Developmental Biology of a Plant-Prokaryote Symbiosis: The Legume Root Nodule

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The development of nitrogen fixing root nodules on the roots of leguminous plants is induced by soil bacteria (for example, from the genus *Rhizobium*). The formation of this plant organ involves specific activation of genes in both plant and bacterium. Analysis of these genes gives insight into the way in which plant and bacterium succeed in coordinating plant development.

A MAJOR FACTOR IN THE ECOLOGICAL SUCCESS OF MEMBERS of the plant family Leguminosae is their ability to enter a beneficial relationship with soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium*. In this association, the bacteria induce the plant to develop a new plant organ, the root nodule. Within this nodule, the ecological niche required for fixation of atmospheric nitrogen by the bacteria is created, thus rendering the plant independent of soil nitrogen (*1*).

Leguminous root nodules are by far the largest sole source of organic nitrogen in the global nitrogen cycle. As early as in ancient Rome, the soil-enriching quality of legumes was recognized, and systems of crop rotations were developed that used legumes for green manuring (*2*). By the 18th century, these systems had greatly increased Europe's agricultural productivity, and they are still in use in large areas of today's world. Symbiotic nitrogen fixation has always attracted a great deal of basic research interest, but recent advances illustrate that the study of root nodule development may be attractive beyond its economical and environmental relevance; this system is likely to contribute considerably to our understanding of plant development in general.

Combined efforts of cytologists, plant physiologists, geneticists and molecular biologists have given insight into the process of nodule formation and function (*3–8*). In short, a particular (*Brady*) *Rhizobium* species is able to interact with one or a limited number of legume species. Rhizobia attach to the roots of their host and cause a characteristic curling of the host's root hairs. The rhizobia then invade the plant by way of a newly formed tube called the infection thread. Meanwhile, cells in the root cortex start to divide and form the nodule primordium (Fig. 1). Infection threads enter individual primordium cells, and bacteria are released from the infection thread into the cytoplasm of the target cell. Bacteria then differentiate into their endosymbiotic forms referred to as bacteroids, and begin to fix nitrogen by the action of the enzyme nitrogenase, a process that is assisted by the morphology and physiology of the root nodule. Ammonia is transported from the bacteroids to the plant cytoplasm, where it is assimilated and then transported to other parts of the plant.

Identification and analysis of plant and bacterial genes that direct nodule formation and function have progressed to a point where these processes can be described in considerable detail at the molecular level. The relative ease by which the "prokaryote inducer" can be studied and manipulated offers an experimental approach to elucidate signal-response pathways involved in plant cell division and differentiation quite unlike any other plant developmental process.

In this review, we will concentrate on the developmental aspects of root nodule formation and the molecular interactions that guide this development, rather than go into details of symbiotic nitrogen fixation. We will describe the genes of both partners that have been found to be important for nodule development and function. Where possible, we will refer to one of the many reviews and monographs available, in which the reader can find an exhaustive coverage of the primary literature.

Plant Nodulin Genes and Bacterial Nodulation Genes

Specific expression of plant and bacterial genes accompanies the development of the symbiosis. The identification of these genes is therefore a prerequisite for insight into root nodule development and the underlying communication between bacterium and plant.

The plant proteins that are specifically formed during the formation and function of a root nodule are called nodulins (*8, 9*). In all *Rhizobium*-legume symbioses examined, nodulin gene expression has been firmly established either by comparison of in vitro translation products from nodule and root mRNA or by the isolation of nodule-specific cDNA clones (*10*). The nodulin genes have been operationally defined as early and late nodulin genes according to the timing of their expression during nodule development (*8, 10*).

The late nodulin genes comprise a large group of genes that are expressed around the onset of nitrogen fixation. Late nodulins aid in the function of a root nodule by creating the physiological conditions required within the nodule for nitrogen fixation, ammonium assimilation, and transport. Among the identified late nodulins are the leghemoglobins, uricase, and subunits of sucrose synthase and glutamine synthetase (*8*). In terms of development, late nodulins are truly late, because the full nodule structure with all its defining characteristics has developed before late nodulin gene expression becomes detectable. Although late nodulin gene expression may well be regulated by rhizobial signals, such signals need not be related to

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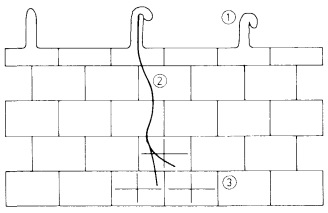

Fig. 1. Schematic representation of the three processes induced by *Rhizobium* in legume roots. (1) root hair deformation and curling, (2) infection thread formation, and (3) mitotic activity in the root cortex.

the signals that trigger the formation of the nodule organ.

Relatively few nodulin genes have been identified that are expressed in the developing root nodule well before the onset of nitrogen fixation. The proteins from these early nodulin genes are most likely involved in the infection process and development of the nodule structure. Most early nodulins are highly proline-rich proteins, and may therefore be cell wall components (*8*). The induction of plant cell differentiation and of early nodulin gene expression is most likely under control of the bacterial partner.

In numerous *Rhizobium* species, genes involved in establishing an effective symbiosis have been identified with the use of transposon mutagenesis, complementation analysis, and other genetic techniques (*7, 11, 12*). In the fast-growing *Rhizobium* species, most of these genes are located on large plasmids (pSyms), whereas the slow-growing *Bradyrhizobium* species carry these genes on the bacterial chromosome. Genes essential for the process of nitrogen fixation include *nif* and *fix*, among which are the structural genes for nitrogenase (*13*). Despite the intrinsic importance of these genes for nitrogen fixation, *nif* and *fix* mutants are able to induce the formation of root nodules in which all nodulin genes are expressed (*8*) and with a morphology like that of nodules induced by wild-type *Rhizobium*. Apparently, nitrogen fixation is the result of and not a prerequisite for nodule development and nodulin gene expression.

The rhizobial genes required for nodule formation and nodulin gene expression include the nodulation (*nod*) genes, several groups of genes concerned with the structure of the outer surface of the bacterium (the *exo*, *lps*, and *ndv* genes), and a number of less well defined genes (*8, 12*). Formal proof for the involvement in the induction of the expression of early nodulin genes has only been demonstrated for the *nod* genes (*8*). Upon transfer of the *nod* gene region, the recipient non-nodulating *Agrobacterium* gained the ability to nodulate. The *nod* gene products, therefore, are the most likely candidates to be signals eliciting nodule development.

The *nod* genes fall in three classes, common, host-specific, and *nod*D. The common *nod* genes include, among others, the *nod*ABC genes that are found in all rhizobial species. The common *nod* genes of different species are structurally very similar and functionally interchangeable (*7, 12*). Mutations in the *nod*ABC genes abolish completely the ability to nodulate, which underlines their pivotal role in nodule development.

The host-specific *nod* genes determine the specificity of nodulation on a particular host. Upon mutation of such a *nod* gene, nodulation is delayed or reduced, or the host range is altered. Host-specific *nod* genes are not functionally interchangeable between rhizobial species, despite a sometimes striking sequence conservation between the respective genes (*11*). Although sequence homologies and protein analyses of Nod proteins hint at a diversity of functions, conclusive evidence for any biochemical function is lacking (*12*).

The third type of *nod* genes is *nod*D (*7, 12*), of which some species carry multiple copies. The *nod*D gene is the only *nod* gene that is constitutively expressed in both the free-living and symbiotic states of *Rhizobium*. In combination with flavonoids excreted by plant roots, the NodD protein probably acts as a transcriptional activator for all other *nod* genes, and the gene is as essential for nodulation as are the common *nod*ABC genes. Because the NodD protein of a particular *Rhizobium* species proves most responsive to the flavonoids excreted by its homologous host, NodD also contributes to host specificity.

Root Hair Deformation and Curling

The first microscopically visible responses of the legume upon contact with *Rhizobium* are deformation and curling of root hairs (*14*). Rhizobia interact with almost all the young emerging root hairs, but only ~25% of the root hairs actually curl. Computer simulation studies suggest that the curling is caused by local stimulation of growth of the root hair tip, rather than by inhibition of growth (*14*). Even at this early stage, nodulation is host-specific, because as a rule only the homologous *Rhizobium* causes deformation and curling. Because *Rhizobium* is chemotactic towards the flavonoid able to induce *nod* gene expression (*15*), host specificity may even be manifested in the rhizosphere before plant and bacterium visibly interact.

Plant gene expression correlated with deformation and curling is difficult to approach experimentally. Only in one study have changes in the expression level of two plant genes been demonstrated (*16*), and no plant genes have yet been analyzed in further detail.

With respect to *Rhizobium*, it was shown over 20 years ago that the physical presence of the bacterium is not a prerequisite for root hair deformation (*17*). More recently, it was shown that sterile culture filtrates of rhizobia grown in the presence of root exudate or *nod* gene–inducing flavonoids contain factors that cause root hair deformation. Genetic analyses indicated that the presence of functional common *nod* genes is required for the production of these extracellular factor (*7*). Recently, Dénarié and co-workers have purified an extracellular compound excreted by the alfalfa symbiont *R. meliloti* and elucidated its structure (*18*). This compound, NodRm-1, is shown by chemical analysis to be a sulphated β-1,4-tetrasaccharide of D-glucosamine, in which three amino groups are acetylated and one is acylated with an unsaturated C_{16} fatty acid chain (Fig. 2). Because the functionally interchangeable common *nod* genes are involved in the synthesis of NodRm-1, it is likely that deformation factors produced by other rhizobial species will have features in common with NodRm-1.

NodRm-1 is only excreted when the common *nod* genes and a host-specific *nod* gene (*nod*H) are functional and the *R. meliloti* bacteria are grown in the presence of the *nod* gene–inducing flavonoid luteolin. Addition of purified NodRm-1 to alfalfa roots in a concentration range of 10^{-8} to 10^{-11} M resulted in root hair deformation, whereas no deformation was observed when this compound was added to vetch roots (*18, 19*). A *R. meliloti nod*H mutant is unable to cause root hair deformation on alfalfa, but does induce root hair deformation on vetch. Analysis of the *nod*H mutant shows it to be defective in NodRm-1 production. Instead, the mutant excretes a more hydrophobic factor, NodRm-2, that, in

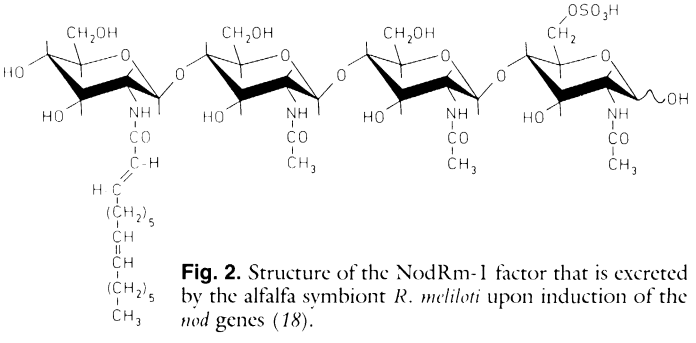

Fig. 2. Structure of the NodRm-1 factor that is excreted by the alfalfa symbiont *R. meliloti* upon induction of the *nod* genes (*18*).

purified form, causes root hair deformation on vetch but not on alfalfa. The structure of NodRm-2 is identical to NodRm-1, except for the presence of the sulphate group. The NodH protein may thus be a specific sulphate transferase (*19*).

The difference in structure between NodRm-1 and NodRm-2 shows that minor structural features in the oligosaccharide molecule can be responsible for the all-or-nothing phenotype. It also suggests that at least some host-specific *nod* genes are involved in modifying a basic compound to form a signal molecule that is specific for the homologous host. The availability of purified NodRm-like factors and mutants for all *nod* genes is likely to result in a rapid elucidation of the biosynthetic pathway for these factors and the functions of the various *nod* genes. It will be interesting to see whether NodRm-like factors are formed by degradation of existing cell wall macromolecules, or by synthesis from glucosamine residues. Moreover, the availability of purified Nod factors will facilitate research into the signal transduction pathways that lead to nodule development and nodulin gene expression.

Infection

Upon root hair curling, rhizobia become entrapped in the curls. The resulting high local concentration of bacterial factors may be the decisive step for the induction of infection, because infection is also observed in root hairs that entrap bacteria while they touch instead of curl (*14*). Actual infection starts with a very localized hydrolysis of the plant cell wall in the root hair curl. The mechanism of this hydrolysis is not clear. The bacteria may produce hydrolytic enzymes that are responsible for the cell wall dissolution. Alternatively, the bacteria may enlist endogenous plant mechanisms, such as, for instance, those used when epidermal cells form root hairs (*14*).

At the site of cell wall hydrolysis, bacteria enter the root hair cell by invagination of the plasma membrane. Around the invaginated membrane, the plant forms a tube-like infection thread by deposition of cell wall-like material (*4, 7*). As the infection thread penetrates the root hair cell, the bacteria proliferate within the thread and become surrounded by a mucopolysaccharide matrix. Most likely both bacterial and plant components contribute to this matrix, although as yet a plant-derived origin has been demonstrated only for one glycoprotein (*20*). The infection threads carry the bacteria from the root hair to the nodule primordia formed in the root cortex.

Detailed cytological analyses of the pea root cortex into which infection threads grow and ramify show that distinct morphological changes occur in cortex cells before they are penetrated by an infection thread (*14*). Microtubules rearrange, the nucleus migrates to the cell center, and an additional cell wall is formed. These morphological changes suggest that a signal from the growing infection thread prepares the cortical cells for penetration. As the morphological changes resemble changes observed in the initial steps of the cell cycle, it has been suggested that the cortex cells are starting cell division and are arrested in the prophase of the cell cycle.

The expression of two early nodulin genes has been correlated with the infection process in pea. The early nodulin cDNA clones pPsENOD5 (*21*) and pPsENOD12 (*22*) were isolated from a pea nodule cDNA library by differential screening. Sequence analysis shows both early nodulins to be proline-rich proteins. The PsENOD12 early nodulin is for the major part composed of two repeating pentapeptides, each of which contains two prolines. This structure suggests that this early nodulin is a hydroxyproline-rich cell wall protein (*22*). The PsENOD5 protein, besides being rich in proline, is relatively rich in alanine, glycine, and serine, suggesting it is related to arabinogalactan proteins (*21*). PsENOD5 lacks the repetitive structure of PsENOD12. Both early nodulins have a putative signal peptide at the amino terminal end of their sequence, and therefore are probably transported across a membrane. In situ hybridization studies with the use of antisense RNA probes show that the PsENOD5 gene is only expressed in cells containing growing infection threads (*21*) (Fig. 3, C and D). Because arabinogalactan-like proteins are known to be components of the plasma membrane (*23*), the PsENOD5 protein may be part of the plasma membrane of the infection thread.

Expression of the PsENOD12 gene is observed in root hairs and in cortical cells that contain a growing infection thread. In contrast to the PsENOD5 gene, the PsENOD12 gene is also expressed in cells that occur several layers in front of the growing infection threads (Fig. 3, A and B) and are undergoing the morphological changes that precede penetration by an infection thread (*22*). The putative cell wall protein PsENOD12 may thus be part of the additional cell wall formed in the cortex cells that prepare for infection thread passage. In addition, the PsENOD12 protein may be a component of the infection thread itself.

Another plant protein for which an involvement in the infection process has been demonstrated is lectin. *R. leguminosarium* biovar (bv.) *viciae* normally infects and nodulates pea and vetch, but not white clover. In partial exception to the barriers of host specificity, *R. leguminosarum* bv. *viciae* is able to induce root hair curling on white clover roots, although infection threads are never formed. However, introduction of a pea lectin gene in transformed hairy roots on white clover allows the infection to proceed beyond root hair curling (*24*). Apparently, pea lectin is in some way involved in specifying the host specificity of infection thread formation. The plant receptor of NodRm-like molecules has been postulated to be a lectin (*18*), although experimental data have not yet been put forward.

The *Rhizobium* genes involved in generation of signals that relate to infection thread formation and growth will be more difficult to identify than the genes and factors involved in root hair deformation. For instance, genetic analysis has indicated that the common *nod* genes are required for infection thread formation. However, the requirement may actually be for root hair curling, which would then allow infection thread formation as a secondary response. Because no nodular structure has ever been described in which an infection thread or a thread-like structure is formed in the absence of the bacterium, the physical presence of *Rhizobium* may be essential for infection thread formation to occur and proceed. Possibly the dividing bacteria are the driving force for infection thread growth.

Application of sterile culture filtrates of *R. leguminosarum* bv. *viciae* to pea seedlings elicits PsENOD12 gene expression in root hairs. The expression of this infection process–related plant gene shows that such a gene can be induced by soluble factors without actual infection thread formation. Application of the purified extracellular factor, NodRlv, also caused root hair deformation (*25*). Although the structure of this NodRlv factor has not been elucidated yet, it is likely to resemble NodRm-1 in several features, as discussed above. Apparently, the oligosaccharide that is responsible for root hair deformation is also involved in triggering at least part of the infection process.

In addition to the *nod* genes, genetic analyses of *Rhizobium* mutants have identified numerous other bacterial genes that are required for normal nodule development (*8, 12*). As a rule, rhizobia mutated in these genes can induce plants to form nodule-like structures, but these fail to form infection threads, form infection threads that abort prematurely, or are defective in bacterial release from the infection threads. Examples include the genes involved in the production of bacterial outer surface components such as exopolysaccharides (*exo* genes), lipopolysaccharides (*lps* genes), and

(cyclic) glucans (*ndv* genes), in addition to genes related to drug resistance, auxotrophy, and carbohydrate metabolism (*8, 12, 26*).

Genetic evidence indicates that the *Rhizobium* genes described above are all essential for proper infection thread formation, and each of them may and often has been claimed to produce a signal that induces a step in this process. However, in our view at least three different classes of genes should be recognized in the interactions between *Rhizobium* and the legume plant.

The first class of genes contributes to the synthesis of signals that induce developmental processes in the plant, as demonstrated for the *nod* genes in the production of NodRm-like factors (*18, 19*). The second class of genes is concerned with gene products that are required for infection, but do not induce a developmental process in the plant. An example is the symbiotic deficiency of auxotrophic mutants (*26*), which indicates that the bacteria need to be metabolically active and multiply in order to infect. The genes producing the rhizobial compounds in the matrix of the infection thread are also likely to belong to this class of genes. The third class of genes is involved in the disguise of *Rhizobium* in order for *Rhizobium* to avoid the plant defense mechanisms and the plant-bacterium interaction to result in a functional root nodule. Plants are able to defend themselves against pathogens by a variety of means (*8*), none of which is observed during nodule development. A perturbation of the normal situation, however, appears to elicit a defense response (*8*). In our opinion, the products of the *exo*, *lps*, and *ndv* genes most likely act as so-called avoidance determinants to make *Rhizobium* a parasite in disguise. Any mutation that unmasks an avoidance determinant will trigger the plant's defense responses and result in abortion of nodule development, even if all signals for proper development are present.

The only bacterial genes for which a direct involvement in generating a signal that triggers development has been demonstrated are the *nod* genes, which produce the Nod factors. Satisfactory evidence has yet to be presented that any bacterial gene other than the *nod* genes is involved in the production of the signals eliciting infection thread formation and growth.

Primordium Formation

The third visible process induced by rhizobia is mitotic activity in the root cortex (*6, 14, 27*). While the infection threads penetrate the root cells and move inward, fully differentiated cells in the root cortex are reactivated and start to divide, thus forming the nodule primordium (*2, 16*) (Fig. 3H). Which root cortex cells divide depends upon the type of nodule a particular host plant forms. In general, temperate legumes, such as pea, vetch, clover, and alfalfa, form indeterminate nodules that are club-shaped and have a persistent apical meristem. In these legumes, the nodule originates from cells located in the inner cortex of the root, in most cases opposite a xylem pole of the root vascular system (*27*) (Fig. 3F). In contrast, most tropical legumes, such as soybean and French bean, form globose-structured determinate nodules, in which mitotic activity ceases during development and cell expansion rather than cell division is responsible for the increase in nodule size. Determinate nodules originate from mitotic activity induced in the root outer cortex (*27*).

In the development of both determinate and indeterminate nodules, the infection threads grow towards the nodule primordia. Upon contact, the threads branch and penetrate the cells of the central region of the primordium. In these cells, the bacteria bud off from the tips of the infection threads into the plant cytoplasm. This release is an endocytotic process in which the bacteria become enclosed by the peribacteroid membrane, which is initially derived from the plasmalemma of the plant cell (*28*).

Concomitantly with bacterial release, some cell layers at the distal site of the nodule primordia develop into the small cytoplasm-rich cells that form the meristem of the indeterminate nodule (Fig. 3H). The meristem generates the different tissue types of the nodule. While the meristematic cells are pushed outwards, the infection threads reverse their growth direction to follow the nodule meristem. Thus, the growth direction of the infection thread appears to be correlated with the position of mitotically active cells (*28*).

In situ hybridization studies show that the nodule primordium

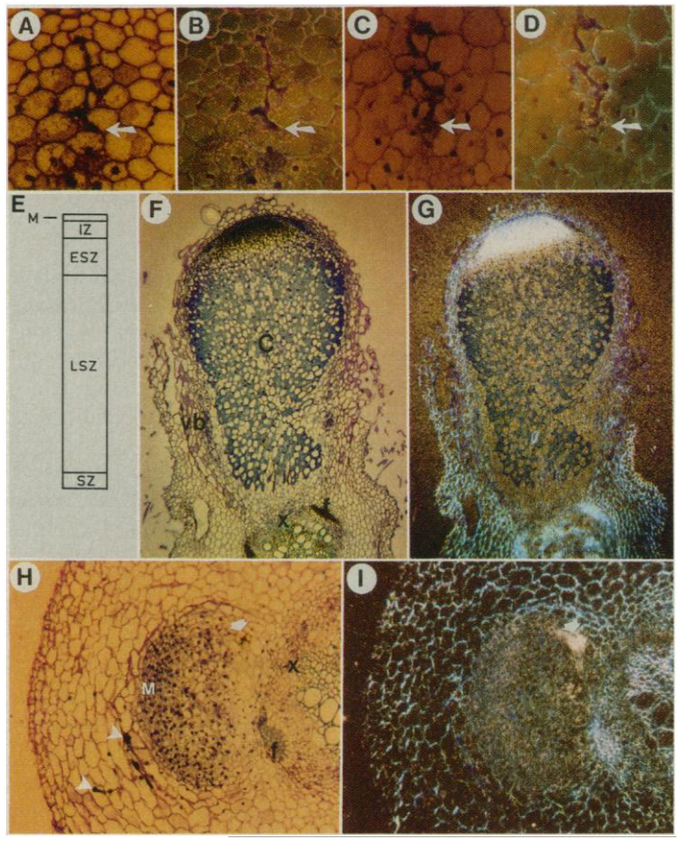

Fig. 3. Light micrographs of the localization by in situ hybridization with antisense RNA of early nodulin mRNAs in pea root nodules at different stages of development. (**A** to **D**) Localization of PsENOD12 (A and B) and PsENOD5 (C and D) mRNA in cross sections of infected pea roots. (A and C), bright-field micrographs corresponding to the dark-field micrographs in (B) and (D), respectively. In the dark-field micrographs, silver grains representing hybridization are visible as white spots. The arrows indicate the tip of the infection thread. PsENOD5 mRNA (D) is located in the cell containing the infection thread tip and in the root cortex cell just passed by the infection thread. PsENOD12 mRNA (B) is located in root cortex cells containing the infection thread as well as in cortex cells in front of the thread. (**E** to **G**) Localization of PsENOD12 mRNA in a mature pea nodule. (F), bright-field micrograph of the corresponding dark-field micrograph in (G). In (E) the approximate position of the different developmental zones of the determinate root nodule shown in (F) and (G) is indicated. The PsENOD12 mRNA is restricted to the invasion zone. (**H** and **I**) Bright-field micrograph (H) and the corresponding dark-field micrograph (I) of a transsection through a pea root containing a nodule primordium. At the distal site of the nodule primordium a meristem (M) is formed. Part of the infection thread that carries *Rhizobium* from the root surface to the nodule primordium is indicated with arrowheads. At this stage the primordium starts to differentiate. The first cell to differentiate into a nodule parenchyma cell hybridizes with antisense PsENOD2 RNA (arrow). Abbreviations: (M), meristem; (IZ), invasion zone; (ESZ), early symbiotic zone; (LSZ) late symbiotic zone; (SZ), senescent zone; (X), xylem pole of the root vascular bundle; (P), phloem pole of the root vascular bundle; (VB), vascular bundle of the nodule; (C), central tissue.

can be distinguished from the nodule meristem by molecular criteria in addition to its distinct morphological features (*22*). The infection-related PsENOD12 gene is expressed in all cells of the primordium, whereas PsENOD12 mRNA is not detectable in the cells of the nodule meristem. Most likely, the expression of the PsENOD12 gene reflects the conditioning of the dedifferentiated primordium cells for infection, but PsENOD12 may also have a defined function in primordium formation.

NodRm-1 applied to alfalfa seedlings in approximately a hundredfold higher concentration than required for curling induces mitotic activity at sites in the inner cortex where nodule primordium formation is normally induced by *Rhizobium* (*19*). As a result of this mitotic activity, nodule-like structures develop (*19*). A single bacterial oligosaccharide is thus able to elicit a wide range of responses from its legume host. The oligosaccharide nature of NodRm-1 is consistent with other observations that oligosaccharides can regulate plant morphogenesis (*29*).

Differentiation into a Root Nodule

After meristem formation, different nodule tissues are formed from the nodule meristem. In fully developed root nodules, two major tissue types can be recognized, the central tissue, and the peripheral tissue (Fig. 3F). The peripheral tissue consists of the nodule cortex and the nodule endodermis, in addition to the nodule parenchyma (inner cortex), which contains the vascular bundles that connect the nodule with the root stele (*28*).

The tissue most characteristic of the root nodule is the central tissue. In the central tissue of the indeterminate nodule, three developmental zones can be distinguished (Fig. 3, E and F). In the invasion zone immediately adjacent to the meristem, release of bacteria from the infection threads continues to establish newly infected cells. Approximately half of the cells are not penetrated by infection threads and remain uninfected. The invasion zone is followed by the early symbiotic zone, in which plant cells elongate and bacteria proliferate. In the late symbiotic zone, the infected cells are completely filled with bacteria that have differentiated into their pleiomorphic endosymbiotic bacteroid form. Nitrogen fixation takes place in this late symbiotic zone. In older nodules, a fourth zone is the senescent zone, in which both plant cells and bacteroids degenerate. Due to the persistence of the apical meristem, all developmental phases of the indeterminate nodule are represented within a single nodule.

Determinate nodules lack a persistent meristem. As a consequence, the development and final organization of a determinate nodule differs from the indeterminate nodule. All cells of the central tissue within a single determinate nodule are progressing through the same stage of development (*28*). The smaller uninfected cells found interspersed among the infected cells in the central tissue of determinate nodules are specialized for the assimilation of fixed nitrogen to ureides (*5*). No evidence has been found for such a specialization of the uninfected cells in indeterminate nodules.

Studies on plant gene expression confirmed the existence of several distinct developmental zones in an indeterminate root nodule (*21*). The developmental zones as defined by cytological criteria coincide more or less with the zones characterized by the expression of particular early nodulin genes. The early nodulin gene PsENOD12 is expressed in all cells of the invasion zone, which is consistent with a function of PsENOD12 in the infection process (*22*) (Fig. 3G). In older parts of the nodule, PsENOD12 mRNA is not detectable. The PsENOD5 gene expression is apparent in the invasion zone of the central tissue, but to the greatest extent in the infected cells of the early symbiotic zone (*21*). In these cells, infection threads have stopped growing, bacteria proliferate, and a very active membrane-synthesizing apparatus surrounds the proliferating bacteria with the peribacteroid membrane. The relatively high activity of the PsENOD5 gene in these cells suggests that the arabinogalactan-like protein PsENOD5 may not only be part of the plasma membrane of the infection thread, but also part of the peribacteroid membrane.

In the early symbiotic zone, two other early nodulin genes, PsENOD3 and PsENOD14, are exclusively expressed in the infected cells of the central tissue (*21*). Sequence analyses of PsENOD3 and PsENOD14 cDNA clones show that the mRNAs encode 6-kD polypeptides that are 55% homologous. They have a putative signal peptide at the amino terminal end and contain four cysteine residues with a spatial distribution resembling that of metal-binding proteins. The PsENOD3 and PsENOD14 nodulins may be metal-binding proteins involved in, for instance, transport of Fe or Mo to the bacteroids which require these metals for a functional nitrogen/hdase. Similar to the PsENOD12 and PsENOD5 genes, the Ps-ENOD3 and PsENOD14 genes are transiently expressed during the development of the nodule. In the late symbiotic zone, the amounts of PsENOD3 and PsENOD14 mRNAs decrease, and late nodulin mRNAs, such as for leghemoglobin, become detectable.

Physiological studies have indicated that the peripheral tissue contributes to the special features of root nodule morphology (*30*). The compact structure of the nodule parenchyma with its few and small intracellular spaces forms an oxygen diffusion barrier. In combination with the high oxygen consumption rate of the bacteria and the presence of leghemoglobin, this barrier helps to maintain the low free oxygen concentration in the central tissue as required for nitrogenase function.

The special characteristics of the nodule parenchyma are also reflected in the pattern of early nodulin gene expression. The early nodulin gene PsENOD2 is specifically expressed in the nodule parenchyma of pea (*31*). Similarly, GmENOD2 gene expression in the determinate soybean nodule is confined to the nodule parenchyma. PsENOD2 (*31*) and GmENOD2 (*32*) are structurally similar to PsENOD12, in that both are mainly composed of two alternating pentapeptides, each of which contains two proline residues. This structure suggests that these early nodulins are cell wall components. Because the cell wall is the major determinant of cell morphology, it is likely that an early nodulin such as ENOD2 contributes to the special morphology of nodule parenchyma cells, which is required for establishing the oxygen diffusion barrier.

Nodule Functioning

The establishment of the early symbiotic zone marks the end of the morphological changes accompanying differentiation into a root nodule. Next, biochemical alterations brought about by, among other things, late nodulin gene expression, create and support the required environment for nitrogen fixation and ammonia assimilation to occur. Late nodulin gene expression has been demonstrated in a large variety of legume species (*8, 10*). Numerous late nodulin genes, including all soybean leghemoglobin (Lb) genes, have been sequenced and a list of all cloned (late) nodulin genes has been presented recently (*33*).

The best studied late nodulin also is the most abundant one: Lb constitutes up to 25% of the total soluble protein in a mature nodule. Lb is an oxyhemoprotein with a high oxygen affinity (*34*), and resembles the vertebrate globins. In combination with the special nodule morphology, Lb controls the concentration of free oxygen in the nodule. It provides a flow of oxygen toward the

bacteroids that balances protection of nitrogenase against oxygen damage with support of respiration. Lb may be a true "symbiotic protein," because the heme group is presumed to be a bacteroid product, whereas the globin part is encoded by the plant genome (*8*).

Other late nodulins have been identified as enzymes or subunits of enzymes that function in nitrogen (glutamine synthetase, uricase) or carbon (sucrose synthase) metabolism (*8*). These late nodulins all perform tasks that individually are not unique to nodule functioning, but occur in other parts of the plant as well. Based on this and other observations not to be discussed here, we have put forward the hypothesis that nodule formation evolved from relatively minor alterations in the pathway of root differentiation, in which common plant genes became adapted to fit the physiological or regulatory constraints of the symbiosis (*35*). Several nodule enzymes have been shown to differ in physical, kinetic, or immunological characteristics from the forms found in roots (*36*): phosphoenolpyruvate carboxylase, choline kinase, xanthine dehydrogenase, purine nucleosidase, and malate dehydrogenase. It is unknown whether the nodule-specific forms of these enzymes originate from the expression of nodulin genes, or result from nodule-specific, post-translational modifications of gene products also found elsewhere in the plant.

Late nodulins are also associated with the peribacteroid membrane (*8*). This membrane encloses the bacteria within the plant cytoplasm, thus forming the physical and metabolic interface between bacterium and plant. The precise biochemical function of most of these peribacteroid membrane nodulins is as yet unknown. The best studied is the soybean nodulin Ngm-26, which probably is a transmembrane protein spanning the full peribacteroid membrane. Its amino acid sequence is homologous to the *Escherichia coli* glycerol facilitator protein (*37*), which is the only known pore-type protein in the *E. coli* cytoplasmic membrane and which functions in transport of small molecules. Recent results demonstrate that Ngm-26 forms an ion channel likely to function in translocation of small compounds, like the carbon source succinate, across the peribacteroid membrane (*38*).

All late nodulin genes appear to be coordinately expressed in time and development (*8, 10*). These genes might thus all be activated as a result of a single signal, perhaps related to release of bacteria from the infection threads, because they are not expressed in nodules without infected cells. The nature of this presumed signal, however, and the involvement of bacterial genes remain unclear.

The importance of the promoter region of nodulin genes for nodule-specific gene expression has been demonstrated with the use of transgenic plants. The promoter region of a soybean Lb gene directed nodule-specific and developmentally correct expression of a reporter gene when transformed into other leguminous plants. Apparently, the Lb promoter region not only carries all required *cis*-regulatory sequences, but all relevant *trans*-acting factors are also conserved among different leguminous species. Deletion analysis of the soybean Lb promoter defines a relatively small region as responsible for the correct expression pattern (*39*). In soybean, a nodule-specific *trans*-acting factor interacting with the Lb promoter has been identified (*40*) and awaits cloning. Molecular analyses of promoters and their *trans*-acting factors will give insight in the mechanisms of nodulin gene regulation and may hint to the nature and origin of the signal presumed to trigger the expression of these genes.

In situ hybridization studies with the use of bacterial antisense RNA showed that the rhizobial *nif* genes, encoding nitrogenase, were expressed later than the late nodulin genes (*41*). This confirmed the notion that the nodule prepares for nitrogen fixation by synthesizing late nodulins. It also suggested yet another step in the communication between plant and bacterium to yield symbiotic nitrogen fixation.

Concluding Remarks

Application of NodRm-1 to legume roots is sufficient to induce root hair deformation, expression of the early nodulin gene PsENOD12 (representative of at least part of the infection process), and mitotic activity in the root cortex followed by formation of a nodule-like structure (*19*). NodRm-like factors thus have a pivotal role in all three processes induced by *Rhizobium* (Fig. 1). The oligosaccharide nature of the NodRm-1 factor is consistent with observations that oligosaccharides can regulate plant morphogenesis (*29*). The nature of the signals involved in induction of late nodulin gene expression is not known, but the presence of infection threads or intracellular bacteria appears to be required. Therefore, the signals triggering late nodulin gene expression are most likely generated by the release of the bacteria from the infection thread.

It is currently an open question how a single factor can induce three apparently different processes. It might be possible that, for example, mitotic activity in the root cortex is induced by a second signal generated in deformed or curled root hairs (*42*). Alternatively, the different processes induced by *Rhizobium* might share certain aspects. Infection and induction of mitotic activity seem rather different, but a relationship between both follows from the hypothesis that cells preparing for infection thread growth begin division and are arrested in the prophase of the cell cycle (*14*).

Another possibility might be that the Nod factor induces the different developmental processes, but plant factors determine the outcome of development. An involvement of plant factors in steps of nodule formation has been demonstrated (*43*). Root nodules are induced predominantly opposite a xylem pole (Fig. 3F), and an alcoholic extract of the root stele is able to influence the pattern of cortical cell divisions in the root cortex (*43*). Therefore, it has been suggested that factors from the xylem are involved in triggering cell division. It is conceivable that a gradient of the Nod factor, or a Nod factor–derived signal, decreasing in concentration from infection site root inwards, interacts with a xylem factor gradient in the opposite direction. This interaction may determine whether cortical cells divide to form the nodule primordium or become arrested in their cell cycle as preparation for penetration by the growing infection thread.

The mechanism by which a Nod factor triggers plant development is unknown, but may involve changes in phytohormone ratios. Addition of auxin transport inhibitors to alfalfa roots gives rise to nodule-like structures, in which early nodulin genes are expressed (*44*) and which cytologically resemble *Rhizobium*-induced nodules. Furthermore, the introduction of a zeatin gene, involved in cytokinin synthesis, can partly complement Nod^- mutants of *R. meliloti* (*45*). The Nod factor may thus accomplish changes in the balance of phytohormones in the root, which secondarily (*46*) result in nodule formation.

To understand more about the mechanism of Nod factor action, the way the signal given by the Nod factor is perceived and transduced by the plant must be elucidated. Identification of the plant receptors involved, as well as research into the signal transduction pathways in the legume plant, will be facilitated enormously by the opportunities to obtain substantial amounts of purified Nod factors from bacterial cultures.

The possible involvement of phytohormones in nodule development suggests that the prokaryote *Rhizobium* has acquired the ability to cope with the endogenous plant systems that regulate development. In terms of symbiosis, it would seem to make sense that the bacterium employs those plant systems as much as possible. The challenge now is to discover what the bacterium apparently already knows. Further exploitation of this prokaryote and its signals will contribute to our understanding of the developmental biology of

root nodule formation, and be of general significance for our insight into the development of plants.

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Molecular Chaperones: The Plant Connection

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Molecular chaperones are a family of unrelated proteins found in all types of cell. They mediate the correct assembly of other polypeptides, but are not components of the mature assembled structures. Chaperones function by binding specifically to interactive protein surfaces that are exposed transiently during many cellular processes and so prevent them from undergoing incorrect interactions that might produce nonfunctional structures. The concept of molecular chaperones originated largely from studies of the chloroplast enzyme rubisco, which fixes carbon dioxide in plant photosynthesis; the function of chaperones forces a rethinking of the principle of protein self-assembly.

THE STUDY OF THE MOLECULAR BIOLOGY OF PLANTS IS undergoing a rapid expansion, fueled both by technical advances and by the realization that there may be economic advantages if plants can be manipulated in new ways. One of the few basic concepts in molecular biology that has originated from research with plants is that of molecular chaperones. This concept developed from studies on the biogenesis of the chloroplast enzyme that fixes carbon dioxide in photosynthesis (ribulose bisphosphate carboxylase-oxygenase or rubisco), but the field now encompasses animal and microbial cells and has medical and biotechnological aspects (*1–10*). In this article I describe how the chaperone concept developed from studies on rubisco and discuss the implications of this concept for future plant research.

Rubisco Biogenesis

Rubisco indirectly or directly plays a vital role in the metabolism of all cells, since it is the principal catalyst that brings carbon into organic combination from atmospheric carbon dioxide during photosynthesis. Its biogenesis is unusually complex and involves the interaction of light as a developmental trigger with two distinct genetic systems, one located within the chloroplast and the other within the nucleus (*11*). Despite its vital role, rubisco is a poor catalyst and has both a low affinity for carbon dioxide and a small turnover number; thus autotrophic organisms devote a major part

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