv. *trifolii*, but as a multi-gene family in other *Rhizobium* species such as *R. meliloti* and *R. leguminosarum* bv. *phaseoli* (for review see Dénarié, *et al.*, 1992; Fischer and Long, 1992; Kondorosi, 1992). The *nod*D genes are different activated by specific flavonoids. Therefore the

presence of several *nod*D genes in certain bacterial species implies the potentiality of interaction with a variety of host plants.

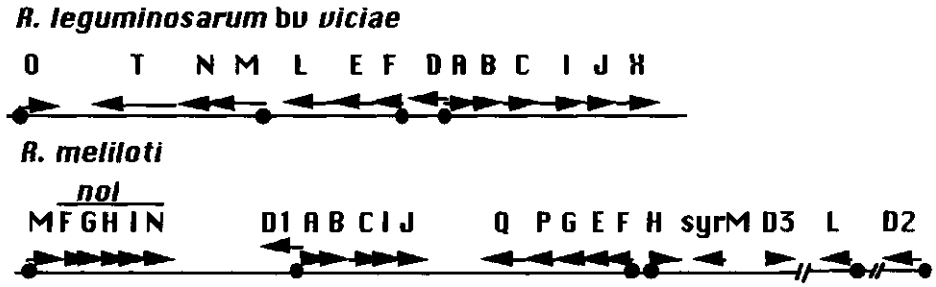


Figure 1. The *nod* and *nol* genes of *R. leguminosarum* bv. *viciae* and *R. meliloti*. Arrows indicate the position of the open reading frames. Solid circles represent the position of the *nod* boxes. The *nod* X gene is only present in *R. leguminosarum* bv. *viciae* strain TOM. For more information see Dénarié *et al* (1992), Kondorosi (1992) and Spaink (1992).

The common *nod*ABCIJ genes are conserved and functional interchangeable between *Rhizobium* species. Mutations in *nod*ABC completely abolish the ability of *Rhizobium* to induce root hair deformation, cortical cell division and infection thread formation (for review see Long, 1989; Hirsch, 1992), while mutations in *nod*IJ cause delayed or less effective nodulation (Dénarié, *et al.*, 1992; Long, 1992). Also mutaions in the host specific *nod* genes (*hsn*) result in delayed or less efficient nodulation, but moreover, mutations in the host-specific *nod* genes can change the host range of the mutated rhizobia (for reviews see Dénarié, *et al.*, 1992; Kondorosi, 1992). For example, *R. meliloti* having a mutation in *nod*H has lost the ability to induce root hair curling (Hac^-), infection thread formation (Inf^-) and nodulation (Nod^-) on the homologous host alfalfa, but has acquired the ability to form nodules on the heterologous host, common vetch (Table 2).

Nod factors

Upon induction of the *nod* genes, *Rhizobium* produces and secretes signal molecules, called Nod factors. All Nod factors have a similar basic structure, a sugar backbone of N-acetylglucosamine residues and a lipid moiety linked to the C2 positon of the non-reducing terminal sugar (Fig.2) (Lerouge, *et al.*, 1990; Spaink, *et al.*, 1991; Price, *et al.*, 1992; Sanjuan, *et al.*, 1992; Mergaert, *et al.*, 1993). Nod factors of different rhizobia can vary in the structure of the lipid moiety and the nature of the substitutions at the reducing and non-reducing terminal sugar residues (Fig. 2). The following rules apply to the nomenclature of Nod factors (Roche, *et al.*, 1991; Spaink, *et al.*, 1991): The bacterial

Gene	Species biovar	Mutant phenotype	Predicted function
nod A	common	Hac-Nod-	unknown
nod B	common	Hac-Nod-	Chitooligosaccharide deacetylase
nod C	common	Hac-Nod-	N-acetylglucosaminyltransferase
nod D	common	Nod-(when all copies are *mutated*)	transcriptional activator
nod E	Rl, Rt, Rm	Nod^{de}; change in host range	ß-ketoacyl synthase
nod F	Rl, Rt, Rm	Nod^{de}; affects infection thread formation	acyl carrier protein
nod G	Rm	Nod^{de}	dehydrogenase, ß-ketoacylreductase
nod H	Rm	Nod-; change in host range to vetch	sulphotransferase
nod I	Rl, Rt, Bj	Nod^{de} in Rl, Rm increase in Hac, Inf in Rm; no effect in Bj	ATP-binding protein
nod J	Rl, Rt, Bj	see nod I	unknown
nod L	Rl, Rt,Rm	Nod- or Nod^{de} in Rl, Rt	acetyltransferase
nod M	Rl, Rt,Rm	Nod^{de}	D-glucosamine synthase
nod O	Rl	Nod+ but reduced	Na+/K+ ion channel
nod PQ	Rm	Nod^{de}	ATP-sulphurylase
nol R	Rm		repressor of nodD
nod X	Rl (Tom)	extend host range to Afghanistan pea	acetyltransferase

Table 2. The predicted function of *nod* and *nol* gene products and their mutant phenotype. Bj=*Bradyrhizobium japonicum*; Nod^{de} =delayed nodulation; Rl=*R.leguminosarum* bv. *viciae*; Rm=*R.meliloti*; Rt=*R.leguminosarum* bv. *trifolil.* Data are from Dénarié *et al* (1992), Kondorosi (1992) and Spaink (1992). The *nod* X gene is only present in *R. leguminosarum* bv. *viciae* strain TOM and encoding for a acetyltransferase (see Firmin, *et al.*, 1993)

source is given with a two or three letter abbreviation, for example, NodRm is a Nod factor from *R. meliloti*, while NodRlv is produced by *R. leguminosarum* bv. *viciae*. The number of N-acetylglucosamine residues is indicated by a roman number and the substitutions are given between brackets starting from the non-reducing terminal sugar. For example, NodRm-IV(16:2, S) is a Nod factor produced by *R. meliloti* with four N-acetylglucosamine residues having a C16:2 acyl group at the non-reducing terminal sugar and a sulphate group at the reducing end.

The possible functions of *nod* genes in the synthesis of Nod factors are presented in Table 2 (for reviews see Dénarié, *et al.*, 1992; Fischer and Long, 1992; Spaink, 1992). NodC protein, based on sequence homology, most likely is a chitin synthase (Bulawa and Wasco, 1991), whereas NodB has chitooligosaccharide deacetylase activity, which only deacetylates the non-reducing N-acetylglucosamine residue (John, *et al.*, 1993). The function of NodA is not yet solved, but since *nod*ABC genes are sufficient to synthesize the core lipo-chitooligosaccharide (Spaink, *et al.*, 1991), it is likely that NodA is involved in the coupling of the acyl moiety to the non-reducing terminal sugar. NodI is homologous to a ATP-binding protein, whereas the function of NodJ is not yet known. *nod*M, encoding a glucosamine synthase, is not essential for the synthesis of Nod factors, but it allows a higher production of these compounds (Baev, *et al.*, 1991).

Modification of Nod factors is carried out by the products of the host-specific *nod* genes and may vary among different rhizobia. For example, NodE and F determine the structure of the acyl moiety; C16:2 in NodRm factors and C18:4 in NodRlv factors (Spaink, *et al.*, 1991; 1992). In *R. meliloti*, NodG most likely plays a role in the synthesis of the acyl moiety as well (see Dénarié, *et al.*, 1992). NodH and NodPQ determine the sulphation of NodRm factors. NodH is most likely a sulphotransferase (Roche, *et al.*, 1991), whereas NodP and NodQ form an ATP sulphurylase (Schwedock and Long, 1990). NodL has acetyltransferase activity *in vitro* and catalyses 6-O-acetylation of the non-reducing sugar residue (Spaink, *et al.*, 1991). NodX is a acetyltransferase which is involved in the 6-O-acetylation of the reducing end sugar (Firmin, *et al.*, 1993).

Bacterial genes encoding surface compounds

Rhizobium cell surface polysaccharides are also important in establishing the symbiosis. Genetic studies on these cell surface components, namely extracellular polysaccharides (EPS), lipopolysaccharides (LPS) and neutral ß-glucans, indicated that they are involved in the infection process and nodule development (for reviews see Bauer, 1981; Carlson, 1981; Leigh and Coplin, 1992; Gonzalez, *et al.*, 1993). In contrast with the diffusible Nod factors, EPS, LPS and ß-glucans remain attached to the cell surface.

The role of EPS has been extensively studied in *R. meliloti*. This bacterium produces two EPS's, EPS I and EPS II. EPS I, a succinoglycan composed of polymerized octasaccharide subunits, is required for the infection process. EPS II is structurally and chemically different from EPS I, but it can substitute for EPS I in nodulation of alfalfa plants, but not of other hosts of *R. meliloti* (Reuber, *et al.*, 1991). EPS mutants (*exo*$^-$) of *R. meliloti* are able to deform root hairs and to induce cortical cell divisions, but are unable to carry out infection. Consequently they form empty nodules which are devoid of bacteria (for review see Leigh and Coplin, 1992; Gonzalez, *et al.*, 1993).

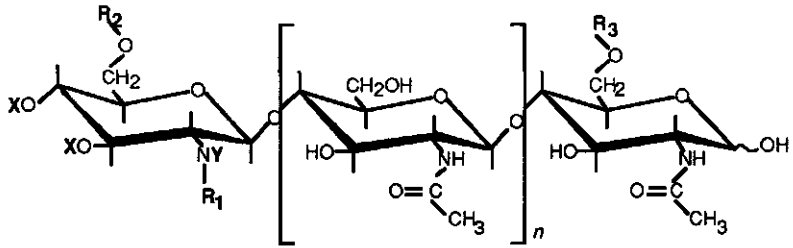
Rhizobium LPS is a component of the outer membrane and consists of a lipid moiety (lipid A) and a polysaccharide O-antigen. The latter is highly variable among *Rhizobium* species. *R. leguminosarum* bv. *phaseoli* mutants lacking the O-antigen are defective in infection thread development (Carlson, *et al.*, 1987), while similar *R. leguminosarum* bv. *viciae lps* mutants are defective in bacterial release from infection threads into the plant cell (De Maagd, *et al.*, 1988).

The involvment of ß(1-2) glucans in nodule formation was first demonstrated by *R. meliloti* mutants (*ndv*) that fail to produce ß(1-2) glucan. Such mutants can still induce nodules but infection does not occur (Dylan, *et al.*, 1986). *R. leguminosarum* bv. *viciae* mutants which do not secrete ß(1-2) glucan and fail to produce the O-antigen containing LPS form nodules that are unable to fix nitrogen. In these nodules only a few infected cells are present (Yang, *et al.*, 1992; Chapter 3).

PLANT GENES INVOLVED IN NODULATION

In the following paragraphs we will discuss the different steps of nodulation and we will especially pay attention to the plant genes involved in passing through this process. Genetic studies revealed the requirement of plant genes (*Sym* genes) in all stages of nodulation (for reviews see Weeden, *et al.*, 1990; Caetano-Anollés and Gresshoff, 1991; Gresshoff, 1993). Up to now none of the *Sym* genes has been cloned, but in some cases significant progress has been made in mapping RFLP markers in the vicinity of interesting *Sym* genes (Landau-Ellis, *et al.*, 1991; 1992; Lu, *et al.*, 1993). Therefore it is to be expected that in the coming years some of these *Sym* genes will be isolated by positional cloning which will provide more information on their role in nodule development. Since none of the *Sym* genes has been isolated so far, we shall not discuss these genes in this introduction.

During the successive steps of nodulation specific plant genes, the so-called nodulin genes (Van Kammen, 1984), are expressed (for reviews see Verma and Delauney, 1988; Nap and Bisseling, 1989; Sanchez, *et al.*, 1991; Franssen, *et al.*, 1992a). Many nodulin genes have been isolated from different legumes by differential screening



Species	R_1	R_2	R_3	X	Y	*n*	Reference
R. meliloti	-C16:2 (2,9) or -C16:3 (2,4,9)	-COCH3 or -H	-SO3H	-H	-H	1, 2 or 3	Lerouge *et al.* 1990 Schultze *et al.* 1992
R. leguminosarum bv *viciae*	-C18:4 (2,4,6,11) or -C18:1 (11)	-COCH3 or -H	-H or -COCH3*	-H	-H	2 or 3	Spaink *et al.* 1991 Firmin, *et al.*, 1993
B. japonicum	-C18:1 (9), or -C18:1 (9,Me), or -C16:1 (9) or -C16:0	-H or -COCH3	2-O-methylfucosyl group	-H	-H	3	Sanjuan *et al.* 1992
Rhizobium sp. strain NGR234	-C18:1 (11) or -C16:0	-H	Sulphated (3) or acetylated (4) 2-O-methylfucosyl group	-H or -CONH2	-CH3	3	Price *et al.* 1992
A. caulinodans strain ORS571	-C18:0 or -C18:1(11)	-CONH2 or -H	D-arabinosyl or -H	-H	-CH3	2 or 3	Mergaert, *et al.*, 1993
R. fredii	-C18:1 (11)	-H	Fucosyl or 2-O-methylfucosyl group	-H	-H	1, 2 or 3	Bec-Ferté, *et al.*, 1993

Figure 2. Basic structure of Nod factors. The number of N-acetylglucosamine residues can vary between 3 to 5. Substitutions at positions R1, R2 and R3 which vary between *Rhizobium* species are indicated. * only present in *R. leguminosarum* bv. *viciae* strain Tom.

of cDNA libraries. Nodulin genes expressed before nitrogen fixation starts are named early nodulin genes (ENOD), while nodulin genes expression of which starts at the onset of nitrogen fixation are called late nodulin genes (NOD) (Nap and Bisseling, 1990). In the nomenclature the plant species is indicated in italics in front of nodulin genes. Some of these nodulin genes will be discussed in the following paragraphs, which deal with the successive steps in nodule formation.

ROOT COLONIZATION

Colonization of legume roots by *Rhizobium* is achieved by the bacteria moving chemotactically to the roots and the potential of the root exudate to stimulate bacterial growth. The involvement of chemotaxis is indicated by the fact that non-motile mutants of *Rhizobium* are less competent in colonizing and infecting roots (Hunter and Fahring, 1980; Ames and Bergman, 1981; Caetano-Anollés *et al.*, 1988a; 1988b; for review see Bauer and Caetano-Anollés, 1990).

The signaling of chemotaxis is not well understood. Studies on the *R. meliloti*-alfalfa interaction have shown that certain *nod* gene inducers like luteolin as well as the *nod*DABC genes are involved (Caetano-Anollés, *et al.*, 1988a). Although this suggests that Nod factors might play a role it is hard to imagine which role that could be. In other systems, like the *R. leguminosarum* bv. *viciae*--pea interaction, *nod* genes are not essential for chemotaxis since bacteria lacking these genes show significantly though decreased chemotaxis (Armitage, *et al.*, 1988).

The growth of *Rhizobium* in the rhizosphere is stimulated by specific compounds secreted by legume roots. The pea compounds that stimulate growth of *R. leguminosarum* bv. *viciae* are homoserine and glutamic acid (Van Egeraat, 1975a; 1975b). The growth of other *Rhizobium* species is not stimulated by these compounds. On the other hand, it has been shown that by flavonoids secreted the plant stimulate growth of *R. meliloti* (Hartwig, *et al.*, 1991; for review see Phillips, *et al.*, 1993), but these compounds have not been tested for a possible effect on growth of other *Rhizobium* species.

So plant secreted flavonoids may have different functions in the *Rhizobium*--legume interaction. In many systems they are inducers of *nod* genes and in addition, they may have a role in stimulating bacterial growth and chemotaxis.

ROOT HAIR DEFORMATION AND CURLING

The first microscopically visible response of the host plant in the *Rhizobium*-plant interaction is deformation and curling of root hairs. Upon attachment of *Rhizobium*, root hairs change their typical cylindrical shape: the root hair tips deform and curl, and form

the so-called Shepherd's crooks (Fahraeus, 1957) by which bacteria become entrapped and infection can start (Dart, 1974).

Root hair deformation and curling are probably the most often described responses of legume roots upon inoculation with *Rhizobium*, but surprisingly root hair deformation has not been studied in any significant detail at a cytological level (Nutman, 1959).

Gene induction

Changes in plant gene expression in deformed root hairs have been studied by comparing both the protein patterns obtained by 2-D gel electrophoresis of isolated proteins and of *in vitro* translated proteins of RNAs isolated from root hairs of inoculated and uninoculated plants. It was found that in pea root hairs several mRNAs are present at markedly elevated levels after inoculation with *R. leguminosarum* bv. *viciae*. However, the majority of these mRNAs are found at similar high levels in root hairs at an early stage of development. This probably reflects that *Rhizobium* stimulates root hair development. Two mRNAs producing the proteins RH-42 and RH-44 respectively, are exclusively present at elevated levels in root hairs of inoculated plants and these proteins may be involved in root hair deformation (Gloudemans, *et al.*, 1989). The induction of the RH-42 gene and the enhanced expression of the RH-44 gene requires active *nod* genes.

In *Vigna unguiculata*, 9 new proteins are formed in root hairs 1 to 4 days after inoculation. Six of these proteins are also present in nodules (Krause and Broughton, 1992), and therefore are probably not involved in deformation but in the infection process. None of these proteins was found in root hairs inoculated with Had$^-$ mutants of *Rhizobium* which are defective in root hair deformation. These proteins supposedly involved in root hair deformation have been named hadulins (Krause and Broughton, 1992). However, a direct relation with root hair deformation has not been demonstrated and it was not checked for instance whether the level of these proteins was elevated in root hairs at early stages of development. It is possible as well that several of these proteins are involved in infection. At present none of the putative root hair deformation related genes has been cloned, nor have antibodies been raised against the encoded proteins. Clones and antibodies will be very useful to study the exact role, if any, of these genes in root hair deformation.

Nod factors induce root hair deformation

Purified Nod factors have the ability to induce root hair deformation at their respective host plants if applied at concentrations higher that 10^{-12}M (Lerouge, *et al.*, 1990; Spaink, *et al.*, 1991; Schultze, *et al.*, 1992). Studies on vetch and alfalfa root hair deformation

have made clear that distinct parts of Nod factors are important in the induction of root hair deformation. NodRlv factors containing 4 or 5 N-acetylglucosamine residues have the same ability to induce root hair deformation on vetch, while a trimer is unable to elicit this response. Alfalfa has more stringent requirements as to the length of the N-acetylglucosamine backbone, since NodRm-IV(C16:2, S) is 100 fold more active than NodRm-V(C16:2, S) in deforming root hairs (Schultze, *et al.*, 1992). The tetramer of N-acetylglucosamine, N,N',N",N'"-tetraacetylchitotetraose which is identical to the sugar backbone of NodRlv-IV without the N-acyl and O-acetyl substitutions, does not elicit vetch root hair deformation (Spaink, *et al.*, 1991). Therefore the presence of a fatty acid chain is essential. The structure of the acyl moiety, at the other hand, appears to be less important. NodRlv factors containing a C18:4, a C18:1 or a C18:0 group as well as desulphated NodRm factors containing a C16:2 acyl moiety are equally able to deform vetch root hairs (Heidstra, personal communication). The presence of the sulphate group at the reducing N-acetylglucosamine unit is of major importance in determining host specificity. NodRm-IV(C16:2, S) can induce alfalfa root hair deformation, but not those of the non-host plant vetch (Lerouge, *et al.*, 1990). In the contrary, NodRm-IV(C16:2), which lacks the sulphate group, is unable to induce alfalfa root hair deformation, but instead is able to deform vetch root hairs (Roche, *et al.*, 1991). In NodNGR factors a 2-O-methylfucosyl group is present at the reducing sugar. This O-methylfucosyl group can be either acetylated or sulphated. The NodNGR factors containing a sulphate group deform alfalfa root hairs, while the ones lacking the sulphate group have the ability to deform vetch root hairs (Price, *et al.*, 1992). Clearly, the presence of a sulphate group appears essential for alfalfa root hair deformation, while the exact position at the reducing sugar seems less important.

EPS I from *R. meliloti* also has the ability to induce root hair deformation in the absence of bacteria (Reuber, *et al.*, 1991). Therefore it is possible that both Nod factors, and EPS, or EPS derived molecules, are involved in root hair deformation.

INFECTION AND INFECTION THREAD FORMATION

Infection thread formation

Infection thread formation may take place if bacteria are entrapped in root hair curls. In the curl local hydrolysis of the plant cell wall occurs (Callaham and Torrey, 1981; Bakhuizen, 1988), and at the site of hydrolysis the plasma membrane grows inward and new wall material is deposited along the invaginating plasma membrane (Callaham and Torry, 1981; Turgeon and Bauer, 1985; Bakhuizen, 1988; for reviews see Bauer, 1981; Newcomb, 1981; Brewin, 1991; Kijne, 1992). In this way, a tubular structure--the so-called infection thread (Dart, 1974)--is formed by which the bacteria enter the plant.

Bacteria inside the infection thread are surrounded by a matrix. The composition of this matrix is unclear, but it appears likely that extracellular proteins of the plant as well as compounds secreted by the bacteria are part of this matrix. The infection thread wall is most likely of plant origin and has a similar ultrastructure as the plant cell wall. The similarity between cell wall and the infection thread wall is further witnessed by the occurrence of common polysaccharides like cellulose, xyloglucan and pectins (VandenBosch *et al.*, 1989; Rae, *et al.*, 1992).

The infection thread grows towards the base of the root hair, and at the sites of infection cortical cells are activated and form radial tracks. The cytoplasm of these activated cortical cells rearranges to form radial transvacuolar cytoplasmic brigdes and the nuclei move to the center of the cell (Bakhuizen, 1988). Such cortical cytoplasmic brigdes are considered to be "prepared for infection thread passage', and have been named "pre-infection threads" (Van Brussel, *et al.*, 1992). The infection thread penetrates root cortical cells by the same mechanism of local hydrolysis of the cell wall used for initiation of the infection thread in root hairs, and it grows through the "pre-infection threads" to the nodule primordium (see below) where bacteria are endocytotically released into the plant cells (Newcomb, 1976; 1981).

Plant genes involved in infection.

The wall of the infection thread is very similar to the plant cell wall (VandenBosch, *et al.*, 1989; Rae, *et al.*, 1992), but possibly the occurrence of nodulins in the infection thread wall contributes to its specific properties. Suitable candidates for such nodulins are the early nodulins *Ps*ENOD5 and *Ps*ENOD12. These nodulins have first been studied in pea (Scheres, *et al.*, 1990a; 1990b). *In situ* hybridization studies demonstrated that *Ps*ENOD12 gene expression is induced by *Rhizobium* in root hairs and root cortical cells harbouring an infection thread. In addition this gene is induced in cortical cells that are in front of the infection thread tip and in nodule primordia as well. Expression of *Ps*ENOD5 genes is only induced in cells containing an infection thread tip. Recently, it was shown that the alfalfa *Ms*ENOD12 gene is induced as early as 3 hr after inoculation, specifically in a zone of root epidermal cells starting just behind the root tip and ending where root hairs reach their mature size (Pichon *et al.*, 1992). From this study it was concluded that ENOD12 is most likely involved in preparing plant cells for *Rhizobium* infection.

The amino acid sequences of *Ps*ENOD5 and *Ps*ENOD12 show that both are proline-rich proteins. The larger part of the *Ps*ENOD12 polypeptide is composed of two repeating pentapeptide units, namely Pro-Pro-Gln-Lys-Glu and Pro-Pro-His-Lys-Lys, and the polypeptide has a putative signal peptide at its N-terminal part. These features suggest that the *Ps*ENOD12 is a (hydroxy)proline-rich cell wall protein and could be a component of the infection thread wall as well as of the wall of epidermal and cortical

cells preparing for infection (Scheres, *et al.*, 1990a; Nap and Bisseling, 1990). The *Ps*ENOD5 polypeptide has a putative signal peptide at its N-terminal part , and it is rich in proline, alanine, glycine and serine, indicating that it might be an arabinogalactan-like protein (Scheres, *et al.*, 1990b). Accordingly it is possible that the *Ps*ENOD5 protein is a component of the infection thread wall or membrane (Scheres, *et al.*, 1990b; Nap and Bisseling, 1990).

In the infection thread matrix a 95 kDa plant glycoprotein was found (VandenBosch, *et al.*, 1989; Rae, *et al.*, 1992). This glycoprotein also accumulates in the intercellular spaces of uninfected root cortex (Rae, *et al.*, 1992). The gene encoding this protein has not yet been characterized and the role of this protein in infection thread formation remains unclear.

Bacterial signal molecules involved in infection

I. Nod factors

Purified Nod factors induce expression of the infection related early nodulin genes, *Ps*ENOD5 and *Ps*ENOD12, but do not achieve infection thread formation. Both *R. leguminosarum* bv. *viciae* NodRlv factors, containing either a C18:4 or C18:1 acyl group induce the expression of these early nodulin genes in pea root hairs, but the kinetics of induction is slightly different. NodRlv-V(Ac, C18:4) induces maximal *Ps*ENOD12 gene expression within 12 hours, while after application of NodRlv-V(Ac, C18:1), expression of this gene only reaches the highest level at about 24 hours (Horvath, *et al.*, 1993).

Recently Van Brussel *et al.* (1992) showed that purified NodRlv factors containing a C18:4 acyl group induce the formation of "pre-infection thread" structures in vetch roots, while NodRlv factors having a C18:1 acyl group are unable to induce these structures. Therefore the lipid moiety seems very important for the induction of "pre-infection thread" formation. Studies on nodulin gene induction as well as "pre-infection thread" formation strongly suggest that Nod factors play a role in the infection process but for the formation of a genuine infection thread something else is apparently required in addition.

II. Bacterial surface compounds

It has been shown by mutagenesis that bacterial surface compounds play a role in the infection process. Mutants disturbed in EPS, LPS and ß(1-2) glucan biosynthesis do often not effectively infect the host plant. The role of these surface compounds in infection is not yet clear. It has been proposed that these compounds 1) are signal molecules inducing infection thread formation, 2) are involved in host-microbe

recognition determining release of bacteria from infection threads and the avoidance of a plant defense response, or 3) are essential to create the proper environment in the infection thread to allow bacterial growth. Here only some arguments will be given that support these different putative roles of the surface compounds. For a more detailed discussion see Brewin (1991) and Kijne (1992) and Pühler *et al.* (1993).

Djordjevic *et al.* (1987) showed that an EPS mutant of *R. leguminosarum* bv. *trifolii* can be complemented by purified EPS for the formation of nitrogen fixing nodules. Similarly the low molecular weight fraction of EPS I from *R. meliloti* is able to rescue invasion defects of *exo* mutants (Battisti, *et al.*, 1992). These observations suggest that EPS molecules function as signal molecules.

LPS mutants of *R. leguminosarum* bv. *viciae* lacking the O-antigen induce small ineffective nodules on vetch and bacteria are not released from infection threads (De Maagd, *et al.*, 1988). Therefore it was concluded that the O-antigen containing LPS of *R. leguminosarum* bv. *viciae* is important for the endocytotic release of bacteria from the infection threads into plant cells.

R. leguminosarum bv. *viciae* mutants unable to secrete the cyclic ß(1-2) glucan form small nodules with a few infected cells. In these nodules a defense-related gene, chalcone synthase (CHS), is induced in cells surrounding the infected cell (Yang, *et al.*, 1992; Chapter 3). Similarly, EPS I mutants of *R. meliloti* induce empty nodules in which callose and phenolics accumulate at the infection sites and a defense-related gene, phenylalanine ammonia lyase (PAL), was induced (Niehaus, *et al.*, 1993; for review see Pühler, *et al.* 1993). It was concluded that the cyclic ß(1-2) glucan and EPS may be involved in avoiding a plant defense response. The cyclic ß(1-2) glucan is also involved in osmotic adaptation (Dylan, *et al.*, 1990), suggesting a role in creating a proper environment for bacteria.

Mechanisms of infection thread formation

It is remarkable that the bacteria induce a very localized hydrolysis of the cell wall in the root hair curls during initiation of infection thread formation (Turgeon and Bauer, 1985; Callaham and Torrey, 1981). Although it has been shown that bacteria secrete hydrolytic enzymes (Hubbell, *et al.*, 1978; Martinez-Molina, *et al.*, 1979), it is hard to imagine that those hydrolytic enzymes can have such local effects (Turgeon and Bauer, 1985). Furthermore, none of the *Rhizobium* genes that play a role in nodulation, encodes a hydrolytic enzyme. Therefore it seems quite possible that the bacteria induce the local secretion of hydrolytic enzymes by the plant (Ljunggren and Fahraeus, 1961; for review see Kijne, 1992). Such a local hydrolysis of the epidermal cell wall happens in root hair formation. Therefore it has been proposed that the mechanism of infection thread formation might be derived from root hair initiation (Van Brussel, *et al.*, 1992; for review

see Kijne, 1992). In this connection it is noteworthy that Nod factors can stimulate root hair formation (Lerouge, *et al.*, 1990; Van Brussel, *et al.*, 1992).

NODULE PRIMORDIUM AND MERISTEM FORMATION

Concommitant with infection, root cortical cells are activated and start to divide (Figure 3). Which of the root cortical cells divide and what type of nodule is formed is determined by the plant, and not by *Rhizobium* (Gresshoff and Delves, 1986; Rolfe and Gresshoff, 1988). In temperate legumes, such as pea, vetch, alfalfa and clover, inner cortical cells of the root divide. In pea and vetch especially the inner cortical cells in the vicinity of a protoxylem pole are mitotically activated (Bond, 1948; Libbenga and Harkes, 1973; Libbenga and Bogers, 1974; Vijn, *et al.*, 1993). Before these cortical cells divide they are easily distinguished from the adjacent cortical cells by their prominent central nucleus and cytoplasmic strands across the central vacuole. First the innermost cortical cells divide and subsequently inner cortical cells more distant from the stele are mitotically activated. The primary division of the cortical cells is predominantly anticlinal, while the following divisions are periclinal and oblique (Libbenga and Bogers, 1974; Newcomb, *et al.*, 1979; Calvert, *et al.*, 1984). The dividing inner cortical cells form the nodule primordium (Libbenga and Harkes, 1973; Newcomb, 1981). In temperate legumes the outer cortical cells are also activated and form the "pre-infection thread" structures (see above). Infection threads grow through the "pre-infection thread" structure towards these primordia, and by this time ramify, and cells at the base of the primordium are infected. At the same time, cells at the distal part of the primordium become small and rich in cytoplasm (Libbenga and Harkes, 1973). These cells constitute the apical nodule meristem, which differentiates basipetally, during the complete nodule life time, into infected and uninfected cells of the central tissue, as well as into cells of the peripheral tissues (see below). In consequence, these nodules have an indeterminate development and represent the indeterminate nodule type.

In tropical legumes, such as soybean, outer cortical cells of the root divide to form the nodule primordium. While the inner cortical cells between the primordium and the nearby protoxylem pole are activated to divide and will then form the connecting vascular bundle. After passing the root hair the infection thread penetrates the central part of the nodule primordium (Bieberdorf, 1938; Dart, 1975; Newcomb, *et al.*, 1979; Turgeon and Bauer, 1982; Calvert, *et al.*, 1984; for review see Rolfe and Gresshoff, 1988). Since the infection threads directly invade meristematic cells after they have penetrated the root hairs, "the pre-infection thread" cell type is not required. As cells at the periphery of the primordium remain mitotically active and become infected later, a spherical meristem is formed in this way (Newcomb, *et al.*, 1979; Calvert, *et al.*, 1984). Such a meristem ceases to divide about 10 days after inoculation (Newcomb, *et al.*, 1979). Since the

meristem loses its meristematic activity at an early stage of root nodule development, nodules have a determinate growth pattern and are called determinate nodules.

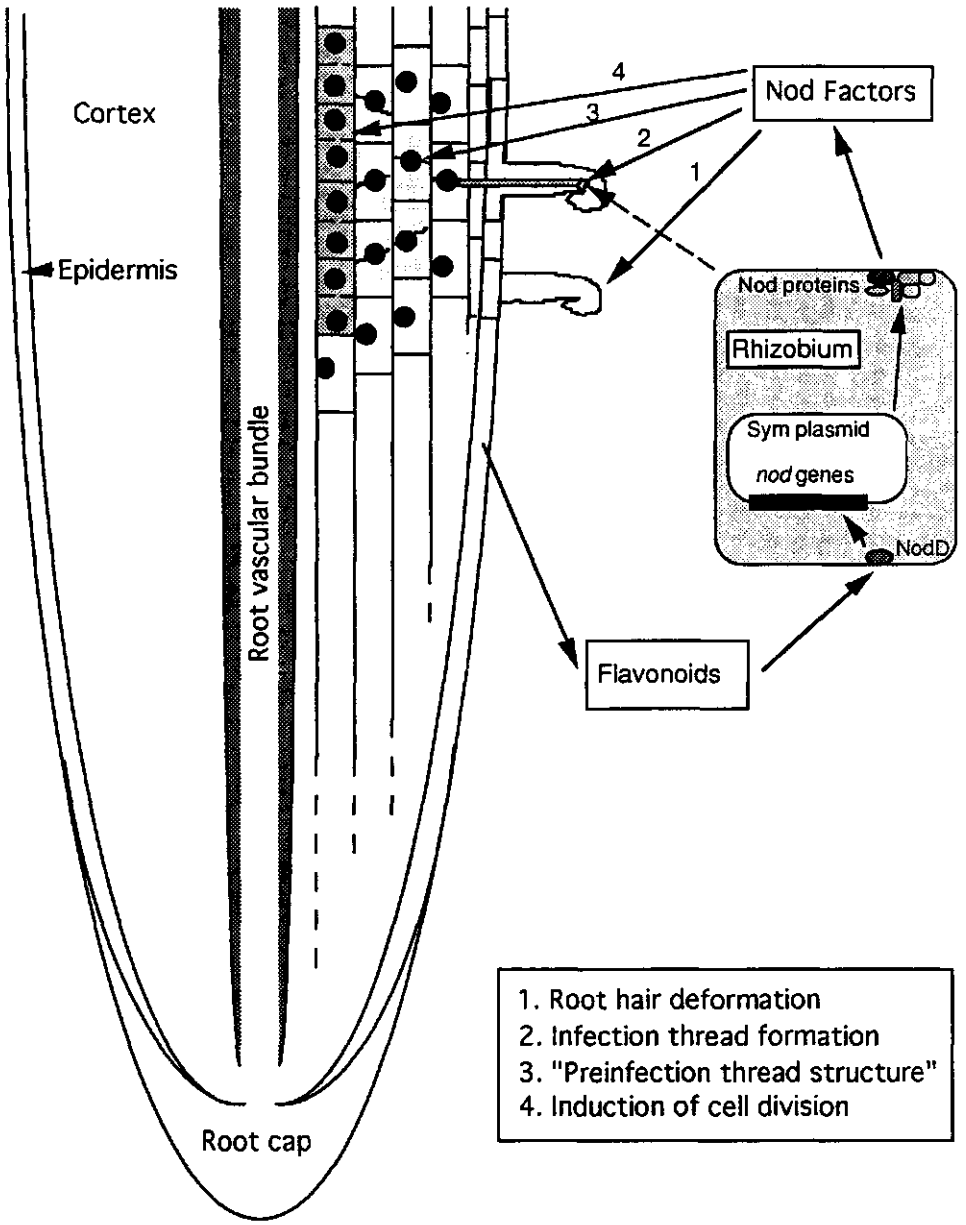


Figure 3. A schematic drawing summarizing the events during early stages of legume-*Rhizobium* interaction

Plant genes induced in nodule primordia

Several plant genes are induced in nodule primordium. Examples are the early nodulin genes ENOD12 (Scheres, *et al.*, 1990a; Pichon, *et al.*, 1992), ENOD40 (Yang, *et al.*, 1993; Chapter 4; Yang, unpublished data) which is homologous to *Gm*N#36 (Kouchi and Hata, 1993), and *Gm*N#93 (Kouchi and Hata, 1993). The *Ps*ENOD12 gene is expressed in pea root cortical cells containing an infection thread (see above), but is also expressed in nodule primordial cells. The pattern of *Ps*ENOD40 gene expression is similar to that of *Ps*ENOD12 (Yang, *et al.*, 1993; Chapter 4). Both genes are not expressed in nodule meristem cells (Scheres, *et al.*, 1990a; Yang, *et al.*, 1993; Chapter 4). So both the *Ps*ENOD12 and the *Ps*ENOD40 genes can be used as molecular marker to distinguish nodule primordium and meristem (Vijn, *et al.* 1993). Beyond being induced in nodule primordia, the soybean and pea ENOD40 genes, but not ENOD12 gene, are also switched on in the pericycle of the root facing the primordium (Yang, *et al.*, 1993; Kouchi and Hata, 1993). Therefore the ENOD40 gene may control transport of compounds between the root central stele and the cortex. The nucleotide sequence of this gene does not show any homology to known genes.

The *Gm*N#93 gene is expressed in the soybean nodule primordium as well as in nodule meristem cells and in the infected cells of the mature nodule (Kouchi and Hata, 1993). Like ENOD40 *Gm*N#93 has no homology to any known proteins.

Ngm-26 is a soybean nodulin which is located in the peribacteroid membrane. Northern blot analysis has shown that this gene is expressed in mature nodules (Fortin, *et al.*, 1987). However, the Ngm-26 promoter fused to a reporter GUS gene was induced in incipient lateral roots as well as in nodule primordia in transgenic *Lotus corniculatus* plants. This suggests that a Ngm-26 (like) gene may be induced in nodule primordia in heterologous genetic background (Miao and Verma, 1993).

Chalcone synthase gene (CHS) is not a typical nodulin gene, but an example of a plant gene induced in nodule primordial cells as well as in the apical part of the nodule meristem (Yang, *et al.*, 1992; Chapter 3). At the other hand, CHS is expressed in lateral root primordia and root meristems. The expression pattern of the CHS gene (in primordial cells and in front of meristems) during the formation of roots and nodules is strikingly similar, suggesting that the two developmental programmes are related.

Bacterial signals

Purified Nod factors are able to induce the formation of nodule primordia in the inner cortex at exactly the same position as *Rhizobium* does, preferentially opposite a protoxylem pole (Spaink, *et al.*, 1991; Truchet, *et al.*, 1991; Vijn, *et al.*, 1993). In the induction of nodule primordia the lipid moiety of the Nod factors is of major importance.

NodRlv factors with a C18:4 acyl group are capable to induce primordium formation, whereas NodRlv factors containing a C18:1 group are unable to do so (Spaink, *et al.*, 1991). The early nodulin genes *Ps*ENOD40 and *Ps*ENOD12 are expressed in the Nod factor induced primordia in a spatial pattern identical to that after *Rhizobium* infection (Vijn, *et al.*, 1993). Therefore the Nod factors appear to be the only bacterial compounds required for the formation of nodule primordia and for early nodulin gene expression in a spatially controlled manner.

Mechanisms of nodule primordium formation

Several hypothesis have been proposed to explain nodule primordium formation. One of the most attractive ones is the so-called gradient hypothesis (Libbenga and Bogers, 1974). This hypothesis implies that two oppositely oriented gradients of morphogens, one originating from the growing infection thread, being most likely the Nod factors (Vijn, *et al.*, 1993), and another from the protoxylem pole, determine the formation and position of the nodule primordium. The morphogen from the protoxylem pole has been named stele factor. Stele factor has been purified from pea root stele and is capable of inducing cell divisions in root cortex explants in the presence of basal phytohormones (Libbenga, *et al.*, 1973; Smit, *et al.*, 1993). The chemical nature of stele factor has not yet been determined.

Several studies indicate that phytohormones play a role in nodule primordium formation. Allen and Allen (1958) showed that compounds like N-1-(naphthyl)phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) that block polar auxin transport induce the formation of nodule-like structures especially on legume roots (Allen and Allen, 1958; Libbenga *et al.*, 1973). Recently it has been shown that early nodulin genes, like *Ps*ENOD12 and *Ps*ENOD2, are expressed in such nodule-like structures (Hirsch, *et al.*, 1989; Van de Wiel, *et al.*, 1990b; Scheres, *et al.*, 1992), indicating that structures induced by auxin transport inhibitors are closely related to *Rhizobium* induced nodules. Since the roots are bathed in auxin transport inhibitors these studies did not distinguish whether the primordia are caused by a relative increase or decrease of auxin. Long and Cooper (1988) showed that *nod*ABC$^-$ mutants of *R. meliloti* can be rescued for the formation of primordia by the introduction of the *tzs* (*trans*-zeatin secretion) gene by which rhizobia secrete cytokinin. This indicates that an increased cytokinin/auxin ratio in the root cortex leads to cell division. Therefores it can be postulated that auxin transport inhibitors cause cortical cell division by decreasing the relative auxin concentration, and this may form part of the signal transduction in Nod factor induced cortical cell division.

NODULE DEVELOPMENT

Organization of nodule tissues

Determinate and indeterminate nodules have a similar tissue organization, a central tissue where bacteria are hosted, surrounded by several peripheral tissues (for review see Rolfe and Gresshoff, 1988; Brewin, 1991; Franssen, *et al.*, 1992b). The peripheral tissues include the nodule cortex, the endodermis and the nodule parenchyma (Van de Wiel, *et al.*, 1990a). The latter tissue harbours the nodule vascular bundles.

The central tissue is composed of two cell types, namely infected and uninfected cells. The infected cell type is fully packed with bacteria and here nitrogen fixation takes place. A few cell layers of uninfected cells--named boundary layers--separate the central tissue from the nodule parenchyma (Gresshoff and Delves, 1986; Franssen, *et al.*, 1992b). Part of the assimilation of the NH_4^+ produced by the bacteria in the infected cells and transport of the fixed nitrogen take place in the uninfectd cell type of the determinate nodules (Newcomb and Tandon, 1981; Nguyen, *et al.*, 1985). The role of uninfected cells in the indeterminate nodule type is not clear.

Meristems of indeterminate nodules continuously differentiate into the different nodule tissues. The effect is that nodule tissues consist of cells at successive stages of development. For example the central tissue can be divided into several zones representing successive stages of development. Different nomenclatures have been used to describe this zonation. Newcomb (1976) divided the central tissue of pea nodules into the following consecutive zones. At the apex of the nodule the meristem is present. The cell layers immediately behind that, where infection occurs, form the infection zone. This zone is followed by the early symbiotic zone in which bacteria proliferate, plant cells elongate and the number of organelles is increased. The late symbiotic zone is marked by changes in the morphology of the bacteria in the infected cells; *Rhizobium* bacteria particularly increase in size and become Y-shaped bacteroids. In addition, plant cells accumulate starch in the amyloplasts. In the senescent zone plant cytoplasm degenerates.

Vasse *et al.* (1990) proposed to use other criteria in characterizing the different zones of an alfalfa nodule. They used ultrastructural changes in plant cell and in bacteroid morphology, starch accumulation and the onset of nitrogen fixation as criteria to classify different zones. The meristem at the apex is designated as zone I. The meristem is composed of small cytoplasmic dense cells that are not infected by rhizobia. This zone is immediately followed by the prefixation zone II. In the distal part of this zone II infection threads penetrate meristematic cells and bacteria are released into plant cytoplasm and differentiation of both symbionts starts. During the release bacteria become surrounded by a plant membrane, the peribacteroid membrane. Bacteria together with the peribacteroid space and membrane form a functional structure called symbiosome (Roth

and Stacey, 1989). In the proximal part of the prefixation zone II plant cells elongate and symbiosomes proliferate. In the nitrogen fixation zone III the plant cells have reached their maximal size and bacteroids begin to fix nitrogen. The interzone II-III is located in between the nitrogen fixation zone III and the prefixation zone II. This zone is characterized by the start of starch accumulation in infected cells and the presence of rhizobia with a specific morphology (Vasse, *et al.*, 1990). In older nodules a senescent zone IV is present. The zonation proposed by Vasse *et al* (1990) is applicable to pea nodules (Franssen, *et al.*, 1992b). We will follow the nomenclature of Vasse in the following part where we summarize what is known about expression of plant and bacterial genes in different nodule tissues.

Gene expression in the central tissue

Expression of plant and bacterial genes has been studied in both determinate and indeterminate nodules. However, the timing of gene expression has been much better studied in the indeterminate nodule type. Therefore we will focus on the expression of plant and bacterial gene expression during development of the different tissues of indeterminate nodules.

I. Nodule meristem

None of the nodulin genes identified so far is expressed in the apical meristem of indeterminate nodules. The nodulin genes, *Ps*ENOD12 and *Ps*ENOD40 are expressed at a maximal level in the first cell layer of the prefixation zone II (Scheres, *et al.*, 1990; Yang, unpublished data), showing that a very clear distinction can be made between the meristem and the prefixation zone II.

CHS genes are expressed in the distal cell layers of the apical meristem of indeterminate nodules and in a few cell layers of the nodule cortex adjacent to the meristem (Yang, *et al.*, 1992; Chapter 3). Therefore the expression of CHS gene marks the distal boundary of nodule meristem. The pattern of CHS gene expression has not been studied during determinate nodule development, but Estabrook and Sengupta-Gopalan (1991) demonstrated by using gene-specific probes that only certain members of the CHS gene family are induced during soybean nodule development.

II. The prefixation zone II

The early nodulin gene *Ps*ENOD12 is only expressed in the distal part of the prefixation zone II. In this region plant cells are penetrated by infection threads and start to differentiate. Therefore this part of the prefixation zone II was previously named

infection zone by Newcomb (1976). In alfalfa *Ms*ENOD10 shows the same pattern of expression as ENOD12 (Löbler and Hirsch, 1993). The *Ps*ENOD5 and *Ps*ENOD40 genes are expressed in infected cells of the whole prefixation zone II. The highest level of expression of the *Ps*ENOD5 is found at the proximal part of the prefixation zone II, whereas the *Ps*ENOD40 transcript is present at an equal level throughout this zone. At the transition of the prefixation zone II into interzone II-III there is a sudden drop in the levels of *Ps*ENOD5 and *Ps*ENOD40 mRNA (Franssen, *et al.*, 1992b; Yang, unpublished data). The high level of the *Ps*ENOD5 mRNA at the proximal part of zone II, where the symbiosomes proliferate, suggests that the *Ps*ENOD5 encoded arabinogalactan-like protein may be part of the peribacteroid membrane (Scheres, *et al.*, 1990b; Nap and Bisseling, 1990).

In soybean two early nodulin genes, *Gm*ENOD55 (Franssen, *et al.*, 1988; De Blanck, *et al.*, 1993) and *Gm*N#315 (Kouchi and Hata, 1993) encode proteins containing Pro/Ser alternating repeat sequences. These early nodulin genes are homologous to *Ps*ENOD5 and are first induced in young soybean nodules in which infection and bacterial release occur. Both *Gm*ENOD55 and *Gm*N#315 gene are expressed only in infected cells (De Blank, *et al.*, 1993; Kouchi and Hata, 1993).

In the distal part of the prefixation zone II, the bacterial *nod* genes are expressed while no expression is detectable in the proximal part of this zone (Schlamman, *et al.*, 1991). This suggests that *nod* genes are transcribed inside the infection threads and/or in bacteria shortly after release from the infection threads and implies that Nod factors can be synthesized in this part of the nodule. Sharma and Signer (1990) demonstrated that *nod* genes are expressed in the distal part of the nodule, but their studies did not allow a precise localization of *nod* gene expression.

The *R. leguminosarum* bv. *viciae* gene *rop*A, encoding a 36kDa outer membrane protein antigen group III, has an expression pattern similar to that of the *Ps*ENOD5 gene. *rop*A gene is transcribed in the prefixation zone II and reaches its highest expression level at the proximal part which subsequently drops dramatically at the transition of the prefixation zone II to interzone II-III (De Maagd, *et al.*, 1993: Chapter 7).

Expression of several nodulin genes is induced in the proximal part of the prefixiation zone II. Examples are the early nodulin genes *Ps*ENOD3 and *Ps*ENOD14 and the late nodulin gene leghemoglobin (Lb). The expression of the *Ps*ENOD3/14 genes starts at the proximal part of the prefixation zone II and reaches its maximal level in the interzone II-III, then decreases around the transition of the interzone II-III into the nitrogen fixation zone III (Franssen, *et al.*, 1992b; Yang, unpublished data). The *Ps*ENOD3/14 early nodulins are 6kDa proteins that are 55% homologous. They have a putative signal peptide at the amino terminal end and contain 4 cysteine residues with a spatial distribution resembling that of metal binding proteins (Scheres, *et al.*, 1990b). The expression of Lb genes starts in the proximal part of the prefixation zone II, it reaches a maximal level at

the beginning of interzone II-III and remains at a constant level throughout the nitrogen fixation zone III (Yang, *et al.*, 1991: Chapter 6; Franssen, *et al.*, 1992b). In older nodules the Lb mRNA predominantly occurs in a few cell layers of the interzone (Kardailsky, *et al.*, 1993; Chapter 5; Yang, unpublished data). Lb is the most abundant nodulin in legume root nodules and functions as oxygen carrier and controls the free oxygen concentration in the nodule central tissue.

III. Interzone II-III

The transition of the prefixation zone II into interzone II-III is marked by a beginning of starch accumulation and a sudden drop in expression of the bacterial *rop*A and several nodulin genes (see above). Furthermore this transition is characterized by the induction of several other genes like late nodulin gene *Ps*NOD6 and the rhizobial *nif* genes. Both the *Ps*NOD6 and *nif* genes are immediately expressed at a very high level in the first cell layer of the interzone II-III which does not increase in further cell layers (Kardailsky, *et al.*, 1993; Chapter 5).

*Ps*NOD6 is homologous to the pea early nodulin genes *Ps*ENOD3 and *Ps*ENOD14. This homology includes the position of the signal peptide cleavage site , the sequence of the signal peptide, the spatial distribution of the 4 Cys residues and amino acids surrounding them (Kardailsky, *et al.*, 1993: Chapter 5). Therefore it is likely that *Ps*NOD6 has a function analogue to *Ps*ENOD3 and *Ps*ENOD14.

IV. The nitrogen fixation zone III

None of the studied genes is induced at the transition of interzone II-III into the nitrogen fixation zone II. But the concentration of *Ps*ENOD3 and *Ps*ENOD14 transcripts decreases around this transition (Franssen, *et al.*, 1992b). Late nodulin genes, like *Ps*NOD6 and Lb, and bacterial *nif* genes are expressed in the nitrogen fixation zone III.

V. The senescence zone IV

Senescence of nodule tissues has hardly been studied at the molecular level. On the analogy of other senescent organs, it is likely that the expression of genes encoding hydrolytic enzymes like proteases and RNases will be active in this zone. Indeed proteases, e.g. thiol proteases, has been found to be active in senescent nodules (Vance, 1986; Peoples and Dalling, 1988). A nodulin gene specifically expressed in senescent nodules has been isolated from winged bean (Manen, *et al.*, 1991). Surprisingly, this gene encodes a 21 kDa protease inhibitor. This protein is exclusively present in senescent infected cells in degenerating bacteroids. Similar protease inhibiting activity is found in

the peribacteroid space in soybean nodules (Garbers, *et al.*, 1988). It suggests that the plant has established a system to control senescence.

Gene expression in the peripheral tissues

The majority of the nodulin genes is expressed in the central tissue, and only two early nodulin genes have been shown to be expressed in the peripheral nodule tissues, namely ENOD2 and ENOD40.

The ENOD2 gene has been identified in several legumes (Franssen, *et al.*, 1987; Allen, *et al.*, 1991; Perlick and Pühler, 1993) and in all cases this gene is specifically expressed in the nodule parenchyma (Van de Wiel, *et al.*, 1990b; Allen, *et al.*, 1991).

It was found by physiological studies that the nodule parenchyma regulates the free O_2 concentration in the nodule (Tjekema and Yocum, 1974; Witty, *et al.*, 1986). The low O_2 concentration in the central tissue of the nodule is achieved by the high O_2 consumption rate of *Rhizobium* at one hand and the O_2 diffusion barrier in the nodule parenchyma at the other hand (Witty, *et al.*, 1986). This O_2 diffusion barrier is established by cell layers which lack intercellular spaces. The ENOD2 protein is composed of two repeating pentapeptides containing two proline residues each: Pro-Pro-His-Glu-Lys and Pro-Pro-Tyr/His-Gln (Franssen, *et al.*, 1987; Van de Wiel, *et al.*, 1990a). And it has been suggested that the ENOD2 protein is located in the cell wall. Since the cell wall is a major determinant of cell morphology, it has further been postulated that ENOD2 contributes to the formation of the O_2 diffusion barrier in nodules (Van de Wiel, *et al.*, 1990a).

Recently, the *Sr*ENOD2 gene from *Sesbania rostrata* has been shown to be induced in roots by exogenously supplied cytokinins (Dehio and De Bruijn, 1992). Other phytohormones, such as indole-acetic acid (IAA), gibberellic acid (GA), abscisic acid (ABA) and ethylene, or the auxin transport inhibitor TIBA, do not induce the *Sr*ENOD2 gene. This indicates that during nodule development cytokinin might regulate the expression of the ENOD2 gene.

The early nodulin gene ENOD40, which is expressed in the prefixation zone II of the central tissue, also belongs to the nodulin genes active in the periphery tissues as it is expressed in pericycle cells of the nodule vascular bundle and the boundary cell layer. The expression in the pericycle of the nodule vascular bundles indicated that this gene might have a transport function (Yang, *et al.*, 1993; Chapter 4; Kouchi and Hata, 1993).

In conclusion, studies on *Rhizobium* genetics and nodulin gene expression as reviewed above have certainly advanced our understanding of root nodule development. In the following part of this thesis the characterization and pattern of expression of CHS, ENOD40, NOD6 and bacterial gene *nif*H and *rop*A are presented.

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Chapter 3

In situ localization of chalcone synthase mRNA in pea root nodule development

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Abstract

In this paper we report studies on the role of flavonoids in pea root nodule development. We followed flavonoid synthesis by localizing chalcone synthase (CHS) mRNA in infected pea roots and in root nodules. In a nodule primordium, CHS mRNA is present in all cells of the primordium. Therefore we hypothesize that the *Rhizobium* Nod factor induces cell division in the root cortex by stimulating the production of flavonoids, that function as auxin transport inhibitors. In nodules CHS mRNA is predominantly present in a region at the apex of the nodule consisting of meristematic and cortical cells. These cells are not infected by *Rhizobium*. Therefore we postulate that CHS plays a role in nodule development rather than in a defense response. In roots CHS mRNA is located at a similar position as in nodules, suggesting that CHS has the same function in both root and nodule development.

When nodules are formed by mutants of *Rhizobium leguminosarum* bv. *viciae* that are unable to secrete ß (1-2) glucan and to synthesize the O-antigen containing LPS I. CHS genes are also expressed in regions of the nodule that are infected by *Rhizobium*. We postulate that the impaired development of nodules formed by these mutants is due to an induction of a plant defense response.

Introduction

In initial stages of the *Rhizobium*-pea interaction the bacterium induces three processes: root hair curling and deformation, infection thread formation, and cortical cell divisions (Nap and Bisseling, 1990a). These processes are the beginning of the development of a root nodule. The induction of mitotic activity leads to the formation of globular nodule primordia in the root cortex. At the apex of these primordia meristems are formed, that remain active during further development of the nodules. From the apical meristem the different nodule tissues develop, and in these differentiation processes the expression of specific sets of nodulin genes are induced (Nap and Bisseling, 1990a, 1990b). Depending on the timing of their synthesis during nodule development, nodulins have been divided into early and late nodulins (Nap and Bisseling, 1990b). The majority of pea nodulin genes are expressed in the infected cells of the central tissue of the nodule, and the development of this cell type involves consecutive expression of specific nodulin genes (Nap and Bisseling 1990a; Scheres *et al.*, 1990a,b). The *Ps*ENOD12 gene is expressed in the invasion zone immediately adjacent to the apical meristem, where infection thread growth and bacterial release occur. *Ps*ENOD5 gene expression occurs in the infected cells of the invasion zone and of the early symbiotic zone. In the latter zone the infected cells elongate and bacteria multiply. The *Ps*ENOD3 and *Ps*ENOD14 genes are first transcribed in the infected cells of the early symbiotic zone and the expression level of these genes decreases when the infected cells have reached their maximum size in the late symbiotic zone. The late nodulin genes, like leghemoglobin (Lb) genes, reach their highest level of expression when the concentration of *Ps*ENOD3 mRNA has already decreased.

Recently, it has been shown that substituted oligosaccharides (Lerouge *et al.*, 1990; Spaink *et al.*, 1991) that are secreted by *Rhizobium* upon induction of the *nod* genes, play a pivotal role in the induction of early steps of nodule formation. These Nod factors are capable of inducing root hair deformation (Lerouge *et al.*, 1990; Spaink *et al.*, 1991) and nodule formation (Roche, *et al.*, 1991) and probably they are also involved in the infection process (Nap and Bisseling, 1990a). Although it is clear that the Nod factors play an important role in the induction of early stages of nodule development, the mechanism by which these factors induce these processes is unknown. Clues about these mechanisms have been obtained from studies in which the induction of nodule development is (partly) mimicked by other compounds.

Hirsch *et al.* (1989) showed that auxin transport inhibitors (ATI's) like N-(1-naphthyl)phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) induce the formation of nodule-like structures on alfalfa roots. These structures have a morphology similar to *Rhizobium* induced nodules and moreover some early nodulin genes are expressed at sites comparable to those in regular nodules (Van De Wiel *et al.*, 1990b).

So ATI's can at least partly mimic the activity of the Nod factor, and thus it is possible that a step in the signal transduction pathway induced by the Nod factor is the accumulation of ATI's. Jacobs and Rubery (1988) have reported that flavonoids might be endogenous ATI's in plants. If so Nod factors might induce in roots a local increase in flavonoid concentration, which contributes to the formation of nodule primordia. To test whether flavonoid synthesis is affected by *Rhizobium*, we studied CHS gene expression during root nodule formation with the *in situ* hybridization technique.

Chalcone synthase (CHS) is a key enzyme of flavonoid biosynthesis (Hahlbrock and Scheel, 1989). We selected CHS mRNA not only because of the pivotal role of CHS in flavonoid biosynthesis, but also because the CHS gene is expressed at the highest level among the genes involved in flavonoid biosynthesis (Hahlbrock and Scheel, 1989).

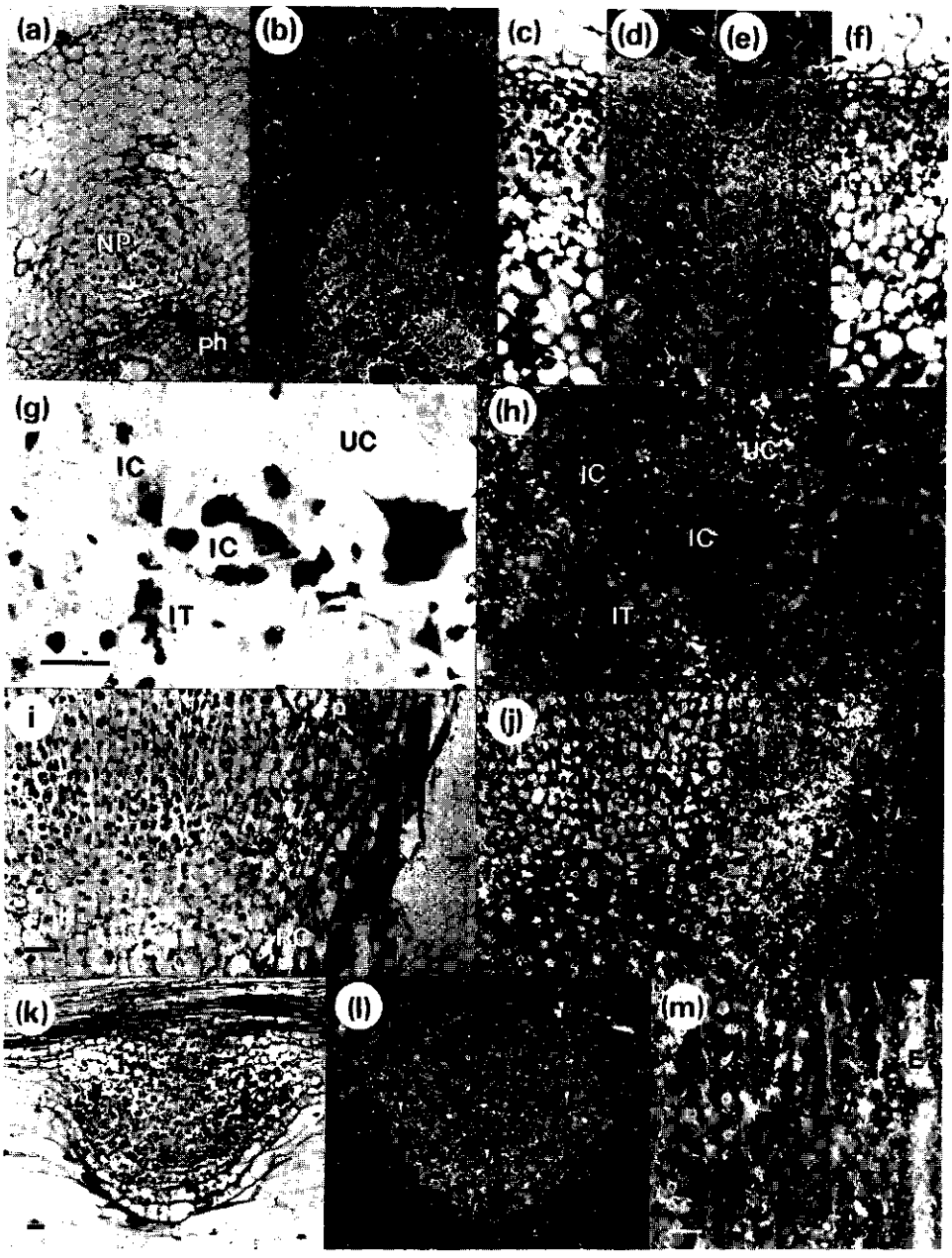
Apart from the hypothetical role of flavonoids in inducing mitotic activity, it has been demonstrated that these molecules are inducers of the *nod* genes of *Rhizobium* (for review see Long, 1989) as well as chemoattractants (Caetano-Anolles *et al.*, 1988). Furthermore it is possible that despite the symbiotic nature of the *Rhizobium*-legume interaction, a defense response is induced at certain stages of the interaction between plant and bacterium (Djordjevic *et al.* 1987a). Such a defense response in pea may include the induction of CHS gene expression (Lamb *et al.*, 1989; Hahlbrock and Scheel, 1989). Thus, hypothetically CHS can be involved in at least three different events during the *Rhizobium*-legume interaction: (1) induction of mitotic activity, (2) production of *Rhizobium nod* gene inducers and chemoattractants, and (3) a defense response.

To determine the role of CHS in the formation of pea root nodules formed by wild type *Rhizobium leguminosarum* bv. *viciae*, we also studied pea nodules formed by *Rhizobium* mutants with different surface properties and defective in symbiotic N_2 fixation. These mutants do not produce the O-antigen containing LPS I (lipopolysaccharide) and fail to secrete ß (1-2) glucan. The cytology and nodulin gene expression pattern of the nodules formed by these mutants are described.

Results

Pattern of CHS Gene Expression During Pea Nodule Development

A pea CHS cDNA clone was isolated from a nodule cDNA library by using a petunia CHS cDNA clone (CHS-A) (Koes *et al.*, 1989) as probe. This pea CHS cDNA clone has an insert of 1.6kb and was shown by sequence analysis to be identical to the pea CHS2 gene described by Harker *et al.* (1990) (results not shown). To study CHS gene expression during nodule formation we localized by *in situ* hybridization CHS mRNA in nodules at different stages of development.


(a)
(b)
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NP
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(k)
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(m)

Figure 1. Localization of CHS mRNA. Longitudinal sections were hybridized with ^{35}S-labeled antisense CHS RNA. DS: dark staining structure, E: epidermis, ES: early symbiotic zone, IC: infected cell, IT: infection thread, IZ: invasion zone, K: columela, M: meristem, NP: nodule primordium, P: protoderm initial, Ph: phloem, RC: root cap, UC: uninfected cell, X: xylem. **a. c. f. g. i** and **k**: bright field micrographs, **b. d. e. j. l** and **m**: dark field micrographs in which white dots are the signal. **h**: epipolarization micrograph. Bar=50μm. The nomenclature of root tissues is according to Popham (1955).
a. Nodule primordium with infection thread (arrow) (8 days after inoculation).
b. CHS mRNA localization in section shown in **a.**
c. Details of a wild type nodule (16 day) apex.
d. CHS mRNA location in the section shiwn in **c.**
e and **f.** ENOD12 mRNA localization of a 16 day old nodule
g and **h.** Details of CHS mRNA localization in VG2 formed nodule. CHS transcripts are present at higher level in uninfected cells.
i.and **j.** Detail of a median longitudinal section of a pea root tip (5 day old) showing that CHS mRNA is present in young root cap cells (arrows). Arrowhead indicates starch grains.
k and **l** CHS mRNA localization in a emerging lateral root. Arrowheads indicate the cell layers with signal.
m CHS mRNA localization in a longitudinal section of the part of the pea root containing emerging root hairs.

In Fig. 1a a section of an infected pea root containing a nodule primordium which is not yet penetrated by an infection thread is shown. This section was hybridized with ^{35}S labeled antisense CHS RNA. As is shown in Fig. 1b. low levels of CHS mRNA are present in all cells of the nodule primordium. Hybridization with sense CHS RNA gave no signal (results not shown). At a slightly later stage of development when an apical meristem is formed in the primordium (Fig. 2a), CHS mRNA is predominantly present in the distal cell layers of this meristem (Fig. 2b). In a mature nitrogen fixing nodule CHS mRNA is also present at the highest level at the apical part of the nodule (Fig. 2c,d); the distal cell layers of the nodule meristem and a few cell layers of the nodule cortex adjacent to the meristem (Fig. 2c,d; Fig. 1c,d). Often a low level of CHS mRNA is also present in the proximal part of the meristem and in the invasion zone directly adjacent to the meristem (result not shown). Fig. 1c,d and 1e,f show magnifications of two adjacent sections hybridized with antisense CHS and *Ps*ENOD12 probes respectively. The *Ps*ENOD12 mRNA is found in the invasion zone of the nodule (Scheres *et al.*, 1990b). Comparison of Fig. 1d and Fig. 1e clearly shows that the highest level of CHS mRNA is present in the distal part of the meristem and the adjacent cortical cell layers.

Thus the *in situ* hybridization studies showed that throughout nodule development the highest expression of CHS genes especially occurs in parts of the nodule where no *Rhizobium* bacteria are present. Therefore it is very unlikely that the expression of CHS genes in nodules reflects a defense response. On the contrary, the location of the CHS mRNA in root nodules suggests that CHS plays a role in the development of this organ. This was further studied by analyzing whether CHS mRNA is found at a similar position in the root,the organ very closely related to root nodules.

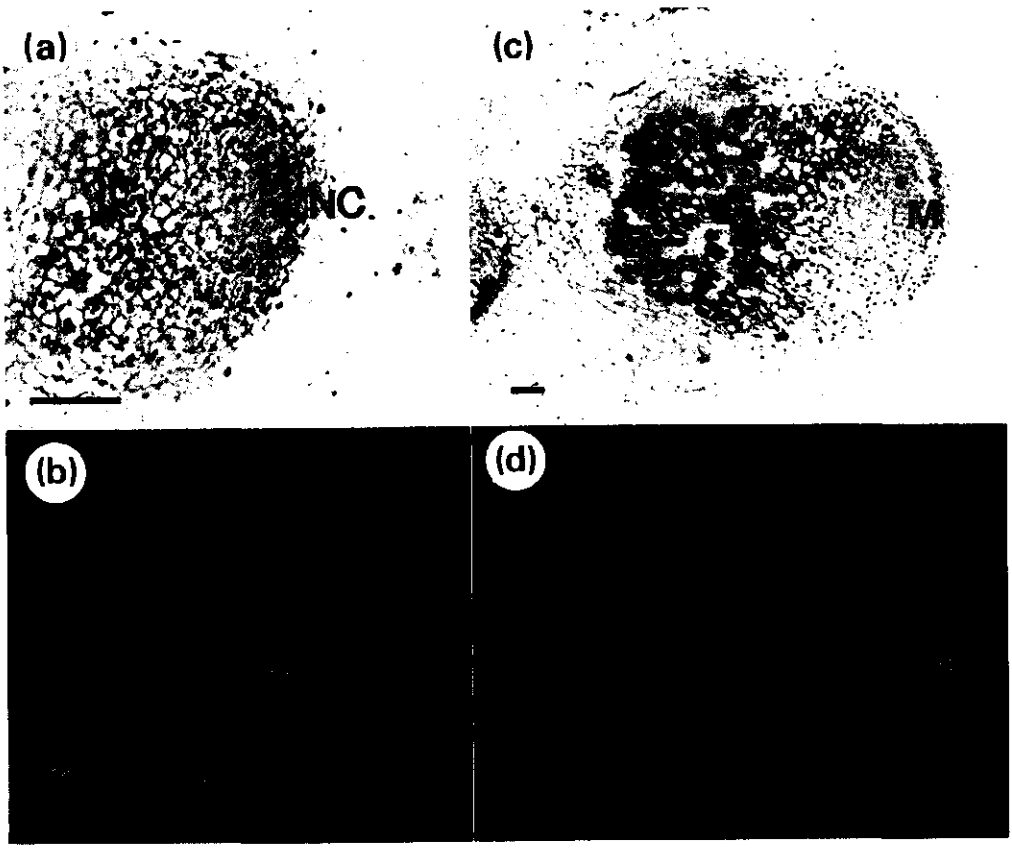

Figure 2. Localization of CHS mRNA in wild type pea nodules. Longitudinal sections were hybridized with ^{35}S-labeled antisense CHS RNA probe. IC: infected cell, M: meristem, NC: nodule cortex. **a** and **c**: bright field micrographs, **b** and **d**: epipolarization micrographs in which hybridization signals are visible as white dots. In the invasion zone a low level of CHS mRNA is present but the singal is too low to be visible in **d**. Bar=100μm.
a. A longitudinal section of a 9 day old nodule.
b. Localization of CHS mRNA in the section shown in **a**.
c. A longitudinal section of a 15 day old nodule.
d. Localization of CHS mRNA in the section shown in **c**.

Localization of CHS mRNA in Pea Roots

Fig. 3a and b show a median longitudinal section of the tip of a pea main root hybridized with antisense CHS RNA. CHS mRNA is present at a relatively high level in young root cap cells which are located at the periphery of the root cap, but the cells of columella (K) (Popham, 1955) do not contain CHS mRNA at a detectable level (Fig. 3j,k). A lower level of CHS mRNA is detectable in the ground meristem. No CHS

transcripts are found in the procambium cells (PC) and protoderm cells (P), (Fig. 1i,j; Fig. 3a,b). In older parts of the root CHS mRNA is only detectable in the zone where root hairs emerge. In this zone the expression of the CHS gene is restricted to the epidermal cell layer (Fig. 1m). In tips of lateral roots the CHS gene is expessed in a similar manner as in the main root.However, at a young stage of lateral root development, before vascular differentiation starts, CHS mRNA is present in a continuous region at the tip (Fig. 1k,l).

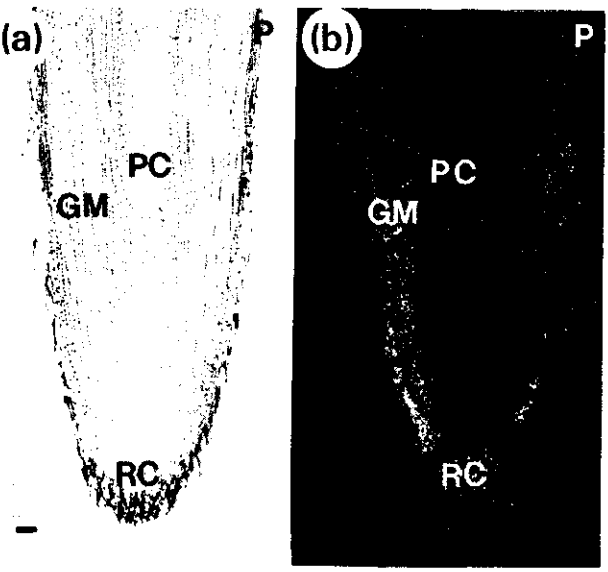

Figure 3 Localization of CHS mRNA in a pea root tip. **a**. Bright field micrograph; **b**. Epipolarization micrograph. Bar=100µm.
a. Median longitudinal section of a pea root apex. GM: ground meristem, P: protoderm, PC: procambium, RC: root cap.
b. CHS mRNA localization in the section shown in **a**.

Pattern of CHS Gene Expression in VG2/VG5 Induced Nodules

Mutants VG2 and VG5 are Tn5 mutants of *R. leguminosarum bv. viciae* strain 248. Both mutants fail to produce O-antigen containing LPS I. Furthermore these mutants do not secrete ß (1-2) glucan. A more detailed description of these mutants is given in Materials and Methods.

The mutants VG2 and VG5 form small white nodules on pea roots that are unable to reduce acetylene. The morphology of these nodules differs markedly from nodules formed by wild type *R. leguminosarum* bv. *viciae* (Figs. 4a,1g). The most striking differences are the occurrence of dark staining thick branched infection threads (Figs. 1g,4a), and the presence of only very few infected cells or even complete absence of infected cells, in the mutant nodules (Figs. 4a). Moreover, dark staining structures are present in these mutant formed nodules (Fig. 4a), which were not observed in wild type nodules. These dark staining structures are packed with small rods (not shown), which

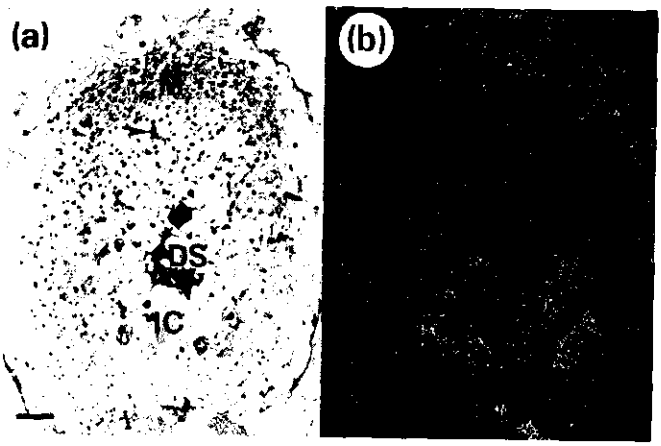

Figure 4. Localization of CHS mRNA in mutant VG2 formed nodules, sections hybridized with ^{35}S-labeled antisense CHS mRNA probe. **a**. Bright field micrograph; **b**. Epipolarization micrograph. Bar=100µm. **a**. Longitudinal section of a 25 day old nodule formed by VG2. Dark staining structure (DS), infected cells (IC), infection thread (arrowhead) and nodule meristem (M) are indicated. **b**. CHS mRNA localization in the section shown in **a**.

could be rhizobia. In 14 day old mutant nodules no infected cells are observed, whereas in 25 day old nodules only a few infected cells are found (Fig. 4a). In contrast in wild type nodules infected cells are already formed in 10 day old nodules.

To determine whether the impaired development of infected cells in the nodules formed by mutant strains VG2 and VG5 might be caused by a defense response, we studied CHS gene expression in these nodules. Longitudinal sections of the mutant formed nodules containing a few (25 days), or no infected cells (14 days) were hybridized with antisense CHS RNA. In both 14 and 25 day old VG2/VG5 nodules the expression of the CHS gene occurs in a zone at the distal part of the nodules. This zone includes a few cortical cell layers, the apical meristem as well as the invasion zone (Fig.4a.d). This is contrast to wild type nodules in which the CHS gene is only expressed at a very low level in the invasion zone. Twenty-five day old VG2/VG5 nodules containing a few infected cells also express the CHS gene in a proximal zone of the nodule (Fig. 4a,b). In the latter zone CHS mRNA is present at a relatively high level in the uninfected cells, whereas a lower level is present in the fully infected cells (Fig. 1 g,h). In 25 day old wild type nodules CHS mRNA is not detectable in infected or uninfected cells (results not shown).

Nodulin Gene Expression in Mutant Nodules

The ability of the VG2 and VG5 mutants to induce pea nodulin gene expression was studied by hybridizing sections of mutant nodules with ^{35}S-labeled antisense probes of nodulin genes. These analysis showed that early nodulin gene transcripts PsENOD12, PsENOD5 and PsENOD3 are present in these mutant nodules (result not shown). In VG2/VG5 nodules containing a few infected cells also the late nodulin gene Lb is expressed in the fully infected cells (Fig. 5).

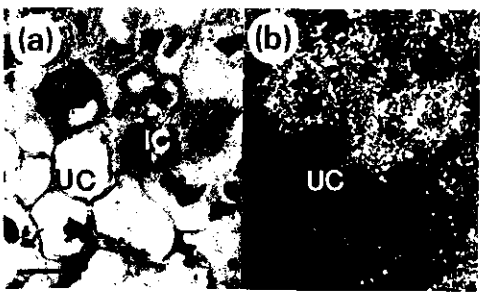

Figure 5. Localization of leghaemoglobin (Lb) mRNA in VG2 formed pea nodule. Bar=50µm.
a. Bright field micrograph indicating infected (IC) and uninfected cells (UC).
b. Epipolarization micrograph of a showing Lb mRNA localization (white dots).

Discussion

CHS Is Involved In Nodule Development

In this paper we studied flavonoid synthesis during pea nodule development by analyzing CHS gene expression *in situ*. Since CHS is the key enzyme in biosynthesis of flavonoids (Hahlbrock and Scheel, 1989), we assume that CHS gene expression can be used as an indirect method to show where flavonoids are synthesized.

We showed that during initial stages of nodule development CHS mRNA is present in all cells of the nodule primordium. Therefore it is very likely that flavonoids accumulate in these dividing cells. Since flavonoids can function as ATI's (Jacobs and Rubery, 1988), we postulate that the *Rhizobium* Nod factor induces cell division in the root cortex by triggering a local accumulation of flavonoids. This accumulation of flavonoids could cause a decreased import of auxin into the primordium cells by which the auxin/cytokinin balance would change in such a way that mitotic activity is induced. At later stages of nodule development, the CHS genes are expressed in a highly spatially controlled manner. In nodules CHS mRNA is predominantly present at the apex, the distal part of the meristem and in a few layers of the nodule cortex. These cells are not infected by *Rhizobium*, which allows the conclusion that also at later stages CHS is involved in nodule development, rather than in a defense response.

Recently, Estabrook and Sengupta-Gopalan (1991) showed by northern blot analysis that during soybean nodule development only certain members of the CHS gene family are induced. Our studies do not provide this type of information since we did not use gene specific probes.

CHS genes are also expressed during root development. In lateral root primordia the CHS transcript is restricted to a few cell layers at the apex. So there is a striking similarity in the spatial distribution of CHS mRNA in a nodule and a lateral root primordiium. Therefore CHS might have a similar role in nodule and root development.

In root tips CHS mRNA is present in two zones, the ground meristem and in young root cap cells. Previously it was shown that a chimaeric gene composed of the bean CHS promotor and the coding region of the GUS gene is expressed in root tips of transgenic tobacco plants (Schmid *et al.*, 1990). These studies did not allow an accurate localization of the GUS activity and so it is unclear whether this chimaeric gene is expressed in the same regions of the root tip as the pea CHS gene.

CHS and *nod* Gene Inducers

Flavonoids play a complex role in nodule development. On one hand they can function as ATI's, whereas they also induce the *nod* genes of *Rhizobium*. Therefore the spatial distribution of flavonoid synthesis determines where *Rhizobium* can produce Nod factors. Consequently, regulation of flavonoid synthesis can be an important tool of the plant to regulate the production of the Nod factors.

In pea roots the CHS gene is expressed in the zone of the epidermis containing developing root hairs. It has been shown that especially this zone of the root secretes *nod* gene inducers (Djordjevic, 1987b; Peters and Long,1988) and so our CHS *in situ* hybridization studies are in agreement with these observations. Van Brussel *et al.* (1990) showed that flavonoid secretion by *Vicia* roots is stimulated upon inoculation with *Rhizobium*, and Recourt (1991) demonstrated that the CHS mRNA level is 1.5 to 2.0 fold increased in these roots. The *in situ* hybridization method is not a very accurate quantitative method and since the increase in CHS mRNA level is rather low we did not compare the amount of CHS mRNA in the epidermis of inoculated and uninoculated roots.

In pea root nodules the highest level of CHS gene expression occurs in a distal zone containing a few cell layers of the nodule cortex and the nodule meristem. In the invasion zone of the wild type nodule very low levels of CHS mRNA are present. Since the *nod* genes are only expressed in this zone where bacteria are released from the infection threads (Sharma and Signer, 1990; Schlaman, *et al.* 1991), we assume that the low level of CHS gene expression is sufficent to produce the flavonoids required for *nod* gene induction.

A Defense Response Is Induced by *Rhizobium* VG2 and VG5 Mutants

The mutants VG2 and VG5 have markedly altered surface properties since they do not form detectable levels of O-antigen containing LPS I and also fail to secrete ß (1-2) glucan. Both mutants form nodules with branched thick infection threads and only a few infected cells are formed. Similar nodule phenotypes have been observed for other *R. leguminosarum* bv. *viciae* LPS mutants (De Maagd *et al.*, 1988, Noel *et al.*, 1986).

Rhizobium ß (1-2) glucan mutants have only been studied in the *R.meliloti*-alfalfa symbiosis. In this system these mutants form nodules but neither infection threads nor intracellular bacteria are present. So the *R.leguminosarum* bv. *viciae* mutants disturb nodule formation at a different stage of development.

Previously we discussed that different classes of *Rhizobium* genes will be involved in the interaction between *Rhizobium* and the legume plant; some genes will contribute to the synthesis of a signal that induces developmental processes in the plant, like the *nod* genes, whereas others are involved in the disguise of *Rhizobium* in order to avoid a defense response of the plant (Nap and Bisseling, 1990b). Plants are able to defend themself against pathogens, but these defense responses are not observed during normal nodule development (Nap and Bisseling, 1990b; Estabrook and Sengupta-Gopalan, 1991; this study).

The aberrant development of VG2/VG5 formed nodules can be due to the inability to produce a signal molecule or to the induction of a defense response. Here we showed that in nodules formed by VG2 and VG5 the CHS gene is expressed at a relatively high level in the invasion zone, the zone where bacteria are released from the infection thread. In wild type nodules only very low levels of CHS mRNA are detectable in this zone. Therefore we postulate that upon release of VG2/VG5 bacteria from the infection threads a plant defense response is induced and this defense response will cause the aberrant development of the infected cells.

In VG2/VG5 nodules containing a few infected cells, the CHS gene is also active in the uninfected cells surrounding these infected cells. Whereas in wild type nodules of the same age CHS mRNA is not dectable in the uninfected cells. In pathogenic plant-microbe interactions CHS gene expression is induced in cells surrounding the cells penetrated by the pathogen. Therefore we assume that CHS gene expression in these uninfected cells also reflects a defense response induced by the *Rhizobium* mutants VG2 and VG5.

25 day old VG2/VG5 formed nodules, containing a few infected cells, express early nodulin genes as well as the late nodulin gene Lb are induced. Consequently these mutants produce all signal molecules that are essential for the induction of expression of nodulin genes, and therefore it is unlikely that putative signal molecules required for the induction of nodulin genes are derived from LPS or ß (1-2) glucans.

Materials and Methods

Characterization of *Rhizobium leguminosarum* bv. *viciae* Mutants VG2 and VG5

All bacteria were grown and maintained on standard laboratory media. *R. leguminosarum* bv. *viciae* strain 248 (Josey, *et al.* 1979) harbors the pRL1JI Sym

plasmid described by Johnston *et al.* (1978). Bacterial matings and Tn5 mutagenesis were performed according to Beringer *et al.*(1978).

R. leguminosarum bv. *viciae* strain 248 was randomly mutagenised with transposon Tn5 and about 6000 mutants were thus obtained. Two of these mutants were used in this study. Here we will give only a short description of the characteristics of these mutants and a more detailed description of these mutants will be published elsewhere. Lipopolysaccharides (LPS) were isolated as described by De Maagd *et al.* (1988), seperated on a SDS-PAGE system (Lugtenberg, *et al.* 1975) and visualized by a silver staining procedure described by Tsai and Frasch (1982). VG2 and VG5 failed to produce the O-antigen containing LPS I. Furthermore, the VG2 and VG5 mutants do not secrete ß (1-2) glucan molecules, whereas the wild type strain does (Canter Cremers, *et al.*, 1991). However, the cells of the two mutant strains, contain a neutral polysaccharide (Batley *et al.*, 1987; Canter Cremers *et al.*, 1991). The ^{13}C NMR spectrum of this polysaccharide isolated from strains VG2 and VG5, was identical to that of neutral ß (1-2) glucan isolated from the culture supernatant of wild type strain 248. We therefore concluded that mutant strains VG2 and VG5 still synthesize ß (1-2) glucan, but fail to secrete it.

Plant Material

Pisum sativum cv. Rondo seeds were germinated and inoculated with *Rhizobium leguminosarum* bv. *viciae* 248 or mutant VG2 or VG5 as described by Bisseling *et al* .(1978).

Preparation of Antisense/sense RNA Probes

A 1.6kb CHS cDNA clone which is identical to the pea CHS2 gene described by Harker *et al.* (1990) was cloned into Bluescript. For antisense RNA probe production, the plasmid was cut with SalI and transcribed with T7 RNA polymerase. For sense RNA preparation the plasmid was cut with BamH1 and transcribed with T3 RNA polymerase. The nodulin antisense/sense RNA probes were prepared according to Scheres *et al.* (1990a,b). All probes were radioactively labelled with [^{35}S]-UTP (1000-1500Ci/mmole, Amersham) and degraded to about 150bp fragments before hybridization (Van De Wiel *et al.*, 1990a).

In Situ Hybridization

Pea roots were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in 50mM sodium phosphate buffer (pH7.2) for 4 hours at room temperature. The preparation of

sections and hybridization conditions are according to procedures described by Cox and Goldberg (1988) (Van de Wiel *et al.*, 1990a).

Microscopy

Sections were stained with 0.025% toluidine blue after one to four weeks exposure at 4°C, and dehydrated and mounted with DPX (BDH). Sections were viewed and photographed with a Nikon microscope equiped with dark field and epipolarization optics.

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Chapter 4

Characterization of *Gm*ENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development

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Abstract

In this paper the soybean "early nodulin" clone p*Gm*ENOD40 is characterized. The *Gm*ENOD40 encoded protein does not contain methionine and does not show homology to proteins identified so far. *In situ* hybridizations showed that this gene has a complex expression pattern, during development of determinate soybean nodules. At early stages of development transcription is induced in dividing root cortical cells, the nodule primordium and the pericycle of the root vascular bundle. In mature soybean nodules, the gene is expressed in the uninfected cells of the central tissue and in the pericycle of the nodule vascular bundles. Studies on nodules devoid of intracellular bacteria and infection threads, showed that the expression of the gene in the nodule primordium is induced in these empty nodules, while the induction of the *Gm*ENOD40 gene in the nodule vascular bundle requires the presence of intracellular bacteria or infection threads. A pea cDNA clone homologous to *Gm*ENOD40 was isolated to enable *in situ* hybridization studies on indeterminate nodules. The expression patterns in both determinate and indeterminate nodules suggests that the ENOD40 protein might have a transport function.

INTRODUCTION

On roots of leguminous plants the formation of highly organized nodules can be induced by soil bacteria of the genera (*Brady*)*rhizobium*. Two types of nodules can be distinguished based on the presence or absence of a persistent meristem: indeterminate or determinate nodules, respectively. Nodules of pea (*Pisum sativum*) and clover (*Trifolium*) species are examples of the indeterminate type whereas the nodules of soybean (*Glycine max*) and bean (*Phaseolus vulgarus*) are of the determinate type. A detailed description of legume root nodule ontogeny and organization is presented in several communications (e.g. Newcomb, 1981; Bergersen, 1982; Calvert, *et al.*, 1984) and an extensive comparison of determinate and indeterminate nodule development is described by Brewin (1992). Here we will confine ourselves to a short description of the formation of the two nodule types. The development of both nodule types begins with the (*Brady*)*rhizobium* induced deformation and curling of root hairs, followed by the formation of infection threads in the curled hairs. The bacteria enter the root through these threads. Concomitantly with the infection process, the bacteria induce cell divisions in the root cortex. In plants on which determinate nodules are formed, cell divisions start in the outer cortical cell layers, whereas in indeterminate nodule formation cell divisions are induced in the root inner cortex. These root cortical cell divisions lead to the formation of nodule primordia and the infection threads grow towards these centers of mitotic activity. After release of bacteria into the plant cells, the primordium differentiates into a root nodule. Both nodule types are composed of a central tissue surrounded by uninfected peripheral tissues. The central tissue contains both infected and uninfected cells and the peripheral tissues (Newcomb, 1981) include the nodule cortex and the nodule parenchyma (Van De Wiel, *et al.*, 1990), separated by the nodule endodermis. The nodule vascular bundles are located in the nodule parenchyma (Van De Wiel, *et al.*, 1990). In determinate nodules the central tissue is separated from the nodule parenchyma by a layer of uninfected cells, the boundery layer (Gresshoff and Delves, 1986).

Legume nodule formation is accompanied by the expression of nodulin (nodule specific) genes (Verma, *et al.*, 1986; Nap and Bisseling, 1990b; Sanchez, *et al.*, 1991). Based on the time point of expression during nodule development, nodulin genes have been divided into early and late nodulin genes. The early genes are already expressed before the actual nitrogen fixation starts, while the expression of the late genes is first detectable around the onset of nitrogen fixation (Govers *et al.*, 1987). Numerous late nodulin genes have been identified. Among them are several genes encoding proteins that are present in the peribacteriod membrane (Fortin, *et al.*, 1987, Jacobs, *et al.*, 1987, Sandal, *et al.*, 1987) and several which encode proteins involved in the carbon, nitrogen, and oxygen metabolism (for review see e.g. Franssen *et al.*, 1992). Only a few early genes have been identified and examples are the pea genes ENOD5 and ENOD12

(Scheres, *et al.*, 1990a and b). The expression of these genes is correlated with the bacterial infection process (Scheres, *et al.*, 1990a and b) . The expression of the early nodulin gene ENOD2 occurs in the nodule parenchyma (Van De Wiel, *et al.*, 1990). None of the early nodulin genes identified so far is expressed in the cortical cells that start to divide as a result of the plant-bacterium interaction.

In this paper we will describe the characterization of a soybean cDNA clone representing a gene specifically induced in dividing root cortical cells. Furthermore we compare the *in situ* expression of this gene in developing soybean (determinate) and pea (indeterminate) nodules.

RESULTS

Isolation and Characterization of p*Gm*ENOD40 Clones

In quest of early processes in nodule development, we searched for nodulin genes markedly expressed before N_2-fixation starts. Therefore a soybean (cv Williams) nodule cDNA library was differentially screened with ^{32}P-labeled cDNA from RNA of nodules from 10-day-old soybean plants and from root RNA. This resulted in 10 cDNA clones hybridizing only to nodule cDNA. Among these clones are the previously described p*Gm*ENOD2, p*Gm*ENOD13 and p*Gm*ENOD55 (Franssen, *et al.*, 1987, 1988).

One of the other nodule specific cDNA clones, p*Gm*ENOD40-1, had an insert of about 300bp. On Southern blots containing HindIII digested soybean DNA, the insert DNA hybridized to 3 fragments (Fig.1a), indicating that there is a small family of ENOD40 genes. On Northern blots *Gm*ENOD40-1 hybridized to an RNA of 700b present at a high level in nodules from 10-day-old plants (7 days after inoculation). *Gm*ENOD40 RNA was found at a similar level in nodules from 14- and 21-day-old plants (Fig.1b). The transcript was not detectable in roots, shoots or leaves but a low level of *Gm*ENOD40 mRNA was observed in stems (Fig.1b). This shows that the *Gm*ENOD40 gene is not a true nodulin gene (Van Kammen, 1984), notwithstanding that it is strongly induced in root nodule tissues formed due to the plant-microbe interaction. Although the *Gm*ENOD40 gene is not a true nodulin gene we will use the term "nodulin" for the sake of convenience. The gene is expressed during nodule development prior to the start of N_2-fixation, which begins at about 14 days, and thus most likely it is not involved in the N_2-fixation process, but probably in the infection process or nodule organogenesis.

To determine in which of these processes the *Gm*ENOD40 gene is involved, we examined *Gm*ENOD40 gene expression in nodules induced by *Bradyrhizobium japonicum* mutant 3160 and *Rhizobium fredii* USDA257, respectively. The nodules produced by both these bacteria are devoid of infection threads and intracellular bacteria (Rossbach, *et al.*, 1989; Franssen, *et al.*, 1987). Expression of the *Gm*ENOD40 gene

was detectable in these nodules (Fig.1c), and this favours a role of the gene in nodule development rather than in the infection process.

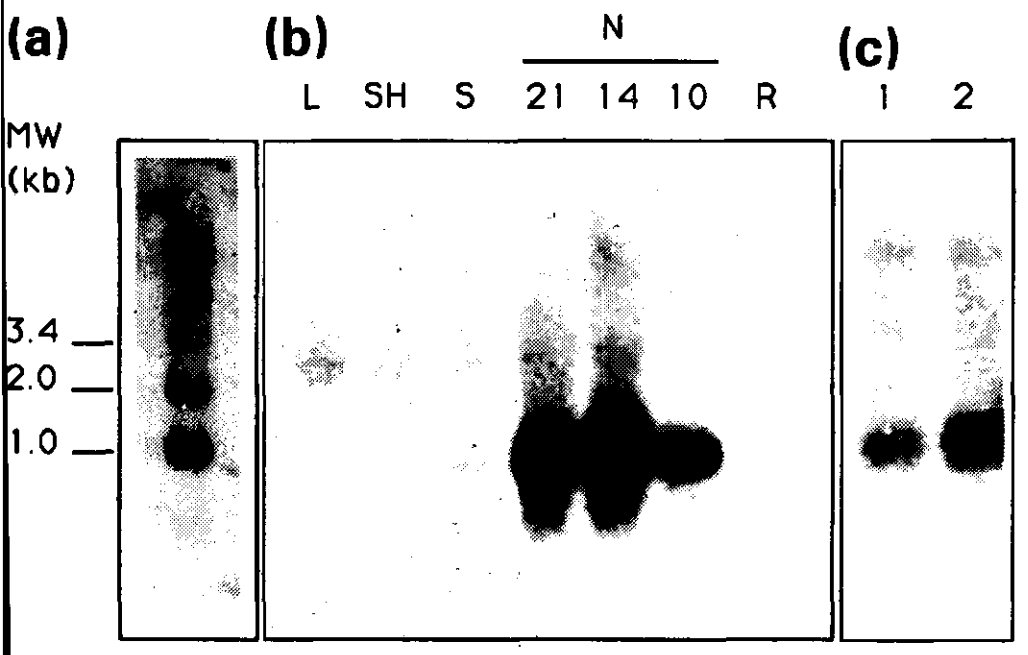

Figure 1. Southern and Northern analysis with p*Gm*ENOD40.
Panel **a**. Autoradiograph of a Southern blot containing 10 micrograms of soybean genomic DNA digested with HindIII.
Panel **b**. Autoradiograph of an RNA transfer blot containing 20 micrograms of total RNA isolated from 3-day-old uninoculated roots (R), nodules (N) harvested at 10, 14, and 21 days after sowing, stems (S), shoots (SH) and leaves (L).
Panel **c**. Autoradiograph of an RNA transfer blot containing 20 micrograms of total RNA isolated from nodules formed by *B. japonicum* mutant 3160 (lane 1) and from nodule-like structures harvested four weeks after inoculation with *R. fredii* USDA257 (lane 2).
All blots were hybridized with ^{32}P-labeled insert DNA from p*Gm*ENOD40-1.

While the p*Gm*ENOD40-1 insert has a length of 300bp, the *Gm*ENOD40 transcript is 700b, and therefore we turned to the isolation of a full-size cDNA clone. Two nodule cDNA libraries, one from RNA isolated from soybean cv Williams and the other from RNA of soybean cv Evans, were screened with ^{32}P-labeled p*Gm*ENOD40-1. This resulted in two cDNA clones with inserts of approximately 700bp , one, designated p*Gm*ENOD40-2, from the cv Williams cDNA library, and the other, p*Gm*ENOD40-3 from the cv Evans cDNA library. Since the length of the inserts of the isolated cDNA

clones was about similar to that of the *Gm*ENOD40 transcript found in nodules, these clones were considered to be usable for further analyses.

Nucleotide Sequences of the *Gm*ENOD40 cDNA Clones

To determine the similarity between the isolated cDNA clones, the nucleotide sequences of the three clones were determined (Fig.2). The PstI insert of p*Gm*ENOD40-2 contained 620 basepairs including a short oligo dA/dT stretch at one end (Fig.2, line b). The sequence of the 300bp insert of p*Gm*ENOD40-1 is identical to the sequence of p*Gm*ENOD40-2 from base 320 to base 620. The EcoRI insert of p*Gm*ENOD40-3 consisted of 670bp also with a short dA/dT stretch at one end. From base 101 to base 653, the nucleotide sequence of this insert is exactly identical to the region of p*Gm*ENOD40-2 from base 1 to base 553 (Fig.2, line a), Further comparison made it clear that the insert of p*Gm*ENOD40-2 is 47bp longer at its 3' end and 100bp shorter at its 5' end than that of p*Gm*ENOD40-3.

We determined the exact size of the *Gm*ENOD40 transcript by primer extension on RNA isolated from nodules of 14-day-old plants (cv Williams) using an oligonucleotide complementary to the sequence between base 40 and 60 of p*Gm*ENOD40-2. This sequence occurs in both cDNA clones. Only one extension product with the size of 160b was detected (data not shown). The single extension product indicated that *Gm*ENOD40-3 is a full size clone, while *Gm*ENOD40-2 is missing 100bp at its 5' end. Subsequently, we showed by reverse transcriptase-PCR experiments (data not shown), that the difference between the 3' ends of *Gm*ENOD40-2 and *Gm*ENOD40-3 is cultivar related and is not due to the excistence of two different ENOD40 transcripts.

Analysis of open reading frames of p*Gm*ENOD40-2/3 .

The longest open reading frame (ORF) that can be derived from the nucleotide sequences of the inserts of both p*Gm*ENOD40-2 and p*Gm*ENOD40-3 encodes a polypeptide of 93 amino acids, containing no methionine. We investigated whether this ORF, positioned between base 106 and 385, could be translated despite the absence of a methionine as a translational start. For that purpose we made a translational fusion between the CaMV PI gene and p*Gm*ENOD40 .This CaMV PI gene has been used for

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Figure 2. Nucleotide sequences of the EcoRI insert of p*Gm*ENOD40-3 (line A) and PstI insert of p*Gm*ENOD40-2 (line B). In the DNA sequences, nucleotides are numbered to the right of the sequence. Dots indicate identical nucleotides in both DNAs. The predicted amino acid sequence of the longest ORF is shown in standard single-letter code above the nucleotide sequence. The amino acids are positioned above the second nucleotide of the triplet. The termination code is indicated with (*).

```
A  CGCTAAACCAATCTATCAAGTCCTGATTAATCTGGTGAGCATGGAGCTTT  50

A  GTTGGCTCACAACCATCCATGGTTCTTGAAGAAGCTTGGAGAGAAAGGGG 100
B

        E  E  R  V  L  T  P  H  T  P  S  L  K  T  V   15

A  TGTGAGAGGAGAGGGTGCTCACTCCTCACACTCCCTCACTTAAAACAGTT 150
B  ..................................................  50

     C  F  G  L  A  L  A  S  L  I  N  K  G  C  V  L  T  32

A  TGTTTTGGCTTAGCTTTGGCTTCTCTGATCAACAAGGGATGTGTTCTAAC 200
B  ..................................................  100

      F  F  L  E  W  R  K  Q  I  H  I  L  R  R  R  R    48

A  ATTCTTTCTTGAGTGGCGGAAGCAGATACACATTCTCCGACGGAGGAGAG 250
B  ..................................................  150

     G  L  A  T  A  W  Q  T  G  K  S  Q  K  R  Q  W  T   65

A  GCTTGGCTACAGCCTGGCAAACCGGCAAGTCACAAAAAAGGCAATGGACT 300
B  ..................................................  200

      P  L  G  S  L  W  L  C  S  A  H  V  V  L  L  A  V  82

A  CCATTGGGGTCTCTATGGCTATGTAGTGCTCATGTAGTTCTTCTTGCTGT 350
B  ..................................................  250

      E  C  N  N  K  Q  S  W  S  S  F  *                 93

A  AGAATGTAATAATAAACAAAGTTGGTCTTCCTTTTGAGAAGTTACCAGCT 400
B  ..................................................  300

A  TTTGCTGTCCAAAATTACTCAATTTGCAGCTGACTAGAATTCCTTTCTCT 450
B  ..................................................  350

A  CTTCAGTTTCTGCAGATGAGTAGGTAGGCAATTTGTGATCACTCCCTTCC 500
B  ..................................................  400

A  CTTTTCATGTCTTCTGTGTTCCCTTTTCCATGCTTGTTTGTGTTGTTAGT 550
B  ..................................................  450

A  TATGACCTTATGAGGAAATAAAAGAATAGTACAATTCTAGTCCCTCAGTT 600
B  ..................................................  500

A  TAGGATTGTATTCTATTGAACTTTATTAGAAAAGTTTCCAGAGTCCTTTC 650
B  ..................................................  550

A  TAAAAAAAAAAAAAAAAAAA                               670
B  ...GGTTGGAGTGAATAATGTTCATGATCCCTCACCCTTTCCCTTTAAAA 600

B  AAAAAAAAAAAAAAAAAAAA                               620
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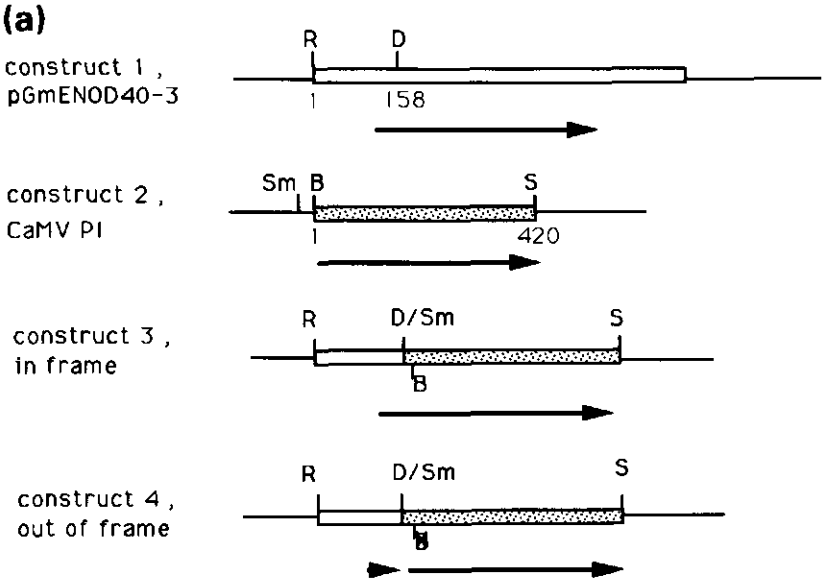
comparable purposes (Verver *et al.*, 1991) and the availability of antibodies directed against the PI protein allows detection of PI peptide sequences in fusion proteins. The 163bp EcoRI-DdeI fragment of p*Gm*ENOD40-3 was cloned in-frame to a 420bp SmaI-SacI fragment of the CaMV PI gene in pBluescript (construct 3, Fig.3a). The out-of-frame construct (construct 4) was generated from construct 3 by cutting the DNA with BamH1 and subsequent filling in of the BamH1 site and religation of the plasmid.

Translation of RNA from CaMV PI DNA (construct 2) resulted in the synthesis of a polypeptide of 14 kDa (marked a; Fig. 2, lane 2) corresponding to the expected size of CaMV PI protein fragment. Indeed the 14 kDa protein was precipitable with PI antibodies. Translation of construct 3 derived RNA yielded two polypeptides with apparent molecular weights of approximately 20 and 30 kDa (marked b and c; Fig.3, lane 3), respectively . The size of the smallest polypeptide is slightly bigger than the predicted size of the chimaeric polypeptide of PI and *Gm*ENOD40-3. Why a second polypeptide is synthesized is unclear. Both the 20 and 30 kDa polypeptides contain PI sequences since both proteins can be precipitated with PI-antibodies (Fig.3, lane 5). In contrast, RNA from the out-of-frame construct 4 (Fig. 3, lane 4) resulted in the 14 kDa protein, precipitable with the PI-antibody. Moreover, RNA transcribed from the p*Gm*ENOD40-3- (construct 1) was not translated into a radiolabeled polypeptide (Fig. 3, lane 1). The protein band present in the upper part of the gel is also present in the absence of exogenously applied RNA (Fig. 3, lane -). Therefore we conclude that the ORF identified in p*Gm*ENOD40-2/3 can be translated *in vitro* although the exact position of this start codon within p*Gm*ENOD40-3 remains unclear. The identification of a cDNA clone homologous to *Gm*ENOD40-3 that contains an ORF homologous to the ORF in p*Gm*ENOD40-3, but starting with an AUG codon (Kouchi, pers. comm.) supports that the identified ORF of p*Gm*ENOD40-3 codes for the *Gm*ENOD40 protein.

The putative *Gm*ENOD40 protein (Fig. 2) has a hydrophilic nature and lacks an N-terminal signal peptide. Therefore, it is most likely a soluble protein. Data base searches with the *Gm*ENOD40 protein did not reveal any significant homology to known proteins but within the amino-terminal part occur several consensus sequences identified as potential phosphorylation sites for various protein kinases, like TPSLK for cdc2 (Sun, *et al.*, 1991), TPHT for the cdc2 related ERK1/2 (Gonzalez, *et al.*, 1991) and SLK for protein kinase C (Sun, *et al.*, 1991). The presence of these sequences indicates that the *Gm*ENOD40 protein might be post-translationally phosphorylated.

In Situ Localization of *Gm*ENOD40 mRNA in Developing Soybean Nodules

*Gm*ENOD40 mRNA was localized in soybean roots at different time points after inoculation with *B. japonicum* USDA110 to determine where and when the gene is



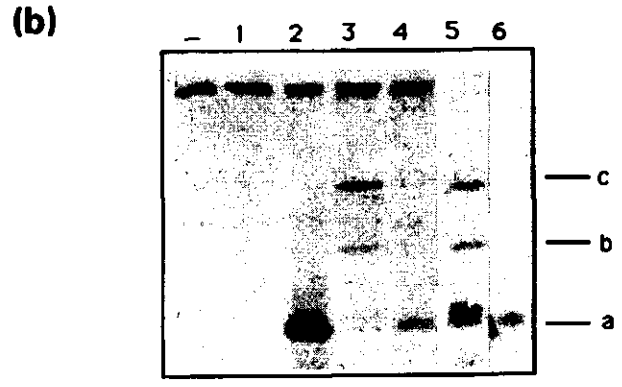


Figure 3. Open reading frames analysis on RNA derived from p*Gm*ENOD40-2/3.
Panel **a**. Schematic presentation of p*Gm*ENOD40 (construct 1), CaMV PI DNA (construct 2) and the in-frame and out-frame constructs between *Gm*ENOD40 and CaMV PI. The positions of the restriction sites for enzymes used in the cloning are indicated.(R= EcoRI; D= DdeI; Sm= SmaI; B= BamHI; S= SacI). The bold arrows indicate the ORFs in the different clones.
Panel **b**. *In vitro* translation products of RNA transcribed from p*Gm*ENOD40-3 (lane 1), CaMV PI gene (lane 2), the in-frame construct (lane 3) and the out-frame (lane 4) construct of p*Gm*ENOD40-3 and the CaMV PI gene. Translation products obtained in the absence of exogenously applied RNA is indicated in lane -.Immunoprecipitations with antibodies against the CaMV PI gene encoded protein of *in vitro* translation products of RNA from constuct 3 or 4 are shown in lanes 5 and 6, respectively. The RNAs were translated in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine. Proteins were separated on a 15% SDS-polyacrylamide gel. At the right, a refers to the position of the 14kDa PI protein and b and c to the extension products generated after translation of RNA transcribed from constuct 3 DNA.

expressed during nodule formation. One day after inoculation the first cell divisions are induced in the subepidermal cell layer (Fig.4, a and b). Hybridizations with ^{35}S-labeled antisense *Gm*ENOD40 RNA showed that *Gm*ENOD40 expression is induced in the dividing root cortical cells. Three days after inoculation a small nodule primordium, composed of small cells with a high cytoplasmic density, is formed. The infection thread has passed the root hair and has reached the primordium cells, where bacteria are released (Fig.4c, d and 4e, which is a magnification of c). At this stage of development, cell divisions also occur in the inner layers of the root cortex (Fig.4c). In cross sections of soybean roots three days after inoculation hybridized to antisense *Gm*ENOD40 RNA, *Gm*ENOD40 mRNA was detected in all cells of the primordium. However, the number of silver grains in the cells containing released bacteria was significantly lower than in the primordium cells containing no bacteria (Fig.4e). Strikingly, *Gm*ENOD40 RNA is present at a markedly lower level in the root hair containing the infection thread than in the adjacent uninfected root hair (Fig.4e; hairs are indicated by arrows). This observation indicates that the *Gm*ENOD40 gene is switched off after infection by bacteria. The *Gm*ENOD40 gene was also expressed in the dividing inner cortical cells but

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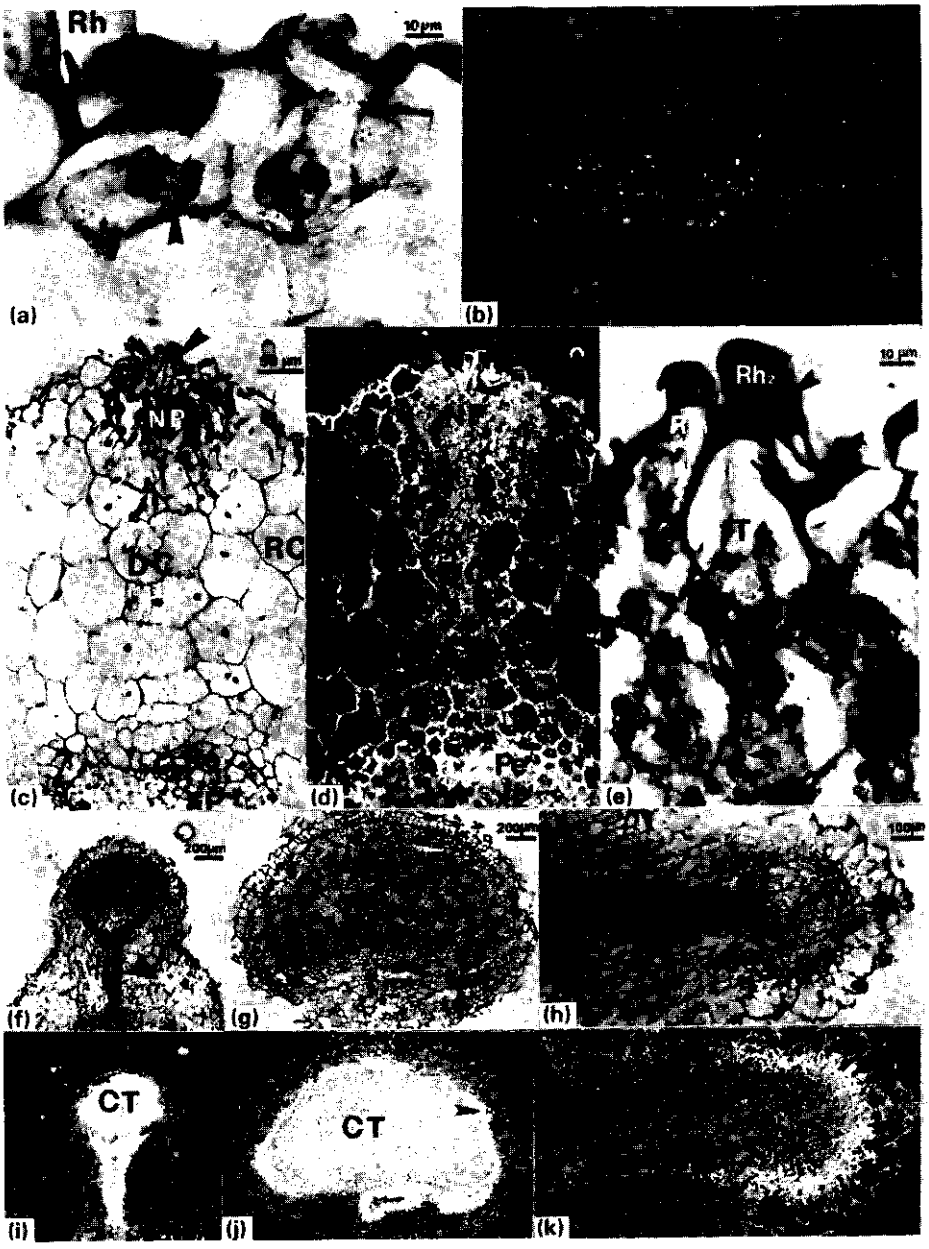
Figure 4. In situ localization of *Gm*ENOD40 mRNA during soybean nodule development.
In situ localization of *Gm*ENOD40 mRNA during soybean nodule development. DC=dividing root cortical cells; CT=central tissue; IT=infection threads; NP=nodule primordium; Pe=pericycle; RC=root cortex; Rh=root hairs and XP=xylem pole. **a**, **c**, **e**, **f**, **g** and **h** are bright field micrographs where signals are visible as black dots. **d**, **i**, **j** and **k** are dark field micrographs where signals are represented by white dots. **b** is an epipolarization micrograph in which white shining dots are signal.
a and **b**. Two dividing subepidermal cells of a 4-day-old root (one day after inoculation) hybridized with ^{35}S-labeled antisense *Gm*ENOD40 RNA are shown (arrowheads), and part of a root hair is visible (Rh).
c and **d**. Section of 6-day-old soybean root (3 days after inoculation) showing a nodule primordium (NP) formed in the outer root cortex, and the dividing root inner cortical cells (DC) connecting the nodule primordium and root stele. An infection thread (small arrowhead) is visible in the infected root hair cell (big arrowhead). **d** is hybridized with the antisense *Gm*ENOD40 RNA probe showing the expression of this gene in uninfected root hair cell (arrow), nodule primordium (NP), dividing root cortical cells and at a high level in root pericycle (Pe) near one of the xylem poles (XP).
e. Magnification of a nodule primordium (NP) shown in **c** showing low expression of *Gm*ENOD40 in root hair (Rh_2) and primordial cells penetrated by infection thread (IT, arrow)), and higher expression in uninfected root hair (Rh_1) and primordial cells.
f and **i**. Section of a 10-day-old nodule hybridized with ^{35}S-labeled antisense *Gm*ENOD40 RNA shows the localization of this gene transcript (black dots in **f** and white dots in **i**) in the nodule central tissue (CT) and pericycle of the connecting vascular bundle.
g and **j**. Section of a 16-day-old nodule shows the organization of a nitrogen-fixing nodule and the localization of this gene transcript in boundary layer (**j**, arrowhead), pericycle of vascular bundle (**j**, arrow) and uninfected cells in central tissue (CT) (for details see Figure 5, **i** and **j**).
h and **k**. The section of an empty nodule formed by *Bradyrhizobium japonicum* mutant 3160 shows a vascular tissue surrounded sclerified cell layer (Sc) and small cells with big nucleus at the tip (arrowhead). This section was hybridized with the same probe as in **c** and **d** showing the expression of *Gm*ENOD40 in the small cells at the tip (arrowhead)


Rh
10 μm
(a)
(b)
NP
RC
bc
(c)
(d)
Rh
T
(e)
(f)
(g)
(h)
CT
CT
(i)
(j)
(k)

the highest level of expression was detected in the root pericycle opposite the nodule primordium. In general nodule primordia are induced opposite one of the xylem poles. Indeed in most cases, the expression of the *Gm*ENOD40 genes was restricted to the part of the pericycle opposite the xylem pole (result not shown). In the exceptional case that a primordium was induced opposite one of the phloem poles the expression of the *Gm*ENOD40 gene extended from the pericycle region opposite the phloem pole to a region opposite a xylem pole. Such an asymmetric pattern of expression in the pericycle is shown in Figure 4c and d.

Expression of the *Gm*ENOD40 gene could not be detected in meristems of lateral roots or shoots (data not shown). In stems the expression is restricted to the cells in the phloem (data not shown).

Seven days after inoculation, the nodule primordium has differentiated into a central region in which the first infected cells can just be recognized, surrounded by the peripheral tissues. The dividing inner cortical cells are now differentiated into a vascular bundle connecting the central tissue and the root stele. *In situ* hybridization showed that the *Gm*ENOD40 gene is expressed in the central tissue especially in the uninfected cells forming the boundary layer and in the cell layer surrounding the connecting vascular bundle (Fig.4f and i). At this stage of development the *Gm*ENOD40 gene is no longer expressed in the root pericycle. In nitrogen-fixing nodules from day 14 onward the expression pattern does not change compared to seven-day-old nodules (Fig.4g and j). A magnificaton of a hybridized section of a nodule of a 16-day-old plant shows that *Gm*ENOD40 mRNA is present in the boundary layer and at a lower level in the uninfected cells of the central tissue (Fig.5i and j). No *Gm*ENOD40 transcript is detectable in the infected cells (Fig.5i, j and d). *Gm*ENOD40 mRNA is present in the connecting vascular bundle (Fig.4f and i) and in vascular bundles surrounding the central tissue (Fig.4g and j; Fig.5a and b). Soybean nodule vascular bundles have an amphicribal organization since the phloem completely surrounds the xylem (Fig.5a and b). The endodermis is the cell layer, that surrounds the vascular bundle and is characterized by the presence of Casparian strips (arrow heads). The pericycle is located between the endodermis and xylem. As is shown in Fig.5a and b, *Gm*ENOD40 gene expression is restricted to the pericycle. A longitudinal section through a developing nodule vascular bundle (Fig.5c) showed that the *Gm*ENOD40 gene is first expressed when the procambial cells differentiate into vascular tissue. Therefore, the induction of this gene is a relatively late step in the formation of these vascular bundles. In summary: The expression of the *Gm*ENOD40 gene starts in the dividing cortical cells and when nodule primordium cells become infected, the level of *Gm*ENOD40 RNA decreases, reflecting that the gene is most likely switched off. Finally, in the central tissue of a mature nodule the expression of this gene is restricted to the uninfected cell type. In root

Figure 5. Localization of *Gm*ENOD40 and *Ps*ENOD40 mRNA during soybean and pea nodule development, respectively. B=boundary layer; CT=central tissue; E=endodermis; IN=infected cell; NC=nodule cortex; NP=nodule primordium; Pe=pericycle; Ph= phloem and N= nodule parenchyma. **a**, **c**, **d**, **e**, **f**, and **i** are bright field micrographs where signals are visible as black dots. **b** is an epipolarization micrograph where signals are white shining grains. **g**, **h** and **j** are dark field micrographs in which signals are represented by white dots.

a and **b**. Detail of a longitudinal section of soybean nodule vascular bundle hybridized with ^{35}S-labeled antisense *Gm*ENOD40 RNA shows the expression of this gene in pericycle (Pe). No expression was detected in endodermis (E) characterized by Casparian strips (arrowheads), phloem (Ph) and xylem (X).

c. Longitudinal section of a nodule vascular bundle showing the localization of *Gm*ENOD40 mRNA in differentiated pericycle cells but not in procambial cells (arrow). Expression of the *Gm*ENOD40 is also seen in boundary layer (BL) and central tissue (CT).

d. Detail of a nitrogen-fixing nodule (16-day-old) shows the localization of *Gm*ENOD40 mRNA represented by black silver grains (arrowhead) in uninfected cells (UC). No signal is detectable above background in infected cells (IC).

e and **h**. Cross section of a pea root 5 days after inoculation shows the presence of two nodule primordia (NP), and the expression of the *Ps*ENOD40 in these primordia (NP) and root pericycle (arrowheads).

f and **g**. Cross section of a pea root 2 days after inoculation shows a nodule primordium (NP) and an infection thread, and the localization of *Ps*ENOD40 mRNA in the primordia (NP) and the low level of expression in cells in front of the infection thread (arrowhead). Root pericycle is indicated by arrow.

i and **j**. Detail of Figure 4**g** showing the different tissues of a soybean nodule, and the localization of *Gm*ENOD40 mRNA in boundary layer (BL) and uninfected cells (UC). No expression of this gene was detectable in nodule cortex (NC), endodermis (E), nodule parenchyma (N) and infected cells (IC).

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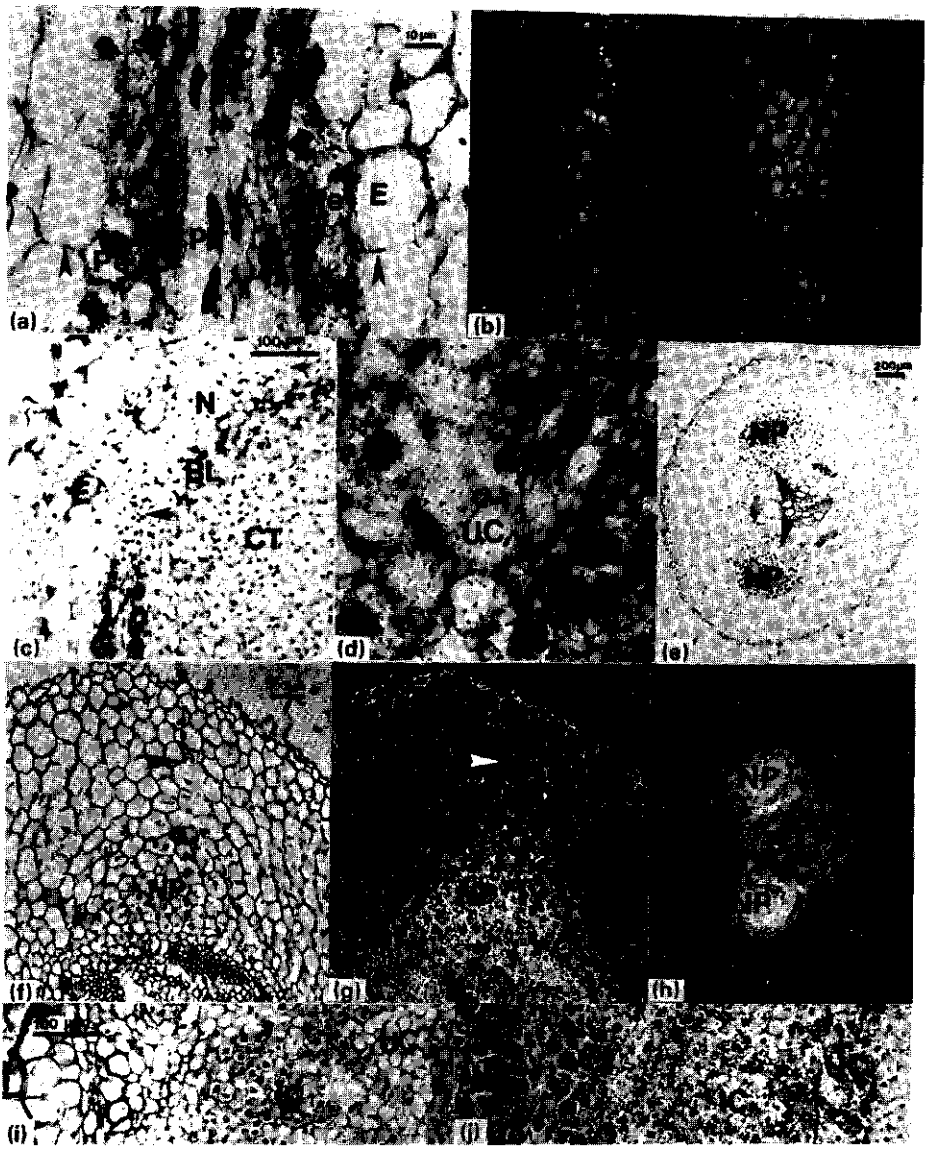
nodules the *Gm*ENOD40 gene is also induced in the pericycle of the nodule vascular bundles.

*Gm*ENOD40 Gene Expression in Empty Soybean Nodule

Since the induction of *Gm*ENOD40 gene expression in dividing root cortical cells is separated in both time and space from that in the pericycle of nodule vascular bundles, we postulate that different mechanisms control the expression of the *Gm*ENOD40 gene in these tissues. To test this hypothesis we analysed the *in situ* expression of the *Gm*ENOD40 gene in so-called empty soybean nodules formed by *B. japonium* mutant 3160. These nodules contain neither intracellular bacteria nor infection threads (Rossbach, *et al.*, 1989). These empty nodules contain at the distal part relatively undifferentiated cells resembling nodule primordium cells and in the proximal part a central vascular bundle (Fig.4h and k). Sections of these empty nodules were hybridized with antisense *Gm*ENOD40 RNA (Fig.4h and k). *Gm*ENOD40 mRNA was found in the cells at the distal part of the nodule, but not in the pericycle of the fully differentiated vascular bundle. Apparently, the induction in the distal region resembling the nodule primordium does not require intracellular bacteria, while the induction of *Gm*ENOD40 in the pericycle requires the presence of intracellular bacteria.


(a)
E
(b)
N
BL
CT
(c)
UC
(d)
(e)
(f)
(g)
(h)
(i)
(j)

*In Situ Ps*ENOD40 Gene Expression During Pea Nodule Development

Since the ontogeny of determinate and indeterminate legume nodules is different, we have examined whether a gene homologous to the soybean *Gm*ENOD40 gene is expressed in indeterminate pea nodules. Northern blot analysis of RNA from pea nodules showed that in pea nodules a ENOD40 transcript of the same size as the *Gm*ENOD40 RNA was present (data not shown).

Screening of the pea nodule cDNA library resulted in the isolation of 4 clones homologous to *Gm*ENOD40-3. Comparison of the nucleotide sequence of one of these pea clones and p*Gm*ENOD40-3 revealed 78% homology over 300bp that were sequenced, indicating that the isolated pea cDNA clone represents *Ps*ENOD40.

To determine the localization of *Ps*ENOD40 gene expression, sections of developing pea root nodules were hybridized to antisense *Ps*ENOD40 RNA (Fig.5e,f,g and h). Two days after inoculation, a nodule primordium is formed in the root inner cortex. In Fig.5f and g a section of such a root is shown and parts of infection threads migrating through the root cortex towards the nodule primordium are visible (Fig.5f and g). The position of the infection thread tip, indicated by an arrowhead, shows that the infection thread has not yet reached the primordium. At this developmental stage the *Ps*ENOD40 gene is expressed in all nodule primordium cells and a low level of expression is detectable in the root pericycle. Five days after inoculation, expression of *Ps*ENOD40 in the root pericycle is stronger while expression in the nodule primordium remains the same (Fig.5e and h). In mature pea nodules the *Ps*ENOD40 gene is expressed in the pericycle of the nodule vascular bundle (data not shown). Therefore, the expression pattern of the ENOD40 gene during pea and soybean nodule development is similar.

DISCUSSION

In this paper we have described the characterization of the soybean "early nodulin" cDNA clone p*Gm*ENOD40. The *Gm*ENOD40 gene has a complex pattern of expression during nodule development. At early stages of development the *Gm*ENOD40 gene is induced in root cortical cells which divide into a root nodule primordium. Furthermore, the *GmENOD*40 mRNA is also found in parts of the root pericycle opposite the nodule primordia. At later stages of development the *Gm*ENOD40 gene is expressed in the pericycle of the nodule vascular bundle as well as in the uninfected cells of the boundary layer and the central tissue. In non-inoculated roots expression of the gene was not observed neither by Northern blot analysis, and reverse transcription-PCR experiments nor by *in situ* hybridization studies, showing that the expression in the root pericycle is induced by *Rhizobium*.

The expression pattern of the *Gm*ENOD40 gene in *B. japonicum* mutant 3160 induced nodules showed that the induction of this gene in nodule primordia does not require an infection process, whereas *Gm*ENOD40 gene expression in the nodule vascular bundle needs infection. The induction of expression of the *Gm*ENOD40 gene in nodule primordia resembles the induction of early nodulin genes such as ENOD2 and N-40' (Franssen, *et al.*, 1987; Moerman, *et al.*, 1987; Dunn, *et al.*, 1988; Govers, *et al.*, 1990; Van de Wiel, *et al.*, 1990), in that the *Gm*ENOD40 gene is induced early in the interaction and does not require the presence of intracellular bacteria or infection threads. In contrast, the transcription of the *Gm*ENOD40 gene in the pericycle of the nodule vascular bundle requires infection and is induced at a relatively late stage of nodule development and as to that resembles expression of late nodulin genes. Thus, the induction of expression of the *Gm*ENOD40 gene in nodule primordium and vascular bundle is separated in time and space, and the induction of expression at these two sites has different requirements with respect to the presence of intracellular bacteria. At present little is known about the mechanisms by which *Rhizobium* regulates nodulin gene expression, but recent studies show that the expression of early nodulin genes *Ps*ENOD5 and *Ps*ENOD12 can be induced with purified Nod factors (Bisseling *et al.*, 1992). Since Nod factors can provoke the formation of nodule primordia (Truchet *et al.*, 1991; Spaink *et al.*, 1991), it seems likely that the expression of the ENOD40 gene can also be elicited with these compound. Therefore, the ENOD40 clones might be usefull tools to study the molecular mechanisms by which Nod factors elicit plant responses. However, since the bacterial *nod* genes are not expressed in mature nodules (Schlamann *et al.*, 1991), it is unlikely that the Nod factors are part of the mechanism controling the ENOD40 gene expression in the mature nodule. Presumably other *B. japonicum* derived signal compounds or physiological conditions, created by the presence of intracellular bacteria, will induce the expression of the *Gm*ENOD40 gene in mature nodules. Together with the n-uricase gene (Bergmann *et al.*, 1983; Newcomb *et al.*, 1990), the ENOD40 genes are so far the only examples of plant genes expressed in uninfected nodule cell types and for both genes the presence of intracellular bacteria is required to become activated. A further similarity with the n-uricase gene expression is that the *Gm*ENOD40 gene expression is also not controled by a metabolite resulting from the nitrogen fixation process, since the *Gm*ENOD40 gene is expressed in nodules formed by a *B.japonicum nif*A mutant (Fischer, *et al.*, 1986; result not shown).

A striking feature of the *Gm*ENOD40 protein is the absence of a methionine residue. In two cDNA clones, isolated from two different cDNA libraries made from RNA from two different soybean cultivars, the longest ORF did not contain a codon for methionine, while AUG codons that might serve as start codons are rapidly followed by stop codons. Therefore, we think that the longest identified ORF represents the amino acid sequence for the *Gm*ENOD40 protein. This conclusion is supported by the identification of a

soybean cDNA clone homologous to *Gm*ENOD40 containing a putative start AUG in an ORF otherwise very similar to the long ORF in p*Gm*ENOD40-2/3 (Kouchi, pers. comm.). In eucaryotes (e.g. Hahn *et al.*, 1987; Yanofsky *et al.*, 1990) and procaryotes (e.g. Kozak ,1983) several genes have been identified in which AUG does not serve as a translation start. In eucaryotes, no specific alternative start codon is used instead of AUG. However, GUG (valine) is preferentially used as a start codon for translation in procaryotic genes when an AUG is lacking. Within the putative *Gm*ENOD40 ORF, GUG is the fourth codon. Therefore it is tempting to speculate that this GUG encodes the N-terminal amino acid of the *Gm*ENOD40 protein. Downstream of this valine several potential phosphorylation sites are present. The potential phosphorylation site for the cell cycle regulating protein $p34^{cdc2}$ is especially of interest, since the *Gm*ENOD40 gene is expressed in dividing cortical cells. The *Gm*ENOD40 protein might be phosphorylated at this site in dividing cells (the nodule primordium), but not in non-dividing cells (uninfected cells). That would provide a mechanism to regulate the activity of the protein in the different tissues.

Since the *Gm*ENOD40 protein is not homologous to any previously described protein, the sequence does not provide a clue to its function. Hence, speculations as to the possible function of *Gm*ENOD40 can only be based on the *in situ* expression pattern. In determinate nodules the uninfected cells form a network and it has been proposed that this network is involved in the transport of metabolites (Pate, *et al.*, 1969). Moreover, it has been postulated that the pericycle of a vascular bundle has a function in transport since it can have a role in loading and unloading of the vascular tissue (Pate, *et al.*, 1969). The *in situ* expression pattern of the *Gm*ENOD40 gene might suggest a role of this "nodulin" in a transport process. Whether the *Gm*ENOD40 protein might also have a similar transport function in the nodule primordium is unclear. Such a role is not suggested by the expression of *Gm*ENOD40 in the developing nodule primordium, but is not necessarily contradicted by that.

The *Gm*ENOD40 gene is not a true nodulin gene since it is also expressed in the stem at a low level. In this respect the *Gm*ENOD40 gene resembles the pea early "nodulin" gene *Ps*ENOD12 (Scheres, *et al.*, 1990b) and the bean "nodule specific" glutamine synthetase gene (Cock, *et al.*, 1991). Neither gene is expressed in uninfected root tissue but a low level of expression of *Ps*ENOD12 is found in stem and flower whereas, the "nodule specific" glutamine synthetase gene is expressed in stem and hypocotyls. Sprent has proposed "that the ancestral nodule may have been formed on stems of legumes growing in marshy areas" (Sprent, 1990), suggesting a stem origin of root nodules. The observation that some "nodule specific" genes are not expressed in roots but are transcribed in stems is consistent with such hypothesis. Furthermore, this observation supports the idea that nodulins are derived from plant genes, which normally encode proteins involved in non-symbiotic processes in plants (Nap and Bisseling, 1990b). The

results on *Ps*ENOD12, n-GS, and *Gm*ENOD40 gene expression show that the original nodulin definition has partly lost its value to classify plant genes induced by *Rhizobium* in legume roots and it becomes clear that the definition to describe these genes might even have to be reassessed.

EXPERIMENTAL PROCEDURES

Growth Condition for Plants and Bacteria

Soybean plants (*Glycine max* (L) Merr. cv. Williams) were cultured at 28°C as described by Gloudeman *et al.* (1987). Soybean seeds were inoculated at the day of sowing (day 0) with *B. japonicum* USDA110, *B. japonicum* mutant 3160 (a generous gift of Hennicke and Rossbach; Rossbach, *et al.* 1989) or *R. fredii* USDA 257 (Franssen *et al.*, 1987). Plants used for *in situ* hybridization were inoculated 3 days after sowing. The bacteria were cultured as described by Bhuvareswari *et al.* (1980). Pea seeds (*Pisum sativum* (L) cv. Rondo) were sown in gravel and grown as described by Bisseling *et al.* (1978). The seeds were inoculated at day 3 with *R. leguminosarum* bv *viciae* strain 248, Plants were grown as described by Josey *et al.* (1979).

Isolation of Nucleic Acids

Total RNAs from plant tissues were extracted as described by Govers *et al.* (1985) and polyA$^+$ RNA was obtained by oligo(dT) cellulose chromatography (Sambrook, *et al.*, 1989). Plasmid DNA was isolated by the alkaline lysis method (Sambrook, *et al.*, 1989) and phage DNA by the plate lysis method (Sambrook, *et al.*, 1989).

Construction and Screening of cDNA Libraries

A cDNA library was constructed in pBR322 from polyA$^+$ RNA isolated from 10-day-old soybean plants (cv. Williams) as described by Franssen *et al.* (1987). A nodule cDNA library of soybean cv Evans in lgt10 was a kind gift of Dr. K. Marcker (University of Aarhus, Denmark) and a lgt11 pea nodule cDNA library was a kind gift of Dr. G. Coruzzi (Rockefeller University, New York, USA). Probes for the differential screening of the cv Williams library were prepared from poly A$^+$ RNA from 5-day-old soybean root segments and from nodules on 10-day-old plants, using 10 µCi a ^{32}P-dATP (specific activity 3200 Ci/mmol, New England Nuclear) as radioactive tracer, as described by Franssen *et al.* (1987). Both lgt10 and lgt11 nodule libraries, were screened with ^{32}P-labeled *Gm*ENOD40 DNA.

DNA Sequencing

The inserts of the isolated cDNA clones were subcloned into pBluescript vector (Stratagene Inc.) using standard techniques (Sambrook, *et al.*, 1989). The nucleotide sequences were determined using the chemical degradation method of Maxam and Gilbert (1980) and the dideoxy chain termination method (Sanger, *et al.*, 1977). Data were stored and analysed by programs written by Staden (1984) on a micro VAX/VMS computer.

RNA and DNA Transfer Blot Analysis

Total RNA was denatured in DMSO/glyoxal and electrophoresed on 1% agarose gels (Sambrook, *et al.*, 1982). The RNA was transfered to GeneScreen (New England Nuclear) filters and was bound to the filters by 1 minute illumination with UV light of 254 nm (Church and Gilbert, 1984). DNA was electrophoresed on 1% agarose gels (Sambrook, *et al.*, 1989) and transferred to GeneScreen plus (New England Nuclear) filters in 0.4M NaOH/0.6M NaCl. Hybridization and washing steps were performed according to the GeneScreen and GeneScreen plus manuals. ^{32}P-labeled DNA probes were obtained by random priming.

Construction of Translation Fusions of p*Gm*ENOD40-3 and CaMV PI Gene

The plasmid containing a BamHI-ClaI fragment of the CaMV PI gene in pBluescript (pBSgI, Verver *et al.*, 1991) was a kind gift of D. Zuidema (Department of Virology, Agricultural University of Wageningen). A translational fusion (construct 3, fig.3A) of p*Gm*ENOD40-3 and CaMV PI gene was constructed by ligating a 163bp EcoRI-DdeI fragment of p*Gm*ENOD40-3 and a 420bp SmaI-SacI fragment of the CaMV PI gene into pBluescript, cut with EcoRI and SacI (Sambrook, *et al.*, 1989). The DdeI site was filled in by Klenow DNA polymerase (Promega). The obtained linearized DNA molecules were circularized by blunt ligation (Sambrook, *et al.*, 1989). The out-frame construct (construct 4, fig.3A) was obtained by cutting the in-frame construct with BamHI and filling in the BamHI site with Klenow DNA polymerase (Promega) followed by blunt ligation (Sambrook, *et al.*, 1989).

In Vitro RNA Transcription and Translation

For sense RNA preparation plasmids were cut with NotI and transcribed with T3 RNA polymerase (Scheres, *et al.*, 1990a,b). The resulting RNA molecules was incubated for 1 hour at 30°C in a rabbit reticulocyte lysate in the presence of 2-5µCi of ^{35}S-methionine (1100Ci/mmol, New England Nuclear). The reaction was stopped by adding

sample buffer and the reaction mix was analyzed on a 15% polyacrylamide-SDS gel. Immunoprecipitation with antibodies against the CaMV PI protein (Zuidema, *et al.*, 1990) was performed as described (Franssen, *et al.*, 1982). Formed proteins were visualised by autoradiography on a Kodak X-omat film.

Reverse Transcription-Polymerase Chain Reaction Analysis

All oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer. The oligo(dT) is a 20-mer. The sequence of oligo 1, is 5'-CTGGTGAGCATGGAG, corresponding to the region between base 32 and 46 in p*Gm*ENOD40-3. The sequence of olig 2 is 5'-TCACTCCAACCTTAG and represents the antisense sequence from base 550 to base 564 (Fig. 2) of p*Gm*ENOD40-2. The sequence of oligo 3 is 5'-GGCAATGGACTCCAT and corresponds to the sequence of the region from base 190 to base 205 present in p*Gm*ENOD40-2 and from base 290 to base 305 of p*Gm*ENOD40-3. cDNA was synthesized on 1 μg RNA using 2U of AMV reverse transcriptase (Life Science) (1 hour at 42°C) and either oligo(dT) or oligo 2 as primer. Subsequently the reaction mixture was split and a polymerase chain reaction was performed in a LEP-PREM PCR machine using 2 units of Taq polymerase (Cetus) and oligo 3 as sense primer. After denaturation at 93°C for 2 minutes the DNA and primers were allowed to anneal at 42°C for 2 minutes. DNA synthesis occurred at 73°C in 2 minutes. After 20 cycles the DNA was electrophoresed on 1% agarose gels, transferred to GeneScreen plus (New England Nuclear) filters. To visualize DNA, the filter was autoradiographed after hybridization to a ^{32}P-labeled insert DNA of p*Gm*ENOD40-1.

In Situ Hybridization

Nodules harvested at different time points after inoculation with *(Brady)rhizobium* were fixed in 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 10mM sodium phosphate buffer for 4 hours (Van De Wiel, *et al.*, 1990). Fixed nodules were dehydrated, embedded into paraffin by routine methods. 7μm thick sections were hybridized with ^{35}S-UTP (1000-1500Ci/mmol, Amersham) labeled antisense or sense RNA probes (Scheres, *et al.*, 1990a) according to a procedure derived from Cox and Goldberg (1988, Van De Wiel *et al.*, 1990). Sections exposed for 2 to 4 weeks were stained with toluidine blue and photographed with a Nikon microscope equipted with epipolarization.

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Chapter 5

The pea late nodulin gene *Ps*NOD6 is homologous to the early nodulin genes ENOD3/14 and is expressed after the leghemoglobin genes

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Abstract

The pea late nodulin gene *Ps*NOD6 has been cloned and sequenced. *Ps*NOD6 is homologous to the pea early nodulin genes *Ps*NOD3 and *Ps*ENOD14. *In situ* hybridization experiments showed that like the *Ps*ENOD3 and *Ps*ENOD14 genes, the *Ps*NOD6 gene is only expressed in the infected cell type. The *Ps*NOD6 gene is first expressed at the transition of the pre-fixation zone II into the interzone II-III (the amyloplast rich zone preceding the fixation zone III), whereas the early nodulin genes *Ps*ENOD3 and *Ps*ENOD14 are already induced in the pre-fixation zone II. Thus these nodulin genes encoding homologous proteins are induced at consecutive stages of nodule development.

The expression of the late nodulin genes encoding leghemoglobin precedes the expression of the late nodulin gene *Ps*NOD6. Therefore these late nodulin genes have to be regulated by different mechanisms despite the fact that they are expressed in the same cell type. This conclusion is consistent with the fact that *Ps*NOD6 lacks one of the conserved regions occurring in the promoters of all other late nodulin genes studied.

Introduction

Pea *(Pisum sativum)* nodules have an indeterminate growth pattern. Consequently nodule tissues are of graded age and can be divided into specific zones; the meristematic zone I at the apex, the pre-fixation zone II where plant cells become infected by *Rhizobium* and subsequently elongate, the fixation zone III where nitrogen fixation occurs and in between zone II and III a few cell layers are present that are marked by the presence of prominent amyloplasts. The latter zone is the so-called interzone II-III [10, 22].

The formation of the infected cell type, present in the central tissue of the nodule, involves consecutive expression of nodulin genes (nodule-specific plant genes) [10, 20, 21]. We previously showed that the *Ps*ENOD12 gene is expressed in the distal part of the pre-fixation zone II of the pea nodules. The *Ps*ENOD5 gene is also transcribed in the pre-fixation zone II, but a maximal level of expression is found in the proximal part of this zone II. The early nodulin genes *Ps*ENOD3 and *Ps*ENOD14 are first expressed in the distal part of the pre-fixation zone II and the level of the corresponding transcripts decreases at the transition of the pre-fixation zone II into the interzone II-III. Finally, the late nodulin gene leghemoglobin (*Ps*Lb) is first expressed in the distal part of the pre-fixation zone II and maximal expression of this gene occurs in the fixation zone III [10, 20].

In this paper we present the characterization of the pea late nodulin gene *Ps*NOD6. Furthermore we studied the *in situ* expression of this gene in pea nodules, showing that this gene is expressed after the *Ps*Lb genes.

Materials and Methods

Plant material

Growth conditions of pea plants (*Pisum sativum* cv Rondo or Feltham First) and inoculation with *R. leguminosarum* bv. *viciae* strain PRE were as described by Bisseling *et al.* [2].

Cloning, DNA and RNA manipulations, sequence analysis

The isolation of the p*Ps*NOD6 cDNA clone was previously described [11]. The *Pst*I-insert of this cDNA clone was subcloned into pBluescript KS(+) and sequenced by the chain-degradation method [15].

The *Pisum sativum* cv. Feltham First genomic library was generously provided by Dr. Anil Shirsat (Durham Univ., UK), it was constructed by partial digestion of leaf DNA with Sau3A and subsequent cloning of fractionated fragments into EMBL3 1 vector. Screening of the library with the labelled p*Ps*NOD6 insert, phage purification, restriction mapping, subcloning,

generation of nested deletions with ExoIII/Mung bean nuclease and chain termination sequencing were done according to standard protocols [19]. Plant RNA was isolated according to the protocol described by De Vries *et al.* [9]. To determine the transcription start of the *Ps*NOD6 gene a kinase-labelled oligonucleotide TTCTGCAATAAGCAAGAG complementary to the 5'-end of the cDNA clone was annealed to nodule RNA and extended with reverse transcriptase. The size of the extension product was resolved on a sequencing gel as described by Scheres *et al.* [21].

The presence of *Ps*NOD6 RNA in different plant organs was studied with the RNase protection assay, using a subclone of the *Ps*NOD6 gene (position -1201 to +490) in pBluescript KS(+). ^{32}P-labeled antisense RNA was *in vitro* transcribed with T7 RNA polymerase from this subclone and used in RNase protection experiments [19].

For sequence assembly, analysis and homology searches the University of Wisconsin Genetic Computer Group package on VAX/VMS was used [8]. Nucleotide sequences of the *Ps*NOD6 gene and cDNA were submitted to the EMBL/GenBank database with accessions X63700 and X63699.

In situ hybridization

The p*Ps*NOD6 insert was subcloned into pBluescript KS(+) and transcribed from the T7 or T3 promoter in the presence of ^{35}S-UTP. Labelling of *Ps*Lb1, *Ps*ENOD3 and *nif*H antisense RNA probes was carried out as described by Yang *et al.* [25]. Pea nodules were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in PBS buffer (pH 7.2) supplemented with 100mM NaCl. Sections were prepared as described before [25]. Hybridization and autoradiography were performed according to a protocol [23] derived from a method described by Cox and Goldberg [4].

Results

Characterization of p*Ps*NOD6 and a *Ps*NOD6 gene

The pea nodulin cDNA clone p*Ps*NOD6 [11] was used to isolate the pea genomic clone lNOD6-2. This clone contains Bam HI (6.2 kb), EcoRI (7.1 kb), and HindIII (2.8 kb) restriction fragments hybridizing strongly to the insert of p*Ps*NOD6. Fragments of the same size are present in pea genomic DNA (data not shown). The 4.3 kb SalI-EcoRI fragment hybridizing with the cDNA was subcloned in pBluescript KS(+), and the nucleotide sequence of the *Ps*NOD6 gene was determined (Fig. 1). The two regions that are 100% homologous to the insert of the cDNA clone p*Ps*NOD6 are underlined in Fig.1, and are separated by a small intron of 103 bp (position (+104)-(+206)). The sequences of the boundaries of this intron are in agreement with the consensus intron junction sites in genes of dicotyledonous plants [12].

The 3'-end of the *Ps*NOD6 sequence contains two putative poly(A)-addition signals [13], which are outlined in Fig. 1.

The transcription start site (Fig. 1) was determined by primer extension experiments (results not shown). An open reading frame (ORF) starting with the first ATG in the transcribed region (position

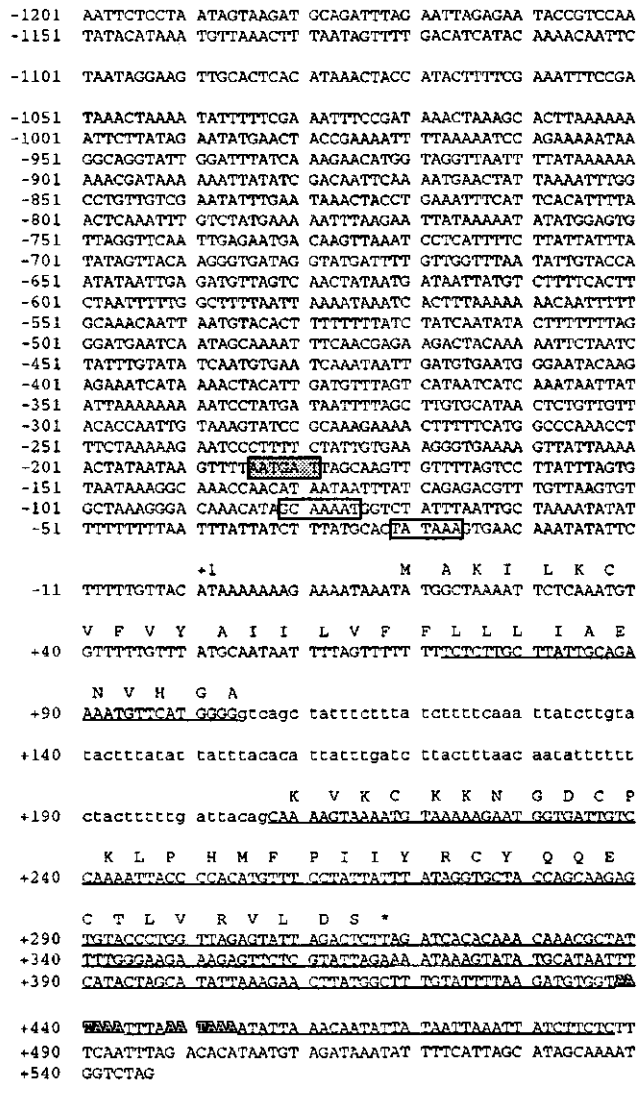


Fig. 1. *Ps*NOD6 gene sequence.
The 5' u*Ps*tream region and coding part of the *Ps*NOD6 gene are shown along with the deduced amino acid sequence (in single-letter code above the first base of each codon). The sequence of the cDNA clone upto the poly(A)-addition site is underlined. Putative poly(A)-addition signals are outlined. The putative intron is shown in small letters. The transcription start is marked with "+1". In the promoter putative TATA- and CAAT-elements are boxed, the nodule-specific element AATGAT is indicated in a shaded box

(+19)) and ending at position (+316) was identified, it encodes a polypeptide of 65 amino acids of which the first 28 amino acids display the characteristics of a signal peptide [24]. Apart from the putative signal peptide, the *Ps*NOD6 polypeptide is rather hydrophilic and has a high positive charge (calculated pI=8.9). The *Ps*NOD6 protein sequence is homologous to two related pea early nodulins, *Ps*ENOD3 and *Ps*ENOD14, described previously [20] (Fig. 2). The most striking feature of these three polypeptide is the conservation of the spatial distribution of the 4 Cys residues and amino acids surrounding them (Fig. 2). The cleavage sites according to Von Heijne [24] are predicted to be between Gly^{28} -Ala^{29} for *Ps*NOD6, between Ala^{24}-Glu^{25} for *Ps*ENOD3 and between Gly^{20}-Asn^{21} for *Ps*ENOD14. We could not detect any significant homology between these three nodulins and protein sequences in the databases.

The 5' u*Ps*tream region of the *Ps*NOD6 gene has the typical characteristics of a plant promoter [16] with "TATA" (position -33 to -28) and "CAAT" (position -83 to -77) elements at proper positions. In all late nodulin genes studied so far an organ specific element (OSE) is found that contains two highly conserved DNA sequences, AAGAT and CTCTT [6]. The CTCTT motif is not present in the *Ps*NOD6 gene promoter region, whereas a sequence close to AAGAT, namely AATGAT, is found at approximately the same relative position as in other late nodulin genes [6]. This sequence also occurs in the OSE of one of the *Sesbania* leghemoglobin (SrLb) genes [6]. Other less conserved sequences of nodulin gene promoters, shown to be binding sites for *trans*-acting factors [6], are not found in the 1.2 kb 5' u*Ps*tream region of the *Ps*NOD6 gene.

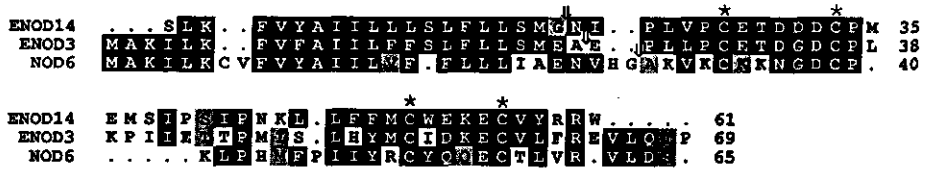


Fig. 2. Homology of *Ps*ENOD3, *Ps*ENOD14 and *Ps*NOD6.
Alignment of *Ps*NOD6 with pea early nodulins *Ps*ENOD3 and *Ps*ENOD14 using the pileup program and displayed using prettybox of the software package by the University of Wisconsin Genetics Computer Group [8]. Shadowed amino acids indicate conservative substitutions. Vertical arrows indicate the predicted positions of the putative signal peptide cleavage sites (24). Cys residues are indicated with *. p*Ps*ENOD14 is not a full-size cDNA clone and lacks Met at the N-terminus of the reading frame [20]. The pairwise similarities, excluding signal peptide, calculated with 'Gap' program [8] are 67% for ENOD3-NOD6 and ENOD14-ENOD3, and 71% for NOD6-ENOD14. The identities are 46% for ENOD3-NOD6, 50% for ENOD14-ENOD3, and 31% for NOD6-ENOD14.

*Ps*NOD6 gene expression is restricted to nodules.

Previously it has been shown that the expression of some nodulin genes is not restricted to nodules, but is also found in other parts of the plant like stem and flower tissues [3, 21]. Therefore we tested by RNase protection whether the *Ps*NOD6 gene is expressed in stem, leaf or flower tissue. ^{32}P labelled antisense RNA (+490 to -1203) was hybridized to total RNA from the various organs. It was subsequently digested with RNase A and RNase T1 and the protected fragments were separated on a sequencing gel (Fig. 3). The most prominent protected fragment after hybridization with nodule RNA (arrowhead, lane 2,3,4 and 7 on Fig. 3) corresponds to the second exon of *Ps*NOD6. Fig. 3 clearly shows that *Ps*NOD6 is expressed exclusively in the nodule.

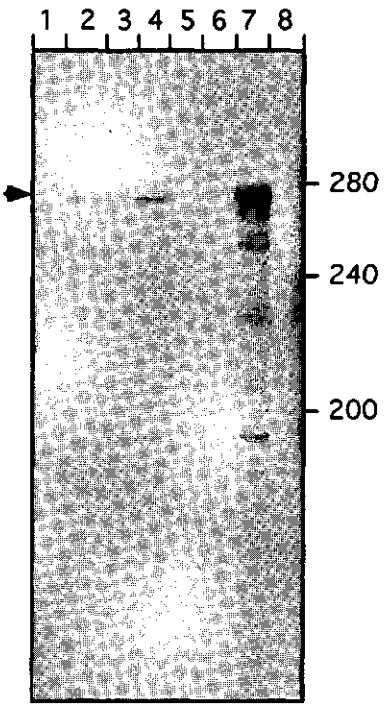


Fig. 3. RNase protection experiment showing nodule-specific expression of *Ps*NOD6 gene
lane 1- no plant RNA added, complete digestion of a probe; **lane 2**- 0.1μg of total nodule RNA; **lane 3**- 0.2mg of total nodule RNA; **lane 4**- 0.5μg of total nodule RNA; **lane 5**- 2μg of total root RNA; **lane 6**- 2μg of total flower RNA; **lane 7**- 2μg of total nodule RNA; **lane 8**- 2μg of total stem RNA. Size markers are in bp. Arrowhead indicates major proteced fragment in nodule RNA which corresponds to the second exon of the *Ps*NOD6 gene.

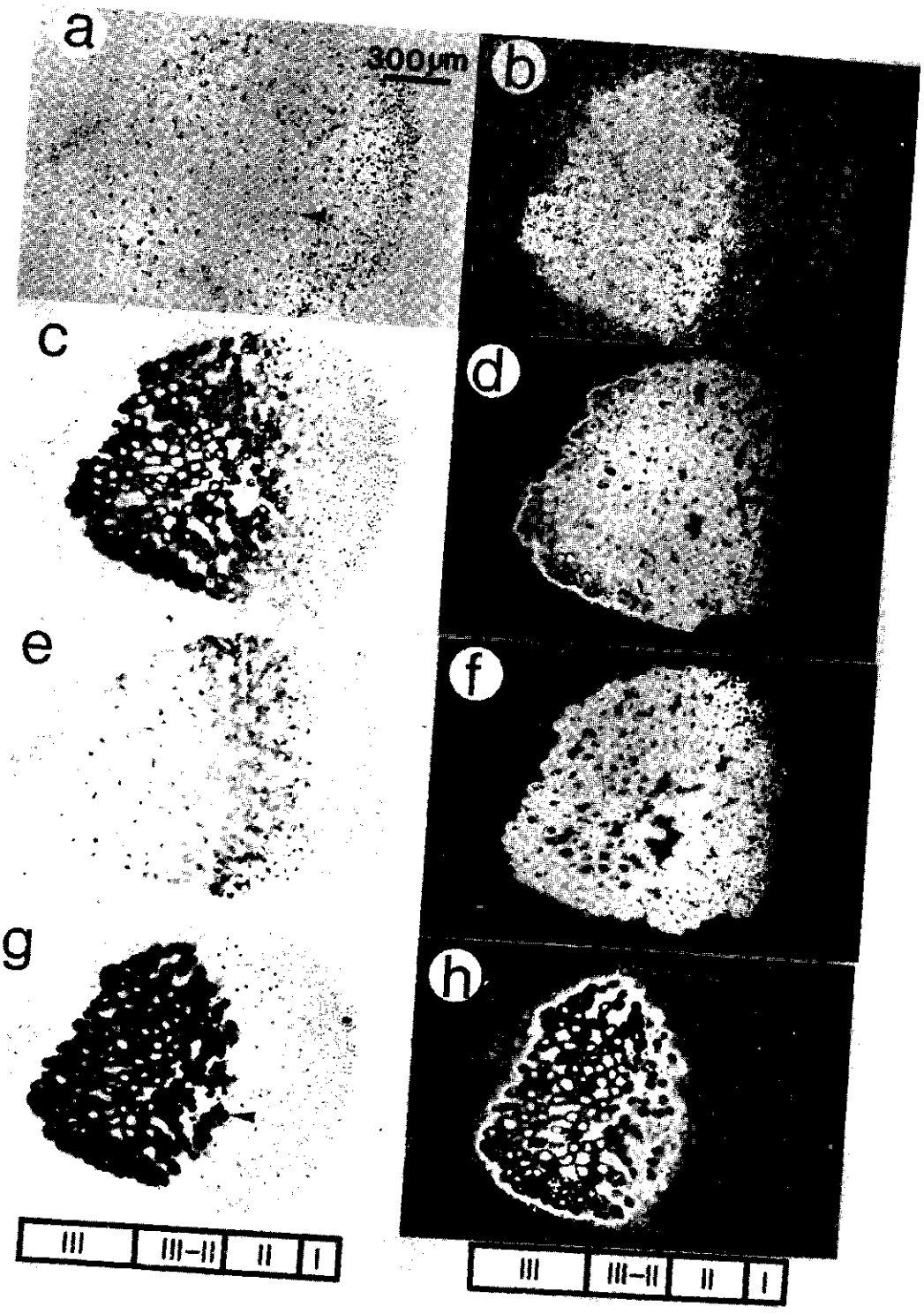
In situ localization of *Ps*NOD6, *Ps*NOD3, *Ps*Lb and nifH mRNA

Longitudinal sections of ea nodules of 14 and 20 day old plants were hybridized with antisense and sense ^{35}S-labeled *Ps*NOD6 RNA. The antisense *Ps*NOD6 RNA hybridized to RNA in cells of the central tissue of the nodule (Fig. 4, a, b), whereas the sense RNA probe gave no signal above background level (not shown). Within the central tissue *Ps*NOD6 mRNA is found in the infected cells, whereas this transcript is not detectable in the uninfected cell type of the central tissue (Fig. 5, a, b).

Figure 5 a-c shows that the infected cells containing *Ps*NOD6 mRNA have amyloplasts, while the adjacent cells not expressing the *Ps*NOD6 gene have none. This demonstrates that the induction of the *Ps*NOD6 gene coincides with the beginning of amyloplasts accumulation, which marks the transition of the pre-fixation zone II into interzone II-III [22]. Since rhizobial *nif* gene induction also occurs at this transition [10, 25], we checked whether the *Ps*NOD6 and *nif* genes are induced in exactly the same cell layer. Adjacent nodule sections were hybridized with *nif*H and *Ps*NOD6antisense RNA probes and it was shown that indeed the *nif*H and *Ps*NOD6 messengers first appear in exactly the same cell layer of the central tissue (Fig. 4, a,b and g,h).

The early nodulin genes *Ps*ENOD3 and *Ps*ENOD14 are first induced in the proximal part of the pre-fixation zone II and the level of their transcripts decreases at the transition of zone II into the interzone II-III in 14-day-nodules (Fig. 4 e and f) [10, 20]. So the *Ps*ENOD3/14 and *Ps*NOD6 gene are induced at different stages of development. To compare the pattern of expression of the *Ps*NOD6 gene with that of the late nodulin genes encoding Lb, adjacent sections were hybridized with *Ps*NOD6 (Fig.4, a, b) and *Ps*Lb antisense RNA probes (Fig.4 c,d). *Ps*Lb gene expression starts in the pre-fixation zone II and reaches a maximal level in the interzone II-III that remains constant in the nitrogen fixation zone III (Fig. 4, c, d), whereas the *Ps*NOD6 mRNA first appears in the interzone II-III (Fig.4, a, b). It is noteworthy that the *Ps*NOD6 (Fig. 4, a,b; Fig. 5, a,b) and *nif*H mRNAs (Fig. 4, g, h) are present at their maximal level in the first cell layer in which they are detectable. In contrast the *Ps*Lb (Fig. 4, c,d; Fig. 5, d,e) and ENOD3 mRNA (Fig.4, e,f) gradually increase in successive cell layers during development [20, 25].

Fig. 4. In situ localization of *Ps*NOD6, *Ps*Lb, *Ps*ENOD3 and Rhizobium nifH mRNA in adjacent nodule (14 day old) sections.
a, c, e and **g** are bright field photographs where signals are visible as black dots. **b, d, f** and **h** are corresponding dark field photographs where hybridization signals are visible as white dots. Arrowheads indicate a corresponding position. The zonation is indicated at the bottom. Adjacent longitudinal sections were hybridized with ^{35}S-labeled antisense RNA of *Ps*NOD6 (**a,b**), *Ps*Lb (**c,d**), *Ps*ENOD3 (**e,f**) and *nif*H (**g,h**), respectively. Note that the decrease of *Ps*ENOD3 mRNA (**e,f**) at the transition of the pre-fixation zone II and the interzone II-III is best visible in the bright field picture shown in **e**. Due to the very high density of silver grains in the infected cells of the *nif*H hybridized section (**g**), the light scattering by dark field illumination (**h**) is impaired. Arrowheads indicating same cells in **a, b, g** and **h**.


a
300µm
b
c
d
e
f
g
h
III
III–II
II
I
III
III–II
II
I

In nodules of 20 day old pea plants the spatial pattern of the *Ps*NOD6 gene expression pattern is not significantly changed. The *Ps*NOD6 mRNA is first detectable at the beginning of the interzone II-III and this transcript is present at a constant level in the older cell layers of the central tissue. In contrast, the distribution of Lb mRNA in a nodule of a 20 day old plant is markedly altered. At this stage of development, this late nodulin mRNA is only present at high levels in the interzone II-III, and its concentration in the pre-fixation zone II and the nitrogen fixation zone III is markedly reduced (results not shown). It is also noted that in nodules older that 20 days the decrease of the *Ps*ENOD3 and *Ps*ENOD14 mRNA starts at the proximal part of the interzone II-III and disappears at the beginning of the nitrogen-fixation zone III (results not shown) [10].

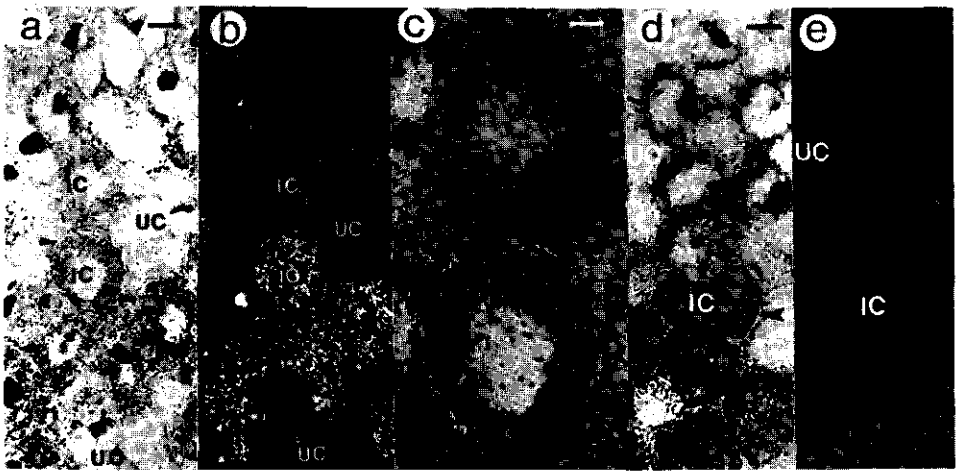


Fig.5. Localization of *Ps*NOD6 and *Ps*Lb mRNA.
Adjacent longitudinal sections were hybridized with ^{35}S-labeled antisense RNA of *Ps*NOD6 (**a**, **b**, **c**) and *Ps*Lb (**d**, **e**). The orientation of the sections is indicated. D=distal, IC = infected cell, P=proximal, UC = uninfected cell.
a. A magnification of part of the central tissue of a nodule (16 day old) at the transition of the pre-fixation zone II into interzone II-III. Amyloplasts are indicated by arrowheads. Bar = 100 µm.
b. Epipolarization micrograph of **a** showing the localization of *Ps*NOD6 mRNA (white grains) in amyloplast-containing infected cells.Arrowheads indicate amyloplasts.
c. Bright field micrograph of a detail of **a** showing two infected cells. The upper cell (*) has no amyloplasts and does not contain *Ps*NOD6 mRNA, whereas the cell containing amyloplasts (arrowheads) has a high level of this messenger RNA. So the *Ps*NOD6 gene is first induced at the transition of the pre-fixation zone II into interzone II-III. The signal is visible as black grains. Bar =10 µm.
d. A magnification of Fig. 4**b** at the transition of the pre-fixation zone II into interzone II-III showing *Ps*Lb mRNA localization (black dots) and amyloplast accumulation (arrowheads). Bar=100µm.
e. Epipolarization micrograph of **d**. White dots represent signals.

Discussion

In this paper we showed that the pea late nodulin gene *Ps*NOD6 is homologous to the early nodulin genes *Ps*ENOD3 and *Ps*ENOD14. *In situ* hybridization experiments showed that the *Ps*ENOD3/14 and *Ps*NOD6 gene are expressed in successive stages of

nodule development. These three nodulins are small peptides of about 6 kDa, all of them containing 4 cysteine residues with a spatial distribution that is found in several metal binding proteins [1]. However, whether or not these nodulins are able to bind a specific metal ion remains to be proven.

The *Ps*NOD6 gene is first expressed at the transition of the pre-fixation zone II into the interzone II-III. At this transition a marked developmental switch in both bacterium and plant occurs. Vasse *et al.* [22] demonstrated cell to cell changes in the ultrastructure of *R.meliloti* bacteroids at this transition. Furthermore *R. leguminosarum* bv. *viciae nif* genes are first expressed at this transition point (Fig. 5, g, h) [10, 25], the expression of an outer membrane protein gene (*rop*A) of *R.leguminosarum* bv. *viciae* is switched off [7], and the lipopolysaccharide of the bacteroids is altered [18]. The switch in plant development is reflected by the marked drop of *Ps*ENOD5 mRNA concentration [10, 22], the start of expression of the *Ps*NOD6 gene as described in this paper and the formation of amyloplasts. The cause of the developmental switch at the transition of the pre-fixation zone II into the interzone II-III is unknown. However, since this change occurs from cell to cell it seems unlikely that physiological changes, like a decrease of the O_2 concentration [14], are sufficient to trigger this switch and we favour the idea that signal molecules from plant and/or bacterium are involved.

De Billy *et al.*[5] showed that Lb mRNA first appears in alfalfa nodules in the first cell layer of the interzone II-III. Our studies showed that in young nitrogen-fixing nodules the *Ps*Lb mRNA is present in the pre-fixation zone II and the mRNA remains present in all older cell layers of the central tissue. In older pea nodules (20-day-old) the *Ps*Lb mRNA is only present at high level in a few cell layers of the interzone II-III, a spatial distribution is similar to the pattern described for alfalfa nodules [5].

The changes in the spatial distribution of *Ps*Lb mRNA during pea nodule development show that pea nodules of 20-day-old plants can no longer be considered to be composed of successive zones with the characteristics of the different ste*Ps* of development. Therefore the timing of *Ps*NOD6 and *Ps*Lb gene expression can best be compared in a relatively young nodules (e.g. 14 day old).

In situ hybridization experiments showed that the expression of the *Ps*Lb genes precedes the transcription of the *Ps*NOD6 genes. Hence these two late nodulin genes have to be regulated by different mechanisms. An organ specific element (OSE) containing two highly conserved DNA sequences AAGAT and CTCTT is found in the promoters of all late nodulin genes that have been studied [6], including a pea Lb gene [17]. In the *Ps*NOD6, only one of the conserved regions (AATGAT) of the OSE is found in the promoter of the *Ps*NOD6 gene. Therefore both the *in situ* expression studies and sequence analysis suggest that *Ps*NOD6 and *Ps*Lb genes are regulated by different mechanisms.

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Chapter 6

In situ localization of *Rhizobium* mRNAs in pea root nodules: *nif*A and *nif*H localization

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Molecular Plant-Microbe Interaction (1991) 4, 464-468

Abstract

Here we demonstrate that *Rhizobium* mRNAs can efficiently be detected in developing root nodules with the *in situ* hybridization technique. We have been able to localize the *Rhizobium nif*H mRNA as well as the transcript of the regulatory *nif*A gene. Therefore we expect that the *in situ* hybridization technique can generally be applied to locate *Rhizobium* mRNAs in root nodules.

In pea nodules the *nif*A and *nif*H mRNAs are first detectable in the third to fourth cell layer of the late symbiotic zone. In these cell layers these mRNAs are detectable immediately at maximal levels. In older parts of the late symbiotic zone the level of *nif*H mRNA remains constant, whereas the level of *nif*A mRNA decreases.

Finally the pattern of *nif* mRNA accumulation was compared with that of nodulin mRNAs.

Additional Keywords: *nif*, nitrogen fixation, nodulin, *Pisum sativum*, *Rhizobium leguminosarum*

The formation of nodules on roots of *Leguminosae* by *Rhizobium* bacteria involves a series of successive steps which require the expression of plant as well as bacterial genes (Long, 1989). The plant genes specifically expressed during nodule formation are the nodulin genes (Van Kammen, 1984). A comprehensive list of nodulins has been published by Delauny and Verma (1988) and these genes have been reviewed extensively by Nap and Bisseling (1989).

In indeterminate nodules, like pea root nodules, a persistent meristem is present at the apex, which continuously generates cells that develop into different nodule tissues. As a consequence the different tissues of a nodule are of graded age and so the central tissue of indeterminate nodules has been divided in the following zones: the apical meristem, the invasion zone, in which the growing infection threads penetrate the meristem cells, the early symbiotic zone, where the bacteria proliferate and the plant cells elongate, and the late symbiotic zone, which harbours infected cells filled by nitrogen fixing bacteroids. In old nodules also a senescent zone is present containing degenerated rhizobia and plant cells (Newcomb, 1976).

Recently, a new set of pea early nodulin cDNA clones was characterized and the location of the corresponding transcripts in specific cells and tissues of infected roots and pea root nodules was determined by *in situ* hybridization (Van De Wiel *et al.*, 1990, Scheres *et al.*, 1990a and b). The ENOD2 mRNA was localised in the nodule parenchyma ("inner cortex") (Van De Wiel *et al.*, 1990), while all other pea early nodulin mRNAs are present in the central tissue. Scheres *et al.* (1990a) showed that ENOD12 gene expression is restricted to the invasion zone. Expression of the ENOD5 gene starts in the invasion zone, but reaches its maximal level in the early symbiotic zone. The ENOD3 and the homologous ENOD14 mRNAs are present at maximal levels in the early symbiotic zone and the first cell layers of the late symbiotic zone (Scheres *et al.*, 1990b), whereas in older parts of the late symbiotic zone the level of these transcripts decreases. The mRNA of the late nodulin leghemoglobin (Lb) is first detectable in the early symbiotic zone, but its maximal level is first reached in the late symbiotic zone. These observations clearly showed that at different stages of root nodule development specific nodulin genes are induced.

Rhizobial genes involved in different steps of the plant-bacterium interaction are the nodulation (*nod*), nitrogen fixation (*nif* and *fix*) genes and genes encoding for surface compounds of the bacteria. Examples of the latter group are genes involved in exopolysaccharide (*exo*), lipopolysaccharide (*lps*) and ß-1,2-glucan (*ndv*) synthesis.

In alfalfa nodules the expression of several *R. meliloti nif* and *nod* genes has been studied *in planta* by using the *gus*A gene (Sharma and Signer 1990) as a reporter gene. Since thick sections (100-250 μm) were used in this study only an inaccurate picture of the spatial distribution of *Rhizobium* gene expression could be obtained. Furthermore, localization data obtained by using a reporter enzyme are obfuscated by the stability of this enzyme. To allow a more accurate localization of *Rhizobium* gene expression, we have used the *in situ* hybridization technique to examine whether bacterial mRNAs can be detected *in planta*. In this paper we report how, using this technique, transcripts of *R. leguminosarum* bv. *viciae nif*A and *nif*H genes can be localized in root nodules. The *nif*H gene encodes a subunit of the nitrogenase enzyme and it is abundantly expressed in nodules, while the *nif*A gene is probably expressed at a relatively low level, as it is a regulatory gene required for the induction of expression of other *nif* and *fix* genes (Hennecke, 1990).

Results and Discussion

Rhizobium mRNAs can efficiently be dectected *in situ*.

To localize *nif*H mRNA by *in situ* hybridization, nodules from 16 day old pea plants were used. Longitudinal sections of nodules were hybridized to ^{35}S-labeled antisense *nif*H RNA. As shown in Fig. 1.C and H the *nif*H mRNA was clearly detectable in the infected cells of the late symbiotic zone. No hybridization was obtained when a ^{35}S-labeled sense *nif*H RNA was used as a probe (data not shown), showing that the signal obtained after hybridization with antisense *nif*H RNA was due to the presence of *nif*H mRNA and not to that of *nif*H DNA of the Sym-plasmid This was further supported by the absence of a hybridization signal in the cells of the youngest cell layers of the late symbiotic zone, though these cells are already fully packed with bacteria (Fig. 1.H. I. J).

The signals obtained after hybridization with antisense *nif*H RNA were just as intense as the signals obtained if antisense leghemoglobin (Lb) RNA was used (Fig. 1.C, E). Since Lb accounts for about 10 % of total soluble nodule protein of the plant and nitrogenase for 10 % the total bacterial protein (Bisseling *et al.*, 1978), we assume that similar amounts of Lb and nitrogenase mRNA are present in the nodule. This indicates that the prokaryotic *nif*H mRNA and the eukaryotic Lb mRNA are detected with the same efficiency.

In situ hybridization using ^{35}S-labeled antisense *nif*A RNA as a probe was similarly carried out to sections of 16 day old nodules. Like the *nif*H mRNA, the *nif*A transcript was detectable in the infected cells of the late symbiotic zone (Fig.1.B,G, I, J) but the intensity of the signal obtained after hybridization with the *nif*A probe was considerably lower than with the *nif*H probe. In most experiments an exposure time of 2-

4 weeks was required for showing *nif*A mRNA localization, while a 2-4 days exposure was sufficient to visualize the *nif*H mRNA hybridization (see legend Fig. 1).
Since even the transcript of the regulatory *nif*A gene, which will be present in low concentrations, can be detected with *in situ* hybridization, it seems plausible that other *Rhizobium* mRNAs can similarly be localized with this method.

*nif*H and *nif*A mRNA accumulation during nodule development

By definition the late symbiotic zone consists of the cells of the central tissue that are fully packed with rhizobia and have already reached their maximal size (Newcomb, 1976). Analysis of serial sections of pea nodules, hybridized with the two *nif* probes, showed that the *nif*H and *nif*A mRNAs (Fig. 1.C and B respectively) are detectable in almost all infected cells of the late symbiotic zone. However, in the first 2-3 cell layers of this zone(LSa) (Fig.1 A) only a small number of silver grains are detectable (Fig. 1.G, H, I,J). To determine whether the *nif* genes are expressed at a low level in these cell layers we determined the number of silver grains in infected cells of the different zones of the nodule central tissue. The silver grains were counted in 5 areas of 400μm^2 in each zone of the central tissue as well as in nodule cortex, root cortex and parts of the slide containing no section.The average values and standard deviations are given in Table 1. The data presented in this table show that on nodule sections hybridized with a *nif*A or *nif*H probe the number of silver grains in nodule meristem, invasion zone, early symbiotic zone and in the first 2-3 cell layers of the late symbiotic zone is not higher than the background level present in nodule cortex, root cortex or areas of the slides containing no section. In the third/fourth cell layer of the late symbiotic zone(LSb)(Fig.1.A) both *nif*A and *nif*H mRNA are present at a maximal level (Fig.1.G.H. I.J, Table 1). In the proximal part of the late symbiotic zone(LSc) (Fig.1.A) the level of *nif*H mRNA remains at a similar level, whereas the number of silver grains above this zone in a *nif*A hybridized section decreases to about 35% of the maximal value. Hybridization with sense *nif*A or *nif*H probe gives a signal that is similar to the background level obtained with antisense probes (result not shown). These observation show that both the *nif*A and *nif*H genes are first expressed in the third or fourth cell layer of the late symbiotic zone. Analyses of 1μm thick sections of technovit imbedded pea nodules showed that the infected cells of the first two cell layers already contained bacteroids with the characteristic Y shaped form (data not shown, see Van De Wiel *et al.*, 1988). This implies that the development into pleiomorphic bacteroids precedes the stage where the *nif* genes are expressed and actual nitrogen fixation can occur.

Recently Vasse *et al.* (1990) proposed a new nomenclature for the zones of the central issue of alfalfa nodules. They name the meristem, zone I, the invasion zone and early symbiotic zone, zone II, the youngest part of the late symbiotic zone interzone II-III

Fig. 1. Localization of *nif* and nodulin transcripts in longitudinal sections of a 16 day old pea nodule. **A,F, I**: bright field micrographs; **B,C,D,E,G,H** : dark field micrographs in which silver grains are visible as white dots. **J**: epipolarization micrograph. **A,B,C,D,** and **E** are adjacent sections. In all cases antisense RNA probes were labeled with ^{35}S-UTP.

A. Nodule meristem (M), invasion zone (arrows), early symbiotic zone (ES) and late symbiotic zone (LS) are indicated.The late symbiotic zone is divided into region a, b and c. Bar=100μm.

B. Section hybridized with antisense *nif*A RNA. Arrowheads indicate the same cells as in **C**. Arrow indicates where the *nif*A mRNA level decreases. Exposure time 4 weeks.

C. Section hybridized with antisense *nif*H RNA. The same cells as in **B** are indicated by arrowheads. Exposure 4 days.

D. Localization of pea ENOD3 mRNA. Exposure time 2 weeks.

E. Localization of pea leghemoglobin mRNA. Exposure time 4 days.

F. Arrows and arrowheads in **F. G. H** indicate the same cells. IC = infected cell; UC = uninfected cell. Bar=100μm.

G. Detail of **B**. Arrowhead indicates the cell without signal, arrow indicates the cells with signal.

H. Detail of **C**.

I. Detail of box region in **G**. Dark dots represent silver grains. Bar=10μm.

J. Epipolarization micrograph of **I**. Bright dots are silver grains.

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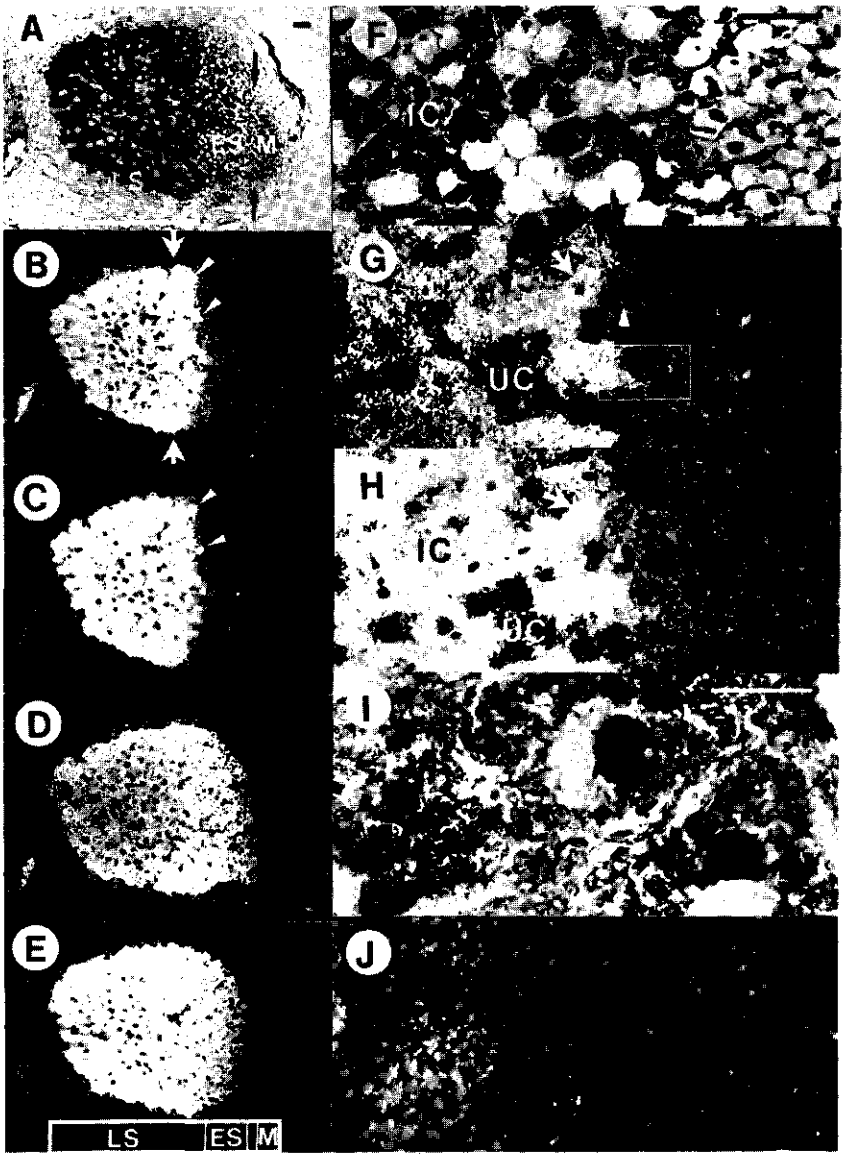
and the rest of the late symbiotic zone, zone III. The interzone II-III is characterized by the presence of prominent amyloplasts and the bacteroids in this interzone have a typical morphology, but most likely do not yet *fix* nitrogen. In zone III the number of amyloplasts is strongly reduced. In pea nodules the amyloplasts are not restricted to a specific zone of the late symbiotic zone, and bacteroid morphology has not extensively been studied. Therefore these criteria can not be used to indicate the interzone II-III in pea nodules. However, the absence of *nif*A and *nif*H mRNA in bacteroids of the youngest cell layers of the late symbiotic zone suggests that these cell layers correspond to the interzone II-III of alfalfa nodules. In Fig. 1.A the part of the late symbiotic zone that could be the equivalent of the alfalfa interzone II-III is marked with LSa.

Both *nif*A and *nif*H mRNA have a striking accumulation pattern during nodule development. Both mRNAs are first found in the third or fourth cell layers of the late symbiotic zone. In these cell layers these *nif* genes are immediately expressed at maximal levels (Table 1). Such a gene expression pattern of *nif*A suggests that a major change occurs in the third/fourth cell layer of the late symbiotic zone, causing *nif*A gene induction. In free-living *R.meliloti* bacteria, expression of the *nif*A gene is induced at microaerobic O_2 concentrations (Ditta *et al.*, 1987). Since microaerobic O_2 concentrations prevail in root nodules it has been postulated that *nif*A gene expression in nodules is also mediated by the O_2 concentration (Hennecke, 1990). The microaerobic O_2 concentration in the nodule is thought to arise by respiratory activity of the bacteria and the presence of an O_2 diffusion barrier in the nodule parenchyma ("inner cortex") (Witty *et al.*, 1986; Van De Wiel *et al.*, 1990). So if the O_2 concentration is the only factor controling *nif*A gene expression in the nodule, a rapid drop in O_2 concentration must occur in the 3rd/4th cell layer of the late symbiotic zone. Although we can not exclude the possibility of this sharp change of O_2 concentration, at this moment there are no cytological or physiological studies that indicate that such a rapid drop occurs.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Therefore it will be essential to demonstrate that the *nif*A gene is regulated by a similar mechanism in free living bacteria and in nodules. The level of the *nif*A mRNA decreases from about the 8th cell layer of the late symbiotic zone (Fig. 1B, arrow). Since the *nif*A gene is autoregulated (Hennecke, 1990) we suppose that this decrease is caused by the accumulation of the *nif*A protein, but this needs to be checked by following the accumulation of the *nif*A protein with immunocytochemical localization methods.

Since *nif*A is a regulatory protein involved in the induction of other *nif* and *fix* genes, we expected that *nif*A gene expression would precede the expression of the *nif*HDK operon. We tried to test this assumption by determining the location of *nif*H and *nif*A mRNA in adjacent sections. To facilitate the comparison of the corresponding cell patterns in these sections we have indicated a few cells with arrowheads (Fig. 1. B,C) and arrows (Fig.1. G,H). These studies showed that cells containing *nif*A mRNA also harbour *nif*H transcripts. Apparently the induction of the *nif*A gene results in a prompt switching on of the *nif*HDK operon.

Our *nif*H mRNA localization studies might be consistent with the studies on *nif* gene expression in alfalfa nodules by Sharma and Signer (1990). They showed by using a *gus*A reporter gene that *nif* H gene expression "occured throughout the nodule, except in the meristematic zone". It is well possible that the "meristematic zone" in their studies includes the meristem, invasion zone, early symbiotic zone and a few cell layers of the late symbiotic zone. However, since thick sections were used to detect *gus*A activity the different zones could not be identified. Recently Boivin *et al.* (1990) used thinner sections to follow *R. meliloti* gene expression using LacZ as a reporter gene. In these studies a more accurate localization was achieved. Sharma and Signer (1990) did not observe any difference in *nif*H and *nif*A gene expression in older parts of the late symbiotic zone. In their studies this difference might have been masked by the stability of the *gus*A protein.

Comparison of accumulation patterns of nodulin and *nif* mRNAs

Clues on possible functions of nodulins and bacterial gene products can be obtained by determining at which stage of development specific gene products are made. For that purpose we compared the spatial distribution of the ENOD3 early nodulin mRNA with that of *nif* mRNAs. Previously we have shown that the amino acid sequence of the ENOD3 polypeptide contains 4 cysteine residues in relative positions characteristic for metal binding proteins (Scheres *et al.*, 1990b). We then proposed that this early nodulin might be involved in transport of molybdenum and/or iron ions into the bacteroids, since the bacteroids require high amounts of these metal ions for the synthesis of the nitrogenase enzyme (Shah and Brill, 1977). As shown in Fig. 1.B.C.and D the maximal level of ENOD3 gene expression coincides with the region of the late symbiotic

zone where the expression of *nif*A and *nif*H genes starts. Therefore we conclude that the mRNA localization studies are consistent with the postulated function of ENOD3 in transport of molybdenum and iron ions towards bacteroids.

Leghemoglobin (Lb) is a nodulin whose appearance during nodule development has been frequently compared with that of *nif* proteins (Bisseling *et al.*, 1986). Since a more accurate comparison of the order of induction of genes can be made by *in situ* hybridization we compared the pattern of Lb and *nif*H mRNA accumulation in longitudinal sections. As shown in Fig. 1.E the Lb mRNA is first detectable in the early symbiotic zone (ES) and gradually reaches a maximal level in the late symbiotic zone (LS). Therefore Lb gene expression markedly precedes *nif* gene expression in pea nodules. This is consistent with most of the previously published biochemical studies (Bisseling *et al.*, 1986).

In this paper we have demonstrated that rhizobial mRNAs can efficiently be detected in root nodules with the *in situ* hybridization technique. Therefore *in situ* hybridization is a very powerful tool to study the sequential order of both plant and bacterial gene expression in plant microbe interactions.

Materials and Methods

Plant materials

Pea (*Pisum sativum* cv. Rondo) plants were cultured and inoculated with *Rhizobium leguminosarum* bv. *viciae* (PRE) as described by Bisseling *et al.* (1978).

In situ hybridization

Pea nodules were harvested 16 days after inoculation and fixed immediately with 4 % paraformaldehyde and 0.25 % glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2) for 4 hours. The nodules were dehydrated by passing through a routine ethanol series and embedded in paraffin. Seven µm thick sections were cut using a Leitz microtome. They were adhered on poly-L-lysine coated slides, and thereafter deparaffinized with graded xylene. The sections were hybridized by a method derived from the procedure described by Cox and Goldberg (1988) (Van De Wiel *et al.*, 1990). In short, sections were hydrated and dried under vacuum. The sections were hybridized with RNA probes as described by Van De Wiel *et al.* (1990). Slides were coated with Kodak NTB2 nuclear emulsion and exposed at 4 ^{o}C. Afterwards the slides were developed in Kodak D19 developer and fixed in Kodak fixer. Sections were stained with 0.25 % toluidine blue and mounted with DPX (BDH). The sections were photographed with a Nikon microscope with dark field and epipolarization optics.

Labelling of antisense/sense RNAs

The 1.8 kb EcoRI-BamHI fragment of pT7.BB containing the coding region of the *nif*A gene (Roelvink *et al.*, 1989) was subcloned in pT7-5 vector (kindly provided by S. Tabor). The pT7-5/*nif*A plasmid was cut with XbaI before antisense *nif*A RNA (from position 893-469 bp) was made with T7 polymerase.

The *nif*H antisense RNA (from position 89 to 433 bp) was transcribed by T7 polymerase from a pTZ19 derivative containing a 518 bp AccI-HpaI fragment of pGBI (Schetgens *et al.*, 1984). For sense *nif*H RNA production, a pTZ18 derivative carrying the same insert was used. The production of antisense *Ps*ENOD3 and Lb RNAs was carried out according to Scheres *et al.* (1990b). The antisense RNA probes were radioactively labeled with [^{35}S] UTP (1000-1500 Ci/mmole, Amersham) as described previously (Van De Wiel *et al.*, 1990), and degraded to about 150 nucleotides long fragments according to Van De Wiel *et al.* (1990) before hybridization.

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Chapter 7

Down-regulation of expression of the *Rhizobium leguminosarum* outer membrane protein gene *rop*A occurs abruptly in interzone II-III of pea nodules and can be uncoupled from nif gene activation

Ruud A. de Maagd, Wei-Cai Yang, Leentje Goosen-de Roo, Ine H.M. Mulders, Henk P. Roest, Herman P. Spaink, Ton Bisseling and Ben J.J. Lugtenberg. Molecular Plant-Microbe Interaction. in press

Abstract

The expression of the *Rhizobium leguminosarum* biovar *viciae* outer membrane protein gene *rop*A during nodule development was studied using immuno-electron microscopy and *in situ* hybridization. Using immunochemical detection in isolated cell envelopes it had been shown earlier that the RopA outer membrane antigen disappears during bacteroid development (de Maagd *et al.*, 1990). In the present study we used immuno-electron microscopy on vetch nodule sections to show that the decrease in RopA protein expression occurs in the nodule after release of the bacteria from the infection thread. Detection of *rop*A mRNA in sections of pea nodules by *in situ* hybridization revealed a sudden decrease in messenger level at the transition from pre-fixation zone II to interzone II-III. This decrease coincided with a sudden increase in *nif*H mRNA levels. Although the decrease in *rop*A messenger and appearance of *nif* messenger are spatially correlated we could show that *rop*A down-regulation can be uncoupled from *nif* gene activation by using a strain that induces non-nitrogen fixing nodules on pea but does develop into bacteroids. The identification of the transition of prefixation zone II to interzone II-III as a developmental switch for bacteroid development is discussed.

INTRODUCTION

The development of an effective symbiosis between plants of the *Leguminosae* family and rhizobia involves a series of steps, in which plant genes as well as bacterial genes play a role. For the bacterium this results in differentiation into a specialized nitrogen-fixing form, the bacteroid, which shows extensive morphological and molecular differences with free-living bacteria. Most notably, the mature bacteroid expresses the *nif* genes which are responsible for the fixation of atmospheric nitrogen. On the plant side, nodulin genes are expressed exclusively in the nodule. According to the timing of their appearance the nodulins can be divided into an early and a late subgroup (Nap and Bisseling, 1989; Franssen *et al.*, 1992).

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

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

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

RESULTS

Immuno-electron microscopic Detection of Group III Antigens in Nodules

Initially, the decrease in antigen group III contents during symbiosis was found by immunochemical comparisons of free-living bacteria with bacteroids from pea nodules (de Maagd *et al.*, 1989). To determine whether decrease of antigen group III expression takes place inside the nodule, immuno-electron microscopy with monoclonal antibody MAb38 (de Maagd *et al.*, 1989) was used to detect this antigen group in thin sections of vetch (*Vicia sativa*) nodules. MAb 38 had been shown earlier to preferentially recognize the non-denatured antigen group III oligomers on Western blots as well as on intact cells of *R. leguminosarum* biovar *viciae* strain 248. The level of labelling of bacteria or bacteroids was quantified for three categories, broadly representing subsequent developmental stages. We compared bacteria from infection threads and infection droplets in the invasion zone, newly released bacteria or "young" bacteroids and mature, nitrogen-fixing bacteroids. A decrease in labelling in mature bacteroids (Fig. 1C) as compared to newly released bacteria (Fig. 1B) and infection thread bacteria (Fig. 1A) was observed.

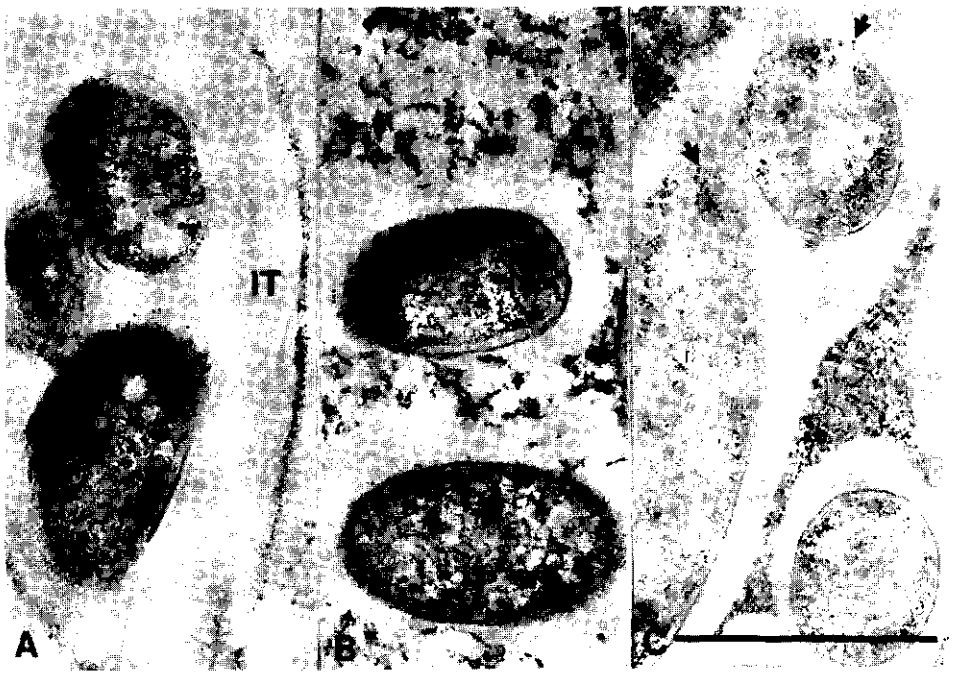

Fig. 1. Indirect immuno-gold labelling with MAb 38 of infection thread-localized bacteria and bacteroids in *vetch* nodules. A, B, and C show the same magnification. Bar = 1 µm. **A.** Bacteria in infection thread (IT). **B.** "Young" bacteroids in plant cytoplasm. **C.** Mature bacteroids (arrowheads indicate the rare gold particles). See text for further details.

To quantify these data, the amount of gold particles per μm^2 section of each category was averaged from a large number of photographs (for details, see Methods section). The results of this quantification are shown in Fig. 2. Statistical comparison of each pair of two categories using the Student's t test showed that while the difference between categories 1 and 2 is not significant, the difference between categories 1 and 3, as well as that between categories 2 and 3, are indeed significant (p..0.01). It can be concluded from these results that decrease in antigen group III expression indeed occurs inside the nodule, between release from the infection thread and occurrence of mature, nitrogen-fixing bacteroids.

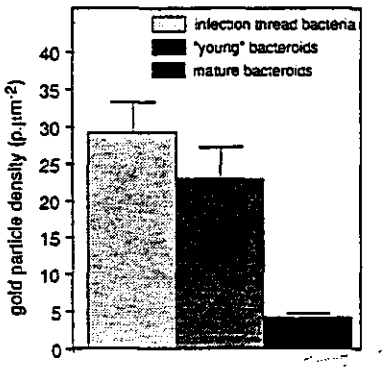

Fig. 2. Mean gold particle density (particles per μm^2) in sections of immuno-gold labelled infection thread and infection droplet bacteria, "young" bacteroids and mature bacteroids. See Methods section for details.

In Situ Localization of *Rop*A Transcripts

To determine whether the decreased detectability of antigen group III in developing bacteroids is the result of an actual decrease in expression rather than of degradation, for example by lytic enzymes in the peribacteroid space, we examined the level of *rop*A mRNA in pea nodules by *in situ* hybridization with a *rop*A probe. This technique would also allow us to determine in what particular developmental stage, if any, down-regulation takes place. Longitudinal sections of 16 day-old pea nodules induced by *R. leguminosarum* biovar *viciae* strain 248 were hybridized with a radioactive antisense-RNA-probe derived from the cloned *rop*A gene. Fig. 3A shows a phase contrast micrograph of a pea nodule section in which the different developmental zones can be identified. The 3 to 4 cell layers of interzone II-III can be easily identified here by their bright white appearance caused by amyloplast accumulation. Fig. 3B shows an overview of a longitudinal section of a whole nodule hybridized with a radioactively labelled *ropA*-probe and with the different developmental zones indicated according to Franssen *et al.*

(1992). Labelling at a low level is detectable in the youngest cells of the pre-fixation zone II. The density of silver particles increases towards the older, proximal part of zone II, probably as a result of bacterial proliferation. However, at the transition of the pre-fixation zone II to interzone II-III the intensity of the signal decreases abruptly. The same section viewed by dark field-microscopy (Fig. 3C) shows the same pattern. It can also be seen here that in the interzone II-III the hybridization signal decreased to a low, though still detectable level that remains constant throughout the rest of the proximal part of the infected tissue of the nodule. A higher magnification of the transition region (Fig. 3E) shows that this decrease occurs abruptly from one cell layer to the next layer, with almost no intermediairy levels. This decrease in *rop*A mRNA level coincides with the appearence of amyloplasts in the infected cells, and so it exactly matches the transition from the pre-fixation II into the interzone II-III. Control sections hybridized with sense-*rop*A RNA probe showed no signal above background levels.

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

Down-Regulation of the *Rop*A mRNA Level Can Be Uncoupled from the Activation of *Nif* Gene expression

The exact coincidence of *rop*A down-regulation and the start of *nif* gene transcription prompted us to address the question whether *nif* gene transcription and *rop*A down-regulation might be regulated by the same mechanism. For this purpose we looked at pea nodules induced by strain K11.pMP258. This is a *nod*E::Tn5 mutant of *R. leguminosarum* biovar *trifolii* strain ANU843 (Djordjevic *et al.*, 1985) containing a cloned *nod*E gene of the *R. leguminosarum* biovar *viciae* strain 248 Sym plasmid pRL1JI, under control of the *nod*A promoter of the same plasmid (Spaink *et al.*, 1989). This strain nodulates various host plants of biovar *viciae* strains such as vetch and pea, but forms ineffective nodules on these plants (Spaink *et al.*, 1989; H.P. Spaink, unpublished results). Light microscopy of 7 μm sections of a pea nodule induced by this

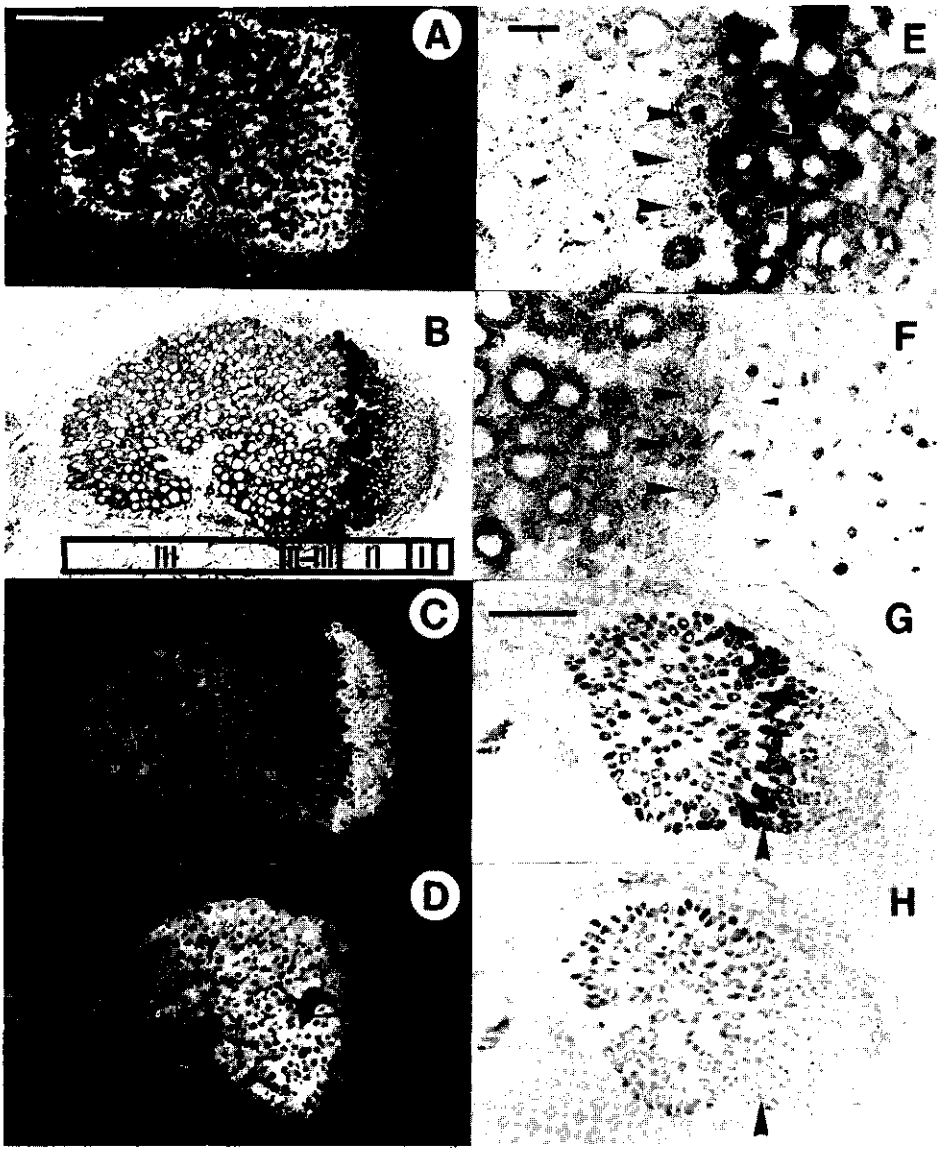

Fig. 3. *In situ* localization of *rop*A and *nif*H mRNA in adjacent longitudinal sections of 16-day-old pea nodules. Nodules were induced by *R. leguminosarum* biovar *viciae* strain 248 (**A-F**). **A.** Phase contrast micrograph showing amyloplast accumulation. Bar=50μm. **B.** Bright field micrograph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

strain showed infected cells with bacteroids (Fig. 3G). This shows that, although the bacteroids are not able to fix nitrogen, release from the infection threads, subsequent proliferation, and to some degree normal bacteroid development do occur. The bacterial strain from which mutant K11 is derived, biovar *trifolii* strain ANU843, has a group of outer membrane proteins related to the group III antigens of strain 248. This relationship consists of immunological cross-reactivity (de Maagd, 1989) as well as of the occurence of two strongly cross-hybridizing DNA fragments in Southern blots of ANU843 DNA probed with *rop*A (Roest *et al.*, unpublished results). Moreover, immunochemical comparison of free-living bacteria and bacteroids from pea nodules of strain ANU843 containing biovar *viciae* Sym plasmid pRL1JI showed that, as in strain 248, this antigen group is severely reduced in bacteroids (Roest *et al.*, manuscript submitted). *In situ* hybridization of 7 μm sections of
K11.pMP258-induced pea nodules with a *rop*A-antisense probe (Fig. 3G) revealed a pattern of mRNA distribution that is similar to that in pea nodules formed by strain 248 (Fig. 3B). However, in adjacent sections of the same nodule we were unable to detect *nif*H mRNA accumulation by *in situ* hybridization (Fig. 3H). These results show that the down-regulation of *ropA* mRNA level during bacteroid development can be uncoupled from the activation of *nif* gene transcription.

DISCUSSION

In this study we have followed the expression of the *ropA* outer membrane protein of *R. leguminosarum* during symbiosis. *Rop*A expression appears to be regulated at the mRNA level, showing a sudden, sharp decrease from one cell layer to the next layer at the transition from zone II to interzone II-III. Although this decrease occurs at exactly the same stage as where *nif* gene transcription is activated, these processes could be uncoupled.

We first addressed the question where the decrease of expression takes place by looking at occurence of the MAb38-epitope of antigen group III. This showed that expression of the epitope decreases inside the nodule, between release from the infection thread and development into mature bacteroids. Subsequently we *in situ* localized the *rop*A messenger in sections of pea nodules. Down-regulation of the *rop*A messenger level occured at the transition of zone II to interzone II-III, clearly showing that this transition is an important region in the nodule for bacteroid differentiation. The interzone II - III can be identified microscopically by the presence of amyloplasts (Vasse *et al.*, 1990; Franssen *et al.*, 1992). During nodule development it is initially a major region of the central tissue, but it decreases to only a few cell layers in mature nodules (Franssen *et al.*, 1992). Whereas in zone II proliferation of the bacteria inside the infected plant cells

appears to be the main feature, in interzone II-III the morphological and - as we propose here - molecular changes start to take place that will eventually give rise to the mature, nitrogen-fixing bacteroid. Interzone II-III represents not only a crucial stage in bacteroid development: expression of plant-derived nodulins also seems to alter in this zone. The early nodulin gene *Ps*ENOD5 is highly expressed in zone II but its expression suddenly drops at the transition to interzone II-III (Franssen *et al.*, 1992). Furthermore the expression of the late nodulin gene *Ps*NOD6 is induced at this transition (Kardailsky *et al.*, pers. commun.). Also, in alfalfa in interzone II-III expression of the late nodulin leghaemoglobin first occurs (De Billy *et al.*, 1991).

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

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

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

METHODS

Plant Materials, Bacterial Strains, Plasmids, and Growth Conditions

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

Electron Microscopy.

Vetch nodules were harvested 21 days after inoculation and fixed overnight at 4°C in 1% glutaraldehyde - 2% paraformaldehyde - 0.1 M sodium cacodylate, pH7.2. Fixed nodules were dehydrated in an ethanol series (30, 50, and 70% at -20°C, 96% and 100% at -35°C) and infiltrated with LR White acrylic resin (Agar Scientific Ltd., Stansted, U.K.) at -35°C. The resin was polymerized using 0.5% benzoinmethylether as a catalyst for 24 hours at -20°C and for 24 hours at room temperature under UV light. Ultrathin sections were collected on collodion-coated nickel grids and immunolabelled as described previously (Goossen-de Roo *et al.*, 1991). Quantification of labelling based on gold particle density was done as described before (Goossen-de Roo *et al.*, 1991). Number of cells for which gold particles were counted: 127, 50 and 70 for infection thread/droplet bacteria, "young" bacteroids, and mature bacteroids, respectively. Number of section areas counted: 19, 14 and 14, respectively. For the two-by-two comparison of different developmental stages a Student's t test was used to determine the statistical significance of observed differences.

In Situ Hybridization

Pea nodules were harvested 16 days after inoculation and fixed immediately with 4% paraformaldehyde and 0.25% glutaraldehyde in 10 mM sodium phosphate buffer (pH7.2) supplemented with 100 mM sodium chloride for 4 hr. The nodules were dehydrated by passing through a routine ethanol series and were embedded in paraffin.

Sections (7 μm thick) were hybridized according to a method derived from Cox and Goldberg (1988) as described before (Van de Wiel *et al.*, 1990; Yang *et al.*, 1991).

Labelling of Antisense/Sense RNAs.

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

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Chapter 8

Concluding remarks

Root nodule formation provides a good system to study general plant developmental processes such as, induction of cell division in terminally differentiated tissues, meristem formation and differentiation of meristems into tissues. Based on the results described in this thesis we will discuss the following aspects in more detail; (1) zonation of indeterminate nodules; (2) a possible mechanism by which cell division is induced in the root cortex (nodule primordium formation), and (3) defense response in nodule development.

Zonation of indeterminate nodules induced in a nodule primordium

When the nodule primordia are penetrated by an infection thread and bacteria are released into the plant cell the nodule primordia form a meristem at their distal part, while simultaneously differentiation into nodule tissues starts at the proximal region. This step in nodule development establishes the first zonation and divides the young indeterminate nodule into a meristem and a prefixation zone II. In alfalfa a meristem is formed in Nod factor induced primordia (Truchet, *et al.*, 1991), while this is not the case in such vetch nodule primordia (Vijn, *et al.*, 1993). This might mean that in some *Rhizobium*--legume interactions additional signals are required to establish a nodule meristem.

Nodule meristematic cells can be distinguished from the prefixation zone II cells by cytological characteristics since they are smaller and more cytoplasmic rich, but it is hard to indicate the exact transition of meristem into the prefixation zone II. Genes which are specifically expressed in dividing cells like B-type cyclins and histone 4 (H4) can be used to distinguish meristematic cells and the cells of the prefixation zone. These genes are expressed predominantly in the nodule meristem, but they are also expressed in some cells of the prefixation zone II (Yang and De Blank, unpublished results). Therefore these genes are not very useful to identify precisely the meristem--prefixation zone transition. In pea (Scheres, *et al.*, 1990a), alfalfa (Pichon, *et al.*, 1992) and vetch nodules (Vijn, personal communication) the ENOD12 gene is expressed in the distal part of the prefixation zone II, but it is not expressed in the meristem. Moreover the ENOD12 mRNA is immediately present at a maximal level in the first cell layer of the prefixation zone II. The ENOD40 gene has a similar expression pattern in nodules. In nodules the ENOD40 gene is expressed in the complete prefixation zone II, whereas this gene is not expressed in the meristem (Yang, *et al.*, 1993; Chapter 4; Yang, unpublished results). Thus ENOD40 and ENOD12 gene expression form good markers to distinguish meristem cells from cells of the prefixation zone II.

We showed that the CHS gene is induced in all nodule primordial cells and at the stage a meristem is established the expression becomes restricted to a few cell layers at the apex (Chapter 3). Therefore the CHS expression pattern is an additional marker to determine whether a meristem has been formed.

When the meristem is formed in the nodule primordium it will differentiate, in a spatially controlled manner, into the different nodule tissues. Since this process will continue throughout the life time of the nodule an organ with a typical indeterminate growth pattern is formed. It is unclear which molecular mechanisms regulate the spatially controlled differentiation of a nodule meristem, but it is striking that the establishment of a nodule meristem and the differentiation into nodule tissues occur concommitantly in a nodule primodium. Similarly, the formation of a root meristem in *Arabidopsis* and pea embryo's is preceded by the formation of root tissues (Scheres, personal communication; Yang, unpublished data). Therefore it is tempting to speculate that the already established spatial distribution of nodule/root tissues determines which part of the meristem differentiates into a specific cell type (Scheres, personal communication).

Zonation in a mature indeterminate nodule

Since the nodule meristem continuously differentiates into nodule tissues in a proximal direction, the indeterminate nodule is composed of zones at consecutive stages of development. In Fig.1 the plant and bacterial genes that mark different stages of development have been indicated.

The CHS gene is expressed in the distal part of the meristem zone I, and this gene is also expressed in the part of the nodule cortex adjacent to the meristem. So this gene is not expressed in a tissue or cell specific manner.

None of the identified plant genes is specifically expressed in the meristem. Therefore this zone is best characterized by the absence of ENOD40 and ENOD12 transcripts, which both are present at a maximal level in the first cell layer of the prefixation zone II. The expression of the rhizobial *nod* genes and the nodulin gene ENOD12 occurs in the distal part of zone II, and this region is named infection zone by Newcomb (1976). The region of expression of the early nodulin genes ENOD40 and ENOD5 as well as the rhizobial *rop*A gene exactly coincides with the prefixation zone II. Expression of Lb and *Ps*ENOD3/14 genes starts in the distal part of the prefixation zone II. Lb gene expression extends to the proximal part of the nitrogen fixation zone III, whereas the concentration of *Ps*ENOD3/14 mRNA decreases dramatically at the beginning of zone III. The late nodulin gene *Ps*NOD6 and the rhizobial gene *nif*H are switched on at a maximal level in the first cell layer of the interzone II-III and the corresponding transcripts are immediately present at a maximal level in this cell layer. These two genes maintain their expression level all over the interzone and zone III. At the moment only one gene has been identified that is expressed in the uninfected cell type of the indeterminate nodule (Van de Wiel, 1991). However this gene has not been cloned and therefore the expression has not been studied.

The studies on the *in situ* expression of *Rhizobium* and plant genes showed that at two positions sudden developmental transitions occur: 1) the transition from meristem zone I to the prefixation zone II, and 2) the transition of the prefixation zone II into interzone II-III. These transitions are marked by changes in gene expression from one cell layer to another. The transition of the prefixation zone II into interzone II-III is furthermore marked by the accumulation of starch granules and a change in bacteroid morphology (Vasse, *et al.*, 1990).

The transition of the prefixation zone II into interzone II-III is an intriguing step in the development of the cells of the central tissue since the developmental change occurs within a single cell layer, and it involves changes in both bacteroid and plant development. These observations can provide some clues on the mechanisms that control the development of the nodule central tissue:

1. At the transition of the prefixation zone into interzone, changes in both bacteroid development and plant gene expression take place within the same cells. This suggests that the same developmental cue controls the development of both bacterium and host plant. Alternatively, a signal molecule can trigger a fast developmental change in one symbiont which then immediately controls the development of the other symbiont.

2. The formation of an infected cell in an indeterminate nodule is probably initiated when a meristematic cell is infected by *Rhizobium*. It is possible that this event initiates a cell autonomous developmental programme, leading to a mature infected cell. We have studied the *in situ* expression of e.g. *nif*H in about 50 pea nodules and we have never observed that an individual cell of the prefixation zone II, so distant to the plane of prefixation zone--interzone transition, expressed *nif*H. Thus in case the development into an infected cell is a cell autonomous response it has to be highly synchronized, which seems unlikely to us. Furthermore it would imply that all the cells of a single cell layer are infected simultaneously, which is also not very probable. Therefore we think it is likely that additional mechanisms control the differentiation of the infected cell; for example mechanisms in which cell-cell communication and/or positional information provided by a gradient of a signal molecule.

When cell-cell communication plays a role in synchronizing the development of the infected cells it must mean that this communication (e.g. by plasmodesmata) predominantly occurs in a direction perpendicular to the distal-proximal axis of the nodule. Since plasmodesmata have not been mapped in a nodule it is unclear whether this is the case.

Since the central tissue has a distal-proximal polarity it is likely that a gradient of a putative regulatory compound is created along this axis. Studies on *Drosophila* development have shown that such gradients of morphogens can be interpreted in zones with very sharp boundaries (Struhl, *et al.*, 1992; Lawrence, 1992). So it is possible that a

distal-proximal gradient of a morphogen controls the development of infected cells and can cause sudden changes in development.

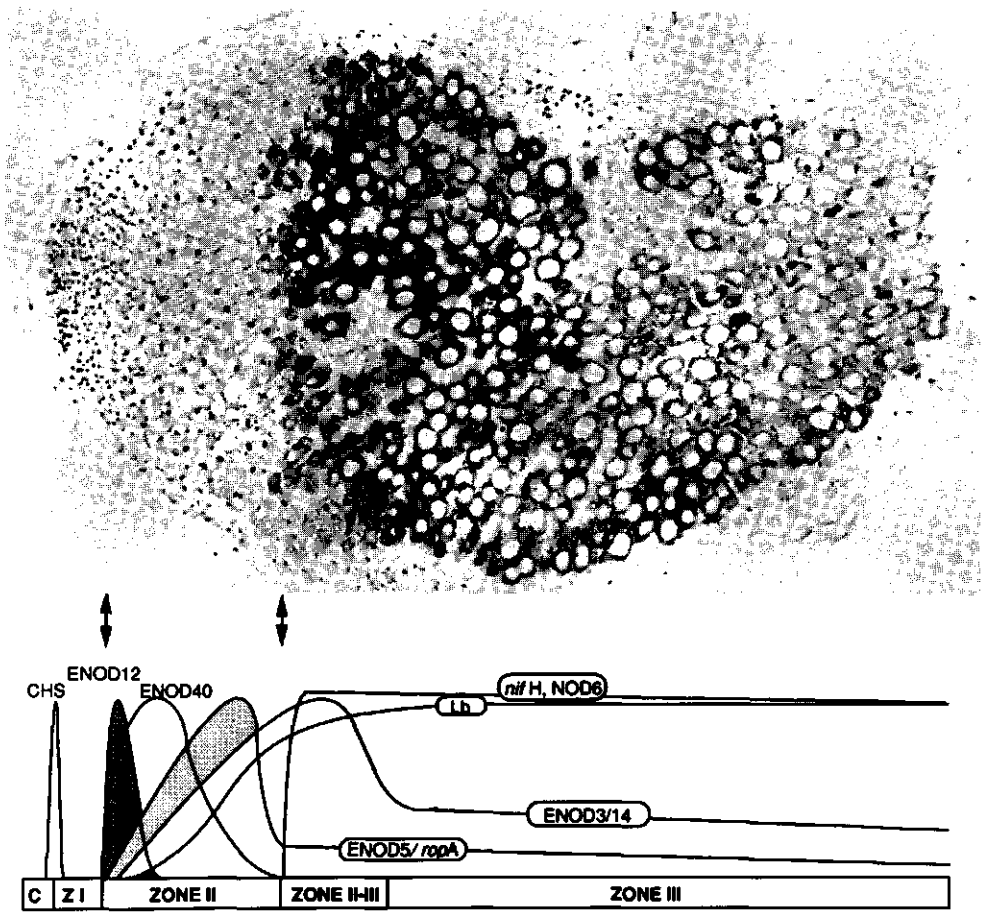

Figure 1. A schematic drawing showing the zonation of the central tissue of a pea nodule and the pattern of plant and bacterial gene expression. The expression level of the different gene is not known therefore the maximal expression level of each gene is indicated by the same height. Arrows indicate two sudden transitions in gene expression. C=nodule cortex, ZI=meristem, ZONE II=prefixation zone, ZONE II-III=interzone, ZONE III=nitrogen fixation zone.

Several molecules can form a gradient in the central tissue: a). Nod factors can only be produced in the distal part of the prefixation zone II since *nod* genes are only expressed in this zone (Schlaman, *et al.*, 1991). Therefore a Nod factor gradient along the distal-proximal axis can be established. b) Meristems are in general sites of phytohormone synthesis and it is likely that this is also the case in a nodule meristem. Therefore it is probably that also the nodule meristem is a source of phytohormones and a phytohormone gradient might be formed. c) Rhizobia proliferate in the infected cells of

the prefixation zone II and therefore O_2 consumption gradually increases in this zone and it can establish an O_2 gradient.

Whether these morphogen gradients are present and can specify patterns of gene expression in nodules is not clear, but the genes that mark the zones of the central tissue might provide tools to study such questions.

Nodule primordium formation

Studies with purified Nod factors have shown that these *Rhizobium* secreted compounds are sufficient to induce the formation of nodule primordia in a spatially controlled manner. For example, in vetch roots primordia induced by either purified Nod factors or *Rhizobium* are formed in the inner cortex, preferentially opposite a protoxylem pole (Spaink, *et al.*, 1991; Van Brussel, *et al.*, 1992; Vijn, *et al.*, 1993). About 20 years ago Libbenga and Torrey have shown that a plant compound, named stele factor, released from the protoxylem poles is involved in the induction of cortical cell division. It is assumed that a gradient formed by the stele factor determines where cell division can be induced (Libbenga and Bogers, 1974). Recently progress has been made in the purification of this compound (Smit, *et al.*, 1993). The availability of purified Nod factors as well as stele factor will make it possible to study how these two morphogens induce cell division in a spatially controlled manner.

Although it is clear that Nod factors are the only bacterial compounds essential for the induction of a nodule primordium, it is unknown by which mechanism these lipooligosaccharides trigger mitotic activity. Studies with compounds that mimic the effects of Nod factors indicate that local changes in phytohormone concentrations are involved in the induction of cortical cell divisions. For example, ATIs (auxin polar transport inhibitors) can induce nodule like-structures on several legume roots (Allen and Allen, 1958) and flavonoids--which probably are endogenous ATIs (Jacobs and Rubery, 1988)--can also trigger the formation of these structures (Hirsch, *et al.*, 1991). However, whether these structures are formed opposite a xylem pole has not been checked. Furthermore, bacteria lacking all *nod* genes but containing the *tzs* gene (encoding an isopentenyl transferase) by which they secrete the cytokinin, zeatin induce nodule-like structures on alfalfa (Long and Cooper, 1988; Cooper and Long, 1994). The latter experiment shows that an increased cytokinin/auxin ratio leads to cortical cell division and suggests that Nod factors induce a similar change to elicit cell division. This hypothesis raises the question how Nod factors establish such a change in the cytokinin/auxin ratio.

According to Morris and Thomas (1978) and Jacobs and Gilbert (1983) auxin is transported from the shoot to the root by the cambium and parenchymatic cells of the stele. Furthermore it is likely that cytokinin is transported from root tip to shoot through

the xylem (Letham, *et al.*, 1978). So it can be questioned whether Nod factors influence the transport of phytohormones from the stele to the cortex or whether phytohormone concentrations are affected by an autonomous response of the cortical cells?

Studies of Pawlowski (personal communication) showed that the expression of a chimaeric gene composed of the CaMV 35S promoter and a soybean ENOD40 cDNA clone (Yang, *et al.*, 1993; Chapter 4), caused a decrease of apical dominance in transgenic tobacco plants. This observation suggests that ENOD40 influences the polar transport of auxin. Interestingly the soybean ENOD40 genes are expressed at a low level in the cambium of the shoots (Yang, unpublished data; Kouchi and Hata, 1993). This location is consistent with a putative role of ENOD40 in auxin transport. Nodule primordium formation induced by *Rhizobium* is preceded by expression of the ENOD40 gene in a region of the root pericycle opposite a protoxylem pole and the adjacent cambium cells (Yang, unpublished data). In Nod factor induced primordia the ENOD40 gene is expressed in the same spatial manner as in *Rhizobium* formed primordia (Vijn, *et al.*, 1993). Based on these observations we postulate the following working hypothesis: The induction of ENOD40 gene expression in the root pericycle, causes a decreased flow of auxin from the cambium to the cortex by which the cytokinin/auxin ratio in the latter tissue increases and this change induces (or sustains) cortical cell division (Fig.2). We showed that the ENOD40 gene is also induced in the primordial cells and furthermore in these primordia the CHS gene is induced. The expression of CHS genes in the dividing primordial cells might result in the production of flavonoids that act as ATIs. Together with ENOD40, these flavonoids might reduce the influx of auxin from the surrounding cortical cells into the primordia, which contributes to the proper cytokinin/auxin balance to maintain mitotic activity in the primordia.

So we propose that instead of a cell autonomous response of the cortical cells, Nod factors influence the communication between stele and cortex as well as between nodule primordium and surrounding cortical cells. The involvement of cell-cell communication in nodule primordium formation would provide the host plant good possibilities to control when and where nodules can develop.

Defense response in nodule development

Genetic analysis of *Rhizobium* mutants have identified several bacterial genes that in addition to *nod* genes are required for normal nodule development (Nap and Bisseling, 1990; Appelbaum, 1989). These genes are involved in the biosynthesis of bacterial outer surface polysaccharides such as exopolysaccharides (*exo* genes), lipopolysaccharides (*lps* genes), and ß-glucans (*ndv* genes). *Rhizobium* mutated in these genes can induce plants to form nodule-like structures, but these form infection threads that abort prematurely, or are defective in bacterial release from the infection threads. The products

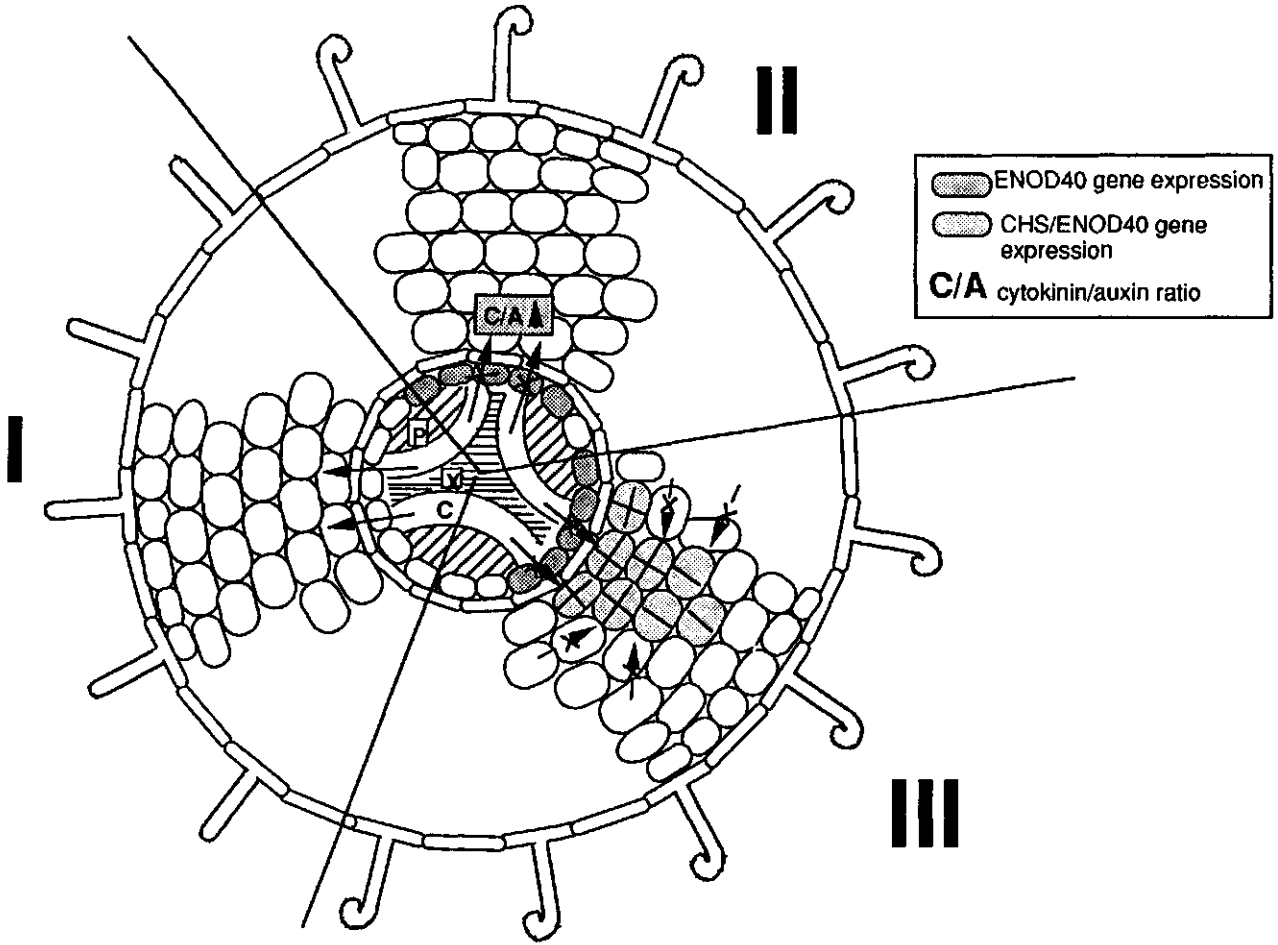

Figure 2. A model for Nod factor action. I: root hairs are present but not yet interact with Nod factors; II: Nod factors induce root hair deformation and ENOD40 gene expression in the pericycle oppsite a xylem pole. III: The ENOD40 gene product can controll the auxin eflux from root stele to cortex, consequently increase the cytokinin/auxin ratio which induces cortical cell division. The induction of ENOD40 as well as CHS would sustain higher cytokinin/auxin ratio in the dividing cells. C=cambium, P=phloem, X=xylem.

of *exo*, *lps* and *ndv* genes most likely act as so-called avoidance determinants to prevent a defense response. Any mutation that unmasks an avoidance determinant will trigger the plant's defense responses and result in abortion of nodule development, even if all signals for proper development are present. For example, *R. leguminosarum* bv. *viciae* mutants which fail to produce the O-antigen containing LPS and do not secrete ß(1-2) glucan form nodules with a few infected cells and the pathogen-related gene, CHS, is induced in uninfected cells surrounding the infected cells which resembles a typical defense response (Yang, *et al.*, 1992; Chapter 3). Recently Vasse *et al* (1993) showed that alfalfa plant can react to infection by its symbiont resulting in either nodule development or a defense response. In this case the plant controls the nodule number by eliciting a hypersensitive defense response to excess infections.

Perspective

The peculiar pattern of expression of plant and bacterial genes in root nodule, as presented in this thesis, is of great interesting in undersanding nodule development and functions. Questions raised from these studies are: How is such a pattern of gene expression established? Is the proceeding gene expression required for the induction of the next genes? What are the key signals involved? To answer these questions several lines of researches in future may be carried out. 1) Identification of transcriptional activators and/or transcription factors which activate these genes; 2) Disruption of expression pattern of these genes by either blocking of a specific gene, e.g. ENOD40, using antisense RNA technique and mutagenesis, or expressing a specific gene at wrong place, for example ENOD40 under the control of Lb promoter; 3) Characterization of functions of genes involved in nodule formation such as ENOD5, ENOD12 and ENOD40; 4) Identification of signal molecules (morphogens?) in nodules. Hopefully, results from these researches will contribute to our undersanding of the mechanisms of root nodule development and of general plant development as well.

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Chapter 9

Summary

SAMENVATTING

Vlinderbloemige planten hebben het vermogen om in symbiose met *Rhizobium* bacteriën stikstofbindende wortelknollen te vormen. Om meer inzicht in de vorming van dit orgaan te krijgen zijn verschillende plante- en bacterie-genen geïsoleerd die een rol spelen bij de knolvorming. De expressie van deze genen gedurende de knolvorming werd met behulp van *in situ* hybridisatie bestudeerd.

Een cDNA kloon van het chalcon synthase [CHS] gen van de erwt werd geïsoleerd en de *in situ* expressie van dit gen werd gedurende de knolontwikkeling bestudeerd. CHS komt tot expressie in knol primordium cellen en wanneer zich in een iets later ontwikkelingsstadium een meristeem heeft gevormd, is de expressie beperkt tot een paar cellagen van de knol cortex die aan het meristeem grensen als ook in de meest apicale cellagen van dit meristeem. In de volgroeide knol blijft dit expressie patroon gehandhaafd. Het is zeer waarschijnlijk dat flavonoïden gesynthetiseerd worden in de cellen waarin CHS tot expressie komt. Aangezien flavonoïden het vermogen hebben het polaire transport van auxine te blokkeren, wordt gepostuleerd dat *Rhizobium* knolvorming initieert door lokaal de biosynthese van flavonoïden te stimuleren, die vervolgens het auxine transport remmen, waardoor de cytokinine/auxine balans verandert.

Rhizobium mutanten die gestoord zijn in de secretie van ß[1-2]glucaan of de synthese van LPS I vormen wortelknollen waarin slechts zeer weing geïnfecteerde cellen voorkomen. In deze knollen komt CHS behalve in de hierboven genoemde cellagen, ook tot expressie in cellen die de paar aanwezige geïnfecteerde cellen omringen. Er wordt gepostuleerd dat de inductie van CHS in deze cellen het gevolg is van de inductie van een afweerreaktie die niet voorkomt tijdens wild type knolontwikkeling.

Er werd een kloon van het erwt vroege noduline *Gm*ENOD40 geïsoleerd en gekarakteriseerd. Het potentiele ENOD40 polypeptide bevat geen enkel methionine en heeft geen homologie met een eerder gekarakteriseerd eiwit. Gedurende vroege stadia van de knolontwikkeling komt ENOD40 tot expressie in de delende cortex cellen en in het deel van de pericykel van de vaatbundel gelegen tegenover de delende cortex cellen die een knolprimordium vormen. In soya wortelknollen komt ENOD40 tot expressie in de niet-geïnfecteerde cellen als mede in de pericykel van de knol vaatbundels. In soya knollen, die noch infectiedraden noch bacteriën bevatten, komt ENOD40 niet tot expressie in de pericykel van de knolvaatbundel, hetgeen suggereert dat de expressie van dit gen de aanwezigheid van bacteriën in de knol vereist. De aanwezigheid van ENOD40 in de wortel pericykel suggereert dat dit noduline de communicatie tussen vaatbundel en cortex beïnvloedt en als zodanig een belangrijke rol bij de initiatie van knolvorming kan spelen.

De zonering van het centrale weefsel van de erwte wortelknol werd bestudeerd en daarom werd een nieuw laat noduline gen, *Ps*NOD6, gekloneerd. *Ps*NOD6 is homoloog met de vroege noduline genen *Ps*ENOD3 and *Ps*ENOD14 van de erwt. *Ps*NOD6 komt alleen tot expressie in de geïnfecteerde cellen van de interzone II-III en de fixatie zone III van het centrale knolweefsel, terwijl de expressie van *Ps*ENOD3 en *Ps*ENOD14 reeds in het distale deel van de pre-fixatie zone II begint. Een vergelijking van de expressie patronen van *Ps*NOD6 en de leghemoglobine genen laat zien dat de laatst genoemde genen eerder in de ontwikkeling geïnduceerd worden en dus zeer waarschijnlijk door een ander mechanisme worden gecontroleerd.

Gedurende de knolontwikkeling ondergaat de bacterie belangrijke veranderingen zowel in vorm en gedaante en in expressie van genen. De expressie patronen van de *nif*H, *nif*A, en *rop*A, de laatste koderend voor een buiten membraan eiwit, werden bestudeerd. *rop*A komt tot expressie in vrij-levende rhizobia en komt ook in de wortelknol tot expressie. Bij de overgang van pre-fixatie zone II in interzone II-III neemt de expressie van dit gen plotseling zeer sterk af. *nif*H en *nif*A beginnen tot expressie te komen precies in de cellaag waar *ropA* expressie ophoudt. *nif*H komt tot expressie in de interzone en de fixatie zone terwijl *nif*A alleen in de interzone tot expressie komt. Aldus markeren het uit- en aanschakelen van *rop*A en *nif*A en *nif*H de overgang van pre-fixatie zone in interzone.

De bestudering van gen expressie in het centrale knolweefsel heeft twee belangrijke controle punten in de knolontwikkeling zichtbaar gemaakt: 1) De overgang van meristeem in pre-fixatie zone die gekarakteriseerd wordt door de inductie van ENOD12 en ENOD40. 2) De overgang van pre-fixatie zone in interzone, die gemarkeerd wordt door het uitschakelen van de expressie van ENOD40 en *ropA* en het aanschakelen van *Ps*NOD6, *nif*A, en *nif*H. Bestudering van de regulatie van de expressie van deze genen tijdens de vorming van het centrale weefsel zal een uitstekend hulpmiddel zijn om de mechanismen die knolvorming controleren te leren begrijpen.

Summary

Formation of symbiotic nitrogen-fixing root nodules is the result of complex interactions between the soil bacterium *Rhizobium* and legume plants. To understand mechanisms by which root nodules are formed several plant genes and bacterial genes were isolated and characterized. The expression pattern of these genes during nodule development was studied by the *in situ* hybridization technique.

A cDNA clone of the pea chalcone synthase gene (CHS), encoding a key enzyme in the biosynthesis of flavonoids, was isolated. CHS is expressed in infected roots and in root nodules. During nodule development this gene was first expressed in nodule primordial cells and at a slightly later stage the expression is restricted to a few cell layers of the nodule cortex adjacent to the nodule meristem as well as in the most apical layers of the meristem.In a mature nodule this latter expression pattern is maintained. During root development a similar pattern of CHS expression was observed. Thus it was concluded that flavonoids are synthesized in the apical part of the nodule. Since flavonoids might function as polar auxin transport inhibitors it has been proposed that *Rhizobium* induces nodule formation by stimulating local biosynthesis of flavonoids that subsequently regulate auxin distribution in the root cortex and then influence the auxin/ cytokinin balance. In ineffective nodules formed by a *Rhizobium leguminosarum* bv. *viciae* mutant which is unable to secrete ß(1-2) glucan and to synthesize the O-antigen containing LPS I, CHS is induced in cells surrounding a few infected cells. It was postulated that the induction of CHS in nodules formed by the *Rhizobium* mutant is due to an induction of a plant defense response which does not occur in normal nodule development.

The early nodulin gene ENOD40 was isolated and characterized. The ENOD40 polypeptide does not start with methionine and does not show homology to known proteins. During early stage of nodule development ENOD40 is expressed in dividing root cortical cells, the nodule primordial cells and the pericycle of the root vascular bundles. In mature soybean nodules *Gm*ENOD40 is expressed in uninfected cells, the boundary cell layers and the pericycle cells of the nodule vascular bundles. In soybean nodules formed by *Bradyrhizobium japonicum* mutant 3160, which lack infection threads as well as intra cellular bacteria the induction of *Gm*ENOD40 expression in the pericycle of the nodule vascular bundles requires the presence of the bacteria inside the nodule The expression of ENOD40 in the pericycle suggests that ENOD40 might play a role in transport of metabolites or regulating communication between the root stele and cortex. Therefore ENOD40 might play an important role in the induction of cortical cell divsions.

To study development of the nodule central tissue, a pea late nodulin gene *PsNOD6* was isolated and characterized. *Ps*NOD6 is homologous to the pea early nodulin genes *Ps*ENOD3 and *Ps*ENOD14. *Ps*NOD6 is expressed in infected cells of the interzone II-III and the nitrogen fixation zone III of the nodule central tissue. While the

expression of *Ps*ENOD3 and *Ps*ENOD14 starts at the distal part of the pre-fixation zone II and reaches the maximal level of expression at the interzone II-III. The homolgy between these genes suggests that they belong to the same gene family, but the expression patterns show that they are activated at different times of development. Comparison of the expression pattern of *Ps*NOD6 and the late nodulin gene leghemoglobin (Lb) suggests that the *Ps*NOD6 and Lb genes are regulated by different mechanisms.

The rhizobia also undergo developmental changes during nodule formation. These include changes in gene expression as well as in morphology. The expression pattern of bacterial genes, *nif*H encoding for a nitrogenase subunit, the regulatory gene *nif*A and *rop*A, which encodes for an outer membrane protein, was studied. *rop*A expression starts in the distal part of the pre-fixation zone II and reaches its maximal level at the end of this zone. The expression of this gene drops dramatically at the begining of the interzone II-III. *nif*H expression starts and immediately reaches its maximal level of expression at the begining of the interzone and maintains the same expression level in the nitrogen fixation zone III. The expression of *nif*A starts exactly at the same cell layer as *nif*H but this gene is switched off after the interzone. Comparison of adjacent sections hybridized with *nif*A, *nif*H and *rop*A, respectively ,indicates that the drop in *rop*A expression matches exactly with the induction of *nif*A and *nif*H expression and the transition of the pre-fixation zone II into the interzone II-III.

The specific regulation of both plant and bacterial genes during the development of the nodule central tissue revealed two major developmental switches. 1) The transition of the meristem into the pre-fixation zone II which is marked by the switching on of the ENOD12 and ENOD40 genes; 2) The transition of the pre-fixation zone II into the interzone. This transition is marked by the switching off of the ENOD40 and *rop*A genes and the induction of *Ps*NOD6, *nif*A and *nif*H. The fact that the described genes can be used to mark specific zones of the central tissueot yet clear, provides good tools to study certain aspects of nodule development.

总结

豆科植物(比如豌豆和大豆)和土壤中的根瘤菌(Rhizobium)相互作用形成一种独特的共生固氮器官--根瘤(Root nodule). 根瘤的形成主要经过四个阶段: 1)诱导根皮层细胞分裂, 2)根瘤原基和分生组织的形成, 3)分生组织分化形成根瘤组织. 一个成熟的根瘤是由中央组织(Central tissue)和外周组织(Peripheral tissue)组成, 中央组织又是由侵染细胞(Infected cell)和非侵染细胞(Uninfected cell)两种类形的细胞构成, 而外周组织则是由皮层组织(Cortex), 内皮层(Endodermis)和根瘤薄壁组织(Nodule parenchyma)组成. 根瘤的器官发生涉及根瘤菌基因和其宿主植物基因在时间和空间上的协调表达和调控. 要弄清楚根瘤器官发生的机理, 就必须研究植物和细菌基因在根瘤形成过程中的表达和调控情况, 正是为了这个目的本论文报导了几个植物和根瘤菌基因的分离鉴定和其原位表达的情况.

在根瘤发育过程中有一些植物基因只在根瘤发育过程表达, 这些基因就是结瘤素基因(Nodulin gene). 在根瘤固氮发生之前表达的结瘤素基因叫做早期结瘤素基因(ENOD)(比如ENOD40), 这类基因主要是在根瘤器官发生中起作用; 而在根瘤固氮发生开始时开始表达的基因则叫做晚期结瘤素基因(NOD), 比如NOD6基因. 晚期结瘤素基因则主要与根瘤的功能有关.

Allen等在五十年代的工作以及Hirsch等近期的报导表明类黄酮(Flavonoid)可能在根瘤发育过程起调节作用. 因此作者通过原位杂交(In situ hybridization)技术研究苯基苯乙烯酮(Chalcone)合成酶(CHS)基因在豌豆根瘤发育过程中的表达来了解类黄酮在根瘤发育过程中的作用. 为此克隆和鉴定了豌豆CHS基因. 结果表明CHS基因在豌豆根和根瘤中都表达了. 在根中CHS基因主要在表皮和根毛细胞以及根尖分生组织中表达. 在根瘤发育过程中, CHS基因主要在根瘤原基细胞中表达, 当根瘤原基开始分化形成分生组织时以及在成熟根瘤中CHS基因表达主要在位于分生组织顶端的一两层细胞中. 因为CHS合成酶是类黄酮生物合成的关键限速步骤, 所以CHS表达的地方代表了类黄酮生物合成的地方. 根据以上结果作者提出一个假说: 即根瘤菌信号分子(Nod factor)通过诱导根皮层细胞合成类黄酮, 而这些类黄酮作为植物生长素(Auxin)极性运输的抑制剂从而影响根皮层的激素平衡, 诱导根皮层细胞分裂形成根瘤. 在正常植物和根瘤菌相互作用的过程中CHS在根瘤发育中起作用, 只有在与根瘤菌表面多糖(如LPS)突变体相互作用时CHS基因表达可能在植物防御反应(Defense response)中起作用

早期结瘤素基因, ENOD40, 编码的多肽不但不以甲硫氨酸开始而且与已知的蛋白没有任何同源性. 在根瘤发育早期, ENOD40基因在正在分裂的根皮层细胞, 根瘤原基细胞和中柱鞘(Pericycle)细胞中表达. 在成熟大豆根瘤中, ENOD40基因在未侵染细胞, 隔离层(Boundary layer)和根瘤维管鞘细胞中表达. 在大豆根瘤菌(Bradyrhizobium japonicum)突变体形成的无中央组织的根瘤(Empty nodule)中, ENOD40基因在根瘤维管鞘细胞中没有表达, 这表明ENOD40基因在维管鞘细胞中的表达须要有根瘤菌的存在. ENOD40基因的细胞特异性表达表明ENOD40基因很可能在中柱(Stele)和根皮层之间起运输或调节通讯的作用.

为了研究根瘤中央组织的分化情况，作者从豌豆中分离和鉴定了一个晚期结瘤素基因NOD6.NOD6和豌豆早期结瘤素基因ENOD3和ENOD14有同源性．NOD6基因只在根瘤中央组织的间隔带(Interzone II-III)和固氮区(Nitrogen fixation zone III)的侵染细胞中表达．而ENOD3和ENOD14基因表达起始于固氮前区(Pre-fixation zone II)的顶端部分，在间隔带达到最高．NOD6和ENOD3及ENOD14之间的同源性和基因表达的连贯性很可能暗示这几个基因是属于同一个基因族(Gene family)但在不同的时间和地点被激活．NOD6和晚期结瘤素基因，豆血红蛋白(Lb)基因表达模式之间的比较表明NOD6和Lb基因是由不同的机理调节的．

除了植物以外，在根瘤形成期间根瘤菌也经历一系列发育变化．这包括基因表达和形态变化，比如根瘤菌基因nifH(编码固氮酶亚基)，nifA和ropA(编码一种细菌外膜蛋白).ropA基因表达开始于固氮前区的顶端部分，在该区的底部达到最大．该基因表达在间隔开始时骤减．超微结构和免疫细胞化学研究证明ropA蛋白存在于位于侵染线(Infection thread)中的细菌的表面，而当细菌从侵染线中释放出来后该蛋白则从细菌表面消失；nifH基因表达开始于间隔区并且立刻达到最大,而在固氮区继续保持最高水平．nifA和nifH表达的区域是一样的，只是在间隔区结束后表达水平下降．通过比较分别和nifA，nifH和ropA杂交的连续相邻切片证明ropA表达骤降和nif基因表达开始发生在相邻的两层细胞之间：即从固氮前区向间隔区过渡

植物和细菌基因表达模式与根瘤中央组织带形的比较表明有两个发育转换：1)从分生组织向固氮前区过渡，2)从固氮前区向间隔区过渡．这个转换是以ENOD40和ropA基因的关闭和NOD6，nifA和nifH基因的表达为标志的．尽管这些基因调控的机理并不清楚，本论文为进一步了解根瘤发育的机理提供了研究的依据．

List of Publications

1. Yang, W.C., Horvath, B., Hontelez, J., Van Kammen, A.and Bisseling, T. 1991. *In situ* localization of *Rhizobium* mRNAs in pea root nodules; *nif*A and *nif*H localization. *Mol. Plant-Microbe Interact.* 4,464-468

2. Yang, W.C., Canter Cremers, H.C.J., Hogendijk, P., Katinakis, P., Wijffelman, C.A., Franssen, H., Van Kammen, A. and Bisseling, T. 1992. *In situ* localization of chalcone synthase mRNA in pea root nodule duveiopment. *The Plant J.* 2,143-151

3. Yang W. C., Katinakis, P., Hendriks, P., Smolders, A., De Vries, F., Spee, J., Van Kammen, A., Bisseling, T. and Franssen, H. 1993. Characterization of *Gm*ENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development. *The Plant J.* 3,573-585

4. Yang, W.C. and Bisseling, T. 1993. Nodulin gene expression during pea nodule development. In: *Current Developments in Soybean-Rhizobium Symbiotic Nitrogen Fixation* (ed. X. Dou), pp55-62. Heilongjiang Science & Technology Publishing House, Harbin

5. Yang, W.C. and Bisseling, T. 1994. Molecular aspects of legume root nodule organogenesis. *Advances in Crop Physiology and Biochemistry.* in press

6. Kardailsky, I., Yang, W.C., Zalensky, A., Van Kammen, A. and Bisseling, T. 1993. The pea late nodulin gene *Ps*NOD6 is homologous to the early nodulin genes PsENOD3/14 and is expressed after the leghemoglobin genes. *Plant Mol. Biol.* 23,1029-1037

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11. Matvienko, M., Van De Sanders, K. Yang, W.C., Van Kammen, A., Bisseling, T. and Franssen, H.J. 1994. Comparison of soybean and pea ENOD40 cDNA clones representing genes expressed during both early and late stages of nodule development. *Plant Mol. Biol.* in press

12. Mylona, P., Moerman, M., Yang, W.C., Gloudemans, T., Van De Kerckhove, J., Van Kammen, A., Bisseling, T. and Franssen, H.J. 1994. The root epidermis specific pea gene RH2 is homologous to a pathogen related gene. *Plant Mol. Biol.* in press

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Curriculum Vitae

Wei-Cai Yang (Yang Wei-Cai in Chinese) was born in Ba county, Sichuan province, China on the 14th of Feburary 1964. From 1970 to 1978 he enjoyed his primary and secondary school life. It was the period in 1978-1980 he received intensive basic high school education in the 11th High School, Ba county after the restoration of the education system in China in 1977. He studied Cell Biology during 1980-1984 for his B.Sc. degrees at the Department of Biology, Lanzhou University. He started his M.Sc. study in the autumn of 1984 and PhD education in 1987 in the Lab of Cell Biology at the Department of Biology, Lanzhou University under the supervision of Professor Gou-Chang Zheng. On the 10th of January 1990 he came to Dr. Ton Bisseling's lab at the Department of Molecular Biology, Wageningen Agricultural University, The Netherlands as a participant of the cooperation project between China and the European Community. He finished his PhD research reported in this thesis at the end of 1992. He continues his postdoctoral research in Dr. Ton Bisseling's lab upto now.