IDENTIFICATION OF *RHIZOBIUM LEGUMINOSARUM* GENES AND SIGNAL COMPOUNDS INVOLVED IN THE INDUCTION OF EARLY NODULIN GENE EXPRESSION

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INTRODUCTION

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

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

We have set out to isolate nodule specific plant cDNA sequences representing genes of which the expression marks different stages of root nodule development. When we started, the already cloned plant nodulin sequences mainly represented genes expressed when nodule

NATO ASI Series, Vol. H 36
Signal Molecules in Plants and Plant-Microbe Interactions
Edited by B. J. J. Lugtenberg

development, according to cytological criteria, is completed (reviewed in Govers *et al* 1987a). We have now isolated cDNA clones representing genes expressed earlier in nodule development and examined whether the expression of these genes correlates to distinct steps in root nodule formation. The information that may be obtained with such marker genes is twofold. First, characterization of the proteins encoded by these genes will provide information about the type of plant proteins essential for different developmental steps. Second, if indeed the expression of a certain gene is characteristic for a step in root nodule development, the expression of such gene may be used to identify bacterial or plant signals necessary to invoke the developmental step in which the gene is involved. Here we shall briefly summarize the characteristics of a set of pea early nodulin cDNA clones. We will describe the correlation of the expression of different genes and different steps in root nodule formation. We further show how expression of these genes can be used to examine whether certain compounds can mimic the effect of signal molecules in the *Rhizobium*-plant interaction. We then focus on the question how one of the cloned cDNAs, pPsENOD12, may be used for developing an assay for purification of bacterial signal molecules.

EARLY NODULINS

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

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

From *Pisum sativum*, the garden pea, we isolated five early nodulin clones pPsENOD2, pPsENOD12, pPsENOD5, pPsENOD3, and pPsENOD14, which could be involved in different developmental steps, judged by the different time courses of accumulation of the corresponding mRNAs during nodule development. pPsENOD12 encodes a protein with a structure similar to that of the pea ENOD2 protein , since both are built up of a series of two repeating pentapeptides each containing two prolines. The three other amino acids in the pentapeptide unit of pPsENOD12 differ from those in ENOD2. In conclusion, the pea ENOD2 and ENOD12 early nodulins are, like the soybean early nodulins ENOD2 and ENOD13, (hydroxy-)proline rich proteins. It is thought that (hydroxy-)proline rich proteins are often associated with cell walls. Therefore we assume that at least part of these proline rich early nodulins are components of cell walls of the different cell types that are formed during symbiosis. The homology of ENOD2, ENOD13 and ENOD12 to the soybean cell wall protein 1A10 (Averyhart- Fullard *et al* 1988) supports this hypothesis. pPsENOD5 encodes a protein with hydrophobic regions at both the C- and the N-terminus, the latter possibly forming a signal peptide. This early nodulin also has a proline rich domain, which moreover has a high content of glycine, alanine, and serine. The amino acid composition of this domain of ENOD5 is reminiscent of that of arabinogalactan proteins (Van Holst *et al* 1981). To date no amino acid sequences of arabinogalactan proteins have been published, which prevents a more detailed comparison of ENOD5 and arabinogalactan proteins. Upon searching different databases no significant homology between ENOD5 and other previously described proteins was found. pPsENOD3 and pPsENOD14 both encode small nodulins, with a molecular weight of about 6 kD. These two early nodulins are 60% homologuous and contain as most striking characteristic a cluster of four cysteins, arranged in such a way that a metal can be bound. This suggests that the ENOD3 and ENOD14 proteins contain a metal ion. Similar motifs of four cysteines are found in the late nodulins Ngm-20, Ngm-23, Ngm26b, Ngm-27, and Ngm-44, forming a small gene family in soybean (Jacobs *et al* 1987). However, there is no significant sequence homology between these or any other proteins from which the sequence is stored in the different databases, and the ENOD3 and ENOD14 proteins.

NODULIN GENE EXPRESSION DURING NODULE DEVELOPMENT

Pea nodules belong to the indeterminate nodule type. These nodules have a persistent meristem. Hence all nodules contain cells at different stages of development: the youngest cells adjacent to the apical meristem and the oldest cells at the basal root attachment point. This type of nodules have the advantage that the presence of transcripts related to particular stages of root nodule development is less dependent on the age of the nodule. A further advantage is that it is possible to compare the expresssion patterns of the nodulin genes in

different cell types during nodule development by comparing the spatial distribution of different nodulin transcripts in serial sections from one nodule using *in situ* hybridization.

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

The transcripts corresponding to four of the five selected early nodulin clones are present in the central tissue of the nodule. For ENOD2, on the other hand, the transcript was specifically located in the nodule inner cortex. The ENOD12, ENOD5, ENOD3, and ENOD14 transcripts are present in successive, but partially overlapping zones of the central tissue. The ENOD12 mRNA is present in a small zone directly adjacent to the meristem, the invasion zone. The ENOD5 transcript starts to accumulate in the cells where ENOD12 mRNA is still present, but the concentration reaches a maximum in the zone of the central tissue that contains enlarging cells. This zone has been named the young symbiotic zone as in this zone the first plant cells that contain bacteria are present (Newcomb 1981). The ENOD5 transcript is present at strongly reduced levels in the cells of the central tissue that have reached their maximum size. This is the symbiotic zone. The ENOD3 and ENOD14 messengers start to accumulate in the cells where the ENOD5 transcript is present at a maximal level and the concentration of these transcripts reaches its maximum in the youngest 3-4 cell layers of the symbiontic zone. In the older cells of the symbiotic zone the ENOD3 and ENOD14 mRNA concentration decreases. In these older cells the level of the transcript of the late nodulin Lb is at its maximum. The ENOD5, ENOD3, and ENOD14 mRNAs are only present in the cells containing rhizobia, while ENOD12 mRNA is present in all the cells of the invasion zone. Therefore ENOD12 transcript is present in cells that will develop in either infected or uninfected cells. At the stage of development of the infected cells when the ENOD3 and 14 mRNA concentrations are decreasing, the nitrogenase mRNA can first be detected in the bacteroid . This proves that the infected cell becomes a functional nitrogen fixing cell at that stage. Therefore the decline of the ENOD3 and 14 mRNA concentrations marks the stage at which the infected cell is fully differentiated into a functional nodule cell.

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

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

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

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

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

EARLY NODULIN GENES AS REPORTERS FOR BACTERIAL OR PLANT SIGNALS

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

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

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

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

Based on the similarities in nodule development induced by anti-auxins and *nod* gene dependent bacterial compounds it is plausible that *Rhizobium nod* gene products interfere with the phytohormone distribution in the legume root. The subsequent change in hormone balance could then induce centres of mitotic activity at certain sites in the root cortex. The observation that *Rhizobium nod* gene mutants can be complemented with the *A.tumefaciens* zeatin gene, which is involved in cytokinin synthesis, is in accordance with this hypothesis (J.Cooper, pers.comm.).

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

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

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

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

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

The common *nod* genes of *R.leguminosarum* are known to be sufficient for excretion of the soluble factor that establishes root hair deformation (Zaat *et al* 1987). We were able to

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

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

CONCLUDING REMARKS

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

The use of ENOD5, ENOD3, and ENOD14 to identify signals involved in later steps of root nodule development will require a search for *R.leguminosarum* mutants unable to elicit expression of these genes, and characterization of the nature of the mutations. Possible

candidate mutant strains for such a study are bacterial release (*bar*$^-$) and bacteroid development (*bad*$^-$) mutants.

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