NODULINS
IN
ROOT NODULE
DEVELOPMENT

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NODULINS IN ROOT NODULE DEVELOPMENT

proefschrift

ter verkrijging van de graad van

doctor in de landbouwwetenschappen,

op gezag van de rector magnificus,

dr. C.C. Oosterlee,

in het openbaar te verdedigen

op vrijdag 27 mei 1988

des namiddags te vier uur in de aula

van de landbouwuniversiteit te Wageningen

ISN= 268163

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

The cover shows a scanning electron micrograph of a nodule section (courtesy C. van de Wiel, and C.J. Keijzer, Department of Plant Cytology and Morphology, Wageningen).

Stellingen

1. De *nod* genen van *Rhizobium* zijn betrokken bij de inductie van de expressie van late noduline genen.

Dit proefschrift.

2. *Agrobacterium* transconjuganten die een deel van het *Rhizobium* sym plasmide bevatten, zijn slechts beperkt bruikbaar in onderzoek naar de regulatie van noduline gen expressie onder invloed van bacteriële signalen.

Dit proefschrift.

3. Leigh *et al.* gaan er in de discussie over de rol van het *exoH* gen product ten onrechte vanuit dat exopolysacchariden actief bij nodulatie betrokken zijn.

Leigh *et al.*, Cell, 51, 579–587, 1987.
Dit proefschrift.

4. De conclusie van Jørgensen *et al.* dat de promoters van de noduline genen Ngm–23 en leg–hemoglobine ongeveer even sterk zijn, valt niet af te leiden uit de door hen gepresenteerde resultaten.

Jørgensen *et al.*, Nucl. Acids Res., 16, 39–50, 1988.

5. De suggestie van Tingey *et al.* dat de aanwezigheid van een wortelknolspecifieke subeenheid van glutamine synthetase samenhangt met de morfologie van de wortelknol is ongegrond.

Tingey *et al.*, EMBO J., 6, 1–9, 1987.

6. Het gegeven dat het proto–oncogen c–*jun* codeert voor de transcriptiefactor AP1 legt een direct verband tussen oncogenese en een verstoring van de regulatie van transcriptie.

Bos *et al.*, Cell, 52, 705–712, 1988.
Bohmann *et al.*, Science, 238, 1386–1392, 1987.

7. Uit de experimentele gegevens van Spicer *et al.* is niet duidelijk dat het door hen gekloneerde cDNA codeert voor een humane weefselfactor.

Spicer *et al.*, Proc. Natl. Acad. Sci., 84, 5148–5152, 1987.
Guha *et al.*, Proc. Natl. Acad. Sci., 83, 299–302, 1986.

8. De bevinding van Odell *et al.*, dat in de CaMV 35S promoter een sequentie aanwezig is, die de transcriptie stimuleert afhankelijk van zijn positie ten opzichte van de start van die transcriptie, zal een belangrijke toepassing vinden in de constructie van promotercassettes die gekloneerde genen op het gewenste niveau tot expressie brengen in transgene planten.

Odell *et al.*, Plant Mol. Biol., 10, 263–272, 1988.

9. De resultaten van Mignery *et al.* met betrekking tot de verhouding waarin twee klassen van patatine genen in de aardappelknol tot expressie komen, zijn zodanig in strijd met de nog geen half jaar eerder gepubliceerde gegevens van diezelfde onderzoeksgroep, dat zowel de integriteit van deze onderzoeksgroep, als de doelmatigheid van het referentensysteem van het tijdschrift Gene, in het geding zijn.

Mignery *et al.*, Gene, 62, 27–44, 1988.
Pikaard *et al.*, Nucl. Acids Res., 15, 1979–1994, 1987.

10. Het model waarin dilauroyl phosphatidylcholine functioneert als zeep in de stimulering van een gereconstitueerd cytochroom P450 systeem, berust op een onjuiste bepaling van de kritische micel concentratie van dit fosfolipide.

Taniguchi *et al.*, Arch. Biochem. Biophys., 232, 585–596, 1984.
Coon, Meth. Enz., 52, 200–206, 1980.

11. De restricties gesteld in Nederland aan het mogen introduceren van genetisch gemanipuleerde planten in het milieu belemmeren het gebruik van dit soort planten zodanig, dat het schrijven van een methodenboek over genetische manipulatie vooralsnog een meer rendabele toepassing van recombinant DNA onderzoek is, dan het maken van een transgene plant.

12. In de recente plannen om het gemengd dubbel uit de Nederlandse tenniscompetitie te schrappen, wordt de sociale betekenis van dat gemengd dubbel ernstig onderschat.

13. De eisen gesteld aan de uitvoering van een proefschrift dienen te worden aangepast aan de salariëring van een promovendus.

14. Dat de wetenschapsbijlage van zowel de NRC als de Volkskrant in de zomermaanden tot één derde wordt gereduceerd, suggereert ten onrechte dat in de zomer nauwelijks onderzoek wordt verricht.

15. Het obligate applaus na een wetenschappelijke voordracht maakt van de spreker een acteur, waarvan het in het merendeel van de gevallen te hopen is dat hij of zij niet tot een toegift besluit.

Stellingen behorend bij het proefschrift **"Nodulins in root nodule development"**

Wageningen, 27 mei 1988 Jan-Peter Nap

aan mijn grootvader

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OUTLINE

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Chapter 1

Outline

Well before the Christian era, leguminous crops have been cultured and appreciated for food. Already in ancient Rome, it was known that leguminous species could be used for soil enrichment by green manuring. As recorded in Roman agricultural writings, *e.g.* Columella's *De Re Rustica* (362), orderly systems of crop rotations based on legumes were developed at that time. Crop rotations with legumes were responsible for a 40 – 50% increase in food production in Europe during the 18th century (13). Although one of the earliest botanical descriptions of structures on the roots of legumes was published in 1587 (70), it was not until a century ago that these structures, so-called root nodules, were identified as nitrogen fixing organs (144). Shortly thereafter, the bacteria responsible for the nitrogen fixation were isolated in pure culture (18). Since then, it has become evident that by far the most significant amount of fixed nitrogen on earth comes from leguminous root nodules. Moreover, the nitrogen fixed by the bacteria is directly available to the legume, which allows the plant to grow without nitrogen fertilizer.

A lot of research has been and still is devoted to the understanding of the symbiosis between *Rhizobium* bacteria and leguminous plants. The successive steps of the formation of a nitrogen fixing root nodule has been well documented microscopically. Four major stages in nodule development can be recognized (357): stage I 'preinfection', stage II 'infection and nodule formation', stage III 'nodule function and maintenance', and stage IV 'nodule senescence' respectively (fig. 1.1.). In stage I, the *Rhizobium* bacteria attach to their particular host and cause characteristic curling of the host's root hairs. Subsequently (stage II), bacteria invade the plant through the root hair, progressively encased by a host-produced cell wall tube called the infection thread. The bacteria are continuously dividing as the infection threads branch and penetrate through several layers of host root tissue. Meanwhile, cells in the root start to divide, and this proliferation results in the formation of the root nodule. Some infection threads penetrate partway into a host cell, stop and bacteria are released from the infection thread into the plant cytoplasm. After release, bacteria often enlarge and/or change shape, and in the endosymbiotic form they are referred to as bacteroids. This final stage of differentiation is the prelude to actual nitrogen fixation (stage III). The nitrogen fixing root nodule contains both infected and uninfected cells, which appear to have distinct functions in the overall process of fixation, assimilation and transport of nitrogen. The thousands of bacteria, the cytoplasm of an infected plant cell may have, can be considered temporary nitrogen fixing plant organelles (350). Each stage in the *Rhizobium*-legume symbiosis is thus characterized by a series of developmental events concerning both bacteria and the plant, resulting in a complex, well organized and well coordinated plant organ.

With the rise of molecular biological techniques, the knowledge of nodule formation and nitrogen fixation at the molecular level has progressed in less than a decade from almost nothing to a point where these processes can be described in considerable detail. It has been found that a number of genes in both plant and bacterium are only expressed in nodules. An effective symbiosis is accomplished by differentiation of bacteria into bacteroids at the one hand, and differentiation of plant cells into a root nodule at the

other. The major part of research activity has concentrated on the nitrogen fixing bacteria, which are more easily accesible to genetic manipulation in comparison with legume genes are the leghemoglobin genes. So far, no nodulin gene expression has been found during stage I and stage IV. Now, the elucidation of both the functions of nodulins and the

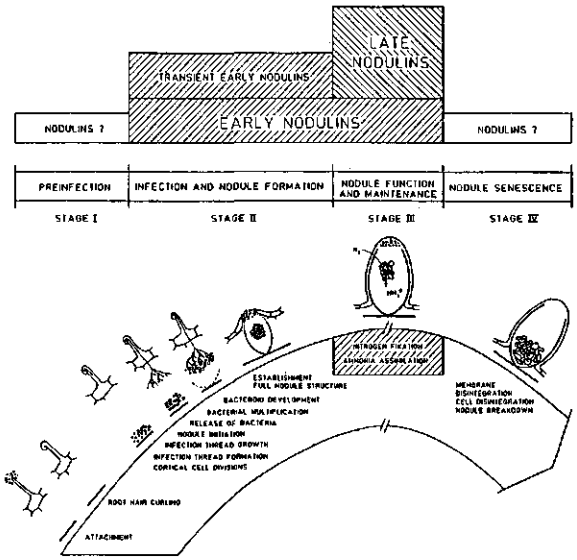


Figure 1.1. Schematic representation of the stages, and sequence of events, in the formation of a nitrogen fixing root nodule , modified after Verma and Long (350), and involvement of nodulins in these stages.

plants. In *Rhizobium*, the genes responsible for nodulation (*nod*), and nitrogen fixation (*nif* and *fix*) are located on large, endogeneous, so-called sym plasmids. Most of these bacterial genes have been isolated and their properties are subject of extensive reseach. Yet the host plant is equally important in the symbiosis. The plant provides the right environment, the energy and uses the fixed nitrogen for its growth and development. Over the last few years, interest in the role of the plant in the symbiosis has considerably intensified, even if the amount of research still represents a small portion of the effort put into the bacterial partner. The existence of plant genome-encoded, nodule specific proteins, nodulins, has been established beyond question, just as the differential expression of nodulin genes during nodule development. The latter has resulted in the distinction between early and late nodulins (fig. 1.1.). Early nodulin genes are expressed in the stage of nodule development during which the nodule structure is formed (stage II). Late nodulin gene expression is associated with the onset of nitrogen fixation (stage III). Type members of late nodulin modes of regulation of the encoding nodulin genes is a major issue in understanding the mechanism of nodule differentiation and functioning.

This thesis is concerned with nodulins, their function in root nodule development and the regulation of the genes that encode nodulins. An early nodulin cDNA clone, pGmENOD2, selected from a soybean cDNA library, has been used to study the expression of the corresponding early nodulin gene, and to characterize the nature of the product of this gene, the nodulin Ngm-75 it codes for. It is shown that this early nodulin Ngm-75 is an extremely (hydroxy)proline-rich protein, that probably represents a cell wall constituent (chapter 2.).

The control of nodule specific gene expression will be exerted at the level of the gene. In an attempt to identify the sequences of the DNA which are responsible for the regulation of late nodulin gene expression, a pea leghemoglobin gene was isolated from a genomic library and analyzed by nucleotide sequenc-

ing. Comparison of the promoter sequence of this pea leghemoglobin gene with known promoter sequences of other leghemoglobin and late nodulin genes revealed two consensus motifs that occur upstream of the transcription initiation site of all sequenced late nodulin gene promoters (chapter 3.). These consensus motifs may be responsible for the tissue specificity of late nodulin gene expression.

Compared with other plant differentiation processes, root nodule development is unique in the involvement of a prokaryote in the induction and control of plant development. In addition to the analysis of nodulin gene expression, elucidation of the signals bacterium and plant exchange to accomplish an effective symbiosis will contribute to our understanding of the symbiosis. In chapters 4. and 5. of this thesis the role of *Rhizobium* in regulating nodulin gene expression is investigated. By Northern blot analyses using nodulin cDNA clones as probes, and by comparing the proteins produced by *in vitro* translation of mRNA from roots and developing nodules of vetch (*Vicia sativa* subsp. *nigra*), several different nodulin gene products were identified (chapter 4.). Nodulin gene expression has been analyzed in vetch nodules disturbed in various stages of development, obtained after inoculation with engineered *Rhizobium* and *Agrobacterium* strains. It is demonstrated that only a limited number of *Rhizobium* genes, the *nod* genes, are indispensable for the induction of early nodulin gene expression (chapters 4. and 5.), and evidence is presented that the same *nod* genes are in some way involved in the induction of the expression of late nodulin genes (chapter 5). The nodules formed were simultaneously investigated microscopically, revealing a correlation between the expression of a certain nodulin gene and the accomplishment of a particular stage in the developmental program of the root nodule. On the basis of expression in nodules disturbed in development, both early (chapter 4.) and late (chapter 5.) nodulin genes can be subdivided into at least two subclasses, the expression of which is regulated differently. The appearance of nodulin gene products can thus be used as markers for development. This provides a basis for speculations about the possible function of nodulins in nodule development.

In chapter 6. of this thesis, the current knowledge on nodulins and the regulation of nodulin gene expression is discussed and integrated with the results presented in the preceding chapters. It is argued that during evolution nodulin genes are derived from normal plant genes and evolved to fit the constraints of the symbiosis.

CHARACTERIZATION OF cDNA FOR NODULIN–75 OF SOYBEAN: A GENE PRODUCT INVOLVED IN EARLY STAGES OF ROOT NODULE DEVELOPMENT

2

Chapter 2

Characterization of cDNA for nodulin–75 of soybean : A gene product involved in early stages of root nodule development

The formation of nitrogen fixing nodules on the roots of leguminous plants induced by bacteria of the genera *Rhizobium* or *Bradyrhizobium* involves the specific expression of a number of plant genes called nodulin genes (117,126,190). In a description of nodule development, Vincent (189) distinguishes between three stages in nodule development denoted as "pre–infection", "infection and nodule formation" and "nodule function". In the pre–infection stage the *Rhizobium* bacteria recognize their host plants and attach to the root hairs, an event that is followed by root hair curling. At the moment, nothing is known about specific plant genes that are involved in this stage. In the next stage, the bacteria enter the roots by infection threads while concomitantly the dedifferentiation of some cortical cells results in the formation of meristems. The infection threads grow towards the meristematic cells and bacteria are released into the cytoplasm of about half of these cells where they develop into bacteroids. In the final stage further differentiation of nodule cells occurs leading up to a nitrogen fixing nodule. Most studies on the expression of nodulin genes so far have been confined to the final stage of root nodule development in which the formation of a nitrogen fixing nodule is accomplished. But the steps involved in root nodule formation show that major decisions determining the development of a root nodule are made in the stages preceding the establishment of a nitrogen fixing nodule. We have shown (128) that nodulin genes are differentially expressed during development and that in pea at least two nodulin genes are transcribed in the second stage of root nodule formation. These genes are referred to as early nodulin genes. Here we report the isolation of soybean cDNA clones representing early nodulin genes and the detailed analysis of one of these clones.

2.1. RESULTS

2.1.1. Isolation of early nodulin cDNA clones.

Six thousand clones out of a cDNA library prepared against poly(A)+ RNA of soybean root nodules were screened by differential colony hybridization for the presence of copies of early nodulin gene transcripts. Using cDNA probes transcribed from poly(A)+RNA isolated from either 5–day–old, uninfected roots or from nodules picked from 10–day–old plants. Ten cDNA clones were isolated that specifically hybridized with the nodule cDNA probe. These clones represent soybean (*Glycine max*) early nodulin genes and will be designated pGmENOD to distinguish them from pGmNOD clones that represent nodulin genes expressed at later stages of development. Cross–hybridization studies of these ten clones revealed two unique cDNA clones, pGmENOD8 and pGmENOD9 having insert lengths of 400 and 950 bp respectively, and eight clones with common sequences, of which the clone pGmENOD2 with an insert length of 1000 bp was chosen for further characterization. Northern blot analyses showed that pGmENOD2 hybridized to a mRNA of 1200 nucleotides and indicated that the concentration of the GmENOD2 mRNA is highest at day 10 and decreases during further nodule development (fig. 2.1A.). Thus the GmENOD2 gene is apparently transiently expressed during soybean nodule develop–

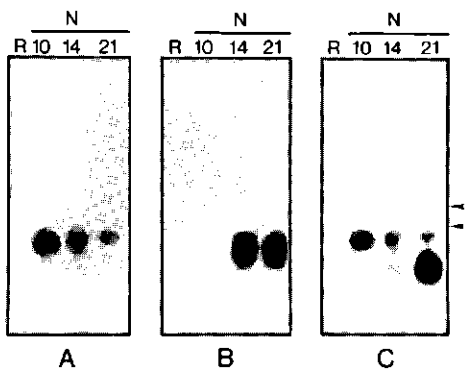


Figure 2.1. Autoradiographs of Northern blots containing 15 microgram of total RNA isolated from 5-day-old uninoculated roots (R) or nodules (N) harvested 10, 14 and 21 days after sowing and inoculation with *B. japonicum* USDA. The blot in A is identical to the blot in C. The blots were hybridized at 42°C with ^{32}P-labeled pGmENOD2 (A), with pGmENOD8 (B) and with pGmENOD2 and pLb in consecutive order (C). The positions of the ribosomal RNAs are indicated by arrowheads.

ment, although in some experiments the concentration of GmENOD2 mRNA remained nearly constant between 10 and 21 days. A similar course of transient expression was found for the GmENOD9 gene that hybridized to mRNA of 1700 nucleotides (not shown). However, the GmENOD9 gene was expressed at considerably lower levels. In contrast, pGmENOD8 hybridized with mRNA of 1000 nucleotides in length that was present at low levels in nodules of 10-day-old plants and reached its highest level at day 14 (fig. 2.1B.). The relative abundance of RNA hybridizing with pGmENOD2 is in agreement with the high frequency by which clones with sequences common to pGmENOD2 were isolated from the cDNA library. For a more detailed analysis we have focussed on pGmENOD2. Since the gene represented by GmENOD2 is abundantly expressed in normal nodules, it appears feasible to analyze the expression pattern of this gene in nodules disturbed in development.

2.1.2. **Characterization of pGmENOD2.**

On Southern blots of *Eco*RI digested soybean and *B. japonicum* genomic DNA five restriction fragments of soybean DNA, 25, 10.6, 5.3, 4.8 and 1.5 kb in size respectively, were found to hybridize with ^{32}P-labeled pGmENOD2, whereas no hybridization was observed with the *B. japonicum* DNA (fig. 2.2.). Hence the cloned GmENOD2 DNA is encoded by the soybean genome and its gene probably is part of a small gene family. On a Northern blot with root and nodule RNA probed with pGmENOD2 under low stringency conditions, pGmENOD2 hybridized not only to a mRNA of 1200 nucleotides but also to a second nodule specific mRNA with a length of 1400 nucleotides (fig. 2.3A.). In addition, a low abundance RNA of about 1000 nucleotides in size was detected under those conditions in uninfected roots (fig. 2.3B.). This observation is consistent with the existence of a small gene family. For comparison of the expression of the GmENOD2 genes with that of nodulin genes expressed later in development, a leghemoglobin (Lb) cDNA clone (pLb) was selected from the cDNA library by hybridization with a soybean Lb cDNA clone (170), made available by K. Marcker (University of Aarhus, Denmark). The difference in time of expression between the GmENOD2 and leghemoglobin genes is illustrated in fig. 2.1C. The Northern blot was first hybridized with pENOD2 and was subsequently probed with pLb. The Lb mRNAs start to appear when the GmENOD2 mRNA concentration is already decreasing.

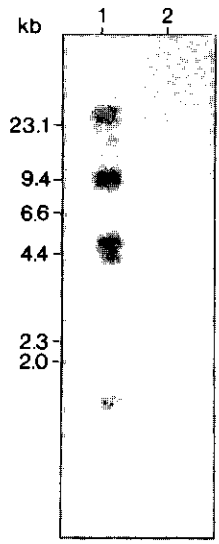


Figure 2.2. Autoradiograph of a Southern blot containing 10 microgram of soybean genomic DNA (lane 1) and 1 microgram of *B. japonicum* USDA110 DNA (lane 2), both digested with *Eco*RI, and hybridized with ^{32}P-labeled pGmENOD2.

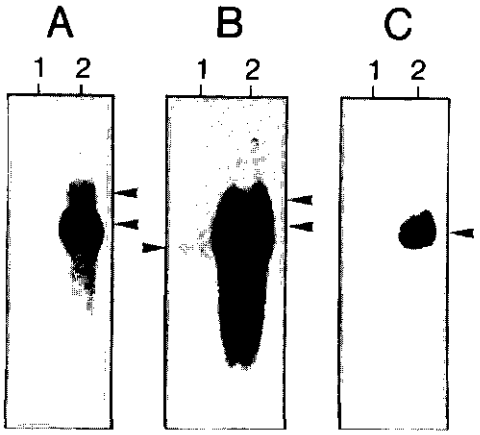


Figure 2.3. Autoradiographs of Northern blots containing 15 microgram of total RNA isolated from (A and B) 5-day-old uninoculated soybean roots (lane 1) and 14-day-old nodules induced by *B. japonicum* USDA110 (lane 2) and from (C) soybean roots (lane 1) and nodule-like structures collected 4 weeks after inoculation with *R. fredii* USDA257 (lane 2). The Northern blots were hybridized under low stringent conditions (35°C; 50% formamide; 1 M NaCl), with ^{32}P-labeled pGmENOD2 as probe. The autoradiograph of the blot shown in B is obtained after an approximately ten fold longer exposure than the autoradiograph of the blot shown in A.

2.1.3. pGmENOD2 codes for nodulin Ngm-75 .

To identify the early nodulin encoded by pGmENOD2, mRNA was hybrid-selected by pGmENOD2 and was translated *in vitro* in the presence of ^{35}S-methionine followed by two-dimensional (2-D) gel electrophoresis. The results showed that the pGmENOD2-encoded polypeptide has an apparent molecular weight of 75,000 with an isoelectric point around 6.5 (fig. 2.4A.). In accordance with the nomenclature previously established for nodulins (345, chapter 6.) the identified polypeptide is named Ngm-75. After *in vitro* translation of the pGmENOD2-selected RNA in the presence of ^{3}H-leucine as the radioactive amino acid, two polypeptides were found, one of which comigrates with the polypeptide detected after translation with ^{35}S-methionine while the other, more prominent, polypeptide is slightly more basic (fig. 2.4A.). This result indicates that the pGmENOD2 hybrid-selected RNA consists of two mRNA species. Under the stringent hybridization conditions used, only mRNA species with a length of 1200 nucleotides will have been selected and the low abundance mRNAs (compare figs. 2.3A. and

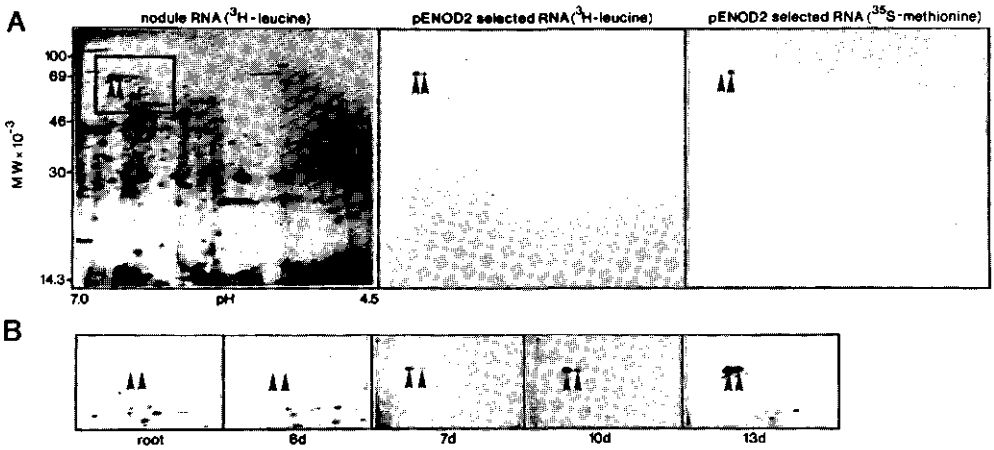


Figure 2.4. Characterization of the early nodulin cDNA clone pGmENOD2 by hybrid-released translation (A) and time-course analysis of the expression of the nodulin Ngm-75 genes during nodule development (B). (A) Total RNA from 16-day-old soybean nodules and RNA eluted from filter-bound pGmENOD2 DNA were translated in a wheat germ extract in the presence of ^{3}H-leucine or ^{35}S-methionine as indicated. The products obtained were separated by 2-D gel electrophoresis and fluorographed. The positions of the nodulins Ngm-75 are indicated by arrowheads. (B) Only the region of the 2-D gels within the square indicated in (A) is shown, that represents the ^{3}H-leucine *in vitro* translation products obtained from RNA of 5-day-old soybean root (root), root segments of infected soybean plants 6 days after sowing and inoculation with *B. japonicum* USDA110, and soybean nodules 7, 10 and 13 days after sowing and inoculation with the same strain.

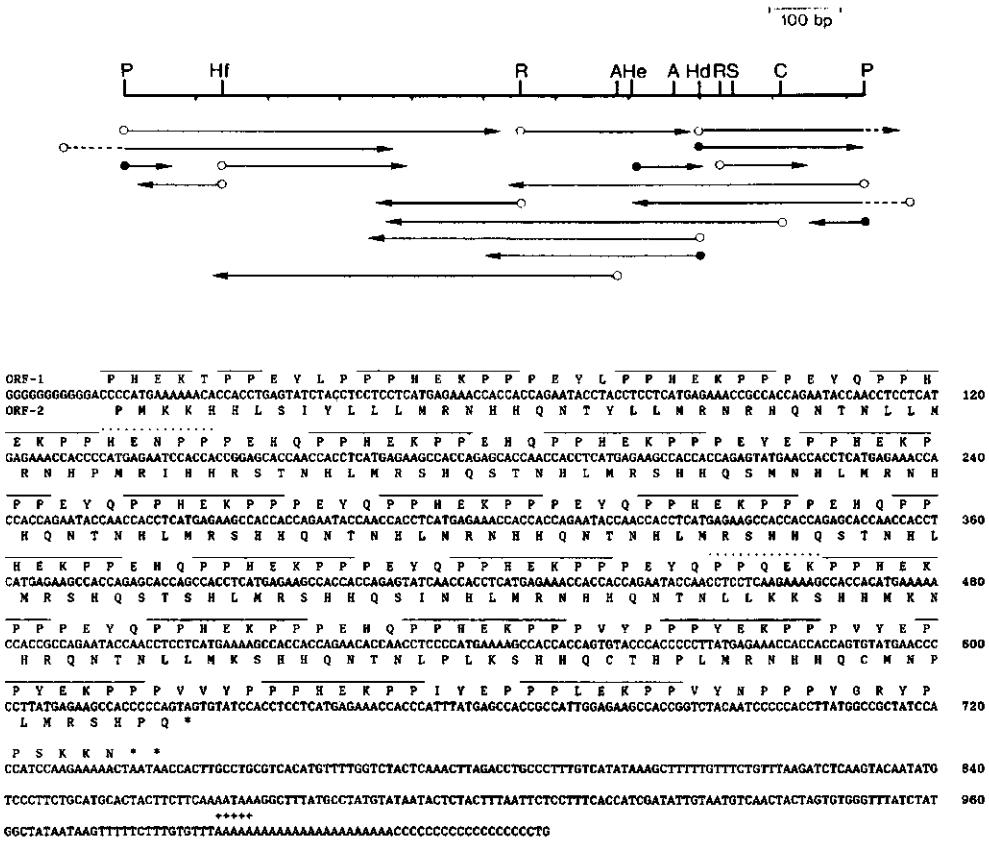


Figure 2.5. Partial restriction map, sequencing strategy and nucleotide sequence of the *Pst*I fragment from pGmENOD2. Sequencing was performed by the Maxam–Gilbert method (226) (open circles), and by the dideoxy method of Sanger (23,285) (closed circles). The arrows depict the direction and extent of sequencing from the indicated site. The dashed lines correspond to sequences originating within the vectors. In the DNA sequence, nucleotides are numbered on the right of the sequence. The predicted amino acid sequence is shown in standard single-letter code for both ORFs and the characteristic heptapeptide repeat is overlined in ORF-1. The two partial repeats are indicated by a dotted line. Termination codons (*) and potential poly(A) addition sites (+++) are also marked. P, *Pst*I; Hf, *Hin*f1; R, *Rsa*I; A, *Acc*I; He, *Hae*III; Hd, *Hin*dIII; S, *Sph*I; C, *Cla*I

2.3B.) will not be present in the pGmENOD2 hybrid selected RNA. The two polypeptides obtained from hybrid-released translation should therefore be closely related and are each encoded by a different member of the GmENOD2 gene family. The polypeptides, both referred to as Ngm-75, were also easily detected as nodulins in the 2-D pattern of the polypeptides obtained upon *in vitro* translation of total RNA from nodules because they are not found among the translation products of RNA from uninoculated roots (fig. 2.4B.). Hence RNA analysis by both Northern blotting and 2-D gel electrophoresis of the products obtained after *in vitro* translation of RNA can be used for the study of the expression of the Ngm-75 genes. The latter proved convenient especially in the case of limiting RNA quantities.

Strikingly, the translation products of these mRNAs of 1200 nucleotides in length have an apparent molecular weight of 75,000, whereas

mRNA of that length has a coding capacity for a polypeptide of, at most, 45 kDa. This notable discrepancy prompted us to sequence the cDNA insert of pGmENOD2 to see if the deduced amino acid sequence could explain the peculiar physical properties of the encoded polypeptides. At the same time information on the structure may provide clues on a possible function of the Ngm-75 nodulins. The *Pst* I insert of the pGmENOD2 cDNA clone was sequenced by both the Maxam-Gilbert (226) and the dideoxy (23,285) sequencing method. The sequencing data (fig. 2.5.) reveal that the insert contains 998 nucleotides including a short 3' poly(A)-tail but excluding the dCdG nucleotides generated in the cloning procedure. At most 200 nucleotides of the 5'-end of the 1200-nucleotide-long mRNA, including the initiation codon and coding sequences for the N terminus of the polypeptide, are thus missing in this cDNA clone. Two open reading frames (ORFs) occur in the same strand (fig. 2.5.). From one ORF, designated ORF-1, 728 nucleotides are found in the cDNA clone (positions 13-741; Fig. 2.5.), and this ORF ends with two successive termination codons. A second ORF, designated ORF-2, not in phase with the first one, comprises 611 nucleotides (positions 14-625; fig. 2.5.) and ends in a single termination codon. Both ORFs are followed by 3'-nontranslated regions of about 250 (ORF-1) and 375 (ORF-2) nucleotides respectively, in which two potential poly(A) addition signals are present (fig. 2.5.).

Although both ORFs seem to be able to code for a nodulin of about the same size, several lines of evidence indicate that only ORF-1 corresponds to a nodulin Ngm-75. First, ORF-1 gives rise to a completely different polypeptide (241 amino acids of which none is methionine) than ORF-2 (203 amino acids of which 20 are methionine). Such entirely different polypeptides will differ in physical properties and will not have almost the same isoelectric point and exactly the same aberrant migration behavior on SDS/polyacrylamide gels. The two Ngm-75 *in vitro* translation products will therefore most likely be related polypeptides with a similar amino acid sequence, derived from two different mRNAs, and not from two ORFs of one mRNA. If then one of the Ngm-75 *in vitro* translation products is shown to contain no methionine (fig. 2.4A.), the other Ngm-75 nodulin may have a low methionine content. But the ORF-2-derived polypeptide contains 20 methionines, indicating that the complete polypeptide will be rich in methionine. This makes it highly improbable that ORF-2 codes for one of the nodulins Ngm-75. We therefore deduce that only ORF-1 encodes a nodulin-75. This conclusion is supported by the absence of methionine in the amino acid sequence deduced from this ORF. In addition, the analysis of the codon usage in both ORFs using a codon frequency table (313) compiled from several published soybean coding sequences indicated that only the codon usage in ORF-1 is in agreement with the average codon usage of soybean (not shown). Moreover, though both ORFs encode a polypeptide containing repeating peptide sequences, the repetitive amino acid sequences occurring in the polypeptide encoded by ORF-1 are better preserved than those present in the ORF-2-derived polypeptide. This indicates that an evolutionary tendency exists for a functional conservation of the polypeptide coded for by ORF-1. We therefore propose that the ORF-1 will exclusively be used for the generation of a nodulin Ngm-75.

ORF-1 shows that the Ngm-75 nodulins are peculiar proline-rich proteins. Ngm-75 contains a repetitive sequence of 10 or 11 amino acids that is repeated at least 20 times. Embedded in this repetitive sequence a heptapeptide sequence is found that is conserved in 19 out of 20 units (fig. 2.5.). This repeated heptapeptide sequence is Pro-Pro-Xaa-Glu-Lys-Pro-Pro, in which 17 times Xaa = histidine and 3 times Xaa = tyrosine or leucine. Three or four amino acids that are not as conserved, mainly proline, glutamic acid/glutamine, and tyrosine flank the heptapeptide repetitive sequence. Two apparent partials of the heptapeptide repeat are found at positions 133 and 451 within the sequence. Neither alpha-helix nor bèta-sheet conformations were found using the method of Lim (211). The high proline content of the Ngm-75 nodulins probably explains the discrepancy between the observed apparent molecular weight of 75,000 and the coding capacity of a 1200-nucleotide mRNA. A similar aberrant migration behavior on SDS/polyacrylamide gels is found for the proline-rich protein collagen (113).

2.1.4. **Ngm–75 is involved in nodule morphogenesis.**

To form an idea of the process in which the proline–rich Ngm–75 protein might be involved, we attempted to correlate the beginning of expression of the Ngm–75 genes with a defined stage in root nodule formation. Seven days after sowing and inoculation, the nodule meristems start to emerge through the root epidermis and become macroscopically visible as tiny brown spots (45,245). Total RNA was isolated from tap root segments of 6–day–old inoculated plants, where nodules are not yet visible, and from nodules harvested 7, 10, and 13 days after sowing and inoculation. RNA preparations were analyzed by *in vitro* translation followed by 2–D gel electrophoresis using ^{3}H–leucine as radioactive amino acid. The area of the 2–D gel where Ngm–75 nodulins are found is shown in fig. 2.4B. Both Ngm–75 proteins first appear at day 7 and then increase in concentration up to day 13 (fig. 2.4B.).

From light microscopic observations (45,245, results not shown) it appears that around the point of time that the Ngm–75 proteins become detectable, both the infection process (defined as the development of infection threads, penetration of infection threads into the nodule meristem and the release of bacteria into nodule cells) proceeds and the differentiation of the nodule meristem into a nodule structure has started. By examining the nodule structures formed by *R. fredii* USDA257, it proved possible to distinguish between the infection process and the differentiation into a nodule structure. On commercial soybean cultivars, this fast–growing *Rhizobium* strain cannot form nitrogen fixing root nodules but forms nodule–like structures that are not able to fix nitrogen. Histological examination of these nodule–like structures revealed that they arise from a combination of cell swellings and randomly oriented cortical cell divisions (fig. 2.6.). In these nodule–like structures, no infected plant cells nor infection threads were observed and none of the structures examined had an organization with vascular bundles at the periphery, similar to normal nodules. Apparently the formation of such a nodule–like structure does not require an infection process. By Northern blot analysis (fig. 2.3C.) Ngm–75 RNA could be detected in RNA isolated from these nodule–like structures. This result indicates that the expression of Ngm–75 genes is not correlated with the infection process.

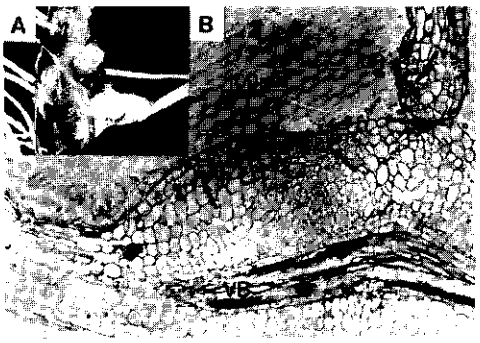


Figure 2.6. (A) Nodule–like structure on soybean roots obtained 4 weeks after inoculation with *R. fredii* USDA257. (B) Part of a longitudinal section of a nodule–like structure is shown. Cell divisions in the outer– and inner cortical cell layers are indicated by arrows. VB, root vascular bundle; LR, lateral root. (Bar = 100 micrometer.)

2.2. **DISCUSSION**

A cDNA library from soybean root nodules has been analyzed for copies of mRNA transcripts of early nodulin genes. These genes are expressed in the early stage of root nodule development when the nodule structure is being formed and the roots become infected. Three non–cross–hybridizing cDNA clones that represent such early nodulin genes were identified and one of these clones, pGmENOD2, was characterized in detail. On Northern blots pGmENOD2 hybridizes to mRNA of 1200 nucleotides in length. Under low stringency hybridization conditions an additional nodule specific RNA of 1400 nucleotides is seen on Northern blots, and under those conditions we could also detect mRNA that cross–hybridized with pGmENOD2 in uninfected soybean roots. (fig. 2.2B). However, this root mRNA was smaller in size than both nodule GmENOD2 mRNAs and Ngm–75 was not found among the *in vitro* translation products from root RNA. Probably the root and nodule RNA sequences code for different but related proteins, and are most likely transcribed from different genes. Ngm–75 might be the nodule specific form of a protein that normally occurs in roots, analogous to the nodule specific form of glutamine syn–

thetase (68). The nucleotide sequence of the *Pst* I insert of pGmENOD2 has been determined and the derived amino acid sequence shows that 45% of the amino acids of Ngm-75 is proline and that the amino acid sequence is organized in highly repetitive units. The repetitive nature suggests that the encoding gene may be derived from numerous gene duplication events.

In an effort to derive a function for Ngm-75 in root nodule development from these sequence data, we have surveyed the occurrence of proline-rich proteins in plants and their assumed functions. All proline-rich proteins that have been described in plants so far contain hydroxyproline that is post-translationally formed and in most cases subsequently glycosylated. Although it remains to be established whether the soybean Ngm-75 proteins become hydroxylated and glycosylated *in vivo*, soybean nodule tissue has recently been described as extremely hydroxyproline-rich (50). Also the apparent absence of a class of proline-rich proteins in plants justifies the assumption that the nodulins Ngm-75 belong to one of the classes of hydroxyproline-rich glycoproteins. There are four major classes of hydroxyproline-rich glycoproteins in plants (228): *(i)* the cell wall structural hydroxyproline-rich glycoproteins (HRGPs) or extensins (56,343), *(ii)* the arabinogalactan proteins (AGPs) (344), *(iii)* the solanaceae lectins (1), and *(iv)* hydroxyproline-rich agglutinins (196). In table 2.1., the amino acid composition of a representative of each of these classes is given and compared to the amino acid composition derived for Ngm-75. Extensins are associated with the cell walls of most dicotyledonous plants (186). They are assumed to play a role in maintaining the integrity of the primary cell wall (61) and they may be important in controlling growth and development. Extensins have been shown to accumulate in plant cell walls upon wounding (58) and pathogen attack (139, 302) in what is considered a defense response. The amino acid sequences of extensins are characterized by the occurrence of a repeating Ser-Hyp-Hyp-Hyp-Hyp pentapeptide. Neither this pentapeptide (fig. 2.5) nor the typical high serine content (table 1.) is found in the amino acid sequence of Ngm-75. The nodulins Ngm-75 are therefore not closely related to extensins. The arabinogalactan proteins (AGPs) are freely soluble, acidic due to uronic acid residues, and alanine rich (105). Ngm-75 does not contain any alanine (table 1.). The overall amino acid composition of neither the solanaceae lectins nor the hydroxyproline-rich agglutinins matches with that of Ngm-75 (table 2.1.). Recently two DNA sequences coding for proline-rich proteins of

Table 2.1. Comparison of the amino acid composition, expressed as mol. %, of typical representatives of plant hydroxyproline-rich glycoproteins and proline-rich proteins, obtained from either amino acid analysis or derived from the DNA sequence. All amino acids not mentioned comprise less than 2 mol. % each in all cases. Putative signal sequences are not included in the amino acid composition.

		Amino acid												
protein	plant	Pro + Hyp	Ser	Glu	Glx	Gln	His	Lys	Tyr	Ala	Val	Gly	Leu	Cys	Reference
extensin	carrot	45.7	14.2		0.4		11.8	6.7	11.0	0.4	5.9	0.4	0.4	0.0	(343)
extensin	carrot	42.2	10.9		2.6		9.5	11.7	12.0	1.4	4.0	0.0	0.0	0.0	(56)
AGP	French bean	29.6	18.2		4.5		0.6	2.6	0.6	16.2	3.2	6.5	3.2	0.6	(344)
lectin	potato	21.9	12.6		6.9		0.0	3.7	3.3	4.1	0.4	12.2	1.2	10.6	(1)
agglutinin	potato	50.9	9.4		1.1		5.1	15.9	6.2	0.9	3.8	1.1	0.2	0.1	(196)
P33	carrot	30.8	2.8	4.7		1.4	12.8	9.5	4.3	4.2	10.0	1.4	1.4	0.0	(57)
"protein 4"	soybean	40.0	0.0	3.9		0.0	0.0	20.0	16.0	0.0	17.4	0.0	0.0	0.0	(157)
N-75	soybean	44.4	0.4	16.6		5.4	9.5	9.5	7.5	0.0	2.1	0.4	1.2	0.4	this report

unknown function have been reported. An auxin regulated soybean gene represented by a cDNA clone (157) codes for a proline-rich protein, designated by us as "protein 4" in table 2.1, with no serines, but a low content of glutamic acid. The relatively high valine content of "protein 4" (table 1) is not found in the amino acid composition of Ngm-75. A carrot cDNA clone was shown (57) to encode a proline-rich 33 kDa polypeptide (designated P33) also with a high valine content. Since the amino acid composition of none of the (hydroxy)proline-rich (glyco)proteins analyzed so far resembles the amino acid composition derived for Ngm-75, we may be dealing with a hitherto unknown class of (hydroxy)proline-rich proteins, characterized by a remarkably low content of serine and a surprisingly high content of glutamic acid. Both the highly repetitive nature of the amino acid sequence and the high proline content suggest that the Ngm-75 nodulins are structural proteins.

To gain a better understanding of the biological function of the Ngm-75 nodulins in root nodule development, we have studied the expression of their genes as a function of time. The expression of the Ngm-75 genes is first detectable at 7 days after sowing and inoculation, when the nodule meristems just emerge through the root epidermis. The detection of the expression of the Ngm-75 genes coincided, however, with a change of RNA isolation procedure since RNA is first isolated from pieces of tap root and then from excised nodule structures (as soon as these become visible). We were not able to detect Ngm-75 RNA in 7-day-old tap root pieces with visible nodule structures, whereas we could detect Ngm-75 mRNA in excised nodule structures of the same age. Therefore it cannot be excluded that these genes are already expressed earlier than 7 days after sowing and inoculation but in fewer cells and at a similar or lower level than at 7 days.

Irrespective of the possible expression of these genes before 7 days, a strong stimulation of Ngm-75 gene expression occurs from 7 to 13 days. The Ngm-75 proteins should therefore be involved in a developmental event that proceeds during this period. Around day 7 in our growth system, the nodules emerged through the root epidermis. This stage of development equals stage VII as described by Calvert et al. (45), and cytological observations have shown that in this stage the meristems start to differentiate into nodule structures (45). Some of the meristem cells have been invaded by infection threads from which the rhizobia are beginning to be released. To examine whether the induction or stimulation of the expression of the Ngm-75 genes is specifically related to the infection process on the one hand, or to the formation of a nodule structure, on the other, we have looked for Ngm-75 gene expression in nodule-like structures formed by *R. fredii* USDA257. This strain induces the formation of nodule structures devoid of intracellular bacteria and infection threads (fig. 2.6.), in which, however, early nodulin Ngm-75 RNA is detectable (fig. 2.3.). The expression of the Ngm-75 genes in nodules without bacteria or infection threads strongly suggests that Ngm-75 is not involved in the infection process, but more likely in nodule morphogenesis.

Quantitative light microscopic observations on nodule initiation have shown (45) that up to the stage in which the meristems emerge through the epidermis, development can stop. However, when a meristem has reached the "emergence stage" it will continue to develop into a mature nodule. The stimulation of the expression of the Ngm-75 genes coincides therefore with the moment the soybean nodule meristems have reached an apparently critical developmental stage. The expression of the Ngm-75 genes might therefore reflect the definitive "commitment" of the meristems to develop into a nodule. The nature of the involvement of the hydroxyproline-rich nodulins Ngm-75 in this commitment remains to be established.

2.3. MATERIALS AND METHODS

Growth conditions for plants and bacteria. Soybean plants (*Glycine max* (L) Merr. cv. Williams) were cultured as described for pea plants (29) but at 28°C. At the time of sowing the soybean seeds were inoculated with *Bradyrhizobium japonicum* USDA110 or *Rhizobium fredii* USDA257. Both strains were cultured as described (22).

Isolation of nodules. Nodules were excised from the roots with a scalpel. For samples prior to 6 days after sowing and inoculation, a 4-cm root segment of the upper part of the main root (where root nodules normally would develop) was harvested. Nodules were frozen in liquid nitrogen and stored at -70°C until use.

Isolation of nucleic acids. Total RNA from nodules and roots was isolated as described (126) and poly(A)+ RNA was obtained by oligo(dT)-cellulose chromatography (218). *B. japonicum* USDA110 and soybean genomic DNA were isolated as described (219, 374) and plasmid DNA was isolated by the alkaline lysis method (24).

Construction of cDNA library. DNA complementary to poly(A)+RNA isolated from nodules from 21-day-old plants was synthesized with reverse transcriptase (Anglian Biotechnology, Essex, England) and second strand synthesis was performed under standard conditions (218). Double-stranded cDNA was treated with S1 nuclease and size-fractionated on a 5-30% sucrose gradient (Beckman SW50; 47,000 rpm; 6 hrs at 4°C). The fractions containing double-stranded cDNA with a length of 500 base pairs or more were collected. The double-stranded cDNA was tailed with dC and then annealed to *Pst* I-cut oligo(dG)-tailed pBR322 (Boehringer Mannheim) in a 1:1 molar ratio. The annealed mixture was used to transform Escherichia coli RR1 (12). On the average 5,000 transformants were obtained per microgram of poly(A)+RNA.

Differential screening of the cDNA library. Individual transformants were picked, transferred to 96-well microtiter plates containing LB medium, 15% glycerol and 12.5 mg/l tetracycline and grown for 16 hr at 37°C. Two replicas, each containing 384 transformants, were made on GeneScreen*Plus* (New England Nuclear) and they were placed on LB agar plates containing 12.5 mg/l tetracycline. The colonies were allowed to grow for 16 hr on the filters. The filters were prepared for hybridization according to the GeneScreen*Plus* manufacturer's manual. Probes for differential screening were prepared from poly(A)+RNA isolated from segments of 5-day-old, uninfected roots and from nodules 10 and 21 days after inoculation, as in the construction of the cDNA library except that 10 microCi ^{32}P-dATP (specific activity 3200 Ci/mmol; 1 Ci = 37 GBq, New England Nuclear) was used. The filters were hybridized for 72 hr at 65°C to either root or nodule ^{32}P-labeled cDNA in 6x SSC (1xSSC = 0.15M NaCl, 0.15M sodium citrate); 5x Denhardt's solution (218); 10 mM EDTA; 0.5% SDS; 100 mg/ml sonicated, denatured calf thymus DNA, and 20 mg/l poly(A). The filters were washed twice in 2x SSC; 0.1% SDS for 15 min at room temperature and twice in 0.5x SSC; 0.1% SDS for 30 min at 65°C.

***In vitro* translation of total RNA.** Total RNA (3 microgam) from roots or nodules was translated *in vitro* in a wheat germ extract (Bethesda Research Laboratories) in a 15 microliter mixture according to manufacturer's manual to which 15-30 microCi ^{35}S-methionine or 6 microCi ^{3}H-leucine was added. Translation products were separated by 2-D gel electrophoresis followed by fluorography of the dried gel to preflashed Kodak XAR5 film (126).

Hybrid-released translation. For hybrid-released translation, the pGmENOD2 insert (10-15 microgram of DNA) was denatured and applied to 0.5 cm^2 discs of diazophenylthioether-paper (Bio-Rad) essentially as described (218). Total soybean RNA from 16-day-old nodules (750 microgram) was then hybridized to the filter-bound DNA in 0,3 ml of 50% (vol/vol) deionized formamide; 0.1% SBS; 0.6 M NaCl; 4 mM EDTA, and 80 mM Tris-HCl (pH 7.8). Hybridization was initiated at 40°C and the temperature was slowly decreased to 37°C over a period of 6 hr. After washing, the bound RNA was eluted (218) and dissolved in 3 microliter of H_2O; 1.5 microliter was translated and analyzed as above.

Northern and Southern blot analysis. Total soybean RNA was denatured in dimethyl sufoxide:glyoxal, electrophoresed in 0.8% agarose gels (218) and transferred to GeneScreen (New England Nuclear) filters as described (126). The blots were prehybridized for 6 hr in 50% (vol/vol) deionized formamide; 1 M NaCl; 0.05 M Tris-HCl (pH 7.5); 5x Denhardt's solution; 0.1% SDS, and 100 mg/l denatured salmon sperm DNA, and hybridized with nick-translated (218) probes. Hybridization was performed for 16 hr at 42°C. Blots were washed twice for 15 min at 42?C in 2x SSC; 0.1% SDS, and twice for 30 min at 42°C in 0.5x SSC; 0.1% SDS. For Southern blot analysis, soybean and *B. japonicum* genomic DNA was digested with restriction enzymes, separated on a 0.7% agarose gel, transferred to nitrocellulose filters (306) and hybridized to ^{32}P-labeled pGmENOD2.

DNA sequencing. Standard techniques were used for cloning into M13- and pUC-vectors (232), for dideoxy (23,285) and for Maxam-Gilbert (226) sequencing. The DNA sequence data were stored and analyzed with programs written by R. Staden (313) on a microVAX/VMS computer.

Cytology .Nodules were fixed for 16 hr in 3% glutaraldehyde in 50mM sodium phosphate buffer (pH 7.2). After fixation, the nodules were rinsed with the same buffer, dehydrated in a graded ethanol series, embedded in Technovit 7100 (Kulzer, Wehrheim, F.R.G.), sectioned into 5 micrometer sections, stained with toluidine blue and examined under a light microscope.

ISOLATION AND ANALYSIS OF A LEGHEMOGLOBIN GENE FROM PEA (*Pisum sativum*)

Chapter 3

Isolation and analysis of a leghemoglobin gene from pea (*Pisum sativum)*

Leghemoglobins are predominant nodulins in the root nodules of leguminous plants. These monomeric hemoproteins, which resemble the vertebrate myoglobins, may constitute up to 25% of the total soluble protein in a nitrogen fixing nodule (354). As oxygen carriers, leghemoglobins control the free oxygen concentration in the nodule (5). In all legume species studied, more than one leghemoglobin is found. The various isoforms of leghemoglobin are encoded by different genes, or result from posttranslational modifications (363). They differ in affinity for oxygen. The Lb proteins occurs exclusively in the infected cells of root nodules (276,378). The Lb genes are activated in a defined order (334), and it has been suggested that during development the leghemoglobin with the largest oxygen affinity eventually becomes predominant (335).

The leghemoglobin gene family in soybean (*Glycine max)* has been studied in considerable detail. In this species, four functional, two pseudo- and two truncated genes have been identified, and their chromosomal arrangement and nucleotide sequence has been elucidated (33,39,40,170,174,198,318, 364). In addition, one of the leghemoglobin genes from French bean *(Phaseolus vulgaris)* (197) and a hemoglobin gene from the non-legumes *Parasponia* (189) and *Trema* (32), respectively, have been sequenced. Recently, it has been shown that a chimeric gene, consisting of the 5' promoter region of the soybean Lbc_3 gene and the chloramphenicol acetyltransferase (CAT) coding sequence, is developmentally correct expressed in root nodules formed on regenerated, transgenic *Lotus corniculatus* plants inoculated with the proper *Rhizobium* strain (314). In the latter experiments, it has unequivocally been demonstrated that a 2 kb region 5' upstream of the start of transcription of the soybean Lbc_3 gene carries the information for nodule specific expression of the gene in the heterologous legume host. Furthermore, these results indicate that the molecular mechanism for regulating Lb gene expression is conserved in different legume-Rhizobium associations.

Comparative studies of promoters of nodulin genes isolated from a number of species may contribute to the understanding of the regulation of late nodulin gene expression. Therefore, the observations on the soybean leghemoglobin and other nodulin genes should be extended to other leguminous plants. Here we describe the isolation and structural analysis of three leghemoglobin cDNA clones and one chromosomal gene from pea (*Pisum sativum*). Sequence analysis revealed that this gene contains all regulatory signals known to be essential for expression in eukaryotes. In the promoter region of the pea leghemoglobin gene some sequence elements occur, comparable with the sequences in the soybean Lbc_3 promoter that have been shown to be important for nodule specific expression.

3.1. **Results and Discussion**

3.1.1. **Analysis of pea leghemoglobin cDNA clones**

From a pea nodule cDNA library constructed in plasmid pBRH2 (*277*) using *Eco*R1 linkers a clone pPsLb101 had been isolated before (28). This clone was proven to contain leghemoglobin sequences by comparing the amino acid sequence derived from the nucleotide sequence (fig. 3.1.) with the published amino acid sequence of pea leghemoglobin (201).

More Lb cDNA clones were obtained by screening approximately 600 cDNA clones from a library of pea nodule cDNA cloned in

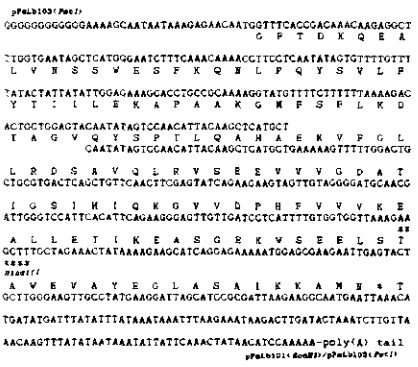


Figure 3.1. Nucleotide sequence of the *Eco* R fragment from pPsLb101 and the *Pst* I fragment from pPsLb103. The derived amino acid sequence is shown in standard single letter code. The sequences are aligned in the identical region.

the *Pst* I site of pBR322 by the dC/dG tailing method (see Govers *et al.* (129), for details) using the 450 bp EcoR1 fragment from pPsLb101 as a probe in colony hybridization experiments. Twenty clones containing Lb cDNA were identified, of which clone pPsLb102 contained the largest *Pst* I insert (500 bp). Northern blot analysis of nodule RNA showed the length of pea Lb mRNA to be approximately 650 nucleotides. Hence, none of the isolated clones contained a full copy of the Lb mRNA.

The cDNA insert of pPsLb102 appeared to have a sequence identical to that of the cDNA insert in pPsLb101 (fig. 3.1.), the 50 bp difference in insert length being due to the dC/dG tails present in pPsLb102 as result of the cloning procedure. Thus pPsLb101 and pPsLb102 presumably contain cDNA of mRNA derived from the same Lb gene.

In an attempt to obtain a Lb cDNA clone covering the 5' end of the Lb mRNA, the plasmids from the twenty Lb cDNA clones were hybridized using the 150 bp fragment from pPsLb102 (fig. 3.1.), containing the 5'– *Pst* I–*Hin*dIII–fragment, as a probe. This yielded clone pPsLb103, which moreover did not hybridize with the fragment from pPsLb102, containing the 350 bp HindIII–PstI–3' fragment. Sequence analysis subsequently showed pPsLb201 to contain the 5' end of the Lb mRNA, including the start codon and 5' untranslated region (fig. 3.1.). The similarity in nucleotide sequence in the overlapping region between pPsLb102 and pPsLb103, however only 30 nucleotides in length, suggests that pPsLb103 is derived from the same Lb gene as pPsLb102.

Hybrid released translation of mRNA selected with pPsLb102, followed by two-dimensional gel electrophoresis of the *in vitro* translation products revealed four polypeptides corresponding to leghemoglobin translation products (fig. 3.2A.). Because pPsLb102 apparently hybrid selected several Lb mRNAs due to sequence homologies, it is impossible to determine which of the three Lb polypeptides is encoded by this cDNA clone. After immunoprecipitation of the *in vitro* translation products of total pea nodule RNA with an antiserum prepared against pea leghemoglobin, the polypeptides indicated in fig. 2B have previously been identified as pea leghemoglobins (126). Surprisingly, the fourth and most acidic Lb polypeptide identified by immunoprecipitation, does not occur among the *in vitro* translation products of the mRNA that was hybrid selected by pPsLb102. This suggests a substantial difference in nucleotide sequence between the mRNA for this translation product and the other Lb mRNAs. Also a minor leghemoglobin *in vitro* translation product (fig. 3.2A.) had not been detected before in the *in vitro* translation pattern of total RNA.

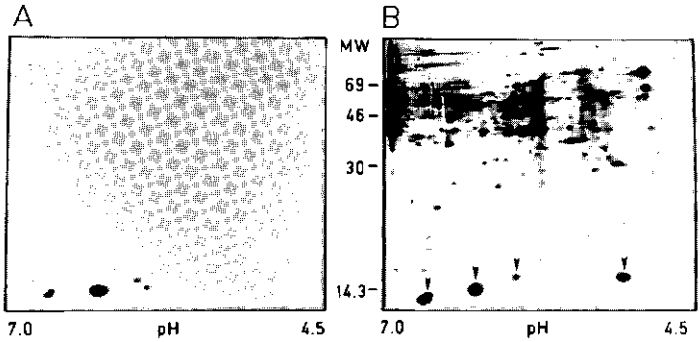


Figure 3.2. Characterization of the Lb cDNA clone by hybrid-released translation. RNA eluted from filter-bound pPsLb102 DNA (A) and total RNA from 17-day-old pea nodules (B) were *in vitro* translated in a wheat germ extract in the presence of ^{35}S-methionine. The products obtained were separated by 2-D gelelectrophoresis and fluorogrphed. In (B) the translation products that previously have been identified by immunoprecipitation to be leghemoglobins are indicated by arrowheads.

3.1.2. **Isolation and sequence analysis of a pea leghemoglobin gene**

Southern blot analysis of *Eco* RI digested pea DNA confirmed that the leghemoglobins in pea are encoded by a multigene family. Using pPsLb102 as probe, eight genomic fragments of circa 40, 9.4, 7.8, 7..1, 4.7, 4.3, 2.7, and 2.5 kb were found to contain Lb sequences (fig. 3.3A.). The large number of fragments is not due to partial digestion of the genomic DNA, because only a single fragment was found if the same blot was hybridized with a probe for pea ribosomal DNA (not shown). We therefore conclude that the leghemoglobin multigene family in pea consists of at least six to eight members.

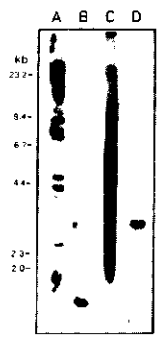
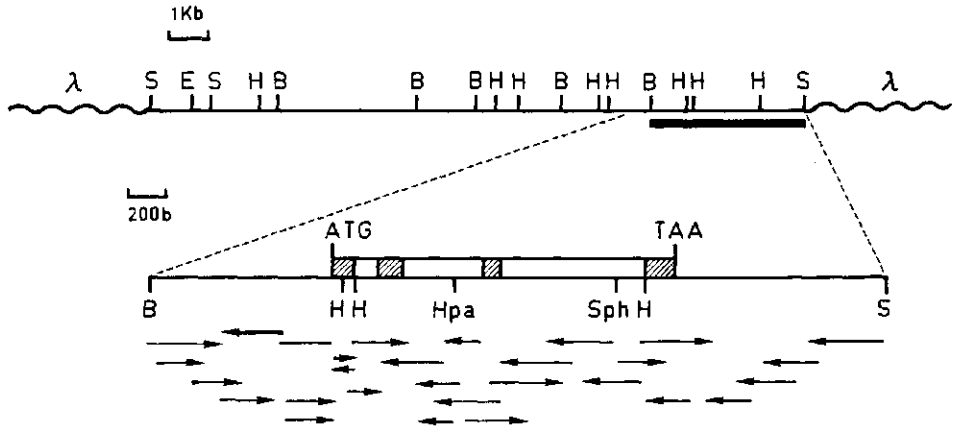


Figure 3.3. Autoradiographs of Southern blots containing pea genomic DNA digested with *Eco* RI (A) or *Hin* dIII (B-D). The blots were hybridized with pPsLb102 (A), the 5' *Pst*I - *Hind* III fragment of pPsLb102 (B), the 5' *Bam* HI - *Sal* I fragment of pPsLb1 (C), and the 3' *Hin* dIII - *Sal* I fragment of pPsLb1.

A pea genomic library of approximately 1 x 10^6 lambda plaques, which represents 1.2 genome equivalents of pea DNA if a haploid genome size of 4.0 x 10^9 nucleotides (79) is assumed (218), was screened by hybridization with a mixture of nick-translated inserts from pea Lb cDNA clones. One hybridizing phage, designated lambda-Lb1, was purified and shown to contain a 16 kb fragment of genomic pea DNA. The physical map of this 16 kb fragment of cloned pea DNA was determined (fig. 3.4.) and by hybridization with a mixture of pPsLb102 and pPsLb103, it was shown that the leghemoglobin gene sequences were contained within a 3.7 kb *Bam* HI-*Sal* I fragment located at the 3' end of the genomic fragment pea DNA. Because the cDNA in pPsLb103 did not hybridize with the 1.2 kb *Hin* dIII-*Sal* I fragment located at the extreme 3' end (not shown), the coding sequence runs from 5' to 3' in the direction from the *Bam* H1 to the *Sal* I site. Hence, the major part of the genomic DNA in Lb1 contains upstream sequences of the Lb gene. In this upstream region only one *Eco* RI site occurred, approximately 12 kb upstream of the leghemoglobin gene. Therefore, the genomic DNA fragment bearing this Lb gene should be larger than 16 kb in length. We therefore expect the gene that we have isolated is located on the 40 kb fragment identified by Southern blot analysis of *Eco* R1 digested pea DNA described above (see fig. 3.3A.).

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GA~AGGATGTCTTTGTGTGATCCCAAAGCTAATCCATAAGATGAGATAGCATGAGGGATC  -880
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AATTGGTCGATGCTTATAGAATAATGACTAAGTTACTTATTTATTTTCCATAAACTTCCA  -400
TTAATCTTAACTTGATTTGAAATGCCATATTATATTTAAAAATAAATAAAATTACCTTCA  -340
TGGTATAAATGACCATTTTAGCCTTAACCTCATATTTACGAAATATTAAAATCTCGATGA  -280
TATAATTAACTCAAGATTATAGATTATCATTTTTATTAGATTAGTTAATAGTTAATTTGA  -220
ATAAACAAAATAAATTTACAATAATGATTAATTTTTTTATTATTAATTATATTTAGTGAA  -160

AAAAGTATATTAAGTTTGGCAAAAGATTTTGTCTCTTTGATTATGTTAATGGACATCTCC  -100

AAGAGCCAATAGATAATTTTACAATAATCATCAGAAATATACTATATTTCTTTAAATCTA   -40
 -----
AATATTTTTTATATAAACAAGTATTGACTGTATTATTAC
      -----

                                                          G   F
   1 TACACTACCAACACAAAAGAAAAATACAAAGAAGAACAAGAGGAGGAGAAATATGGGTTT

       T   E   K   Q   E   A   L   V   N   S   S   W   E   L   F   K   Q   N   W   E
  61 CACTGAGAAGCAAGAAGCTTTAGTTAATAGCTCATGGGAATTATTTAAACAGAATTGGGA

       L   F   K   Q   N   P   N   Y   S   V   L   F   Y   T
 121 ATTATTTAAACAGAATCCTAATTATAGTGTTTTGTTCTACACCA/GTAAGCTTGTATCCTT
 181 GGCTTTTAGTTTTTTTTTTTTGTTAATTATTGTTTTATAAGAAGTGGTGTGAACGAAAAG
```

```
                                                  I  I  L  E  K  A  P  A  A
241 CATTAATAATTCTGGCTGATTTACTCAAAATAG/TAATATTGGAAAAAGCACCCGCAGCAA

    K  G  M  F  S  F  L  K  D  S  A  E  V  V  D  S  P  K  L  Q
301 AAGGAATGTTCTCATTTCTTAAGGATTCGGCTGAAGTAGTGGATAGTCCTAAACTTCAAG

    A  H  A  E  K  V  F  G  M
361 CCCATGCTGAAAAAGTTTTTGGAATG/GTGAGTGAATCCGTATTCAATAACTTGGGCTTCA
421 TATTTTAGTTCAACTCTCAAAATACATGGTTGTTAAATCTTATTTTAAATAATAATTGTT
481 ACAAACATAATGTTTCATTTCCAATTTCAATGAAATATTTAATAAAATTATTAAATTTTG
541 ATTTTCCATGTTCACCTTATGTAAAGTAGATTTTCTATGAATATATAAAGTTATATTGCA
601 TTCTTTATTTTAATCTAAATTTTACCTATATGATCTTATATATAGCCAAAAACATAGACC
661 ATATTCAAAAGTTAACGGGGTCAAAAAAGGTCCTTCGAGCATGATACTTCTTAGGTTGTG
721 TTTAGTTTGTGAACTATATAACTTTAAATCTTAAAATATTTATACCTTCTTAAATTACAG/

     V  R  D  S  A  I  Q  L  R  A  S  G  E  V  V  V  G  D  A  T
781 GTGCGAGACTCAGCTATTCAACTTCGAGCCTCAGGAGAAGTGGTTGTAGGGGATGCAACA

     L  G  A  I  H  I  Q  K  G  V  V  D  P  H  F  V
841 TTGGGTGCCATTCACATTCAGAAGGGAGTTGTTGATCCTCATTTTGTG/GTACGTAATATA
901 ACAAAAAAGACGCATTCTTAATTTTTTATTGGCTAATGGTCACCTATTGTCAAATTCAAT
961 AATACAACAATATATAATTGAATAAATTTATGTAAAATTAATTTTGAAAAATAAAAATAT
1021 GTCATATATTTAATCATTATTTAGCTCGGGTATATAGTATAAGTTCTTAAAGTGAGAAAA
1081 CAATAGTTGATATGATAATCAACCATGATATACTATGATTGGTCTATATACTACTAATCA
1141 TTGATTTATTAACAAAAATTGATTTCACTTCTTTTTTATACTAATGCTATATATCTCTCT
1201 ATTCTCTAAAGTTACTTGTTAATTTTAGAAGAGTCTTTCCAATAAAAAATAAATTTAATG
1261 GGACGTAACTAACAAGTTTGCAATAAAGTTATGAATTATTGCTATAAAGTTATGTTGTTT
1321 TTACTTCTTATTATGTATTCCATTTAAAAATATTATTGGCTTTAAATTAATTATATATAT
1381 TTTTTAAATTTAATAATTAAAAGTATACATTTTCAAATAAAATGACATATTTAATTTTAT
1441 ACTACTATATTGGGTCTATTAACTAATAAAGAAGCATGCAAAGGAAAATAATATTATAAA
1501 GATATTAATAATTAGATATTTTTTATTTTACAAACATACCTATTTTCAAATGTCTAAAAC

                                                  V  V  K  E  A  L  L  E
1561 TCTAACCTAAATTTAATGCTTTTGGTGCCTTGCAG/GTGGTTAAAGAAGCTTTGCTAGAAA

    T  I  K  E  A  S  G  E  K  W  S  E  E  L  S  T  A  W  E  V
1621 CCATAAAAGAAGCATCAGGAGAAAAATGGAGCGAAGAATTGAGTACTGCTTGGGAAGTTG

    A  Y  E  G  L  A  S  A  I  K  K  A  M  N  *
1681 CCTATGAAGGATTAGCATCCGCAATTAAGAAGGCAATGAATTAAACATGATATGATTTAT
1741 ATTTATAAATAAATTTAAGAAATAAGACTTGTATAACTAAATCTTGTTAAACAAGTTTAT
1801 ATAATAAATATTATTGAAAGTATAAGATCCAAATTCTCATATTACATTAGTTGTTGTTAC
1861 TTTTCTGTTCCTCTGCTAGTATGATGAGTTAGGGTATATTAGTCTAGATATATATACTAT
1921 TAGGACTTTATGGAG
```

Figure 3.4. Partial restriction map of lambda Lb1 and of the 3,7 kb *Bam* HI–*Sal* fragment containing the Lb gene. The sequencing strategy for the latter fragment is shown below the restriction map. The arrows depict the direction and the extent of sequencing. In the DNA sequence of the *Bam*HI–*Sal* fragment, the nucleotides are numbered relative to the putative point of transcription initiation, which is indicated with an arrow. The consensus sequences TATAA and CCAAT are underlined. The sequences AAAGAT and CTCTT from the organ specificity box are overlined. Amino acids are indicated in single letter code, above the coding sequence. Termination codons (*) and potential poly(A) addition sites (+++) are also marked. H, *Hin* dIII, B, *Bam* HI, E, *Eco* RI.

The 3.7 kb *Bam* HI–*Sal* I fragment subcloned into pBR322 was designated pPsLb1. Smaller fragments were subsequently subcloned in appropriate M13 vectors and sequenced using the dideoxy method. The fragments *Bam* HI–*Hin* dIII, 1.5 kb *Hin* dIII–*Hin* dIII and *Hin* dIII–*Sal* I were also subcloned into pUC vectors. Exonuclease III–generated deletions of these subclones were obtained and cloned in M13 phages for sequencing. Figure 3.4 shows the sequencing strategy and complete sequence of the *Bam* HI – *Sal* I fragment carrying the pea Lb gene.

The nucleotide sequence of the coding sequence in the leghemoglobin gene present in this genomic clone differs from the nucleotide sequence of the cDNA clones pPsLb102 and pPsLb103, indicating that the Lb mRNAs corresponding to these cDNA clones do not originate from the gene we have isolated. Strikingly, the nucleotide sequences of the 3' non-translated region are identical in the cDNA clone pPsLb101 and the genomic clone. The consensus sequences identified in the 3' non-translated region of several Lb mRNAs (183) are also present in this pea Lb mRNA. Comparison of the genomic sequence with the cDNA sequences revealed that the Lb gene contains three introns, all of which obey the Breatnach–Chambon rules for intron/exon boundaries. The positions of the introns coincide with the positions of the three introns found in other (leg)hemoglobin genes from soybean, French bean, *Parasponia* and *Trema*. Except for the central intron, which appears to be unique for plant hemoglobin, the other two are also found in the animal globins at corresponding positions. The conserved position of these introns is an indication of the existence of a common ancestral gene present well before the radiation of plants and animals.

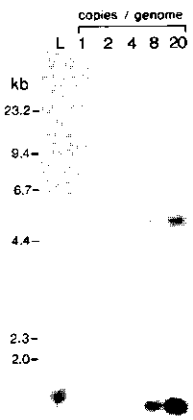


Figure 3.5 Reconstruction analysis to estimate the number of genes that contain the 1.5 kb *Hin* dIII fragment. Autoradiograph of a Southern blot containing *Hin* dIII–digested genomic pea DNA and different amounts of *Hin* dIII– digested DNA from clone pPsLb1, corresponding to the genome equivalents indicated. The blot was hybridized with nick-translated pPsLb102 as probe. Comparison of the intensities of hybridization of the 1.5 kb *Hin* dIII fragment from genomic DNA with the hybridization of the cloned fragment, indicates that six to eight genes have this *Hin* dIII fragment in common.

When *Hin* dIII digested pea DNA was hybridized with the 150 bp fragment of pPsLb102 covering the 5' end of this cDNA clone, only one hybridizing fragment of 1.5 kb was found (fig. 3.3B.). This result suggests that all members of the pea Lb gene family have this fragment in common. Indeed six to eight genes were estimated to contain this 1.5 kb fragment in a reconstruction experiment in which a concentration range of *Hin* dIII–digested pPsLb1 was used to calibrate the number of genes (fig. 3.5.). Since the 1.5 kb *Hin* dIII fragment contains all three introns, we conclude moreover that the intron size in the pea Lb gene family does not vary as strong as in Lb genes of soybean.

Southern blot analysis of *Hin* dIII digested pea DNA was also used to explore the flanking regions of the isolated pea Lb gene. After hybridization of Southern blots containing *Hin* dIII–digested pea DNA with the 1.0 kb *Bam* HI–*Hin* dIII fragment, covering the 5' end of the gene as probe, a smear was obtained (fig. 3.3C.). Such a hybridization signal may indicate that a highly repetitive sequence is present 5' upstream of the pea Lb gene. Hybridization of Southern blots of HindIII digested pea DNA with the 1.2 kb *Hin* dIII–*Sal* I fragment covering the 3' end of the gene revealed a major hybridizing fragment of 2.6 kb (fig. 3.3D). These findings show that a number of Lb genes have a *Hin* dIII site at a conserved site, approximately 2.5 kb downstream from the stop codon, suggesting that the 3' downstream region of these genes is conserved as well.

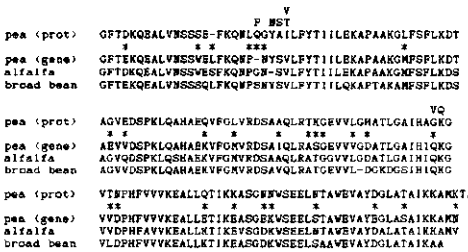


Figure 3.6. Comparison between the amino acid sequences of pea leghemoglobin, as determined by protein sequencing (prot), as derived from the nucleotide sequence of the coding region of the pea Lb gene (gene), and the amino acid sequences of broad bean and alfalfa leghemoglobin. The amino acid sequences are shown in single letter code. The published ambiguities in the pea protein sequence (201) are incorporated in the figure. The differences between the two pea amino acid sequences are indicated by *.

3.1.3. **Amino acid sequence of pea leghemoglobin**

The amino acid sequence for pea Lb derived from the nucleotide sequence of the gene differs considerably from the amino acid sequence of Lb obtained by direct protein sequencing (24). The insertion, deletion and-substitutions of different amino acids are shown in fig. 3.6. Such differences may be explained by assuming that the two amino acid sequences concern different genes from the pea leghemoglobin gene family, or they may result from protein sequencing errors. Based on the protein sequence data, an evolutionary tree has been constructed (222), in which the pea leghemoglobin is most closely related to the broad bean (*Vicia faba*) and the alfalfa (*Medicago sativa*) leghemoglobins, in accordance with supposed taxonomical relationships between these legumes. The corrections on the pea leghemoglobin amino acid sequence presented here result in an even greater homology between the leghemoglobin protein sequences of these leguminous plants (fig. 3.6).

3.1.4. **Analysis of the promoter region of the pea leghemoglobin gene**

The canonical sequence TATAA is located 80 nucleotides upstream from the ATG initiation codon of the coding sequence (fig. 4). In soybean, the cap addition site, as determined by S1 nuclease mapping (39) or primer extension (314), occurs 31 or 32 nucleotides downstream from the TATAA box. In analogy, we estimate the starting point of transcription of this pea gene to be at position −50 relative to the initiation codon. Following the conventions in the sequence analysis of genes, this putative point of transcription initiation will be indicated as position +1. Upstream from the TATA box the CCAAT consensus sequence is found. Thus, the consensus *cis*-acting regulatory sequences characterizing a functional promoter are present in the isolated pea Lb gene.

For the soybean Lbc_3 gene, it has unequivocally been demonstrated that the 2 kb region upstream of the start of transcription carries all information for the nodule specific and developmentally correct expression of the soybean gene in heterologous legume hosts (314,315). This promoter region has been characterized in deletion studies in which the various shortened promoters were cloned before the CAT coding sequence (316). A strong enhancer activity was found in the sequence between the positions −1100 and −950. The presence of this region stimulated the expression of the chimeric gene, measured as CAT activity, ten fold. The CaMV enhancer was able to substitute the natural enhancer (316). The sequence of the enhancing element has not been published yet, so it is impossible to decide whether a similar enhancer may be present in the promoter of the pea Lb gene. A *cis*-regulatory element involved in organ specificity was shown to be located within the −139 to −102 region of the soybean Lbc_3 promoter (316). In this region, the sequences 5'AAAGAT and 5'CTCTT are present. These two sequences occur in the 5'upstream regions of all late nodulin genes, *i.e.* the leghemoglobin genes, the N23 gene and four genes encoding proteins of the peribacteroid membrane (284). These so-called late nodulin genes are practically simultaneously expressed in the root nodule during

nodule development. It is assumed that they share common regulatory sequences. The occurrence of the two sequences from the 'organ specificity box' in all late nodulin genes analyzed strongly indicates their involvement in the nodule specificity of gene expression. Both sequences also occur in the promoter region of the pea Lb gene (fig. 3.4). This adds to the notion that the mechanism of the regulation of nodulin gene expression is conserved in different leguminous species.

Recently two A–T rich sequences, 5'CTTAAATTATTTATTT and 5'GATATATTAA-TATTTTATTTTATA respectively, have been identified in front of the "organ specificity box" of the promoter of the soybean Lbc_3 gene. These two A–T rich sequences were shown to bind a protein by gel retardation studies. Synthetic oligonucleotides of these sequences could each compete out the binding of the transacting factor to either sequence, indicating that both sequences bind the same *trans*–acting factor (E.Ø. Jensen and K.A. Marcker, pers. com.). The sequences involved in binding the soybean *trans*–acting factor do not match exactly with sequences in the promoter of the pea Lb gene, although also the pea promoter region is extremely A–T rich. The soybean *trans*–acting factor is nodule specific and occurs in relatively high concentrations in the nodule. Extracts from alfalfa and *Sesbania* nuclei also cause gel retardation of the DNA fragment containing the soybean Lb promoter, indicating that these legumes possess a similar *trans*–acting factor (F. de Bruijn, pers. com.). It seems likely, therefore, that also in pea a similar *trans* –acting factor is present. The sequences in the promoter of the pea Lb gene that bind this factor are unknown.

The structural comparison of the pea Lb gene with the soybean Lbc_3 gene conducted here has defined several putative regulatory elements in the pea Lb gene that may be involved in the nodule specific regulation of transcription. A functional analysis of these elements will contribute to our understanding of the mechanism of transcription of leghemoglobin. The evidence presented in this paper favors the view that the leghemoglobin gene analyzed here is an actively expressed gene. The final proof can, however, only be obtained by transfer of the isolated gene into a legume plant. The expression of this pea leghemoglobin gene in heterologous leguminous species is currently under investigation.

3.2. Material and methods

Plant material. Pea plants *(Pisum sativum* cv. Rondo, Cebeco Lelystad, The Netherlands) were cultured and inoculated with wild-type *Rhizobium leguminosarum* PRE as described. Seventeen days after sowing and inoculation, nodules were picked and immediately frozen in liquid nitrogen and stored at -80°C.

Bacterial strains, plasmids and phages. *E. coli* strains JM109 (232) and DH5alpha-F' were used for propagation of recombinant derivatives of pUC and Bluescript plasmids and M13 bacteriophages. Growth conditions, transformation and transfection were essentially as described (232). *E. coli* RR1 (28) was used for propagation of cDNA clones and clones containing pBR322 derivatives. *E. coli* K803 was used for initial plating of the library in the lambda replacement vector EMBL3 (114). Plaque purifications were performed with the *E. coli* strains K803, Q359 and Q364 (114,218).

DNA technology. Restriction enzymes, T4 ligase, Exonuclease III and Klenow polymerase were obtained from Boehringer, BRL or Promega Biotec, and were used according to standard procedures (218). Southern analysis of DNA was performed by digesting DNA with restriction endonucleases, separating the DNA fragments by agarose gel electroforesis and blotting the DNA fragments onto nitrocellulose using standard techniques. Blots were hybridized at 65°C with nick-translated DNA probes or labeled M13 probes as previously described (126). Pea DNA was isolated according to (374). Plasmid DNA was isolated by the alkaline lysis method (24), and large scale plasmid preparations were further purified by CsCl density gradient centrifugation (218). Standard techniques were used for obtaining deletions by exonuclease III treatment (153). Plaque purifications and large scale DNA isolation from liquid lysates of recombinant phage were performed according to (218).

cDNA library screening and hybrid-released translation. Colonies from a nodule cDNA library were screened with nick translated insert from the pea Lb cDNA clone pPsLb101 as described (129). Characterization of the Lb cDNA clone pPsLb102 by hybrid released translation was performed according to Govers *et al.* (129).

Construction and screening of a pea genomic library. Pea DNA was partially digested with Sau3a, and size-fractionated by centrifugation on 10–40% sucrose gradients. Fractions containing DNA of 15–20 kb were pooled and the DNA was precipitated with ethanol. The lambda vector EMBL3 was digested with BamHI and EcoRI, phenol extracted and precipitated with isopropanol. After ligation, and packaging according to standard procedures (218), the packaging mix was plated onto square petri dishes using *E. coli* K803 as a host. The resulting phage lawn was replicated onto nitrocellulose and screened with nick translated leghemoglobin cDNA clones. Pure plaques were obtained after 3 rounds of purification.

DNA sequencing. Standard techniques were used for cloning into M13 and plasmid vectors and for dideoxy sequencing (232, 285). Analysis of the DNA sequence was performed on a microVax/VMS computer using the Staden DNA sequence analysis programs (313).

RHIZOBIUM NOD GENES ARE INVOLVED IN THE INDUCTION OF TWO EARLY NODULIN GENES IN *VICIA SATIVA* ROOT NODULES

Chapter 4

Rhizobium nod genes are involved in the induction of two early nodulin genes in *Vicia sativa* root nodules

By using *Rhizobium* mutants (for review, see 357) and *Agrobacterium* strains that contain parts of the *Rhizobium* genome (152,163,330,368) different groups have demonstrated that it is possible to arrest root nodule formation at different stages of development. Most of these studies were aimed at understanding the *Rhizobium* part of symbiotic nitrogen fixation. But on the premise of a correlation between a blockade in development and nodulin gene expression, the same *Rhizobium* and *Agrobacterium* strains can provide insight into the bacterium-legume interaction from the plant's point of view.

Studies on nodulin gene expression in non-effective nodules induced by different types of *Rhizobium* symbiotic mutants have shown the expression of all nodulin genes known so far (116,126,190,296). However, in the case of non-effective nodules induced in pea by *A. tumefaciens* carrying a *R. leguminosarum* sym plasmid, we have found the exclusive expression of the early nodulin gene PsENOD2, a nodulin gene that is expressed well before the leghemoglobin genes (128). The nodules formed by this *Agrobacterium* transconjugant are devoid of intracellular bacteria and are therefore called "empty". On common vetch *(Vicia sativa*, subsp. *nigra)* the same *Agrobacterium* transconjugant is able to form nodules in which, in contrast, a number of plant cells is filled with bacteria (163). Thus the developmental program of the vetch nodules is arrested at a later stage than that of the pea nodules. This difference could provide a clue for the requirement of an intracellular location of bacteria in the induction of expression of nodulin genes. We, therefore, studied vetch root nodule formation in more detail.

We report the identification and time-course of expression of nodulin genes during development of vetch wild-type nodules. Also the expression of nodulin genes both in nodules induced by *Rhizobium* strains carrying essentially the nodulation region of a sym plasmid and in nodules formed by an *Agrobacterium* transconjugant with a complete sym plasmid was studied. The use of such an engineered *Agrobacterium* strain to study nodulin gene expression requires knowledge on the inductive capacities of the *Agrobacterium* genome itself. Therefore, nodulin gene expression in tumors formed on vetch was surveyed.

4.1. RESULTS

4.1.1. Nodulin mRNAs in vetch nodules.

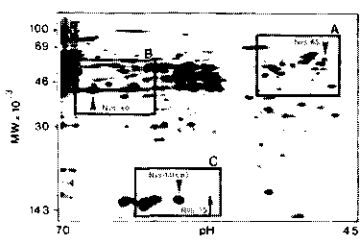
Nodulin mRNAs in vetch root nodules induced by *R. leguminosarum* PRE were identified by comparison of the 2-D gel electrophoresis pattern of the *in vitro* translation products of root (not shown) and nodule RNA (fig. 4.1.) respectively. This comparison showed that 15 polypeptides were exclusively detected in the translation products profile of nodule RNA, whereas all other polypeptides that can be identified are present in both roots and nodules. Hence these 15 polypeptides represent nodulin mRNAs.

The major nodulins that we will focus our analyses on, are indicated by arrowheads in fig. 4.1. They have apparent molecular weights of 14.000, 40.000, and 65.000 respectively. Also a nodule-reduced polypeptide with a molecular weight of 15.000 is indicated by an arrow, that is present in root and diminishes during nodule development. Adding the plant species initials in lower case to the nodulin

nomenclature established earlier (345, see chapter 6.), we indicate these nodulins by Nvs–14, Nvs–40 and Nvs–65 and the nodule–reduced polypeptide by Rvs–15. The Nvs–14 nodulin was selected out of a group of four prominent nodulin spots, that most probably are the vetch leghemoglobins because of their relative abundance and molecular weight.

About 10 to 11 days after sowing and inoculation wild–type *Rhizobium* nodules on vetch start to fix nitrogen under our growth conditions. This coincides with the time at which Nvs–14 (vetch Lb) can be first detected (fig. 4.1C.). Already 8 days after sowing and inoculation both Nvs40 and Nvs–65 (figs. 4.1A. and 4.1B.) are detectable. But Nvs–65 is only a minor spot, while Nvs–40 is already present at its maximal intensity. It follows from these observations that the Nvs–40 gene is expressed well before the Nvs–65 and Nvs–14 genes. According to previously published classifications (125), Nvs–40 can therefore be regarded as an early nodulin.

The cDNA clones pGmENOD2 (112, chapter 2.) and pPsLb101 (28, chapter 3.) represent nodulin genes expressed at different stages of nodule development. Clone pGmENOD2 was isolated from a soybean (*Glycine max*) nodule cDNA library and it was shown that in both pea (128) and soybean (112) the corresponding gene is expressed one week before the Lb genes. Hence ENOD2 can be regarded as a marker for early processes in root nodule development. Clone pPsLb101 is a pea (*Pisum sativum*) leghemoglobin cDNA clone (28), representing the class of nodulin genes expressed at later stages of development. Both cDNA clones strongly cross–hybridize with vetch nodulin mRNAs with a length of 1500 and 700 nucleotides respectively (fig. 4.4.). The vetch ENOD2–homologous gene is expressed well before the vetch Lb–genes, indicating that in vetch nodule development also an ENOD2–like early nodulin gene



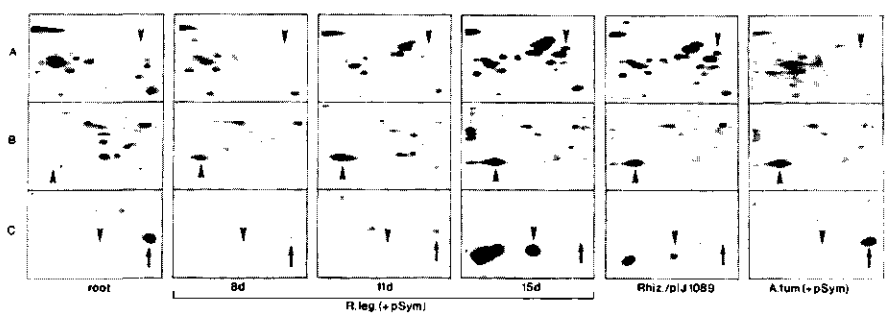


Figure 4.1. Identification of vetch nodulin mRNAs and the expression of three major nodulin genes and a nodule–reduced gene in nodules induced by *R. phaseoli* pIJ1089 and *A. tumefaciens* LBA2712 and in the development of nitrogen fixing vetch root nodules as a function of time.

In the upper part of the figure a fluorograph is shown of a 2–D gel of *in vitro* translation products from total RNA isolated from effective vetch root nodules 15 days after sowing and inoculation with *R. leguminosarum* PRE. The major nodulin spots Nvs–65, Nvs–40 and Nvs–14 are indicated by arrowheads and the nodule–reduced polypeptide Rvs–15 is indicated with an arrow. ^{14}C–methylated molecular weight markers included phosphorylase b, bovine serum albumine, ovalbumin, carbonic anhydrase and lysozyme, and are indicated in kilodalton.

In the lower part of the figure fluorographs are shown of *in vitro* translation products of total RNA isolated from 8–day–old, uninfected vetch roots, from vetch root nodules 8, 11 and 15 days after sowing and inoculation with *R. leguminosarum* PRE, and from nodules induced on vetch roots by *R. phaseoli* pIJ1089 and *A. tumefaciens* LBA2712 as indicated. Only the parts of the gels within the squares indicated in the upper part are shown, as these contain the major nodulin spots. The comparison is shown for Nvs–65 in (A), Nvs–40 in (B) and Nvs–14 (vetch Lb) and Rvs–15 in (C).

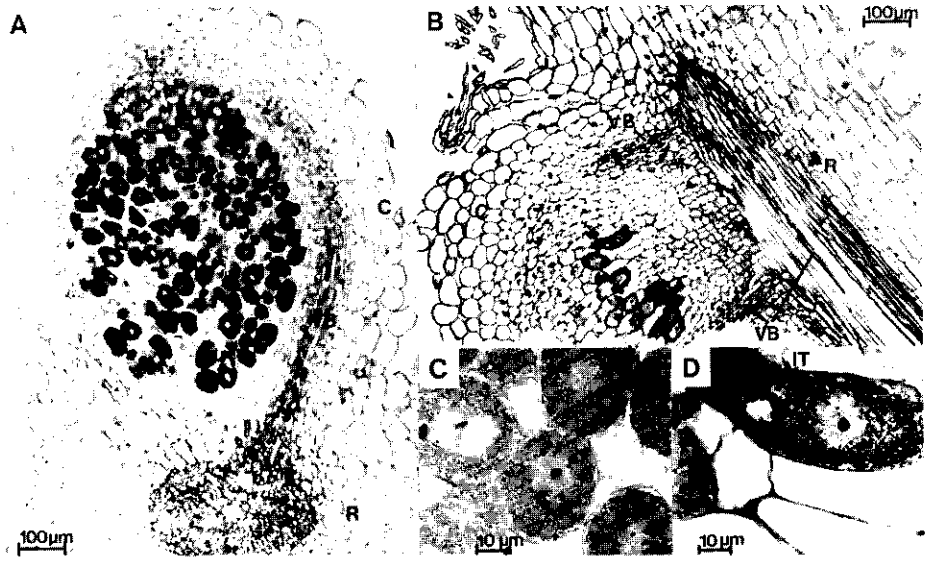


Figure 4.2. Cytology of nodules on vetch roots induced by *R. phaseoli* plJ1089 and the *Agrobacterium* transconjugant LBA2712.

Light micrograph of a longitudinal section through (A) a nodule isolated 15 days after sowing and inoculation with *R. phaseoli* plJ1089 and (B) nodule isolated 14 days after sowing and inoculation with LBA2712. The vascular bundle (VB), root (R) and cortex (C) are indicated. In (C) and (D) a magnification of the infected area is shown. Infected cells (IC) and infection threads (IT) are indicated.

operates.

4.1.2. Involvement of the sym plasmid in inducing nodulin gene expression

To assess the potentials of the sym plasmid in the induction of vetch nodulin gene expression, we studied nodules formed by a *R. phaseoli* strain cured of its sym plasmid, but containing a cosmid derived from a *R. leguminosarum* sym plasmid gene library (table 4.1.). Two cosmids, plJ1085 and plJ1089, contain a 10 kb overlapping part of the *R. leguminosarum* sym plasmid pRL1JI (91). Upon transfer of either cosmid to a cured *R. phaseoli* strain, the recipient strain regains the ability to form nodules on pea (91.128) as well as on vetch. These nodules are non-effective due to the absence of *fix* genes, but their morphology and development appear to be normal (fig.4.2.). The 2-D pattern of the major (fig. 4.1.) and minor nodulin spots obtained after the *in vitro* translation of RNA from 15-day-old nodules induced by these cosmid containing rhizobia was found identical to the pattern obtained from vetch wild-type nodule RNA. Northern blot analysis of the RNAs also showed the presence of both early nodulin (vetch ENOD2) and Lb transcripts (not shown).

4.1.3. Role of the *Rhizobium* chromosome in inducing nodulin gene expression

The contribution of genes on the *Rhizobium* chromosome to the induction of nodulin genes was studied by analyzing nodules formed by an *A. tumefaciens* cured of its Ti-plasmid but bearing a *R. leguminosarum* sym plasmid, pSym1, instead (table 4.1.) (161). This *Agrobacterium* transconjugant, designated LBA2712, has been shown to form "empty" nodules on pea roots (128), *i.e.* nodules without cells infected with rhizobia. The nodules on the roots of vetch, however, do contain infected cells (fig. 4.2.) in which the intracellular bacteria are surrounded by a peribacteroid membrane (163). Infection threads

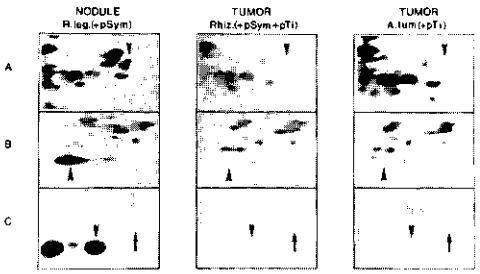


Figure 4.3. Expression of nodulin genes in vetch root nodules and tumors induced by different *Rhizobium* and *Agrobacterium* strains. Fluorographs of the *in vitro* translation products of total RNA isolated from nodules and tumors induced by the strains indicated. Only the parts of the 2-D gels within the squares indicated in the upper part of Fig. 1. are shown as these contain the nodulins of interest. The comparison is shown for Nvs-65 in (A), Nvs-40 in (B) and Nvs-14 and Rvs-15 in (C).

are present and vascular bundles are found at the periphery of the nodule, which is characteristic for normal nodules. The size of the infected cells is comparable to that of infected cells in normal nodules, but they are fewer in number relative to the nodule area.

We examined nodulin gene expression in nodules induced by LBA2712 on vetch plants isolated 14 (fig. 4.1.) and 20 (not shown) days after inoculation. The 2-D gel pattern shows the exclusive expression of the early nodulin gene Nvs-40, that is expressed at wild-type levels. None of the other nodulins can be detected (fig. 4.1.), whereas the nodule-reduced polypeptide Rvs-15 is present in amounts comparable to uninoculated roots. Northern blot analyses also showed (fig. 4.4.) the presence of the early (vetch ENOD2) nodulin transcript, whereas Lb transcripts could not be detected in the RNA from nodules induced by the Agrobacterium transconjugant, even after prolonged exposure (fig. 4.4.).

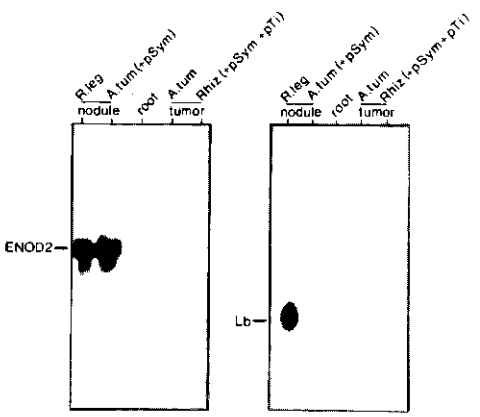


Figure 4.4. Expression of ENOD2 and Lb genes in vetch roots, root nodules and tumors. Autoradiographs of Northern blots containing RNA from roots, nodules and tumors induced by the strains indicated, hybridized with pGmENOD2 and pPsLb101 as indicated.

4.1.4. Role of the *Agrobacterium* chromosome in inducing nodulin gene expression in tumors

Because early nodulin genes are expressed in nodules formed by the *Agrobacterium* transconjugant, we examined the ability of the *Agrobacterium* chromosome to induce the genes in tumors that have been identified as early nodulin genes in nodules. Neither the analysis of the *in vitro* translation products from tumor RNA (fig. 4.3.) nor Northern blot analysis with pENOD2 as probe (fig. 4.4.) revealed the presence of any early nodulin transcripts. Also in tumors formed by *R. trifolii* LPR5076, a strain containing both a sym- and a Ti-plasmid (this strain is LPR5055 (163) with an additional *R. leguminosarum* sym plasmid) no expression of the early nodulin genes Nvs-40 (fig. 4.3.) and VsENOD2 (fig. 4.4.) was found, although the same *Rhizobium* strain is able to induce the formation of nitrogen fixing nodules on roots of vetch (not shown).

4.2. DISCUSSION

On vetch roots an *Agrobacterium* transconjugant harbouring a *R. leguminosarum* sym plasmid forms nodules in which cells are filled with bacteria, whereas the same transconjugant forms "empty" nodules on pea roots. Both early nodulin genes Nvs-40 and VsENOD2 are expressed in the nodules with infected cells formed on vetch *(V. sativa)*, while in the "empty" pea *(P. sativum)* nodules the early nodulin Nps-40' cannot be detected.

The vetch nodulin Nvs–40 can be immunoprecipitated with an antiserum directed against pea Nps–40' (25) so Nvs–40 and Nps–40' are structurally and functionally identical nodulins. The difference in occurrence of these Nvs–40/Nps–40' nodulins correlates with the difference between both types of nodules regarding the presence of cells infected with bacteria. The expression of the vetch Nvs–40 gene may therefore be linked to the release of the bacteria into the plant cells.

No expression of the Lb genes, or of all other nodulin genes normally expressed concomitantly with the Lb genes, was observed in the vetch nodules formed by the *Agrobacterium* transconjugant. Since the number of cells infected with bacteria in these nodules is about 30% of the number that occurs in wild-type nodules (fig. 4.2.), Lb–mRNA would have been detected by our methods of analysis, provided there was wild–type level of Lb gene expression in the infected cells. Therefore, it can be concluded that despite the intracellular location of the bacteria, the genetic information of the total sym plasmid in an *Agrobacterium* chromosomal background is not sufficient to induce Lb genes. Apparently the signal that is responsible for the induction of these genes is not related to an intracellular location of the bacteria, and differs from the signal that triggers early nodulin gene expression. Interestingly, a root polypeptide that diminishes during normal nodule development, did not diminished in the nodules formed by the *Agrobacterium* transconjugant.

In vetch nodules formed by two cured *R. phaseoli* strains containing respectively the cosmids pIJ1085 and pIJ1089 – which have a 10 kb *nod* region of a *R. leguminosarum* sym plasmid in common (91) – all known nodulin genes are normally expressed. It follows that the 10 kb *nod* region of the sym plasmid in a *R. phaseoli* chromosomal background carries all information required for the induction of the expression of nodulin genes. In combination with the analyses of the vetch nodules formed by the *Agrobacterium* transconjugant it can be concluded that the *nod* genes encode the signal for the induction of the expression of the two early nodulin genes, Nvs–40 and VsENOD2. It appears that the *nod* genes are not involved in the induction of the Lb genes. Since an *Agrobacterium* equipped with a sym plasmid does not give rise to normal nodules and *A. tumefaciens* itself does not elicit nodulin gene expression in tumors, it is unlikely that the *Agrobacterium* chromosome makes any contributions to the induction of the expression of nodulin genes. Consequently, the signal involved in the induction of the Lb genes has to be encoded by *Rhizobium* genes located outside the sym plasmid.

Although these conclusions seem justified by the results presented, two points may be raised. First, analyses of RNA from tumors induced by a *Rhizobium* carrying both a sym and a Ti plasmid showed no nodulin gene expression while the same *Rhizobium* induces effective nodules on vetch roots. Apparently a bacterium harboring a complete and functional set of genetic information for nodule formation is not able to induce the expression of any nodulin gene in a tumor. This result might weaken the conclusion that the *Agrobacterium* chromosome does not contribute to the expression of early nodulin genes. It is known, however, that the expression of both the *vir* genes of *Agrobacterium* (312) and the *nod* genes of *Rhizobium* (236,281) are induced by compounds excreted by the host plant. Acetosyringone, which induces the *vir* genes, has been shown to be preferentially synthesized in wounded plant tissue (311). The *nod* genes are not induced by acetosyringone, but by flavones (106,371). In fact, the expression of the *nod* genes appears to be repressed by acetosyringone (106), so it is unlikely that upon formation of a tumor the *nod* genes are expressed in *Rhizobium* carrying both a sym and a Ti plasmid. The lack of *nod* gene products could explain the absence of early nodulin gene expression in tumors.

Second, in the "empty" pea nodules induced by the *Agrobacterium* transconjugant only the PsENOD2 gene was found to be expressed, while Nps–40' was not detectable. Using the same rationale as above, we have concluded that in pea the *nod* genes are only responsible for the induction of the expression of the early nodulin gene PsENOD2. We now conclude that in vetch the *nod* genes are involved in the induction of the expression of both Nvs–40 and VsENOD2. The reason for this difference between pea and vetch is not clear. Since Nps–40' is similar to Nvs–40, it seems likely that both early nodulin genes will be subject to the same regulation mechanism(s). Consequently, the *Agrobac–*

Table 4.1. Rhizobium and *Agrobacterium* strains used in this study.

Chromosomal background	Strain	Relevant characteristics				Reference or source
Rhizobium leguminosarum	PRE	pSym			$nod^+fix^+vir^-$	(210)
Rhizobium phaseoli	pIJ1085		pcR1		$nod^+fix^-vir^-$	(91)
	pIJ1089		pcR1		$nod^+fix^-vir^-$	(91)
Rhizobium trifolii	LPR5076	pSym		pTi	$nod^+fix^+vir^+$	Hooykaas
Agrobacterium tumefaciens	LBA2712	pSym			$nod^-fix^-vir^+$	(161)
	T37			pTi	$nod^-fix^-vir^+$	Hooykaas

pSym, sym plasmid from *R. leguminosarum*; nod, ability to nodulate; fix, *in planta* nitrogen fixation; pcR1, cosmid from a *R. leguminosarum* sym plasmid gene library; vir, virulent.

terium transconjugant will, in principle, be able to induce the expression of the Nps–40' gene in pea, an induction for which the *nod* genes are responsible. Extrapolating, this might implicate that the *nod* genes may be involved in the induction of the expression of all nodulin genes. The absence of the expression of the Nps–40' gene in pea suggests that there may be a difference in necessity of the presence of *Rhizobium* chromosomal genes. These chromosomal genes need not be essential in all host plants, analogous with what has been shown for some *nod* genes of *Rhizobium* (87,164) and *vir* genes of *Agrobacterium* (160,369).

Alternatively, the *Agrobacterium* transconjugant may be recognized by the plant in a different way than *Rhizobium* and may be subject to plant defense mechanisms. If this is the case, the importance of such transconjugants in the study of the *Rhizobium*–legume interaction becomes limited. If the presence of genetic information for the induction of a (part of the) developmental program is no guarantee for the actual realization of that induction – because it can be counteracted by the defense response of the host plant – then such a system does not allow conclusions with respect to the *Rhizobium* genes that encode the signals responsible for the induction of the plant genes that are found to be ***not*** expressed.

4.3. MATERIALS AND METHODS

Cultivation of plants and bacteria. *Vicia sativa* L. subsp. *nigra* (L.) seeds were sterilized and germinated as described (341). Germinated seeds with roots approximately 1 cm in length were placed in gravel trays, inoculated and cultured as described for pea plants (29). In table 4.1. the relevant characteristics of the *Rhizobium* and *Agrobacterium* strains used in this study are listed. Strains were maintained as –80°C stocks frozen in 10% glycerol and were grown as described (22). Nodules were excised with a scalpel, together with small pieces of root, or, depending on their size, were picked from the root with tweezers. Root tips from uninfected plants were isolated 8 days after sowing. Tumors were induced by wounding the stem of 8-day-old vetch plants with the appropriate strain. Tumor tissue was harvested 14 days after wounding. All tissues were immediately frozen in liquid nitrogen and stored at –80°C until use.

RNA isolation, *in vitro* translation and 2-D gel electrophoresis. Total RNA from plant tissue, isolated as described (126) was translated *in vitro* in a rabbit reticulocyte lysate. Typically 2 microgram total RNA was translated in a 6 microliter reaction mixture according to standard procedures (256). The *in vitro* translation products were separated by 2-D gel electrophoresis essentially according to O'Farrell (21) as described previously (126).

Northern blotting and hybridization. Total vetch RNA was denatured in DMSO/glyoxal (218), separated on agarose gels and subsequently transferred to GeneScreen membranes (New England Nuclear). The membranes were hybridized with ^{32}P-labeled probes (218) under the conditions previously described (128).

Cytology. Nodules were picked and fixed for 16 hr in 3% glutaraldehyde buffered with sodium phosphate at pH = 7.5. After washing and dehydration in an ethanol series, the nodules were embedded in Technovit 7100 (Kulzer), cut into 5 micrometer sections, stained with toluidine blue and examined under a light microscope.

THE RELATIONSHIP BETWEEN NODULIN GENE EXPRESSION AND THE *RHIZOBIUM NOD* GENES IN *VICIA SATIVA* ROOT NODULES

Chapter 5

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

Leguminous plants are distinguished from other plant families by their ability to form nitrogen fixing root nodules in close cooperation with bacteria from the genera *Rhizobium* or *Bradyrhizobium*. In several leguminous species, approximately thirty plant genes, so-called nodulin genes (345), have been identified that are exclusively expressed in the root nodule (126,200,349). In recent years, it has been found that root nodule formation is attended by a differential expression of nodulin genes. This has resulted in a division of nodulin genes into two distinct classes, early and late nodulin genes.

Early nodulin genes are expressed well before the onset of nitrogen fixation, and early nodulins are most likely involved in the formation of the nodule structure and/or the infection process (122,128). The best studied example, so far, is the soybean early nodulin Ngm-75. Ngm-75 has a protein sequence containing about 45 % (hydroxy)proline and it probably is a cell wall constituent (112, chapter 2.). Late nodulin gene expression starts around the onset of nitrogen fixation, and late nodulins probably function in establishing and maintaining the proper conditions within the nodule that allow nitrogen fixation and ammonia assimilation to occur. Type members of the class of late nodulin genes are the leghemoglobin genes.

Insight in the mechanisms of nodulin gene regulation may be gained by studying the involvement of *Rhizobium* in the induction of nodulin gene expression. The *Rhizobium* genes required for nodulation (*nod* genes) are located on a large plasmid, the so-called sym plasmid. Based on analyses of the phenotypes of Tn5 mutants, four genes, *nodD, A, B,* and *C,* have been found to be absolutely essential for nodulation. Mutations in the *nodE, F, I,* or *J* genes do not cause an inability to induce a nitrogen fixing root nodule, but result in a delayed nodulation and a smaller number of nodules (90). It thus appears that *Rhizobium* gene-derived signals are important in the regulation of nodulin gene expression.

In the present study, we aim at proving that the *Rhizobium nod* genes are involved in the induction of nodulin gene expression. For that purpose, we have analyzed nodulin gene expression in nodules induced by engineered *Rhizobium* and *Agrobacterium* strains carrying the *nod* region. As a test plant, we have used vetch (*Vicia sativa* subsp. *nigra*), because this small leguminous plant responds rapidly to inoculation with such engineered bacteria (340).

By analyzing the *in vitro* translation products of vetch nodule and root RNA respectively, on two-dimensional (2-D) gels, we have previously identified one early, Nvs-40, and 15 late nodulin genes (234, chapter 4.). Late nodulin genes included the genes for the leghemoglobins, and Nvs-65. A second early nodulin, VsENOD2, was identified by Northern blot analysis using the soybean early nodulin cDNA clone pGmENOD2 (chapter 2.), which encodes Ngm-75, as a probe. Investigations using overlapping cosmid clones allowed to draw the conclusion that 10 kb *nod* region of the sym plasmid is sufficient for the induction of early and late nodulin gene expression, if present in a rhizobial chromosomal background. Because in nodules induced by an *Agrobacterium* strain carrying the complete sym plasmid, early but no late nodulin genes were expressed (chapter 4.) we tentatively concluded that the 10 kb *nod* region on the

sym plasmid carries at least the information for the induction of early nodulin gene expression.

Here we present definitive evidence that the *Rhizobium nod* genes are involved in the induction of the expression of early nodulin genes. Our results further indicate that the *nod* genes are in some way involved in the induction of the expression of late nodulin genes. Analysis of nodulin gene expression in nodules increasingly disturbed in development, shows that the process of root nodule development can be divided in successive steps each characterized by the start of expression of defined nodulin genes.

5.1. RESULTS

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

We have used strain *R. leguminosarum* 248^{c}(pMP104) which contains the cloned 12 kb *nod* region of the sym plasmid of *R. leguminosarum* . A physical and genetic map of the 12 kb *nod* region carrying the *nodE, F, D, A, B, C, I,* and *J* genes, inserted into a low-copy-number *Inc* P vector to yield plasmid pMP104 (308), is given in fig. 5.1. Strain 248^{c}(pMP104) has the ability to induce nodule structures on both vetch and pea. After introduction of pMP104 in the sym plasmid-cured *R. trifolii* strain ANU845, the resulting strain ANU845(pMP104) also obtained the ability to induce nodules on vetch and pea. The histology of the nodules induced on vetch by ANU845(pMP104) (fig. 5.4A) and by 248^{c}(pMP104) is similar to that of the nodules induced by wild-type *R. leguminosarum* (243). These nodules have an apical meristem, peripherally located vascular bundles and a central tissue containing uninfected cells and infected cells fully packed with bacteroids. The bacteroids develop into Y-shaped forms (fig. 5.4D), but fail to fix nitrogen due to the absence of *nif* and *fix* genes.

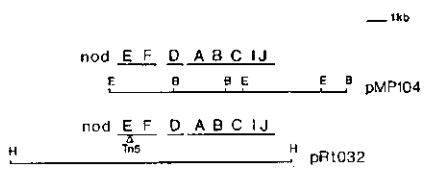


Figure 5.1. Simplified physical and genetic maps of the *nod* region derived from the sym plasmid pRL1JI of *R. leguminosarum*, as present in pMP104, and the *nod* region derived from the sym plasmid pANU843 of *R. trifolii*, as present in pRt032. With the Tn5 in *nodE* at position K11, the latter plasmid becomes pRt032(*nodE* K11::Tn5). The maps are aligned to stress their similarities. The nomenclature of nod genes is according to (308). H, *Hin*dIII; E, *Eco*R1, B, *Bam*H1.

Irrespective of the *Rhizobium* chromosomal background, both early and late nodulin genes are expressed in the nodules induced on vetch by each of the strains containing pMP104. In fig. 5.2. the expression of three major nodulin genes, Nvs-40, Nvs-65 and vetch Lb, is shown for the ANU845(pMP104)-induced nodules. All other identified late nodulin mRNAs are also present (data not shown). The presence of the early nodulin VsENOD2 is demonstrated by Northern blot analysis using pGmENOD2 as probe (fig. 5.3.). These results prove conclusively that the *nod* region is the only part of the sym plasmid that is essential for the induction of early and late nodulin gene expression.

5.1.2. The role of the Rhizobium chromosome in nodulin gene induction

To examine any role of the *Rhizobium* chromosome in the induction of nodulin gene expression, we used the *Agrobacterium* transconjugant LBA4301(pMP104). This Ti plasmid-cured *Agrobacterium* contains plasmid pMP104 with the 12 kb *nod* region and efficiently induced nodules on vetch. These nodules have an apical meristem, and vascular bundles at the periphery (fig. 5.5A.). Thus, such nodules are anatomically organized like wild-type nodules, but remain smaller. In the early symbiotic zone, bacteria are released from the infection threads into the cytoplasm of the host cells, and become surrounded by a peribacteroid membrane (fig. 5.5D.). However, after release from the infection threads further development of the infected cells is severely disturbed. Unlike wild-type nodule development, some bacteria are observed within the central vacuole (fig. 5.5D.). Bacteria

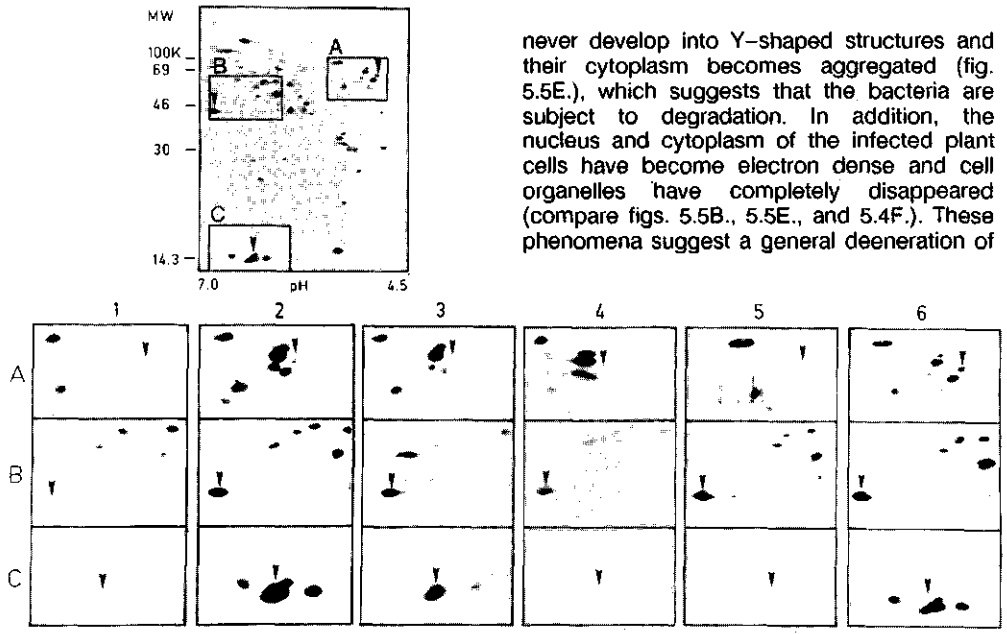


Figure 5.2. Expression of three major nodulin genes induced in vetch by *R. trifolii* ANU845(pMP104), *R.trifolii* ANU843(*nodE* K11::Tn5), *R.trifolii* ANU845(pRt032)(*nodE* K11::Tn5), *A. tumefaciens* LBA4301(pMP104), and *R. leguminosarum* PRE, respectively.

The upper part of the figure shows the fluorograph of a 2-D gel of *in vitro* translation products obtained with total RNA that was isolated from nitrogen fixing vetch root nodules, 15 days after sowing and inoculation with *R. leguminosarum* PRE. The major nodulin spots Nvs-65, Nvs-40 and vetch leghemoglobin (VsLb) are indicated by arrowheads. In the lower part of the figure, fluorographs are shown of *in vitro* translation products obtained from total RNA isolated from vetch root tips (1), and from nodules induced on vetch by (2) *R. trifolii* ANU845(pMP104), (3) *R.trifolii* ANU843(*nodE* K11::Tn5), (4) *R.trifolii* ANU845(pRt032)(*nodE* K11::Tn5), (5) *A.tumefaciens* LBA4301(pMP104) and (6) *R. leguminosarum* PRE. Only the parts of the 2-D gels within the squares in the upper part are shown, as these contain the major nodulin *in vitro* translation products. The comparison is made in (A) for Nvs-65, in (B) for Nvs-40, and in (C) for VsLb.

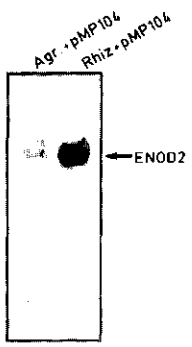


Figure 5.3. Expression of the VsENOD2 gene in vetch root nodules. Autoradiograph of a Northern blot containing RNA isolated from four-week-old nodules induced on vetch by *A. tumefaciens* LBA4301(pMP104), indicated as Agr+pMP104, and in three-week-old nodules induced by *R. trifolii* ANU845 (pMP104), indicated as Rhiz+pMP104. The blot was hybridized with nick translated pGmENOD2.

never develop into Y-shaped structures and their cytoplasm becomes aggregated (fig. 5.5E.), which suggests that the bacteria are subject to degradation. In addition, the nucleus and cytoplasm of the infected plant cells have become electron dense and cell organelles have completely disappeared (compare figs. 5.5B., 5.5E., and 5.4F.). These phenomena suggest a general deeneration of the plant cells. In contrast, the uninfected cells appear to be undegraded. They have a prominent central vacuole and they contain cell organelles, like plastids with starch granules (fig. 5.5D), just as uninfected cells in nodules induced by wild-type *Rhizobium.*

Analysis of the RNA isolated from these LBA4301(pMP104)-induced nodules shows that the two early nodulin mRNAs, VsENOD2 (fig. 5.3.) and Nvs-40 (fig. 5.2.) respectively, are present, but late nodulin mRNAs are not detectable (fig. 5.2.). This result proves that the *nod* region is the only *Rhizobium* DNA that in combination with the *Agrobacterium* chromosome is necessary for the induction of early nodulin gene expression. Although this result might imply that the *Rhizobium* chromosome has to be involved in the induction of late nodulin gene expression, such a conclusion is not warranted, because the degrada-

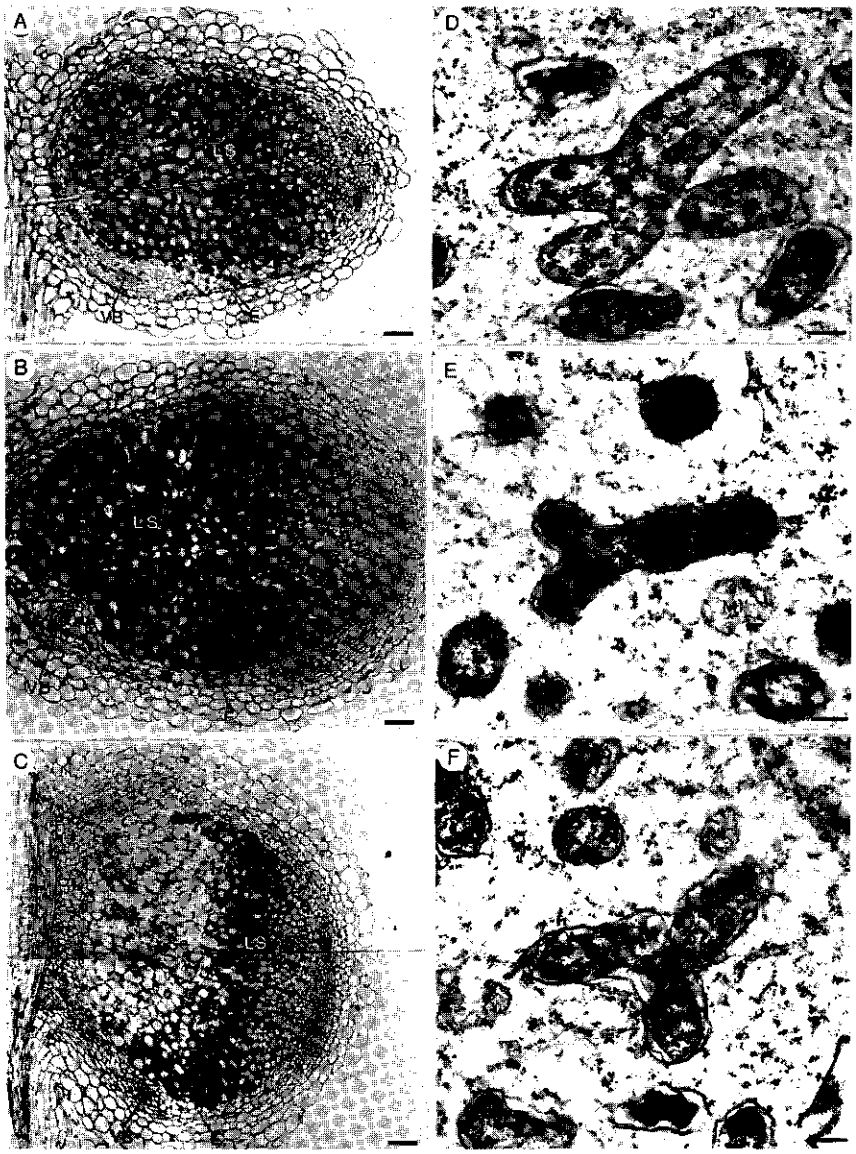


Figure 5.4. Light micrograph montages (A - C) of nodules induced on vetch by (A) *R. trifolii* ANU845(pMP104). (B) *R.trifolii* ANU843(*nodE*K11::Tn5), and (C) *R.trifolii* ANU845(pRt032) (*nodE*K11::Tn5) respectively, and electron micrographs (D - F) of bacteroids of these strains.

A) Three-week-old nodule induced by *R.trifolii* ANU845(pMP104). D) Bacteroid of *R.trifolii* ANU845(pMP104).B) Four-week-old nodule induced by *R. trifolii* ANU843(*nodE*K11::Tn5). E) Bacteroid of *R.trifolii* ANU843(nodE K11::Tn5). C) Four-week-old nodule induced by *R.trifolii* ANU845(pRt032)(*nodE* K11::Tn5). F) Bacteroid of *R.trifolii* ANU843(pRt032)(*nodE*K11::Tn5). All three nodules (A - C) exhibit the characteristics of an indeterminate nodule: an apical meristem (M), peripheral vascular bundles (VB), an endodermis (E), an early symbiotic zone (ES) and a late symbiotic zone (LS). In addition, the ANU845(pRt032)(*nodE*K11::Tn5)-induced nodule (C) shows an large senescent zone (SE). Bar = 100 micrometer. The electron micrographs (D - F) show that in all cases the bacteroids have differentiated into the characteristic Y-shaped form. Mi, mitochondrion. Bar = 0,3 micrometer.

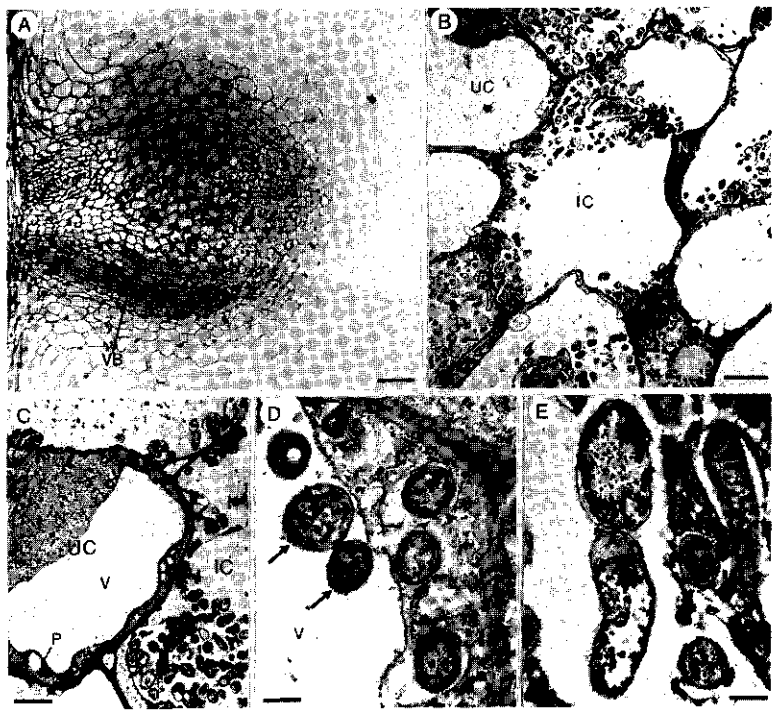


Figure 5.5. Light micrograph montage (A) and electron micrographs (B - E) of a four-week-old nodule induced on vetch by the *Agrobacterium* transconjugant LBA4301 (pMP104).

A) Section showing the apical meristem (M), vascular bundles (VB) , endodermis (E), early symbiotic zone (ES), and late symbiotic zone (LS). Bar = 100 micrometer. B) Detail of the late symbiotic zone. The contents of the infected cell (IC) have deteriorated. No organelles can be discerned, except for a very dark staining nucleus (N). The uninfected cell (UC) appears normal. Bar = 5 micrometer. C) Detail of an uninfected cell in the late symbiotic zone. The contents of the uninfected cell (UC) appear unaffected, in contrast to the contents of the infected cells (IC). The uninfected cell shows a prominent central vacuole (V), a nucleus (N), and plastids with starch granules (P). Bar = 3 micrometer. D) Detail of an infected cell in the early symbiotic zone. In addition to bacteria in the cytoplasm surrounded by a peribacteroid membrane (arrowhead), several bacteria (arrows) are found within the central vacuole (V). Bar = 0,4 micrometer. E) Detail of an infected cell showing bacteroids in the late symbiotic zone. The bacteroids exhibit a condensed cytoplasm, , and the plant cytoplasm is staining dark, which is a sign of severe degradation. Bar = 0,4 micrometer.

tion of the infected cells shortly after release indicate the elicitation of a defense response in the LBA4301(pMP104)-induced nodules.

5.1.3. *Nod* genes relate to the induction of late nodulin gene expression

Analogous to the 12 kb *nod* region of *R. leguminosarum*, a 14 kb *nod* region of *R. trifolii* contains all essential functions for the induction of a nodule on clover (*Trifolium*) roots (292). A physical and genetic map of the 14 kb region, in plasmid pRt032, is shown in fig. 5.1. It has been found that a Tn5 insertion in *nodE* of *R. trifolii* extends the host range of the recipient mutant strain to the pea/vetch cross-inoculation group (85). After introduction of plasmid pRt032(*nodE* K11::Tn5), containing a Tn5 insertion in the *nodE* gene at position K11, in the sym plasmid-cured strain ANU845, the resulting strain ANU845(pRt032) (*nodE* K11::Tn5) differs only from ANU845(pMP104) in the *R. trifolii* origin of its *nod* region. Thus, the capacities of two different *nod* regions in the induction of nodulin gene expression can be analyzed in nodules induced by each of

the strains on the same host plant species. Strain ANU845(pRt032) (*nodE* K11::Tn5) induces nodules on vetch with a frequency of only about one nodule per five plants. The few nodules formed develop without delay like wild-type nodules (fig. 5.4C.) up to the stage in which infected cells become fully packed with rhizobia. Bacteria develop into Y-shaped forms (fig. 5.4F.), but, in contrast with wild-type nodule development, senescence occurs soon afterwards. In a four-week-old nodule, only a few layers of fully packed cells and a large zone of senescence are observed (fig. 5.4C.).

Analyses of RNA isolated from these nodules show that both the Nvs-40 (fig. 5.2.) and the VsENOD2 (fig. 5.3.) gene are expressed. Also the late nodulin gene Nvs-65 is expressed (fig. 5.2.), but expression of other late nodulin genes, including the leghemoglobin genes, is not detectable in the nodules induced by ANU845(pRt032)(*nodE* K11::Tn5). These observations do not exclude that late nodulin genes are still expressed in the few fully infected cells observed in the ANU845(pRt032)(*nodE*K11::Tn5)-induced nodules (fig. 5.4C.), because their expression might not be detectable in a total RNA preparation. Therefore we examined the presence of leghemoglobin by means of immunocytochemistry. Immunogold silver stained sections showed only a low background staining in the infected cells of the ANU845(pRt032)(*nodE*K11::Tn5)-induced nodules (figs. 5.6A. and 5.6B.) after incubating with an antiserum directed against pea leghemoglobin followed by silver staining. In contrast, high levels of silver staining are found in the plant cytoplasm surrounding wild-type *R. leguminosarum* bacteroids (figs. 5.6C. and 5.6D.). These analyses of individual cells provide solid evidence that the leghemoglobin genes are not expressed in the fully infected cells of the ANU845(pRt032)(*nodE* K11::Tn5)-induced nodules at levels found in infected

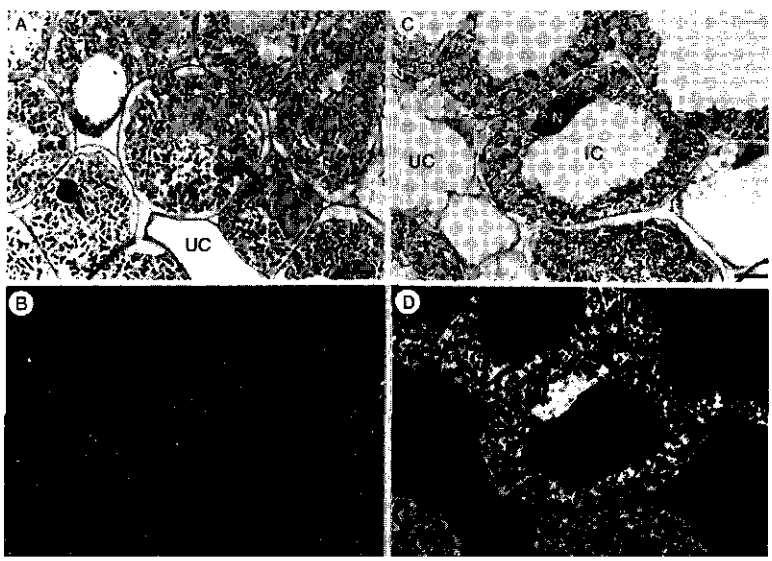


Figure 5.6. Localization of leghemoglobin by immunogold silver staining in a four-week-old nodule induced on vetch by *R.trifolii* ANU845(pRt032)(*nodE* K11::Tn5) (A, B) and in a three-week-old nodule induced on vetch by wild-type *R. leguminosarum* PRE (C, D).

A and C are the toluidine blue-stained, bright field micrographs of the epipolarization micrographs shown in B and D, respectively. The strong signal observed in the infected cells in the nodule induced by wild-type *R. leguminosarum* PRE (D) can be seen as a dark silver stain in the bright field micrograph C. No signal above background is observed in the cells of the nodule induced by ANU845(pRt032)(*nodE* K11::Tn5) (B). IC, infected cell, UC, uninfected cell, N, nucleus. Bar = 10 micrometer.

cells of nitrogen fixing nodules.

Strain ANU845(pRt032)(*nodE* K11::Tn5) induced the expression of early nodulin genes and a single late nodulin gene, Nvs-65. As discussed in the previous section, strain ANU845(pMP104) induced nodules in which all early and late nodulin genes examined, including the leghemoglobin genes, are expressed. Since the only difference between the two strains is the origin of the *nod* regions, the *nod* region appears to be involved in the induction of the expression of late nodulin genes.

5.1.4. *R. trifolii* can induce early and late nodulin genes in vetch nodules

The absence of most late nodulin gene transcripts in the vetch nodules formed by ANU845(pRt032)(*nodE* K11::Tn5) indicates that the genetic information on this mutated *nod* region is deficient in inducing the expression of late nodulin genes in vetch. To investigate further the genetic potentials of the *R. trifolii* sym plasmid, we used strain ANU843(*nodE* K11::Tn5), which contains a complete *R. trifolii* sym plasmid with a Tn5 in *nodE* at position K11. Per vetch plant, strain ANU843(*nodE* K11::Tn5) induced on the average one nodule, which occurred primarily at lateral root emergences. Wild-type *R. trifolii* strain ANU843 very rarely induced a nodule on vetch, confirming the influence of the Tn5 mutation in *nodE* on host range (85).

The histology of the nodules induced on vetch by ANU843(*nodE* K11::Tn5) was similar to the histology of nodules induced by wild-type *Rhizobium* (fig. 5.4B.). The Y-shaped morphology of the ANU843(*nodE* K11::Tn5) bacteroids (fig. 5.4E.) was also similar to that of the ANU845(pMP104) bacteroids (fig. 5.4D.).

Analysis of RNA from the vetch nodules induced by ANU843(*nodE* K11::Tn5) revealed that all nodulin genes are expressed (figs. 5.2. and 5.3.). This shows that the *R. trifolii* sym plasmid genes are equivalent to *R. leguminosarum* sym plasmid genes in establishing late nodulin gene expression. It indicates furthermore that the *nodE* mutation is not the cause for the failure of strain ANU845(pRt032)(*nodE* K11::Tn5) to induce late nodulin gene expression in vetch nodules.

The nodules induced on vetch by ANU843(*nodE* K11::Tn5) do not fix nitrogen, while all *nif* and *fix* genes are present in this strain. Because all early and late nodulin genes, as far as identified, are expressed in these vetch nodules, it appears unlikely that the ineffective nature of the nodules is due to the absence of certain nodulins. For a better understanding of the Fix phenotype of these nodules, we examined whether the enzyme nitrogenase was produced. Western blots of total protein isolated from wild-type *R. trifolii* bacteroids, from clover nodules, and from ANU843(*nodE* K11::Tn5) bacteroids, from vetch nodules, were incubated with antisera against the components CI and CII of the *R. leguminosarum* nitrogenase complex. The bound antibodies were visualized with ^{125}I-protein A (fig. 5.7.). The fluorograph shows that no detectable levels of CI and CII are present in the ANU843(*nodE* K11::Tn5) bacteroids, whereas the *R. trifolii* nitrogenase is easily detected in clover nodules by the antisera used. The lack of nitrogenase was confirmed in immunocytological studies. No silver staining above background was detectable in the ANU843(*nodE* K11::Tn5) bacteroids after incubation of nodule sections with CI anti-

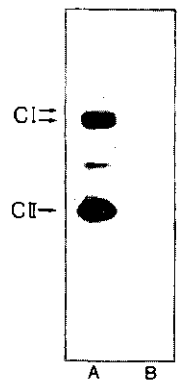


Figure 5.7. Autoradiograph of a Western blot containing bacteroid proteins from ANU843(*nodE* K11::Tn5) bacteroids isolated from vetch nodules (A) and from wild-type *R. trifolii* bacteroids isolated from clover nodules (B). The blot was incubated with antiserum raised against purified *R. leguminosarum* nitrogenase components CI and CII and ^{125}I-labeled protein A to detect the immune complexes.

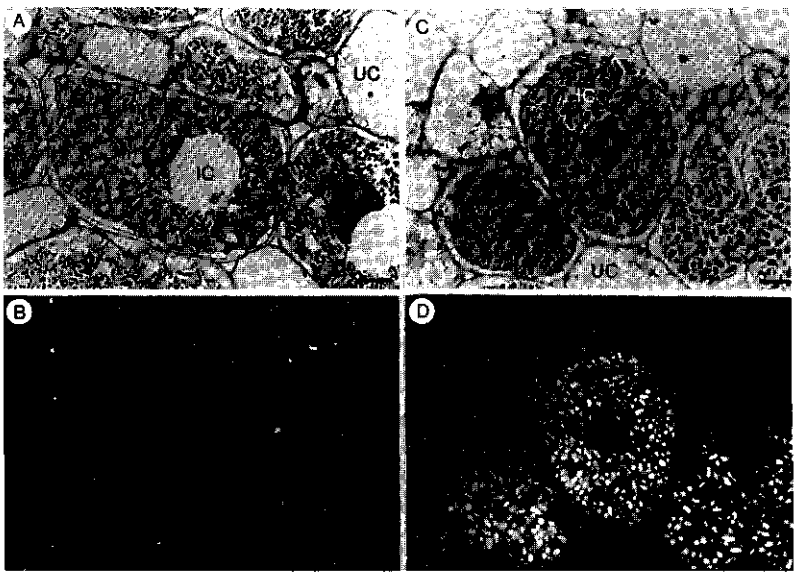


Figure 5.8. Localization of nitrogenase by immunogold silver staining in a four-week-old nodule induced on vetch by *R.trifolii* ANU845(*nodE*::K11::Tn5) (A, B) and in a three-week-old nodule induced on white clover by wild-type *R. trifolii* ANU843 (C, D).

A and C are the toluidine blue-stained, bright field micrographs of the epipolarization micrographs shown in B and D, respectively. The strong signal observed in the bacteroids in the nodule induced by wild-type *R. trifolii* ANU843 (D), to be seen as a dark silver stain in the bright field micrograph C, shows that the antiserum used is able to visualize nitrogenase in sections of clover. No signal above background is observed in the infected cells of the nodule induced by ANU845(*nodE*::K11::Tn5) (B). IC, infected cell. UC, uninfected cell. N, nucleus.

serum (figs. 5.8A. and 5.8B.). In contrast, after the same treatment a high level of silver staining is observed in wild-type *R. trifolii* bacteroids in white clover (*Trifolium repens*) nodule sections (figs. 5.8C. and 5.8D.) and wild-type *R. leguminosarum* bacteroids in vetch nodule sections (not shown). These observations indicate that despite the expression of all early and late nodulin genes, nitrogenase protein is not synthesized in ANU843(*nodE*::K11::Tn5)-induced nodules.

5.2. **Discussion**

Strains of *Rhizobium* carrying only the *nod* region from a *R. leguminosarum* sym plasmid are able to induce nodules on the roots of vetch. In such nodules, both early and late nodulin genes are expressed. Furthermore, we have shown that if the *nod* region from a *R.leguminosarum* sym plasmid is present in an *Agrobacterium* transconjugant, this *nod* region confers upon the *Agrobacterium* the ability to induce a nodule on vetch. In these nodules only early and no late nodulin gene expression is found. These results confirm and extend our previous findings (chapter 4.). Similar results have recently been found in nodules induced on alfalfa (*Medicago sativa*) by an *Agrobacterium* transconjugant containing the cloned *nod* region of the *R. meliloti* sym plasmid (80).

Eight *nod* genes, *nodE, F, D, A, B, C, I,* and *J,* have been identified in the *nod* region of *R. leguminosarum.* Mutations in *nodD, A, B,* or *C* abolish nodulation. The *nodD* gene encodes a regulatory protein required for the expression of all other *nod* genes (280). The *nodA, B,* and *C* genes are essential for root hair curling, formation of the infection thread and the induction of cortical cell divisions (76,90,93). Therefore, the gene products of the *nodA, B,* and *C* genes are likely to be responsible for the generation of one or more signals that result in these three phenomena, followed by induction

of early nodulin gene expression and formation of a nodule. It is unclear whether the *nodA, B,* and *C* gene products accomplish these effects directly or by initiating a cascade of reactions. Transfer of a fragment carrying exclusively the *nodD,A,B,C,E,* and *F* region into a sym plasmid-cured *Rhizobium* does not confer upon the recipient strain the ability to induce nodules, although such a strain still causes root hair curling (Knight et al, 1985). This observation suggests that the *nodD,A,B,* and *C* genes are not sufficient by themselves, and additional information encoded by the *nod* region is required, for the induction of early nodulin gene expression and the formation of root nodules. Indeed, mutations in the *nodE, F, I,* or *J* genes located on plasmid pMP104, if present in a sym plasmid-cured *Rhizobium*, result in a Nod-phenotype (A.A.N. van Brussel and coworkers, in preparation). The gene products of the *nodE, F, I,* and *J* genes thus appear indispensable for nodulation and nodulin gene expression. On the other hand, if these genes are part of the complete sym plasmid, mutations in the *nodE, F, J,* or *I* genes result only in a delayed nodulation and a reduction of the number of nodules. Possibly the function of the mutated *nodE, F, J,* or *I* gene is complemented by another sym plasmid gene, that, however, is not detectably homologous with the mutated *nod* gene. This question needs further clarification. The results of the phenotypical analysis of mutations do not allow the decision whether the *nodE, F, J,* and *I* gene products have any role in the induction of early nodulin gene expression.

As reported previously, early nodulin gene expression is not detectable in tumors, and the *Agrobacterium* chromosome does not appear to contribute signals involved in the induction of early nodulin genes (234). Recently, it was shown that the *nod* genes from *R. meliloti* are inducible to levels comparable with the level found in wild-type *R. meliloti,* if these genes are present in *A. tumefaciens*, but not if they are present in other Gram-negative bacteria such as *Escherichia coli* or *Pseudomonas savastanoi* (370). If the *Rhizobium* and *Agrobacterium* chromosome have common characteristics that allow the induction of the *nod* genes, the common chromosomal genes will be essential for root nodule formation. It seems unlikely that these common genes have a role in generating signals towards the plant for the induction of early nodulin gene expression. Chromosomal genes will rather support the basic physiology of the bacterium, which in turn will be important for creating the conditions allowing the interactions between bacterium and host plant.

Whereas the evidence of the *nod* region being sufficient for the induction of early nodulin gene expression is unequivocal, it is not clear whether the *nod* region alone, or in cooperation with non-sym plasmid genes, also regulates the induction of late nodulin gene expression. None of the late nodulin genes is expressed in nodules induced by the *Agrobacterium* transconjugant LBA4301 (pMP104) carrying the *nod* region. Our cytological data indicate that infected plant cells and the agrobacteria start to degenerate after release of the agrobacteria from the infection threads. This degeneration may be atrributed to a plant defense response (see Van de Wiel *et al* (342)). The outer membrane of the *Agrobacterium* transconjugant is likely to differ from the *Rhizobium* outer membrane, and bacterial membrane components become part of the peribacteroid membrane (36). Upon release of bacteria from the infection threads, the plant might thus detect an aberrant bacterial surface and react with a defense response. As a consequence, the lack of late nodulin gene expression in the nodules induced by LBA4301(pMP104), the *Agrobacterium* carrying the 12 kb *nod* region, does not prove that additional genes besides the *nod* region are required for the induction of late nodulin gene expression. Due to an interference of nodule development by host defense, late nodulin gene expression might have been prevented or aborted.

Evidence that the *nod* genes are indeed involved in the induction of late nodulin gene expression can be derived from our studies of nodules induced by *Rhizobium* strains containing the *nod* regions from *R. trifolii* and *R. leguminosarum* respectively. Strains ANU845(pMP104) and ANU845(pRt032)(*nodE* K11::Tn5) differ only in the *nod* region they contain, and both strains are able to nodulate vetch. In the nodules induced on vetch by ANU845(pMP104), all early and late nodulin genes are expressed, whereas in the nodules induced by ANU845(pRt032)(*nodE* K11::Tn5) the majority of the late nodulin genes is not

expressed. This difference in the pattern of late nodulin gene expression should be attributed to the only difference between the two strains, *i.e.* to the *nod* region. Hence, the *nod* genes have a role in the induction of the late nodulin genes. This conclusion is supported by the observation (289) that the *nodA* and *nodC* genes are expressed in *R. meliloti* bacteroids, thus at a relatively advanced stage of nodule development. Although formal proof for the involvement of the *Rhizobium nod* genes in the induction of late nodulin gene expression cannot be obtained, these *nod* genes may very well be the only *Rhizobium* genes essential for the induction of the expression of all nodulin genes.

The reason for the absence of the transcripts from most late nodulin genes in nodules induced by the strain with the *R.trifolii nod* region is unclear. The mutation in *nodE* seems not responsible for the failure to induce the expression of late nodulin genes, because *R. trifolii* strain ANU843(*nodE* K11::Tn5), carrying the *nodE* K11 mutation in the complete sym plasmid, induces nodules on vetch in which all late nodulin genes are expressed. The amount of *nod* gene products appears critical for the proper development of nodules. If a high-copy number plasmid carrying the *nodA,B,C* genes transcribed constitutively from a vector promoter, was introduced in a *R. leguminosarum* with a complete sym plasmid, nodulation ability was abolished completely (180). A two fold enhancement of *nod* gene expression was sufficient to result in a strain which induced on vetch only twenty procent of the number of nodules compared to wild-type strains and these nodules were ineffective (155). A difference in copy number between pMP104 and pRt032(*nodE* K11::Tn5) may therefore explain the observed differences in the nodulin gene expression pattern.

A remarkable finding is the ineffective nature of the nodules induced by *R. trifolii* ANU843(*nodE* K11::Tn5) due to the lack of nitrogenase. The *nif* and *fix* genes were present in this strain, and both early and late nodulin genes were expressed in the nodules formed, so all prerequisites for nitrogen fixation on vetch seem fulfilled. The same strain induces nitrogen fixing nodules on subterranean clover (*Trifolium subterraneum*) (85), proving that ANU843(*nodE* K11::Tn5) has all genetic potentials for nitrogen fixation. Possibly vetch nodules lack a factor which is present in subterranean clover nodules and which is involved in the induction of bacterial nitrogenase gene expression. This presumably plant species-specific factor may be a second regulating factor in the induction of bacterial nitrogenase gene expression, in addition to the recently suggested role of low oxygen concentrations (81,107).

The *Rhizobium* and *Agrobacterium* strains used in this study induce nodules in which development is increasingly disturbed. Combining the histological data of the various nodule types with the pattern of nodulin gene expression in these nodules, a correlation is found between nodule structure and nodulin gene expression. The nodules induced on vetch by strain ANU845(pRt032)(*nodE* K11::Tn5) contain only 2-4 layers of fully packed infected cells. The absence of most late nodulin gene expression in these nodules suggests that these late nodulin genes are not expressed in the youngest cells that are cytologically fully differentiated into infected and uninfected cells. This conclusion is in agreement with our immunocytological localization studies of leghemoglobin in wild-type pea nodules (342).

In nodules induced by LBA4301(pMP104), bacteria were released from the infection threads, but late nodulin gene expression was not detectable. Apparently release from the infection threads is not sufficient to induce the expression of late nodulin genes. Comparison of the histology of the nodules induced by ANU845(pRt032)(*nodE* K11::Tn5) and LBA4301(pMP104) showed that in the LBA4301(pMP104)-induced nodules fully packed infected cells were not found, whereas some fully packed infected cells were found in the nodules induced by strain ANU845(pRt032)(*nodE* K11::Tn5). The presence of fully packed infected cells correlates with the expression of the Nvs-65 gene, suggesting that the Nvs-65 gene is probably first expressed in the youngest cells which are completely filled with bacteria. In view of the time course of expression of the Nvs-65 gene, this gene is member of the class of late nodulin genes (234). Because the Nvs-65 gene is expressed in the nodules induced by ANU845(pRt032)(*nodE* K11::Tn5), whereas leghemoglobin gene expression is not detec-

table, it appears likely that the Nvs-65 gene is regulated differently from the leghemoglobin genes. Consequently, late nodulin genes must be subdivided into two subclasses, the expression of which is regulated differently and correlates with a step in the developmental program of the root nodule.

Electron microscopical observations indicate that uninfected cells in the LBA4301(pMP104)-induced nodules develop like uninfected cells in wild-type nodules. The absence of expression of the identified late nodulin genes in nodules induced by LBA4301(pMP104) may indicate that these late nodulin genes are not expressed in uninfected cells. Correlations like these between the expression of a particular nodulin gene on the one hand, and nodule development up to a certain stage on the other, may be of use in determining the cell type in which a particular nodulin gene is expressed and must be borne in mind in speculations about the function of nodulins.

Table 5.1. Bacterial strains and their relevant characteristics

Strain	Characteristics			Reference
Rhizobium leguminosarum				
PRE (wild-type)	pSym	nod^+fix^+		(210)
248 (wild-type)	pSym	nod^+fix^+		(175)
248^c (cured 248)		nod^-fix^-		(372)
248^c(pMP104)	pRlnod	nod^+fix^-	Tc^r	this study
Rhizobium trifolii				
ANU843 (wild-type)	pSym	nod^+fix^+		(291)
ANU843(*nodE* K11::Tn5)	pSym	nod^-fix^-	Km^r	(85)
ANU845 (cured ANU843)		nod^-fix^-		(291)
ANU845(pMP104)	pRlnod	nod^+fix^-	Tc^r	this study
ANU845(pRt032)(*nodE* K11::Tn5)	pRtnod	nod^+fix^-	$Km^r Cb^r$	(85)
Agrobacterium tumefaciens				
LBA4301 (cured Ach5)		nod^-fix^-		(161)
LBA4301(pMP104)	pRlnod	nod^+fix^-	Tc^r	this study

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

5.3. MATERIALS AND METHODS

Plants and bacteria. Vetch seeds were sterilized, germinated, inoculated and cultured as described (chapter 2.) Bacterial strains and their relevant characteristics are listed in table 5.1. Bacterial crosses were performed as described (308) using pRK2013 (81) as helper plasmid. Bacteria were grown in YEM medium as described (122) with 2,5 mg/l tetracycline for pMP104 selection and 75 mg/l kanamycine for Tn5 selection. Nodules were excised from the roots with a scalpel. Root tips from uninfected plants were isolated 8 days after sowing. All tissues were immediately frozen in liquid nitrogen and stored at -80°C until use.

RNA isolation, in vitro translation and 2-D gel electrophoresis. Total RNA from plant tissue was isolated according to Govers et al. (1985). Approximately 2 microgram total RNA was translated *in vitro* in a rabbit reticulocyte lysate in a 6 microliter reaction mixture as described (234). Translation products were separated by 2-D gel electrophoresis, followed by fluorography of the dried gel to preflashed Kodak XAR5 film (126).

Northern blot analysis. Total RNA was denatured in dimethyl sulfoxide/glyoxal, electrophoresed in 0.8% agarose gels (218) and transferred to GeneScreen (New England Nuclear) membranes as described (126). The membranes were hybridized with ^{32}P-labeled probes (218) under the conditions previously described (112).

Protein isolation and Western blot analysis. Bacteroid proteins were isolated and separated by SDS/polyacrylamide electrophoresis as previously described (26). Proteins were transferred to nitrocellulose by electroblotting (373) and after incubation with antiserum they were visualized with ^{125}I-protein A (26). Preparation of the antisera against leghemoglobin and the components CI and CII of the *R. leguminosarum* nitrogenase has been described before (27,342).

Microscopy and immunocytochemistry. Nodules were fixed with 2,5 % glutaraldehyde and 1 % osmiumtetroxide and embedded in LR White resin as described previously (342). Sections were cut with glass knives on an LKB Ultrotome V. Semithin sections (0.5-2.0 micrometer) were stained with 1% toluidine blue 0. Ultrathin sections were stained at room temperature in an LKB Ultrostainer 2168 with uranyl acetate for 20 min. and then with lead citrate for 40 sec. Sections were examined using a Philips EM 301 transmission electron microscope operated at 60 kV. For immunocytochemistry, nodules were fixed in 4 % paraformaldehyde, embedded in LR White resin, sectioned and attached to slides as described (342) Semithin sections (0.5 micrometer) were incubated with antiserum, followed by incubation with 10 nm gold particles coupled to protein A (Janssen Pharmaceutica), and the signal was silver enhanced using the IntenSEII™ silver enhancement kit (Janssen Pharmaceutica) according to the manufacturer's manual. After treatment, sections were stained with 0.1 % toluidine blue 0 for 1 minute and mounted in Euparal (Chroma) and examined under a Nikon microscope equipped with epipolarisation optics (Philips 100 Watt halogene lamp).

NODULINS AND NODULIN GENE REGULATION IN ROOT NODULE DEVELOPMENT: OVERVIEW AND DISCUSSION

6

Chapter 6

Nodulin function and nodulin gene regulation in root nodule development: overview and discussion

6.1. INTRODUCTION

The interactions leading to a nitrogen fixing nodule have been well analyzed from a morphological point of view (12,71,124, 206,243) In general, the sequence of events is as follows. Rhizobia interact with epidermal cells in the region where root hairs are beginning to emerge (20). The root hairs respond by a marked curling due to uneven growth of the root hair, thereby entrapping bacteria within a "pocket" (332,333). Bacteria penetrate the plant cell wall through partial dissolution of the host cell wall (44,268,269,333). Subsequently, bacteria invade the root hair cell and then the root cortex through a tube-like structure, the infection thread. As the infection threads ramify, the bacteria proliferate within the thread and become surrounded by mucopolysaccharide (244). Meanwhile, but independently from the infection process (207,245), cells of the root cortex enter the new developmental program of root nodule formation. At several places, cortical cells start dividing. From these centers of mitotic activity, the nodule primordia are formed (45). Infection threads grow towards these meristematic centers, and upon contact, rhizobia bud off from the tips of the infection threads into the cytoplasm of the plant cells. This release is an endocytotic process (11) in which the bacteria become enclosed by a membrane, the so-called peribacteroid membrane, that is initially derived from the plasmalemma of the host cell (275). After release, bacteria and peribacteroid membranes divide in a coordinated fashion to fill the host cell cytoplasm (272). The bacteria differentiate into pleiomorphic bacteroids which synthesize the nitrogen fixing system. Not all nodule cells are invaded by rhizobia. About half of the nodule cells remain uninfected. These uninfected cells occur among the infected cells (243).

Numerous variations on nodule development have been described (71). For example, not in all leguminous plants infection threads are observed. In peanut *(Arachis hypogea)*, infection by rhizobia is not via root hairs but by inter- and intracellular invasion (53). Differences also exist in the susceptibility of root hairs to become infected. In alfalfa (*Medicago sativa*), the epidermal cells in the region of rapid root elongation can be infected, whereas in white clover *(Trifolium repens)* also the mature root hairs are susceptible to infection (21). The way *Rhizobium* invades its non-legume host *Parasponia* differs substantially from the infection pathways of most legume hosts (187,188). The initial infection involves intercellular penetration of the epidermis, frequently accompanied by degrada-tion of cortical cells. Eventually infection threads develop. Rhizobia are not released from this infection thread, do not differentiate into bacteroids and fix nitrogen while retained within the infection threads. Persistent infection threads without bacterial release are also observed in certain tree legume nodules *(Andira* species). These nodules may represent a primitive stage in the evolution of root nodules (77).

By their morphology, two main categories of leguminous nodules can be recognized, determinate and indeterminate nodules, although more refined classifications have been proposed (65). In general, temperate legumes, such as *Pisum, Vicia, Trifolium,* and *Medicago* species, develop indeterminate nodules, while determinate nodules occur on the roots of tropical legumes such as *Glycine, Phaseolus, Vigna* and *Arachis* species. The

morphology of the *Parasponia* nodule is indeterminate but differs from legume nodules in that it has a central vascular bundle (188,260). Nodule morphology is the result of a developmental program under control of the host plant, because for a number of *Rhizobium* strains it has been demonstrated that one and the same *Rhizobium* strain can induce determinate nodules on one host, indeterminate nodule on the other (71), and can also nodulate *Parasponia* (328).

A schematic representation of a longitudinal section of an indeterminate nodule, modified after Sutton (319), is shown in fig. 6.1. Uninfected tissue, known as the nodule cortex, surrounds the central infected zone of the nodule. A peripheral endodermis divides this nodule cortex in outer- and inner layers. Within the latter tissue, the vascular bundles are located. Two cell layers composed of small uninfected cells, termed the boundary cell layer (131), separate the inner cortex from the central tissue of the nodule. In the mature nodule, the central tissue is divided in distinct zones which differ in developmental stage (242). The most distal zone is the apical meristem, which is adjacent to enlarging cells that may become infected by *Rhizobium* (the invasion zone). In the early symbiotic zone, host cells differentiate into infected and uninfected cells, while in the late symbiotic zone uninfected cells and fully packed infected cells are found. In this late symbiotic zone, nitrogen fixation and ammonia assimilation occur. The most proximal zone in the nodule is the senescent region where both plant cells and bacteroids degenerate.

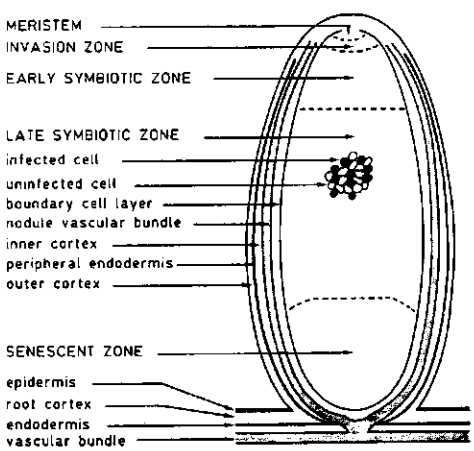


Figure 6.1. Overall organization of an indeterminate nodule.

In contrast to indeterminate nodules, determinate nodules exhibit a different orientation and duration of meristematic activity within the nodule. Determinate nodules do not have a persistent meristem (245). After release of rhizobia from the infection thread, the infected cells continue to divide till about one week after the onset of nitrogen fixation. When mitotic activity has ceased, increase in nodule size is caused by cell expansion rather than by cell division. As a consequence, the developmental phases in a determinate nodule are separated in time rather than in space. All cells of the central tissue within a single nodule are progressing through the same stage of development.

The different nodule tissues described above are all initiated from the nodule meristem. In an orderly fashion one stage of development follows another in the proper sequence. And at each stage, cells which hitherto had shown a common lineage diverge into alternative pathways of differentiation. Unlike the developmental program of a lateral root, the legume root nodule meristem originates from cells of the root cortex and not from the pericycle. The vascular strands are positioned peripherally and not centrally. These two arguments do not apply to the *Parasponia* nodule, in which nodule growth starts in the pericycle (187) and the vascular bundle is central (188). For this reason, the *Parasponia* nodule seems similar to a modified lateral root. The nodule meristem differentiates into nodule cells in one direction only. Also, the nodule does not form a root cap. Therefore, the nodule can be considered a unique organ, different from lateral roots (206).

If the legume nodule is considered as a unique organ, the developmental program leading to root nodules will be as complex as other developmental programs in plants (176,177), and involve numerous genes. Central to the problem of development is the mechanism whereby the right genes are activated in the right cells at the right time. A useful first approach to understand the nature of root nodule development seems therefore to be the identification of the genes, the expression of which distinguishes a root nodule from

other plant organs. In many *Rhizobium*-legume symbioses, the occurrence of plant-encoded, nodule-specific proteins, the so-called nodulins (199), has now been firmly established (349). It has become evident that differential expression of nodulin genes accompanies the development of root nodules. Considering nodulin gene expression as the most specific aspect of nodule differentiation, the study of nodulin gene expression may provide a path towards an understanding of root nodule development.

In this chapter we will discuss nodulins, nodulin genes, the relation between nodulin gene expression and nodule development, and the communication between the two partners in the symbiosis correlated with regulation of nodulin gene expression.

6.2. NODULINS AND NODULIN GENES

6.2.1. Nodulin nomenclature

By definition, nodulins are plant gene-encoded proteins, which are found only in root nodules and not in uninfected roots nor in other parts of the host plant (345). Nodulin genes are, by consequence, plant genes exclusively expressed during the development of the symbiosis. Until a defined biochemical function has been assigned to the protein, the identified nodulins are indicated by the letter N and the molecular weight as determined by SDS polyacrylamide gelelectrophoresis. In addition, we propose to add the plant genus and species initials in lower case to the N in order to facilitate the discrimination between nodulins of different plant species. If the protein turns out to be a nodule-specific form of an enzyme that also occurs elsewhere in the host plant, like glutamine synthetase (see 6.2.3.3.), addition of the prefix "n" to the name of the protein is recommended. *In vivo* nodule proteins, that, by analysis with *e.g.* antisera, are shown to be nodule specific, should be indicated by Nsp and their molecular weight, until their nodulin nature has been ascertained (see Govers *et al.* (125) for discussion). In this chapter we will adopt this nomenclature, implying that some nodulins will be named differently from what previously has been published.

Nodulin genes are differentially expressed during nodule development (116,117,122, 126,129,200,296). The majority of nodulin genes is expressed around the onset of nitrogen fixation. Typical representatives of these nodulin genes are the leghemoglobin genes. Few nodulins are detectable at earlier stages of development, at the stage in which the nodule structure is being formed (112,128, 238, see chapter 2.). To account for the apparent difference in timing in expression, nodulin genes have been classified in class I and class II (125), and in class A and class B nodulin genes (122), but for class I/class A nodulins the term 'early nodulins' is frequently used. Therefore, we will adopt the term 'late nodulin' for class II/class B nodulins (239). Early nodulin cDNA clones are designated by ENOD (112, see chapter 2.), while late nodulin cDNA clones are best designated by NOD (117), to avoid confusion with the bacterial *nod* genes. The names of both NOD and ENOD clones should be preceded by the plant genus and species initials.

It should be noted that the factor time as discriminator between nodulins may prove to be inadequate when steps of development that are successive in one plant species, are synchronized in another species. Furthermore, there may be early nodulins yet undetected, that are involved in stages earlier than the early nodulins identified so far. Some nodulins may also be involved in nodule senescence, so play a role later than the identified late nodulins. Bearing all this in mind, the proposed terminology of early and late nodulins is an operational one; one that serves our purposes for the time being, but will have to be changed as more data become available.

Histological analyses of nodule development in combination with studies on the timing of nodulin gene expression have shown that the complete nodule structure with all its defining characteristics is formed, when only early nodulin genes are expressed and no late nodulin gene expression is yet detectable This applies to both determinate (soybean) (122) and indeterminate (pea) (128) nodules. Therefore, early nodulins can be involved in nodule organogenesis and the infection process, but late nodulins are not. The expression of late nodulin genes during wild-type nodule development is correlated with the onset of nitrogen fixation (116,126). Thus, late nodulins will most probably function in establishing and maintaining a proper environment within the nodule that allows nitrogen fixation and

Leghemoglobin is supposed to be a true "symbiotic protein" in the sense that the heme moiety is a presumed product of the bacteroid (237), whereas the globin part is plant-genome encoded (see Bisseling *et al.* (30) for discussion). In contrast to a *R. meliloti* strain carrying a mutation in the gene encoding delta-aminolevulinic acid synthetase that induces white, ineffective nodules (204), *Bradyrhizobium japonicum* strain MGL1, mutated in the same gene, still induces fully effective nodules, that apparently do not suffer any heme deficiency (134). Moreover, there is no evidence for heme transport across the peribacteroid membranes. Therefore, it is still an open question as to whether the bacterium indeed excretes the heme required for leghemoglobin synthesis.

In all legumes studied to date, more than one leghemoglobin is found in the root nodule, and the leghemoglobins are encoded by more than one gene. Soybean nodules contain four major leghemoglobins in addition to several minor components (114). The minor components are probably the result of post-translational modifications of the major leghemoglobins (363). Slight differences have been observed in the time of synthesis of the different leghemoglobins during nodule development for both soybean (220,351) and pea (334). The functional significance of the occurrence of different leghemoglobins and the differences in timing of the expression of the leghemoglobin genes is unclear. It has been suggested (334,335) that the increase in nitrogen fixing activity in nodules is paralleled by an increase in the amount of the leghemoglobin component with the higher oxygen affinity, resulting in a more efficient nitrogen fixation.

The non-legume *Parasponia* has only one hemoglobin gene (189). Two hemoglobins are found in *Parasponia* nodules, a major and a minor component, that by consequence are both derived from the unique hemoglobin gene. The oxygen affinity of the major component was found to be sufficiently high to allow the non-legume hemoglobin to function in a similar way to that of leghemoglobin (182).

6.2.3.2. *Uricase*

The second most abundant protein in the cytoplasm of soybean root nodules is Ngm-35 (199). By protein purification it was shown that Ngm-35 is the 33 kDa subunit of n-uricase (or uricase II) (16), a key enzyme in the ureide biosynthetic pathway used in soybean to assimilate ammonia. Uricase activity in root or leaf tissue is due to a diamine-oxidase/peroxidase system (321), requiring a soluble cofactor (322). This two-enzyme system has no immunological cross-reactivity with n-uricase. Therefore, n-uricase is the product of a totally different gene than the root and leaf uricase. However, using an antiserum directed against n-uricase, low concentrations of n-uricase have been observed in soybean roots (16) and callus tissue (194). In contrast, no hybridization with RNA from any uninfected soybean tissue could be detected using a n-uricase cDNA clone as probe (247). The reason for the apparent discrepancy between the results obtained with the n-uricase antiserum and the n-uricase cDNA clone is not clear. Southern blot hybridizations of soybean genomic DNA using n-uricase cDNA clones as probes indicated several fragments homologous with Ngm-35 sequences, suggestive of the existence of a small number of genes (247).

By immunocytochemistry, soybean n-uricase was shown to be localized in the peroxisomes of the uninfected cells (16,247). The apparent metabolic specialization of uninfected cells in determinate nodules is also indicated by biochemical (141) and ultrastructural (240,241,347) data. Sequence analysis of the soybean n-uricase cDNA clone did not reveal a signal peptide, so information for transport into peroxisomes must reside in the protein itself. Two hydrophobic domains in the amino acid sequence may facilitate translocation across the peroxisomal membrane (247).

6.2.3.3. *Glutamine synthetase*

Various glutamine synthetase (GS) isozymes are found in different organs and cell compartments of plants. These octameric proteins catalyze the first reaction in the assimilation of ammonia into organic nitrogen (233). The GS isozymes of leguminous plants are immunologically related with the GS isozymes of non-legume plants (147,227), but not with bacterial or mammalian GS (66). In nodules, GS is located in the cytoplasm of the

infected cells (355). Two isozymes of GS are present in the nodules of French bean (*Phaseolus vulgaris*) (67,193). Several different GS subunits have been identified, ranging from 41 to 45 kDa, only one of which is exclusively found in nodules. One of the nodule enzymes is composed primarily of a subunit also found in the root, but the other GS is composed mainly of the nodule-specific subunit (192,270). In contrast, all GS subunits found in pea nodules are also detected in roots and leaves (325), while conflicting data have been reported for soybean (146,147,295,355).

Sequence analysis of various GS cDNA clones from leguminous plants revealed 70-90% homology in the coding sequences for different GS from the same species and for GS from different species, in agreement with the immunological data. This high homology has hindered the identification of cDNA clones representing the gene for the n-GS subunit. A French bean GS cDNA clone hybridized exclusively to nodule RNA under high stringency conditions, indicating that this clone is derived from a gene for a n-GS subunit (68). In addition, it was found that there are at least two n-GS genes (119). In contrast to the coding sequences, the 5'- and 3'-untranslated regions of GS genes have diverged highly. The divergence in these regions of the GS cDNA clones has been used as a confirmation for the existence of nodule specific GS genes. An alfalfa n-GS cDNA clone has been isolated that could be distinguished from other GS sequences because of a unique sequence in the 3'-untranslated region (95).

The amino acid homology between root and nodule GS subunits, and hetero-oligomeric composition of the GS proteins (270), are both in agreement with the remarkably similar biochemical properties of these GS isozymes (67). The differential expression of GS genes in different parts of the plant therefore results in functionally similar enzymes.

6.2.3.4. *Other metabolic nodulins*

Robertson and Farnden (273) have presented a list of enzymes assayed in the plant- and bacteroid fraction from nodule tissue, a list that has been extended steadily. Several of these enzymes have a substantially higher activity in nodules compared to roots. Although the conclusion seems justified that nodulins are involved not only in nitrogen assimilation but in all aspects of nodule metabolism, only in few cases has the occurrence of nodule specific forms of these enzymes been ascertained.

The soybean nodulin Ngm-100 has been shown to be the subunit of sucrose synthase (323). Nodule specific forms of enzymes that differ in physical, kinetic or immunochemical properties from the corresponding enzymes in roots have been found for phosphoenolpyruvate carboxylase (78), choline kinase (230), xanthine dehydrogenase (246), purine nucleosidase (195) and malate dehydrogenase (4). They may prove to be the result of the expression of nodule specific genes, but at the moment it is too early to conclude whether these nodule specific forms are true nodulins or the result of nodule specific modifications of root enzymes.

6.2.3.5. *Peribacteroid membrane nodulins*

In soybean, a number of nodulins have been characterized, that are associated with the peribacteroid membrane, but have not yet been assigned a clear biochemical function. The peribacteroid membrane is formed during release of rhizobia from the infection thread. After endocytosis, the total amount of peribacteroid membrane increases extensively, indicating a very active membrane synthesizing apparatus. The peribacteroid membrane is initially derived from the plasmalemma, but its chemical composition suggests that also the endoplasmatic reticulum and the Golgi apparatus contribute to peribacteroid membrane biogenesis (127,231 ,275,331,352). The peribacteroid membrane is the physical and metabolic interface between the *Rhizobium* and its eukaryotic partner (274). As such the involvement of peribacteroid membrane-associated nodulins in the symbiosis seems selfevident.

Nodulin Ngm-24 was proven to be part of the peribacteroid membrane through the use of an antiserum directed against a synthetic peptide representing the repeated hydrophobic region of the Ngm-24 protein (111). Ngm-24 was first identified as the 24 kDa hybrid-released translation product of a soybean nodulin cDNA clone (117). The protein contains a signal peptide that can co-transla-

minosae it became advantageous to have the synthesis of glutamine synthetase regulated to fit the conditions of the symbiosis. In both alfalfa (95) and French bean (193,253) the gene for the n-GS subunit is expressed in ineffective nodules induced by bacterial strains that are defective in nitrogenase activity. This shows that ammonia, the result of the symbiotic nitrogen fixation, is not involved in the regulation of the expression of the n-GS genes. On the other hand, in soybean it has been shown that the expression of a non-nodule specific form of GS is regulated by ammonia (146). This illustrates that n-GS genes are subject to a different regulation mechanism, possibly due to the presence of *Rhizobium*. Because it appears that some legumes possess a n-GS, whereas others might not (see 6.2.3.3.), the requirement for a nodule-specific regulation of GS by *Rhizobium* must have come up relatively late in evolution. More precisely, the n-GS will have been evolved *after* the divergence of the taxonomically closely-related soybean and French bean, because it is claimed that soybean has no n-GS (146) whereas French bean does (119). Because alfalfa/pea diverged from French bean/soybean before they diverged from each other (65), a n-GS gene must have evolved in different legumes independently, for alfalfa has a n-GS (95), whereas pea appears to have none (325). We feel that a seemingly arbitrary and independent evolution of different n-GS genes is quite unlikely. Therefore, the absence of n-GS genes in soybean and pea would seem to deserve a careful re-evaluation.

Less obviously derived from an already existing plant gene is the n-uricase gene. The n-uricase is well suited to function in the special physiological conditions in the nodule, *i.e.* a high pH due to ammonia and a low free oxygen tension (16). The main uricase activity in roots is catalyzed by a diamine-oxidase/peroxidase system (321) that seems less advanced because it uses two enzymes with each a broad substrate specificity. This two-enzyme system requires a low catalase activity, whereas catalase activity is high in peroxisomes (142), the place where n-uricase is operational. Therefore, it seems that two entirely different systems have evolved, each fulfilling its task under different physiological conditions. However, n-uricase is also detected in callus (194) and low activities are observed in uninfected roots (16), showing that n-uricase activity is not unique for the root nodule. It is not known whether the n-uricase found in roots and callus is encoded by the same gene as the protein found in the nodule, or by a different gene encoding a similar protein. If it is the same gene, the n-uricase gene can no longer be considered a true nodulin gene, and need not be considered in an evaluation of the origin of nodulin genes. Assuming that the root and callus "n-"uricase activities are derived from different genes, a non-symbiotic form of uricase is obviously present. The n-uricase gene then is an example of a plant gene evolved to be expressed at markedly higher levels in the root nodule, most likely as an adaptation to the different physiological conditions in the root nodule. Because n-uricase is also synthesized in ineffective nodules (194, 199), these physiological conditions are independent of the nitrogen fixation process.

The early nodulin Ngm-75 is related to the soybean SbPRPs (see 6.2.2.) and bears resemblance to the extensins. In several tissues, different SbPRP genes are expressed (156,157), indicating that related molecules may function in different developmental programs, controlled by distinct regulatory mechanisms. The striking structural, and therefore presumably also functional, similarity of the nodulin Ngm-75 to the non-nodule specific SbPRPs suggests a relationship between the corresponding genes. The nature of this relationship, however, is not obvious. On the basis of the structural similarities between Ngm-75, the SbPRPs and extensin, a common ancestor seems likely. The genes for these cell wall proteins may have been derived from a common plant gene encoding a small, proline-rich protein that acquired a function as a cell wall constituent. Numerous gene duplications and divergence then resulted in proteins that function more or less similarly in different developmental situations. On the other hand, on an evolutionary time scale, hypocotyl and root differentiation are older than nodule formation, so the Ngm-75 genes may be derived from the SbPRP genes. Irrespective of the exact evolution of the Ngm-75 genes, it complies with the hypothesis that also the genes for the early nodulin Ngm-75 have been derived from an already functioning plant gene.

So far, most nodulin genes can be assigned a non-symbiotic counterpart. What then about the archetype of the nodulins, leghemoglobin? Leghemoglobin gene expression has never been detected in any part of a leguminous plant other than the nodule, so the absence of a non-symbiotic counterpart seems fairly well established. There has been a great deal of speculation whether the leghemoglobin genes were acquired by horizontal genetic transmission from either *Rhizobium* itself or from an animal vector (205). Detailed analyses of the soybean leghemoglobin gene family (42) suggested, however, that globin genes were already present in the common ancestor of present-day plants and animals. The presence of a hemoglobin gene in the non-legume *Parasponia,* that has a high sequence homology and the same gene structure as the leghemoglobin genes in legumes (189) has profound consequences for considering the evolution of these genes. The *Parasponia* hemoglobin sequence substantiates the likelihood of a vertical evolution of the globins starting from a hypothetical ancestor before the radiation of animals and plants. In principle all plants could have the globin sequence. Indeed, the presence of leghemoglobin-like sequences in various non-legumes has been reported (143). In fact, a *Parasponia* cDNA clone for hemoglobin hybridizes to hemoglobin genes in the distantly-related *Casuarina*, which has a nitrogen-fixing symbiotic association with the actinomycete *Frankia* (189). It also hybridizes to presumably related sequences in the DNA of *Trema*, a close relative of *Parasponia,* that does not nodulate (189). Moreover, it has been found that in *Parasponia* and *Trema* roots hemoglobin gene expression can be detected at a low level (32). Because there occurs only one hemoglobin gene in *Parasponia* DNA (189), this same gene must be expressed in roots as well as in nodules. Thus, both in *Parasponia* and *Trema* roots the expression of a hemoglobin gene can be detected in non-symbiotic tissue. Therefore, also in legumes there may be a non-symbiotic counterpart for leghemoglobin that is transcribed in non-symbiotic tissue, although expression of such a gene has not been detected yet.

Various laboratories have encountered problems in identifying leghemoglobin sequences in one species using a cDNA clone from another species (pea/soybean, *Parasponia*/soybean, alfalfa/pea) despite the structural homologies between the various leghemoglobins. For instance, *Parasponia* leghemoglobin is 40% homologous with soybean leghemoglobin, yet a cDNA-clone for soybean does not crossreact with a *Parasponia* leghemoglobin cDNA clone (189). Therefore, it appears conceivable that the hypothetical non-symbiotic form of a leghemoglobin gene has diverged beyond the point of recognizability. On the other hand, the hypothetical non-symbiotic form may be expressed at very low levels. Soybean contains a leghemoglobin gene that seems to meet all requirements for a functional gene, but is nevertheless thought to be a pseudogene, because the encoded leghemoglobin contains methionine and a methionine containing leghemoglobin has never been found in soybean nodules (39,364). This Lb pseudogene may, in fact, represent a non-symbiotic leghemoglobin. Sequence analysis of the 5'-flanking region showed some minor deviations from leghemoglobin genes functional in the nodule. These alterations may withdraw the gene from nodule specific regulation of expression.

Thus, also the leghemoglobin genes may fit in the hypothesis that there is a form that is non-symbiotic, with or without a function. In this concept, the encoding nodulin genes have evolved from common plant genes in an adaptation to the specific regulatory and/or physiological requirements of root nodule formation and symbiotic nitrogen fixation.

Non-symbiotic counterparts have not been identified for peribacteroid membrane nodulins, but it should be realized that the analysis of plant membranes is a relatively new field of research (250), and the relevant analogous genes may simply not have been identified yet. The gene for Ngm-24 is characterized by three direct repeats arranged in tandem and gene duplica-tion has been implicated in the generation of this gene (178). Because there is very little sequence divergence between the three repeated units of the Ngm-24 gene, the duplication events must have taken place relatively recently in evolution. The peribacteroid membrane nodulins Ngm-20, Ngm-23, Ngm-26b, Ngm-27 and Ngm-44 all have regions in common, and regions unique to each nodulin (167,284). Whereas the regions in common may originate from duplication

Heme has been shown to regulate the expression in yeast of a chimeric gene consisting of the 5'-flanking region of the soybean Lbc_3 gene and the coding sequence of the neomycin phosphotransferase gene at the post-transcriptional level (169). Free heme may decrease the enzyme activity of the nodulin sucrose synthase, because it was found that in the presence of free heme sucrose synthase dissociates rapidly *in vitro* (323). Although heme may thus influence the activity of nodulins, the significance of a heme-mediated mechanism *in vivo* is unknown. There is no evidence that heme is involved in the induction of nodulin gene expression.

Ammonia has been implicated in the regulation of expression of glutamine synthetase genes in soybean (146), but the glutamine synthetase genes investigated appeared not to be nodulin genes. Three lines of evidence strongly suggest that ammonia is not involved in the induction of the expression of nodulin genes. First, in normal nodule development, all nodulin genes are expressed before nitrogen fixation starts (122,126). Second, all nodulin genes are expressed in nodules induced by *Rhizobium* and *Bradyrhizobium nif* and *fix* mutants (122,126). Third, on the roots of cowpea (*Vigna unguiculata*) grown in an argon/oxygen environment containing negligible amounts of nitrogen gas, nodules could be induced by *Rhizobium* in which both nitrogenase activity and leghemoglobin were detectable (7). Neither the availability of nitrogen, nor the result of nitrogenase activity, ammonia, are thus essential for the induction of nodulin gene expression.

Phytohormones affect processes of a wide diversity in plants and they are considered crucial to the developmental programming of plants (136). As such, the involvement of phytohormones in nodule development seems not controversial. Exogenous application of hormones to pea roots and root explants resulted in the induction of cortical cell divisions similar to those found in early nodule development (208,209). Auxin, auxin-like substances, and kinetin cause hypertrophies on the roots of leguminous and non-leguminous plants that are easily mistaken for nodules (2,6,278). Analysis of the expression of early nodulin genes in these structures deserves attention.

The root nodule contains up to a hunderd-fold higher concentration of the three major groups of plant hormones, auxins (9,146,212), cytokinins (10,265,320) and gibberillic acid (94), relative to the hormone content of the uninfected root. Because auxins (101,360) and cytokinins (259) are known to be produced by *Rhizobium* in pure culture, the hypothesis is appealing that these phytohormones are produced by the infecting *Rhizobium* and trigger cell division (206,208). Analysis of the signalling between plant and *Rhizobium* has indicated that the *Rhizobium* genes required for nodulation may be producing signals that interfere with the hormone housekeeping of the root (see 6.4.2.). A group of soybean proteins which appeared three days after infection with *Bradyrhizobium* also appeared after treatment of roots with auxin (353). On the other hand, *Rhizobium* mutants completely ineffective in phytohormone synthesis have not been identified. Mutants producing only a small amount of auxin are for the greater part not symbiotically defective (158,360). Therefore, the data available to date do not allow a conclusion with respect to the role of *Rhizobium*-produced phytohormones in nodulin gene expression.

6.2.6.3. *Rhizobial signals*

When nodulin gene expression is not induced via alterations in the physiological conditions of the nodule, the invading *Rhizobium* itself will be the factor that does deliver inducing signals for the expression of nodulin genes. In the sections to follow, we will survey the rhizobial genome as the origin of causative signals that induce and regulate nodulin gene expression. The nature of those signals is unknown, but the mechanism of nodulin gene expression, and hence the signals regulating them, appear to be conserved in different species.

The most convincing evidence for the conservation of the regulation of Lb gene expression is the nodule specific and developmentally correct expression of a chimeric soybean leghemoglobin gene (see 6.2.6.1.) in transgenic birdsfoot trefoil plants (314) and white clover nodules (222). These results indicate a conserved mechanism for the induction of leghemoglobin gene expression independent from differences in the developmental program of determinate (soybean and birdsfoot

trefoil) and indeterminate (clover) nodules. Some naturally occurring, broad host range rhizobia induce nitrogen fixing, *i.e.* with all their nodulin genes properly expressed, root nodules on the roots of a variety of legumes as well as on the roots of the non-legume *Parasponia* (328). The very same *Rhizobium nod* genes are involved in the nodulation of all these host plants (see 6.4.2.). Therefore, irrespective of their exact nature, the signals from the invading rhizobia that regulate the induction of nodulin gene expression should be very alike in different plants.

6.3. NODULE DEVELOPMENT AND NODULIN GENE EXPRESSION

Two classes of nodulin genes, early and late nodulin genes, have been revealed by a first analysis of wild-type nodule development. The analysis can be refined by examining in more detail the coupling of histological and molecular biological data through the study of nodules blocked at different stages of development (239,357). Correlations between nodule structure and nodulin gene expression may provide clues to the role of nodulins in the developmental program of the nodule. To date, analyses have predominantly been done with nodules that are formed by mutated and engineered bacterial strains. This is because the *Rhizobium* genome can easily be manipulated in comparison to the plant genome. By classical genetic experiments, several plant genes involved in nodulation and symbiotic nitrogen fixation have been identified in pea (132,154), soybean (43,49, 131,132,356), clover (14,251) and alfalfa (337,338). Similar to the control of nodule development by *Rhizobium*, mutations in the plant genome can also result in disturbed nodule development, varying from the absence of nodules to the development of wildtype-like but ineffective nodules (357). Generally, the nature of the plant mutation is not known, and nodulin gene expression has not been analyzed. Therefore, we will not take these plant-conditioned disturbance of nodule development into account.

In the following paragraphs we will discuss the relationship between nodule development and nodulin gene expression, itemized for the different plant species pea, vetch, alfalfa and soybean. Despite the many reports of clover nodules disturbed in development, the plant species clover is omitted in the following discussions because knowledge about nodulin gene expression in clover is lacking.

6.3.1. Pea and vetch

The correlation between nodule structure and nodulin gene expression is best studied in the plant species, vetch and pea, both belonging to the same cross-inoculation group. On the roots of these plants *R. leguminosarum* induces the formation of indeterminate nodules. In nodules induced on vetch by wild-type *R. leguminosarum*, two early nodulins ENOD2 and Nvs-40, and 15 late nodulins have been identified (chapter 4.). In nodules induced on pea (*P. sativum*), two early, ENOD2 and Nps-40', and 20 late nodulins have been found (126). *Rhizobium* strains mutated in one of the *nif* or *fix* genes (see 6.4.1.) induce the formation of nodules on pea and vetch that are morphologically similar to nodules induced by wild-type *Rhizobium*. In these nodules, rhizobia differentiate into the characteristic bacteroidal shape and all nodulin genes are expressed (126,129,234). Nitrogen fixation *per se* is apparently not essential for the induction of the expression of nodulin genes.

Strain P8 is a *Rhizobium* wild-isolate that induces ineffective nodules on pea, in which bacteria are released from the infection threads, but do not differentiate into the characteristic Y-shaped bacteroids as do wild-type bacteria. All pea nodulin genes are expressed in the nodules formed by strain P8 (126). Thus, induction of the expression of nodulin genes does not depend on bacteroid development.

Strain 248^{c}(pMP104) is *R. leguminosarum* 248 (175), containing essentially 12 kb *nod* region from a *R. leguminosarum* sym plasmid (308). Strain 248^{c}(pMP104) forms nodules on pea and vetch that have the same histological organization as wild-type nodules, including the development of infected and uninfected cells. In this case, also all early and late nodulin genes identified are expressed (chapter 5.). The same is true for strain ANU845(pMP104), which contains the 12 kb *nod* region in a *R. trifolii* chromosomal background. These results show conclusively that the *nod* genes are the

only sym plasmid genes required for the induction of nodulin gene expression (see 6.4.2.).

Strain ANU845(pRt032)(*nodE* K11::Tn5) contains essentially 14 kb *nod* region from a *R. trifolii* sym plasmid (292) with a Tn5 insertion in the *nodE* gene. The mutation in *nodE* extends the host range of this *R. trifolii* strain to the pea/vetch cross-inoculation group (85). The nodules formed on vetch by this strain deviate from nodules induced by wild-type strains in the structure of the late symbiotic zone. The late symbiotic zone contains two to four layers of infected and uninfected cells. In the proximal part of the nodule, a large area of senescing tissue is present almost without any bacteria. In these nodules, both early nodulin genes ENOD2 and Nvs-40 are expressed. Only the late nodulin gene Nvs-65 is transcribed, but no mRNA from the other late nodulin genes, including the leghemoglobin genes is detectable (chapter 5.). Apparently the class of late nodulin genes must be divided in two subclasses that seem to be regulated in a different manner. The absence of Lb transcripts in the nodules induced by ANU845(pRt032)(*nodE* K11::Tn5) suggests that the Lb genes are first expressed when the nodule meristem cells are fully differentiated into infected and uninfected cells. Immunocytological localization of leghemoglobin in pea wild-type nodules supports this suggestion. In these nodules leghemoglobin is not detectable in the early symbiotic zone, nor in the first two cell layers of the late symbiotic zone (342).

Strain LBA4301(pMP104) is a Ti plasmid-cured *Agrobacterium tumefaciens* (161) containing the same 12 kb *nod* region from a *R. leguminosarum* sym plasmid as strain 248^{c} (pMP104)(chapter 5.). Strain LBA4301 (pMP104) forms nodules in which bacteria are released from the infection threads. The bacteria then become surrounded by a peribacteroid membrane. However, upon release from the infection thread, the bacteria are degraded despite the presence of a peribacteroid membrane, and they never develop into bacteroid-shaped structures. At the same time, also the organelles in the plant cytoplasm of these "infected" cells disintegrate. The uninfected cells appear normal, as judged by electron microscopical observations and the early nodulin genes ENOD2 and Nvs-40 are expressed. On the other hand, none of the late nodulin transcripts, including Nvs-65 and Lb, are detectable. Because differentiation into uninfected cells seems normal, the late nodulin genes found expressed in these vetch nodules are apparently not expressed in uninfected cells. Absence of the late nodulin Nvs-65 in the nodules formed by LBA4301(pMP104) suggests that release of bacteria from the infection thread is not sufficient for the induction of expression of the Nvs-65 gene. Comparison between the nodules formed by ANU845(pRt032)(*nodE* K11::Tn5) and LBA4301(pMP104) indicates that the Nvs-65 gene is probably first expressed in the youngest cells of the late sym-biotic zone that are completely filled with bacteria (chapter 5.).

To date, no bacterial strains are available that induce on vetch roots the formation of nodules without release of the bacteria from the infection threads. Such nodules are formed on pea roots (128) by the *Agrobacterium* transconjugant LBA2712, containing a complete *R. leguminosarum* sym plasmid instead of the Ti plasmid. The vetch (*V. sativa*) early nodulin Nvs-40 can be immuneprecipitated with an antiserum raised against the pea (*P. sativum*) early nodulin Nps-40' (25), and the soybean ENOD2 cDNA clone cross-hybridizes with both a pea (129) and a vetch (chapter 4.) ENOD2-like early nodulin mRNA. This shows that the early nodulins Nps-40'/Nvs-40 and PsENOD2/VsENOD2 are closely related. Therefore, the expression of the two early nodulin genes Nps-40' and PsENOD2 could be studied in pea nodules as substitute for vetch nodules. Only the PsENOD2 gene is expressed in LBA2712-induced pea nodules, while the Nps-40' gene and all pea late nodulin genes are not transcribed in these nodules (128). If one assumes that the Nvs-40 and Nps-40' genes are regulated similarly, the difference in Nvs-40/Nps-40' gene expression observed between nodules induced on vetch by LBA4301(pMP104) and nodules induced on pea by LBA2712 suggests that Nvs-40 gene expression is related to release of the bacteria from the infection threads and/or to the subsequent differentiation into infected and uninfected cells (chapter 4.). In view of the results obtained by analyses of nodulin gene expression in alfalfa nodules induced by *Rhizobium exo* mutants (see 6.3.2.), it is most likely that the expression of the Nvs-40 gene is related to the differentiation into infected and uninfected cells.

From the detailed studies on pea and vetch, it can be concluded that now at least four classes of nodulin genes can be distinguished on the basis of their expression in developmentally disturbed nodules. Both early and late nodulin genes can each be divided into two subclasses, the expression of which correlates with a stage of root nodule development, the formation of a nodule structure (ENOD2), differentiation into infected and uninfected cells (Nvs–40, Nps–40'), packing of the infected cells (Nvs–65) and subsequent processes (Lb and other late nodulins), respectively.

6.3.2. **Alfalfa**

R. meliloti induces on alfalfa (*M. sativa*) roots the formation of indeterminate nodules. In nodules induced on alfalfa by a wild–type *R. meliloti* about twenty nodulins have been identified (95,191,217). Two early nodulin genes, Nms–30 and MsENOD2, have been found. The alfalfa ENOD2–like early nodulin has approximately 80% amino acid homology with the soybean early nodulin Ngm–75 (80).

Detailed electron microscopical observations on alfalfa nodules induced by various *R. meliloti nif* and *fix* mutants revealed some minor deviations in structure compared to wild–type nodules (149,150). These deviations occur after release and maturation of the bacteroids. Nodules remain small due to rapid senescence. A *R. meliloti nifA* mutant induced nodules in which bacteroid maturation appeared interrupted. The bacteroids rarely attained the dimensions or appearance of wild–type bacteroids (149), but the nodules themselves elongated for the greater part to wild–type nodule dimensions. Whatever deviations in structure, all nodulin genes were expressed in nodules induced by the *nif* and *fix* mutants investigated (95,217), as is the case in pea and vetch nodules induced by *Rhizobium nif* and *fix* mutants.

The *R. meliloti* mutants *exoA* through *exoF* lack an acidic extracellular polysaccharide in their cell wall (104,202). These strains induce nodules on alfalfa totally devoid of infection threads and intracellular bacteria (104). This nodule phenotype will be referred to as empty. The rhizobia are restricted to the intercellular spaces in the nodule outer cortex. Only the early nodulin genes MsENOD2 and Nms–30 were expressed in these empty nodules (80,95), whereas none of the late nodulin transcripts were detected (95,217). The same pattern of nodulin gene expression is found in nodules induced by *Agrobacterium* strains carrying the *R. meliloti* sym plasmid 217, or the cloned *nod* genes (80), which both induce empty nodules resembling the nodules induced by *exo* mutants, and in nodules induced by a *R. meliloti exoH* mutant which fails to succinylate its acidic extracellular polysaccharide and induces nodules containing aborted infection threads and infrequently released bacteria (203).

The alfalfa early nodulin Nms–30 (also named Nms–38 (203)) immunoprecipitates with antiserum directed against pea Nps–40' (25), just as the vetch early nodulin Nvs–40 does. Apparently, a structurally similar early nodulin occurs in all three species. Assuming that pea Nps–40', vetch Nvs–40 and alfalfa Nms–30 are not only structurally but also functionally related, the pattern of expression of Nvs–40 and Nms–30 in vetch and alfalfa nodules respectively allows a potential role for this nodulin to be deduced. From the studies on vetch nodulin gene expression, it is concluded that the expression of nodulin Nvs–40 is related to the release of bacteria from the infection thread and/or to the differentiation into infected and uninfected cells (see 6.3.1.) Infection threads and infected cells are not present in the nodules induced on alfalfa by *Rhizobium exo* mutants, but the related Nms–30 gene is expressed. Therefore, the expression of the Nvs–40/Nms–30 nodulin gene is related to the differentiation into uninfected cells.

The absence of Nps–40' in the nodules induced on pea by *Agrobacterium* LBA2712 (128), in which bacteria are not released from the infection threads, seems contradictory to this relationship. The apparent contradiction can however be explained in the following way. The cells in the LBA2712–induced pea nodule may be without bacteria and morphologically resemble the uninfected cells in the empty alfalfa nodule, but differ on the molecular level. It is conceivable that the cells without bacteria in the pea nodule have not differentiated into uninfected cells, whereas the cells without bacteria in the alfalfa nodule are similar to the uninfected cells occurring in mature nodules. Because the ENOD2 gene is expressed in nodules without infection threads, the early nodulin gene ENOD2 is in–

volved in the establishment of a nodule structure and not in the infection process *per se*.

6.3.3. **Soybean**

Bradyrhizobium japonicum induces on soybean (*G. max*) roots the formation of determinate nodules. In nodules induced on soybean by a wildtype *B. japonicum*, more than twenty nodulin mRNAs have been identified (8,122,). At least four nodulins, Ngm-75, Ngm-44b (nomenclature changed to distinguish Ngm-44b from the peribacteroid membrane nodulin Ngm-44, see 6.2.3.5.), Ngm-41 and Ngm-38, are already found at the time of development when a globular meristem has been formed. These early nodulin genes are transiently expressed during nodule development. Except for the early nodulin Ngm-44b, their mRNAs increase in concentration up to the stage in which the complete nodule structure is established and then decrease in concentration. The concentration of Ngm-44b mRNA remains constant in this period and then decreases as well. Because Ngm-44b gene expression follows meristematic activity, the expression of the Ngm-44b gene may be correlated with meristematic activity in the nodule (122). Also, several late nodulin cDNA clones have been isolated (116,296).

Nodules induced on soybean by *B. japonicum* strains mutated in genes for *nif* and *fix* functions develop similar to wild-type nodules (135), just as is the case for pea, vetch and alfalfa. All nodulin genes are expressed in these ineffective nodules(116,122, 296). This also applies to nodules formed by a *B. japonicum nifA* mutant (317), but the nodules induced by this strain are severely disturbed in nodule development (108). The bacteria are released from the infection thread, but do not multiply extensively. Moreover, the infected cells collapse at an early stage of development. On the other hand, the uninfected cells in these *nifA* mutant-induced nodules appear normal. Although all nodulin genes are expressed, the concentration of leghemoglobin (mRNA) is reduced drastically (317). These data will be discussed in 6.4.1.

Strain HS124 is an ill-defined *B. japonicum* mutant obtained by UV irradiation (248). In the nodules formed by this mutant, bacteria are rarely released from the infection thread and the few cells that have become infected, appear to degenerate immediately (122,248). The uninfected cells in the HS124-induced nodules appear normal. All early nodulin genes are expressed, but interestingly their expression is no longer transient (122). Most of the identified late nodulin genes are expressed at approximately the same level of expression as found in wild-type nodules, but the expression of five late nodulin genes is not or hardly detectable. *E.g.*, leghemoglobin mRNA is hardly detectable. Apparently, late nodulin genes in soybean nodule development can be subdivided into two groups. On the one hand the group of late nodulin genes that is expressed in HS124-induced nodules and on the other the group of genes that is not or barely expressed in these nodules. Because infected cells develop hardly or not at all in nodules induced by HS124, the nodulin genes not expressed in HS124-induced nodules are most likely transcribed in infected cells during normal nodule development. The group of nodulin genes fully expressed in HS124-induced nodules is transcribed in uninfected cells or in both cell types. However, n-uricase, a marker for the uninfected cells, could not be detected immunologically (296). Therefore, the expression of some nodulin genes expressed in uninfected cells is affected as well. The concept of two subclasses of late nodulin genes has been confirmed by the isolation of late nodulin cDNA clones, some of which represented mRNA absent in HS124-induced nodules (296).

B. japonicum mutant T8-1 induces normal-sized nodules that contain infection threads, but almost completely lack intracellular bacteria due to a block in bacterial release (235). Because the marker proteins for the infected and uninfected cells, leghemoglobin and n-uricase respectively, could be detected, the differentiation into these two cell types appears undisturbed. Whereas the mRNA for most late nodulins was present at reduced levels in these nodules, mRNA for Ngm-26 was present at concentrations found in wild-type nodules (110,235). In wild-type nodules, nodulin Ngm-26 gene is associated with the peribacteroid membrane. However, the expression of the Ngm-26 gene in these developmentally disturbed nodules shows that this gene is also expressed when the peribacteroid membrane is not formed (110,235). Although these results have been taken to

suggest that there are at least two developmental stages in peribacteroid membrane biosynthesis (235), the apparent contradiction of the expression of the peribacteroid membrane nodulin gene Ngm-26 in nodules induced by *B. japonicum* T8-1 questions the exact location of this nodulin. Ngm-26 gene expression is not detected in nodule-like structures devoid of infection threads (235), therefore Ngm-26 may be located in the infection thread as well as in the peribacteroid membrane.

6.3.4. **Conclusions**

Various studies on nodulin gene expression and nodule development with specific *Rhizobium* mutants have shown that nodule formation can be arrested at different stages of development as judged by histological criteria, and that these stages of development can be correlated with the expression of different sets of nodulin genes. On the basis of their expression in developmentally disturbed nodules, early and late nodulins can each be divided into at least two subclasses, the expression of which is regulated differently. Each subclass may reflect the occurrence of a different step in the developmental program of the root nodule.

6.4. **RHIZOBIUM GENES INVOLVED IN NODULIN GENE EXPRESSION.**

Both early and late nodulin genes can be subdivided into subclasses and each subclass correlates with the attainment of a defined stage in root nodule development beyond which further development is blocked by the mutation in the *Rhizobium*. This suggests that the bacterium delivers signals to the plant for the induction of expression of the successive subclasses nodulin genes. For a characterization these putative *Rhizobium* signals, the *Rhizobium* genes required for nodulin gene induction have to be identified. In fast growing rhizobia (genus *Rhizobium*) the majority of the genes essential for nodulation and symbiotic nitrogen fixation are located on a large plasmid (17), the so-called sym plasmid, whereas in slow growing rhizobia (genus *Bradyrhizobium* (173)) the genes involved in the symbiosis are located on the bacterial chromosome (145,310). The bacterial strains used to correlate nodulin gene expression with nodule development (section 6.3.), will now be discussed with the aim to identify the *Rhizobium* genes required for the induction of the expression of nodulin genes.

6.4.1. **Nitrogen fixation genes**

The *Rhizobium* genes essential for symbiotic nitrogen fixation are the *nif* and *fix* genes. *Nif* genes have been defined on the basis of structural or functional analogy with the *nif* genes in the free-living nitrogen fixing species *Klebsiella pneumoniae* (83,283). *Fix* genes are also required for nitrogen fixation because nodules induced by strains mutated in these genes do not fix nitrogen, but *fix* genes share no homology with *K. pneumoniae* genes. In both *Rhizobium* (64,97,133, 286,287) and *Bradyrhizobium* (98,145). clusters of *nif* and *fix* genes have been identified.

All investigated nodulin genes are expressed in nodules induced on various legume plants by all *nif* and *fix* mutants examined so far. Therefore, these *Rhizobium nif* and *fix* genes are not essential for the induction of nodulin gene expression. The observation that all nodulin genes are expressed in nodules formed on pea and vetch by *Rhizobium* strain 248^{c}(pMP104), containing only a small region of the sym plasmid (see chapter 5.) without *nif* and *fix* genes, is the most conclusive evidence that *nif* and *fix* genes are not required for the induction of nodulin gene expression.

The *nif* and *fix* genes do, however, appear to influence the level of expression of late nodulin genes. In nodules formed on pea by *nif* and *fix* mutants the amount of mRNA of the late nodulin genes is 10-40% of the amount found in wild-type nodules (126). This phenomenon might for the greater part be attributed to impaired nodule growth resulting in a change in the ratio of different cell types. On the other hand, in pea the amount of Lb protein in ineffective nodules is not in proportion to the amount of Lb mRNA (126). Therefore a post-transcriptional regulation of leghemoglobin formation is likely.

Whereas *nif* and *fix* mutants of *Rhizobium* do not influence the development of the root nodules they induce, a few notable exceptions have been described for *Bradyrhizobium nif* and *fix* mutants. A *B. japonicum nifA* mutant induces nodules severely disturbed in the later stages of nodule development (108)(see 6.3.3.). All nodulin genes are expressed, but leghemoglobin is present at an extremely reduced concentration compared to the concentration in nodules induced by other *nif* and *fix* mutants (317). Whereas in other *B. japonicum nif* and *fix* mutants investigated, the concentration of leghemoglobin protein is about 50% of the concentration in wild-type nodules, the leghemoglobin concentration in the *nifA*-mutant induced nodules is less than 1% of the concentration found in nitrogen fixing nodules. The decrease in leghemoglobin concentration is partly due to a decrease in transcription, since the relative concentration of leghemoglobin mRNA in the *nifA*- mutant-induced nodules is about 5–10% of that found in wild-type nodules. The relative concentration of two other late nodulin mRNAs, Ngm-23 and n-uricase, in the *nifA* mutant-induced nodules is reduced to about 30% of that in wild-type nodules, while the expression of the early nodulin gene Ngm-75 is not decreased at all (317). The very strong decrease in concentration might thus be unique for leghemoglobin. The dramatic effect of the *B. japonicum nifA* mutation on nodule development and in particular on the accumulation of leghemoglobin suggests that the *nifA* gene product not only regulates the expression of the *nif* genes in *Bradyrhizobium* but is also in some way involved in the regulation of nodulin gene expression in the plant cells. Because all nodulin genes are expressed in the *nifA* mutant-induced nodules, it can be excluded that the *nifA* product is required for the actual induction of nodulin gene expression. However, the *nifA* product appears to be required for the accumulation of leghemoglobin during nodule development. Besides the *nifA* mutant, a few *B. japonicum fix* mutants have been isolated that are phenotypically impaired in free-living nitrogen fixation, but have no auxotrophic defects (267). Counterparts for this type of *fix* genes have not been found in fast growing rhizobia. Just as the *nifA* mutant, these *fix* mutants induce nodules severely disturbed in development, but nodulin gene expression in these nodules has not yet been studied.

The effect of *nifA* and certain *fix* genes on soybean nodule development, shows that gene products involved in building up the nitrogen fixing system have a regulatory role in nodule development and nodulin gene expression in the *B. japonicum*-soybean symbiosis, whereas there is no evidence for such a regulatory role of *nif* and *fix* gene products in the symbiosis of fast growing rhizobia and legumes.

6.4.2. **Nodulation genes**

The *Rhizobium* genes required for, or involved in nodulation of legume hosts, the *nod* genes, have been identified by a variety of genetic means (214). So far the genes *nodA* through *nodJ* have been identified in at least one of the various *Rhizobium* and *Bradyrhizobium* species studied (88,90,168,181,185,215, 282,293,294,365). Of these *nod* genes, the *nodDABC* genes are found in all species. These four genes are functionally interchangeable between different species of *Rhizobium* (88,90,109,181,365) and are therefore called common *nod* genes. The common *nod* genes appear to be absolutely essential for nodulation in all *Rhizobium*-legume symbioses, because mutations in these genes result in a Nod- phenotype (214). The same four genes are also essential for nodulation of the non-legume *Parasponia* (224). The common *nod* genes fall within one region of about 14 kb in *R. leguminosarum* (90) and *R. trifolii* (292), and within two regions separated by about 12 kb in *R. meliloti* (215).

The other genes, called host-specificity *nod* genes, delay or reduce nodulation or alter host range when mutated, but these mutations do not cause a complete inability to form nitrogen fixing root nodules (74–76,87,91,165). Therefore, the genes *nodE* through *nodJ* are involved in a fine tuning of the regulation of nodulation, but they are *not* essential for the induction of nodulin gene expression.

The *nodDABC* genes are thus the most prominent candidates for generating the signal(s) involved in the induction of the expression of nodulin genes. The *nodABC* genes constitute one operon, the constitutively expressed *nodD* is transcribed separately in the reverse direction (86,100,281,290,307). In the presence of flavonoids excreted by the

root, the *nodD* gene product induces the expression of all other *nod* genes (86,106,166,236,257,266,371), possibly as a positive transcriptional activator (216). The *nodD* gene products have been shown to differ in responsiveness to different flavonoids in a host-specific way (164,309). These observations imply that the interchangeability, hence the common *nod* gene status of *nodD*, has to be questioned.

Genetic analyses indicated already the pivotal role of the *nodDABC* genes in the establishment of the symbiosis. The *nodDABC* genes have been shown to be essential for root hair curling (90,92,168), formation of the infection thread (85,76) and the induction of cortical cell division (93), thus for the earliest steps in the developmental program of the root nodule in which no nodulin gene expression has been identified yet.

In reaction to the plant-excreted flavonoids, *Rhizobium* produces low molecular weight, soluble factors that cause a thick and short root (Tsr) phenotype on vetch (340,341), and root hair deformations on vetch (372) as well as on clover (20). The branching factor produced by *R. trifolii* also causes root hair deformations on vetch (47). Mutations in the common *nod* genes abolish the ability of the bacterium to produce these factors (19,372), demonstrating a direct effect of *nodABC* gene products in the production of a return signal from bacterium to plant. In view of the reaction of the plant to this signal, it is likely that the signal is hormone-like in nature. The sequence of the Rhizobium *nod* genes does not resemble sequences of phytohormone synthesis genes, but complementation of a *R. meliloti* Nod- mutant with an *A. tumefaciens* cytokinin gene resulted in a strain capable to induce an empty nodule, suggesting that indeed alterations in the hormone housekeeping of the legume root, in some way brought about by the *nod* gene products, are sufficientfor the formation of a nodule structure (213). Overproduction of the *nodABC* gene products either by increased gene copy number or from strong promotors proved deleterious to nodulation (155,180). Thus, the concentration of the *nodABC* gene products is critical for the proper development of the symbiosis.

Upon introduction of a fragment carrying exclusively the *nodDABC* region into a sym plasmid-cured *Rhizobium* strain, the recipient strain acquires the ability to curl root hairs (180), but a nodule structure is not formed. Whereas the *nodDABC* region by itself is not sufficient for the formation of a nodule structure, upon introduction of 12 kb *nod* region from a *R. leguminosarum* sym plasmid into a sym plasmid-cured *Rhizobium,* the recipient strain regains the ability of the donor strain to induce nodules on pea and vetch. In these nodules, the expression of all nodulin genes is induced (chapter 5.). Apparently, the host specificity *nod* genes present on this 12 kb region, in addition to *nodDABC*, pave the way for development, without being essential in themselves, because mutations in these additional host specificity *nod* genes do not result in a Nod- phenotype. This result proves conclusively that 12 kb *nod* region in a *Rhizobium* chromosomal background is sufficient for the induction of the expression of all nodulin genes identified.

The strain obtained by introducing the same 12 kb *nod* region into a Ti plasmid-cured *Agrobacterium* induces nodules on vetch in which only the early nodulin genes, Nvs-40 and ENOD2, are expressed, but no late nodulin mRNAs are detectable (chapter 5.). Thus, the presence of the 12 kb nod region in an *Agrobacterium* chromosomal background is sufficient for the induction of early nodulin gene expression. The *Agrobacterium* chromosome itself is unlikely to contribute signals specifically involved in the induction of nodulin gene expression, because nodulin gene expression is not detectable in tumors formed on the stem of vetch plants after wounding with *A. tumefaciens* (chapter 2.). Therefore, the 12 kb *nod* region must be responsible for the induction of the expression of early nodulin genes. In view of the results of the mutation analyses, the *nodABC* genes must be the gene involved in this induction. Because the expression of the identified early nodulin genes becomes first detectable after the nodule primordia have been formed, the induction of their expression is part of the developmental stage following the induction of cortical cell divisions. Therefore, the *nodDABC* genes are also involved in a stage of development beyond the induction of cortical cell divisions.

The *nodABC* genes seem not sufficient for the induction of the expression of the late

nodulin genes, because late nodulin gene expression is not detectable in the nodules induced by the strain containing the 12 kb *nod* region in an *Agrobacterium* chromosomal background. Although this result suggest that chromosomal or non-sym plasmid genes are essential for the induction of late nodulin gene expression, in 6.5.2. will be discussed that such a conclusion cannot be drawn.

Indirect evidence indicates that the *nodABC* genes are indeed involved in the induction of late nodulin gene expression. Two *Rhizobium* strains that only differ in the origin of the cloned *nod* genes they contain were used to induce nodules on one and the same host plant, vetch. Strain ANU845(pMP104) contains the 12 kb *nod* region discussed above in a *R. trifolii* chromosomal background. Strain ANU845(pRt032)(*nodE*::Tn5) contains 14 kb *nod* region from a R. *trifolii* sym plasmid in the same *R. trifolii* chromosomal background. Due to a Tn5 mutation in *nodE*, the host range of this *R. trifolii* strain is extended to vetch (85). Analyses of the pattern of nodulin gene expression in nodules induced on vetch by both strains showed that in nodules induced by ANU845(pMP104) the expression of all nodulin genes is induced, whereas in nodules induced by ANU845(pRt032)(*nodE*::Tn5) the late nodulin gene transcripts, except for Nvs-65 mRNA, were not detectable (chapter 5.). The marked difference between these two strains with respect to late nodulin gene expression will be related to the only difference between the strains: the apparently distinguishing characteristics of the 12 kb *nod* region in the one strain *versus* the 14 kb *nod* region in the other. Irrespective of the exact cause of the observed difference in the induction of late nodulin gene expression, this difference indicates an involvement of *nod* genes in a stage of nodule development associated with the induction of late nodulin gene expression. This conclusion is supported by the observation that the *nodA* and *nodC* genes are expressed in *R. meliloti* bacteroids (289).

6.4.3. **Surface determining genes**

The *Rhizobium* surface has been supposed to be involved in adhesion to the root hair surface, determination of host range, and nodulation (73,137,304). A firm relation between the *Rhizobium* surface and the development of a nitrogen fixing root nodule was established by the isolation of well-defined mutants that fail to produce extracellular polysaccharide and form a developmentally disturbed nodule (52). Like other Gramnegative bacteria, *Rhizobium* has an outer membrane outside the peptidoglycan cell wall. External to the outer membrane, but tightly associated with it through 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: exo-polysaccharides (EPS), and the more tightly bound capsular polysaccharides (CPS). The EPS contain a fraction of heteropolysaccharides, the majority of these being acidic, and a fraction of homopolysaccharides which are mainly neutral glucans (48). The various surface polysaccharides are a complex mixture of different oligomers and polymers, every one of which may be of importance in the symbiosis.

Several genetic loci for *Rhizobium* surface determinants have been identified, and the genes are being characterized as detailed as the *nif*, *fix* and *nod* genes. Eight loci affecting acidic EPS have been identified in *R. meliloti* (104,202). Strains mutated in the loci *exoA* through *exoF* fail to produce a particular acidic EPS (104). In addition to acidic EPS, the *exoC* mutant also lacks cyclic glucan (80). These *exo* mutants induce nodules devoid of intracellular bacteria and infection threads. Some of the mutations could be complemented by genes from *A. tumefaciens* (46). A mutation in the eighth exo locus, *exoH*, resulted in a strain that produces a slightly modified acidic EPS in which the succinyl modifications are absent (203). In the nodules induced by the *exoH* mutant, infection threads are present but bacterial release is rarely observed (203). Similarly, *R. meliloti* mutants that either lack acidic EPS or have an acidic EPS without pyruvate modifications, and elicit empty or *exoH* mutant-like nodules respectively, have been isolated (262). These results show that the acidic EPS and its noncar-bohydrate substitutions have a role in the infection process. In all *exo* mutant-induced nodules on alfalfa, the expression of two early nodulin genes, MsENOD2 and Nms-30, was induced, but late nodulin gene transcripts were absent (80,203).

In addition to the *exo* loci, two *ndv* (nodule development) loci, *ndvA* and *ndvB*, have been identified in *R. meliloti* (96). These genes are homologous to and functionally interchangeable with the chromosomal virulence genes *chvA* and *chvB* of *A. tumefaciens*. *R. meliloti ndv* mutants induce nodules with a morphology similar to that of the *exo* mutant-induced nodules (80,96). In contrast to *R. meliloti exo* mutants, these mutants are not impaired in the synthesis of acidic EPS, but they are defective in the biosynthesis of cyclic glucan (80,120), just as the *Agrobacterium chv*-mutants (264). As is the case in the *exo* mutant-induced nodules, the expression of the early nodulin genes, Nms-30 and ENOD2, is induced, but late nodulin gene expression could not be detected (80).

Also in other *Rhizobium*-legume symbioses, a relation between EPS and the formation of a nodule has been demonstrated (34,55,89,339). In addition, surface components like LPS and CPS have been shown to affect nodule development (118,249,263). In all these cases, nodulin gene expression has not been studied, so the involvement of these surface components in the induction of the expression is not known.

Besides phenotypical analyses of mutations, complementation studies with mixed inoculations have demonstrated the importance of surface determinants in nodule development and nodulin gene expression. Coinoculation of a sym plasmid-cured Exo$^+$ *nod* mutant of the broad host range, fast-growing *Rhizobium* strain NGR234 with a Nod$^+$ *exo* mutant of the same *Rhizobium* that formed severely disturbed nodule-like structures on *Leucaena* plants resulted the induction formation of nitrogen fixing root nodules on *Leucaena* plants ((54). Both original mutants could be isolated from the nodules induced. This observation suggests that the EPS contributed by the Exo$^+$ *nod* mutant complements the defect of the Nod$^+$ *exo* mutant. Moreover, the addition of the correct EPS, purified from the parent strain NGR234, to the *exo* mutant has been reported to cause the formation of nitrogen fixing root nodules (89). This confirms that the EPS is essential for effective nodulation. Also on alfalfa, coinoculation of *R. meliloti nod* mutants with *exo* mutants resulted in the induction of nitrogen fixing root nodules (179, 262), but suppression of symbiotic deficiency on alfalfa by the addition of EPS purified from the parental strain failed in the case of *R. meliloti exo* mutants (203).

6.4.4. **Other genes**

It has been shown that the *R. melilioti nod* genes are expressed at normal levels in various *Rhizobium* chromosomal backgrounds and in *Agrobacterium tumefaciens*, but not in other Gram-negative bacteria (370). Apparently, the *Agrobacterium* chromosome contains genes that are essential for the induction of the *nod* genes. A mutation in these genes will result in a Nod phenotype. Introduction of the *Rhizobium nod* region in an *Agrobacterium* chromosomal background results in a strain that is able to induce a nodule structure in which early nodulin genes are expressed (see 6.4.2.). It cannot be excluded that the *Agrobacterium* chromosome contains genes essential for the induction of early nodulin gene expression. However, if the *Agrobacterium* chromsosome were to contain essential genes for this induction, this implies that *Agrobacterium* has retained genetic inflormation it never uses in its natural situation.

Because the expression of late nodulin genes is not detected in nodules induced by *Agrobacterium* containing the *nod* region, the *Agrobacterium* chromosome is not sufficient for the induction of late nodulin gene expression, which suggest that *Rhizobium* chromosomal genes are involved in the induction of the expression of late nodulin genes (see, however, 6.5.2.). Various other genes have been shown to be involved in nodule development. A *R. meliloti leu* mutant induced on alfalfa roots small white nodules, in which bacteria were not released from the infection threads (329). When leucine or one of its precursors was added to the plant growth medium, bacterial release from the infection thread was restored, and nitrogen fixing root nodules developed. This result suggests an involvement of leucine in nodule development. Several drug-resistant mutants, carbohydrate metabolis mutants, and other auxotrophic mutants have also been reported to induce symbiotically deficient nodules (184,366), suggesting a role in nodule development for the mutated gene. In most cases, neither nodule morphology nor nodulin gene expres-

sion have been studied.

It seems doubtful that all these various genes are responsible for signals towards the plant that are involved in nodule development. Obviously, certain basic physiological requirements must be met in order for the *Rhizobium* to grow. Active growth of *Rhizobium* seems a prerequisite for proper development of nitrogen fixing root nodules. Mutations that affect these basic physiological requirements will only as a secondary consequence result in a symbiotically deficient strain. The role of the *Rhizobium* chromosome in nodule development and the induction of nodulin gene expression is probably for the greater part the support of the basic physiology of the bacterium.

6.4.5. **Conclusions**

Several *Rhizobium* genes that affect nodule development and nodulin gene expression have been identified by phenotypic analysis of mutant-induced nodules. The effect of some auxotrophic mutants on nodule development shows that the mere disturbance of nodule development does not necessarily imply that the *Rhizobium* gene mutated is actually responsible for a signal essen-tial for the induction of nodulin gene expression. Of the *Rhizobium* genes identified, the *nod* and surface determining genes are the most obvious can-didates to encode proteins providing signals for induction of the successive phases of nodule development. In 6.5 we will discuss the role of these genes in relation to the induction of nodulin gene expression.

6.5. **RHIZOBIUM AND THE REGULATION OF NODULIN GENE EXPRESSION**

The correlation between nodulin gene expression and nodule development (6.3), together with the identification of the Rhizobium genes affecting nodule development (6.4), provide the basis for the discussion how many *Rhizobium* signals are involved in the induction of nodulin gene expression, and which genes generate them. However, in the previous sections, the possibility of another plant reaction interfering with nodule formation, *i.e.* a plant defense response has been left outside of consideration. Yet such an alternative plant reaction may be an essential factor in identifying the (rhizobial) signals for the induction of nodulin gene expression. Therefore, we will first take the role of a defense response during nodule development into account. We will discuss briefly the role of a plant defense response in nodule development and outline the consequences of that role for *Rhizobium* signals in the induction of nodulin gene expression. Then we will discuss the role of the *Rhizobium nod* and surface determining genes in the interplay of plant and bacterium resulting in a root nodule.

6.5.1. **Defense response**

Plants are able to defend themselves against plant-pathogens by a variety of means (60,297). Some of the defenses are general, in that they provide protection from infection by a range of pathogens. These defenses include phytoalexin accumulation (72), extensin accumulation, and other responses called collectively the hypersensitive response (297). Other defense responses are highly specific, detected only in response to attack by a particular pathogen. Although root nodule development has repeatedly been considered as a special kind of plant-pathogen interaction (84, 172,300,336), establishment of a nitrogen fixing root nodule does not appear to provoke any known defense response. In soybean root nodules the concentration of the phytoalexin glyceollin is even lower than in uninfected roots (361). In preliminary studies in our laboratory no increase of extensin-related RNA in nodules compared to uninoculated roots was observed (25). Also stress-related RNA, detected on RNA transfer blots using a soybean general stress cDNA clone as probe (68), was not enhanced in concentration (25). These results suggest that the known defense mechanisms are not operating during normal root nodule development. Furthermore, none of the nodulins identified in vetch and pea nodules are detectable in tumors formed on the stem of vetch and pea plants by *Agrobacterium tumefaciens*

(chapter 4.) while *Agrobacterium* is a well-recognized plant-pathogen. This result shows that none of the identified nodulins functions in defense mechanisms.

A perturbation of the normal situation during nodule development appears to elicit a defense response in the host plant. In nodules formed on soybean by a *B. japonicum* mutant that forms unstable peribacteroid membranes, the phytoalexin concentration increases fifty fold (361). The nodules senesce prematurely and the necrotic appearance of the degenerated nodule is reminiscent of a hypersensitive response to pathogenic infection. Up to now, this is the only case in which a recognized parameter of defense has been measured. Further evidence for a defense response is circumstantial. A *R. trifolii* mutant that overproduces exopolysaccharides induces a disturbed infection process in which infection thread growth is aborted in the root hair cell (279). The reaction of the plant is interpreted as a hypersensitive response, because electron dense material is deposited around the infection site. Nodules induced on the roots of vetch plants by an *Agrobacterium* transconjugant carrying the complete *R. leguminosarum* sym plasmid exhibit a clearly dark center in the nodules. This dark center may be similar to the phenomenon of browning, associated with the hypersensitive response (3). Structural analyses of these nodules at the light and electron microscopical level indicated that some nodule cells contain bacteria, but the bacteria degenerate directly after release from the infection thread and plant cells collapse (342). Thus, although in normal nodule development no indications of a defense response are apparent, such a defense response seems to be present in the development of some ineffective nodules. It can be argued that in these ineffective nodules the symbiosis must be considered as a classical parasitic interaction (336).

In view of the apparent absence of a plant defense response during normal nodule development, we hypothesize the presence of a system in the plant to which we will refer as sensor system, that is probing the performance of the symbiosis. The bacterial surface is constantly, or at defined stages of development, evaluated. The available data on nodule development indicate that the sensor system is active at least at two different stages of development. First at the initial growth of the infection thread, and second at the release of bacteria from the infection thread. When this sensor system detects an aberration of the permitted surface, a defense response is elicited. As a result, further nodule development is impaired.

6.5.2. **Rhizobial signals**

The triggering of a defense response in root nodules induced by deviating bacteria puts the communication between *Rhizobium* and the plant in a different perspective, because it implies the existence of two types of signals. On the one hand, there are signals that actively cause the induction of nodule differentiation and nodulin gene expression. To these signals we will refer as inductive signals. On the other hand, there are signals which permit nodule development and achieve avoidance of a defense response. Because the latter type of signal will only be a passive one, we prefer the term avoidance determinant rather than signal. Avoidance determinants turn *Rhizobium* into a "parasite in disguise".

In terms of *Rhizobium* signals involved in the induction of nodulin gene expression, the requirement of correct avoidance determinants runs up a fundamental limitation of what can be concluded from developmentally disturbed nodules induced by engineered rhizobia and agrobacteria. When a mutation changes an avoidance determinant into a component that triggers the defense mechanism, the developmental program of the nodule will be aborted, while the mutant still has all genetic information for the inductive signals. In this case, a blockade of development does not indicate a lack of an inductive signal, but is only due to the unmasking of the engineered bacterium as result of incorrect avoidance determinants.

In the following two sections we will discuss these consequences of an interfering defense response for the roles of the *Rhizobium* nodulation and surface determining genes in the induction of nodulin gene expression. Are these genes merely encoding avoidance determinants, or are their products more directly involved in the induction of the

developmental program of the root nodule?

6.5.2.1. *Surface determining genes*

A relation between microbial outer surface and plant defense responses has been well established (299). For instance, cell surface oligo- and polysaccharides of *Phytophtera* are able to elicit plant defense responses (72). In the interactions between *Pseudomonas solanacearum* and potato or tobacco, a plant defense response can be generated by living or dead bacteria or their lipopolysaccharides (130,298). In normal nodule development, a defense mechanism is not elicited. Therefore, the *Rhizobium* surface components are likely to function as avoidance determinants. On the other hand, however, it has been shown that oligosaccharins can regulate developmental programs in plants directly (327). Hence, surface determinants may also have inductive capacities. Legume roots excrete enzymes that are able to degrade rhizobial polysaccharides (305), which appears a plausible way of producing oligosaccharins. However, we will show that most data available can be explained by assuming that the surface determining genes code for avoidance determinants.

A mutation in the chromosomal *pss* (Poly-Saccharide Synthesis) gene of *R. phaseoli* in combination with the *R. phaseoli* sym plasmid does not affect the capacity to form nitrogen fixing nodules on French bean (35), but when the same chromosomal mutation is combined with a *R. leguminosarum* sym plasmid, the ability to nodulate peas is completely blocked. Normally, a strain containing a *R. leguminosarum* sym plasmid in a *R. phaseoli* chromosomal background nodulates pea. Therefore, the *pss* gene product would be essential for the induction of nodule formation and nodulin gene expression on pea, while on the natural host French bean this gene product is not important. In view of the apparent conservation of the mechanisms involved in nodulin gene expression (see 6.2.6.3.), it is unlikely that a gene product absolutely essential for nodulation of the one legume (pea), has no importance at all for nodulation of another legume (French bean). Therefore, *pss* most likely codes for an avoidance determinant and is not involved in the generation of an inductive signal. The failure to nodulate pea shows that only the heterologous host plant does not tolerate the bacterial surface components that are exhibited in the absence of the *pss* gene product.

The results obtained with the *pss* gene may show that some plants accept more differences in the bacterial surfaces than others, or react more slowly to an aberration. An *Agrobacterium* transconjugant harbouring a *R. leguminosarum* sym plasmid forms nodules on vetch, in which most likely a defense response is elicited after release of bacteria from the infection thread (342). The same *Agrobacterium* transconjugant forms nodules on pea plants that are totally devoid of intracellular bacteria, despite the presence of infection threads (128). Thus, on two different legumes *Agrobacterium* transconjugant-induced nodules differ in the stage where development is arrested. On alfalfa roots, an *Agrobacterium* transconjugant with the *R. meliloti* sym plasmid, or cloned *nod* genes, forms nodule-like structures that are even more disturbed in development than the nodules formed on pea, since infection threads are not or only in the root hair cell observed (80,151,330,368). In clover, infected cells are observed in nodules induced by an transconjugant carrying the *R. trifolii* sym plasmid (162). A transconjugant harbouring the *R. phaseoli* sym plasmid is capable of inducing nitrogen fixing root nodules on the roots of French bean plants, unless plants were grown at 21°C instead of 26°C (159 ,223). A similar range in nodule morphology has been described for nodules induced by *Agrobacterium* transconjugants on other leguminous plants (41,340). These results can be interpreted as a different tolerance of the sensor system of different legumes towards the (slightly) deviating surface determinants of *Agrobacterium* compared to the surface of the taxonomically closely-related *Rhizobium*.

The best studied surface determining genes are the *R. meliloti* genes for the synthesis of acidic exopolysaccharides. Mutants are available that either lack acidic EPS, or have a modified form (104,202,203,262). Most *exo* mutants of R. meliloti form empty nodules on alfalfa. In coinoculation experiments using a *nod* mutant of *R. meliloti* in combination with an *exo* mutant, wildtype-like nodules were obtained, showing that the defects are mutually restored (262). However, coinoculation of the *nod* mutant with a strain having a

modified EPS lacking pyruvate residues was unsuccessful (262). Although the correct EPS is contributed by the *nod* mutant, the presence of modified EPS is apparently sufficient to arrest development. This observation strongly suggests that the blockade in development is not due to the absence of an EPS-derived signal molecule. It is most likely that EPS functions as an avoidance determinant.

The nodules induced on alfalfa by *exo* mutants lacking acidic exopolysaccharides, by an *Agrobacterium* transconjugant carrying the *R. meliloti* sym plasmid or cloned *nod* genes, or by *ndv* mutants impaired in cyclic glucan synthesis, are all devoid of intracellular bacteria. Only early nodulin gene expression is induced in these nodules (80). The different bacterial strains appear to differ only in their outer surface. Because nodule morphology and the pattern of nodulin gene expression in these nodules are similar, all these different bacteria would produce the signals required at the same stage of development. But it seems very unlikely that the various surface components are all involved in the generation of signals required at the same time of development. A more simple explanation is that all these surface components are required as avoidance determinants at the same stage of nodule development. In view of the phenotype of the *ndv* mutation and the interpretation here described, it should be noted that the designation *N*odule *De V*elopment for this gene (96) is unfortunate and misleading. The observed nodule morphology is most likely only an indirect effect of the mutation.

In the interpretation of all data available, it can not be excluded that the bacterial surface components, in addition to functioning as avoidance determinants, are also responsible for the generation of inductive signals. These two possible modes of action of surface determinants are not mutually exclusive. A change in a surface determinant can change an avoidance determinant, and at the same time destroy a (saccharide) signal that is essential in the developmental program of the nodule. At the moment, it seems premature to assume that rhizobial surface determinants have such an active role. Satisfactory evidence has yet to be provided that surface determining genes are responsible for signals involved in the induction of nodulin gene expression. The only indication that surface components have an active role, is the observation that the oligosaccharide repeat unit of acidic EPS complemented an *exo* mutant of the broad host range *Rhizobium* strain NGR234 (89), when added to the culture medium. Future experiments need to be designed in which the differences between avoidance determinants and inductive signals can be more clearly assessed.

6.5.2.2. *Nodulation genes*

Mutations in the common *nod* genes, *nodDABC*, abolish the ability to induce nodules and nodulin gene expression completely. These studies already suggested that the *nodDABC* genes are essential for establishing a nodule and for the induction of nodulin gene expression. The most conclusive evidence that the nod genes are involved in nodulin gene expression can be inferred from DNA transfer studies. Transfer of a limited piece of sym plasmid DNA carrying essentially the *nod* genes to *Agrobacterium*, conferred upon the recipient stain the ability to induce a nodule structure in which early nodulin genes are expressed (80, chapter 5.). The *nod* genes are the only *Rhizobium* genes so far, for which such a positive correlation between the presence of genetic information and the induction of the expression of (early) nodulin genes has been established. Of the nod genes, only *nodDABC* are essential for nodulation (see 6.4.2.). The *nodDABC* genes may therefore well be the minimum genetic requirement needed for the induction of the developmental program up to and including early nodulin gene expression. In view of the regulatory role of *nodD*, the *nodABC* genes are thus the most likely candidates for the generation of one or more signals towards the host plant that result in the expression of early nodulin genes. It cannot be totally excluded, however, that chromosomal genes are also responsible for inductive signals.

The mode of action of the *nodABC* gene products is still largely unknown. The *nodC* protein is a hydrophobic protein that is an integral part of the bacterial outer membrane and this protein may be involved in transmembrane signalling (171). Although the *nodA* gene product contains hydrophobic regions (326), it has been localized in the cytosol (289). Upon induction, these gene products cause the production of low molecular weight,

soluble factors, that have hormone-like activities. Both the *nodA* and *nodC* gene products are detectable in bacteroids (289), suggesting that these gene products also function in the mature nodule. The different processing of the *nodC* gene product in free-living bacteria, in which the *nod* genes are induced, compared to bacteroids (289) may indicate that varying *nodC* gene products have different functions at subsequent stages of nodule development. These observations support the notion that the *nod* gene products are actively involved in later stages of nodulin gene expression as well. Because the late nodulin genes are not expressed in the nodules formed by *Agrobacterium* carrying essentially the nod region, direct evidence for the involvement of the nodABC genes in the induction of late nodulin gene expression is lacking (chapter 5.). Just as the surface determining gene products, the *nodA* and *nodC* gene products in bacteroids may be concerned with the outer surface structure of *Rhizobium*. The role of the nodABC genes in late nodulin gene expression may thus be a role in avoiding plant defense reactions.

6.6. CONCLUSIONS

During development of effective nodules, *Rhizobium* succeeds in bypassing the plant defense mechanisms that normally protect a plant against invading pathogens. However, when plants are infected by mutated or engineered strains, a plant defense response can be induced. *Rhizobium* genes involved in nodule formation and nodulin gene expression can therefore be involved in either the avoidance of the plant defense mechanism (avoidance determinants), or in the generation of a signal responsible for nodulin gene expression (inductive signal), or in both at the same time. A serious consequence of the involvement of a host defense mechanism is that conclusions with respect to the genetic potentials of a bacterial strain are no longer allowed for developmental stages this strain does not induce. A strain may have the genetic information for all inductive signals, but at the same time lack an avoidance determinant. As a result, expression of the genetic information for the inductive signals is obscured. Put differently, not every blockade in development needs to be due to the absence of a signal of

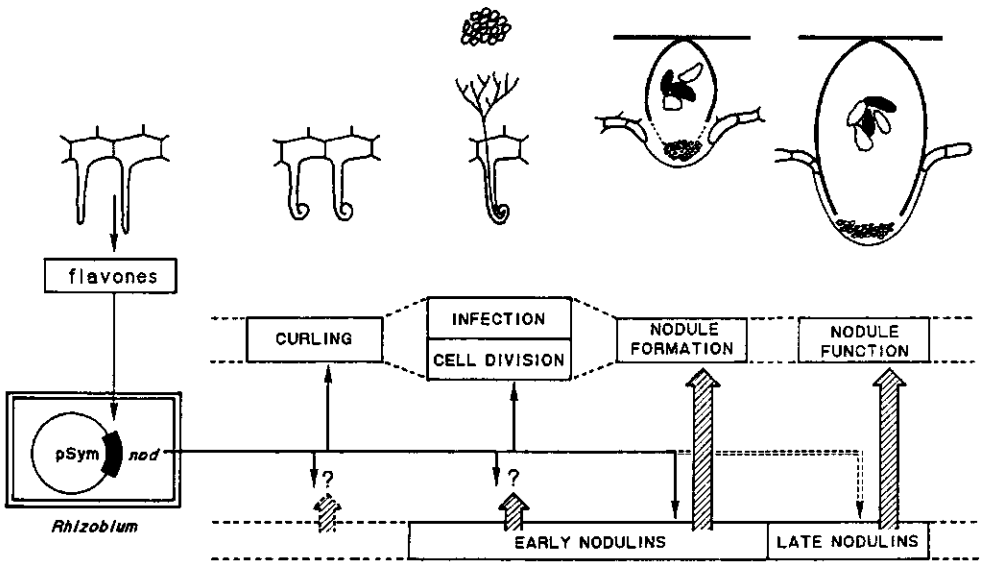


Figure 6.2. Schematic representation of the relationsjhips between nodulins, the *Rhizobium nod* genes, and the successive steps in root nodule development. See text and *figure 6.1.*

Rhizobium.

Evaluation of the data available indicates that the genes responsible for the bacterial outer surface most likely code for avoidance determinants, while the *nodABC* genes are more likely responsible for the induction of the expression of early nodulin genes and possibly also of late nodulin genes. A model outlining the involvement of the *Rhizobium* nod genes is presented in fig. 6.2. Plant root secreted flav(an)ones induce, via *nodD*, the expression of the other *nod* genes, upon which the *nod* gene products produce a return signal. The *nodABC* genes are essential for root hair curling, the infection process and the induction of cortical cell division. Early nodulins have not been identified in these very early stages of the interaction, and it is not clear whether nodulins are involved in these early stages. The expression of the early nodulin genes that have been identified, is first detectable when the nodule primordia have been formed, so the induction of their expression is part of a stage of development succeeding to the induction of cortical cell division and the formation of a nodule meristem. The *nod* genes are also responsible for the induction of the expression of these early nodulin genes. In fig. 6.2 this is indicated by a solid arrow for the signal, and a hatched arrow for the resulting early nodulin gene products. The involvement of the *nod* genes in the induction of the late nodulin genes is less clear. Therefore, this relation is indicated by a dashed arrow in the figure. The involvement of late nodulin gene products in the functioning of a nodule is also indicated by a hatched arrow.

CONCLUDING REMARKS

Concluding remarks

The formation of nitrogen fixing root nodules is attended by differential expression of nodulin genes. Early nodulins are involved in the organogenesis of the nodule (112, chapter 2). Late nodulins, on the other hand, most likely function in creating the physiological conditions that allow nitrogen fixation and ammonia assimilation. In the promoter sequence of several late nodulin genes a nodule specificity box has been identified, that is involved in the regulation of the nodule specific gene expression of these late nodulin genes (316, chapter 3). The expression of the genes encoding early nodulins is first detectable when the nodule meristem is differentiating into a nodule structure. Little is known about specific plant gene expression before that stage. Preliminary data from our laboratory indicate that early nodulins are already present in root hairs as early as 20 hours after inoculation of the plant with *Rhizobium* bacteria (25). These early nodulins may be involved in root hair curling and/or the infection of the root hair cell. Early nodulin gene expression has not been identified in the stages of nodule development, in which cortical cell divisions occur and the formation of the nodule meristem is established. The failure to detect early nodulin gene expression in these stages might be due to technical limitations of the detection methods used, but another possibility is that the nodule meristem does not differ from other plant meristems. In the latter case, nodule specific genes are not yet expressed. If indeed first a normal meristem is generated in the interaction of *Rhizobium* and the leguminous plant, the questions arise when and how is determined that this meristem enters the developmental program leading up to a root nodule.

In a first approach to answering these questions, it may be relevant to compare the legume root nodule with the root nodule of the non-legume *Parasponia*. The morphology of the *Parasponia* root nodule differs substantially from legume root nodule morphology, because the vascular bundle is positioned centrally and not peripherally (188). Also, nodule growth starts in the pericycle and not in the root cortex (187). Therefore, the *Parasponia* root nodule is considered to be a modified lateral root. The same *nodABC* genes of one and the same *Rhizobium* strain are equally essential for legume and for *Parasponia* root nodule induction (224). Thus, the same rhizobial signals might trigger the developmental program for both a legume and the *Parasponia* nodule type. It is unlikely that the same signals trigger two totally different developmental programs.

Moreover, *Agrobacterium* and *R. trifolii* transconjugants carrying cloned pieces of the nodulation region of the *R. meliloti* sym plasmid are capable of inducing on clover roots the formation of hybrid structures intermediate between a nodule and a lateral root (151). Similar structures have been reported to be formed occasionally on the roots of alfalfa after inoculation with a *R. meliloti* strain that at the same time induces morphologically normal nodules (93). Also these observations indicate that the developmental program underlying legume root nodule formation is more close to the program of lateral root formation than previously thought. It is feasible that the developmental program of a root nodule is the outcome of relatively little changes in the developmental program of a lateral root.

In general, the formation of a plant organ is thought to involve huge numbers of tissue-specific genes, which undoubtedly will hinder the detailed understanding of the underlying developmental programs. If the differences between lateral root and root nodule formation are relatively small, than root nodule formation becomes an attractive system to study plant development. Not because the differentiation into a root nodule will be less complex than other plant differentiation processes, but because the differences between the two developmental programs seem more accessible to understanding than a developmental program as a whole.

A unique feature of root nodule development, as opposed to other plant developmental processes, is the involvement of a prokaryote in the induction and control of development. The regulatory role of *Rhizobium* offers unique possibilities for dissecting this plant differentiation process (chapters 4. and 5.). Moreover, it offers an entry to the elucidation of the signals that guide root nodule development by allowing the identification of the *Rhizobium* genes responsible for these signals (chapter 5.). An amazingly limited number of bacterial genes, the *nod* genes, appear to generate the signal(s) for the induction of early nodulin gene expression. The same genes are also in some way involved in the induction of late nodulin gene expression. Elucidation of the nature and mode of action of the signals involved will contribute to our understanding of root nodule development. Also by virtue of the relative ease of manipulation of the inducing *Rhizobium*, root nodule development is a highly attractive system for the study of plant developmental biology, apart from the intrinsic fascination of symbiotic nitrogen fixation.

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SAMENVATTING

Samenvatting

In de bodem zijn *Rhizobium* bacteriën in staat de wortels van vlinderbloemige planten (erwt, boon, klaver) te infecteren en aan te zetten tot de vorming van knolletjes. In die wortelknolletjes zijn de bacteriën in staat om stikstof uit de lucht te binden en om te zetten in ammonia. Met de ammonia kan de plant zich in belangrijke mate voorzien in haar stikstofbehoefte. Op hun beurt krijgen de bacteriën voedingsstoffen van de plant, zodat beide partners profiteren van deze symbiose.

Een stikstofbindende wortelknol is een uiterst gespecialiseerd plante-orgaan, dat gevormd wordt in een aantal opeenvolgende stappen, waarin rhizobia de plant binnendringen, en deze aanzetten tot de vorming van een wortelknolstructuur. Uiteindelijk vullen de *Rhizobium* bacteriën ongeveer de helft van de cellen in de wortelknol, veranderen van vorm en beginnen vervolgens met de stikstofbinding. Gedurende dit proces wisselen plant en bacterie waarschijnlijk voortdurend signalen uit om het goede verloop van het proces te bewerkstelligen.

Het onderzoek naar het mechanisme van wortelknolvorming en stikstofbinding op moleculair niveau heeft een hoge vlucht genomen. Zowel in de bacterie als in de plant zijn genen geïdentificeerd, die alleen in de wortelknol tot expressie komen. *Rhizobium* bacteriën bezitten naast hun chromosoom een zogenaamd sym plasmide, waarop de genen liggen die betrokken zijn bij de symbiose. Geïdentificeerd zijn de genen voor wortelknolvorming (*nod* genen) en stikstofbinding (*nif* en *fix* genen). Over de functie van de genproducten en de regulatie van de expressie van deze genen is veel bekend, maar wat met name de *nod* genen precies teweegbrengen is nog onduidelijk.

Een twintig- tot dertigtal genen van de plant komen uitsluitend in de wortelknol tot expressie; dit zijn de zogenaamde noduline genen. Onderzoek naar de expressie van noduline genen in relatie tot de ontwikkeling van de wortelknol heeft laten zien dat er tenminste twee klassen noduline genen onderscheiden kunnen worden: vroege en late noduline genen. De vroege noduline genen komen ruim voor de stikstof-binding tot expressie, als het orgaan de wortelknol wordt aangelegd. Vroege nodulines spelen daarom waarschijnlijk een rol bij het vormen van de structuur van de wortelknol. Late noduline genen komen tot expressie rond het tijdstip dat de wortelknol met stikstofbinding begint. Het is dan ook aannemelijk dat late nodulines betrokken zijn bij het functioneren van de wortelknol. Mogelijk scheppen zij de voorwaarden voor stikstofbinding en het transport van gebonden stikstof. Omdat de late noduline genen min of meer tegelijkertijd tot expressie komen, is het waarschijnlijk dat deze genen op één en dezelfde wijze worden gereguleerd.

Het merendeel van de noduline genen die tot nu toe geïdentificeerd zijn, behoort tot de klasse van late noduline genen. Het best bestudeerde late noduline is leghemoglobine, een myoglobine-achtig eiwit dat de zuurstofhuishouding in de wortelknol regelt. Van slechts een gering aantal van de overige nodulines is de functie in de wortelknol bekend. Ook over de manier waarop de plant ervoor zorgt dat noduline genen op het juiste moment en op de juiste plaats, dus alleen in de wortelknol, tot expressie komen, en over de rol van de *Rhizobium* bacteriën bij dit pro-

ces, is de kennis nog gering.

Dit proefschrift beoogt een bijdrage te leveren aan de kennis over nodulines en noduline genexpressie. De beschreven experimenten hebben tot doel inzicht te krijgen in het mechanisme van de regulatie van noduline genexpressie. Vooral de rol van *Rhizobium* genen bij de inductie van noduline genexpressie staat daarbij centraal. Anderzijds komt ook de functie van vroege nodulines tijdens de vorming van een wortelknol ter sprake.

Na een korte algemene inleiding over wortelknolvorming (hoofdstuk een) wordt in hoofdstuk twee een cDNA kloon beschreven die een vroeg noduline gen representeert. Deze cDNA kloon, pGmENOD2, is geïsoleerd uit een cDNA bank gemaakt tegen wortelknolRNA van soja. Het ENOD2 DNA blijkt te coderen voor een noduline met een molecuulgewicht van 75.000, aangeduid met Ngm-75. De aminozuurvolgorde van dit noduline, afgeleid uit de DNA sequentie, laat zien dat Ngm-75 een zeer proline-rijk eiwit is, met een repeterend motief in zijn primaire structuur. Dit duidt erop dat Ngm-75 een structureel eiwit zou kunnen zijn. Het gen dat codeert voor Ngm-75 komt tot expressie in wortelknol-achtige structuren, die weliswaar door *Rhizobium* bacteriën geïnduceerd zijn, maar waarin geen bacteriën aangetroffen worden, zogenaamde 'lege' knollen. Ngm-75 lijkt daarom geen functie te hebben in het proces waarbij de *Rhizobium* bacteriën de plant binnendringen, maar eerder lijkt dit vroege noduline een bijdrage te leveren aan de vorming van de wortelknolstructuur.

In hoofdstuk drie wordt de regulatie van de expressie van late noduline genen op het niveau van het DNA onder de loep genomen. Leghemoglobine cDNA kloons, geïsoleerd uit een cDNA bank gemaakt van wortelknolRNA van de erwt, zijn gebruikt om een leghemoglobine gen te isoleren uit een genomische bank gemaakt van erwteDNA. Van dit leghemoglobine gen van de erwt is de DNA sequentie bepaald. Uit de analyse van deze sequentie blijkt dat het geïsoleerde gen volledig is en alle kenmerken bezit van een actief gen. De vergelijking van de promotergebied van het uit de erwt geïsoleerde leghemoglobine gen met de promotergebieden van soja leghemoglobine genen laat zien dat er overeenkomstige sequenties voorkomen. Omdat de overeenkomstige sequenties ook worden aangetroffen in de promotergebieden van andere late noduline genen, zijn deze sequenties wellicht betrokken bij de wortelknolspecifieke expressie van alle late noduline genen.

In de hoofdstukken vier en vijf staat de communicatie tussen plant en bacterie centraal. Gepoogd wordt te achterhalen welke genen van *Rhizobium* betrokken zijn bij het aanschakelen van noduline genen. Hierbij is gebruik gemaakt van de mogelijkheden die de bacteriële genetica biedt om bacteriën te construeren die weliswaar een gedefiniëerd deel van het totale *Rhizobium* genoom missen, maar toch nog wortelknollen kunnen induceren. Deze studies zijn uitgevoerd met wikke (*Vicia sativa* subsp. *nigra*), omdat dit kleine vlinderbloemige plantje snel reageert op inoculatie met (genetisch veranderde) *Rhizobium* bacteriën. In hoofdstuk vier is de basis gelegd voor de analyse door het identificeren van de noduline genen van wikke. Uit wortelknollen werd RNA geïsoleerd, en *in vitro* vertaald in eiwitten, welke werden gescheiden op tweedimensionale polyacrylamide gels. Door het vergelijken van het aldus verkregen eiwitpatroon met dat van wortelRNA zijn vijftien noduline mRNAs geïdentificeerd, waaronder één vroeg noduline mRNA. Een tweede vroeg noduline mRNA van wikke is geïdentificeerd op Northern blots met behulp van de in hoofdstuk twee beschreven soja cDNA kloon pGmENOD2.

De expressie van de noduline genen van wikke is vervolgens bestudeerd in wortelknollen geïnduceerd door een *Rhizobium* stam waarin het sym plasmide is vervangen door een plasmide met alleen 12 kb van het *nod* gebied. Alle noduline genen bleken tot expressie te komen. Kennelijk speelt de informatie op het sym plasmide buiten deze 12 kb *nod* gebied geen enkele rol bij de inductie van noduline genexpressie. In wortelknollen geïnduceerd door een *Agrobacterium* transconjugant, waarin het Ti plasmide is vervangen door dezelfde 12 kb van het *nod* gebied, bleken alleen de twee vroege noduline genen tot expressie te komen. Het *nod* gebied is dus het enige *Rhizobium* DNA dat betrokken lijkt te zijn bij de inductie van vroege noduline genexpressie. Hoewel dit resultaat tegelijkertijd suggereert dat het *Rhizobium* chromo-

soom betrokken moet zijn bij de inductie van late noduline gen expressie, mag die conclusie niet zomaar getrokken worden, want cytologisch onderzoek laat zien dat de *Agrobacterium* transconjugant een afweerreactie van de plant oproept. Het is dus mogelijk dat de genen op het *nod* gebied weliswaar in staat zijn om late noduline genen aan te schakelen, maar dat de verdere ontwikkeling van de wortelknol al gestopt is door de tussenkomst van het afweermechanisme, vóórdat die late noduline genen aangeschakeld konden worden. *Agrobacterium* transconjuganten zijn dus niet bruikbaar om de rol van de nod genen bij de inductie van late noduline genexpressie te onderzoeken.

In hoofdstuk vijf worden experimenten besproken die, zij het indirect, laten zien dat de *nod* genen inderdaad betrokken zijn bij de expressie van late noduline genen. Gezien het fenotype van mutaties in de diverse *nod* genen aanwezig in de 12 kb van het *nod* gebied zijn het waarschijnlijk de *nodA, B* en *C* genen, die één of meer signalen aan de plant geven, waardoor de expressie wordt geïnduceerd van de vroege en vervolgens mogelijk ook van de late noduline genen.

Uit bovenstaande experimenten bleek dat er een correlatie bestaat tussen de ontwikkeling van een wortelknol, zoals die op microscopisch niveau gevolgd kan worden, en de expressie van noduline genen. De correlatie tussen de expressie van een bepaald noduline gen en het bereiken van een bepaald ontwikkelingsstadium biedt de mogelijkheid om te speculeren over de functie die dat noduline zou kunnen hebben. Op grond van de experimenten beschreven in de hoofdstukken vier en vijf kunnen vroege zowel als late noduline genen verder onderverdeeld worden in subklassen, die ieder correleren met een stap in de ontwikkeling van een wortelknol. Hoofdstuk zes, tenslotte, is een overzicht van de huidige kennis over nodulines, noduline genen en de regulatie van noduline genexpressie. De resultaten gepresenteerd in de hoofdstukken twee tot en met vijf worden in dit laatste hoofdstuk met deze kennis geïntegreerd.

Account

Most of the results presented in this thesis have been published before, or will be published. The contents of the preceding chapters have been based on these publications.

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Curriculum vitae

Jan-Peter Nap werd op 10 juni 1958 geboren te Rotterdam. Hij behaalde in 1976 het eindexamen gymnasium ß aan het Marnix Gymnasium te Rotterdam en begon datzelfde jaar te studeren aan de toenmalige Landbouwhogeschool te Wageningen. In 1983 werd de ingenieursstudie in de richting Moleculaire Wetenschappen *cum laude* afgesloten met als hoofdvakken moleculaire biologie (Prof. Dr. A. van Kammen), erfelijkheidsleer (Prof. dr. ir. J.H. van der Veen) en voorlichtingskunde (Prof. Dr. Ir. A.W. van den Ban). Oktober 1983 trad hij in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.). Hij was verbonden aan de vakgroep Moleculaire Biologie van de Landbouwuniversiteit, waar het in dit proefschrift beschreven onderzoek is uitgevoerd. Vanaf februari 1988 is hij als moleculair bioloog werkzaam op het onderzoeksinstituut ITAL.

Nawoord

De volledige auteurslijst van dit proefschrift zou niet minder dan negentien namen hebben geteld, en dat is nog maar een klein gedeelte van de velen die mij de afgelopen jaren met raad en daad hebben bijgestaan. Hen allen ben ik dank verschuldigd.

Ab van Kammen, jouw bereidheid om op de onmogelijkste tijden en plaatsen manuscripten te bespreken ("omdat ze de deur uit moeten") is maar een klein teken van het enthousiasme dat je voor dit onderzoek hebt gehad. Ik heb dat enthousiasme als zeer plezierig en stimulerend ervaren. Ton Bisseling, jouw onophoudelijke stroom van ideeen, die onstuitbare drang tot weten, de manier waarop jij het onderzoek van een hele groep stuurt, bindt en leidt, en daarbij ruimte laat voor ieder afzonderlijk, het is meer dan ongelofelijk. Ik heb een hoop van je geleerd en ik denk dat ik steeds meer zal gaan beseffen welk een voorrecht het is geweest met je te hebben mogen werken. Francine Govers, Marja Moerman, Ben Scheres, Clemens van de Wiel, Henk (J.) Franssen, en Ton Gloudemans, jullie weten maar al te goed wat er van al die artikelen, 2-D gels, sequenties en cytologische waarnemingen (van mij ?) terecht was gekomen, als jullie er niet waren geweest. *In vele opzichten was het goed toeven in jullie midden* !

Verder wil ik met name bedanken : Jos van der Meer, voor zijn helaas korte maar daarom niet minder belangrijke bijdrage aan het maken van een genoombank; Paul Hooykaas, Herman Spaink, en Michael Djordjevic voor het construeren en beschikbaar stellen van *Rhizobium* en *Agrobacterium* stammen; Ton van Brussel voor het afstaan van *Vicia* zaadjes; Willem Stiekema en Wim Dirkse van het ITAL voor hun gastvrijheid op het ITAL en hulp bij het Maxam-Gilbert sequencen; Ann Hirsch en Jeremy Weinman voor het kritisch doorlezen van manuscripten; Sjra Maessen, Elly Speulman, Ruud van den Bulk, Siebe van Genesen, Herman Scholthof, Henk-Jan Bussink, en Michel de Kok, die in het kader van hun studie bij het onderzoek betrokken waren; Piet de Kam voor het inoculeren en verzorgen van de vele planten die in het onderzoek gebruikt zijn; Piet Madern, Reindert de Fluiter, Job Tielens, Peter van Druten en Sybout Massalt voor het teken- en fotowerk; Marie-José van Neerven, Angélique Jonkers, Hedy Adriaansz en Gré Heit-kÖnig voor het bevrouwen van het secretariaat en het voor jullie rekening nemen van zoveel typewerk; *en ook* Els Hulsebos, Fred van Engelen, Geertje Rezelman, Jacques Aarts, Jacques Hille, Jan Hontelez, Jan Verver, Joan Wellink, Juan Garcia, Martine Jaegle, Peter Roelvink, Peter Sterk, Pieter Vos, Pim Zabel, Raymond van Daelen, Rene Klein-Lankhorst, Rik Eggen, Rob Goldbach, Rommert van den Bos, Sacco de Vries, Takis Katinakis, en al die studenten die de labs en koffiekamer hebben bevolkt. Jullie allemaal maken "Molbi" tot die heerlijke bende die zo vaak mijn 'thuis' is geweest.

Tot slot wil ik mijn ouders en grootouders noemen. Ik weet dat ik mij naar jullie idee met nagenoeg onbegrijpelijke zaken heb bezig gehouden. Toch hebben jullie mij daar toe in staat gesteld en er al die jaren veel belangstelling voor getoond. Ik wil jullie daarvoor danken en met jullie verzuchten "Het is af....". Vrienden en vriendinnen, kennen jullie me nog ?