

REVIEW ARTICLE

Signals in Root Nodule Organogenesis and Endocytosis of *Rhizobium*

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INTRODUCTION

The rhizobia comprise a diverse group of organisms that elicit hypertrophic growth on the roots of legume plants to form a new organ, the root nodule, which they inhabit to fix nitrogen. This endosymbiotic association makes legume plants autotrophic for external nitrogen, an essential nutrient for plant growth. Because the interaction with rhizobia is highly beneficial, legume plants have evolved a set of genes encoding nodule-specific proteins (nodulins; Legocki and Verma, 1980); expression of these genes is required for the development and function of root nodules. A cascade of events controlling the expression of these genes allows for the development of infection, nodule organogenesis, the release of rhizobia from the infection thread, and the establishment of a symbiotic state. Although the root nodule structure can develop without bacterial infection (see below), infection and organogenesis normally proceed simultaneously. Furthermore, neither nodule organogenesis nor endocytosis of rhizobia depends on the ability of the bacteria to fix nitrogen.

Intensive study over the past two decades has led to an understanding of many of the molecular events in early plant-*Rhizobium* communication leading to nodule organogenesis. It is now clear that nodule development is under the control of specific nodulation (nod) signal(s) produced by rhizobia in response to the host, although hypertrophic growth can also occur autonomously on roots of certain legumes (Truchet et al., 1989; Caetano-Anolles and Gresshoff, 1991). Nod signals have recently been purified from several rhizobia, and their structures have been determined (see below); these may allow elucidation of the molecular basis for host specificity. The application of purified nod factors triggers nodule ontogeny, and these signals can be modified chemically or by mutagenesis of bacteria, providing an excellent system to study the development of this plant organ. Because generation of the nodule requires new cell division in root cortical cells, nod factors may control events leading to the initiation and/or regulation of cell division. Uncoupling of nodule organogenesis from endocytosis of bacteria may allow identification of the signal(s) that operate once the nodule developmental program is triggered. Understanding the signal transduction pathways that

control the expression of host genes required for the initiation of nodule primordia may reveal how the development of a plant organ can be regulated by internal or external factors and how bacteria have acquired the ability to trigger this organogenesis program.

Meristems are generally refractive to bacterial infection; however, rhizobia invade the newly divided cells of the nodule primordium. Invasion of legume roots occurs through a highly orchestrated infection process that does not provoke host defense reactions, and the bacteria are released via the infection thread into the "target cell." Little is known about the endocytosis of bacteria in plant cells, the nature of the subcellular compartment that harbors the bacteria, and the biogenesis of this compartment. The formation of such a compartment is essential to all endosymbioses; the failure to form this compartment may evoke pathogenic responses in the host. *Rhizobium* has evolved a mechanism to counter the host defense response (Verma and Nadler, 1984), but it elicits a pathogenic response in certain ineffective conditions (Werner et al., 1985; Djordjevic et al., 1987). Current interest in extending symbiosis to other crop plants requires understanding of host specificity, organogenesis of nodule structure, invasion and endocytosis of bacteria, and formation of a new subcellular compartment able to sustain a foreign organism endosymbiotically. Some of the key issues yet to be understood in our efforts to uncover molecular events in the control of nodule development and endocytosis of rhizobia are discussed here.

NODULE ORGANOGENESIS

Events in the Genesis of Root Nodules

Sensitivity to oxygen, the fundamental constraint on the process of nitrogen fixation, keeps rhizobia confined to a specialized organ, the root nodule, and does not allow them to integrate into the entire cell lineage of the plant as has been

achieved by chloroplasts and mitochondria. This organ has evolved to support optimal interdependence between the plant and *Rhizobium*. Root nodule development involves a series of interactions between the two organisms (see for reviews, Verma and Long, 1983; Long, 1989; Nap and Bisseling, 1990; de Bruijn and Downie, 1991; Verma et al., 1992) that begins with chemotaxis of rhizobia and continues through the establishment of the symbiotic state and senescence of the nodules. The model in Figure 1 shows how these interactions are mediated by specific molecular signals that are precisely titrated and elicit temporally and spatially distinct events in nodule organogenesis. Recent molecular genetic studies have allowed the dissection of these events and the dissociation of the nodule organogenesis program from endocytosis and nitrogen fixation.

Early Signal Exchange(s) between Plant and Bacteria

A precise exchange of molecular signals between rhizobia and the host plant over space and time is essential to the development of an effective root nodule. One important early exchange of signals involves the secretion of phenolic compounds, flavones and isoflavones, by the plant (Peters and Verma, 1990). These signal compounds are often exuded by the portion of the root with emerging root hairs, a region that is highly susceptible to infection by rhizobia. These compounds activate

the expression of *nod* genes in rhizobia, stimulating production of bacterial nod factors (Long, 1989; de Bruijn and Downie, 1991; Kondorosi, 1992). The initiation of the meristematic activity that gives rise to the nodule occurs before the infection thread is formed and can occur prior to root hair curling (Libbenga and Harkes, 1973; Newcomb et al., 1979; Dudley et al., 1987; Guinel and LaRue, 1991), which is an early host response to rhizobia. These observations suggest that diffusible nod factors produced by rhizobia are responsible for initiating the cascade of events in the host that lead to nodule organogenesis.

The nod signal compound from *Rhizobium meliloti* (NodRm-1) has been identified as a sulfated and acylated oligo-*N*-acetylglucosamine (Lerouge et al., 1990). The activity of this compound depends upon the common *nodABC* genes and the host range-determining genes *nodH* and *nodQ*. The latter genes participate in the addition of sulfur to the nod factor. The nod signals are effective at very low concentrations (10^{-11} M) and initiate cortical cell division in alfalfa leading to the formation of nodule-like structures when added to the medium (Truchet et al., 1991) or applied locally (D. Ehrhardt and S. Long, personal communication). The signal molecule from *Bradyrhizobium japonicum* is very similar to NodRm-1, having a backbone of pentaglycosamine, but it is not sulfated (G. Stacey, personal communication). *R. leguminosarum* bv *viciae* also produces nonsulfated nod signals, but these molecules contain a unique, highly unsaturated fatty acid and differ in the

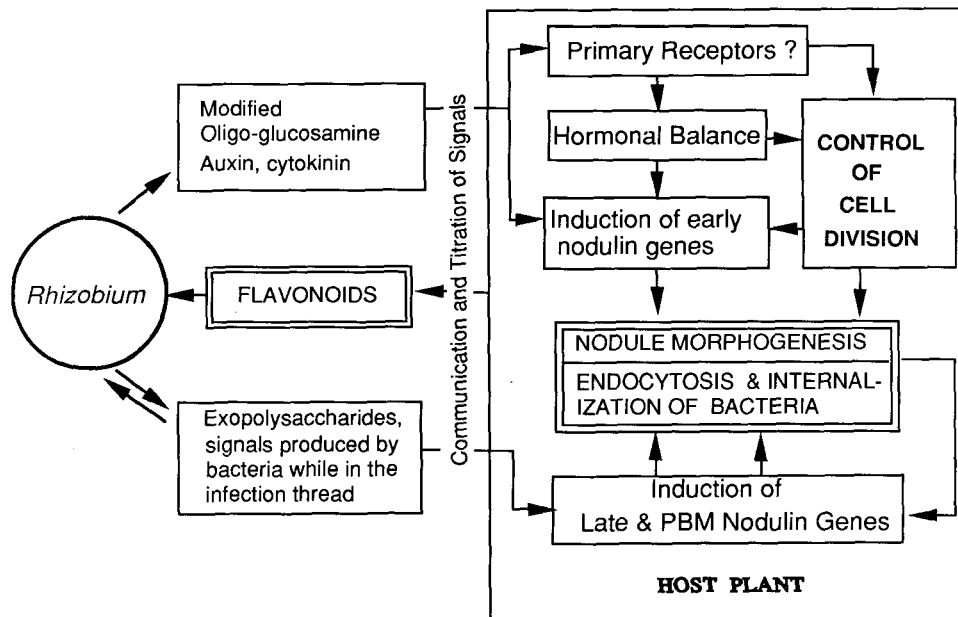


Figure 1. Early Interactions and Signal Exchange(s) between the Legume Plants and Rhizobia Leading to Endocytosis and Establishment of the Symbiotic Association.

The lipo- and exo-polysaccharide signals appear to play a more important role in the infection thread.

number of *N*-acetylglucosamine units (Spaink et al., 1991). Both the fatty acid moiety (*nodE*-dependent) and *O*-acetyl substitution (*nodL*-dependent) are essential for the initiation of the meristematic activity that gives rise to nodule-like structures on the host plant.

Different *Rhizobium* species apparently make different modifications to the core structure, which may provide host specificity for the initiation of cortical cell divisions in nodule organogenesis. Whereas the nod compounds from *R. meliloti*, *B. japonicum*, and *R. leguminosarum* have an oligosaccharide core, several factors secreted by *R. trifolii* bear no resemblance to the nod compounds. *N*-Acetylglutamic acid induces nodule-like primordia on clover (Philip-Hollingsworth et al., 1991), whereas diglycosyldiacylglyceride causes uniform cell division in the cortex, leading to a thick and short root phenotype (F. Dazzo, personal communication). Rhizobia are also known to secrete cytokinins (Sturtevant and Taller, 1989). These compounds apparently have distinct receptors and use separate signal transduction pathways that may converge to elaborate their responses via a common route.

Nodule Initiation

Prior to the entry of bacteria into the host, initiation of cell division occurs. Purified nod compounds can initiate cell division, giving rise to nodule-like structures (Spaink et al., 1991; Truchet et al., 1991) analogous to those produced by the application of cytokinins (Arora et al., 1959; Libbenga et al., 1973) or auxin transport inhibitors (e.g., triiodobenzoic acid; Hirsch et al., 1989). These results suggest that nod factors act by altering phytohormone levels in some way. Nodule organogenesis is an independent process but normally occurs along with infection. Cells in the cortical tissue of soybean root begin to divide 12 to 24 hr after inoculation with rhizobia (Calvert et al., 1984). This response is specific for compatible strains of rhizobia.

In the case of determinate nodules, hypodermal cells of the cortex are the first to respond to infection. In indeterminate nodules, by contrast, cells of the inner cortex appear to be the primary target for initiation of meristematic activity (Libbenga and Harkes, 1973; Newcomb et al., 1979). How cell division is triggered by the nod signal molecules is not known. These signals may interact with the cell cycle control mechanism so that resting cortical cells enter mitosis (see below). Although many infections occur on the root, only a small fraction of them give rise to cortical cell divisions, suggesting that further titration of some factor(s) along the path of the infection process is essential for eventual success in generating nodule primordia. Many bacterial (Long, 1989) and plant (Gresshoff and Delves, 1986; Guinel and LaRue, 1991) mutants are known that abort this developmental program, presumably by perturbing communication and signal transduction pathways, but the precise steps in the process are not understood.

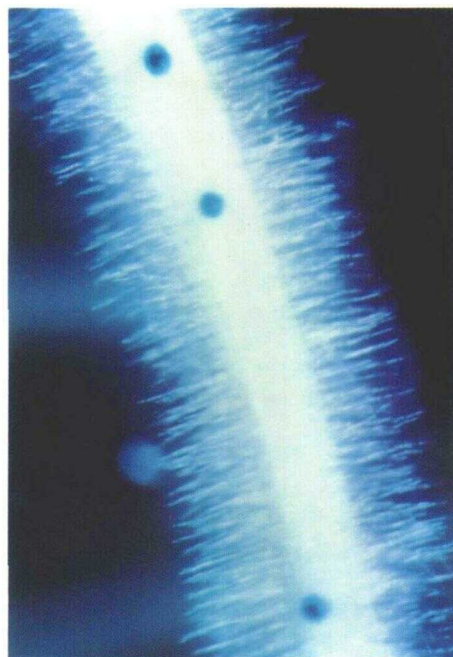


Figure 2. Induction of Soybean Nodulin-26 Promoter in Incipient Lateral Root Primordia of Transgenic *L. corniculatus*.

The nodulin-26 promoter was fused with the GUS reporter gene and introduced in *L. corniculatus* via *Agrobacterium rhizogenes*, as described elsewhere (Miao et al., 1991). Blue spots indicate incipient lateral root primordia.

Although lateral root emergence and nodule meristem initiation have many features in common, these two organs have distinct ontogenies. The nodule meristem originates from cortical cells, whereas lateral root primordia have their origins in the pericycle. Legume nodules are not lateral roots, but they share a part of their genetically controlled developmental program with lateral roots. As Figure 2 shows, when a soybean late nodulin (nodulin-26) gene promoter fused to a reporter gene (GUS) is introduced into *Lotus corniculatus*, GUS activity is localized to incipient lateral roots. The nodulin-26 gene promoter is also expressed in nodule primordia (data not shown). These data suggest a common requirement for the expression of this gene in both types of meristems. Although nodulin-26 is not expressed in soybean roots (Fortin et al., 1987), homologs of this gene that have been isolated from tobacco and *Arabidopsis* are found to be expressed in root tips (Yamamoto et al., 1990).

Although rhizobia do not invade root and nodule meristems, they do invade dividing cells in the cortex, indicating that the dividing cells of the nodule primordium differ from those of root and nodule meristems. The cessation of meristematic activity gives rise to determinate nodules. Because the same bacteria can form determinate or indeterminate nodules in different hosts, the persistence of the meristematic activity depends upon the host. Factors controlling the continuity of this

activity are not known. Recent genetic approaches to obtain mutants have identified many loci affecting root development (Schiefelbein and Benfey, 1991). Some of these mutations may have relevance to early events in the *Rhizobium* infection process, such as root hair curling. Mutants in lateral root emergence are known in nonlegumes but have not yet been identified in legumes (Schiefelbein and Benfey, 1991). It is not known whether such mutants would affect nodule organogenesis. Infection of *Parasponia* (a nonlegume) by *Bradyrhizobium* gives rise to nodules that resemble modified lateral roots with a central vascular system. These nodules initiate from the pericycle rather than the cortex, as they do in legumes (Gresshoff and Delves, 1986). The *Parasponia*-*Rhizobium* association may be a progenitor of modern legume nodules.

Control of Cortical Cell Division

Plants are able to form new organs throughout their lives by initiating cell division in distinct sites. In the organogenesis of a nodule, the cells of the inner cortex begin dividing in response to signals from bacteria. Some cells of the legume root cortex have a 4C DNA content and are arrested in the G2 phase of the cell cycle (Gresshoff and Mohapatra, 1981); these cells appear to act as foci for nodule meristem initiation. The cell cycle arrest in these cells has been suggested to be due to the translocation of 7-methyl nicotinate, a compound found in cotyledons of beans but not of nonlegume plants (Evans and Van't Hof, 1973). Genistein, an isoflavone produced by soybean roots, may also be involved in the early signal transduction pathway because it is a potent inhibitor of protein tyrosine kinase (Akiyama et al., 1987), and protein kinases are involved in cell cycle progression (see below). Nod signal compounds may directly or indirectly (by modulating hormonal balance) regulate genes that control the progression of cells through mitosis. Such a mechanism could allow cortical cells arrested in G2 to enter mitosis rapidly. Release of G2 arrest by nod signal(s) would provide a mechanism by which the host could respond rapidly to incoming signals from rhizobia, initiating cell division without the need for DNA synthesis. Figure 3 outlines a possible mechanism for the control of specific genes and the role of nod factors in regulating cell division in the root cortex.

The basic machinery of the cell cycle is conserved in eukaryotes (Nurse, 1990), including higher plants (Doonan, 1991), although its regulation may differ because many signals are able to trigger cell division in plants. The product of the yeast *cdc25* gene and its homologs in higher eukaryotes control the activity of maturation promoting factor (MPF, a complex of p34^{cdc2} and cyclin B; Lewin, 1990), which is essential for cells to enter mitosis. The *cdc25* gene encodes a tyrosine phosphatase that dephosphorylates p34^{cdc2} protein kinase, activating MPF (Gautier et al., 1991). Homologs of *cdc25* have been isolated from animals (Gautier et al., 1991), and the conservation of this gene in plants is likely. MPF is regulated by both protein kinase and phosphatase (Lewin, 1990). Genistein,

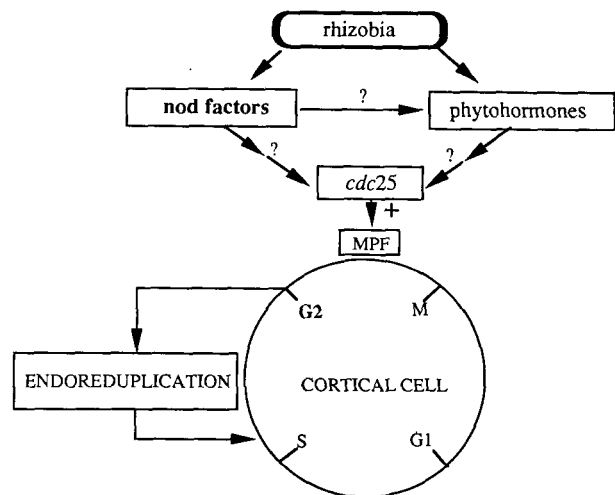


Figure 3. Proposed Interaction of Nod Factors with the Cell Cycle Control Machinery Leading to Initiation of Nodule Primordia.

MPF, maturation promoting factor; G1, S, G2, and M, phases of the cell cycle.

the tyrosine kinase inhibitor found in soybean root, may interact with *cdc2* kinases, affecting MPF activity. In addition to being a mitotic inducer, the *cdc25* gene product also acts as a nutrient sensor (Malone, 1990), and it is considered a key regulator for cell division (Edgar and O'Farrell, 1989). Because both external and internal signals can control cell division in plants, these pathways may converge on the same downstream gene(s) to initiate cell division.

The pathway that controls cell division in plants is not known. In the initiation of nodule primordia, nod factors may possibly participate in the cascade that leads to *cdc25* gene expression (Figure 3). Homologs of the yeast *cdc2* gene have been isolated from alfalfa (Hirt et al., 1991), pea (Feiler and Jacobs, 1990), and soybean (G.-H. Miao, Z. Hong, and D. P. S. Verma, manuscript in preparation). In soybean, two functional homologs of *cdc2* exist that appear to be differentially expressed in different meristems. A cyclin B cDNA has also been cloned from soybean (Hata et al., 1991; D. P. S. Verma, unpublished data), and this gene has been shown to be expressed at a very high level in early nodule development, which correlates with cell proliferation. A number of other cyclin genes that may play roles in different stages of the cell cycle have been isolated from eukaryotes (Hunter and Pines, 1991). The availability of genes that regulate cell cycle progression in plants will help to elucidate how they are controlled in legumes and how they interact with nod factors to initiate cell division in the root cortex. The initiation of cortical cell division is a nonsynchronous event; therefore, spot application of purified nod signal compounds and the use of other markers (such as the nodulin-26-GUS gene fusion) may help delineate nodule primordia at an early stage and make it possible to monitor the expression of various cell cycle genes.

Once bacteria enter newly divided cells, further cell division often ceases. Cessation of cell division may be due to interference with the normal cell cycle control mechanism. The cell cycle does not proceed toward condensation of chromatin and spindle formation; instead, it reenters the DNA synthesis phase, resulting in endoreduplication and an increase in DNA content up to 64C. During endoreduplication in nodules, no selective gene amplification occurs. Instead, the entire genomic DNA is multiplied with fidelity (Brisson et al., 1982). Treatment of cortical cells with auxins has been shown to cause endoreduplication (Libbenga and Torry, 1973), suggesting that an alteration in the levels of phytohormones in nodules may be responsible for endoreduplication in infected cells. In addition, the physical presence of bacteroids may interfere with microfilaments, thus preventing cytokinesis. With the availability of plant cell cycle genes as probes, dissection of the early events leading to the expression of these genes and their role in endoreduplication in nodules is now possible. These approaches may also allow determination of the differences in the signal transduction pathways of root and nodule meristems as well as the role of nod factors in the initiation of the nodule primordium.

Differentiation of the Root Nodule

Nodule-like structures induced by nod factors or by bacterial mutants that fail to enter the host cell are highly differentiated, consisting of enlarged cells (that would normally contain bacteroids) and small interstitial cells containing more starch. Differentiation of nodules without the entry of rhizobia suggests that nodule development is largely under the control of an organogenesis program that is triggered by nod factors. The entry of bacteria and their release from the infection thread completes the differentiation process, and nitrogen fixation commences.

Rhizobia are released from the infection thread only in newly divided cells, possibly due to an extensive need for endoplasmic reticulum (ER) and Golgi activity to generate large amounts of membrane necessary to enclose bacteria. The differentiated cortical cells of root do not have significant ER and Golgi activity. Cell division ceases in infected cells, although in indeterminate nodules, the nodule meristem persists, generating new cells that continue to be infected. In determinate nodules, cell division ceases before the commencement of nitrogen fixation, ~10 days after infection in soybean, for example. Nodule growth continues by cell enlargement. Prior to the commencement of nitrogen fixation, the bacteria also cease dividing. In fact, cell division and nitrogen fixation have been suggested to be incompatible processes (Ludwig, 1984), and only those bacteria that are differentiated into bacteroids may fix nitrogen.

Many early nodulin genes are expressed sequentially during nodule differentiation, and some of these genes can be induced in nodules devoid of bacteria (Nap and Bisseling, 1990). These studies suggest that some of the early nodulins

are involved in the early infection process, whereas others participate in root nodule morphogenesis. The expression of one of the early nodulin genes, ENOD2, is correlated with early differentiation. This gene is expressed in many hypertrophic growths on the root. ENOD2 gene expression has been shown to be induced by high levels of cytokinin, which regulates the activity of the ENOD2 gene promoter (Dehio and de Bruijn, 1992). The late nodulin genes are expressed concomitant with or following the release of bacteria from the infection thread but prior to the induction of nitrogenase and the commencement of nitrogen fixation (Verma and Delauney, 1988). These genes are regulated by a variety of factors, but many late nodulin genes are not expressed in nodules devoid of bacteroids (Verma et al., 1988).

Because certain cells are infected whereas others remain uninfected, a symbiotic zone is formed in which infected and uninfected cells assume different roles to meet the metabolic needs of the nodule. This zone is surrounded by nodule parenchyma and endodermis. The latter seems to prevent diffusion of ammonia (Miao et al., 1991). The nodule parenchyma controls diffusion of oxygen, and the ENOD2 gene product is localized in this region (Nap and Bisseling, 1990). The infected cells produce leghemoglobin, which allows the maintenance of oxygen flux under low oxygen tension. The oxygen tension in infected and uninfected cells may differ, as evidenced by monitoring the expression of an oxygen-responsive promoter (the alcohol dehydrogenase gene promoter) in a transgenic legume (Verma et al., 1992). The organization of nodule tissue into infected and uninfected cells and the exquisite adaptation of carbon and nitrogen metabolic pathways (Vance and Heichel, 1991) provide maximum energy under microaerobic conditions. The ureide-producing nodules also utilize low amounts of carbon to transport fixed nitrogen, making them highly energy-efficient organs.

ENDOCYTOSIS OF RHIZOBIUM

The endocytosis of bacteria inside the host cell without provoking host defense reaction(s) is the most crucial but least understood aspect of the legume–*Rhizobium* association. The formation of a subcellular compartment housing the bacteria inside the infected cell is the final stage of successful infection. The failure to form this membrane compartment or its disintegration renders the association pathogenic (Werner et al., 1985). Many rhizobial mutants are known (including exo- and lipo-polysaccharide mutants; Noel, 1992) in which bacterial entry does not take place but nodule morphogenesis proceeds to different stages; however, the specific interactions of these mutants with the host are not known.

Entry of Bacteria in Eukaryotic Cells

A successful pathogen is able to enter a host by avoiding or exploiting the host's defense mechanisms. It finds a unique

niche within the host and finally exits to infect a new host. Rhizobia are refined parasites (Djordjevic et al., 1987) that provide an essential nutrient to the host and have, thus, become symbionts. Recent studies on the interaction of pathogenic bacteria with their animal hosts suggest a possible molecular mechanism by which some prokaryotic organisms enter the eukaryotic host cells (Falkow, 1991). Several animal pathogenic bacteria, such as *Yersinia* and *Legionella*, enter their hosts by way of an outer membrane protein, invasins, that interacts with the integrin family of cell receptor molecules involved in phagocytosis. *Escherichia coli* expressing the invasins gene are able to enter mammalian cells. This mode of invasion may be common to organisms that gain entry through phagocytosis and then persist inside their hosts as either parasites or endosymbionts. There is no evidence that such a mechanism exists in *Rhizobium*; however, analogies with bacterial parasites suggest that receptor-mediated endocytosis is likely (Morrison and Verma, 1987) and worth experimental pursuit. No information is available as yet on the transmembrane signaling system that triggers endocytotic or phagocytotic events.

A specific interaction of the infecting bacteria with the host cell was observed in nodules formed by a *Tn5* mutant of *B. japonicum* (Morrison and Verma, 1987). When bacteria of this strain infect host plants, infection and nodule differentiation proceed normally. However, the bacteria are not released from the infection thread, and bacterial entry is stopped at the stage of endocytosis. The resulting nodules appear almost normal but are largely devoid of bacteria. Additional evidence of interactions between the plant membrane and bacteria is provided by the attachment of the peribacteroid membrane (PBM) to components of the bacterial surface (Robertson et al., 1985; Verma and Fortin, 1989; Brewin, 1991). Moreover, an antibody prepared against the PBM also recognizes a protein in purified bacteroids (Fortin et al., 1985). Such interactions may act as "proximity sensors" to allow adequate membrane deposition around the bacteria.

Biogenesis of the PBM

Infection by rhizobia triggers proliferation of the membrane system that generates components of the PBM. Continued proliferation of this membrane is essential to enclose rhizobia so that contact of the bacteria with the host cytoplasm is avoided. The study of this membrane may give insight into the evolution of cellular compartmentation, including the origin of organelles. In soybean root nodules, almost 30 times more membrane is generated in the form of PBM than in the form of plasma membrane (Verma et al., 1978). The PBM has properties of both tonoplast and plasma membranes, and its biogenesis is not well understood. Proliferation of the PBM begins concomitant with the release of bacteria from the infection thread. This requires redirecting some of the plasma membrane proteins and all of the PBM nodulins to this newly formed

subcellular "organelle." In nodules induced by the *B. japonicum* strain defective at the stage of endocytosis, a large number of membrane vesicles was found to fuse at the tip of the infection thread (Morrison and Verma, 1987). PBM nodulins are induced at this stage of development but are differentially expressed in nodules formed by the mutant. Transduction of all the signals necessary for the induction of PBM nodulins apparently precedes the release of bacteria from the infection thread (Verma et al., 1988), but these signals may vary for each PBM nodulin (Morrison and Verma, 1987).

Although the PBM arises from the plasma membrane during endocytosis (Verma et al., 1978), it undergoes significant alterations and appears to be a mosaic membrane with properties common to both plasma and vacuolar membranes. Nodulin-26, an intrinsic membrane protein of the PBM (Fortin et al., 1987), resembles an integral tonoplast membrane protein (TIP) and a plasma membrane protein of the bovine eye lens (MIP) (Pao et al., 1991). A nodulin that resembles protease inhibitor, a vacuolar protein, has recently been identified in the PBM compartment of *Psophocarpus* (Manen et al., 1991). α -Mannosidase (a vacuolar marker enzyme) is also found inside the PBM compartment (Kinnback et al., 1987). Placing rhizobia in a subcellular compartment with lysosomal properties may allow the host cell to exert further control over this invasion in the event nitrogen fixation does not occur and the bacteria need to be destroyed. Lysis of bacteria does occur in some incompatible associations (Werner et al., 1984). It is not known whether the lysosomal features of the bacteroid compartment become pronounced only in the absence of nitrogen fixation or whether they are active throughout the symbiotic phase.

Functional complementation of certain yeast mutants (Herman and Emr, 1990) that are blocked in membrane biosynthesis and intracellular vesicular transport may allow some of the genes involved in PBM biogenesis to be cloned. The yeast SEC4 protein may be responsible for unidirectional transport of vesicles from the Golgi complex to the plasma membrane (Salminen and Novick, 1987). This protein binds GTP and has GTPase activity. Similarly, yeast YPT1 protein controls an early step in vesicular transport between the ER and the Golgi complex. Both Ca^{2+} and GTP are essential for vesicular transport (Hall, 1990). Mammalian homologs (*rab1-7*) of YPT1 and SEC4 have been isolated and shown to be located in the early and late endosomes, the intermediate compartments between the ER and the Golgi complex, suggesting that these genes participate in the endocytic and exocytic pathways; *rab5* was shown to be specifically involved in endosome fusion (Gorvel et al., 1991). Recently, a plant cDNA was isolated that encodes a small GTP binding protein (*Rha1*) that shows high homology with members of the Ras family and 60% homology with mammalian *rab5* protein (Anuntalabhochai et al., 1991). Isolation of legume homologs of yeast genes involved in membrane biosynthesis and trafficking may allow delineation of the endocytotic route of rhizobia and the role of various pathways that contribute to the formation of the PBM.

Targeting of Nodulins to the Bacteroid Compartment

Membrane biogenesis in eukaryotes follows a common path. The movement of proteins from the ER to the Golgi complex and then to the plasma membrane is by bulk flow (default pathway), whereas those directed to specific subcellular compartments require specific topogenic information (Chrispeels, 1991). It is not fully understood how the PBM, which is derived from the plasma membrane, acquires some of the properties of the vacuolar membrane (Verma and Fortin, 1989). The specific targeting of nodulins to the PBM and the peribacteroid space needs to be analyzed in detail to gain insight into the genesis of this subcellular compartment. Individual nodulins appear to be targeted to the PBM by different mechanisms. Nodulin-24 of soybean has an amino-terminal signal sequence that is cleaved cotranslationally (Katinakis and Verma, 1985). The cleaved peptide is further processed post-translationally, which increases its molecular weight by almost 12 kD. The nature of this modification is not known. *In vitro* cotranslational processing and membrane protection experiments suggest that no part of nodulin-24 is exposed outside the vesicle and that it has no transmembrane regions; following signal removal, this peptide is free in the lumen of the ER (C.-I. Cheon and D. P. S. Verma, unpublished data). Attachment of this nodulin to the membrane occurs during its passage through the Golgi complex. Nodulin-24 forms an amphipathic α -helix that appears to face the bacteroid side of the PBM. Amphipathic molecules may act as antibody paratopes (antigenic recognition sites; Kauvar et al., 1990). Such recognition may facilitate the attachment of bacteroids to the PBM surface, which may be necessary to coordinate PBM synthesis with bacterial cell division during the early stages of infection. The relationship between PBM synthesis and bacterial cell division has not yet been experimentally determined.

Nodulin-26 contains noncleavable internal signal sequences and thus differs from nodulin-24 in its mode of targeting to the PBM. It is glycosylated cotranslationally as evidenced by ConA binding, and the glycosylation site faces the bacteroid. The orientation of the carboxyl and amino termini was determined by chemical cleavage mapping at cysteine residues of untreated and trypsinized *in vitro* synthesized peptide (G.-H. Miao, Z. Hong, and D.P.S. Verma, manuscript submitted).

"Internalization" of the Bacteroid Compartment

Because the rhizobia are encapsulated within the PBM, the space between the bacteria and the PBM, which is known as the peribacteroid space, must be equilibrated with certain metabolites, including dicarboxylic acids (used as carbon sources by bacteroids), to eliminate the concentration gradient between the host and the rhizobia. In a sense, this internalizes the organism and brings it into the closest association possible with the host. Equilibration of the peribacteroid space is apparently accomplished by opening specific channels in the PBM.

Nodulin-26 appears to be such a channel. This nodulin has homology with a group of proteins conserved from bacteria to mammals (Pao et al., 1991; Verma, 1991). Two members of this group have been shown experimentally to form channels. The nature of the compounds transported through these channels, including the putative nodulin-26 channel, is not known. Nodulin-26 may facilitate active transport of metabolites such as dicarboxylic acids across the PBM and into the peribacteroid fluid for uptake by the bacteria. Nodulin-26 is phosphorylated by a kinase located in the PBM (G.-H. Miao, Z. Hong, and D.P.S. Verma, manuscript submitted). The presence of this type of channel in the PBM demonstrates a previously unknown dimension of symbiosis, in which the essentially extracellular compartment that encloses the microsymbiont must be physiologically internalized, bringing the foreign organism spatially and functionally into closer proximity with the host. Because the peribacteroid space in nodules occupies as much or more space as does total cytoplasm in an infected cell, equilibration across the gradient becomes even more essential. The peribacteroid compartment must be tightly regulated by the appropriate osmoticum, which may help control the flow of specific metabolites. The presence of such channels in the membrane that separates eukaryotic and prokaryotic cells thus seems essential to the efficiency of endosymbioses.

FUTURE DIRECTIONS

Root nodule organogenesis is an ideal system for developmental studies because it occurs under the control of defined signal molecules produced by foreign organisms, rhizobia. The oligosaccharide nature of nod signals suggests a diverse role of these molecules in morphogenesis in plants because such compounds have been found to be involved in flower development (Marfa et al., 1991). The diversity of the signals able to trigger nodule organogenesis and their mode of action may also allow elucidation of the signal transduction program underlying lateral root organogenesis because the two organs share many similarities. Molecular characterization of plant mutations that block specific steps in the nodule developmental pathway or that affect root morphogenesis (Schieffelbein and Benfey, 1991) would be highly beneficial for these studies.

Plant cell division is triggered by seemingly different stimuli that may operate through a common signal transduction pathway to generate different organs. Because nodule and lateral root meristems differ in their ontogenies, specific receptors for nod signals may be involved in initiating nodule meristematic activity. Isolation of bacterial or plant mutants blocked in the early signal transduction pathway for nodule meristem initiation will help to elucidate the mechanism that controls lateral root and nodule meristems. Many currently available bacterial mutants affecting the infection process need to be analyzed for their ability to induce cell cycle genes in the host plant. The tools to address these questions are now

available. Rapid changes in the phosphorylation of specific proteins have been observed in the transduction of elicitor signals for pathogenesis (Felix et al., 1991), and it is possible that similar events take place in response to nod signals.

Endocytosis of bacteria is another poorly understood facet of the *Rhizobium*-plant association that needs to be addressed with new approaches. The need to produce enormous amounts of membrane to enclose bacteria provides a model system for membrane biogenesis in plants. This membrane proliferation amplifies the basic machinery 20 to 30 times over that in a normal root cell (Verma et al., 1978; Verma and Fortin, 1989). Because some of the steps in membrane biogenesis may be conserved between yeast and plants, the use of a functional complementation strategy could prove invaluable for finding plant homologs of yeast genes that control membrane traffic. The availability of yeast mutants blocked in vesicular transport (Herman and Emr, 1990) may allow plant homologs of yeast genes to be isolated, helping to dissect the pathway of membrane biogenesis in plants. Finally, understanding subcellular compartmentalization, metabolite flow, and the adaptation of various biosynthetic pathways to optimize the symbiotic interaction may reveal how plants deal with the nodule environment, e.g., high pH, low O₂, and high osmolarity.

In light of the requirements of endosymbiosis and the role of the host in elaborating the nodule structure, recent studies attempting to broaden the interaction of rhizobia to cereal plants require careful evaluation. Specific parameters governing the nodule organogenesis program that allows a foreign organism to coexist within the living plant cell need to be analyzed. Some bacteria have been shown to be engulfed nonspecifically by plant protoplasts (Davey and Cocking, 1972), but such entry does not ensure that the bacteria will survive and multiply, and the invasive bacteria often kill the plant cells. Death of the plant cell could be a hypersensitive reaction to the invader. This appears to be the case in nodule-like structures formed on rice and wheat roots because the contents of the infected cells disintegrate (cf. Cocking et al., 1990). The application of both existing molecular tools and those under development will allow the evaluation of new symbioses that may widen the host range of rhizobia to improve plant productivity.

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