nodulin gene expression in the developing pea root nodule

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De lichtmicroscopische opname op de omslag toont een wortelknol op een tien dagen oude erwteplant.

ter nagedachtenis aan mijn moeder

contents

stellingen

1. Er zijn tenminste twee signalen van Rhizobium nodig om expressie van noduline genen in de plant te induceren.

Dit proefschrift.

2. De suggestie van Lang-Unnasch en Ausubel dat B-66, een eiwit dat voorkomt in zowel vrijlevende Rhizobium meliloti bacteriën als in luzerne wortelknollen, een functie heeft bij de nodulatie is ongegrond.

Lang-Unnasch & Ausubel, Plant Physiol. 77, 833-839 (1985).

3. De waarnemingen van Hirsch et al. dat Escherichia coli transconjuganten wortelknollen kunnen vormen op luzerne zijn in strijd met het gegeven dat nod gen promoters niet functioneel zijn in E. coli.

Hirsch et al., J. Bacteriol. 158, 1133-1143 (1985).

4. De experimentele gegevens van McNeil et al. rechtvaardigen niet de conclusie dat zure polysacchariden van Rhizobium geen rol spelen in het bepalen van de gastheerspecificiteit bij de nodulatie van vlinderbloemige planten.

McNeil et al., Carbohydr. Res. 146, 307-326 (1986).

5. De benaming "overdrive" voor een bepaalde nucleotiden volgorde op het Ti plasmide van Agrobacterium tumefaciens die transmissie van T-DNA naar het plantegenoom stimuleert is een incorrecte poging tot popularisering van wetenschap.

Peralta et al., The EMBO J. 5, 1137-1142 (1986).

6. De bewering van Borkird et al. dat in de Daucus carota cellijn W001C bijna 100% van de suspensie cellen differentiëert tot somatische embryo's is niet alleen onjuist, maar ook in strijd met de door henzelf getoonde resultaten.

Borkird et al., Plant Physiol. 81, 1143-1146 (1986).

7. De waarneming dat α en β ketens van "leucocyte function-associated antigen 1" (LFA-1) van mens en muis heteroloog kunnen associëren zodat deficiëntie van LFA-1 wordt opgeheven, opent mogelijkheden voor gen therapie.

Marlin et al., J. Exp. Med. 164, 855-867 (1986).

8. Het voorkomen van humaan T4 antigeen op de plasmamembraan van T lymfocyten is niet voldoende voor een productieve infectie van deze cellen met HTLV III (AIDS virus).

Klatzmann et al., Nature 312, 767-768 (1984),
McDougal et al., Science 231, 382-385 (1986).

9. Met het recent gekloneerde retinoblastoom gen kan de rol van een zogenaamd anti-oncogen bij het onstaan van embryonale tumoren worden bestudeerd.

Friend et al., Nature 323, 643-646 (1986).

10. De toevoeging aan personeelsadvertenties dat bij gelijke geschiktheid van kandidaten vrouwen de voorkeur genieten wekt ten onrechte de suggestie dat geschiktheid van personen objectief te bepalen is.

11. Veel wit stucwerk wordt zwart gemaakt.

12. De plaats van leestekens is soms discutabel.
Voor het puntje op de i geldt dit zeker niet.

Stellingen behorend bij het proefschrift
"Nodulin gene expression in the developing pea root nodule"

Wageningen, 16 januari 1987 Francine Govers

I

scope of the investigation

SCOPE OF THE INVESTIGATION

The association between legumes and *Rhizobium* bacteria results in the development of nitrogen fixing nodules on the roots of host plants. Several plant and bacterial genes are specifically involved in the formation of these nodules. The experiments reported in this thesis were aimed at characterizing the plant genes which are specifically expressed during root nodule development, so called nodulin genes, and at the role of the *Rhizobium* bacteria in regulating the expression of these genes.

By comparing the proteins produced by *in vitro* translation of mRNA from uninfected pea roots and developing pea root nodules at least twenty different nodulins were identified (chapter III). From a root nodule cDNA library various nodulin cDNA clones were selected. These clones were used for a further characterization of the corresponding genes and nodulins (chapter IV).

In root nodules the free oxygen concentration is much lower than in other plant cells. To see whether such a condition by itself induces the expression of specific genes, the RNA content of pea roots grown under microaerobic conditions was studied, both by comparing the *in vitro* translation products with those from nodule RNA and by Northern blot analyses using leghemoglobin and alcohol dehydrogenase cDNA clones as probes (chapter V).

The role of *Rhizobium* in regulating nodulin gene expression was studied by analysing the occurence of nodulin mRNAs in non-effective root nodules of which the development was disturbed at certain stages. Non-effective pea root nodules were obtained by infecting plants with either *Rhizobium* strains which, due to mutations, are unable to fix nitrogen (chapter III and IV), or by engineered *Rhizobium* and *Agrobacterium* strains that still have the genetic information that enables the bacteria to form root nodules but lack the genes which are required for fixing nitrogen (chapter VI). As a first step towards

elucidating the mechanisms by which Rhizobium induces nodulin gene expression, evidence is presented that Rhizobium nodulation (nod) genes are involved in the expression of at least one nodulin gene (chapter VI).

Because Rhizobium genes appear to play an essential role in regulating nodulin gene expression, the current knowledge on bacterial genes involved in nodule formation is reviewed in chapter II. In chapter VII the results are summarized and the possible functions of different nodulins in root nodule development are discussed.

II

interaction between plants and nitrogen fixing microbes

INTERACTION BETWEEN PLANTS AND NITROGEN FIXING MICROBES

1. INTRODUCTION

In nature the capacity to fix nitrogen is restricted to certain prokaryotes which have the ability to synthesize nitrogenase, the enzyme that catalyses the reduction of dinitrogen (N_2) to ammonia (NH_3). Whereas some nitrogen fixing bacteria, like for example Klebsiella pneumoniae and Azotobacter vinelandii, and the cyanobacterium Anabeana cylindrica are capable of fixing nitrogen in the free living state, others only fix nitrogen in symbiotic relation with certain host plants. A well known example is the symbiosis between leguminous plants and bacteria of the genera Rhizobium and Bradyrhizobium. Some Bradyrhizobium species can also fix nitrogen in a free living state but only if the cultures are grown under specific conditions, particularly in N-limiting media and a microaerobic environment. Under natural conditions rhizobia and bradyrhizobia infect their host plants and induce the formation of root nodules. Once the bacteria have penetrated the nodule cells they are able to fix nitrogen. Apparently the appropriate ecological niche is created in the plant cell which enables the rhizobia to express their nitrogen fixing capacity.

A century ago Hellriegel (1886) revealed that the nodules on the roots of leguminous plants function as nitrogen fixing organs and shortly thereafter Beyerinck (1888) isolated the bacteria that inhabited the nodules in pure culture. Since then the importance of symbiotic nitrogen fixation for agriculture has become evident and attempts have been and are still made to improve the yield of agriculturally important legume crops by enhancing the efficiency of the symbiotic nitrogen fixation. Moreover, by growing crops that fix nitrogen the need for fertilizers diminishes which on the one hand provides an economical advantage and on the other hand it has a positive impact on the environment. Pollution of surface water due to excessive

manuring of the fields is a serious ecological problem nowadays. In this respect, the idea of making better use of biological nitrogen fixation is most attractive. Improvement of legumes for enhanced symbiotic nitrogen fixation is the objective of several breeding programmes (Nutman, 1981; Phillips and Teuber, 1985) and during the past decade research on biological nitrogen fixation has been considerably intensified. This has, among other things, resulted in a rapid increase of the knowledge of Rhizobium and in the possibilities to manipulate these bacteria genetically (Beringer et al., 1982).

Other symbiotic nitrogen fixers which presently receive increasing attention for their use as biological fertilizers are Azospirillum species, which associate with the roots of certain grasses and cereals, Anabeana, the symbiotic partner of the waterfern Azolla that grows in rice paddies, and Frankia species which induce nitrogen fixing nodules on the roots of certain trees and shrubs. The potential use of Azospirillum and Anabeana as symbiotic nitrogen fixers was only recognized a decade ago (Dobereiner, 1977; Peters, 1977) and the genetic analyses of Azospirillum have just started (Elmerich et al., 1985). Actinorhizal nodules have already been identified as nitrogen fixing associations in the late 19th century, but research was hampered by the inability to isolate nitrogen-fixing actinomycetes in pure culture. Now this has been accomplished (Akkermans et al., 1984), the microsymbiont becomes accessible to genetic analyses. However, compared to legumes actinorhizal plants have the disadvantage of being woody, tough and often slow growing, factors which can slow down the progress in research on this symbiosis.

Nitrogen fixing plant-microbe associations vary in complexity. The most simple one seems to be colonization of the rhizosphere of certain cereals (e.g. corn and sorghum) and grasses by the soil bacterium Azospirillum. In this case the two partners live side by side without the formation of specific structures. However an exchange of metabolites is thought to take place (Okon, 1984). In the case of the symbiosis between the waterfern Azolla and the cyanobacterium Anabeana

azollae the plant develops cavities in the dorsal lobes of its leaves in which the cyanobacteria live and reproduce but the association remains extracellular (Tel-Or et al., 1984). The most complex associations, in which specialized structures are formed on the host plants, involve actinomycetes (Frankia) and bacteria of the genera Rhizobium and Bradyrhizobium. The microbes penetrate the plant and only in the newly formed root nodules nitrogen is fixed. Whereas actinorhizal nodules are only found on non-legume angiosperms, for example Alnus and Casuarina species, Rhizobium and Bradyrhizobium induced nodules are restricted to members of the family Leguminosae. The only exception known sofar is the non-legume Parasponia which can be nodulated by Bradyrhizobium as well.

All the relationships mentioned above have in common that the plant is a host in the sense that it provides carbon sources for the microsymbiont. In return the guest "pays" with fixed nitrogen which is an essential nutrient for the host. The striking difference between the various relationships is the complexity which increases from loose interactions to the development of specialized nitrogen fixing organs. The various plant-microbe associations are the result of a specific interaction between the genomes of the two symbiotic partners and this is a fascinating aspect of the symbioses. During the development of the symbiosis microbes differentiate from free living organisms to symbiotic nitrogen fixers and plant cells differentiate to accomodate the microbes. However differentiation of one of the partners is only induced upon signals derived from the other partner. Genetic analysis provides an entry to study the microbe-host signal system and of the mechanism of differentiation. The genes of Rhizobium involved in the interaction have been subject of many studies and also some information on symbiosis specific host plant genes has been obtained.

In the subsequent sections I will review the current knowledge on plant and microbe genes involved in the nitrogen fixing associations. It will be focussed mainly on the Rhizobium-legume symbiosis but if information on other associations is available, similarities and dif-

ferences with the Rhizobium-legume symbiosis will be mentioned. The data presented in this thesis are integrated in the overview. The implications of the experimental results reported in chapter III trough VI are discussed in chapter VII and to avoid duplication the text will refer to chapter VII whenever necessary. Moreover, chapter VII.3 contains a concised description of the developmental stages in the Rhizobium-legume symbiosis. Hence, in this chapter I will confine this aspect by presenting a table with the distinct developmental steps and their phenotypic codes according to Vincent (1980) (table 1).

2. PLANT GENES

During the development of a nitrogen fixing root nodule a number a plant genes are specifically expressed. The proteins encoded by these genes are called nodulins (Van Kammen, 1984). Methods employed to detect nodulins and to study expression of nodulin genes are described in chapter VII.2 of this thesis. Nodulin gene expression has been studied in soybean (Legocki and Verma, 1980; Gloudemans et al., 1986), pea (Bisseling et al., 1983; chapter III of this thesis), alfalfa (Lang-Unnasch and Ausubel, 1985; Vance et al., 1985) and yellow lupin (Strózycki et al., 1985). These studies have shown that leguminous root nodules contain at least twenty different nodulins. By following the appearance of nodulin mRNAs during the formation of pea and soybean nodules, induced after inoculation with Rhizobium and Bradyrhizobium respectively, it was found that the nodulin genes are differentially expressed (chapter III of this thesis, Gloudemans et al., 1986). Two nodulin genes in pea and five in soybean are expressed during the stage that nodule structures are formed (stage II in table 1) but expression of the majority of the nodulin genes in pea as well as in soybean starts a few days later, just before or just after the onset of nitrogen fixing activity in the nodules (stage III in table 1). These observations have resulted in the classification of nodulins

into two groups. Class I nodulins, also termed early nodulins, which are involved in early steps of root nodule development and class II nodulins which apparently are required for supporting nitrogen fixation.

Table 1. Stages in root nodule development[+]

Stage	Abridged description	Phenotypic code
I. Preinfection		
1. Multiplication on root surface	root colonization	roc
2. Attachment to root surface	root adhesion	roa
3. Branching of root hairs	hair branching	hab
4. "Marked" curling of root hairs	hair curling	hac
II. Infection and nodule formation		
5. Formation of infection thread	infection	inf
6. Meristem development; nodule development and differentiation	nodule initiation	noi
7. "Intracellular" release of rhizobia from infection thread	bacterial release	bar
8. "Intracellular" multiplication of rhizobia and development of full bacteroid form	bacteroid development	bad
III. Nodule Function		
9. Reduction of N_2 to NH_4^+ (nitrogenase)	nitrogen fixation	nif
10. Complementary biochemical and physiological functions	complementary functions	cof
11. Persistence of nodule function	nodule persistence	nop

[+]from Vincent (1980)

The genes encoding class II nodulins are expressed simultaneously with the genes for leghemoglobin (Lb) and since the expression of Lb genes is easily detectable, Lb can be considered as a type member of class II. Therefore, the class II nodulins are sometimes denoted as Lb-class nodulins. Since the expression of the two classes of nodulin genes coincides with particular stages in the development of the symbiosis the functions of class I and class II nodulins must be related to specific events that take place during these stages. Hence, early nodulins will probably have a role in the formation of a nodule or support, for example, the infection process or the bacterial release (table 1: inf, noi and bar). Experimental data that support this suggestion are discussed in chapter VII.4.1. of this thesis. On the other hand, class II nodulins may have a role in establishing symbiotic nitrogen fixation and enable, for example, development and maintenance of the bacteroids or assimilation and transport of ammonia excreted by the bacteroids (table 1: bad, cof and nop). The functions assigned to some of the class II nodulins are summarized in chapter VII.4.2. of this thesis.

Nitrogen fixing nodules on the roots of the non-legume Parasponia contain an oxygen binding protein that has approximately 40% homology to soybean and lupin Lbs (Kortt et al., 1985) and a Parasponia gene encoding such a hemoglobin (Hb) has been cloned (Landsmann et al., 1986). The overall gene structure of the Parasponia Hb gene with regard to the position of the three introns, is similar to the structure of soybean Lb genes and the DNA sequence of the coding region is 50% homologous to soybean Lb coding sequences. Also in actinorhizal nodules induced by Frankia species Hbs have been found (Appleby,1984). Expression studies on Hb genes or other plant genes specifically induced in root nodules on Parasponia and actinorhizal plants have not yet been reported but the discovery of Hbs suggests that also nonleguminous root nodules have a specific set of plant proteins (nodulins) that fulfil the physiological and structural requirements for the symbiotic interaction.

3. BACTERIAL GENES

3.1. Rhizobium

The symbiotic partnership of Rhizobium bacteria and legumes is highly specific. Rhizobium species have even been taxonomically classified based on their ability to nodulate certain groups of legume plants. Rhizobium leguminosarum, for example, will nodulate pea and vetch but not clover or alfalfa. The last two are nodulated by R. trifolii and R. meliloti, respectively. Rhizobium harbours high molecular weight plasmids one of which is designated symbiotic (sym) plasmid because it carries genes essential for nodulation and nitrogen fixation. The sizes of sym plasmids range from 150 to 1000 kilobases (kb). Hybridization experiments with structural nitrogenase genes of Klebsiella pneumoniae as probes and extensive Tn5 mutagenesis in R. leguminosarum, R. trifolii and R. meliloti, have led to the identification of two gene clusters on the sym plasmids which are both required for fixing nitrogen. Rhizobium mutants with Tn5 insertions in these genes, called nif and fix genes, retain the ability to induce nodules on their specific hosts and the bacteria are always released into the plant cells (Hirsch et al., 1983; Aquilar et al., 1985), but the nodules are non-effective i.e. they are not capable of fixing nitrogen. However, in non-effective pea nodules formed by nif^- and fix^- Rhizobium mutants all nodulin genes, the early nodulin genes as well as those of the Lb class, are expressed (chapter III and IV of this thesis). Therefore, nif and fix gene products are required neither for the induction of nodulin gene expression nor for the steps leading to nodule formation and bacterial release (table 1: stage 1-7). For the developmental steps following bacterial release (table 1: stage 8-11) nif and fix genes need to be functional. Their possible roles will be discussed in 3.1.1. Tn5 mutations in another gene cluster on the sym plasmid produced strains that could not induce nodules anymore and the genes involved were termed nodulation (nod)

genes. They will be reviewed in 3.1.2. Several chromosomal genes or genes on plasmids other than the sym plasmid have a role in the symbiosis as well. A few of these have been genetically characterized and cloned and they are the subject of discussion in section 3.1.3.

3.1.1. Genes involved in symbiotic nitrogen fixation

The Rhizobium genes involved in symbiotic nitrogen fixation can be divided in two classes: nif and fix genes. Nif genes have been defined on the basis of analogy with nif genes in the free living nitrogen fixing bacterium Klebsiella pneumoniae. Till now, seven Rhizobium genes with homology to Klebsiella nif genes have been detected: nif K, D, H, A, B, E and Q. The first three encode the structural components of nitrogenase and all other microbes that have a symbiotic relationship with plants have nif K, D and H in common. Nif B and E are known to be involved in the synthesis of the iron-molybdenum cofactor of nitrogenase. Nif Q mediates molybdenum uptake and nif A encodes a regulatory protein which is a transcriptional activator required for the expression of all other nif operons in K. pneumoniae. Also in Rhizobium nif A has a regulatory function. Fix genes are also required for nitrogen fixation but they share no homology with K. pneumoniae genes and no counterparts of fix genes have been detected in other free living nitrogen fixers. The role of fix gene products has not yet been determined but speculating on their function one can assume that they might be involved in, for example, bacteroid development, support of the fixation process or maintenance of the bacteroid function.

As stated in section 3.1. the nif and fix genes are not required for the developmental steps prior to bacteroid development (table 1). Mutations in nif genes can cause, however, some abnormalities in the development of bacteroids. For example nodules induced by R. meliloti nif H mutants have bacteroids containing compact electron-dense bodies, and bacteroids of R. meliloti nif DK mutants age prematurely (Hirsch et al., 1983).

After the bacterial release the symbiosis has reached the stage in which one partner functions within the cells of the other one. Both partners exchange metabolites and hence still have a close interaction but, compared to the foregoing, a kind of steady state has been reached. In chapter VI of this thesis experimental results are described which show that in the absence of nif and fix genes Rhizobium is still able to activate the plant to form morphologically rather normal nodules that contain all the nodulins. So neither nitrogen fixing activity of bacteroids nor gene products of nif and fix genes elicit completely new responses of the plant in the sense that nodulin genes are expressed. Fix and nif genes do, however, influence the level of expression of nodulin genes. This regulation though is limited to the class II nodulin genes which most likely have a role in supporting the nodules to function as nitrogen fixing organs. Expression of class I nodulin genes, which are involved in the nodule formation, is not influenced in nodules formed by rhizobia that lack functional nif or fix genes (chapter IV). It seems that the role of nif and fix genes is restricted to the ultimate goal of the interaction, symbiotic nitrogen fixation, whereas other bacterial genes have functions in the developmental steps leading to it.

3.1.2. Nodulation genes

The genes on the Rhizobium sym plasmid which are essential for the initial stages of nodule development appear to be located on a very restricted piece of DNA. This was already suspected from the relative scarcity of nodulation mutants found after random mutagenesis of Rhizobium strains. Usually, less than five out of several thousand random Tn5 mutants were found to be nod$^-$ (Long, 1984). The presumption was confirmed by cloning in suitable vectors fragments of the sym plasmid which, upon transfer to sym plasmid cured parental strains, conferred the ability to form nodules. On the R. trifolii sym plasmid a 14 kb region (shown in figure 1 as the Rt HindIII fragment) encodes

all the functions required for host specificity, infection, nodule initiation, nodule development and bacterial release (Schofield et al., 1984). However, in the clover nodules formed by rhizobia containing from the sym plasmid only this 14 kb region, stages which follow the bacterial release are disturbed. Bacteroid development is somewhat aberrant, especially the packaging of the bacteria in peribacteroid membranes, and due to the absence of nif and fix genes no dinitrogen is reduced. Experiments with cloned fragments from R. leguminosarum have led to similar conclusions. Here two cosmid clones (pIJ1089 and pIJ1085) containing 30 kb of the pRL1JI sym plasmid with a 10 kb overlapping region were each transferred to cured Rhizobium strains. Both types of transconjugants regained the ability to nodulate pea and from these results Downie et al. (1983) deduced that the 10 kb overlap (figure 1, Rl two linked EcoRI fragments) is sufficient for infection and initiation of nodule development on pea.

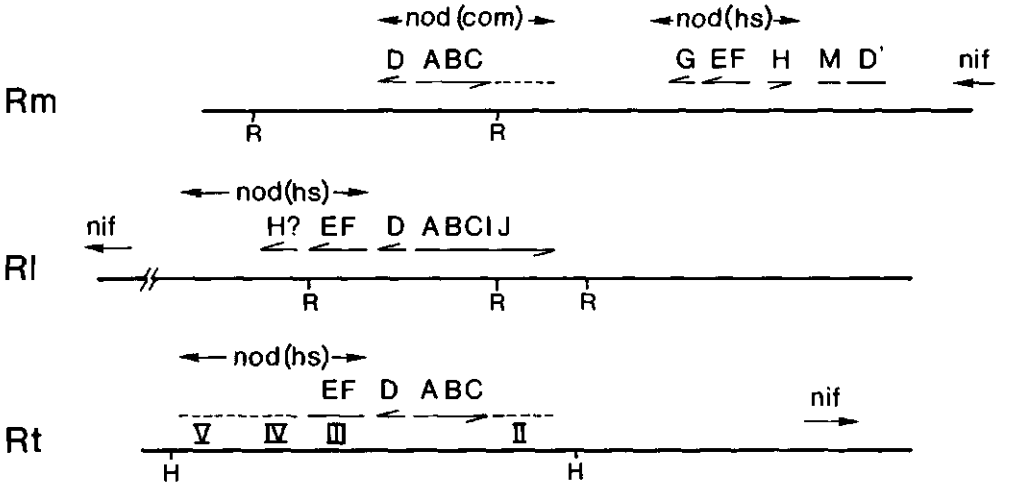

Figure 1. Simplified maps showing the organization of common (com) and host-specific (hs) nod genes on symbiotic plasmids of R. meliloti (Rm), R. leguminosarum (Rl) and R. trifolii (Rt). Thin lines with partial arrows represent transcriptional units. Restriction sites, EcoRI and HindIII, are indicated by R and H respectively and nif shows the position of the structural nitrogenase genes.

As in nodules induced by wild type R. leguminosarum strains, bacteria are normally released and enclosed in membranes (Downie et al., 1983) and expression of all nodulin genes, those of class I as well as of class II, is induced in such nodules (chapter VI of this thesis). Sym plasmid cured R. trifolii strains which contain a 12 kb fragment from the R. leguminosarum sym plasmid covering the 10 kb overlap from the cosmid clones pIJ1085 and pIJ1089, induce normal nodules on pea as well (Spaink et al., 1986) providing the definite proof that the 20 kb regions located up- and downstream from the 10 kb nod region are not essential for nodule formation.

In the last few years complementation studies and sequence analyses of the cloned nodulation region have resulted in an overall picture of the organization of nodulation genes in three different Rhizobium species, R. leguminosarum, R. trifolii and R. meliloti. An overview is shown in figure 1. All three species contain a cluster of so-called common nodulation genes nod A, B, C and D, flanked by host specificity genes nod E, F, G, H and M in R. leguminosarum and R. meliloti or regions III, IV and V in R. trifolii. Nod A, B, C and D are functionally interchangeable between the three Rhizobium species (Djordjevic et al., 1985a) and their DNA sequences are highly conserved (Schofield and Watson, 1986). Nod A, B and C constitute one operon whereas nod D is a separate transcription unit which is transcribed in the reverse direction. For R. leguminosarum it has been shown that the nod ABC operon contains two additional nodulation genes, nod I and J, directly upstream of nod C (Spaink et al., 1986). Region II in R. trifolii, which is also located upstream of nod ABC, most likely carries genes similar to nod I and J. A deletion of this region can be functionally complemented by DNA fragments from R. leguminosarum and R. meliloti. Therefore nod I and J must be considered as common nod genes as well (Djordjevic et al., 1986; Spaink et al., 1986).

Expression analyses of the nodulation genes using fusions of potential promotor sequences with the coding sequence of β-galactosidase, revealed that nod D is constitutively expressed in all three Rhizobium

species. In the presence of plant root exudate nod D controls the expression of the nod ABC operon (Mulligan and Long, 1985; Innes et al., 1985; Rossen et al., 1985). As mentioned before, in R. leguminosarum this operon is extended with nod I and J and hence expression of these genes is regulated by the nod D gene product and plant root exudate as well. The same mode of regulation has been observed for several nodulation genes which determine host specificity like nod FE in R. leguminosarum (Shearman et al., 1986) and region II, III and IV genes in R. trifolii (Innes et al., 1985).

The molecules present in plant root exudate that activate nod gene expression are all flavones but each Rhizobium species is activated most efficiently by a different one. The inducer molecule for R. meliloti nod genes, isolated from alfalfa root exudate, is 3',4',5,7-tetrahydroxyflavone (luteolin) (Peters et al., 1986). In clover root exudate 7,4'-dihydroxyflavone (DHF) is the most active compound but two closely related flavones have a stimulatory effect on R. trifolii nod gene expression as well (Redmond et al., 1986). The natural inducer of R. leguminosarum nod genes has not yet been identified but from several commercial available flavonoids, naringenin (5,7,4'-trihydroxyflavanone) appeared to be the most efficient (Zaat et al., 1986). Comparison of the structures of flavones which induce nod gene expression and those which do not, suggests that hydroxylation at the 7 and 4' positions is required for activity. Also plant root exudates from various non-host legumes were found to contain the appropriate compound for activating nod gene expression in the three Rhizobium species, indicating that at least in this step the plant does not discriminate between species.

Sequence analyses showed that the 5' flanking regions of the nod ABC and nod FE operons in all three Rhizobium species have a highly conserved DNA sequence of 25 basepairs (bp) which is usually located between 150 and 250 bp upstream of the translational start codon and which is not found in front of nod D. Downstream of these 25 bp are two smaller conserved sequences with a length of 7 and 5 bp, respec-

tively (Rostas et al., 1986; Schofield and Watson, 1986; Spaink et al., 1986). The three conserved sequences together are called nod box. Hybridizations with nod box sequences showed that R. meliloti has four additional nod boxes on the sym plasmid, one preceding nod H whereas the three others could not yet be linked to any known functional gene (Rostas et al., 1986). In R. leguminosarum nod H is preceded by a nod box as well (Spaink et al., 1986) while in R. trifolii an additional nod box is found in region IV but the exact position relative to functional genes in this region is not known yet (Schofield and Watson, 1986). It is remarkable that all nod genes which are activated by the nod D gene product and plant root exudate are all preceded by a nod box. This correlation suggests a role for the conserved sequence in the induction of the expression of genes coding for nodulation functions. The plant root exudate might, for example, change the nod D gene product into an activator which subsequently binds to the nod box. The amino acid sequence of the R. leguminosarum nod D protein has indeed some homology with a putative DNA binding region of araC, the regulatory protein of the E. coli arabinose operon (Shearman et al., 1986), but the significance of this homology remains to be established.

Nod D is clearly a regulatory gene. In R. leguminosarum it even regulates its own expression (Rossen et al., 1985). But what is the function of the other nod genes in the nodulation process? R. leguminosarum and R. trifolii mutants with Tn5 insertions in one of the common nod genes D, A, B or C have a nod^- phenotype which indicates that these genes are essential for nodulation. In R. meliloti Tn5 insertions in nod A, B and C also abolish nodulation ability but nod D mutants are leaky (Jacobs et al., 1985) probably because there is a second nod D gene, nod D', located elsewhere on the R. meliloti sym plasmid, which is functionally similar to the nod D gene that flanks nod ABC. Tn5 insertions in the other nodulation genes, nod E, F, G, H, I, J and M, do not abolish the nodulation ability but cause delayed nodulation on their specific hosts and sometimes also a change in host range (Downie et al., 1983; Djordjevic et al., 1985b; Innes et al.,

1985; Horvath et al., 1986). Legume species that normally are non-hosts for the wild-type Rhizobium strains can be nodulated by the mutants. In other cases mutants become nod$^-$ on their normal host but nod$^+$ on non-hosts. Such results suggest that these genes are not absolutely required for nodule initiation. Single Tn5 insertions in either nod A, B or C result in mutant strains which are unable to induce normal root hair curling on the host plant. Root hair curling is the first visible reaction of the plant upon infection with a wild type Rhizobium strain. In the curled root hairs infection threads are formed through which the rhizobia enter the plant. Nod A, B and C mutants do not establish infection thread formation nor cortical cell divisions, which are the initial steps in the formation of a nodule structure. These mutants thus have a hac$^-$ inf$^-$ noi$^-$ phenotype (table 1) and functions of nod A, B and C will be related to these steps.

DNA sequence analyses of nod ABC genes have as yet given few clues on the functions of proteins encoded by these genes and no significant homologies have been found with registered DNA and protein sequences. Because the nod genes appear to be involved in the initial steps of the nodule development, i.e. attachment, root hair curling and infection, it is not unlikely that their gene products are transported out of the bacterial cells. However, no known consensus signal sequence for transport across membranes has been found (Egelhoff et al., 1985). The nod C protein is hydrophobic at its carboxy terminal end indicating that it might be inserted into the cell membrane (Torok et al., 1983). This is supported by the observation that antibodies against the nod C protein inhibit nodulation (John et al., 1985).

Mutations in the R. leguminosarum nod I and J genes, the two other common nodulation genes, cause on pea a delay of nodulation but the nodules formed have a normal morphology and fix nitrogen (Downie et al., 1985). Mutations in region II of R. trifolii, which carries genes equivalent to nod I and J, cause exaggerated root hair curling on clover, the nodulation is delayed and fewer nodules are formed in comparison to inoculation with wild type strains (Djordjevic et al.,

1985b). The sequence of R. leguminosarum nod I and J has recently been published (Evans and Downie, 1986) and the deduced amino acid sequence of nod I shows a high degree of homology with known transport proteins. The nod J protein has a highly hydrophobic nature strongly indicating that it is an integral membrane protein. The nod IJ proteins might thus be involved in transport. Because Tn5 mutations in nod I and J do not totally block nodulation, another transport system might take up the substrate usually transported by nod I and J proteins or, alternatively, the substrate transported is not absolutely required for nodulation but only improves its efficiency.

The modified host range phenotype of nod E, F, G, H and M mutants suggests that these genes are involved in prevention of nodulation on non-host plants. They may also be required, together with nod ABC, for root hair curling and infection thread development in the root hairs. The amino acid sequence deduced from the DNA sequence of the R. leguminosarum nod F gene shows homology with that of acyl-carrier proteins from E. coli and barley and, therefore, the nod F protein could be involved in acetylation, synthesis of fatty acids or lipopolysaccharides (Shearman et al., 1986). None of the mutations in the host specific nodulation genes result in a complete inability to form nodules. So nod E, F, G, H and M appear to be involved in a fine regulation of the host specificity.

From the foregoing it can be concluded that nod A, B, C and D are the only genes on the sym plasmid that are absolutely required for nodule formation. This conclusion is supported by studies of Hirsch et al (1985) who transferred cloned R. meliloti sym plasmid fragments containing nod DABC and no other known nodulation genes (Rm EcoRI fragment in figure 1) to Agrobacterium and to a sym plasmid cured R. trifolii strain. The transconjugants induced on alfalfa the formation of nodules which, like normal nodules, had peripherically located vascular bundles but in contrast with normal nodules had several discrete meristematic regions without infection threads and intracellular bacteria. So the phenotype of the transconjugant is noi$^+$

inf . The complete sym plasmid of R. meliloti or R. leguminosarum in an Agrobacterium chromosomal background induced similar nodule structures on alfalfa and pea, respectively (Truchet et al., 1984; chapter VI of this thesis) and this shows that for the formation of a complete nodule structure, including infection thread formation and bacterial release, the sym plasmid by itself is not sufficient. For the complete developmental programme also chromosomal Rhizobium genes or genes located on other plasmids are required. The noi$^+$ phenotype of Agrobacterium containing only the common nod genes (Hirsch et al., 1985) strongly suggests that a cured Rhizobium strain carrying the same genes must at least have a noi$^+$ phenotype. Since there is no evidence that the host specific nodulation genes are required for the infection process and the nodulation, one would expect that a sym plasmid cured Rhizobium having only the nod DABC region of the sym plasmid is capable of inducing the formation of normal nodules filled with bacteria. Surprisingly, this type of experiments has not been published yet. Knight et al. (1986) recently showed that transfer of a cloned DNA fragment (pIJ1216, Rl EcoRI fragment, figure 1) carrying nod A, B, C, D, E and F genes from R. leguminosarum to a cured Rhizobium strain did not result in nodule formation on pea but early meristematic cell divisions were observed. However in the same series of experiments they introduced nod ABC on a multicopy plasmid into wild type R. leguminosarum and showed that this strain became nod$^-$. Apparently a concentration of nod ABC proteins above wild type level leads to an inhibition of nodulation. Since pIJ1216 is a derivative of a multicopy plasmid this might explain why no nodule initiation is observed even when all the genes that appear to be essential (i.e. nod D and nod ABC) are present. Preliminary data from A. Downie indicate that indeed a low copy plasmid with the same nod fragment as present in pIJ1216 confers on a cured Rhizobium strain the ability to form pea nodules. However, whether nod E and F are required for nodule formation or whether nodulation also occurs without them remains to be tested.

In chapter VI of this thesis experimental data are presented which show that nod genes of R. leguminosarum are essential for inducing expression of at least one early nodulin gene in the host plant. The sym plasmid fragment that is able to achieve this carries nod EF, nod D and nod ABCIJ (two linked EcoRI fragments, figure 1). The same arguments that led to the conclusion that only the nod DABC fragment is required for nodule formation are conclusive to claim that from the nod genes only nod D, A, B and C can be involved in the induction of nodulin gene expression.

3.1.3. Genes involved in exopolysaccharide synthesis

Exopolysaccharides are part of the Rhizobium cell surface. Like other Gram-negative species Rhizobium has an outer membrane outside the peptidoglycan cell wall. External to the outer membrane, but tightly associated with it via covalent linkage to lipid A, are the lipopolysaccharides (LPS). More loosely bound are the extracellular polysaccharides which consist of two types defined by the tightness of adhesion to the bacterial surface: exopolysaccharides (EPS), and the more tightly bound capsular polysaccharides (CPS). The EPS contain a fraction heteropolysaccharides, the majority of these being acidic, and a fraction homopolysaccharides which are neutral and mainly glucans (Carlson, 1982).

Quite often it has been postulated that Rhizobium surface components are involved in specific recognition of, and binding of bacteria to their hosts (reviewed by Bauer, 1981) but till now no conclusive evidence for this has been published. Recently McNeil et al. (1986) showed that the structural features of acidic EPS secreted by R. trifolii, R. leguminosarum and R. phaseoli are identical. Even a sym plasmid cured R. trifolii strain secreted acidic EPS with the same glycosyl sequence and identical locations of non-glycosyl substituents as in acidic EPS from the three wild type strains. Because R. trifolii, R. leguminosarum and R. phaseoli only nodulate their specific

host plants, clover, pea and bean respectively, it was concluded that acidic EPS secreted by free living rhizobia is not the determinant of host specificity. This conclusion was supported by the identical EPS structures of sym plasmid cured and wild type strains because in Rhizobium the sym plasmid has been shown to control host specificity (Hooykaas et al., 1982). The results, however, do not exclude the possibility that a minor acidic EPS component is involved but then the quantity of such a component is below the detection level. Moreover, it remains to be studied what happens with the acidic EPS when nodulation genes are activated. The nod gene products might modify the EPS structure thus still making it a determinant of host specificity.

Even if EPS do not determine host specificity they still have an essential function in the development of the symbiosis. Exopolysaccharide deficient mutants (exo$^-$) induce the formation of nodules but no nitrogen is fixed in these nodules. A R. trifolii Tn5 mutant deficient in the synthesis of EPS induced small, white non-effective nodules containing only a few intracellular bacteria. These bacteria did not differentiate into bacteroids and were not surrounded by membranes (Chakravorty et al., 1982). The mutation could be complemented by introducing a cloned DNA fragment of 14.5 kb that carried the wild type locus indicating that a single Tn5 mutation caused the exo$^-$ and fix$^-$ phenotype. In R. phaseoli three Tn5 induced mutants had an exo$^-$ fix$^-$ phenotype. Root hair curling and initiation of root cortical cell divisions occurred normally in bean seedlings inoculated with these mutants but no infection threads were formed and the rhizobia did not invade the plant cells (Vandenbosch et al., 1985).

An extensive set of R. meliloti Tn5 mutants has been isolated which all fail to produce a particular acidic exopolysaccharide (Leigh et al., 1985). Based on the ability of cloned R. meliloti DNA fragments to complement the exo$^-$ phenotype it was concluded that the mutants belong to six distinct genetic loci designated exo A, B, C, D, E and F. Three of these loci, exo A, B and F, are genetically linked to each other and located on a megaplasmid other than the sym plasmid whereas

exo D is located on the chromosome. All the exo$^-$ mutants attach to the root hairs and induce nodule formation but after inoculation with these mutants neither root hair curling nor infection thread formation is observed and the nodules formed are devoid of intracellular bacteria. The nodules consist of a meristematic region, a region with cells derived from the meristem and vascular bundles positioned along the periphery of the nodules, features which characterize a nodule structure (Finan et al., 1985). From these studies it appears that the symbiotic function associated with acidic exopolysaccharides is invasion of the roots and nodules, and this seems to be related to the ability to form infection threads. Besides a role in the infection process EPS appear to have another role in root nodule development. Klein et al. (1986) inoculated alfalfa with mixtures of R. meliloti mutants deficient in nodulation (nod$^-$), nitrogen fixing (nif$^-$) and acidic exopolysaccharide production (exo B). They showed that if an exo$^-$ nif$^+$ strain has invaded the nodule, probably with the help of the coinoculated exo$^+$ nif$^-$ strain, then still no nitrogen is fixed. Apparently the mutation in exo B prevents proper differentiation of the exo$^-$ nif$^+$ strain into bacteroids. Therefore, these results suggest a role for exopolysaccharides in bacteroid development as well.

Nodules induced by two of the R. meliloti exo$^-$ mutants described above, exo A and exo B, have been analysed for the presence of nodulin mRNAs (Dunn et al., 1986). It was shown that in the empty exo$^-$ nodules expression of only one nodulin gene is induced, instead of the approximately twenty nodulin genes normally expressed in alfalfa nodules. This suggests that exopolysaccharides are in some way involved in the induction of host plant genes, although simply a blockade in the infection process caused by the exopolysaccharide deficiency might prevent the rhizobia from reaching the proper location where they can induce plant gene expression.

Another R. meliloti Tn5 mutant, designated ndv B, induces nodules on alfalfa with the same morphological characteristics as the exo$^-$ nodules described in the previous paragraphs (Dylan et al., 1986): a

nodule structure with vascular bundles but without infection threads and intracellular rhizobia. In contrast to the exo$^-$ strains, the ndv B mutant is still able to induce root hair curling. Isolation of the ndv B mutant started with the finding that Rhizobium contains sequences on the chromosome homologous to the chromosomal virulence genes of Agrobacterium tumefaciens, chv A and chv B. Chv A and chv B are known to be essential for oncogenic transformation of plant cells. R. meliloti cosmid clones carrying the chv homologous sequences were selected by the ability to restore upon transfer the virulence of A. tumefaciens chv A and chv B mutants. By inserting Tn5 in the chv A and chv B equivalent regions in R. meliloti two symbiotically deficient strains, ndv A and ndv B, were generated. The terminology ndv, an abbreviation for nodule development, is misleading because the ndv B mutant still induces normal nodule structures and only the bacterial invasion seems disturbed. In fact, ndv B mutants have to be called nod$^+$ inf$^-$. The effect of a ndv A mutation has not been studied yet. In contrast to the exo$^-$ mutants ndv mutants exhibit normal fluorescence with the dye Calcofluor indicating that they are not deficient in the production of normal amounts of acidic exopolysaccharides. However, the Agrobacterium chv mutants lack the surface component (1-2)β-glucan, an homoexopolysaccharide, and it is conceivable that the Rhizobium ndv mutants have a similar deficiency. It is remarkable that from all bacteria studied only Rhizobium and Agrobacterium species synthesize (1-2)β-glucan (Bauer et al., 1981) but, as yet, no specific correlation has been found between the presence of these glucans and the ability of bacteria to interact with plants.

How exopolysaccharides are involved in the regulation of bacterial invasion and differentiation into bacteroids is unknown. The finding of Albersheim and coworkers (Tran Thanh Van et al., 1985) that oligosaccharides derived from plant cell walls are able to control the morphogenesis of tobacco plants suggests that these small sugar chains are important signalling molecules in plants. Once degraded, either by the host plant or by the rhizobia themselves, Rhizobium exopoly-

saccharides might function as oligosaccharins (oligosaccharides with regulatory activity) and as such induce processes like infection and bacteroid development.

3.2. Bradyrhizobium, Frankia and Azospirillum

In contrast to Rhizobium, Bradyrhizobium species do not contain symbiotic plasmids, but genes involved in the symbiotic interaction with plants are part of the chromosome. Bradyrhizobium japonicum which nodulates soybean, contains two gene clusters with nif and fix genes (Hennecke et al., 1985). B. japonicum deletion mutants missing nif H, D and K induce the formation of morphologically normal nodules and in these fix$^-$ nodules all nodulin genes are expressed (Gloudemans et al., 1986). In soybean nodules formed by B. japonicum nif A mutants the bacteria are released in the plant cells and differentiate into bacteroids but the bacteroids are subject to severe degradation (Fischer et al., 1986). It is likely that similar to Rhizobium, Bradyrhizobium nif and fix genes are not required for developmental steps prior to bacteroid development.

In B. japonicum a region with putative nodulation genes has been identified at a distance of only 10 kb from one of the nif/fix gene clusters. A clone containing this region was selected from a gene bank by colony hybridization using R. phaseoli nod genes as a probe. The B. japonicum nod region hybridized to specific nod D, A, B, C, I and J probes from R. leguminosarum genes and the homologous sequences are arranged in this order. Tn5 insertions in B. japonicum nod A and nod D resulted in mutants deficient in nodulating soybean. Mutations in any of the other putative nod genes have not been tested yet. The nod A and nod D genes are, as in Rhizobium, transcribed from opposite strands and in between lies an intergenic region of 1700 bp (Lamb et al., 1986). In Rhizobium nod A and nod D are only 360 bp or less apart from each other (Shearman et al., 1986; Schofield and Watson, 1986; Egelhoff et al., 1985).

DNA regions having homology with nod DABC have also been cloned from two broad host range Bradyrhizobium sp., RP501 and ANU289, which nodulate among others cowpea (Marvel et al., 1985; Scott, 1986). The putative nod region from ANU289 was sequenced. The deduced amino acid sequence of four open reading frames strongly resembles the sequence of the nod D, A, B and C proteins from Rhizobium species and the genes are arranged in the same order. From the sequence it appeared that the intergenic region between nod A and D is 850 bp. This region contains another open reading frame directly upstream of nod A, which has been assigned as nod K (Scott, 1986). However, the effects of mutations in nod K or in nod D, A, B and C on the nodulation phenotype have not yet been studied. Upon transfer of a plasmid containing the ANU289 nod region, a R. trifolii nod D mutant regained the ability to nodulate clover. This shows that the Bradyrhizobium nod D gene can functionally complement a Rhizobium nod D mutation.

Whereas the nod clone of Bradyrhizobium sp. ANU289 was selected by hybridization with R. trifolii nod genes, the nod region from the other broad host range Bradyrhizobium sp. strain RP501 was selected by the ability of clones to complement insertion and point mutations in nod ABC of R. meliloti (Marvel et al., 1985). This functional complementation and additional hybridization and complementation studies showed that besides conservation of the function, also the organization of the nod ABC operon is the same in Rhizobium and Bradyrhizobium.

In the broad host range Bradyrhizobium sp. strain ANU289 as well as in B. japonicum the region between nod A and D contains the conserved nod box sequence. This suggests that if the nod box is involved in transcriptional regulation, the nod genes in Rhizobium and Bradyrhizobium are regulated in a similar way.

Both Bradyrhizobium strains, ANU289 and RP501, are able to nodulate several other legumes than cowpea e.g. the tropical legume siratro (Macroptilium atropurpureum), but they can also nodulate the non-legume Parasponia. Since mutations in the nod genes of the two strains have not been tested for their effect on the nodulation phenotype,

there is no direct evidence that these nod genes are indeed required for nodulation. However, based on the conserved amino acid sequence, the functional complementation studies and the nod$^-$ phenotype of nod A and D mutants of the closely related B. japonicum, one can assume that the cloned nod region from RP501 and ANU289 contains functional genes. These genes must therefore be considered as common nod genes, which are at least essential for nodulation of the legume hosts. Whether the same set of nod genes has a role in the nodulation of the non-legume host Parasponia is unknown. Hybridization of the fragment containing the ANU289 nod region with genomic ANU289 DNA did not reveal other restriction fragments having homology with the nod genes. This suggests that genes required for Parasponia nodulation are either completely different from the common nod genes or, more likely, that the Bradyrhizobium broad host range species have only one set of common nod genes that function for nodulation of leguminous plants as well as Parasponia.

There is quite some difference in the infection process and in the morphogenesis of nodules formed on the legume siratro and on the non-legume Parasponia. As in most legumes, infection of siratro occurs via root hair curling and infection thread formation in the root hairs. The infecting bradyrhizobia induce cortical cell divisions and the infection threads grow towards the newly formed meristem that finally develops into a nodule. The mature nodule has several peripherically located vascular bundles and a central tissue containing cells filled with bacteroids and uninfected cells (Ridge and Rolfe, 1986; see also table 1). When Parasponia plants are inoculated with bradyrhizobia, root hair curling does not occur. Instead, formation of multicellular root hairs is induced and at the same time, or shortly thereafter, cell divisions are initiated in the outer root cortex always subjacent to the multicellular root hairs. In the early stages of infection no infection threads are observed but the bradyrhizobia enter via intercellular invasion at the bases of the multicellular root hairs. In the dividing root cortical cells intracellular infection threads are

formed and the bradyrhizobia move from the intercellular space to the intracellular infection threads. The dividing cortical cells do not differentiate into a characteristic nodule structure as is the case in legumes, but remain an unorganized callus-like mass, the prenodule. The bradyrhizobia induce besides cortical cell divisions also mitotic activity in the pericycle, the cell layer where normally lateral root meristems are formed. From the dividing pericycle cells the so-called nodule lobes develop which are in fact modified lateral roots having one central vascular bundle. The intracellular infection threads grow from the cortical cells towards the cells derived from the nodule lobe meristem. The apical meristem itself is never infected but a zone of cells surrounding the central vascular bundle becomes fully packed with intracellular infection threads. The nitrogen fixing bradyrhizobia remain in the infection threads, in contrast to legume nodules where the (brady)rhizobia are always released from the threads into the plant cells (Lancelle and Torrey, 1984a; 1984b).

So the same Bradyrhizobium strain is able to induce developmental programmes that result in two morphologically completely different nodule types. If Bradyrhizobium uses the same common nod genes to initiate the nodule development in legumes and non-legumes the nod gene products must cause a different response in legumes versus non-legumes. In view of this it will be of interest to see whether similar genes of the host plants are involved in the steps leading to nodule formation in legumes and Parasponia.

The development and structure of Parasponia nodules greatly resembles nodules on the roots of actinorhizal plants, e.g. Alnus glutinosa, which are formed upon infection with Frankia species (Akkermans and Van Dijk, 1981). In actinorhizal plants hyphae enter via deformed root hairs and grow intracellular towards the prenodule. As in Parasponia the prenodule arises from cortical cell divisions induced by the infecting microbes and also nodule lobes or modified lateral roots are formed from the pericycle. The growing hyphae move from the prenodule to the nodule lobe and a zone of cells that

surround the central vascular bundle is infected. The tips of the hyphae differentiate into vesicles wherein nitrogen is fixed. In search for homology at the genetic level, DNA restriction fragments hybridizing to a nod C probe from R. meliloti have been found in Frankia. A R. meliloti nod AB probe hybridized to Frankia DNA as well but to a lesser extent than the nod C probe (Drake et al., 1985). Frankia might thus have genes similar to Rhizobium nod genes. This observation supports the assumption that Bradyrhizobium will use common nod genes for the nodulation of Parasponia instead of a completely different set of nodulation genes. By further investigating Bradyrhizobium nod genes required for Parasponia nodulation and Frankia genes essential for nodulation, the found similarities might provide clues for the common pattern of nodule development.

The association of Azospirillum species with their hosts, grasses and cereals, does not lead to the formation of differentiated structures comparable to nodules. It remains a loose, extracellular interaction although upon inoculation root hair deformations are observed and the number of root hairs and lateral roots is enhanced. Surprisingly, probes containing R. meliloti common nod genes as well as host specific nod genes hybridize to DNA isolated from Azospirillum species (Fogher et al., 1985). As in Frankia it needs to be investigated whether the hybridizing sequences contain functional and essential genes required for the symbiotic interaction.

To my knowledge, studies on homology of Rhizobium nod genes with DNA of cyanobacteria that associate with plants have not been reported. The Anabeana interaction with the waterfern Azolla leads to leaf deformations instead of root deformations and there are also cyanobacteria that induce root nodules on some rare Cycadales species (Akkermans and Van Dijk, 1981), which makes them interesting to analyse.

In summary, observations on the interaction between Bradyrhizobium and Parasponia, Frankia and actinorhizal plants and Azospirillum and grasses, indicate that these seemingly more primitive nitrogen fixing plant microbe associations have features in common with the Rhizobium-

legume and Bradyrhizobium-legume symbioses. Besides the structural nif H, D and K genes which are similar in all nitrogen fixing microbes, Frankia and Azospirillum have DNA sequences which are homologous to nod genes of Rhizobium. If these sequences carry genes that are required for the interaction with plants, the mechanism by which the microbes elicit responses of the host plant might also be similar. All four, Rhizobium, Bradyrhizobium, Frankia and Azospirillum, cause root hair deformations on their respective hosts. In this view it is interesting to note that a sym plasmid cured R. trifolii strain carrying nod DABC on a multicopy plasmid, is able to induce root hair deformations on maize and rice plants (Planzinski et al., 1985). Induction of cortical cell divisions in legumes, formations of modified lateral roots resulting in nodule lobes in Parasponia and actinorhizal nodules, and the enhancement of the number of lateral roots on grasses and cereals might be responses of the different host plants on the same primary stimulus given by the microbe. In all cases this primary stimulus must be accompanied or followed by reactions which are specific for each association.

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III

expression of plant genes during the development of pea root nodules

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Expression of plant genes during the development of pea root nodules

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The expression of plant genes involved in the pea-*Rhizobium* symbiosis was studied by analysing mRNA from root nodules. The RNA was translated *in vitro* and the translation products were separated by two-dimensional gel electrophoresis. The results show differential expression of nodulin genes during root nodule development. One gene encoding N-40′ is expressed at a significant level 5 days before the leghemoglobin genes. Most other nodulin genes are expressed more or less concomitantly with the leghemoglobin genes whereas the N-21 mRNA is only present late during the development. In the development of ineffective root nodules induced by infection with different *nod*$^+$*fix*$^-$ mutants of *R. leguminosarum* all nodulin genes are expressed except for the N-21 gene. The results suggest that neither bacteroid development, heme excretion nor nitrogen fixation are essential for the induction of nodulin gene expression in the host plant. Further, it appears that the amount of leghemoglobin in ineffective nodules is regulated at a post-transcriptional level.

Key words: ineffective root nodules/leghemoglobin/nodulin gene expression/pea-*Rhizobium* symbiosis/*Rhizobium*/root nodule development

Introduction

Symbiotic nitrogen fixation involving *Rhizobium* and legumes depends on genetic properties of both the bacteria and the host plant. The *Rhizobium* genes involved in host specificity, nodulation and nitrogen fixation are located on a large plasmid, the so-called *sym*-plasmid and the genes concerned are the subject of intensive study (Ausubel, 1982; Pühler *et al.*, 1984; Rolfe and Shine, 1984). Classical genetic experiments have revealed that plant genes play an important role in the symbiotic process from the pre-infection stage to the assimilation of ammonia in a nitrogen-fixing root nodule (for reviews, see Vincent, 1980; Nutman, 1981). The nature and number of plant genes which are essential for symbiotic nitrogen fixation have hardly been studied. Recently, molecular hybridization and immunological techniques have been used by Verma and co-workers (Auger *et al.*, 1979; Legocki and Verma, 1980; Auger and Verma, 1981; Fuller *et al.*, 1983; Fuller and Verma, 1984) for the identification of mRNAs and polypeptides specifically synthesized in soybean root nodules, whereas Bisseling *et al.* (1983) used a nodule-specific antiserum preparation for the detection of nodule-specific proteins in pea root nodules (for reviews, see Bisseling *et al.*, 1984b; Verma and Long, 1983). Nodule-specific proteins that are encoded by the plant genome, are called nodulins (Van Kammen, 1984). Over 20 different nodulins have been detected so far. Among the nodulins are leghemoglobin (Appleby, 1984) and a nodule-specific form of uricase (n-uricase) (Bergmann *et al.*, 1983), but the possible function of most nodulins is as yet not clear. Nodulins may have specific functions in the formation of nodule tissue after de-differentiation and proliferation of cortical cells, in the transport of substrates to the bacteroids, in assimilation of ammonia excreted by the bacteroids or even in the senescence of the nodule tissue. In view of the specific interaction between *Rhizobium* and its host plant it appears likely that the expression of nodulin genes is controlled in some way by *Rhizobium*. However, the molecular mechanisms of this regulation are completely unknown.

Previously, we have reported the detection of 20 – 30 nodule-specific proteins in pea root nodules using a nodule-specific antiserum preparation and the appearance of these proteins during the development of the nodule (Bisseling *et al.*, 1983). Although this approach proved valuable for our understanding of root nodule development, the method has several limitations. It has been shown that *Rhizobium* bacteroids excrete some proteins into the cytoplasm of the plant cells and the nodule-specific antiserum preparation does not discriminate between host plant and *Rhizobium* encoded proteins (Bisseling *et al.*, 1984b; and in preparation). Further, the titer of the antibodies raised against each protein varies depending on the antigenicity of the protein and, therefore, variation in reaction with the complex nodule-specific antiserum preparation did not necessarily reflect the relative amounts of the proteins present in root nodules. Relatively abundant but less antigenic proteins may have remained undetected.

Here we report our analysis of nodule mRNA at different stages of nodule development by *in vitro* translation of the RNA followed by two-dimensional separation of the translation products on polyacrylamide gels. The data give information on the relative amounts of mRNA present for different nodulins and the rate of expression of the genes. Furthermore, we have studied the expression of nodulin genes in three different types of ineffective, i.e., not nitrogen-fixing, pea root nodules produced by different mutants of *R. leguminosarum*. The results of these experiments have led us to hypothesize on the regulation of nodulin gene expression in the *Rhizobium*-host plant interaction.

Results

Nodulin mRNAs

Total RNA isolated from mature, wild-type pea root nodules was translated in a reticulocyte lysate and the translation products were separated by two-dimensional (2-D) gel electrophoresis (Figure 1A). Using this method ~500 polypeptides could be identified in a reproducible manner. When bacteroid RNA was translated in the same eukaryotic translation system no detectable polypeptides were synthesized, indicating that all *in vitro* translation products derived from total nodule RNA and visualized by this method are plant encoded. This was confirmed by translation and analysis of nodule polyA$^+$ RNA which resulted in a polypeptide pattern identical to total RNA. The polypeptides

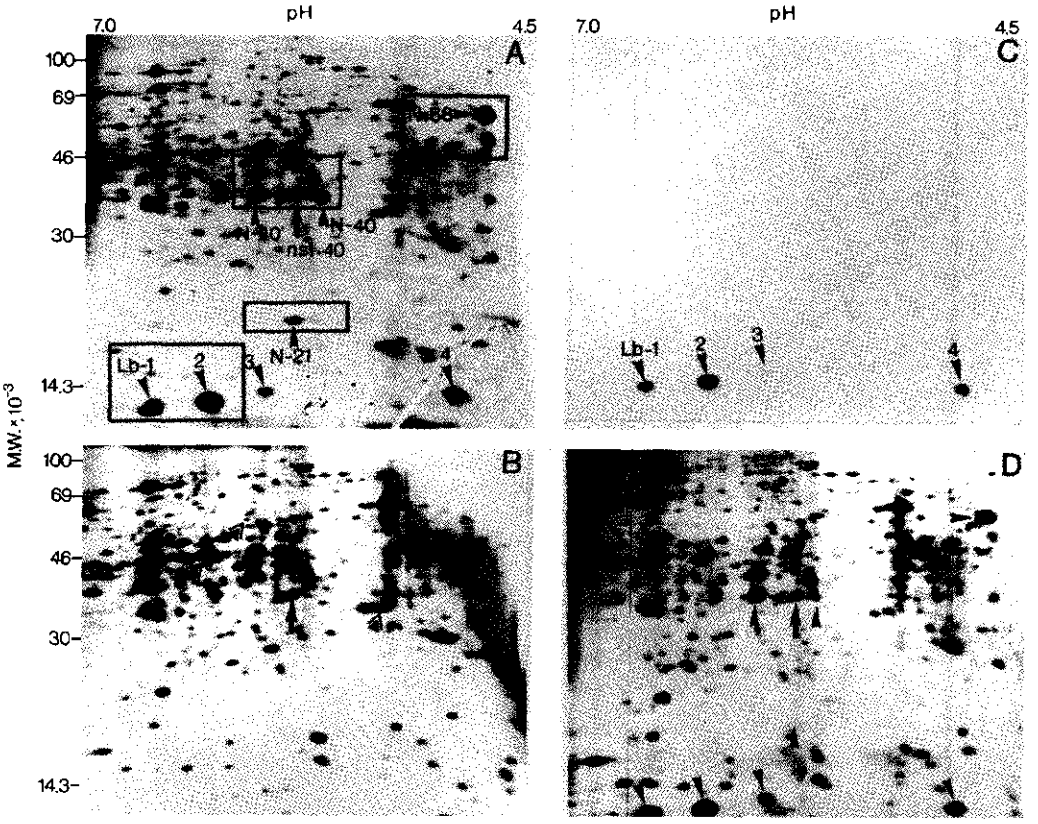


Fig. 1. Identification of nodulin mRNAs. Fluorographs of two dimensional (2-D) gels of *in vitro* translation products from total RNA isolated from **(A)** wild-type effective pea root nodules induced by *R. leguminosarum* PRE, 15 days after sowing, **(B)** 3-day-old uninfected pea roots and **(D)** ineffective pea root nodules induced by *R. leguminosarum* (PRE)2(Tn5::*nifD*), 15 days after sowing. In **(C)** the *in vitro* translation products from RNA isolated from effective pea root nodules were immunoprecipitated with anti-Lb serum and the precipitate was separated on a 2-D gel. The major nodule-specific spots, N-68, N-40', N-40, N-21, Lb-1, Lb-2, Lb-3 and Lb-4 are indicated by ►, the nodule stimulated spot, nst-40, by →, and some root stimulated spots by ►. Mol. wt. markers included ^{14}C-methylated phosphorylase b (100 000 and 92 500), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000) and lysozyme (14 300).

detectable in this way reflect the abundant and middle abundant mRNAs present in the total RNA population (Davidson and Britten, 1979). The RNA concentration used for *in vitro* translation was the same in all experiments and was chosen in the range of a linear relationship between the amount of RNA added and the incorporated radioactivity ([^{35}S]methionine). The intensity of a particular spot will therefore be directly proportional to the relative amount of mRNA present for that particular polypeptide. Changes in the intensities of spots during nodule development can be interpreted as increases or decreases in the expression of the corresponding plant genes, assuming that the translation efficiencies of the mRNAs remain at the same level during nodule development.

Comparison of the 2-D patterns of nodule polypeptides with those obtained from 3- and 8-day-old uninfected pea roots showed that the majority of proteins was present in both roots and nodules (Figure 1A and 1B). However, 21 spots were only observed in the pattern of polypeptides obtained after translation of nodule mRNA and presumably are nodulins. The mol. wts. of these nodulins vary between 15 000 and 80 000 and the group can be divided in 13 minor and eight major spots (the arrowheads in Figure 1A point to the major spots). Four of the conspicuous nodulin spots are leghemoglobin, as shown by immunoprecipitation of the *in vitro* translation products with anti-leghemoglobin serum, and are indicated as Lb-1, Lb-2, Lb-3 and Lb-4 (Figure 1C). The four other major nodulins have apparent mol. wts. of 68 000, 40 000, 40 000 and 21 000 and are indicated as N-68, N-40, N-40' and N-21, respectively, in agreement with the notations proposed recently (Van Kammen, 1984).

Eleven other polypeptides occur both in the pattern from uninfected roots and root nodules, but these spots are more intense in the pattern from root nodules, indicating that the relative amounts of the corresponding mRNAs have increased in the nodules. A clear example of such a nodule-stimulated (nst) polypeptide is nst-40 with a mol. wt. of 40 000 (Figure 1A). Further comparison of the 2-D gel patterns showed that 13 proteins *in vitro* translated from root mRNA are present at a much lower concentration or are even below the level of detection

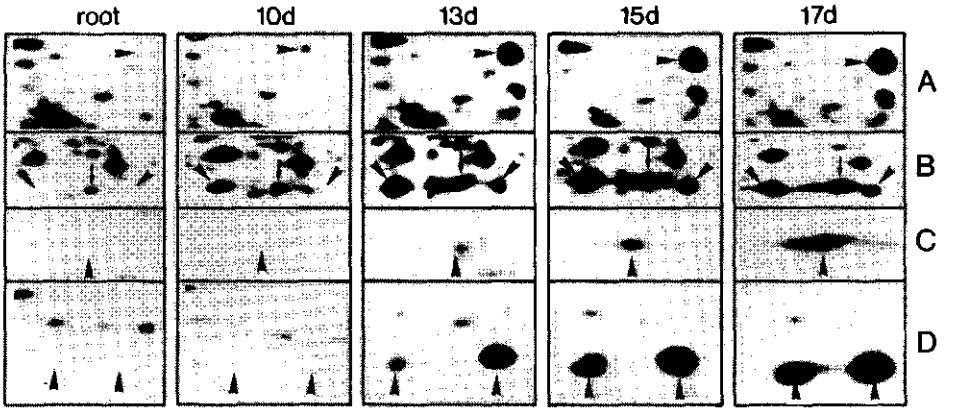


Fig. 2. Expression of nodulin genes during development of nitrogen fixing pea root nodules. Fluorographs of *in vitro* translation products from total RNA isolated from uninfected pea roots and from root nodules, 10, 13, 15 and 17 days after sowing and inoculation. Only the parts of the gels within the squares indicated in Figure 1A are shown as these contain the major nodulin spots. Nodule-specific and nodule-stimulated polypeptides are indicated by ► and →, respectively. The appearance after infection is shown for N-68 in (**A**), N-40', nst-40 and N-40 from left to right in (**B**), N-21 in (**C**) and Lb-1 and Lb-2 from left to right in (**D**).

among the translation products from root nodule mRNA (Figure 1B).

These observations indicate that a number of plant genes are expressed in root nodules but not in uninfected roots, whereas the expression of some genes is increased and of some others decreased.

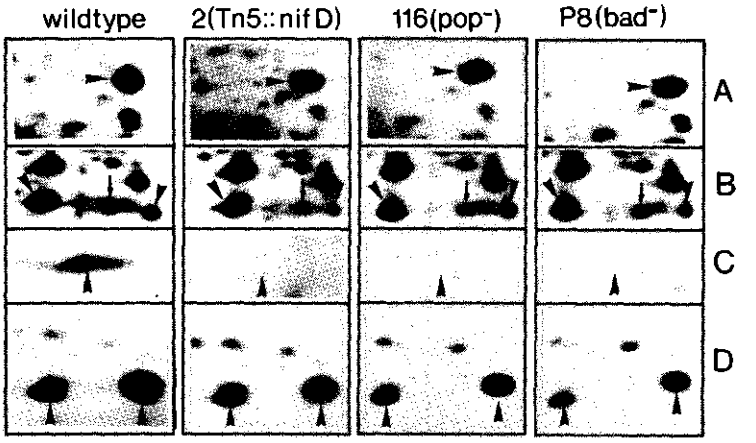
Nodulin mRNAs during nodule development

To study whether the expression of all nodulin genes starts at the same time after infection of the roots with *Rhizobium* or alternatively whether some nodulin genes are expressed early and others later, we followed the appearance of the nodulin mRNAs during development of a nitrogen-fixing root nodule.

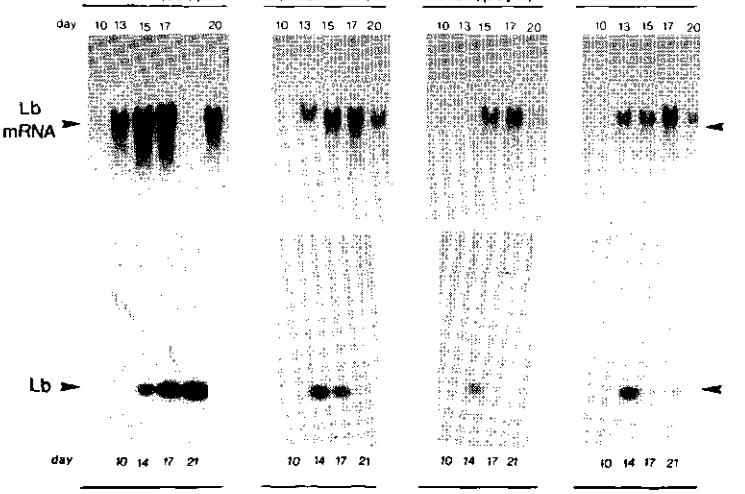
In the pea-*R. leguminosarum* symbiosis nodules appear, under our growth conditions, only on a restricted part of the main root. The first nodule-like structures are visible 10 days after sowing and inoculation. At day 12 leghemoglobin is present and one day later nitrogenase can be detected (Bisseling *et al.*, 1980). After this the nitrogen-fixing activity increases rapidly and reaches its maximum between 3 and 4 weeks after sowing whereupon it starts to decrease (Bisseling *et al.*, 1979). At day 7, 8, 9 and 10 after sowing and inoculation we collected 2.5 cm pieces of roots where nodules normally appear and at day 13, 15 and 17 we harvested root nodules. From these tissues RNA was isolated, translated and analysed on 2-D gels. We focused our analysis on the major nodulin spots N-68, N-40', N-40, N-21, Lb-1, Lb-2, Lb-3 and Lb-4 and on the major nodule-stimulated spot nst-40 (Figure 2). On 2-D gels from 8-day-old infected tissue N-40' was the only detectable nodule-specific translation product (data not shown). The mRNA of this nodulin increased in amount and produced a rather intense spot at day 10. At that time N-68 appeared as a minor spot (Figure 2A, B). The concentration of N-40' mRNA further increased during the development until day 15, after which it remained constant. N-68 changed from a minor spot at day 10 to a major spot at day 13, so its mRNA concentration increased drastically during these 3 days. During the next 2 days, N-68 further increased only slightly. Nodule-specific spots which were still absent at day 10 but present in 13-day-old nodules are N-40 and the four *in vitro* translation products from Lb mRNAs (Figure 2B, D; Lb-3 and Lb-4 not shown). The amounts of mRNA for both N-40 and nst-40 rapidly increased between day 13 and 15, but then the increase slowed down and the amounts reached a maximum at day 17. At day 13 all four Lbs occurred in the 2-D gel pattern, but Lb-1 and Lb-3 as minor spots compared with Lb-2 and Lb-4.

The intensities of the Lb-1, Lb-2 and Lb-4 spots strongly increased from day 13 to day 15 whereas the Lb-3 spot did not display a comparable increase, neither did it reach the same intensity as those of the other Lbs (data not shown). At a certain spot intensity it is no longer possible to estimate visually the rate of increase of the intensities of the different nodulin spots. Therefore we also determined the relative mRNA concentrations for each of the major nodulins during nodule development by measuring the radioactivities in equal-sized pieces cut from the 2-D gels (data not shown). This allows a more precise estimation of the period during nodule formation in which the concentration of a nodulin mRNA rapidly increases. For Lb-1, Lb-2 and Lb-4, which are found at well-separated positions on the gels, we used these data to determine the beginning of the expression of the respective Lb genes by extrapolating to the time of zero incorporation. It appeared that expression of the genes coding for Lb-2 and Lb-4 starts before that of Lb-1. A comparable differential appearance of Lb components has also been found in soybean root nodules (Fuchsman and Appleby, 1979; Verma *et al.*, 1979) and expression studies at the RNA level confirmed this observation (Marcker *et al.*, 1984). Finally N-21 is hardly visible in the 2-D pattern from 13-day-old nodules; it is a minor spot at day 15 and a major spot at day 17 (Figure 2C), so the rapid increase in concentration of N-21 mRNA occurs at least 2 days later in comparison with the other nodulin mRNAs.

In summary, the group of eight major nodulins comprises one early nodulin, N-40' and one late nodulin, N-21. N-40 and the Lbs appear and increase during a 4-day period from day 11 to day 15, whereas the appearance of N-68 is intermediate between the early nodulin N-40' and the Lbs. The minor nodule-specific spots, which have not been studied in detail, also appear and increase from day 11 to 15. These results indicate that there is dif-



. 3. Expression of nodulin genes in ineffective root nodules. A comparison of *in vitro* translation products from total RNA isolated from effective and ffective pea root nodules, 15 days after sowing and inoculation with either the effective *R. leguminosarum* strain PRE or the ineffective strains :E)2(Tn5::*nifD*), (1062)116(*pop*⁻) and P8(*bad*⁻). Only the parts of the gels within the squares indicated in Figure 1A are shown as these contain the major lulin spots. The comparison is shown for N-68 in (A), N-40', nst-40 and N-40 from left to right in (B), N-21 in (C) and Lb-1 and Lb-2 from left to right D).



ɟ. 4. The occurrence of Lb and Lb-mRNA in pea root nodules induced by infection with the effective strain *R. leguminosarum* PRE and the ineffective ains (PRE)2(Tn5::*nifD*), (1062)116(*pop*⁻) and P8(*bad*⁻). (A) Autoradiographs of Northern blots containing RNA isolated from effective and ineffective pea ɪ nodules at different stages during the development and hybridized with ^{32}P-labeled pPsLb101 as a probe. The predominant band corresponds to a mRNA ~700 bases in length. (B) Autoradiographs of Western blots containing cytoplasmic proteins from effective and ineffective pea root nodules at different ges of nodule development and incubated with antiserum raised against purified pea leghemoglobins and ^{125}I-labeled protein A to detect immune complexes. e band on the autoradiographs corresponds to a 14 000 mol. wt. protein.

ential expression of nodulin genes during nodule development. It seems likely that the first signal that activates expression of nodulin gene(s) will be derived from the infecting rhizobia. hether further signals that lead to expression of nodulin genes e derived from plant-encoded genes or also from the *Rhizobium* symbiont is a matter of speculation. If such signals are related to the expression of symbiotic genes of *Rhizobium*, one or more nodulin genes may not become active in nodules induced by rhizobia mutated in one of the known symbiotic genes. We have therefore analysed nodulin gene expression in ineffective nodules

produced by different *nod*$^+$*fix*$^-$ *Rhizobium* mutants.

Nodulin mRNAs in ineffective root nodules

Three mutant *R. leguminosarum* strains were used to obtain ineffective root nodules. (PRE)2(Tn*5*::*nifD*) is a *nod*$^+$*fix*$^-$ mutant of the wild-type *R. leguminosarum* PRE used in our experiments to produce effective nodules. This mutant has a Tn*5* insertion in *nifD*, one of the structural genes of nitrogenase and consequently (PRE)2 bacteroids lack component 1 of nitrogenase (Schetgens *et al.*, 1984). (1062)116(*pop*$^-$) is a *nod*$^+$*fix*$^-$ mutant of *R. leguminosarum* 1062. The *pop*$^-$ mutation is manifested by the accumulation of porphyrin. (1062)116 apparently has a defect in the biosynthesis of heme and therefore the bacteroids excrete a low amount of heme (Nadler, 1981). The third *nod*$^+$*fix*$^-$ *R. leguminosarum* used, is a wild isolate referred to as P8(*bad*$^-$). P8 bacteria are released from the infection threads and invade root nodule cells, but appear not to differentiate into characteristic Y-shaped bacteroids as do the wild-type PRE and the other two mutants. It seems plausible that these three *nod*$^+$*fix*$^-$ mutants disturb the development of an effective root nodule at different stages. (PRE)2 is clearly defective in one of the last steps before nitrogen fixation can start.

Since leghemoglobin (Lb) synthesis precedes the synthesis of nitrogenase (Bisseling *et al.*, 1980; Bergersen and Goodchild, 1973) mutant (1062)116, which has a defect in the synthesis of heme required for functional Lb, may disturb nodule development at an earlier stage than (PRE)2. The differentiation of *R. leguminosarum* bacteria into Y-shaped bacteroids normally occurs shortly after the bacteria have entered into the plant cells and therefore P8 may disturb root nodule development at a rather early stage.

When total RNA from effective nodules and from the three types of ineffective nodules was translated *in vitro*, the incorporated radioactivity was in all cases about similar indicating that the available amount of translatable mRNA is the same in both effective and ineffective. The 2-D gel patterns of the *in vitro* translation products from the ineffective nodules were for the greater part similar to those from effective nodules. An example is shown in Figure 1D and a composition of details showing the major nodulins in Figure 3. In 15-day-old nodules induced by each of the three *nod*$^+$*fix*$^-$ mutants all major nodulins, except the late nodulin N-21, are present. Even in still older nodules this translation product remained below the level of detection. The minor nodulin spots were also detectable in the three types of ineffective nodules. This is not clearly shown in Figure 3, but the presence of a minor nodulin spot with a somewhat lower mol. wt. than N-68 and the same isoelectric point (Figure 3A) may serve as an example. Whereas the results demonstrate that all but one of the nodulin genes are expressed in ineffective nodules the major nodulin spots had less intensity in the 2-D pattern from ineffective nodules than the corresponding spots in the pattern from effective nodules. Measuring the radioactivity incorporation in individual nodulin spots confirmed this observation.

The radioactivity in the major nodulin spots was reduced to 15 – 30% of that in the corresponding nodulins translated from RNA of effective nodules, indicating that the relative amount of translatable nodulin mRNA is considerably decreased in ineffective nodules in comparison with effective nodules. Although it is tempting to assume that nodulin gene expression is less active in ineffective nodules the observed relative decrease in mRNA is probably due to a smaller zone of bacteroid containing cells or a lower ratio of infected to uninfected cells within this zone in ineffective nodules (Bisseling *et al.*, unpublished data; Newcomb *et al.*, 1977).

Post-transcriptional regulation of leghemoglobin

In contrast with previously published results (Bisseling *et al.*, 1983, 1984a) which showed that in protein preparations from ineffective nodules Lb is found at very reduced levels, the analyses presented here indicate the presence of a considerable amount of mRNA capable of directing the synthesis of Lb. Therefore we compared the relationship between the amount of Lb mRNA and leghemoglobin proteins during development of effective and ineffective nodules by Northern and Western blotting, respectively (Figure 4). In effective nodules induced by *R. leguminosarum* PRE, Lbs and Lb mRNA were detected in 13-day-old nodules and both increased in amount till 17 days after inoculation whereupon both Lb mRNA and the protein remained at a constant level during further nodule development. In ineffective nodules induced by mutants (1062)116 or P8, Lb mRNA was found in 13-day-old nodules and increased in amount till day 17. Densitometric scanning of the autoradiographs showed that the amount of Lb mRNA in P8-induced nodules reached 30% of the amount found in effective nodules; in nodules induced by strain (1062)116 Lb mRNA reached 60% of the amount present in effective nodules.

In both types of ineffective nodules Lb was detectable in 14-day-old nodules but at day 17 and following days the amount of Lb appeared strongly reduced, mostly below the detection level, although mRNA was detectable even until day 20 after inoculation. The same phenomenon occurred in ineffective nodules induced by mutant (PRE)2, with the difference that the amount of Lb mRNA now reached 70 – 80% of the level in effective nodules and that the protein decreased in amount after day 17.

These results confirm the previously published observations of strongly reduced amounts of Lb in ineffective nodules (Bisseling *et al.*, 1983, 1984b) and demonstrate that the continuous synthesis of Lb mRNA in ineffective nodules does not result in accumulation of Lb in the nodules as is the case in effective nodules. It is clear that the synthesis of Lb is not only regulated at the level of transcription or processing of primary transcripts to mature mRNA but also at later stages, presumably at the post-transcriptional level.

Discussion

To elucidate the molecular mechanisms of the development of nitrogen-fixing root nodules as a result of the symbiotic interaction between *Rhizobium* bacteria and leguminous plants, it is as important to analyse the plant genes involved in this process as it is to study the concerned *Rhizobium* genes. We have tried to survey the expression of plant genes involved in pea root nodule development using *in vitro* translation of mRNA and analyses of the *in vitro* translation products by two-dimensional gel electrophoresis. In this way we were able to detect the specific expression of 21 different plant genes during nodule development and, in addition, increased and decreased activities of plant genes which are also expressed in non-infected root tissue. Previously, 20 – 30 nodule-specific proteins have been detected by using a nodule-specific antiserum preparation (Bisseling *et al.*, 1983) but in that study no clear distinction could be made between pea and *Rhizobium* encoded proteins, between the relative amounts of the different proteins and between the rate of expression of different nodulin genes. Here we have shown that eight genes, encoding nodulins N-21, N-40, N-40', N-68 and four Lbs are strongly expressed. Among these nodulins the Lbs have a well-

defined function in O_2-transport in nodules (Appleby, 1984) whereas the function of the four other nodulins is as yet unknown. The appearance of the eight major nodulins showed that there is differential expression of specific plant genes during nodule development and this result may therefore help in the selection of plant genes worth isolating for further study of their structure and regulation of expression.

Our results indicate that the N-40′ gene is strongly expressed from the very beginning of the development of nodule tissue, even before nodule-like structures are visible and that N-40′ might be involved in the formation of nodule meristem. Since we have not been able to detect N-40′ mRNA in the meristematic tissue of root tips, N-40′ may be a specific marker for nodule meristem formation. The expression of the N-40′ gene is not repressed during further nodule development which is in agreement with the fact that pea nodules have a zone of actively dividing cells. The expression of the N-68 nodulin gene appears to start ~2 days later than that of N-40′ but is clearly prior to the expression of most of the other nodulin genes such as the genes for Lbs, N-40 and many nodulins occurring in minor amounts. This suggests that N-68 marks a further specialization of the nodule cells while the Lbs, N-40 and the other nodulins might be involved in creating an appropriate environment for nitrogen fixation, since the stage at which the genes for these nodulins are expressed is characterized by the beginning of symbiotic nitrogen fixation. N-21, the gene of which is expressed rather late during nodule development, is distinct from the other nodulins. A zone of senescent tissue has been observed in pea root nodules ~20 days after inoculation of the roots (Kijne, 1975) and it might therefore be argued that N-21 is involved in the senescence process. This appears, however, very unlikely since the expression of the N-21 gene is repressed in ineffective nodules which show signs of senescence at an earlier stage than effectively nitrogen-fixing nodules (Bisseling *et al.*, unpublished data; Newcomb *et al.*, 1977). A common feature of the three types of ineffective nodules used for our analyses, is their inability to fix nitrogen and one might assume that N-21 is involved in the assimilation or transport of ammonia.

Our observations that nodulin genes are expressed in ineffective root nodules are in agreement with those of Fuller and Verma (1984), who showed that in two kinds of ineffective soybean nodules the Lb genes and the genes of four other nodulins were expressed. The amounts of the relative nodulin mRNAs in such ineffective soybean nodules was considerably reduced compared with the level in effective nodules. Likewise in ineffective pea root nodules the expression of the nodulin genes is reduced. Since pea root nodules are composed of different types of cells (Newcomb, 1976) and specific nodulin genes might only be expressed in one cell type, the decrease of nodulin mRNAs in ineffective nodules may be due to a change in the ratio of different cell types resulting in, for example, less bacteroid-containing cells in ineffective nodules.

In ineffective nodules the amount of some nodulins appears to be regulated by a mechanism other than transcriptional control of the corresponding genes as is illustrated here by the amount of Lbs and Lb mRNA found at different stages of nodule development. We demonstrated that Lb mRNA is present in ineffective nodules in considerable amounts from about day 13 till day 20 after inoculation of the roots, which was the latest stage tested. In contrast, Lbs were detected in ineffective nodules only during the first 3–4 days of that period but were virtually undetectable from day 17 after inoculation. On the other hand, in effective nodules both the amount of detectable Lb and the relative amount of Lb mRNA remained at a constant level under comparable conditions. Therefore, it appears that the amount of Lb in root nodules is regulated at a post-transcriptional level. The translation experiments with RNA isolated from nodules demonstrate that there is no change in *in vitro* translatability of the Lb mRNA. If this reflects the situation *in vivo* the disappearance of the Lbs in ineffective nodules can only be explained by rapid turnover of synthesized Lbs. A similar post-transcriptional regulation for Lb does not seem to exist in soybean nodules. In cases where strongly reduced amounts of Lbs are found in ineffective soybean nodules it is low throughout the development and is in approximate proportion to the amount of Lb mRNA present. A marked decrease of the protein compared with the mRNA at later stages of ineffective nodule development has not been detected (Verma *et al.*, 1981). Whether this reflects an essential difference between ineffective soybean and pea nodules is unknown.

Not only Lb but also other nodule-specific proteins or nodule-stimulated proteins have been found in decreased amounts in ineffective nodules as compared with effective nodules. In ineffective pea nodules several nodule-specific proteins were decreased or even missing (Bisseling *et al.*, 1983, 1984b). In ineffective soybean nodules the activity of glutamate synthase was not detectable and that of glutamine synthetase considerably decreased (Sen and Schulman, 1980). Ineffective *Phaseolus* root nodules contained only trace amounts of nodule-specific glutamine synthetase activity whereas the normal glutamine synthetase activity was reduced (Lara *et al.*, 1983). Such decreases may be due to decreases in the relative amounts of mRNAs such as we have observed here in ineffective pea nodules and Fuller and Verma (1984) in ineffective soybean nodules. On the other hand it may also be a matter of increased turnover of some nodule-specific proteins in ineffective nodules.

The results of our experiments show that there is successive expression of different nodulin genes during pea root nodule development indicating that the expression of all nodulin genes is not induced at the same time. Since the expression of nodulin genes is effected by interaction with infecting rhizobia it seems plausible to assume that signals from *Rhizobium* play a role at an early stage of nodule development for example in the induction of early pea nodulin genes like N-40′. Such signals might be generated by information encoded by the *nod* genes on the *sym* plasmid of *R. leguminosarum*, which are essential for the induction of nodule formation on the pea roots (Ausubel, 1982; Rolfe and Shine, 1984). It is not clear whether at later stages of root nodule development, when *Rhizobium* bacteria differentiate into nitrogen-fixing bacteroids, the bacteria again give signals to the pea genome for further nodulin gene expression. We used three different mutated rhizobia, which produced ineffective root nodules, but no differential effect on the expression of most nodulin genes was observed. We can therefore conclude that neither nitrogen fixation, heme secretion nor bacteroid development are essential for the expression of these genes. In all three types of ineffective, not nitrogen-fixing root nodules the late nodulin gene N-21 was not expressed. This suggests that the expression of the N-21 gene is in some way controlled by the nitrogen fixation process since the expression of the gene is only started concomitantly with or after the onset of symbiotic nitrogen fixation.

Further studies with *R. leguminosarum* mutants with defects in symbiotic genes other than those in the mutants used in the present experiments, are in progress to establish whether or not the expression of *R. leguminosarum* genes is essential for the induction of nodulin genes at different stages of pea root nodule development.

Materials and methods

Root nodules

Pea plants (*Pisum sativum* var. Rondo) were cultured and inoculated with wild-type and mutant *R. leguminosarum* strains as described by Bisseling *et al.* (1978). At early stages during the development, 2.5 cm pieces where nodules normally appear were cut from the main root whereas at day 13, 15 and 17 root nodules were removed. Uninfected plants were cultured in the same way and total roots or pieces from the main root were harvested 3 and 8 days after sowing. The roots and nodules cultured for the isolation of RNA, were immediately frozen in liquid nitrogen and stored at −70°C. Freshly harvested material was used for isolation of cytoplasmic proteins.

Isolation of total RNA

Total RNA was isolated essentially as described by De Vries *et al.* (1982). Frozen tissue was ground in a mortar to a fine powder under liquid nitrogen. Portions of >4 g were ground in a Waring Blender. To 1 g of material a mixture of 2.5 ml 0.2 M sodium acetate, pH 5.0, 1% SDS, 10 mM EDTA and 2.5 ml distilled phenol containing 0.1% 8-hydroxyquinoline was added. After vigorously shaking for 5 min, 2.5 ml chloroform (chloroform:isoamylalcohol = 24:1) was added and the suspension was shaken for another 5 min. After centrifugation the aqueous phase was removed, re-extracted twice with phenol:chloroform (1:1) and chloroform, respectively, and 8 M LiCl was added to a final concentration of 2 M. The RNA was precipitated overnight at 4°C, collected by centrifugation, washed once with 2 M LiCl and twice with 70% ethanol. The dried pellet was dissolved in double-distilled water and stored in portions at −70°C.

In vitro translation

Total RNA isolated from effective and ineffective pea root nodules and uninfected pea roots was translated in a mRNA-dependent rabbit reticulocyte lysate. Typically 2 μg total RNA was translated during 60 min at 30°C in a 6 μl incubation mixture containing cell-free rabbit reticulocyte lysate with additives according to standard procedures (Pelham and Jackson, 1976) [^{35}S]Methionine (6 μCi per 6 μl reaction mixture) was used as radioactive amino acid. The radioactivity incorporated in translation products was counted after precipitation of 1 μl reaction mixture in 10% trichloroacetic acid on Whatmann 3 MM filters.

Immunoprecipitation

For immunoprecipitation of *in vitro* translation products with antiserum raised against purified Lb components (Bisseling *et al.*, 1979), 4 μg RNA isolated from 15-day-old nodules was translated in a 12 μl incubation mixture. After translation, 10 μl anti-Lb serum and 500 μl RIA buffer (10 mM Tris-HCl pH 7.5, 0.9% NaCl, 1% BSA, 0.05% Triton X-100) was added and incubated for 16 h at 4°C. Subsequently, 50 μl of a 10% (w/v) suspension of *Staphylococcus aureus* cells (IgGsorb from the Enzyme Center Inc., Boston, MA) in RIA buffer was added and incubation at 4°C was continued for 1 h by constantly shaking on a rotary mixer. Samples were then centrifuged through a sucrose cushion consisting of a 0.5 ml layer of 1 M sucrose and a 0.2 ml layer of 0.5 M sucrose both in RIA buffer, at 17 000 *g* for 20 min. The precipitate was washed twice in RIA buffer and finally resuspended in sample buffer for isoelectric focusing.

Two-dimensional gel electrophoresis

The *in vitro* labeled translation products were separated in two dimensions, the first according to isoelectric point and the second according to mol. wt. The procedure was performed essentially as described by O'Farrell (1975). Amounts of protein corresponding to 20 000−300 000 c.p.m. in the trichloroacetic acid-precipitable fraction were applied to the gel. For the isoelectric focusing, 1.6% ampholines pH 5−7 and 0.4% ampholines pH 3.5−10 were used and for the SDS-electrophoresis, 12.5% polyacrylamide slab gels. The translation products were visualized by fluorography using preflashed Kodak XAR-5 films.

Northern blotting and hybridization

Total RNA isolated from root nodules was separated under denaturing conditions on 1.5% agarose gels containing 6% formaldehyde. To each lane 25 μg total RNA was applied. After electrophoresis the RNA was blotted onto Gene Screen paper (New England Nuclear Corp.). The electrophoresis and diffusion blotting procedure were performed as described in detail in the Gene Screen information booklet. The RNA was hybridized to pPsLb101, a plasmid consisting of a 400-bp insert of Lb cDNA in the *Eco*RI site of pBRH2. ^{32}P-Labeling of pPsLb101 was performed by nick translation. The Northern blot was hybridized with the denatured DNA probe at 42°C in a hybridization mixture containing 50% formamide, 10 x Denhardt's solution, 50 mM Tris-HCl pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 0.1% SDS, 10% dextran sulfate and 100 μg/ml denatured salmon sperm DNA. After hybridization the filter was washed twice for 30 min at 65°C in 2 x SSC, 0.5% SDS and 30 min at room temperature in 0.1 x SSC, 0.1% SDS and exposed to Kodak XAR-5 film.

Western blotting and immunological detection

Cytoplasmic proteins from root nodules were isolated and separated by SDS-gel electrophoresis in 15% polyacrylamide gels. The proteins were blotted onto nitrocellulose and this was incubated with anti-Lb serum and [^{125}I]protein A. All procedures were performed as described previously by Bisseling *et al.* (1983).

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IV

cDNA cloning and developmental expression of pea nodulin genes

submitted for publication

cDNA CLONING AND DEVELOPMENTAL EXPRESSION OF PEA NODULIN GENES

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SUMMARY

A cDNA library prepared from pea nodule poly(A)$^+$ RNA was screened by differential hybridization with cDNA probes synthesized from root and nodule RNA respectively. From the cDNA clones that hybridized exclusively with the nodule probe five clones, designated pPsNod 6, 10, 11, 13 and 14 and each containing unique sequences, were further characterized together with one leghemoglobin and one root specific cDNA clone. *In vitro* translation of RNA selected by the pPsNod clones showed that the corresponding genes encode nodulins with molecular weights ranging from 5,800 to 19,000. During pea root nodule development expression of the five PsNod genes starts more or less concomitantly with the onset of nitrogen fixing activity in the nodules and the time course of appearance and accumulation of the nodulin mRNAs is similar to that of leghemoglobin mRNA. In non-effective pea root nodules expression of the PsNod genes is induced but the final accumulation levels of the mRNAs are markedly reduced to various degrees. The expression of another nodulin gene, designated ENOD2, was followed using a heterologous soybean cDNA clone as probe. In pea root nodules the ENOD2 gene is expressed at least five days before the PsNod and leghemoglobin genes and in contrast to the PsNod mRNAs the concentration of the ENOD2 mRNA is the same in wild type and *fix*$^-$ nodules. The results described suggest that in root nodules several regulatory mechanisms exist which determine the final nodulin mRNA amounts accumulating in the root nodule.

INTRODUCTION

Nodulins are plant proteins that are specifically synthesized during the formation of nodules on the roots of leguminous plants, a process elicited by Rhizobium and resulting in a symbiotic association between bacteria and plant (6, 19, 20, 28). The best studied nodulins are the oxygen binding protein leghemoglobin (1) and two enzymes involved in ammonium assimilation, uricase II (2) and a nodule specific form of glutamine synthetase (8). Recently one additional nodulin, nodulin-24 which is associated with the membrane that surrounds the bacteroids, has been studied in more detail (10). The genes or cDNAs encoding leghemoglobin, uricase II and nodulin-24 have been cloned from soybean (27, 24, 12) and the cDNA encoding glutamine synthetase from Phaseolus vulgaris (9).

In this report we describe the construction of a cDNA library from pea nodule RNA and the isolation and characterization of five nodulin cDNA clones. In addition we present data on the expression of the corresponding pea nodulin genes during the development of effective root nodules and in non-effective nodules produced by Rhizobium mutants.

MATERIALS AND METHODS

Plant material

Pea plants (Pisum sativum var. Rondo) were cultured and inoculated as described by Bisseling et al. (3). Pea root nodules were induced by either wild type Rhizobium leguminosarum PRE or the mutant Rhizobium strains (PRE)2(Tn5::nifD) (24), (1062) 116 (pop$^-$) (23) and P8 (bad$^-$). During early stages of nodule development 2.5 cm pieces where nodules normally appear, were cut from the main root whereas from 12 days after sowing and inoculation root nodules were removed. From unin-

fected plants, total roots or pieces from the main root where nodules appear on infected plants, were harvested 3 and 8 days after sowing, respectively. The harvested material was immediately frozen in liquid nitrogen and stored at -70°C.

Isolation of RNA

Total RNA from roots and root nodules was isolated as described by Govers et al. (13) and poly(A)$^+$ RNA was selected by chromatography on oligo(dT)cellulose according to Maniatis et al. (21).

cDNA synthesis and cloning

Double stranded (ds) cDNA was synthesized from poly(A)$^+$ RNA from 14 day old pea root nodules using a combination of the methods described by Land et al. (18), Maniatis et al. (21) and Murray et al. (22). The reaction mixture for the first strand synthesis contained 100 mM Tris-HCl pH 8.3, 140 mM KCl, 10 mM $MgCl_2$, 4 mM NaPPi, 1.5 mM DTT, 0.2 mM dATP/dGTP/dCTP/dTTP, 0.1 µg/µl oligo(dT)$_{12-18}$, 0.1 µg/µl poly(A)$^+$ RNA and AMV reverse transcriptase (obtained from Dr. J.W. Beard, St. Petersburg) at 5 U/µg poly(A)$^+$ RNA in a total volume of 10 µl. This was incubated for 2.5 h at 42°C. After hydrolysis of RNA (40 mM NaOH, 60 min, 65°C) and gelfiltration on Sephadex G50 the single stranded (ss) cDNA was dC-tailed using terminal deoxyribonucleotidyl transferase (TdT) (PL-Biochemicals) in a reaction mixture of 100 µl containing the ss cDNA, 120 mM sodium cacodylate pH 7.2, 0.1 mM DTT, 0.1 mM dCTP, 0.5 µg/µl bovine serum albumin and 1 mM $CoCl_2$. After preincubation for 5 min at 37°C 7.5 U TdT per pmol cDNA were added and the mixture was incubated for 60 min at 37°C. Oligo(dG)$_{12-18}$ was hybridized to the dC-tailed ss cDNA to prime the synthesis of the second-strand. Hybridization and second-strand synthesis was carried out according to Land et al. (18) except that the DTT concentration was lowered to 1.5 mM and 12.5 U reverse transcriptase per pmol ss cDNA were used. The 3'

recessed termini were filled using the Klenow fragment of DNA polymerase I (Boehringer) followed by treatment of the ds cDNA with S1 nuclease (Sigma) and a second incubation with Klenow. The incubations were performed according to standard procedures (21). Double stranded cDNAs shorter than 200 basepairs were removed from the mixture by chromatography on a Sepharose 4B column. The remaining ds cDNAs were dC-tailed as described above except that the incubation time varied from 1 to 60 min and 50 U TdT per pmol ds cDNA were added. During the whole procedure aliquots of the reaction mixtures or duplicate mixtures were incubated with radioactive nucleotides, $\alpha[^{32}P]$dATP or $\alpha[^{32}P]$dCTP, to calculate the efficiency of the first- and second-strand synthesis and the length of the dC-tails. Pst1 digested dG-tailed pBR322 (BRL) was annealed to the dC-tailed ds cDNA in a 1:1 molar ratio using the conditions described by Land et al. (18). The annealing mixture was used to transform Escherichia coli RR1. Per ng ds cDNA 1500 tetracycline resistant transformants were obtained, of which 90% was sensitive to ampicillin. The cDNA clones were grown at 37°C in LB medium with 12.5 µg/ml tetracycline (21).

Colony screening

For screening by differential hybridization 10% of the colonies was transferred to 96-well microtiter plates and grown overnight. From each masterplate three replicas were made on Gene Screen Plus membranes (NEN), put on agar plates and incubated overnight. The colonies were lysed and the DNA was fixed to the membrane according to the procedure described by Maniatis et al. (21) except that the baking step was omitted. Two filters were hybridized with cDNA probes complementary to poly(A)$^+$ RNA from 21 day old root nodules and from 8 day old uninfected roots, respectively. The cDNA probes were synthesized in a reaction mixture of 50 µl containing 50 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 100 mM HCl, 4 mM NaPPi, 20 mg/ml oligo$(dT)_{12-18}$, 1 mM dGTP/dCTP/dTTP, 0.015 mM dATP, 40 µCi $\alpha[^{32}P]$dATP

(3200 Ci/mmol), 1 µg poly(A)$^+$RNA and 10 U reverse transcriptase, incubated for 60 min at 42^{o}C. The third replica was hybridized to a ^{32}P-labeled M13 clone containing the insert from the pea leghemoglobin cDNA clone pPsLb101 (7). Prehybridization was for 8 hours at 65^{o}C and hybridization with the denatured cDNA or M13 probes for 20 hours at 65^{o}C in a mixture containing 1 M NaCl, 0.05 mM Tris-HCl pH 7.5, 0.1% NaPPi, 1% SDS, 10x Denhardt's solution and 100 µg/ml denatured salmon sperm DNA. The filters were washed (30 min in 2x SSC, 0.5% SDS and 30 min in 0.5x SSC, 0.1% SDS at 65^{o}C) and exposed to Kodak XAR-5 film. Clones considered to be of interest were transferred from the masterplates to liquid LB medium and plasmid DNA was isolated according to the alkaline lysis method (21).

Northern blotting and hybridization

Samples of total RNA isolated from effective and non-effective root nodules were denatured in DMSO/glyoxal, electrophoresed on 1.2% agarose gels and blotted onto Gene Screen membranes (NEN). The Northern blots were hybridized with plasmid DNAs labeled by nick translation (21). Electrophoresis, blotting and hybridization procedures were as previously described (14).

Hybrid released translation

For hybridization selection of specific mRNAs the method described by Maniatis et al. (21) was used with some minor modifications. Inserts of nodule specific cDNA clones were isolated from agarose gels, purified, denatured and spotted on diazophenylthioether (DPT) filters, obtained by activation of aminophenylthioether paper (BIORAD). To each filter disc (0.3-0.5 cm^2) 10-20 µg insert DNA was applied. After drying and washing, 5 to 8 filters were incubated in 300 µl hybridization buffer containing either 1 mg total RNA or 130 µg poly(A)$^+$ RNA from 17 day old pea root nodules. Hybridization was ini-

tiated at 50°C and then the temperature was slowly decreased to 38°C over a period of 6 hours. After washing and elution of the selected RNA from the filters, 5 µg calf liver tRNA (Boehringer) was added. The RNA was ethanol precipitated and dissolved in 3 µl twice-distilled water; 1.5 µl was translated in a wheat germ cell-free system (BRL) according to the manufactors manual using ^{35}S-methionine as radioactive amino acid. The translation products were analysed on 12.5% SDS-polyacrylamide gels (acrylamide:bisacrylamide = 40:1) (18) and radioactive polypeptides were visualized by fluorography. For proteins with molecular weights smaller than 10,000, 15% gels were used, the length of the separating gel was enlarged from 13 to 19 cm and the running time lengthened from 4 to 36 hours. For selection of mRNA by heterologous cDNA the hybridization temperature was 31°C during 6 hours and the NaCl concentration in the washing buffer was 10 fold higher.

Isolation of genomic DNA, Southern blotting and hybridization

DNA was isolated from young pea leaves and from Rhizobium leguminosarum as described by Zimmer et al. (30) and Krol et al. (16), respectively. DNA was digested with restriction enzymes and electrophoresed on a 0.7% agarose gel (21).The gel was stained and then placed in 0.25 M HCl for 15 min followed by denaturation in 0.4 M NaOH, 0.6 M NaCl for 30 min. The DNA was blotted onto Gene Screen Plus membrane using 0.4 M NaOH, 0.6 M NaCl as transfer solution (alkaline transfer). Following capillary transfer for 16 hours the membrane was washed in 0.5 M Tris-HCl pH 7.0, 1 M NaCl for 15 min, dried for 60 min at room temperature and prehybridized at 65°C in 5x SSC, 10x Denhardt's solution, 40 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS, 100 µg/ml denatured salmon sperm DNA for 8 hours whereupon denatured plasmid DNA, ^{32}P-labeled by nick translation, was added. For hybridization the membrane was incubated further for 24 hours at 65°C. The filters were washed twice for 30 min in 2x SSC, 0.5% SDS and 30 min in 0.5x SSC,

0.1% SDS at 65°C and exposed to Kodak XAR-5 film. Hybridizations with heterologous probes were at 57°C while the blots were washed at less stringent conditions (0.5x SSC, 42°C).

Light microscopy

Nodulated roots were fixed in 3% glutaraldehyde for 2 hours, dehydrated in a graded ethanol series and embedded in Technovit 7100; 4 µm cut sections were placed on slides, stained with toluidine blue and examined with a light microscope.

RESULTS AND DISCUSSION

Construction and screening of the nodule cDNA library

A cDNA library was constructed from poly(A)$^+$ RNA of 14 day old pea root nodules. Of 6000 colonies approximately 600 were screened by differential hybridization with ^{32}P-labeled cDNA synthesized from root and nodule poly(A)$^+$ RNA respectively. Forty percent of the clones that hybridized exclusively with the nodule probe appeared to contain leghemoglobin (Lb) sequences as shown by hybridization with pPsLb101 a Lb cDNA clone isolated previously (7). One Lb clone (pPsLb102) and five nodule-specific cDNA clones, that did not hybridize with the Lb clone or with each other (pPsNod6, pPsNod10, pPsNod11, pPsNod13 and pPsNod14), were randomly chosen for further investigation. In addition one root-specific cDNA clone, pPsRt1, was selected based on its much stronger hybridization to the root probe than to the nodule probe.

Characterization of nodule-specific clones and a root-specific clone

Northern blot analyses confirmed that the five selected nodule-specific cDNA clones specifically hybridized with RNA from nodules and

not with root RNA (Fig. 1 and 5A). Southern blot analyses showed that all clones hybridized only with pea DNA and not with Rhizobium DNA, even though the relative amount of bacterial DNA on the Southern blots was ten fold higher than the relative amount of pea DNA (Fig. 3). The pPsNod clones contain therefore sequences corresponding to plant genes that are specifically expressed in nodules i.e. they represent nodulin genes. In Table 1 the sizes of the hybridizing mRNAs and genomic fragments are summarized together with other characteristics of the selected cDNA clones including the insert size and the molecular weights of the in vitro translation products from RNA selected by hybridization with the cDNA clones.

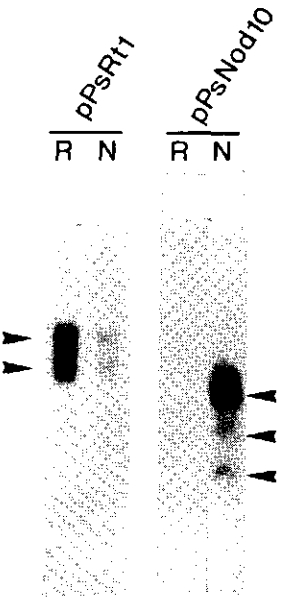

Figure 1. Expression of nodulin gene PsNod10 and "root" gene PsRt1 in roots and nodules. Autoradiographs of Northern blots with RNA from (N) nitrogen-fixing pea root nodules (20 days after sowing and inoculation) and (R) uninfected pea roots (3 days old) hybridized with nick translated plasmid DNA isolated from the indicated clones. The length of the hybridizing mRNAs (arrowheads) is given in Table 1 (PsNod10) or in the text (pPsRt1).

The mRNAs hybridizing to pPsNod6, pPsNod13 and pPsNod14 have a length of 560, 800 and 540 nucleotides (n) respectively (Table 1), and they each encode one polypeptide with an apparent molecular weight of 9,000, 14,000 and 7,500 respectively (Fig. 2A and 2B). The 14,000 dalton in vitro translation product derived from RNA selected by pPsNod13 comigrated on a SDS-polyacrylamide gel exactly with the Lb polypeptide (Fig. 2B). However the pPsNod13-encoded polypeptide does

not precipitate with anti-Lb serum while the pPsLb102-encoded polypeptide does (data not shown) showing that pPsNod13 indeed is not a Lb cDNA clone. pPsNod6 and pPsNod13 hybridized each to one EcoRI and one HindIII fragment of the pea DNA, while pPsNod14 hybridized with four EcoRI fragments and four HindIII fragments (Fig. 3 and Table 1). These results indicate that PsNod6 and PsNod13 sequences are encoded on the pea genome by single copy genes, whereas pPsNod14 represents a member of a small gene family, like the Lb genes.

Clone pPsNod11 hybridized on Northern blots with a 650 n long mRNA and on genomic blots with one EcoRI and one HindIII fragment (Table 1 and Fig. 3). The mRNA selected by hybridization was translated into two proteins with apparent molecular weights of 19.000 and 17,500 respectively (Fig. 2B) indicating that the 650 n long mRNA comprises two different RNA species.

Table 1 Characterization of selected clones.

Clone	Insert size (basepairs)	RNA size (bases)	Size of genomic fragments (kb)		Translation products (D)
			EcoRI	HindIII	
pPsNod6	450	560	4.0	2.6	9,000
pPsNod10	620	710 570 440	8.5	3.6	8,000 6,800 5,800
pPsNod11	300	650	1.5	7.0	19,000 17,500
pPsNod13	230	800	9.6	5.0	14,000
pPsNod14	230	540	6.1 4.4 2.4 2.0	10.5 7.2 3.9 3.8	7,500
pPsLb102	480	700	x	x	14,000

x several bands see reference 7

Clone pPsNod10 hybridized with three RNAs of different lengths (Fig. 1). On the autoradiograph the hybridization signal with the largest RNA (710 n long) was much more intense than the signal with the two smaller RNA species (570 and 410 n long). It seems unlikely that the 570 and 440 n long RNAs are degradation products of the 710 n long mRNA because hybridization of the same Northern blot with another cDNA clone gave only one sharp band on the autoradiograph. In vitro translation of RNA selected by pPsNod10 resulted in 8,000, 6,800 and 5,800 dalton polypeptides (Fig. 2A). These three polypeptides might represent translation products of the three different mRNAs.

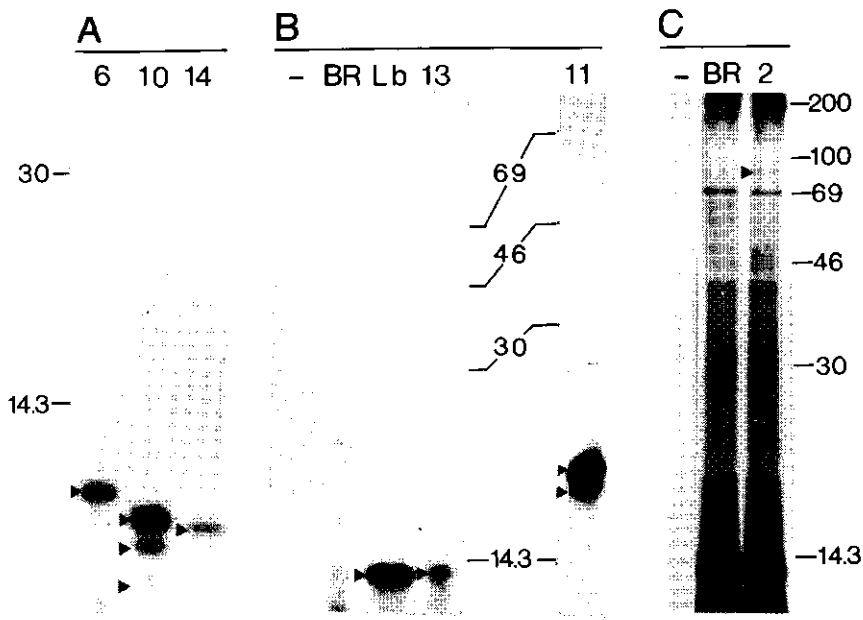

Figure 2. In vitro translation products of mRNAs selected by hybridization to nodulin cDNA clones.
Fluorographs of (A) 15% SDS-polyacrylamide gels and (B) and (C) 12.5% SDS-polyacrylamide gels. Arrowheads indicate major products translated from RNA selected by the indicated clones: 6, 10, 14, 13 and 11 are the pPsNod clones with that number, Lb is the pPsLb102 clone and 2 is the pENOD2 clone. The molecular weights are given in Table 1. In (A) and (B) ^{35}S-methionine was used as radioactive amino acid, in (C) ^{3}H-leucine. Control translations are RNA selected by pBR322 (BR) and background (-) from the wheat germ cell-free system. In panel (C) the pBR322 filter was hybridized and washed at low stringency at the same conditions as the pENOD2 filter. ^{14}C-methylated molecular weight markers are in kilodalton.

The root-specific clone pPsRt1 hybridized with two RNAs (Fig. 1) of 800 and 1300 n respectively. The intensity of the two bands on the autoradiograph of the Northern blot was almost equal and both RNAs appear to be present in large amounts in roots whereas only low concentrations are detectable in the nodule RNA preparation (Fig. 1). Whether the low PsRt1 RNA concentration in nodules reflects a low expression level of the corresponding gene in nodules or whether the RNA is derived from root cells that surround the pea nodules, can not be concluded.

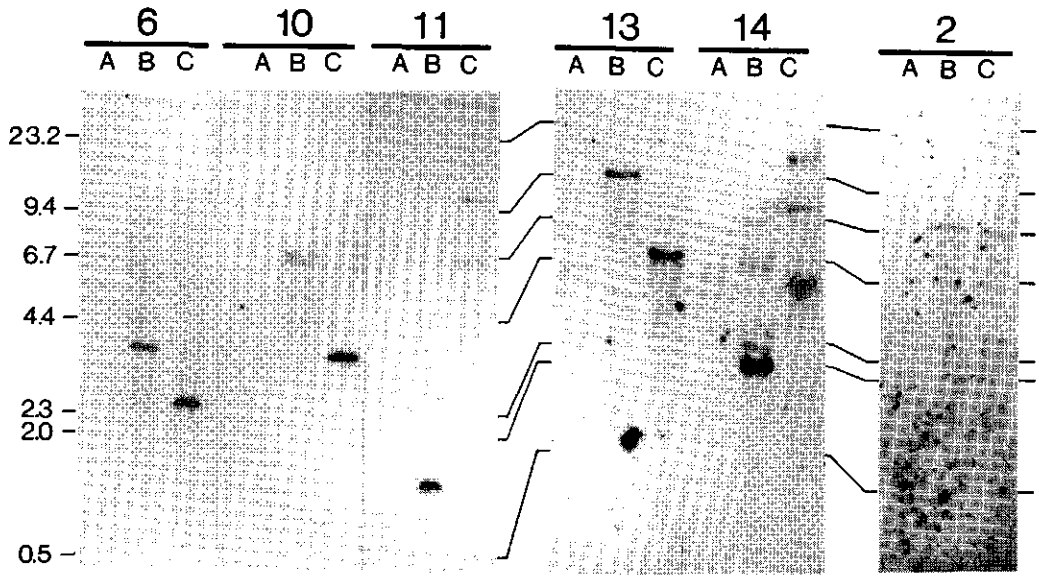

Figure 3. Hybridization of nodulin cDNA clones to genomic pea DNA and Rhizobium DNA. Autoradiographs of Southern blots containing Rhizobium DNA digested with EcoRI (A) and nuclear DNA isolated from pea leaves and digested with EcoRI (B) and HindIII (C). The blots were hybridized with nick translated plasmid DNA isolated from the indicated clones. The numbers 6, 10, 11, 13 and 14 refer to pPsNod clones and 2 to pENOD2. The hybridization temperature was 65°C. For hybridization with the heterologous clone pENOD2 the temperature was lowered with 8°C. For further conditions see MATERIAL AND METHODS. DNA markers are in kilobases. The length of the hybridizing fragments is given in Table 1 or in the text.

Expression of nodulin genes during nodule development

To elucidate possible functions of nodulins it is important to know in which stage(s) of development nodulin genes are expressed. Therefore we examined the development of pea root nodules histologically in parallel to the search for specific nodulin mRNAs.

Root nodule development can be split up into three major stages (29). The first is the preinfection stage, in the second stage infection takes place and nodule structures are formed and in the third stage the formation of a functional nitrogen fixing organ is accomplished. Under the conditions we use for growing pea plants the first nodule structures are macroscopically visible as small swellings on the main root, 10 days after sowing and inoculation with Rhizobium leguminosarum (Fig. 4A). Examination of these initial nodules by light microscopy revealed the presence of peripherically located vascular bundles. Infected as well as uninfected cells have developed from the apical meristem (Fig. 4A and 4C). Apparently all features of a nodule with the cell types belonging to it are already present 3 days before the onset of nitrogen fixation (5). Eight days after sowing and inoculation, when nodules are not yet macroscopically visible, several cells from the nodule meristem have developed into non-dividing cells, some of which contain already a few bacteria while others are (still) uninfected (Fig. 4B). At day 13, when nitrogen fixation starts, more pronounced nodules are visible on the main root and the infected cells are now fully packed with bacteroids (Fig. 4D). From these histological observations it can be concluded that 8 days after inoculation pea nodule development has reached the second stage (29) while the conversion from the second to the third stage happens between 10 and 13 days.

The five nodulin cDNA clones selected from the cDNA library (Table 1) were used to follow the expression of the corresponding PsNod genes during nodule development. Northern blots containing total RNA isolated from segments of 10 day old infected roots and 13, 15, 17 and 20 day old nodules were hybridized with the ^{32}P-labeled pPsNod clones.

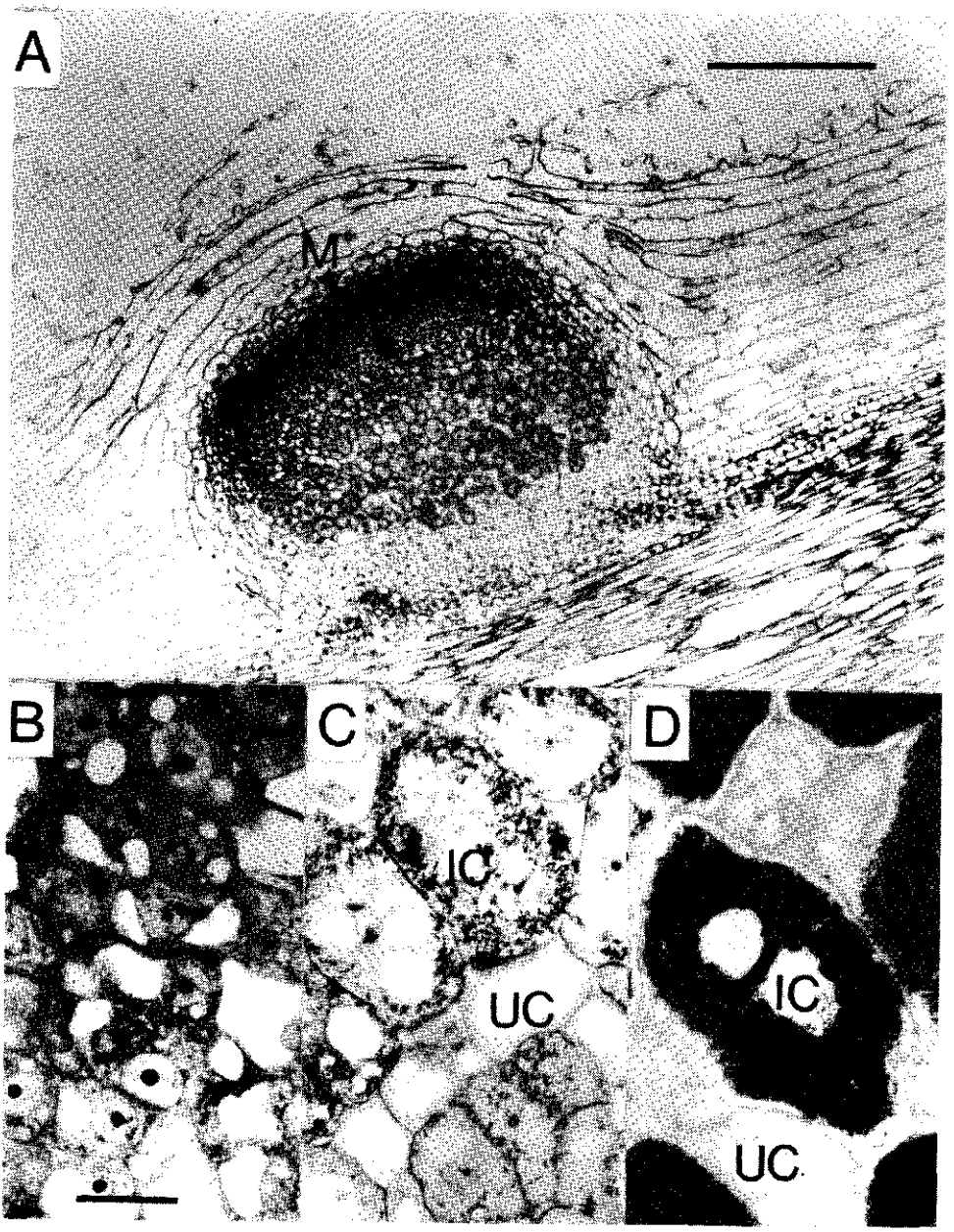

Figure 4. Structural analysis of wild type pea root nodules.
(A) Longitudinal section through an infected pea root, 10 days after sowing and inoculation with R. leguminosarum. M, nodule meristem; bar, 300 µm.
(B), (C) and (D) Light micrographs showing the central part of nodules, 8, 10 and 13 days after inoculation respectively. IC, infected cell; UC, uninfected cell; bar, 600 µm.

As shown in Figure 5A there was no hybridization to RNA from 10 day old infected roots indicating that at this stage none of these nodulin genes are yet expressed. In 13 day old nodules all five pPsNod mRNAs were present and each mRNA increased in concentration during the next 2 to 4 days, the period of time in which nitrogen fixing activity accumulated rapidly (4). By the time nitrogenase activity has almost reached its maximum, day 17, the PsNod mRNA levels remained constant. The time course of appearance and accumulation of the mRNAs of these five nodulin genes is similar to that of Lb mRNA (Fig. 5A). So, the 5 PsNod genes and the Lb genes belong to a class of nodulin genes that are expressed just before nitrogen fixation starts, i.e: in the third stage of nodule development (29). Since the nodule structure is formed in the second stage the nodulins encoded by the PsNod genes can not be involved in the differentiation processes leading to a nodule structure. Probably they play a role in the nitrogen fixation process, for example, in the assimilation and transport of ammonia, as glutamine synthetase, or in support and maintenance of the bacteroids, as leghemoglobin.

Characterization of an early nodulin cDNA clone

In previous analyses of the in vitro translation products from nodule RNA by two-dimensional gelelectrophoresis, we have shown that there is successive expression of nodulin genes during pea root nodule development (13). One nodulin, N-40', was detectable as early as 8 days after sowing and inoculation while another nodulin, N-80, was present in 10 day old infected tissue. Most other nodulins first appeared at day 13. This showed that at least two nodulin genes, called early nodulin genes, are expressed during the second stage of the nodule development, while the majority is expressed in the third stage.

Apparently none of the pPsNod clones represents an early nodulin gene such as the genes encoding N-40' and N-80. Recently Franssen et

al. (11) have isolated several clones from a soybean cDNA library that represent early nodulin genes. One clone, pENOD2, hybridized strongly with RNA from pea root nodules whereas no hybridization was observed with RNA from pea roots (Fig. 5A). Therefore this soybean clone can be used as a probe to study expression of the corresponding nodulin gene in pea. Northern blot analysis showed that in 10 day old infected pea root tissue the ENOD2 gene is expressed (Fig. 5A) and even 8 days after sowing and inoculation RNA hybridizing with pENOD2 can be detected in root segments (data not shown). During development of the nodules the ENOD2 mRNA concentration increases until in 15 day old nodules it has reached its maximum (Fig. 5A). The length of the pea mRNA hybridizing with pENOD2 is 1400 n. Clone pENOD2 hybridized to single EcoRI and HindIII restriction fragments of genomic pea DNA and no hybridization was observed with Rhizobium DNA (Fig. 3). By hybridization of the pENOD2 clone with total pea nodule RNA, a mRNA was selected which translated in vitro into a protein with an apparent molecular weight of 80,000 (Fig. 2C). Since it concerned a hybridization with heterologous cDNA the washings were performed at low stringency resulting in a high background. However, in vitro translation from RNA selected by pBR322 DNA produced the same proteins except the translation product of 80,000 dalton present in the pattern of ENOD2 selected RNA. It can not be excluded that ENOD2 selected RNA gives rise to more translation products which are masked by the high background. The molecular weight of the pea ENOD2 nodulin suggests that the pea ENOD2 gene encodes N-80, the early nodulin that has been identified among nodule-specific in vitro translation products on 2-D gels (13) but further characterization of the protein is required to ascertain this.

The striking cross-hybridization between ENOD2 sequences on soybean and pea, which is much stronger than the cross-hybridization between Lb sequences from both species (unpublished results), suggests that the ENOD2 gene is strongly conserved. In both species the ENOD2 gene is an early nodulin gene that is expressed during the formation of a

nodule structure and the ENOD2 nodulin might be involved in this process. This is supported by the observation that the ENOD2 gene is expressed in pea nodules induced by an Agrobacterium strain harbouring a Rhizobium sym plasmid (15). These so-called "empty" pea nodules, in which no other nodulin mRNAs are detectable, have the characteristic nodule structure but the rhizobia are not released from the infection threads into the plant cells.

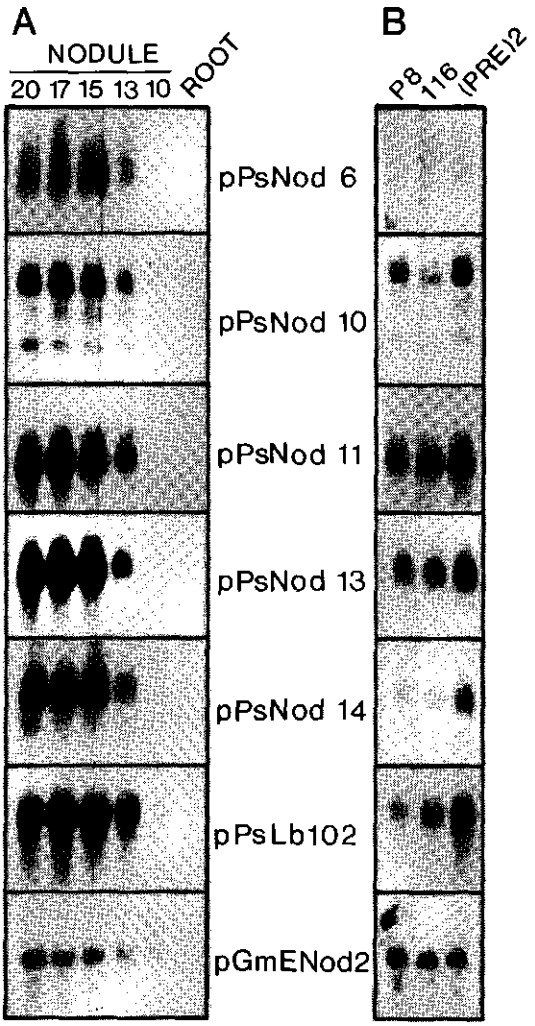

Figure 5. Expression of nodulin genes and Lb genes during pea root nodule development and in non-effective pea root nodules.
Autoradiographs of Northern blots containing in panel (A) RNA from 20, 17, 15, 13 and 10 day old pea root nodules and as a control 8 day old uninfected pea roots (R), and in panel (B) RNA from 15 or 17 day old non-effective pea root nodules induced by fix$^-$ Rhizobium strains (P8(bad$^-$), (1062)116(pop$^-$) and (PRE)2(Tn5::nifD)).The blots were hybridized with nick translated plasmid DNA isolated from the indicated clones. The hybridization temperature was 42°C. For hybridization with the heterologous clone pENOD2 the temperature was lowered with 7°C. For further conditions see MATERIAL AND METHODS. The length of the hybridizing mRNAs is given in Table 1.

Expression of nodulin genes in non-effective nodules

Expression of the PsNod genes and the Lb genes starts concomitantly with or one day before the onset of nitrogen fixation. To study whether the fixation process influences the expression of these genes we determined the mRNA concentrations in root nodules unable to fix nitrogen. Non-effective nodules were induced by the three *nod*$^+$ *fix*$^-$ *Rhizobium* strains also used in previous studies (6, 13). These nodules have a morphology similar to wild type nodules as they contain infected and uninfected cells. The mutations become only manifest after 11 days. In nodules induced by P8(*bad*$^-$) the bacteria do not differentiate, *R. leguminosarum* (1062)116(*pop*$^-$) forms nodules lacking functional Lb and *R. leguminosarum* (PRE)2(Tn5::*nifD*) fails to synthesize nitrogenase. Northern blots of RNA isolated from 15 or 17 day old non-effective nodules were hybridized to ^{32}P-labeled nodulin cDNA probes. As shown in Fig. 5B expression of both the early nodulin gene ENOD2 and the PsNod genes is induced in all three different *fix*$^-$ nodules. The concentration ENOD2 mRNA is the same in the *fix*$^-$ and wild type nodules while the levels of the PsNod and Lb mRNAs are lower in the *fix*$^-$ nodules in comparison with wild type nodules (Fig. 5A). The amounts PsNod6- and PsNod14-mRNA detectable in all three types of non-effective nodules are reduced more than the PsNod10, PsNod11, PsNod13 and the Lb RNA levels, and more RNA of all PsNod and Lb genes is detectable in (PRE)2 nodules than in nodules induced by the strains P8 (*bad*$^-$) and 116(*pop*$^-$).

The observation that all nodulin genes are expressed in *fix*$^-$ nodules is in agreement with the previous reported results in which the same types of non-effective nodules were analysed for the occurrence of nodulins by *in vitro* translation of nodule mRNA (13). Also in soybean (13), alfalfa (29) and *Vicia sativa* (M. Moerman, unpublished results) all nodulin genes are expressed in non-effective nodules. Although the induction of nodulin gene expression does not depend on the nitrogen fixation process, there is apparently an effect on the

final accumulation of the nodulin mRNAs since the mRNA levels of the PsNod and Lb genes are markedly reduced. In contrast, the accumulation of ENOD2 mRNA is not influenced by the nitrogen fixation process.

Whether the decreased levels of PsNod and Lb RNAs are due to a lower expression activity of these genes or to a decrease in the relative number of nodule cells in which these genes are expressed, can not be decided. But from our results it may be concluded that Rhizobium not only induces nodulin gene expression but after the induction more signals have to follow that regulate the final accumulation level. The three different Rhizobium mutants form nodules with different nodulin mRNA concentrations and furthermore, the mRNA levels of the PsNod6 and PsNod14 genes are decreased more drastically than the levels of PsNod10, PsNod11, PsNod13 and Lb RNA in all three fix$^-$ nodules, while the ENOD2 mRNA concentration is not influenced in any of the three. Therefore we conclude that several regulatory mechanisms must exist that determine the final level of different nodulin mRNAs.

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microaerobiosis is not involved in the induction of pea nodulin gene expression

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MICROAEROBIOSIS IS NOT INVOLVED IN THE INDUCTION OF PEA NODULIN GENE EXPRESSION

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ABSTRACT

Gene expression in pea roots grown in a medium with a low oxygen concentration was compared to that in nitrogen-fixing pea root nodules induced by Rhizobium bacteria. The results show that during microaerobiosis the expression of eight genes is increased. None of these belong to the group of genes earlier identified as nodulin genes. On the other hand, no enhanced transcription of microaerobic genes can be detected during nodule development and hybridizations of Northern blots containing nodule RNA and RNA isolated from oxygen-stressed roots, show that the alcohol dehydrogenase genes are not expressed at a higher level in pea root nodules whereas a higher expression is observed during microaerobiosis. From these observations it can be concluded that it is unlikely that a low concentration of free oxygen induces the expression of nodulin genes. Furthermore, genes that are activated as a result of oxygen deficiency are not expressed in pea root nodules, indicating that if the concentration of free oxygen is low the nodule cells do not suffer under microaerobic conditions. Probably, leghemoglobin functions as an efficient oxygen buffer for the energy-generating process in both the plant cells and the bacteroids.

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INTRODUCTION

Nitrogen-fixing nodules on the roots of legumes are specialized organs in which both, plant and bacterial cells are highly differentiated. Plant genes that are specifically expressed in root nodules are called nodulin genes (reviewed by Bisseling *et al.*, 1984b; Verma and Nadler, 1984). Some proteins encoded by nodulin genes have well defined functions in the symbiosis, for example the oxygen binding protein leghemoglobin (Lb; reviewed by Appleby, 1984). However, the possible function of the majority of the nodulins is as yet not clear and the mechanisms by which the synthesis of nodulins is induced are also still unknown. Although there is no doubt that *Rhizobium* induces nodule formation and nodulin gene expression, the nature and the specificity of the signals involved are fully unknown.

The aim of the present study was to examine whether the free oxygen concentration in the root nodule is a physiological factor that is involved in the induction of nodulin gene expression. In root nodules the concentration of free oxygen is low. In soybean nodules for example, it is assumed to be only 10 nM (Tjepkema and Yocum, 1974; Bergersen and Turner, 1975). In pea nodules it is thought to be slightly higher since the major pea Lb component has an affinity for oxygen three times lower than the major soybean Lb component (Uheda and Syono, 1982). It has been suggested by De Vries *et al.* (1980) that oxygen stress is responsible for enhanced enzyme activities of phosphoenolpyruvate carboxylase, malate dehydrogenase and alcohol dehydrogenase (ADH) in pea root nodules, whereas Tajima and LaRue (1982) showed that soybean nodules contain enzymes and substrates typical for an anaerobic metabolism (see also LaRue *et al.* 1984). In several plants, including pea, a set of genes coding for so-called anaerobic proteins is specifically induced when these plants are exposed to anaerobic conditions (Sachs *et al.* 1980). Whether some of these genes are expressed in root nodules and should be considered as nodulin genes, is unknown. Therefore, we have studied gene expression

in pea roots grown in a microaerobic environment by analysing the *in vitro* translation products from root RNA on two-dimensional gels. We have then compared those products with the polypeptides obtained by translation of nodule RNA as described in a previous study (Govers *et al*. 1985).

One of the anaerobic proteins is ADH. In most plants studied so far, there are at least two ADH genes which are expressed in a tissue specific way (Hanson *et al*. 1984; Dennis *et al*. 1985). We have chosen the ADH genes as a model in order to find out if there are nodulin genes which code for nodule-specific forms of anaerobic proteins. Furthermore, the level of expression of ADH genes can be used as a measure for the oxygen concentration in root nodules.

MATERIAL AND METHODS

Nodules on pea (*Pisum sativum* var. Rondo) plants and root tips from uninfected plants were obtained as described by Govers *et al*. (1985). For exposure of pea roots to a microaerobic environment, 14-d-old plants were placed in holders that covered the upperside of beakers containing nutrient solution (in mg per litre 287 $Ca(NO_3)_2.4H_2O$; 80 KNO_3; 737 $MgSO_4.7H_2O$; 19 $NaH_2PO_4.2H_2O$; 191 Na_2SO_4; 15 KCl; 18.6 EDTA-Fe-Na; 0.5 H_3BO_3; 5.3 $MnSO_4.H_2O$; 0.25 $Na_2MoO_4.2H_2O$; 2.1 $ZnSO_4.7H_2O$ and 0.025 $CuSO_4$). The roots were exposed to different concentrations of O_2 by bubbling through the nutrient solution a mixture of air, N_2 and CO_2. The CO_2 content of the gas mixture was kept at 0.5% whereas the ratio air/N_2 varied for the different groups of plants. The O_2 concentration of the nutrient solution was measured polarographically with a Clark-type oxygen electrode using an YS1 model 53 oxygen monitor (Yellow Springs Instruments, Yellow Springs, Ohio, USA) and ranged from 6 µM to 19 µM. As a control, plants were grown on nutrient solution bubbled through with air; the concentration O_2 in the control solutions was 240 µM . After 48 h the roots were cut

off and immediately frozen in liquid nitrogen. The material was stored at -70°C.

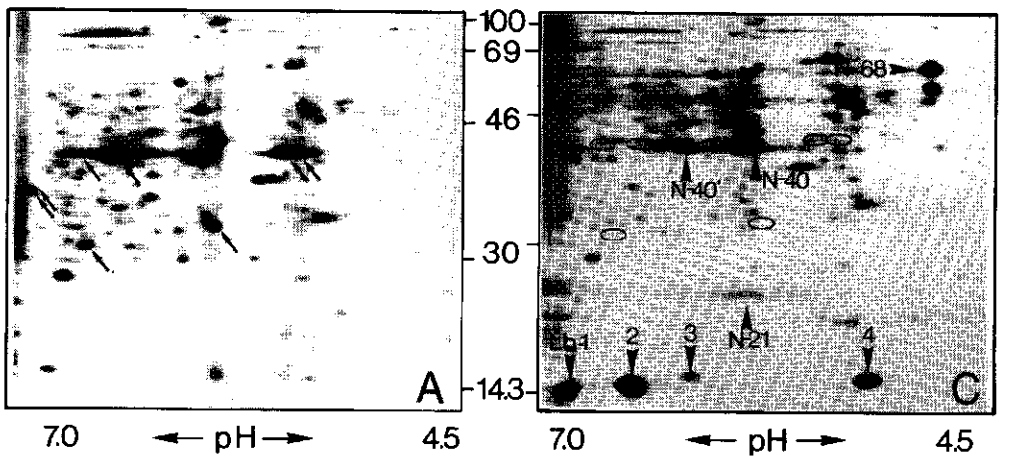
Total RNA was isolated from nodules and roots, translated *in vitro* in a mRNA-dependent rabbit reticulocyte lysate with ^{35}S-methionine as radioactive amino acid, and the *in vitro* labeled translation products were separated on two-dimensional gels. The methods used are desribed by Govers *et al.* (1985).

For Northern blot analyses, samples of 25 µg total RNA were denatured in 0.5 M glyoxal, 50% dimethyl sulfoxide (DMSO), 0.01 M sodium phosphate pH 6.5 for 60 min at 50°C, separated on 1.2% agarose gels and transferred to Gene Screen paper (New England Nuclear Corp., Boston, Mass., USA) using 25 mM sodium phosphate pH 6.5 as blotting buffer. The hybridization mixture consisted of 50% formamide, 10 x Denhardt's solution, 50 mM Tris-HCl pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 0.1% SDS, 10% dextran sulfate and 100 µg ml^{-1} denatured salmon-sperm DNA. The hybridization temperature was 35°C when the 900-basepair insert of pZML84, a maize Adh1 copy-DNA (cDNA) clone (Gerlach *et al.*, 1982), was used as a probe, but was 42°C with pPsLb101, a pea Lb cDNA clone (Bisseling *et al.*, 1984a). The probes were labeled by nick translation (Maniatis *et al.*, 1982). After prehybridization for 8 h and hybridization for 24 h the filter was washed twice for 30 min at 60°C in 2 x SSC, pH 7.0, 0.5% SDS and 30 min at room temperature in 0.5 x SSC, 0.1% SDS (pZML84) or twice in 2 x SSC, 0.5% SDS at 65°C and 30 min at room temperature in 0.1 x SSC, 0.1% SDS (pPsLb101). The filters were exposed to Kodak XAR-5 film.

RESULTS

In order to study the effect of microaerobic conditions on gene expression in pea roots, total RNA was isolated from root tissue exposed to different concentrations of oxygen (6, 7, 19 or 240 µM) and was translated in a reticulocyte lysate. The translation products were

separated by two-dimensional (2-D) gel electrophoresis. In Fig. 1A, the 2-D pattern of 14-d-old roots cultured for 48 h in a solution containing 6 μM O_2 is shown. An identical polypeptide pattern was obtained from roots grown in a solution containing 7 μM O_2, whereas the 2-D pattern from roots exposed to 19 μM O_2 could not be distinguished from that of the control tissue at 240 μM O_2 (Fig. 1B). When exposed for 48 h to less than 6 μM O_2 in the nutrient solution the pea roots died and no RNA could be isolated.



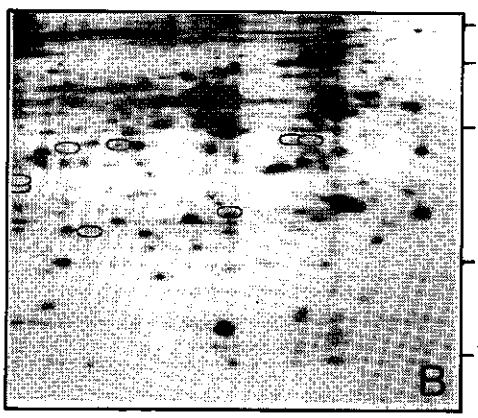


Fig. 1A-C. Identification of microaerobic mRNAs and comparison of these mRNAs with nodulin mRNAs. Fluorographs of in vitro translation products separated by two-dimensional gel electrophoresis. (A) RNA from uninfected pea roots cultured aerobically for 14 d and then exposed to a microaerobic environment (6 μM O_2) for 2 d. The microaerobic spots are indicated by arrows. (B) RNA from 3-d-old uninfected pea roots exposed to an aerobic environment (240 μM O_2). In vitro translation of RNA from 16-d-old uninfected roots exposed to an aerobic environment results in an identical pattern. The positions of the spots that are new or stimulated in a microaerobic environment are indicated by circles. (C) RNA from 17-d-old pea root nodules grown at 240 μM O_2. The major nodulin spots are indicated by arrowheads while circles mark the positions of the spots that are new or stimulated in a microaerobic environment. The ^{14}C-methylated molecular-weight markers are in kilodalton.

Comparison of the 2-D patterns of polypeptides from microaerobically grown roots with those obtained from roots grown in a normal atmosphere (Fig. 1A and 1B) shows that most of the proteins were present in both with no dramatic differences in the intensities of the spots. However, eight polypeptides either appeared as new spots or were much more intense in the pattern of polypeptides from the microaerobically grown roots, indicating that the relative amounts of the corresponding mRNAs were increased in the roots upon exposure to O_2 concentrations below 7 μm compared with the control tissue. These so-called microaerobic proteins varied in molecular weight between 30,000 and 40,000 and their location is marked by arrows in Fig. 1A.

To study whether a microaerobic environment induces the expression of nodulin genes, we compared the translation products of the mRNAs present in uninfected pea roots exposed to oxygen concentrations of 6 and 7 μM with those of pea root nodules grown in normal atmosphere (240 μM O_2). A 2-D gel of *in vitro* translated RNA from 17-d-old pea root nodules is shown in Fig. 1C and it is obvious that, again, most spots are identical to those in the gel for uninfected pea roots (Fig. 1B). Previously we have shown that 21 new mRNAs can be detected in root nodules: eight major (indicated by arrowheads in Fig. 1C) and 13 minor spots. Leghemoglobin mRNAs are represented by four major spots (Govers *et al.*, 1985). Close examination of the 2-D patterns shown in Fig. 1A and 1C and also of root nodules in different stages of development (data not shown) revealed that the new spots on the root-nodule gels were at different positions from those on the gels for microaerobically grown pea roots. This indicates that the genes encoding nodulins were not expressed in roots exposed to low oxygen concentrations.

The possibility remains, however, that some nodulin genes code for nodule-specific microaerobic proteins. Because each tissue-specific form of a protein can have its own characteristic isoelectric point or molecular weight, 2-D analysis is not the appropriate technique to recognize microaerobic and nodule-specific forms of polypeptides with

the same catalytic activity or function but derived from different (microaerobic and nodulin) genes. Instead, hybridization of Northern blots, containing RNA from root nodules and from uninfected roots exposed to varying concentrations of oxygen, with probes derived from microaerobic genes can be used to study tissue-specific expression. The Northern blots were hybridized with a maize ADH cDNA clone at low stringency to be sure that all the ADH mRNA present would hybridize to the heterologous maize probe. As shown in Fig. 2A the hybridization with RNA from roots grown at the normal O_2 concentrations of 240 µM was very weak indicating that only minor amounts of ADH mRNA were present. However, by decreasing the concentration of O_2 to microaerobic conditions the amount ADH mRNA was clearly increased. Total RNA isolated from root nodules contained a concentration of ADH mRNA similar to that of uninfected roots grown under aerobic conditions, demonstrating that there was no increase in the expression of ADH genes in root nodules compared with uninfected roots. Using nodule polyadenylated RNA instead of total RNA - the former should allow a more sensitive detection of ADH mRNA - gave the same result. The Northern blots were also hybridized with pea Lb cDNA (Fig. 2B) and, as

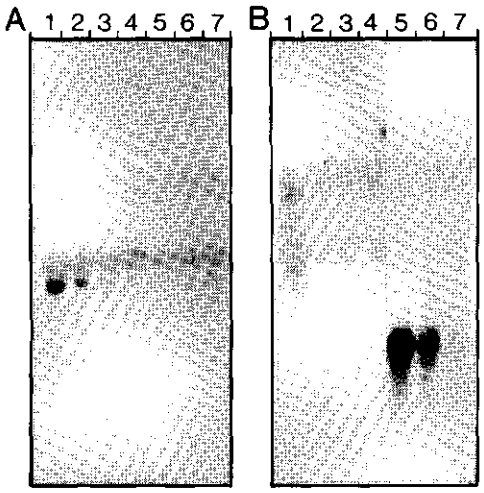


Fig. 2A, B. Expression of ADH genes and Lb genes in microaerobically grown pea roots and pea root nodules. Autoradiographs of Northern blots containing RNA isolated from uninfected pea roots grown in a nutrient solution containing: lanes (1) 6 µM O_2, (2) 7 µM O_2 and (3) 19 µM O_2; from control tissue: (4) uninfected pea roots exposed to a normal atmosphere (240 µM O_2); and from pea root nodules in different developmental stages: (5) 17, (6) 13 and (7) 10 d after sowing and inoculation. The blots were hybridized with (A) a ^{32}P-labeled maize Adh1 cDNA clone (pZML84) and (B) a ^{32}P-labeled pea Lb cDNA clone (pPsLb101). The predominant bands correspond to mRNAs of ~ 1600 bases (ADH) and ~ 700 bases (Lb) in length.

shown previously (Govers et al., 1985), RNA from 13- and 17-d-old root nodules hybridized strongly, indicating that the Lb genes were highly expressed in these stages of development. In 10-d-old nodules and in uninfected roots, no Lb mRNA was detectable and decreasing the O_2 concentration did not lead to induction of Lb-gene expression in uninfected roots. These observations show that the expression of ADH genes is increased under microaerobic conditions but not in root nodules.

DISCUSSION

In order to see if one or more nodulin genes are activated under microaerobic conditions we compared the mRNA population in pea roots exposed to low concentrations of O_2 with mRNAs in pea root nodules. The results show that none of the genes that have been identified as nodulin genes are expressed in uninfected roots grown in a microaerobic environment, but the expression of eight other genes was specifically induced or stimulated. These genes presumably code for proteins with a specific function under such conditions, but only for some microaerobic or anaerobic proteins is the function known. One of these proteins is ADH and, indeed, we found that the concentration of ADH mRNA was at least 10 times higher in pea roots exposed to 6 μM oxygen compared to roots grown under normal conditions. Therefore, one of the eight new or stimulated polypeptides on the 2-D gels probably represents the translation product of ADH mRNA. The increased mRNA level is in agreement with the increase in ADH activity observed in the root samples (data not shown) and also with the finding of Smith and ap Rees (1979) who have shown that in pea roots the major route of carbohydrate breakdown during anaerobiosis is alcoholic fermentation mediated by ADH.

During pea root nodule development no translation products could be detected from genes that are activated under microaerobic conditions. No differences were found in the amount of ADH mRNA or in the size of

the ADH mRNA from roots or root nodules. In addition, there were no indications of an increase of ADH gene expression in nodules of different ages from very young to mature nodules, or for the presence of a nodule-specific form of ADH. These observations are seemingly in disagreement with the results published by De Vries et al. (1980) who measured an increased ADH activity in pea root nodules. However, results obtained by different groups are not consistent. The most thorough study seems to be the recently published work of Smith (1985) who showed that there are no significant differences in ADH activity between the pea root nodule and the meristematic tissue at the root apex of the uninfected pea root, observations that are in agreement with the results presented here.

The ADH genes studied so far are always expressed at a higher level in tissues that are deprived of oxygen (maize: Gerlach et al., 1982; barley: Hanson et al., 1984; pea: this study). Since there is no increase in ADH gene expression in pea root nodules it can be concluded that - despite the observation that the concentration of free oxygen is low in root nodules - the amount of oxygen that is available in the cytoplasm of nodule cells is not limiting for the oxidative generation of energy. When Lb is present in the cytoplasm of the infected nodule cells the oxygen-binding properties of this protein presumably protect the bacteroid-encoded nitrogenase from inactivation of free oxygen, while at the same time providing for the efficient supply of oxygen to both the bacteroids and the plant cell. At the stage when Lb is not yet present in the nodule we find the expression of the early nodulin gene, N-40' (Govers et al., 1985), but no stimulation of expression of ADH genes or other microaerobic genes. Although in this early stage the multiplication of the bacteria in the infection thread is an energy-consuming process, this apparently does not result in an environment wherein the supply of oxygen is limited. We do not find expression of any of the nodulin genes in a microaerobic environment and therefore it is unlikely that a low concentration of free oxygen is involved in induction of expression of these genes.

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VI

Rhizobium nod genes are involved in the induction of an early nodulin gene

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Rhizobium nod genes are involved in inducing an early nodulin gene

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Rhizobium **bacteria can invade the roots of leguminous plants and elicit the formation of root nodules. This process involves the expression of at least 20 nodule-specific genes encoded by the host plant, the so-called nodulin genes, which include the leghaemoglobin genes[1–3]. During development of the root nodules the nodulin genes are differentially expressed[1]. In fast-growing *Rhizobium* species several essential symbiotic genes are located on a large plasmid, and when fragments of the plasmid which contain the genes essential for nodulation (the *nod* region) are cloned and transferred to *Rhizobium* strains lacking the symbiotic plasmid ('cured' strains), the recipients regain the ability to form nodules. However, these nodules cannot fix nitrogen because essential nitrogen-fixation genes are absent[4–6]. We show here that transfer of the *nod* region (10 kilobases; kb) alone from a *Rhizobium leguminosarum* symbiotic plasmid into cured *Rhizobium* strains is sufficient to elicit nodulin gene expression in the host *Pisum sativum*. We also show that pea root nodules induced by an *Agrobacterium* strain containing the *R. leguminosarum* symbiotic plasmid express an early nodulin gene but no other nodulin genes. These results show that at least two signals are involved in the induction of expression of nodulin genes and the presence of the *Rhizobium* nodulation genes seems to be required to elicit the first signal.**

A 60-kb region of DNA from the *R. leguminosarum* symbiotic plasmid pRL1JI carries a cluster of nodulation genes which is flanked by two groups of genes involved in nitrogen fixation[7]. Downie *et al.*[4] found that two overlapping cosmid clones of pRL1JI—pIJ1089 and pIJ1085—containing a 10-kb region in common, enabled a cured *R. phaseoli* strain to form nodules on pea; the development of these nodules appeared normal and bacteroid forms surrounded by a peribacteroid membrane were present, although the nodules did not fix nitrogen because of nitrogen-fixation genes were absent. Transfer of the same cosmid clones to a cured *R. leguminosarum* strain also restored the pea nodulation ability of this strain. If the 10-kb region was absent no nodules were formed and it was concluded that the 10-kb region is the only fragment of the pRL1JI symbiotic plasmid which is essential for pea nodule formation. We therefore used the strains containing pIJ1089 or pIJ1085 to determine whether pea nodulin genes are induced. Nodulin gene expression was studied in two ways, by Northern blot analyses and separation of *in vitro* translation products from nodule RNA on two-dimensional gels. We have shown previously that *in vitro* translation of pea nodule RNA gives rise to at least 21 nodulins[1]. Figure 1*a* shows a two-dimensional gel of *in vitro* translation products of nodule RNA while Fig. 1*b* shows six major nodulin spots, including two leghaemoglobin (Lb) spots. Nodulin N-40' was detected on the gels 5 days before Lb whereas nodulins N-40 and N-68 appeared during nodule development simultaneously with the Lb spots. N-21 was only detectable 2 days later.

For the Northern blot analyses nodulin complementary DNA clones were selected representing either messenger RNAs that appear at the same day as Lb mRNA (pPsNod clones; F.G., unpublished results) or mRNAs that appear several days before Lb mRNA (pENOD clones, H.J.F., manuscript in preparation). The pPsNod clones are of pea origin whereas the pENOD clones were isolated from a soybean nodule cDNA library; however, one of these, pENOD2, cross-hybridizes strongly with RNA from pea nodules and represents a pea nodulin gene that is expressed 5 days before the Lb genes (H.J.F., manuscript in preparation), that is, at the same time as the *in vitro* translation product N-40' is first observed[1]. Nodulin genes can thus be divided into two classes: the early nodulin genes, such as N-40' and ENOD2, and the nodulin genes that are expressed just before or after the onset of nitrogen fixation, such as N-68, N-40, N-21, Lb and PsNod genes.

In 21-day-old pea root nodules formed by a cured *R. phaseoli* strain containing pIJ1089 or pIJ1085 and no other parts of a symbiotic plasmid, the pattern of major (Fig. 1) and minor nodulin spots (data not shown) was similar on two-dimensional gels to that produced by the wild-type *R. leguminosarum* strain. N-68, N-40', N-40 and Lbs were present at wild-type levels, but the amount of N-21 was reduced in the nodules formed by the *Rhizobium* containing pIJ1085 or pIJ1089. On Northern blots, RNAs hybridizing to pENOD2 and the pPsNod clones were also detected (data not shown), and thus all the identified nodulin genes in pea are expressed. This shows that, apart from the *nod* region, *nif, fix* and other putative genes encoded by the symbiotic plasmid are not essential for the induction of expression of these nodulin genes. In previous experiments using *Rhizobium* mutants that produce ineffective (*fix*$^-$) root nodules,

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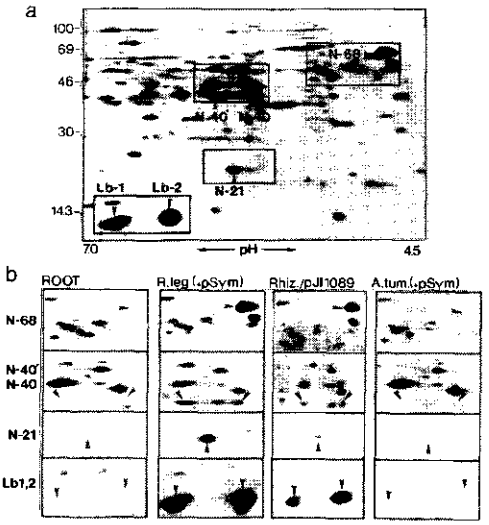

Fig. 1 Expression of nodulin genes in pea root nodules. Fluorographs of two-dimensional gels of *in vitro* translation products from total RNA isolated from: *a*, effective pea root nodules 17 days after sowing and inoculation with *R. leguminosarum* (PRE); *b*, uninfected pea roots from 8-day-old plants, pea root nodules induced by wild-type *R. leguminosarum* (PRE) (15 days), *R. phaseoli* cured of its own symbiotic plasmid and containing the cosmid pIJ1089 (21 days), and an *Agrobacterium* strain, LBA 2712, carrying a symbiotic plasmid (28 days). Nodules formed by LBA 2712 were first detected on roots of 21-day-old pea plants and were collected from 21-, 28-, 35- and 42-day-old plants, respectively. In none of these nodules were major nodulins detected by *in vitro* translation and in all cases bacteria isolated from surface-sterilized nodules were resistant to the appropriate antibiotics. The major nodulin spots N-68, N-40, N-40', N-21, Lb-1 and Lb-2 are indicated by arrowheads. In *b* only the parts of the gels within the squares indicated in *a* are shown. The nodulin pattern induced by *Rhizobium*/pIJ1085 (result not shown) is similar to that elicited by *Rhizobium*/pIJ1089. In *a*, ^{14}C-methylated M_r markers are shown on the left ($\times 10^{-3}$).

Methods. Nodulated pea plants (*Pisum sativum* var. Rondo) were grown in Leonard jars filled with Perlyte under the growth conditions described by Bisseling *et al.*[14]. Total RNA was isolated from uninfected roots and nodules, and *in vitro* translated in the presence of ^{35}S-methionine in a mRNA-dependent rabbit reticulocyte lysate as described previously[1]. The *in vitro* labelled translation products were separated by two-dimensional gel electrophoresis according to O'Farell[15], and 3-[(3-chloroamidopropyl)dimethylammonio]-1-propanesulphonate was used in the isoelectric focusing sample buffer[16].

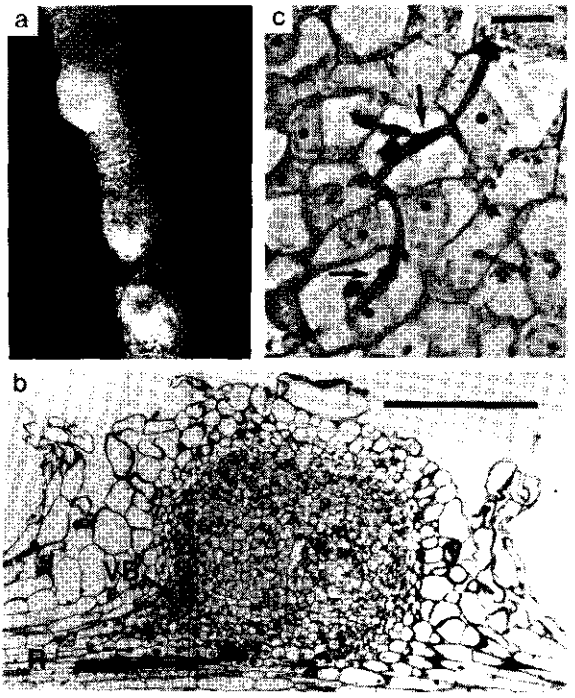

Fig. 2 Morphology and cytology of 'empty' nodules on pea roots induced by the *Agrobacterium* transconjugant LBA 2712. *a*, Part of a pea root with nodules (28 days). *b*, Light micrograph of a longitudinal section through a nodule (28 days). The vascular bundle (VB) connection to the main root (R) is maintained. Scale bar, 400 μm. *c*, Light micrograph of a part of the central nodule tissue in which infection thread-like structures are visible (arrows). Scale bar, 40 μm.

Methods. Pea plants were grown as described in Fig. 1 legend. Four-week-old nodules were fixed in 3% glutaraldehyde for 2 h, dehydrated in a graded ethanol series and embedded in Technovit 7100; 4-μm sections were cut with steel knives, placed on slides and stained with toluidine blue[17].

nodulin N-21 was never found among the *in vitro* translation products and it was suggested that the expression of the N-21 gene was controlled by the nitrogen-fixation process[1]. However, the present results refute this conclusion. The difference is probably due to the plant growth conditions. By growing the plants in Leonard jars containing perlyte instead of trays containing gravel, we can detect N-21 at low levels.

The *nod* region is clearly essential for nodule development and, as shown above, the presence in *Rhizobium* of only this part of the *R. leguminosarum* symbiotic plasmid is sufficient to induce nodulin gene expression in pea. It is becoming increasingly evident that *Rhizobium* chromosome and non-symbiotic plasmids contain genetic information that is essential for effective nodule development. For example, mutations in the

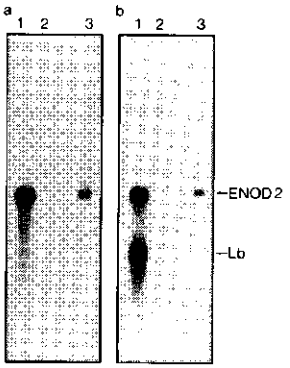

Fig. 3 Expression of leghaemoglobin and ENOD2 genes in pea root nodules. Autoradiographs of a Northern blot containing RNA from wild-type pea root nodules (17 days, lanes 1) induced by *R. leguminosarum* (PRE), 8-day-old uninfected pea roots (lanes 2) and 'empty' nodules induced by the *Agrobacterium* transconjugant LBA 2712 (28 days; lanes 3). The blot was hybridized with pENOD2 (*a*) and subsequently with pPsLb101 (*b*). ENOD2 RNA corresponds to 1,400 bases in length. Because the blot was not deprobed, the hybridization of the heterologous soybean cDNA clone pENOD2 with pea RNA shown in *a* is still visible in *b* but the intensity is reduced due to higher stringency washings after hybridization with the homologous Lb cDNA clone.

Methods. Samples of RNA (10 μg) were denatured in 0.5 M glyoxal, 50% dimethyl sulphoxide, 0.01 M sodium phosphate *p*H 7.0 for 60 min at 50 °C and separated on a 1.2% agarose gel in 10 mM sodium phosphate *p*H 7.0. After electrophoresis, the RNA was transferred to GeneScreen paper (NEN) using 25 mM sodium phosphate *p*H 6.5 as blotting buffer. The filter was prehybridized for 8 h in HB (50% formamide, 10 × Denhardt's solution, 50 mM Tris-HCl *p*H 7.5, 1 M NaCl, 0.1% SDS, 10% dextran sulphate and 100 μg ml^{-1} denatured salmon sperm DNA) and hybridized for 24 h in HB plus denatured pENOD2 DNA labelled with ^{32}P by nick translation[18]. The hybridization temperature was 35 °C and the filter was washed twice for 30 min at 42 °C in 2 × SSC, 0.5% SDS and for 30 min at room temperature in 0.5 × SSC, 0.1% SDS. After exposure to Kodak XAR-5 film the filter was prehybridized for 8 h, hybridized for 24 h at 42 °C with ^{32}P-labelled pPsLb101, washed twice for 30 min at 65 °C in 2 × SSC, 0.5% SDS followed by 30 min at room temperature in 0.5 × SSC, 0.1% SDS, and again exposed to Kodak XAR-5 film.

genes involved in the exopolysaccharide synthesis in *Rhizobium meliloti* result in nodules lacking intracellular bacteria[8]. Because it is not known whether the chromosome is involved in the induction of nodulin genes, we studied nodulin gene expression in pea root nodules formed by a strain (LBA 2712) that contains an *Agrobacterium* chromosome and a *R. leguminosarum* symbiotic plasmid (pSym1)[9]. It has been reported recently that 'empty' root nodules are formed on alfalfa roots by *Agrobacterium tumefaciens* carrying clones containing the *nod* region of *R. meliloti*[5,10–12], and we observed similar empty nodules on pea roots inoculated with LBA 2712. Histological examination of these small ineffective nodules showed that the plant cells lack bacteria, although some structures similar to infection threads were observed (Fig. 2). Vascular bundles were present at the periphery of the nodule, characteristic of normal nodules. These empty nodules first appeared 3 weeks after inoculation, and analyses of *in vitro* translation products from RNA isolated from empty nodules on 3-, 3.5-, 4- and 5-week-old plants revealed no Lb or other major nodulins (N-68, N-40, N-40' and N-21) (Fig. 1*b*). Hybridization of a Northern blot with pPsLb101, a pea Lb cDNA clone[13], showed that Lb mRNA is found only in the wild-type nodules and not in the empty nodules (Fig. 3*b*), confirming the results found by *in vitro* translation of the RNAs. However, hybridization of the same blot with pENOD2 showed that ENOD2 mRNA, an early nodulin mRNA, is present in 3.5-week-old empty nodules (Fig. 3*a*). Indeed, close examination of several two-dimensional gels revealed that a minor nodulin of the same relative molecular mass (M_r) as the hybrid-released translation product of pENOD2 (M_r 80,000; F.G., unpublished results) is consistently present in empty nodules. Hence, in empty pea root nodules only the early nodulin gene ENOD2 is expressed while the other nodulin genes, including that encoding the early nodulin N-40', are not activated. In pea tumours induced by *A. tumefaciens* no ENOD2, Lb or other nodulin mRNAs are detected (data not shown). In such tumours, the agrobacteria do not penetrate the plant cells and wounding is necessary for causing tumour development, so that the bacteria can reach the intercellular spaces. Furthermore, empty nodules contain no intracellular bacteria, although some infection threads with bacteria are observed (Fig. 2*c*). As the *Agrobacterium* strain carrying the symbiotic plasmid induces expression of the ENOD2 gene whereas *A. tumefaciens* does not, it seems unlikely that the *Agrobacterium* genome itself harbours genes involved in the induction of this gene. Such genes must be located on the symbiotic plasmid and, as shown by analyses of nodules induced by *Rhizobium* strains carrying pIJ1089 or pIJ1085, only a 10-kb region from the symbiotic plasmid is required for expression of the ENOD2 gene. This region contains the *nod* genes and we suggest that the *nod* gene products are responsible, directly or indirectly, for induction of ENOD2 gene expression.

We conclude from the present results that: (1) An intracellular location of *Rhizobium* is not required for the expression of the ENOD2 gene in pea, although this may be essential for expression of the other nodulin genes. (2) At least two signals from *Rhizobium* seem to be involved in expression of pea nodulin genes, one inducing the expression of the early nodulin gene ENOD2 and a second for the other nodulin genes; the generation of the latter signal might be dependent on the effects produced by the first signal. (3) The nodulation genes of *R. leguminosarum* are involved in the induction of the ENOD2 gene in pea. (4) *Rhizobium* genes not located on the symbiotic plasmid seem to be essential for the expression of other pea nodulin genes.

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VII

nodulins in the developing root nodule : an overview

plant physiology and biochemistry, in press

NODULINS IN THE DEVELOPING ROOT NODULE: AN OVERVIEW

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1. INTRODUCTION

Infection of leguminous plants with bacteria of the genus *Rhizobium* results in localized root cell de-differentiation followed by the formation of a meristem and *de novo* development of a specialized organ, the root nodule, in which the rhizobia can display their capacity to fix atmospheric nitrogen. Root nodule formation can be regarded as a plant differentiation process and as such it has the advantage over other systems that the program for root nodule development is induced by bacteria of which the genetics relating to the symbiotic interaction has been well developed. Engineered bacterial strains are available that cause defects in the developmental program which facilitates the identification and analysis of the successive steps leading to the formation of a root nodule.

This paper deals with the nodulin genes, the host plant genes which are specifically expressed during the development of the symbiosis, and it is organized into four main sections. After a discussion of the different methods used for identification of nodulins, the expression of nodulin genes during root nodule development is described. We then focus on possible functions of nodulins and on the involvement of *Rhizobium* in the induction and regulation of nodulin gene expression. Finally a model is presented in which the current knowledge on induction and expression of nodulin genes is integrated and correlated with functions of nodulins in the differentiation of nodule cells and in the fixation process.

2. IDENTIFICATION OF NODULINS

Till now we have applied three methods for the identification of nodulins and the analyses of nodulin gene expression in pea root nodules. The first method uses antisera against nodule specific proteins for the analyses of *in vivo* proteins. In the second method, nodule RNA is translated *in vitro* after which the translation products are analysed by two dimensional (2-D) gel electrophoresis. Finally, the expression of nodulin genes is studied using nodulin cDNA clones as probes.

Antisera were raised against total protein extracts from the plant fraction of pea root nodules and then made nodule specific by titration with proteins from uninfected roots (Bisseling *et al.*, 1983). By analysing Western blots of pea nodule proteins after incubation with the nodule-specific antiserum preparation, 30 different nodule-specific polypeptides could be detected. Some of these proteins were shown to be located in the peribacteroid space but the majority was found in the cytoplasmic fraction of the nodules, indicating that a considerable part of the nodule-specific proteins is encoded by the plant genome. However, at least two cytoplasmic proteins reacting with the antiserum preparation appeared to be encoded by *Rhizobium*. They are excreted by the bacteroids into the cytoplasm (Bisseling *et al.*, 1984b) and this limits the utility of such an immunological assay for identifying specifically the nodulins apart from bacterial proteins. Unfortunately, nodule-specific antiserum preparations have still more disadvantages. It is for instance possible that the antiserum recognizes antigenic determinants occurring on both, root proteins and nodule-specific proteins. If the antiserum is absorbed with root proteins the reaction with some nodule-specific proteins might be lost and as a result these proteins remain undetected. This is illustrated by an antiserum raised against a nodule-specific form of glutamine synthetase that also reacts with a functionally similar protein present in uninfected roots (Cullimore and Miflin, 1984a). Furthermore, a

nodule-specific antiserum preparation contains a mixture of antibodies, the titers of which need not reflect the relative amounts of the proteins in root nodules because of differences in antigenicity. Relatively abundant but less antigenic proteins may therefore remain undetected as well. The most serious disadvantage of the use of nodule-specific antiserum preparations is that antisera produced by different rabbits immunized with protein preparations from the same type of nodules can recognize a different set of nodule-specific proteins. Also the intensity of the immunological reaction with particular proteins can change, dependent on the protein amount injected and the methods used for the titration. This makes it impossible to compare results of different research groups studying the same plant but using their own nodule-specific antiserum preparations (see e.g. Lang-Unnasch and Ausubel, 1985a and Vance et al., 1985).

To overcome some of the drawbacks of nodule-specific antiserum preparations, antisera were raised against purified nodule-specific proteins with either known (leghemoglobin, Bisseling et al., 1979) or unknown functions (Bisseling et al., 1984a and b). By incubating Western blots of soluble cytoplasmic proteins and peribacteroid space proteins with antisera against four purified nodule-specific proteins it was shown that two of them were located exclusively in the peribacteroid space while the two others were cytoplasmic proteins (Bisseling et al., 1984a). In vivo labeling of isolated bacteroids followed by immunoprecipitation showed that the two peribacteroid space proteins are encoded by Rhizobium, which again illustrates a major pitfall of studies on nodulins by means of analyses of in vivo proteins: the plant or bacterial origin of a nodule-specific protein can not be stipulated beforehand. However, once a monospecific antiserum against a nodulin is available it can be very useful for expression studies. Using leghemoglobin (Lb) antiserum the appearance of Lb during effective nodule development (Bisseling et al., 1980) and in non-effective nodules was analysed (Bisseling et al., 1984a), and by comparing Western and Northern blots it was shown that there is a posttranscrip-

tional regulation of Lb in non-effective pea root nodules (Govers et al., 1985). In addition, localization of a nodulin within the complex nodule structure contributes to the identification of its function and for localization studies monospecific antisera are essential tools. Immunocytological studies with Lb antiserum and nodulin-35 antiserum, confirmed that Lb is localized in the cytoplasm (Robertson et al., 1984b) while uricase II (nodulin-35) is exclusively found in the peroxisomes of uninfected cells (Nguyen et al., 1985). Moreover, monospecific antisera can be used for selection of corresponding nodulin cDNA clones as was demonstrated with the isolation of a soybean nodulin-35 cDNA clone (Nguyen et al., 1985) and a Phaseolus vulgaris glutamine synthetase cDNA clone (Cullimore et al., 1984b).

To focus on the contribution of the plant to the symbiosis we decided to use methods that exclusively give information on plant gene expression. In vitro translation of total RNA isolated from pea root nodules and from the bacteroid fraction of the nodule, followed by electrophoresis of the translation products on 2-D gels, showed that in eukaryotic in vitro translation systems only plant encoded RNAs are transcribed. This procedure gives an overview of the expression of ~ 500 most abundantly transcribed plant genes and it requires only small amounts of total RNA (1-2 μg). So analysis is even possible when minor quantities of tissue are available, for instance from non-effective nodules or from root hairs. Using this method we have shown that in pea root nodules at least 21 plant genes are expressed which are not expressed in uninfected roots (Govers et al., 1985). From the nodulins encoded by these genes eight give rise to major spots on 2-D gels (fig. 1A), four of which are leghemoglobins (Lb-1, 2, 3 and 4), while from the other four (N-68, N-40', N-40 and N-21) the function is not known yet. A disadvantage of this method might be that unstable RNAs or RNAs that are translated in vitro with low efficiency are not detected. Moreover, by in vitro translation in the presence of ^{35}S-methionine, the methionine content of a protein determines the intensity of a spot and the major spots are not necessarily derived

from the most abundant RNAs. Since *in vitro* translation products from pea nodule RNA containing ^{3}H-leucine produce a similar 2-D pattern we do not consider this as a serious problem. However, by incorporating only one radioactive amino acid, nodulins can be missed as is illustrated by soybean Lbs which have no methionine residue (Fuchsman, 1985).

Another approach we apply to study plant gene expression in root nodules is Northern blot analysis using cloned nodulin sequences as probes. To obtain the probes we have constructed a cDNA library from pea nodule poly(A^+) RNA and selected several nodule-specific cDNA clones by differential screening. Six of these were studied in more detail (table 1) (Govers *et al.*, 1986b). In a hybrid released translation experiment two cDNA clones selected mRNA that was translated *in vitro* into nodulins that belong to the group of 21 nodulins identified

Table 1. Nodulin cDNA clones[o]

Class	Clone	RNA size (bases)	Translation products (D)
I	pENOD2[•]	1,400	80,000
II	pPsNod6	560	9,000
	pPsNod10	710	8,000
		570	6,800
		440	5,800
	pPsNod11	650	19,000
			17,500
	pPsNod13	800	14,000
	pPsNod14	540	7,500
	pPsLb102	700	14,000

[o]from Govers *et al.*, 1986b.
[•]a soybean cDNA clone (Franssen *et al.*, 1986) hybridizing to a pea nodulin mRNA of 1,400 bases.

on 2-D gels. For one of these clones, pPsNod11, this is shown in figure 1B. The other four cDNA clones encode nodulins with molecular weights smaller than 14,000 and these were not yet identified by 2-D analyses of in vitro translation products of nodule RNA. Northern blot analyses require more RNA than in vitro translation studies, and in one experiment expression of only one nodulin gene is analysed in contrast to the overview of nodulin gene expression obtained by 2-D analyses. However, once a cDNA clone appears to represent a nodulin gene of interest, one can directly isolate the gene and via sequencing and peptide synthesis antiserum can be produced.

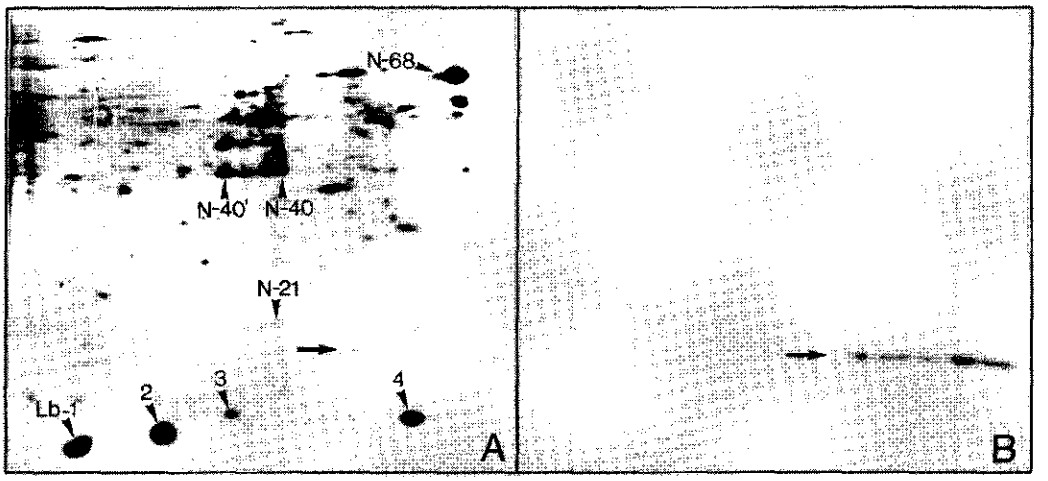


Figure 1. Identification of nodulins and of the hybrid released translation products of a nodulin cDNA clone.
Fluorographs of two dimensional gels of in vitro translation products from (A) 17 day old pea nodule RNA and (B) RNA selected from 17 day old pea nodule RNA by hybridization with pPsNod11. In (A) the 8 major nodulin spots are indicated by arrowheads. In (B) the translation products of RNA selected by pPsNod11 are indicated by an arrow as are the corresponding spots in (A). When the RNA selected by pPsNod11 is translated in a wheat germ system and separated on a one dimensional SDS-polyacrylamide gel it gives rise to two proteins with molecular weights of 19,000 and 17,500 (tab. 1). The in vitro translation products shown in this figure are synthesized in a cell-free rabbit reticulocyte lysate and no clear distinction can be made between two proteins with different molecular weights. It is possible that one of the products is not synthesized in the reticulocyte lysate or the two proteins are not well enough separated on this gel. Why the nodulins on these gels are not focussed at one isoelectric point is unclear, but the minor nodulins comigrating in (A) with the hybrid released translation products in (B) are in one experiment distinct spots while in other experiments they produce a smear as shown here.

In summary, each method employed with the purpose to identify nodulins and nodulin genes has its own pros and cons. Nevertheless, it is possible by using the different techniques side by side to select those nodulin genes of which it is worth studying the structure and regulation of expression and the function of the corresponding nodulins in the symbiosis.

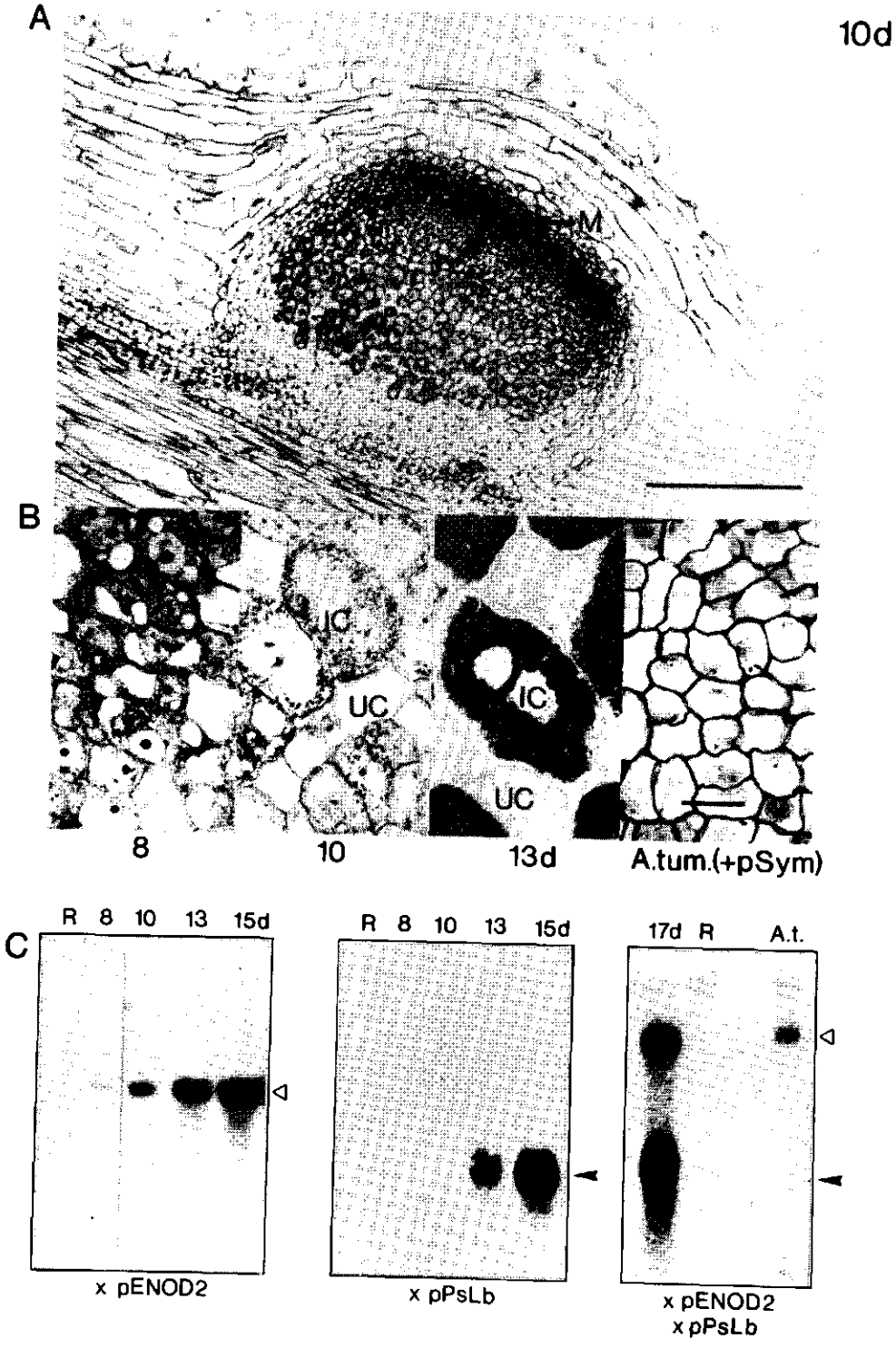
3. EXPRESSION OF NODULIN GENES DURING ROOT NODULE DEVELOPMENT

The mature nitrogen fixing nodule is the result of a multistep developmental process. For elucidating the possible functions of nodulins in this process it is essential to know in which developmental stage nodulin genes are expressed.

Upon inoculation of a leguminous plant with its compatible Rhizobium species the first visible reaction of the host is curling of the root hairs followed by formation of infection threads through which the rhizobia invade the root. Already at that time, before infection threads have reached the cortex, cell divisions are induced in the cortex. Meanwhile the rhizobia in the threads multiply (Libbenga and Bogers, 1974). The growing infection threads pass from cell to cell until they reach the centers of the cortical cell divisions where by that time a nodule meristem is formed. Then, an unknown cue causes a switch from infection thread growth to dissolution and the rhizobia are released into the target cells of the evolving nodule. Here they are surrounded by a peribacteroid membrane derived from the plasmalemma of the host plant cell (Brewin et al., 1985). Following differentiation into bacteroids, nitrogenase is synthesized that catalyses the reduction of dinitrogen. The ammonia produced is excreted by the bacteroids, assimilated by the plant cells and transported to other parts of the plant. In pea the site of meristem formation is the inner cortical cell layer and in our growth system (Bisseling et al., 1978) the first small swellings on the main pea

root are macroscopically visible 10 days after sowing and inoculation, while nitrogen fixation activity starts 3 days later. Light microscopic studies of infected pea roots show that the small swellings already have the morphological characteristics of what defines a nodule (fig. 2A). These swellings contain an apical meristem, a zone with infected and uninfected cells (detail in fig. 2B) and infection threads, peripherically located vascular bundles and cortical cell layers surrounding the central part of the nodule. Eight days after sowing and inoculation (fig. 2B) the differentiation process in the central zone has started but the distinction between infected and uninfected cells is not yet clear. When nitrogen fixation starts (13 days) more pronounced nodules are visible on the roots, this in consequence of both an increase in cell number in the symbiotic zone and an enlargment of infected cells which are now fully packed with bacteroids (fig. 2B). After 13 days the nodules still increase in size. Between 3 and 4 weeks after inoculation the nitrogen fixation activity reaches its maximum whereupon it starts to decrease.

Our 2-D analyses of _in vitro_ translation products from RNA isolated from infected pea roots and pea root nodules have shown that there is differential expression of nodulin genes during nodule development (Govers _et al._, 1985). One nodulin, N-40' (represented by a major spot) was detectable as early as 8 days after sowing and inoculation and another, minor nodulin, N-80, was first observed in 10 day old infected tissue. Most other nodulin genes were expressed concomittantly with the leghemoglobin genes, that is from 12 days on, just prior to the commencement of nitrogen fixing activity. Also Western blot analyses of soluble nodule proteins with the nodule-specific antiserum preparation showed the successive appearance of nodule-specific proteins during nodule development (Bisseling _et al._, 1983). Six cDNA clones (5 pPsNod clones and a pPsLb clone, tab. 1) selected from a pea nodule cDNA library represent nodulin genes that are expressed in 13 day old nodules and not before that time (Govers _et al._, 1986b). Based on these observations we have classed nodulin genes


A
10d
M
B
IC
UC
IC
UC
8
10
13d
A.tum.(+pSym)
C
R 8 10 13 15d
x pENOD2
R 8 10 13 15d
x pPsLb
17d R A.t.
x pENOD2
x pPsLb

into two categories, class I nodulin genes encoding so-called early nodulins that are detectable during the formation of a nodule structure (such as N-40' and N-80) and class II nodulin genes that are not expressed until a complete nodule structure is formed, just before or after the onset of nitrogen fixation (such as the Lb and PsNod genes). In order to isolate cDNA clones from class I nodulin genes Franssen *et al*. (1986) screened a soybean cDNA library with RNA from 10 day old soybean nodules. Three class I cDNA clones were selected, one of which, pENOD2, had a high degree of sequence homology with a nodulin mRNA species in pea. Northern blot analyses showed that pENOD2 hybridized to RNA from 8 and 10 day old infected pea roots and mature pea root nodules (fig. 2C) and in a hybrid released translation experiment with pea nodule RNA, pENOD2 selected a mRNA that was translated into a protein with a molecular weight of 80,000 which might be N-80, one of the *in vitro* translation products on 2-D gels identified as an early nodulin (Govers *et al*., 1986b).

So, for the two classes of nodulin genes we now have a representative set of which the expression can easily be detected either by 2-D analyses of *in vitro* translated nodule RNA (the 8 major nodulin spots) or by Northern blot analysis (pENOD2 and pPsNod clones, table 1). Expression of these genes coincides with successive steps in the development of the symbiosis and it will be convenient if one of each group can be used as a marker for a particular stage of development.

Figure 2. Structural analyses (light microscopy) of pea root nodules and the expression of a class I and a class II nodulin gene during nodule development and in "empty" pea root nodules.
(A) Longitudinal section through an infected pea root, 10 days after sowing and inoculation with *R. leguminosarum*. M, nodule meristem; bar, 300 μm.
(B) Light micrographs showing the central part of wild type pea root nodules (8, 10 and 13 days after inoculation) and of an "empty" pea root nodule induced by the *Agrobacterium* transconjugant LBA 2712 (28 days after inoculation). IC, infected cell; UC, uninfected cell; bar, 600 μm.
(C) Autoradiographs of Northern blots containing RNA from 8 day old uninfected pea roots (R), 8 and 10 day old infected pea roots (8, 10), 13, 15 and 17 day old pea root nodules (13, 15, 17) and 28 day old "empty" pea root nodules formed by the *Agrobacterium* transconjugant LBA 2712 (A.t.). The three blots shown here were hybridized with respectively the class I cDNA clone pENOD2, the class II cDNA clone pPsLb102 and with both pENOD2 and pPsLb102.

4. FUNCTIONS OF NODULINS

In this section we will first focus on the possible involvement of nodulins in the successive steps leading to the formation of a nodule and discuss the role of two early nodulins ENOD2 and N-40'. Secondly, we will briefly describe functions of class II nodulins in the nitrogen fixation process.

4.1. Early nodulins

The first visible reaction of a leguminous plant upon infection with Rhizobium is curling of the root hairs but even before that, when rhizobia colonize the roots and attach to the root surface, the host plant exerts its influence on the symbiosis and this continues throughout the nodule development (Vincent, 1980). By analysing plant mutants in classical genetic experiments several host genes have been identified that affect the initial stages of the symbiotic interaction (Nutman, 1981; LaRue et al., 1985). In our expression studies however, pea nodulins are not observed until 8 days after sowing and inoculation (see section 3) and these observations raise the intriguing question how the plant handles the symbiotic interaction during the initial developmental stages when it synthesizes infection threads for the entry of the rhizobia and at the same time starts with the formation of meristems from which nodules will develop. Is it necessary that the plant cells extend their abilities by expressing a new set of genes, nodulin genes, or does the microsymbiont redirect functions of normal plant cells and exploit these for the establishment of the symbiosis? In fact, infection threads are composed of primary cell wall material and there is no evidence that meristems induced by Rhizobium differ from other plant meristems. So, seemingly there is no requirement for the synthesis of nodulins although it can not absolutely be excluded that nodulin genes are expressed during the first 7 days of the symbiotic interaction. Since only a relatively small number of

plant cells is involved in the initial interaction and since these plant cells can not be separated from root cells that are not affected by Rhizobium, we might have missed specific expression of genes with the methodology we use to identify nodulins and nodulin genes.

By the time that cells derived from the meristem become infected with rhizobia, we observe the first appearance of the nodulins ENOD2 and N-40' which are classified as early nodulins to distinguish them from the others that appear at least 5 days later (class II nodulins). Experimental data on ENOD2 and N-40' are the results of studies in which the expression of the corresponding genes is compared in effective and non-effective nodules or nodule-like structures. The expression pattern of the early nodulin genes during normal nodule development suggests that N-40' and ENOD2 are involved in nodule morphogenesis since their appearance coincides with the nodule formation (fig. 2B and C). However, at the same time highly branched infection threads are observed in the young nodules from which rhizobia are released into the target cells and the appearance of N-40' and ENOD2 can also be related to either the infection process or the bacterial release which is inherent to the formation of differentiating infected and uninfected cells.

The soybean nodulin cDNA clone pENOD2 crosshybridizes on Northern blots strongly with nodulin mRNA from pea, alfalfa and common vetch (Vicia sativa) showing that a homologous nodulin gene is expressed in different legumes and we assume that the function of the encoded protein is similar in these species. On soybean roots inoculated with R. fredii (USDA 257) the formation of nodule-like structures is induced but in these structures neither infection threads nor intracellular bacteria are found (Franssen et al., 1986). Also in alfalfa nodules induced by R. meliloti mutants that have lost the ability to produce exopolysaccharides (Finan et al., 1985) infection threads and cells containing bacteria are missing. In both cases ENOD2 mRNA has been detected in the nodule tissue (Franssen et al., 1986; T. Bisseling, unpublished results) while it was not found in root hairs from soybean

seedlings infected with wildtype Bradyrhizobium (Franssen et al., 1986). These observations support the hypothesis that ENOD2 has a function in nodule morphogenesis and not in the infection process while release of rhizobia into the nodule cells is not required for expression of the ENOD2 gene.

In nodules induced on pea roots by an Agrobacterium strain (LBA 2712) that harbours a R. leguminosarum sym plasmid (Hooykaas et al., 1982), infection threads are observed but intracellular bacteria are not (Govers et al., 1986a). As shown in figure 2B such nodules have the same organization as nodules induced by wildtype R. leguminosarum: a central tissue surrounded by cortical cell layers and peripherically located vascular bundles. However, in contrast to nodules induced by wildtype Rhizobium the central tissue consists of only one type of cell: relatively small non-dividing cells that do not resemble the infected nor the uninfected cells found in normal nodules (fig. 2B). In these "empty" pea root nodules only ENOD2 was detectable but not the other early nodulin N-40' nor any of the class II nodulins. The absence of N-40' in the "empty" pea root nodules, even though infection threads are present, suggests that also N-40' is not essential for the infection process. Moreover, N-40' is not required for the formation of the type of nodule structure as induced by the Agrobacterium transconjugant LBA 2712. During normal nodule development the appearance of peripherally located vascular bundles coincides more or less with the appearance of differentiating infected and uninfected cells. Since these two cell types as well as N-40' are absent in "empty" pea nodules we speculate that N-40' is in some way involved in the formation of different cell types.

Unfortunately no cDNA clone is available for N-40' and studies on the expression of this gene are confined as yet to pea root nodules. However, in Vicia sativa which is closely related to pea and belongs to the same cross-inoculation group, an early nodulin with a molecular weight of 40,000, N-40(V. sativa), has been identified among in vitro translation products on 2-D gels (M. Moerman, unpublished results).

Its isoelectric point is similar to that of N-40' in pea and it is tempting to assume that N-40' and N-40(V. sativa) are nodulins with a comparable function. The Agrobacterium transconjugant LBA 2712 which harbours a R. leguminosarum sym plasmid forms nodules on V. sativa that consist of infected and uninfected cells, in contrast to the "empty" nodules induced on pea by the same strain. In such Vicia nodules not only ENOD2 mRNA is present, as in "empty" pea nodules, but also N-40(V. sativa). So the N-40(V. sativa) gene is expressed in nodules in which bacteria are released into the nodule cells. If in V. sativa and pea the genes encoding N-40 and N-40' respectively, are regulated similar, then bacterial release might be required for expression of N-40' in pea and this supports the hypothesis that the appearance of N-40' in pea is related to the part of the differentiation process which involves the formation of the two cell types, infected and uninfected cells, found in the symbiotic zone of a root nodule.

4.2. Class II nodulins

The majority of the nodulin genes identified so far belong to class II. Their expression starts after the formation of a complete nodule structure is accomplished, so just prior to or after the commencement of nitrogen fixation. Therefore class II nodulins can not be involved in the differentiation processes leading to a nodule structure but they probably have to do with the nitrogen fixation process.

The leghemoglobins (Lbs) are not only the most abundant class II nodulins but also the most striking because of the reddish color that marks their presence and they have been the subject of many investigations (reviewed by Appleby, 1984). By their oxygen binding properties Lbs control the concentration of free oxygen in the nodule cells and provide for an efficient supply of oxygen towards the bacteroids (Appleby, 1984) and probably also towards the host part of the nodule cells (Govers et al., 1986c).

Other class II nodulins may have a role in maintenance of the nodule structure or, as Lb, support of the functioning of the bacteroid. Furthermore, they can be involved in the specific assimilation of reduced nitrogen. Nodulin-35 of soybean and nodule-specific glutamine synthetase are two such nodulins. Nodulin-35 is the subunit of a nodule-specific uricase (uricase II; Bergmann et al., 1983) which is exclusively found in the uninfected nodule cells. In soybean several enzymes involved in the ureide synthetic route are localized here (Hanks et al., 1983) and uninfected cells appear to have a particular role in the nodule physiology. A nodule-specific form of glutamine synthetase has now been identified in several plant species (Cullimore et al., 1983; Lang-Unnasch et al., 1985b; Sengupta-Gopalan and Pitas, 1986; T. Bisseling, unpublished results). This new form is probably required for assimilating the large quantities of ammonium produced by the bacteroids. By turning on expression of nodulin genes involved in the ammonium assimilation the plant has the ability to boost its assimilation capacity within the nodule cytosol several fold and it seems very likely that more class II nodulins have an enzymatic function in the assimilation pathways.

For one soybean class II nodulin it has been demonstrated that it is located in the peribacteroid membrane (pbm) but the function it serves is presently unknown (Fortin et al., 1985). Because of the numerous metabolic exchanges between plant and bacteroid cells (Dilworth and Glenn, 1984) it seems plausible that some nodulins in the pbm will be involved in transport but they might also have structural roles for allowing the rhizobia to function, in combination with this plant membrane, as a kind of intracellular organelle (Robertson et al., 1984a).

5. INDUCTION AND REGULATION OF NODULIN GENE EXPRESSION

5.1. Nodulin gene expression is only induced by Rhizobium

In order to study the mechanisms by which nodulin gene expression is induced we searched for the origin of the signals that are involved. Although the Rhizobium-legume interaction leads to a symbiotic relation it can not be excluded that infection with Rhizobium elicits a defence reaction of the plant. Some of the proteins that are identified as nodulins could be involved in this host defence mechanism implicating that phytopathogenic organisms might just as well be able to induce expression of these genes. To test this hypothesis we have analysed the gene expression in pea tumors induced by Agrobacterium tumefaciens which is closely related to Rhizobium, but no nodulins or nodulin mRNAs were detected (Bisseling et al., 1985). Once nodules are formed the physiological situation within the nodule cell is different from those in root cells. The free oxygen concentration is very low and it has been suggested that these microaerobic conditions might cause enhanced activities of enzymes involved in an anaerobic metabolism (De Vries et al., 1980; LaRue et al., 1984). In pea roots grown in a microaerobic environment the expression of at least 8 genes is specifically induced but none of these are nodulin genes (Govers et al., 1986c).

From these observations we concluded that nodulin gene expression is not related to a general host defence mechanism and only microaerobiosis is not sufficient for the induction. Apparently Rhizobium delivers *inducing signals*.

5.2. Expression of nodulin genes in non-effective nodules

In our first approach to investigate what kind of signals from Rhizobium are possibly involved in inducing or regulating nodulin genes, we analysed the nodulin gene expression in non-effective pea

root nodules formed by three mutant R. leguminosarum strains: (PRE)2(Tn5::nif D) (Schetgens et al., 1984), (1062)116(pop$^-$) (Nadler, 1981) and P8(bad$^-$) (wild isolate). In all three nodule types the cells are normally invaded by the rhizobia so the infection process and the bacterial release is not disturbed, and one might expect that expression of class I nodulin genes is not influenced. However, in nodules induced by P8(bad$^-$) the bacteria do not differentiate into the characteristic Y-shaped bacteroids, in 116(pop$^-$) nodules no functional Lb is synthesized and (PRE)2(nif$^-$) nodules lack component I of nitrogenase. These defects result in all three cases in nodules unable to fix nitrogen (nod$^+$ fix$^-$) and this might affect the expression of class II nodulin genes.

In vitro translation of RNA followed by analyses on 2-D gels showed that in the fix$^-$ nodules expression of all nodulin genes is induced so neither nitrogen fixation, heme secretion nor bacteroid development are essential for the induction (Govers et al., 1985). These observations are in agreement with the results of Fuller and Verma (1983) and Lang-Unnasch and Ausubel (1985) obtained by studying non-effective soybean and alfalfa nodules, respectively. However, the relative intensity of some of the major pea nodulin spots was markedly reduced in the 2-D pattern from non-effective nodules in comparison to wild-type nodules indicating that in fix$^-$ nodules less of these nodulin RNAs is available for translation. Using the cDNA clones that represent the two classes of nodulin genes we could determine the RNA levels of class I and class II nodulins in non-effective nodules. From our results it appeared that the concentration of ENOD2 RNA was the same in fix$^-$ and wild-type nodules but the levels of the RNA transcribed from class II PsNod and Lb genes were decreased in the fix$^-$ nodules (Govers et al., 1986b). PsNod6 and PsNod14 RNA were hardly detectable while all fix$^-$ nodules contained still reasonable amounts of RNA from the other 3 PsNod genes, PsNod10, 11 and 13, and the Lb genes (representative examples of each group are shown in fig. 3). Furthermore, the concentration of all class II nodulin RNAs was

consistently found to be higher in (PRE)2 nodules than in nodules induced by P8(bad$^-$) and 116(pop$^-$) (fig. 3). These data indicate that apart from the induction of nodulin genes, Rhizobium also regulates, directly or indirectly, the accumulation level of the nodulin mRNA. The Rhizobium genes involved in this regulation are probably the ones that contribute to the bacteroid development and to the nitrogen fixing capacity of the Rhizobium strains. Since different RNA levels are found in the three types of non-effective nodules and for different nodulin genes (fig. 3) we suggest that several regulatory mechanisms must exist at the transcriptional level.

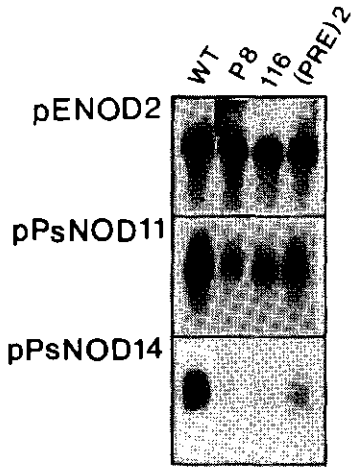


Figure 3. Expression of class I and class II nodulin genes in effective and non-effective pea root nodules.
Autoradiographs of Northern blots containing RNA from wild-type pea root nodules (WT) and non-effective nodules induced by fix$^-$ Rhizobium strains (P8(bad$^-$), (1062)116(pop$^-$) and (PRE)2(Tn5::nifD)). The blots were hybridized with nick-translated DNA from a class I cDNA clone pENOD2 and two class II cDNA clones, pPsNod11 and pPsNod14. The latter two represent two groups within the class II nodulin genes based on the level of expression in non-effective nodules.

Comparison in non-effective pea root nodules of the Lb mRNA levels with the Lb protein concentrations by Northern and Western blot analyses respectively, showed that the final concentration of the gene product is also regulated at the posttranscriptional level (Govers et al., 1985). Recently Jensen et al. (1986) reported that the expression in yeast of a chimaeric gene consisting of the 5' flanking region of a soybean Lb gene and the coding sequence of the neomycin phosphotransferase gene appears to be regulated by heme at a posttranscriptional level. So far there is however no evidence that a similar heme-specific regulatory mechanism functions in the natural situation, the

soybean nodule. Our results obtained with the non-effective pea nodules show that, if such a heme-specific regulation exists in nodules, then this surely is not the only mode of regulation because also in nodules induced by nif⁻ and bad⁻ strains posttranscriptional regulation of Lb synthesis is observed.

5.3. Rhizobium genes involved in the induction of nodulin gene expression

Since the symbiotic genes mutated in the three Rhizobium strains described above are not involved in the induction of nodulin gene expression, other Rhizobium genes have to function as producers of inducing agents. In search for the genes involved, nodulin gene expression in nodules induced by engineered Rhizobium and Agrobacterium strains was analysed.

In fast growing Rhizobium species genes essential for nitrogen fixation and nodulation are organized in clusters which are located on a large plasmid, the so-called sym plasmid (Rolfe and Shine, 1984). Rhizobia cured of the sym plasmid are unable to induce root nodule formation but after introduction of only a fragment of the symbiotic plasmid, namely one that contains the nodulation gene cluster (the nod region), the recipient Rhizobium regains the ability to form nodules (Downie et al., 1983; Hirsch et al., 1984; Schofield et al., 1984). These nodules are non-effective because nif and fix gene clusters are absent. The nod region consists of 4 "common" nod genes (nod A, B, C and D) and several "hsn" genes, host specificity genes (nod E, F, G, H, I, J) (Kondorosie et al., 1985; Downie et al., 1985b; Rolfe et al., 1985). The "common" nod genes are functionally interchangeable between R. meliloti, R. leguminosarum and R. trifolii and their DNA sequences are highly conserved. The expression of nod A, B and C as well as the expression of some of the "hsn" genes is induced by flavones excreted by the plant (Peters et al., 1986; Redmond et al., 1986) and this induction is dependent on the regulatory nod D gene, which is

expressed constitutively. Mutations in the "common" nod genes have shown that nod A, B, C and D are absolutely essential for nodulation while Tn5 insertions in other R. leguminosarum nod genes caused only delayed nodulation. However, reintroduction into cured rhizobia of a fragment containing only nod A, B, C and D does not result in a nodulating strain (Downie et al., 1985a) indicating that besides the "common" nod genes the other nod genes have a crucial role in root nodule formation as well.

By using two overlapping cosmid clones pIJ1089 and pIJ1085, Downie et al. (1983) showed that a 10 kb fragment containing a part of the nod region of the R. leguminosarum sym plasmid pRL1JI, in a R. phaseoli or R. leguminosarum chromosomal background is sufficient for root nodule formation on pea and we showed (Govers et al., 1986a) that in the pea nodules induced by R. phaseoli/ pIJ1089 or R. phaseoli/pIJ1085 all nodulin genes are expressed. Hence, it was concluded that besides 10 kb of the nod region nif, fix and also other putative genes encoded by the sym plasmid are not involved in the induction of nodulin genes. The nod region might thus be involved in the induction although the results do not exclude a role of the Rhizobium chromosome. An Agrobacterium strain harbouring a complete R. leguminosarum sym plasmid (LBA 2712) (Hooykaas et al., 1982) induces nodules on pea in which the exclusive expression of the early nodulin gene ENOD2 is demonstrated (see section 4.1., fig. 2C). Since in pea tumors formed by Agrobacterium tumefaciens the ENOD2 gene is not expressed it seems unlikely that the Agrobacterium genome itself carries genes involved in the induction of the ENOD2 gene. Therefore the sym plasmid in LBA 2712 must be responsible for the induction and from this sym plasmid only the 10 kb nod-region is essential. The Rhizobium genes involved in inducing ENOD2 expression are thus the nod genes located on the 10 kb fragment.

The other early nodulin N-40' is not expressed in the "empty" nodules on pea, so one might argue that the nod region is not sufficient for the induction of this nodulin gene. However, a similar early nodu-

lin gene N-40(V. sativa) is expressed in nodules formed on V. sativa by the same Agrobacterium transconjugant (see section 4.1.) It is not found in tumors on V. sativa, so the expression of N-40(V. sativa) is also regulated by the nod genes. With the plausible, but unproven assumption that N-40(V. sativa) and N-40' are similar, we speculate that expression of the N-40' gene in pea is regulated by the nod genes as well, but bacterial release is required for expression. The striking differences between the V. sativa and pea nodules formed by the Agrobacterium transconjugant LBA 2712 concerning their morphology (nodules with infected cells versus "empty" nodules) and expression of the early nodulin genes, N-40(V. sativa) versus N-40', shows that in pea the nodule development is blocked at an earlier stage than in Vicia sativa. This can be the consequence of either the reaction of the host plant or the genetic potential of the nodulating strain. Vicia sativa might allow the bacteria to enter the cells while pea defends itself by raising a barrier before the phytopathogenic agrobacteria can reach the cytoplasm. Alternatively, LBA 2712 might lack the genes involved in the release from the infection threads or expression of such genes is suppressed, while activation of these genes is not required for the bacterial release in Vicia nodules. This implies that it may concern either host specificity genes located on the Rhizobium chromosome which are thus different from the nod hsn genes, or sym plasmid genes regulated by the chromosome in a host specific way.

Induction of expression of the class II nodulin genes is observed neither in "empty pea" root nodules nor in Vicia nodules induced by the Agrobacterium transconjugant LBA 2712. Apparently bacterial release is not the only requirement for induction of class II genes because in Vicia, nodule cells are infected and still no expression is observed. If the expression fails because the genetic information of the nodulating strain is not sufficient, then Rhizobium genes located on the chromosome or on non-symbiotic plasmids are involved in the induction of class II nodulin genes.

6. CONCLUDING REMARKS

Complex interactions between a procaryote, Rhizobium, and an eucaryote, the host plant, accomplish the formation of root nodules (a simplified model is shown in fig. 4). The crucial importance of molecular signalling in the interaction is obvious from the induction of bacterial nod genes by plant root exudate substances (Peters et al., 1986; Redmond et al., 1986). The nod gene products are in turn required for infection as well as for induction of mitotic activity in the cortex of the roots, two independent processes which occur within 24 hours after inoculation with Rhizobium and which have to coincide for successful nodulation (Dudley et al., 1986; Bauer et al., 1985).

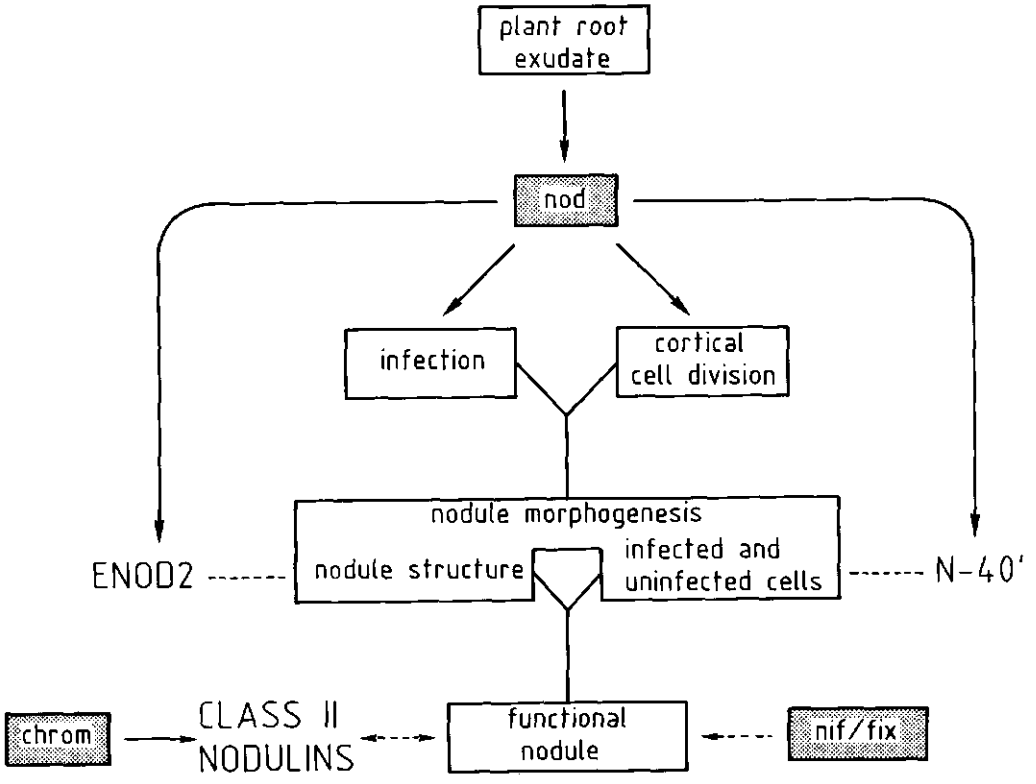


Figure 4. A schematic representation of the relation of nodulins (capitals) with successive and parallel steps in the nodule development (boxes), and the involvement of Rhizobium genes (shaded boxes).

As discussed in the foregoing sections, the nod genes are also involved in expressing two early nodulin genes, ENOD2 and N-40'. Expression of these genes in pea is first detected when differentiation into a nodule structure is observed and this event occurs at least 6 days after the first cortical cell divisions, indicating that nod genes influence several subsequent steps in the early stages of the developmental program. The mechanism of nod gene action is still an open question. Do nod gene products produce substances which influence directly gene expression in the plant? Or do they cause an initial recognition event on which the host plant responses by transduction of that signal into a secondary message in the plant? And then, are the reactions of the plant specific for the nodule development or are the effects achieved be changing the concentrations of certain regular plant enzymes or hormones? We searched for expression of nodulin genes in infected root segments during the first cortical cell divisions, but without success. Even if certain nodulin genes are expressed in the initial stages, techniques more sensitive than in vitro translation of RNA or Northern blot analysis are probably required for detecting the nodulins or the corresponding RNAs. Because only a few host cells are involved, the problem must be addressed at the cellular level by in situ hybridization with RNA or by immunocytology.

In our expression studies the first specific reaction of the plant is the appearance of ENOD2 and N-40'. The data on the two early nodulin genes favour a role for these nodulins in nodule morphogenesis. N-40' is only detectable in nodules containing differentiating infected and uninfected cells whereas ENOD2 is also found in nodules that have no intracellular bacteria. We suggest that ENOD2 and N-40' are related to different steps in the developmental program: formation of a nodule structure and formation of the two cell types, respectively. In some nodule types induced by engineered Rhizobium or Agrobacterium strains peripherically located vascular bundles, which characterize a nodule structure, are formed without invasion of nodule cells by rhizobia. The reverse, the appearance of infected cells

without the formation of a nodule structure, has to our knowledge not been observed so far. The two steps are either independent processes which have to coincide to achieve the functional nodule stage or rhizobia are not released into the nodule cells until vascular connections to the roots are formed, which may be required for the supply of nutritional substances to the rhizobia. We can, however, not discriminate between these two possibilities.

When the formation is completed the nodule becomes a nitrogen fixing organ by switching on class II nodulin genes in the host and nif and fix genes in the bacteroids. Class II nodulins function in the nitrogen fixation process by assimilating ammonia, supporting the bacteroids or maintaining the nodule structure. The nature of the Rhizobium genes responsible for induction of class II nodulin genes is still unclear. So far however, we have only detected expression of class II nodulin genes in nodules induced by strains having at least a Rhizobium chromosome and the nod region, indicating that chromosomal genes are involved, but the nod region can have a role in this part of the process as well. Expression of nodulin genes does not necessarily lead to synthesis of functional nodulins. For class II nodulins several regulatory mechanisms are operative, both at a transcriptional and post-transcriptional level and signals from Rhizobium appear to be involved in this regulation, either directly or indirectly via the products of the nitrogen fixation process.

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summary

Infection of leguminous plants with bacteria of the genus Rhizobium results in a symbiotic interaction which brings about the development of an entirely new organ on the plant, the root nodule. Within this organ about half of the plant cells are inhabited by bacteroids, the endosymbiotic form of the bacterial partner. The bacteroids reduce atmospheric nitrogen (N_2) to ammonia (NH_3) which the plant uses as a nutrient. The host plant, on the other hand, provides the rhizobia with carbon compounds as an energy source for the nitrogen fixation process. Development of a nitrogen fixing root nodule is a true co-operation between two genomes. In chapter II the plant and bacterial genes involved in this symbiotic interaction are reviewed.

The research reported in this thesis deals with the identification of plant genes which are specifically expressed during the development of the symbiosis, the so-called nodulin genes and, in addition, bears upon the question which bacterial genes are involved in regulating the expression of nodulin genes.

By comparing the RNA content of pea root nodules induced by Rhizobium leguminosarum and uninfected pea roots by means of in vitro translation, we have been able to demonstrate the expression of twenty one nodulin genes (chapter III). Out of a nodule cDNA library seven pea nodulin cDNA clones were selected (chapter IV). The appearance of nodulin mRNAs during pea root nodule development, studied by analyses

of *in vitro* translation products and Northern blots, showed that nodulin genes are differentially expressed. Thus, nodulins could be classified into two groups: class I or early nodulins and class II nodulins which are expressed later in nodule development (chapter III and IV).

To determine the origin and the nature of signals responsible for the induction of nodulin genes, we mimicked the microaerobic conditions supposed to exist in root nodules and showed that in pea roots microaerobiosis by itself is not sufficient to trigger the induction of nodulin gene expression (chapter V). Also, in pea tumors formed by *Agrobacterium tumefaciens* no expression of nodulin genes was detected (chapter VI). Therefore, it seems likely that nodulin gene expression is induced by specific signals from *Rhizobium* and we have searched for the *Rhizobium* genes responsible for such signals.

Analysis of nodulin gene expression in non-effective nodules, formed by *Rhizobium* mutants and engineered *Rhizobium* and *Agrobacterium* strains, showed that *Rhizobium* genes required for nodulation (*nod* genes) are involved in the induction of at least one early nodulin gene (chapter VI), whereas *Rhizobium* genes essential for the nitrogen fixation process (*nif* and *fix* genes) are not required for the induction of nodulin gene expression (chapter III, IV and VI). In non-effective nodules the final accumulation level of class II nodulin mRNAs is reduced, indicating that the gene products of the *Rhizobium nif* and *fix* genes, directly or indirectly via the products of the nitrogen fixation process, regulate the level of nodulin gene expression.

Finally, in chapter VII the implications of the reported results are discussed in the perspective of the possible functions of nodulins in the developing root nodule and the way nodulin gene expression may be induced and regulated.

samenvatting

Vlinderbloemige planten en Rhizobium bacteriën kunnen samen wortelknollen vormen waarin de bacteriën in staat zijn stikstof uit de lucht om te zetten in ammonia. De ammonia wordt uitgescheiden door de bacteriën en is dan beschikbaar voor de plant, die op deze manier voor een belangrijk deel in zijn stikstofbehoefte kan voorzien. De bacteriën krijgen, op hun beurt, voedingsstoffen van de plant die ze gebruiken als energiebron om ondermeer de stikstofbindingsreactie te laten verlopen.

Stikstofbindende wortelknollen zijn zeer gespecialiseerde planteorganen. Bij de vorming van deze organen zijn een aantal stappen te onderscheiden. De eerste stap bestaat uit aanhechting van de Rhizobium bacteriën aan de wortels van de plant. De wortelharen gaan krullen en de bacteriën dringen de plant binnen via infectiedraden die in de gekrulde wortelharen gevormd worden. Tegelijkertijd beginnen in de wortelschors cellen te delen. De infectiedraden gevuld met bacteriën groeien door de wortelschors tot aan de delende cellen, en de bacteriën komen vrij uit de infectiedraad om vervolgens de delende plantecellen te infecteren. In de geïnfecteerde cellen veranderen de bacteriën van vorm en beginnen de enzymen te produceren die nodig zijn voor het omzetten van stikstof in ammonia. De stikstofbinding kan nu beginnen. De plantecellen verwerken de door de bacteriën geproduceerde ammonia en transporteren deze in de vorm van aminozuren naar andere delen van de plant. Op de erwt (Pisum sativum), de plantesoort die gebruikt werd voor de experimenten beschreven in

dit proefschrift, verschijnen tien dagen na infectie met Rhizobium leguminosarum de eerste zichtbare uitstulpingen op de wortels. De wortelknolvorming is dan al in volle gang en er zijn reeds plantecellen geinfecteerd met bacteriën. Twaalf dagen na infectie is voor het eerst stikstofbinding meetbaar.

Plant en bacterie zijn dus twee samenwerkende organismen die ieder hun eigen functies hebben zowel bij de vorming van de stikstofbindende organen als tijdens het binden van stikstof. Echter, de ene partner kan die functies alleen uitvoeren als de andere partner ook zijn functies uitvoert en waarschijnlijk worden voortdurend signalen over en weer gestuurd om een goed verloop van het proces te bewerkstelligen. De experimenten beschreven in dit proefschrift zijn enerzijds gericht op het bestuderen van plantegenen die specifiek tot expressie komen in wortelknollen, de zogenaamde noduline genen, en anderzijds op de rol van Rhizobium genen in het aanschakelen van noduline genen.

Het doel van het onderzoek, alsmede de samenhang tussen de verschillende hoofdstukken staat beschreven in hoofdstuk I. Hoofdstuk II beschrijft de interactie tussen planten en stikstofbindende microorganismen in het algemeen en in het bijzonder de plantegenen en bacteriële genen die een rol spelen bij de wortelknolvorming en de stikstofbinding. In hoofdstuk III tot en met VI staan de experimentele resultaten van het onderzoek weergegeven.

In hoofdstuk III wordt beschreven hoeveel plantegenen er specifiek tot expressie komen in wortelknollen van de erwt en op welke dag na infectie met Rhizobium deze genen aangeschakeld worden. Boodschapper RNA (mRNA) geïsoleerd uit wortelknollen van verschillende leeftijden werd in vitro vertaald in eiwitten. Vervolgens werden deze eiwitten elektroforetisch gescheiden op gels. Uit analyse van de eiwitpatronen bleek dat wortelknollen tenminste 21 nieuwe mRNAs bevatten. Hieruit werd geconcludeerd dat er in de erwt tenminste 21 noduline genen tot expressie komen. Deze genen zijn onderverdeeld in twee klassen: klasse I of vroege noduline genen waarvan het mRNA reeds tijdens de wortelknolvorming, vanaf 8 dagen na infectie, te detecteren is en klasse II

noduline genen die tot expressie komen als de stikstofbinding op gang komt, dus vanaf 12 dagen na infectie. De meerderheid van de noduline genen valt onder klasse II.

Om hulpmiddelen in handen te krijgen voor een nadere bestudering en karakterisering van individuele noduline genen, zijn uit een copie DNA (cDNA) bank, gemaakt van wortelknol RNA, een zevental cDNA klonen geselecteerd (hoofdstuk IV). Deze klonen bevatten cDNA van mRNA dat alleen in wortelknollen te detecteren was. Tussen de geselecteerde noduline cDNA klonen bevond zich één vertegenwoordiger van een klasse I noduline gen, terwijl de rest van de klonen afkomstig bleek te zijn van klasse II noduline genen.

Vervolgens werd bestudeerd welke signalen nodig zijn voor het aanschakelen van noduline genen en of deze signalen noodzakelijkerwijs afkomstig zijn van Rhizobium. Daarom werd onderzocht of interactie van erwteplanten met andere bacteriën ook leidt tot het activeren van een of meerdere noduline genen. In tumoren op de erwt die ontstaan na infectie met Agrobacterium tumefaciens bleek dit niet het geval te zijn (hoofdstuk VI). Daarnaast is de genexpressie bekeken in erwtewortels die blootgesteld werden aan een lage zuurstofspanning. In geïnfecteerde wortelknolcellen is ten gevolge van de aanwezigheid van Rhizobium de vrije zuurstof concentratie veel lager dan in andere plantecellen en dit zou kunnen leiden tot het aanschakelen van specifieke genen. Daarom hebben we de lage zuurstof condities nagebootst zonder de aanwezigheid van Rhizobium, maar ook dan bleken geen noduline genen tot expressie te komen (hoofdstuk V). Deze resultaten maken het aannemelijk dat alleen Rhizobium specifieke signalen produceert waardoor noduline genen aangeschakeld kunnen worden.

Om te weten te komen welke genen van Rhizobium betrokken zijn bij het aanschakelen van noduline genen, werd gebruik gemaakt van Rhizobium stammen die gemuteerd zijn in bepaalde genen of van stammen waarin een groot stuk genetische informatie verdwenen is. Rhizobium bacteriën hebben naast het chromosoom een zogenaamd symbiontisch (sym) plasmide. Op dit plasmide liggen een groot aantal genen die essentieel zijn voor

de symbiose met vlinderbloemige planten en van een deel van deze genen is de functie bekend. Zo zijn er een aantal sym genen betrokken bij de wortelknolvorming (nod genen), terwijl andere een rol spelen bij de stikstofbinding (nif en fix genen). Infectie van planten met Rhizobium stammen die mutaties hebben in nif of fix genen, of met stammen die van het sym plasmide alleen de nod genen bevatten, leidt tot de vorming van wortelknollen, waarin geen stikstofbinding plaatsvindt. Uit eiwitpatronen van translatie produkten van mRNA uit deze niet-effectieve wortelknollen (hoofdstukken III en VI) én uit hybridisatie proeven van hetzelfde RNA met noduline cDNA klonen (hoofdstukken IV en VI) bleek dat in deze wortelknollen wel alle noduline genen tot expressie komen. Hieruit werd geconcludeerd dat Rhizobium sym genen die essentieel zijn voor stikstofbinding, niet verantwoordelijk kunnen zijn voor het aanschakelen van noduline genen en dat van het sym plasmide alleen de nod genen hierin een functie kunnen hebben. De rol van chromosomale Rhizobium genen werd onderzocht door het Rhizobium chromosoom te vervangen door een Agrobacterium chromosoom. Als erwteplanten geïnfecteerd worden met een Agrobacterium stam die een sym plasmide van Rhizobium bevat, worden door de aanwezigheid van nod genen wortelknollen gevormd. Echter, de wortelknolcellen zijn niet gevuld met bacteriën zoals dat normaal het geval is. Hieruit blijkt dat het Rhizobium chromosoom in ieder geval noodzakelijk is voor een normale wortelknolvorming met geïnfecteerde cellen. In de lege wortelknollen kon de expressie van één vroeg noduline gen aangetoond worden terwijl géén expressie van een ander vroeg noduline gen noch van klasse II noduline genen gevonden werd (hoofdstuk VI). Hieruit werd geconcludeerd dat Rhizobium nod genen in ieder geval betrokken zijn bij de expressie van een vroeg noduline gen terwijl voor het aanschakelen van de andere noduline genen andere of meer signalen noodzakelijk zijn.

In hoofdstuk VII, tenslotte, worden de resultaten die beschreven zijn in dit proefschrift bediscussieerd binnen een algemene beschouwing over nodulines in de zich ontwikkelende wortelknol.

curriculum vitae

Francine Govers werd op 19 april 1955 in Reek geboren. In 1972 behaalde zij het HBS-B diploma aan het Titus Brandsma Lyceum te Oss. In augustus 1972 werd gestart met de opleiding tot radiologisch laborante. Zij was gedurende deze opleiding en na het behalen van het diploma werkzaam op de afdeling Radiodiagnostiek van het Radboud-ziekenhuis te Nijmegen. In 1976 ging zij aan de Landbouwhogeschool in Wageningen studeren en behaalde daar in januari 1980 het kandidaats-examen planteziektenkunde. De ingenieursstudie, die cum laude werd afgesloten in september 1982, omvatte de hoofdvakken virologie en moleculaire biologie en het bijvak erfelijkheidsleer. In oktober 1982 begon zij op de vakgroep Moleculaire Biologie van de Landbouwhogeschool met het onderzoek waarvan de resultaten in dit proefschrift staan beschreven. Het onderzoek werd uitgevoerd onder leiding van Prof.Dr. A. van Kammen en Dr. T. Bisseling en met financiële steun van de Stichting voor Biologisch Onderzoek Nederland van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek. Sinds november 1986 participeert zij weer in het onderzoek naar de vorming van stikstofbindende wortelknollen dat uitgevoerd wordt op de vakgroep Moleculaire Biologie van de Landbouwuniversiteit.

nawoord

Het titelblad van dit proefschrift suggereert, volkomen ten onrechte, dat de inhoud het werk van mij alleen is geweest. Als ik terugkijk hoe het geheel tot stand gekomen is, zou ik dat willen vergelijken met de vorming van een stikstofbindende wortelknol. Mijzelf beschouw ik dan als één van de noduline genen en, zoals bij de wortelknolvorming, is expressie van meerdere noduline genen noodzakelijk om het beoogde eindresultaat te bereiken.

Wortelknollen zijn planteorganen en zonder een plant zullen nooit wortelknollen gevormd worden. Zo is er zonder een promotor geen proefschrift. Ab, jij bent in deze vergelijking de plant en jij bent dan ook essentieel geweest voor het tot stand komen van dit proefschrift. Je hebt mij de kans gegeven om tot expressie te komen en je hebt dat op een goede en prettige manier en met veel enthousiasme gereguleerd. Daarvoor ben ik je zeer erkentelijk.

Ton, jij bent in deze parabel ongetwijfeld een vroeg noduline gen: een voorloper, steeds eerder dan de anderen. Altijd kom jij tot expressie. Ook in omstandigheden waarin andere noduline genen het niet meer zien zitten, ben jij nog vol enthousiasme en je probeert dat ook uit te dragen. Jij weet maar al te goed welke essentiële functies wij toekennen aan vroege nodulines. Op een vergelijkbare wijze ben jij heel belangrijk geweest voor mijn vorming. Ik heb veel van je geleerd en heel prettig met je samengewerkt.

Jan-Peter, ik moet jou een rol toebedelen waarmee je eigenlijk liever niet geassocieerd wordt. Jij was namenlijk "abundant" aanwezig tijdens het tot stand komen van dit proefschrift. Bij jou kon ik steeds naar zuurstof happen als ik weer iets op mijn hart had. Vooral ook in de beginperiode van mijn promotieonderzoek, toen jij als student de eerste stappen zette in de moleculaire biologie, heb ik veel steun aan je gehad.

Marja, jou beschouw ik als een noduline gen dat een functie heeft bij de stabiliteit en het onderhoud van de wortelknol. Daar heb jij heel goed voor gezorgd. Je hebt oneindig veel 2-D gels gerund die mede bepalend zijn geweest voor de inhoud van dit proefschrift. Dankzij jouw inzet is deze wortelknol nu goed te plukken, ondanks het feit dat tijdens de ontwikkeling de stabiliteit soms wankelde door overexpressie van ons beide.

Ton (G.), jouw expressie was al vrij vroeg tijdens de ontwikkeling van dit proefschrift merkbaar. Als doctoraalstudent heb jij een belangrijke bijdrage geleverd aan het erwteonderzoek. Gelukkig bleef je ook daarna deel uit maken van onze club zodat je deskundigheid niet verloren ging.

Henk, jouw verschijning heeft iets van een laat noduline gen. We konden eerst de expressie van dit gen niet detecteren in ineffectieve knollen. Nu weten we dat het wel tot expressie komt. Het is ook ondenkbaar dat jouw inzet en enthousiasme niet merkbaar zouden zijn. Zelfs met rondvliegende mediumflessen ben jij niet uit te schakelen. Naast jou aan de labtafel was het goed vertoeven.

Sommige noduline genen komen ook "transient" tot expressie. Willem, ik heb van jou veel geleerd wat betreft "echte" moleculair-biologische technieken. Het was erg plezierig om met je samen te werken. Sacco, jouw inbreng in het onderzoek is veelvuldig in dit proefschrift terug te vinden. Het opzetten van een efficiënte methode om 2-D gels te runnen heeft zijn nut goed bewezen. Jeanine, ook jij hebt duidelijke sporen achtergelaten. De cytologische opnames geven een bijzonder cachet aan dit proefschrift. Rita, jouw expressie was kort maar hevig.

Bedankt voor je bijdrage aan het onderzoek en vooral voor de "plezante" samenwerking. Studenten zijn gedoemd een "transient" expressiepatroon te hebben. Tijdens mijn promotieonderzoek hebben veel studenten een plaatsje gehad op lab 1 en ieder beïnvloedde op zijn of haar eigen manier de sfeer. Slechts enkelen wil ik noemen. Ingrid, Marcel, Jaap en Erik, bedankt voor jullie inbreng en ondersteuning.

Wortelknollen hebben naast geïnfecteerde cellen ook niet-geïnfecteerde cellen die noodzakelijk zijn om de gebonden stikstof te verwerken. De experimentele resultaten verkregen aan de labtafel, moesten op het secretariaat, aan de tekentafel en in de doka verwerkt worden tot leesbare manuscripten met mooie figuren. Gré, Marie-José, Angélique, Piet en Rijndert, hartelijk bedankt voor jullie ondersteuning bij het tot stand komen van dit proefschrift.

Om een wortelknol ammonia te laten produceren is energie nodig in de vorm van koolstofbronnen. Ook moet de atmosfeer stikstof bevatten die gebonden kan worden. De koolstofbronnen voor mijn promotieonderzoek waren de financiële middelen die verstrekt werden door de Stichting voor Biologisch Onderzoek Nederland. Bovendien werd ik met subsidie van het Fonds Doctor Catharina van Tussenbroek in staat gesteld een studiereis te maken naar de Verenigde Staten. De atmosferische stikstof werd geleverd in de vorm van plantemateriaal en bacteriestammen. Ik wil Anton Houwers en Piet de Kam bedanken voor het kweken, verzorgen en inoculeren van de vele erwteplanten, Jeanne Hooymans voor het kweken van wortels onder lage zuurstofspanning en Resie Schetgens en Paul Hooykaas voor het beschikbaar stellen van Rhizobium en Agrobacterium stammen. Also Allan Downie and Ken Nadler are acknowledged for providing Rhizobium strains.

Naast noduline genen komen in een wortelknol vele andere genen tot expressie die een rol spelen bij het normaal functioneren van plantecellen. Collega's van de vakgroep Moleculaire Biologie, symbolisch gezien zijn jullie allemaal zo'n dikke spot op een 2-D gel. Bedankt voor het creëren van de juiste sfeer op het lab en de gezellige praatjes tijdens de koffiepauzes.

De grote vraag die in dit proefschrift steeds naar voren komt is: hoe wordt de expressie van noduline genen gereguleerd en door welke signalen worden ze aangeschakeld. Ik, als noduline gen, heb voor mijn regulatie wel een tipje van de sluier opgelicht. Het eerste signaal dat mij ertoe bracht om te gaan studeren in een biologisch getinte richting kwam ongetwijfeld van jou, Ad. Jouw enthousiasme voor het wetenschappelijk onderzoek werkte aanstekelijk en jij hebt mij ook steeds gestimuleerd om verder te gaan. Door jouw ondersteuning heb ik dit punt kunnen bereiken. Bedankt,

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