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Developmental biology of legume nodulation

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CONTENTS

SUMMARY

Many legumes respond to *Rhizobium* inoculation by developing unique structures known as nodules on their roots. The development of a legume nodule in which rhizobia convert atmospheric N_2 into ammonia is a finely tuned process. Gene expression from both partners of the symbiosis must be temporally and spatially coordinated. Exactly how this coordination takes place is an area of intense study. Nodule morphogenesis appears to be elicited by at least two distinct signals: one from *Rhizobium*, a product of the *nod* genes (Nod factor), and a second signal, which is generated within plant tissues after treatment with Nod factor. The identity of the second signal is unknown but changes in the balance of endogenous plant hormones or the sensitivity of plant tissues to these hormones are likely to be involved. These hormonal changes may be triggered by endogenous flavonoids produced by the root in response to inoculation with *Rhizobium*. There is some controversy as to whether the legume nodule is an organ *sui generis* or a highly derived lateral root. A resolution of this question may become more critical as attempts to induce nodules on non-legume hosts, such as rice or maize, increase in number and scope.

Key words: Legume nodulation, development, *Rhizobium*, lectin, flavonoids.

I. INTRODUCTION

A large number of dicotyledonous plants, from several different families, establish symbiotic associations with specific N_2-fixing bacteria. In these associations, a unique structure (the nodule) develops on the root of the plant after the diazotroph and its host positively recognize each other. The root nodule is the site where N_2 gas is reduced to ammonia, which is assimilated into amino acids; these are then used to synthesize other nitrogen-containing compounds (for reviews, see Schubert, 1986; Cullimore & Bennett, 1992). Although considerable progress has been made in understanding the molecular

biology, genetics, and biochemistry of the bacterial partner, we still have an imperfect understanding of the plant's role in the establishment of the symbiosis.

The best studied N_2-fixing association is that between plants of the Fabaceae and members of the Gram-negative Rhizobiaceae. Three genera, *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* specifically associate with legumes. These three genera are taxonomically distinct, but for ease of discussion, are described as *Rhizobium* (*sensu lato*) in this review. The one exception to the *Rhizobium*–legume 'rule' are members of the genus *Parasponia* (Ulmaceae), the only non-legume to form N_2-fixing nodules in association with *Rhizobium* (Trinick, 1979). However, *Parasponia* nodules differ from legume nodules in a number of important features. First, the nodule is a modified lateral root, and has a central vascular bundle. Also, the rhizobia are not surrounded solely by host-derived membrane as are the bacteria that infect the nodules of herbaceous legumes. Rhizobia that infect *Parasponia* remain confined to threads in a manner similar to *Andira* and other woody legumes (Sprent, 1989). These threads are suberized and are restricted to an individual host cell (Smith, Skvirsky & Hirsch, 1986). They are known as fixation threads because they enclose bacteria that are actively fixing N_2 (Price, Mohapatra & Gresshoff, 1984). Other threads, which travel from cell to cell, have been called infection threads. They have lignified cell walls (Smith *et al.*, 1986).

In addition to the legumes, a diverse group of unrelated plants in eight different families fix N_2 symbiotically with the filamentous Gram-variable actinobacterium *Frankia* (Simonet *et al.*, 1990; Baker & Mullin, 1992). These plants, called actinorhizal plants, are woody trees and shrubs like *Casuarina* (Casuarinaceae), *Alnus* (Betulaceae), *Ceanothus* (Rhamnaceae), *Myrica* (Myricaceae), and *Elaeagnus* (Elaeagnaceae), among others. In a situation analogous to the *Rhizobium*–legume symbiosis, *Frankia* filaments enter curled root hairs via an infection thread-like structure. However, like *Parasponia*, the bacterial cells are not released from the threads. In addition, as in *Parasponia*, the actinorhizal nodule is a modified lateral root.

Although the different types of root nodules, either *Rhizobium*- or *Frankia*-induced, serve the same function (N_2 fixation and ammonia assimilation), there is considerable variation in their development and final morphology. As described earlier, some nodules, like those of *Parasponia* and actinorhizal plants, closely resemble lateral roots. In contrast, legume nodules differ significantly from lateral roots not only in lacking a root cap and having a more stem-like vascular arrangement (Sprent, 1989), but also in their initial pattern of development. These differences from lateral roots suggest that the legume nodule is distinct from other organs normally present on plants (Libbenga & Bogers, 1974). The question of whether the nodule is truly an organ *sui generis* or a highly modified lateral root becomes relevant when we consider the evolution of symbiotic N_2 fixation and also ponder such questions as: What is the morphogen(s) that triggers the change from differentiated cortical cell to nodule primordium? How do *Rhizobium*-produced signal(s) induce the normally quiescent, non-dividing root cortical cells to change their developmental fate and to follow a new pathway? What is the number of genes and the sequence of gene expression essential for nodule morphogensis? This latter question is particularly relevant if we wish to manipulate agronomically important nonlegumes to associate with N_2-fixing bacteria in an effective symbiosis.

In addition to such practical issues, root nodule formation offers an alternative system to such paradigms as flowering, embryogenesis, or vegetative shoot meristem formation for studying plant development. Nodulation is relevant to such overall developmental questions as cell–cell interactions, pattern formation, and the involvement of signal transduction pathways in triggering morphogenesis. Other topics that are germane to the study of plant development and which can be investigated using nodule development as a model include: the types and timing of appearance (as well as numbers) of specific ligands and receptors, the identification of transcriptional activators, the role of cytoskeletal elements in changing cell fate, and effects of signal molecules on the cell cycle. Moreover, studying nodule development offers the advantage of studying two distinct genomes (one from a eukaryote and the other from a prokaryote) as well as the interplay that goes on between the two partners to produce a new, highly specialized organ.

However, what makes nodule development unique in contrast to such developmental processes as embryogenesis or flowering is that nodule formation is not absolutely essential for the life of the plant. If adequate amounts of combined nitrogen are provided, nodule development is repressed; such repression is not completely understood. Nodule formation proceeds only when the plant is grown in soil that is nitrogen-deficient and then only when a bacterium that is compatible with the host plant is present.

This review will cover the various mechanisms that can be invoked to explain how plant cells respond to a *Rhizobium*-produced signal. This signal, the product of *nod* gene expression, is designated the primary signal in this review because it is the first trigger for a new pattern of plant morphogenesis. After describing mature nodules in terms of structure and nodulin gene expression, the possible role of the plant hormones in nodule development will be explored, as will whether changes in endogenous hormone balance and/or sensitivity serve as a second signal for morphogenesis. Lastly, some of the

arguments regarding the *sui generis* nature of the nodule will be reviewed, keeping in mind the different sequence of events that take place during nodule and lateral root development.

This review concentrates on the early stages of nodule development. Other reviews that deal with the early stages of nodule development have been written recently (see Brewin, 1991; Kijne, 1992). In addition, reviews concerning the expression of genes specific to root nodules (the nodulin genes) have also been published (Nap & Bisseling, 1990; Sanchez *et al.*, 1991; Verma, Hu & Zhang, 1992). Additional reviews (in chronological order) include those by Bauer (1981), Newcomb (1981), Meijer & Broughton (1982), Sutton (1983), Sprent (1989), Long (1989), de Bruijn & Downie (1991), Fisher & Long (1992), and the reader should consult these for more information. For a review of the earlier literature on nodule development, see the articles by Libbenga & Bogers (1974) and Dart (1977).

II. NODULE DEVELOPMENT

1. *Initial interactions*

(*a*) *Stages of pre-infection.* Nodule development can be arbitrarily divided into stages of pre-infection, nodule initiation, and differentiation. The pre-infection stages commence even before the host plant and its compatible *Rhizobium* strain recognize each other as potential partners on a cellular basis. Legume seed coats, like those of many plants, contain large quantities, as well as a diversity, of flavonoids. In alfalfa, the types of flavonoids found in the seedling root differ from those present in the seed coat (Maxwell *et al.*, 1989; Hartwig *et al.*, 1990). Flavonoids released by the plant serve as chemo-attractants and also induce *Rhizobium nod* genes, which are not expressed or expressed at very low levels in free-living rhizobia in the absence of a plant (see references in Long, 1989).

After chemotaxis, rhizobia attach to root hairs all over the root, but the hairs that are the most responsive are those that have recently emerged (Bhuvaneswari, Turgeon & Bauer, 1980). The rhizobia attach to susceptible root hairs via a two-step attachment process (Dazzo *et al.*, 1984; Smit, Kijne & Lugtenberg, 1987). First, they loosely attach to a plant receptor via a protein on the bacterial surface known as rhicadesin (Smit *et al.*, 1987). Rhicadesin is a calcium-binding protein that appears to be common among Rhizobiaceae. Then, tighter adherence occurs either by means of cellulose fibrils (Smit *et al.*, 1987) or fimbriae (Vesper & Bauer, 1986). Often, the rhizobia are seen to attach to the root hair in a polar or end-on fashion. Entry of bacteria appears to occur at the root hair tip, probably because the cell wall is thinner and less cross-linked there than elsewhere. Depending on the host, root hair deformation takes place 6–18 h after inoculation. Susceptible root hairs deform into a number of unusual shapes after inoculation with rhizobia, including corkscrews, branches, twists, and spirals. A few of the deformed root hairs coil 360° and form diagnostic curls known as shepherd's crooks. Root hair deformation is dependent on the presence of functional *Rhizobium nod* genes.

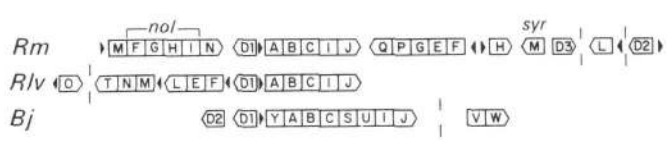


Figure 1. Organization of the *nod* genes in *Rhizobium meliloti* (*Rm*), *R. leguminosarum* bv. *viciae* (*Rlv*), and *Bradyrhizobium japonicum* (*Bj*). With the exception of *nol* (an additional three-letter designation given for nodulation genes beyond the letter 'z') and *syr* (symbiotic regulator), all the letters refer to *nod* genes. The black triangles refer to *nod* boxes, while the broken vertical lines designate large interruptions in the map. The genes are not drawn to scale. Modified from Györgypal *et al.* (1991).

(*b*) Rhizobium *nodulation genes.* In the fast-growing *Rhizobium* species, the *nod* genes are located on a large plasmid, known as pSym. Specific flavones, flavanones, and chalcones are the inducers of *nod* genes in the fast-growing species. In the slow-growing *Bradyrhizobium* species, the *nod* genes are chromosomally borne. Besides flavonoids, a wide range of compounds interact with the *Bradyrhizobium nodD* gene (Györgypal, Kiss & Kondorosi, 1991). Figure 1 illustrates the organization of the *nod* genes in two fast-growing species, *Rhizobium meliloti* and *R. leguminosarum* bv. *viciae*, and in the slow-growing *Bradyrhizobium japonicum*.

The common *nod* genes (*nodABCIJ*) as well as nodulation genes involved in host specificity (*nodFE*, *nodG*, *nodH* and *nodL*) not only play a major role in root hair deformation (*Had*; *ha*ir *d*eformation) and shepherd's crook formation (*Hac*; *ha*ir *c*urling), but also in the initiation of *c*ortical *c*ell *d*ivisions (*Ccd*) which establish the nodule primordium (*Noi*; *no*dule *i*nitiation). The common *nod* genes are so-called because they have been detected in all rhizobia examined so far, and also because *nod* genes of one species, for example, *R. meliloti*, functionally complement comparable genes in other *Rhizobium* species. If any one of the *nodABC* genes is mutated, the ability of *Rhizobium* to deform root hairs and to initiate cortical cell divisions on its host is eliminated (see references in Kondorosi *et al.*, 1991; Long, 1992).

Flavonoids act together with the product of the regulatory gene *nodD*, which is found in all rhizobia that have been analyzed. Although *nodD* is constitutively expressed, the genes of the *nod* operon are normally not expressed if host-derived molecules are absent. To some extent, *nodD* functions in host specificity. Chimeric genes have been constructed

Table 1. *Common and host specificity nodulation genes of* Rhizobium meliloti *and their possible functions*

Gene	Effect of mutant gene on alfalfa	Characteristics	Homology	Representative references
nodD	Nod$^+$; if all 3 copies mutated, then Nod$^-$	Transcriptional regulator	*lysR*	Mulligan & Long (1985); Honma & Ausubel (1987)
		Common *nod* genes		
nodAB	Hac$^-$Nod$^-$	Detected in cytoplasm; *nodA* and *nodB*-encoded factors stimulate cell division	Unknown	Török *et al.* (1984); Eglehoff *et al.* (1985); Schmidt *et al.* (1986); Schmidt *et al.* (1988)
nodC	Hac$^-$Nod$^-$	Localized to bacterial membrane	Chitin synthase	John *et al.* (1985); Johnson *et al.* (1989); Atkinson & Long, pers. comm.; Debellé, Rosenberg & Dénarié, pers. comm.
nodI	Increase in Hac and Inf, but Nod^d	Presumed to be in *R. meliloti*, but not yet completely characterized; detected in cytoplasmic membrane of *R.l.* bv. *viciae*	ATP binding protein involved in transport	Higgins *et al.* (1986); Evans & Downie (1986); Schlaman *et al.* (1990)
nodJ	See *nodI*	See *nodI*; membrane protein; may function together with *nodI*	Unknown	Evans & Downie (1986)
		Host specificity *nod* genes		
nodF	Nod^d; affects infection thread formation	Localized to cytoplasm	Similar to acyl carrier protein	Debellé *et al.* (1986); Shearman *et al.* (1986); Spaink *et al.* (1991*a*)
nodE	Nod^d; change in host range	Cytoplasmic membrane protein	*fabB*	Horvath *et al.* (1986); Bibb *et al.* (1989)
nodG	Nod^d		Sequence similar to ribitol or glucose dehydrogenase	Debellé *et al.* (1986); Horvath *et al.* (1986)
nodH	Nod$^-$; change in host range to vetch	Localized to the bacterial membrane	Sulphotransferase	Kondorosi *et al.* (1984); Faucher *et al.* (1989); Roche *et al.* (1991); Schmidt *et al.* (1991)
nodL	Nod$^-$ or Nod^d in *R. leguminosarum* bv. *viciae* and *trifolii*	Predicted to be localized to the cytoplasmic membrane	*lacA*	Downie (1989)
nodPQ	Nod^d	ATP sulfurylase activity	*cysDNC*	Cervantes *et al.* (1989); Schwedock & Long (1990); Fisher & Long (1992)
nodM	Nod^d	Glucosamine synthetase activity	*glmS*	Baev *et al.* (1991)

from *nodD* genes of rhizobia that nodulate different hosts. These studies have shown that the C-terminal end of the NodD protein determines flavonoid specificity, while the N-terminal region is involved in binding to regions of DNA known as *nod* boxes (Horvath *et al.*, 1987; Spaink *et al.*, 1987). The *nod* box is a highly conserved 47 bp long, *cis* regulatory region found in the promoters of *nod* operons (Rostas *et al.*, 1986) (Fig. 1). Although it is not exactly known how flavonoids interact with NodD, it is thought that the protein binds to the *nod* box more tightly after interaction with the correct flavonoid (Györgypal *et al.*, 1991).

Other *nod* genes also mediate host specificity. Mutations in *nodH* enable *R. meliloti* to deform root hairs of white clover and vetch, species not normally compatible with that *Rhizobium* (Faucher *et al.*, 1988). The host specificity *nod* genes are not functionally conserved among the various *Rhizobium* species: they cannot be genetically complemented by genes from other species. Host specific *nod* genes are also induced by plant-derived molecules. Table 1 summarizes the proposed functions of the *nod* genes in *Rhizobium meliloti* and the effects of *R. meliloti* with specific *nod* gene mutations on alfalfa (*Medicago sativa* L.).

(*c*) *The primary signal for nodule morphogenesis comes from* Rhizobium. Both the common and host specificity *nod* genes are involved in the production of a

factor, identified as a lipo-oligosaccharide (glycolipid), that causes root hair deformation and cortical cell divisions in a compatible host. The chemical structure of the root hair deformation factor of *R. meliloti* (designated NodRm-1) was the first to be identified (Lerouge *et al.*, 1990). NodRm-1 is a sulphated β-1,4-tetra-D-glucosamine with three acetylated amino groups. A C16 unsaturated fatty acid occupies the non-reducing end of the molecule, while the reducing end contains a sulphate group.

Recently, a uniform method for naming Nod factors has been proposed (Roche *et al.*, 1991). The naming is based on the various substitutions present on the glucosamine backbone. For example, NodRm-1 is now described as NodRm-IV(S) – Rm signifies *R. meliloti*, IV the four glucosamine residues, and S the sulphate on the reducing end of the molecule. *R. leguminosarum* bv. *viciae* Nod factors, which are either tetra- or pentaglucosamines, are acetylated and lack a sulphate group. They are designated Rlv-IV(Ac) or Rlv-V(Ac).

Other lipo-oligosaccharides besides NodRm-1 have been recently identified from *R. meliloti*. Truchet *et al.* (1991) have found a NodRm-1-like factor with an O-acetyl group at carbon 6 of the terminal sugar at the nonreducing end (Fig. 2). Another factor, designated NodRm-2 is structurally related to NodRm-1, but lacks the sulphate group (Lerouge *et al.*, 1990). Other Nod factor molecules contain five instead of four glucosamine residues (Schultze *et al.*, 1992; E. M. Atkinson & S. R. Long, personal communication). In addition, a trisaccharide-containing Nod factor has been isolated from *R. meliloti* (Schultze *et al.*, 1992).

The synthesis of the Nod factors is mediated by specific *nod* genes (Fig. 2). For example, *nodC*, which has been shown to be similar in DNA sequence to yeast chitin synthase (E. M. Atkinson & S. R. Long, personal communication; F. Debellé, C. Rosenberg & J. Dénarié, personal communication), may be involved with linking individual glucosamine units synthesized by the activity of *nodM*. The DNA sequence of *nodM* is homologous to the *glmS* gene in *E. coli* which encodes D-glucosamine synthetase (Baev *et al.*, 1991). The *nodL* gene product is thought to acetylate the glucosamine residues (Downie, 1989) while *nodFE*-encoded proteins are involved in synthesizing the fatty acid side-chain (Horvath *et al.*, 1986; Shearman *et al.*, 1986) (Fig. 2).

When purified *R. meliloti* Nod factor is applied to roots of its host alfalfa, it elicits root hair deformation at 10^{-11} M, while at 10^{-7} M, it stimulates cortical cell divisions in alfalfa roots (Truchet *et al.*, 1991). Non-host plants, such as vetch (*Vicia sativa* L.), do not respond to the addition of NodRm-1 at these concentrations. However, when *R. meliloti* Nod factor lacking the sulphate group (NodRm-2) is applied to *V. sativa* (Lerouge *et al.*, 1990) or when pentasaccharide-containing Nod factor is supplied at

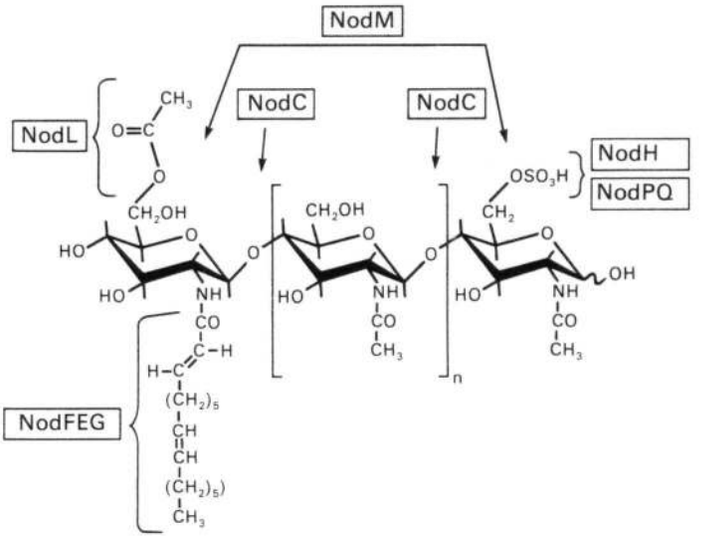


Figure 2. Generalized structure of a Nod factor; n refers to the number of glucosamine residues. The lipid moiety shown here is typical of Nod factor secreted by *Rhizobium meliloti*. The proposed roles of the *nod* gene products in the synthesis of Nod factor are indicated by the arrows (see text for details). NodL is proposed to add an acetyl group to NodRm-1. Modified from de Bruijn & Downie (1991).

10^{-8} to 10^{-9} M (Schultze *et al.*, 1992), root hair deformation takes place on vetch. A non-sulphated Nod factor is produced by either *nodQ* or *nodH* mutants of *R. meliloti*. *nodH* is homologous to a sulphotransferase (Roche *et al.*, 1991), while Schwedock & Long (1990) have shown that in *R. meliloti*, *nodP* and *Q* specify ATP sulphurylase activity. The presence of the sulphate group appears to be critical for recognition by an alfalfa receptor.

Recently, lipo-oligosaccharides from other *Rhizobium* species have been chemically characterized (Spaink *et al.*, 1991*a*, *b*). In the case of the *R. leguminosarum* Nod factors, of which there are at least five, the most obvious difference from the *R. meliloti* Nod factors is the lack of a sulphate group. Genes homologous to *nodH* and *nodPQ* do not appear to be present in *R. leguminosarum* bv. *viciae*. Other differences between NodRm-1 and the *R. leguminosarum* bv. *viciae* Nod factor are the length of the fatty acid side chain and its extent of unsaturation (Spaink *et al.*, 1991*b*). NodRm-1 contains 16 carbons and two double bonds while the *R. leguminosarum* bv. *viciae* Nod factor has 18 carbons and four double bonds. Mutations in *nodE*, a gene with sequence similarity to *fabB*, a gene in *E. coli* that encodes an enzyme for fatty acid synthesis (Bibb *et al.*, 1989), results in a *R. leguminosarum* bv. *viciae* Nod factor that induces root hair deformation, but which no longer elicits cortical cell divisions in its own host. The lipid tail of this Nod factor contains one instead of four double bonds (Spaink *et al.*, 1991*b*). Another difference from NodRm-1 is the presence of an O-acetyl group on the non-reducing end of the *R. leguminosarum* bv. *viciae* Nod factor (Spaink *et al.*, 1991*b*) although, as mentioned

earlier, NodRm-1 is sometimes acetylated in this position (Truchet *et al.*, 1991). *R. leguminosarum* bv. *viciae nodL* mutants make a Nod factor that lacks the O-acetyl group.

The slow-growing *Bradyrhizobium* species also secrete Nod factors with a glucosamine backbone. However, the various substitutions on the backbone differ. For example a 5-*o*-methyl fucose substituent is present on the reducing end (G. Stacey, personal communication).

(*d*) *The nod factor-receptor model for rhizobial invasion.* How does Nod factor induce root hair deformation and cortical cell divisions? The following model is proposed: (1) the *N*-glucosamine residues of the Nod factor react with a sugar-binding site of a receptor, presumably a lectin; and (2) the strength of the interaction between Nod factor and its receptor regulates early events in nodulation. The strength of the interaction between Nod factor and receptor depends on several properties: the length of the glucosamine backbone, the presence or absence of various substituents like sulphate, and the composition of the lipid side chain. The extent of unsaturation, as well as the number of carbons in the fatty acid, are proposed to influence the mobility and orientation of the glucosamine residues. However, the lipid itself does not bind to the plant receptor.

Although Nod factor is secreted into the medium by rhizobia, the Nod factor is proposed to function *in situ* as part of the bacterial membrane with the lipid moiety inserted into the membrane. Two pieces of information support this proposal: (1) root hair curling factors added to Nod$^-$ bacteria do not restore the wild-type conditions (Banfalvi & Kondorosi, 1989; A. Hirsch, unpublished results), and (2) the molecular structure of the Nod factors suggests a membrane location. The failure of secreted Nod factor molecules to complement Nod$^-$ *Rhizobium* may indicate that either (1) a soluble form of the factor is insufficient by itself, or (2) an exact orientation of the Nod factor is essential for the full response of the plant. The length of the NodRm-1 lipid moiety is estimated to be 2 nm, which is approximately equivalent to one-half of a lipid bilayer, strongly suggesting that the lipid tail of the Nod factor is embedded in the rhizobial membrane. Thus, the polar glucosamine headgroup should extend either intra- or extracellularly from the lipid bilayer. Because the Nod factor is a signal molecule, the glucosamines are assumed to extend extracellularly.

Receptor molecules that bind the lipo-oligosaccharide are presumed to be present on the root hairs. The chemical nature of the receptor molecule is so far unknown, but it has been postulated to be a lectin (Lugtenberg *et al.*, 1991). Previously, lectins were thought to play a major role in specific attachment of *Rhizobium* to its host. Bohlool & Schmidt (1974) and Dazzo & Hubbell (1975) proposed the lectin recognition hypothesis, which stated that lectins with unique sugar-binding properties would interact with specific saccharides on the rhizobial surface. Lectins are attractive candidates for receptors also because some are located in the region of the root that is most susceptible to *Rhizobium* infection (Díaz *et al.*, 1986). There have been numerous studies on the role of lectins in attachment, but until now their role in host recognition remains elusive and controversial. Recently, Kijne *et al.* (1986) proposed that lectins are more likely to be involved in invasion rather than attachment of rhizobia. The reader is referred to reviews by Kijne (1992) and Roth & Stacey (1991) for descriptions of some of the previous lectin research and for more recent views on the lectin hypothesis.

The best genetic evidence for the involvement of lectin in host specificity has been presented by Díaz *et al.* (1989), who made transgenic plants by introducing the pea lectin gene into clover (*Trifolium repens* L.) via *Agrobacterium rhizogenes*. Normally, *R. leguminosarum* bv. *viciae* does not infect or nodulate clover, although this strain curls clover root hairs (Yao & Vincent, 1969). When transformed hairy roots of clover were inoculated with *R. leguminosarum* bv. *viciae*, some of the roots developed red, N_2-fixing nodules, implying that the presence of the pea lectin gene led to a change in host range.

It has been suggested that a lectin is unlikely to function as a receptor for NodRm-1 or any other Nod factors because such proteins lack a transmembrane domain, a feature essential for eukaryotic receptor proteins (Alberts *et al.*, 1989). However, lectins, which are multivalent, are likely to be part of a receptor complex. Another possibility is that lectins could interact with transmembrane proteins in the root hair membrane once Nod factors were bound. Some lectins have protein-binding as well as sugar-binding domains (Barondes, 1988). Alternatively, glycoproteins other than lectins may be involved. Whatever the receptor is, it must be similar to lectin in being produced by root hairs that are susceptible to infection and in exhibiting specificity differences according to cross inoculation groups (see references in Kijne, 1992).

Figure 3 illustrates the key points of a Nod factor-receptor model for *Rhizobium* invasion. The receptor proteins are assumed to be equally distributed on the plasma membrane at the growing end of the root hair. Several studies have shown that lectins are localized at the tips of growing root hairs and thus fulfill this criterion (Dazzo, Yanke & Brill, 1978; Law & Strijdom, 1984). Rhizobia attach to the thin, unpolymerized cell wall at the tip of the emerging root hair. Receptor molecules, which can diffuse laterally in the plane of the root hair membrane, bind

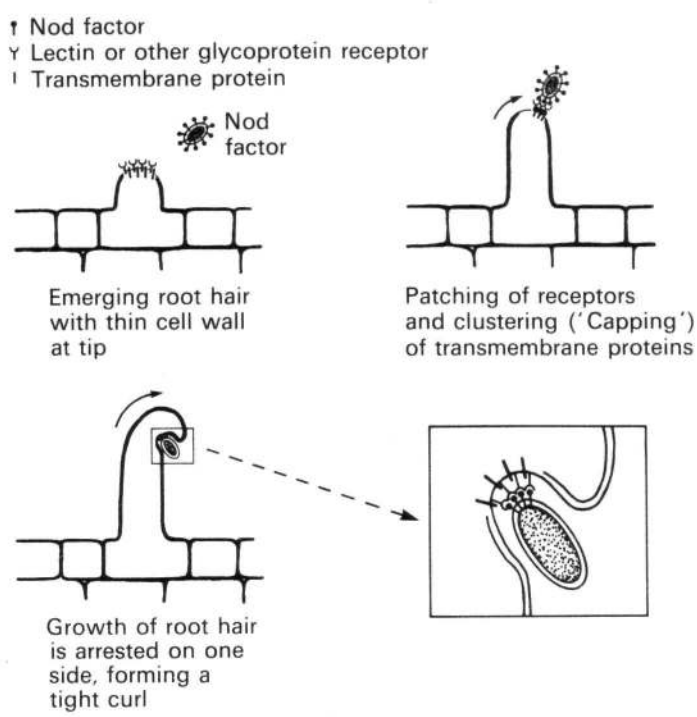


Figure 3. The Nod factor-receptor model for rhizobial invasion (see text for details).

Nod factor on the *Rhizobium* membrane. Because rhizobia are attached to specific points on the root hair membrane, the receptor molecules patch and form a 'cap', as has been postulated for antibodies binding to specific proteins on the plasma membrane (Alberts *et al.*, 1989). As the receptors form the cap, transmembrane proteins attached to the lectins, also 'cap' in the membrane. Receptor-mediated endocytosis, which is the basis of infection thread formation, then follows. Coated vesicles, typically found in cells undergoing receptor-mediated endocytosis, are associated with infection thread penetration (Robertson & Lyttleton, 1982). After the rhizobia are taken in, a number of subcellular changes take place within the root hair cell, including changes in membrane permeability and cytoskeletal arrangement, each of which may be part of or may generate a 'second signal' for inducing cortical cell divisions as postulated by Long & Cooper (1988). The second signal could also be independent of receptor-mediated endocytosis and may be propagated to cortical cells via other molecules that stimulate cell division (see Section IV).

As a consequence of 'capping' at the infection site, a change in growth direction at the point of rhizobial attachment occurs such that the root hair cell membrane invaginates to form the infection thread. The rest of the root hair continues to elongate, eventually growing around the infection site. Computer simulations by van Batenberg, Jonker & Kijne (1986) suggest that rhizobial attachment elicits an increase in the rate of plant cell wall expansion on the opposite side. The physical attachment of living rhizobia is required for 'tight' or 360° curling of the root hair. When purified Nod factor is added in solution, root hair deformation occurs, but no shepherd's crooks are formed. This argues that tight curling sets up a focused site (a specifically sized cap) for the initiation of the infection thread and subsequent *Rhizobium* invasion. So far, no soluble factors have been shown to induce infection thread formation, thus rhizobia are required for this step.

Differences in plant response to the amount and molecular structure of purified Nod factor are also explained by the model presented in Figure 3. As mentioned earlier, 10^{-11} M NodRm-1 in aqueous solution triggers root hair deformation, while a concentration three orders of magnitude greater (10^{-7} M) is required for the induction of cortical cell divisions. When NodRm-1 is dissolved in water, it probably forms micelles with the glucosamine residues on the outside. The micelles are likely to be of different sizes, especially if detergent is used to solubilize the Nod factor. In any case, the micelles can interact with receptors on the plasma membrane because the glucosamine backbone is exposed. There are several ways that the concentration of Nod factor could influence the plant's response.

One possibility is that there are two distinct receptors, each of which has a different affinity for NodRm-1, i.e. one for low and another for high concentrations of the molecule (10^{-11} M *vs.* 10^{-7} M). Alternatively, there may be one receptor solely for the root hair deformation response and another one for cell division. The simplest explanation, however, is that a different plant response occurs depending on the degree of receptor cross-linking. Root hair deformation, which is elicited by 10^{-11} M Nod factor, requires a minimum number of receptors cross-linked, while cortical cell divisions (10^{-7} M Nod factor is needed) require a larger number of receptors cross-linked, i.e. a large cap. The small cap may influence cytoskeletal arrangement thereby allowing root hair deformation to occur, while the large cap generates a second signal, which in turn triggers cell division.

The model also explains the effect on legume roots of Nod factors with alterations in the length or degree of unsaturation of the fatty acid chain. Nod factor from *R. leguminosarum* bv. *viciae nodE* mutants, which produce a more saturated fatty acid, or from *R. meliloti nodH* or *nodPQ* mutants, which has 16 carbons in its fatty acid tail but no sulphate on the glucosamine backbone, elicits root hair deformation, but no cortical cell divisions on vetch (Lerouge *et al.*, 1990; Spaink *et al.*, 1991*b*). It is likely that changes in the fatty acid chain result in an imprecise orientation of the glucosamine residues so that only a small number of receptors are able to bind the Nod factor and become cross-linked. On this basis, cortical cell divisions, which require a greater degree of receptor cross-linking in order to generate a second signal, do not take place. A minimum

number of receptor molecules might have to be cross-linked to generate the physiological responses of root hair deformation, cortical cell division, and infection thread formation, the latter response occurring only when *Rhizobium* are present. If Nod factor results in a below-threshold cross-linking of receptors, the second signal is not generated and only the initial plant response, root hair deformation, takes place.

Support for the model should come from studies of plant mutants in which root hair deformation, cortical cell divisions, and infection thread formation are uncoupled. Caetano-Anollés & Gresshoff (1991) have prepared a list of symbiotic legume mutants, a number of which are non-nodulating. Recently, Miller, Viands & LaRue (1991) have described five loci in non-nodulating *Melilotus albus* Desr. mutants. In some of these mutants, root hair deformations occur, but no subepidermal cell divisions take place in response to inoculation with *R. meliloti* (J. H. Norris, personal communication). Similarly, two soybean (*Glycine max* L. Merr.) mutants exhibit an uncoupling of root hair deformation and cortical cell divisions (Carroll, McNeil & Gresshoff, 1986). Other interesting mutants to study are temperature sensitive mutants where nodulation occurs at one temperature and not at another (Fearn & LaRue, 1991*a*). Temperature may affect either lipid phase transition or cytoskeletal components as well as the rates of specific enzymes involved in nodule formation. LaRue and colleagues (personal communication) have found that ethylene production increases if pea roots are warmer.

The test of whether lectins are the receptor for Nod factors will occur if plant mutants with defective root lectin are found, and these have altered host specificity or are defective in nodulation. In addition, the above model should be applicable to those plants where root hair curling does not take place, as in peanut and *Stylosanthes* as well as the nonlegume *Parasponia*. In these plants, rhizobia enter the roots between epidermal cells where lateral roots emerge (see references in Dart, 1977; Torrey, 1986). The rhizobia that infect these plants carry the common *nod* genes, and it is assumed that the Nod factor produced following the induction of these genes will be structurally related to NodRm-1. The Nod factor of *Rhizobium* species NGR234, which nodulates at least 35 different genera of legumes as well as the nonlegume *Parasponia*, is chemically related to NodRm-1 (Broughton *et al.*, 1991). However, it is not known whether rhizobial *nod* gene products are required for non-root hair cell invasion or whether specific receptor molecules are localized on the root at the invasion sites. Too little information is available regarding these less typical *Rhizobium*–host plant symbioses.

(*e*) Rhizobium *cell surfaces*. How is Nod factor related to the lipopolysaccharides (LPS), exopolysaccharides (EPS) and capsular polysaccharides (CPS) that are associated with rhizobial surfaces? The relationship of these cell surface components and Nod factor remains undefined. *R. meliloti exo* mutants, which induce small, white nodules on alfalfa (Finan *et al.*, 1985; Leigh *et al.*, 1987), have functioning *nod* genes (Klein, Walker & Signer, 1988). They elicit root hair cell deformation and can initiate infection thread formation. However, the threads abort in the peripheral cells of the bacteria-free, 'empty' nodule (Finan *et al.*, 1985). Numerous, small vesicles have been observed adjacent to the outer membrane of *R. meliloti exo* mutants within the aborted infection thread (Yang, Signer & Hirsch, 1992). These are not observed in Exo^+ bacteria, either because the vesicles are normally masked by the EPS of the wildtype *R. meliloti* or because they are produced only by Exo^- bacteria. It is not known whether these vesicles are related to the production of exopolysaccharides or other factors.

(*f*) *Other factors*. Several factors, exclusive of the lipo-oligosaccharides just described, influence root hair proliferation, branching or deformation, cortical cell divisions or cause a phenotype known as the thick, short root response (Tsr) (van Brussel *et al.*, 1986). The chemical structures of some have been elucidated. Dazzo and colleagues have identified a number of bacterial factors (BF) that are produced by *R. leguminosarum* bv. *trifolii*. Some promote root hair proliferation or work synergistically to elicit hair deformation and cortical cell divisions on clover (Hollingsworth, Philip-Hollingsworth & Dazzo, 1990). One of these factors, BF-5, which is dependent on *nod* gene induction by flavonoids, has been identified as *N*-acetylglutamic acid (Philip-Hollingsworth, Hollingsworth & Dazzo, 1991). BF-5, when added to clover roots, causes root hair branching and tip swelling. It also increases the number of foci of cortical cell divisions. BF-5 does not elicit these responses on alfalfa or *Lotus* (Philip-Hollingsworth *et al.*, 1991).

A factor produced by *R. meliloti* competes with radioactive NPA (*N*-1-(naphthyl)pthalamic acid) for its binding site (A. M. Hirsch, H. I. McKhann & M. Jacobs, unpublished results). NPA, an auxin transport inhibitor, elicits the formation of nodule-like structures on alfalfa roots; these nodule-like structures contain transcripts for early nodulin genes (Hirsch *et al.*, 1989). This *Rhizobium*-derived factor, which has not yet been identified, is produced by Nod^- as well as Nod^+ strains of *R. meliloti*. However, luteolin is required for secretion of the NPA-competing factor. Alfalfa roots develop a short root phenotype when treated with a partially purified culture filtrate from *R. meliloti* that contains this factor (A. Hirsch, unpublished results). However, it is not known whether the genes required for

production of the factor are essential for the symbiosis.

2. *Root hair responses*

Inoculation with wild-type *Rhizobium* brings about expression in the root hairs of genes that are not expressed in untreated roots. The products of these genes have been called 'hadulins' by some workers and are detected as proteins whose presence differs in inoculated *vs.* uninoculated roots.

Twelve symbiosis-specific proteins have been identified by two-dimensional electrophoresis in root hairs of cowpea (*Vigna unguiculata* L. Walp.) inoculated with the broad host range *Rhizobium* sp. NGR234 (Krause & Broughton, 1992). Three proteins (15, 31 and 33 kDa) are transiently expressed. Five proteins, including the 15 and 31 kDa proteins, were detected 24 h after inoculation. Surprisingly, the 15 kDa protein is not induced by *R. fredii* USDA 25751, a *Rhizobium* strain that also induces N_2-fixing nodules on *V. unguiculata*. Thus, the role of the 15 kDa protein relative to symbiosis is unclear.

In pea (*Pisum sativum* L.), several *in vitro* translation products from root hair mRNA have been identified. RH-44 is present in uninoculated plants at low levels, but is inoculation-enhanced. On the other hand, RH-42 is induced in root hairs following infection with *R. leguminosarum* bv. *viciae* (Gloudemans *et al.*, 1989). In addition, the early nodulins PsENOD12 and PsENOD5 are expressed in pea root hairs that have been inoculated with *R. leguminosarum* bv. *viciae* or treated with soluble root hair deformation factors (see references in Franssen *et al.*, 1992).

In summary, soon after the initial interaction between *Rhizobium* and host, detectable biochemical changes are occurring within root hairs, at least in the two plant systems studied (pea and cowpea).

3. *Infection*

(*a*) *Role of* Rhizobium *genes*. After inducing shepherd's crook formation, the rhizobia penetrate the root hair cell by means of an infection thread. *Rhizobium* genes, including *ndv*, *lps*, and *exo*, appear to be essential for successful infection thread formation. *R. meliloti ndv* and *exo* mutants induce bacteria-free, 'empty' nodules on alfalfa. The *exo* mutants fail to synthesize acidic EPS (Finan *et al.*, 1985), while *ndv* mutants are unable to make β-1,2 glucans (Dylan *et al.*, 1986). The *ndv* mutants also show decreased motility and increased phage sensitivity. The *ndvA* and *ndvB* genes are homologous to *chvA* and *chvB* of *Agrobacterium tumefaciens* (Dylan *et al.*, 1986). Recently, *ndv*-caused defects in motility have been suppressed by isolating second-site pseudorevertants. However, these bacteria are not completely restored to symbiotic competence. On the other hand, *ndv* pseudorevertants selected for their ability to induce normal N_2-fixing nodules on alfalfa, do not recover the ability to synthesize β-1,2 glucan (Dylan *et al.*, 1990). Thus, the effects of the *ndv* locus on nodule development, particularly with regard to facilitating rhizobial entry into plant tissues, are unclear.

(*b*) exo *and* lps *mutants*. Djordevic *et al.* (1987) showed that high-molecular-weight EPS could restore symbiotic function to Exo$^-$ bacteria of *R. leguminosarum* bv. *trifolii*. In contrast, high-molecular-weight EPS does not restore symbiotic effectiveness to *R. meliloti exo* mutants. Battisti *et al.* (1992) have found that normal N_2-fixing nodules which contain typical infection threads and bacteroids develop if low molecular weight EPS is added to *exoA* mutants. An interesting finding is that only low molecular weight EPS from *R. meliloti* is effective. EPS from heterologous species does not promote invasion, implying that there is some type of molecular specificity in host plant recognition of rhizobial EPS. It is not known how low molecular weight EPS serves as a signal molecule for host plant infection.

Several recent studies indicate that rhizobial acidic EPS is essential for the establishment of N_2 fixation in indeterminate nodules, e.g. alfalfa or pea, but is not as important for the formation of determinate nodules, e.g. *Phaseolus* or *Lotus* (see Section III). Like *R. meliloti exo* mutants, *R. leguminosarum* bv. *viciae* or bv. *trifolii* with defective EPS do not establish normal symbiotic associations with their preferred host, i.e. pea (Borthakur *et al.*, 1986) or clover (Chakravorty *et al.*, 1982). In contrast, normal N_2-fixing nodules are formed on soybean, bean, or *Lotus* after inoculation with EPS mutants of *R. fredii*, *R. leguminosarum* bv. *phaseoli*, or *R. loti*, respectively (Borthakur *et al.*, 1986; Kim, Tully & Kiester, 1989; Hotter & Scott, 1991).

Defects in lipopolysaccharide (LPS) synthesis in *Rhizobium* species also affect nodule development. Although *R. meliloti* that are deficient in LPS induce N_2-fixing nodules on alfalfa (Clover, Kieber & Signer, 1989), *lps* mutants of *R. leguminosarum* bv. *viciae* elicit nodules on pea or vetch, which, while appearing to be normal overall, are Fix$^-$ because the rhizobia are not released from the infection threads (Priefer, 1989; Brewin *et al.*, 1990). In contrast, rhizobial LPS defects are very pronounced on a host that forms determinate nodules. For example, *R. leguminosarum* bv. *phaseoli* with defective LPS elicits the formation on bean (*Phaseolus vulgaris* L.) of small, bump-like nodules, devoid of bacteria. Infection threads abort within the root hairs (Noel, VandenBosch & Kulpaca, 1986; Diebold & Noel, 1989).

The reason for the different responses to *exo* or *lps* mutants in the two nodule types (determinate *vs.*

indeterminate) has not yet been explained. Each nodule type has a different mode of invasion, e.g. infection threads travel a longer distance in indeterminate nodules, infection threads are broader in indeterminate vs. determinate nodules, etc. Reviews by Brewin (1991) and Kijne (1992) discuss some of the possibilities. In addition, unlike determinate nodules, a persistent nodule meristem is initiated secondarily in indeterminate nodules, after the nodule primordium has already formed (see Section III). The situation is further complicated in *R. meliloti* by a second polysaccharide EPSII (also known as EPSb) which can function in place of missing acidic EPS (EPSI) in *R. meliloti* strain 1021 (Glazebrook & Walker, 1989; Zhan *et al.*, 1989). Furthermore, modified LPS, a product of the *lpsZ* gene of *R. meliloti* strain 41, can substitute for absent acidic EPS (Williams *et al.*, 1990).

In summary, the *Rhizobium* cell membrane and cell surface components encoded by *lps*, *exo* and *ndv* are in some way involved with the entry of rhizobia into plant cells. If or how Nod factor interacts with these rhizobial surface components is not known. Moreover, there are significant differences in the way a particular host plant responds to infection with *exo* or *lps* mutants and the importance of this interaction with regard to bacterial-produced signals also remains undiscovered. For more details about the roles of exopolysaccharide in invasion, the reader is referred to Gray & Rolfe (1990).

(*c*) *Infection thread formation.* Several studies have shown that the infection thread is a continuation of the plant cell wall. In their electron microscopic study, Callaham & Torrey (1981) postulated that rhizobia cause the dissolution of the plant cell wall at a specific point, while others (Nutman, 1956) proposed that the infection thread forms via a process of cell wall invagination (see references in Pueppke, 1986). Recent studies by Bakhuizen (1988) support Callaham and Torrey's observations.

A hyaline spot is usually the first sign of infection thread penetration. Marked cytoplasmic streaming occurs in response to the attachment of rhizobia to the root hair. The nucleus migrates towards the refractile spot. Following dissolution of the cell wall, the plasma membrane of the root hair invaginates and cell wall material is deposited around it and the rhizobia within. The invagination with the newly formed cell wall forms the infection thread (see references in Kijne, 1992). The host cell nucleus, attached by microtubules to the infection thread tip, precedes the infection thread as it passes through the root hair cell (Lloyd *et al.*, 1987; Bakhuizen, 1988). The bacteria travel from host cell to host cell via the infection thread and its branches.

Although numerous root hairs deform, very few of them form *bona fide* shepherd's crooks. Moreover, infection thread formation and penetration are rare events. From their studies of alfalfa, Wood & Newcomb (1989) found 52 infection threads in ten different seedlings; two were initiated in branched hairs, 17 in intertwined hairs, and 33 in shepherd's crooks. The ten seedlings were estimated to have more than 80000 root hairs among them. Surprisingly, only 27 root nodules were formed. The frequency of infection events that actually result in the formation of N_2-fixing nodules in alfalfa is thus very low. The reasons for this low rate of infection success are unknown. Infection threads often abort in the root hair cells, although some penetrate into the root cortical cells (Bond, 1948; Libbenga & Harkes, 1973; Dart, 1977).

Several studies have suggested that infection thread abortion is associated with a hypersensitive (HR)-like response by the plant. Recently, after studying aborted infection threads in alfalfa cv. 'Gemini' after inoculation with wild-type *R. meliloti*, G. Truchet (personal communication) observed that the threads terminate their growth within a cell that is usually positioned in the mid-cortex. By immunolocalization, this cell is observed to contain hydroxyproline-rich glycoproteins as well as several enzymes, including phenylalanine ammonia lyase and chalcone synthase, two key enzymes of the phenylpropanoid biosynthetic pathway, the pathway for phytoalexin production.

Other studies with *exo* mutants of *R. meliloti* by Pühler *et al.* (1991) have indicated that cells containing aborted infection threads, and adjacent cells, autofluoresce, indicating the presence of phenolic compounds in their cell walls. Phenolic accumulation in cell walls usually foreshadows the lignification of the responding cells. Lignification as well as phytoalexin accumulation are symptoms of an HR.

In summary, infection involves the continued interplay between the two symbiotic partners; it is at this point that host specificity is likely to be determined. Infection is a multi-stepped process which fails more frequently than it succeeds. Although much is known from a descriptive standpoint, very little is known of the mechanism of infection thread formation and elongation. For more details about the infection process in legumes, the reader is referred to the review by Kijne (1992).

III. NODULE INITIATION

1. *Types of nodules*

After the preinfection stages, cortical cell divisions take place several cells distant from the advancing infection thread. The location of the initial cell divisions presage the type of nodule that forms. Cell divisions occur either in the outer or inner cortex of the root. Unlike the divisions involved in lateral root formation, the cell divisions that initiate nodule formation are initially anticlinal.

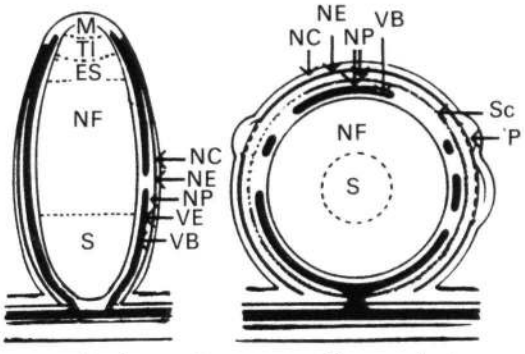
The type of nodule that develops depends on the host plant, not on the rhizobial strain (Dart, 1977; Newcomb, 1981). There are two major types of nodules that are found on the roots of herbaceous legumes: they are distinguished from one other in a number of ways. The indeterminate type is characterized by a persistent nodule meristem, while the determinate nodule type lacks such a meristem. In this review, the term 'meristem' is reserved for a group of cells that, by mitosis, give rise to derivatives, some of which differentiate into specific cell types while others (initials) remain as part of the meristem. 'Meristem' thus differs from 'meristematic region' which refers to an area or zone of cells that actively divide for awhile and subsequently differentiate. Thus, determinate nodules lack a meristem: a self-perpetuating assembly of dividing cells is not initiated.

The persistent meristem causes indeterminate nodules to be elongate and club-shaped because new cells are constantly being added to the distal end of the nodule. All stages of nodule development are represented in one nodule because an age gradient occurs from the distal meristem to the proximal point of attachment to the parent root. Plants having indeterminate nodules include clover, alfalfa, vetch, and pea. In contrast, determinate nodules are spherical. Cell divisions cease early during nodule development and the final form of the nodule results from cell enlargement rather than cell division. Nodules of soybean, mungbean, or common bean are examples of determinate nodules.

As discussed earlier, another difference between legumes with either determinate or indeterminate nodules is their response to *Rhizobium exo* or *lps* mutants. Like *R. meliloti nif* or *fix* mutants, *exo* mutants elicit ineffective nodules on alfalfa. However, the nodules induced by *nif* or *fix* mutants, have normal, distally localized nodule meristems (Hirsch, Bang & Ausubel, 1983; Hirsch & Smith, 1987). In alfalfa cv. 'Iroquois', a persistent nodule meristem is not initiated after inoculation with *R. meliloti exo* mutants (C. Yang *et al.*, 1992). Nodules induced by *exo* mutants of *R. meliloti* are broadly based; a region of diffuse meristematic activity is positioned at their distal ends. They lack the typical cylindrical shape of an alfalfa nodule with its focused meristem. Whether the lack of meristem persistence represents an additional hypersensitive-like response, besides infection thread abortion and phenolic accumulation, is currently unclear.

The salient features of the two nodule types are summarized in Table 2.

Table 2. *Major differences between indeterminate and determinate nodules (modified from Sutton, 1983)*



	Indeterminate	Determinate
Site of initial cell divisions	Inner cortex	Outer cortex
Nodule growth	Cell division; persistent meristem	Cell expansion
Effect of *exo* mutant	Fix$^-$, 'empty' nodules	Fix$^+$, normal nodules
Effect of *lps* mutant	Fix$^-$ or $^+$, normal in overall appearance	Fix$^-$, abnormal nodules
Infection thread	Broad	Narrow
Origin	Temperate regions	Subtropical and tropical regions
Transport	Amides	Ureides
nod gene inducers	Flavones, flavanones	Isoflavones, mainly
Examples	Alfalfa, pea, vetch	Soybean, bean, *Lotus*

M, meristem; TI, thread invasion zone; ES; early symbiotic zone; NF, nitrogen-fixing zone; S, senescent zone; NC, nodule cortex; NE, nodule endodermis; NP, nodule parenchyma; VE, vascular endodermis; VB, vascular bundle; Sc, sclerenchyma; P, periderm.

2. *Indeterminate nodules*

To initiate an indeterminate nodule, anticlinal cell divisions take place in the inner cortex of the root (Libbenga & Harkes, 1973; Newcomb, Sippel & Peterson, 1979). The dividing cortical cells are separated from the root hair containing an infection thread by several cell layers. The derivatives of the inner cortical cells eventually form the nodule primordium. In pea, although anticlinal cell divisions initiate the formation of the nodule, cell divisions also take place in other planes, resulting in the formation of 'multicellular colonies' within the confines of the original cortical cell. These multicellular packets of cells are not separated by intercellular spaces as are the original root cortical cells (Newcomb *et al.*, 1979). The initial cell divisions usually occur opposite a protoxylem point, suggesting that factors (hormones or others?) moving in the stele influence the site of nodule formation (Libbenga *et al.*, 1973). Díaz *et al.* (1986) have also found that lectin is concentrated on the surface of root hairs opposite protoxylem points.

As cortical cell divisions ensue, the infection thread elongates, leaves the root hair cell, and

penetrates the highly vacuolated cells of the root cortex. In preparation for infection thread penetration, a cytoplasmic bridge forms in the outer cortical cells (Bakhuzien, 1988). Radially orientated cytoplasmic strands fuse into a centrally positioned bridge structure which is in the path of the advancing infection thread. The infection thread grows through these cells towards the inner cortical derivatives which constitute the nodule primordium. Eventually, branches of the infection thread invade cells of the nodule primordium; these cells stop dividing and begin differentiating (Newcomb *et al.*, 1979). Mitoses in cells adjacent to the nodule primordium, near the middle of the root cortex, generate the nodule meristem. The nodule meristem gives rise to all of the tissues of the nodule, except the cells of the nodule cortex and the tissues at the base of the nodule. The nodule cortex consists of cells that are distal to the nodule meristem and external to the nodule endodermis. They are highly vacuolated and separated from each other by intercellular spaces. The nodule cortex results from enlargement as well as division of the cells of the outer cortical layers of the root (Bond, 1948). Some of the tissues at the base of the nodule are derived from divisions of the pericycle and associated tissues. The root endodermal cells divide tangentially and extend into the developing nodule. As the nodule increases in size, mitoses gradually cease in the cells near the stele, but continue in the apical end of the nodule (Bond, 1948).

As mitoses continue, the nodule meristem grows away from the stele of the parent root. Infection threads, which had invaded the nodule primordium, branch and extend towards the nodule meristem. Rhizobia are released into cells from wall-less branches of the infection threads, the infection droplets (Newcomb, 1976), probably via a mechanism that is related to phagocytosis (Kijne, 1992). However, the infection threads never enter the cells of the meristem proper. Eventually, bacteria from the infection droplets become enclosed by plant-derived membrane, the peribacteroid membrane (Newcomb, 1976; Robertson *et al.*, 1978; Verma *et al.*, 1978).

The nodule meristem gives rise to cells that differentiate into the central tissue, which consists of infected and uninfected cells, the peripheral vascular bundles surrounded by vascular endodermis, and the nodule parenchyma (formerly called 'inner cortex'; van de Wiel *et al.*, 1990*b*). Differentiation of these tissues is acropetal. Thus, indeterminate nodules exhibit a gradient of differentiation from the distal nodule meristem to the older, proximal tissues attached to the parent root (Fig. 4). This gradient is reflected in a distinct cytological zonation that is especially obvious in mature N_2-fixing nodules. Relating to the cytological changes that are evident in the different tissues are the patterns of nodulin gene expression (Fig. 4). The genes that are expressed can be used as molecular markers to delimit the various cell types within the nodule, providing insight into the physiological and biochemical status of certain tissues within the nodule.

Inside the nodule endodermis, a number of different cell types make up the central region of the nodule. Proximal to the nodule meristem is the invasion zone. Some cells in this region are invaded by infection threads, while others are not. The cells of the invasion zone are larger and slightly more vacuolate than the cells of the nodule meristem. In mature pea nodules, these cells express the early nodulin gene PsENOD12, which is also found 24 h after inoculation in pea root hairs (Scheres *et al.*, 1990*a*). Three days after inoculation, mRNA encoding PsENOD12 is detected in cells containing infection threads as well as in uninvaded cells preparing for infection thread passage. The nodule primordium also contains PsENOD12 transcripts. PsENOD12 is a proline-rich protein, and is similar to proteins that have been identified as components of soybean cell walls (Scheres *et al.*, 1990*a*).

In alfalfa cv. 'Iroquois', a gene similar to PsENOD12, MsENOD12A, is expressed in the invasion zone of the nodule. Both ENOD12 proteins have a putative signal peptide with 91 % identity at the amino acid level (M. Löbler and A. M. Hirsch, unpublished results). However, the amino acid sequence of the coding region of MsENOD12A is only 43 % similar to that of PsENOD12. Also, in contrast to PsENOD12, which is expressed 24 h after inoculation as detected by hybridization to PCR products amplified from root hair mRNA (Scheres *et al.*, 1990*a*), mRNA encoding MsENOD12A has not been detected earlier than 4–6 d post-inoculation. However, the method used was northern analysis of RNA isolated from alfalfa roots (M. Löbler and A. M. Hirsch, unpublished results): PCR analysis may demonstrate an earlier timepoint of MsENOD12A expression. On the other hand, ENOD12 from the diploid *Medicago truncatula* L. is more similar to pea ENOD12. The mRNAs for MtENOD12 and MtENOD11, the genes of which are 65–70 % homologous to the PsENOD12 gene, are detected by *in situ* hybridization in a zone present in young nodules that is probably equivalent to the invasion zone (D. Barker, personal communication). Transcripts of these two nodulin genes are detected by northern analysis 4 d after inoculation with *R. meliloti*. It is not yet known whether MtENOD12 and MtENOD11 genes are expressed in root hairs as early as the PsENOD12 genes are expressed in pea or whether NodRm-1 treatment induces their expression in *M. truncatula* root hairs in the same way that Nod factors from *R. leguminosarum* bv. *viciae* induce PsENOD12 expression in pea root hairs (see references in Franssen *et al.*, 1992).

A zone of larger, more vacuolate cells is located

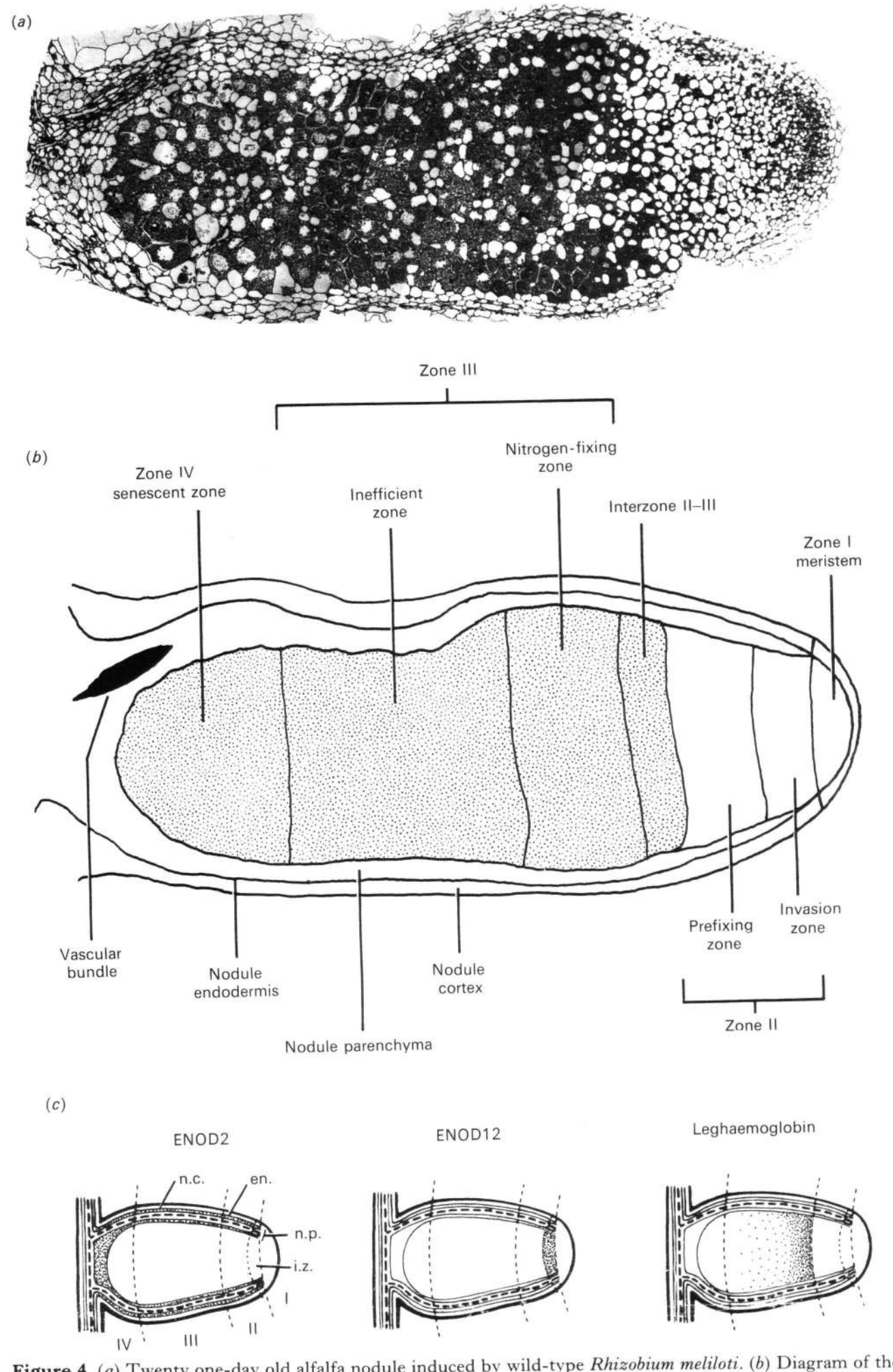


Figure 4. (*a*) Twenty one-day old alfalfa nodule induced by wild-type *Rhizobium meliloti*. (*b*) Diagram of the nodule in (*a*) showing the different zones and tissues. The central region is shaded to show the zone of cells that contain rhizobia. (*c*) Diagrams of alfalfa nodules showing the localization of mRNAs (shaded) for ENOD2 (nodule parenchyma, n.p.), ENOD12 (invasion zone, i.z.), and leghaemoglobin (central tissue). En., endodermis; n.c., nodule cortex.

proximally to the invasion zone. This zone has been called the early symbiotic zone. Recently, Vasse *et al.* (1990) established a new nomenclature, based on the stages of bacteroid development, to describe the histology of indeterminate nodules. They have assigned the designation 'prefixing zone II' to the invasion and early symbiotic zones. The late symbiotic zone (zone III) is subdivided into N_2-fixing and inefficient zones. Zone IV is the senescent zone. In this review, the invasion zone is delimited from 'prefixing zone II' even though the two intergrade, especially in immature nodules. As described above, the invasion zone is characterized by infection threads, and the expression of the early nodulin ENOD12. The proximal end of the invasion zone contains rhizobia that have recently been released from the infection thread. The bacteroids are rod-shaped, still dividing, and surrounded by peribacteroid membrane. They correspond to the type 1 bacteroids described by Vasse *et al.* (1990).

Type 2 bacteroids, which are more elongate, are found in the prefixing zone. The host cells, populated with bacteroids, are more differentiated than the cells of the invasion zone. The mRNA of PsENOD5, an arabinogalactan-type protein, is detected in the cells at the proximal end of the prefixing zone in pea (Scheres *et al.*, 1990*b*). Although PsENOD5 may also be a cell wall protein, its function in nodule development is so far unknown. It may also be part of the plasma membrane of the infection thread as well as part of the peribacteroid membrane (Franssen *et al.*, 1992).

Proximal to the prefixing zone is interzone II–III, a sharply defined region in which cells are enlarged and contain type 3 bacteroids. Type 3 bacteroids have elongated to their full size and display cytoplasmic heterogeneity. A diagnostic feature of the plant cells of interzone II–III is that they contain numerous amyloplasts. Several nodulin genes are expressed in this region in pea nodules: PsENOD3, PsENOD14, and NOD6 (Scheres *et al.*, 1990*b*). *In situ* hybridization studies show that *Rhizobium nif* gene transcripts are also detected in interzone II–III (W. C. Yang *et al.*, 1992). PsENOD5 gene expression drops off sharply in this area (Scheres *et al.*, 1990*b*). In alfalfa nodules, leghaemoglobin (Lb) mRNAs are detected in interzone II–III (de Billy *et al.*, 1991; Reddy, Bochenek & Hirsch, 1992), while in pea nodules, Lb mRNAs are first detectable in the prefixing zone II (Scheres *et al.*, 1990*b*).

Although the mRNA products of the late nodulin genes have not yet been localized to specific nodule zones by *in situ* hybridization, it is likely that many will be found in the same location as the products of the Lb genes. Most of the late nodulins are expressed at the same time or slightly after the induction of the Lb genes (see references in Verma *et al.*, 1992).

Interzone II–III appears to be a major transition region with regard to patterns of gene expression. The change in pattern no doubt reflects the changes in metabolism that occur as each symbiotic partner differentiates to commence N_2 fixation. However, not all cells in interzone II–III show these changes; some remain uninfected. In determinate nodules, uninfected cells have a specific function: assimilation of ammonia into ureides (Newcomb & Tandon, 1981). It is not known whether the uninfected cells of indeterminate nodules perform a similar function.

The distinction between infected and uninfected cells becomes more obvious in zone III, formerly called the late symbiotic zone, because the infected cells are packed with elongated rhizobia. Amyloplast accumulation is significantly decreased in this zone in N_2-fixing nodules. Zone III consists of the distally located N_2-fixing zone and the more proximally positioned inefficient zone, where nitrogenase activity is curtailed. Type 4 bacteroids, characterized by marked cytoplasmic heterogeneity, are found only in the N_2-fixing zone. The inefficient zone is occupied by bacteroids that show increasingly homogeneous cytoplasm (type 5). Lastly, at the proximal end of the nodule, closest to the point of attachment to the parent root, is the senescent zone (zone IV), where both plant and bacterial cells degenerate.

Surrounding the invasion zone and the rest of the central region of the nodule are cells that consist of a variety of cell types, including vascular tissues, a vascular endodermis, and adjacent parenchyma cells (Bond, 1948). The nodule parenchyma, which is delimited on its outer edge by the nodule endodermis, completely surrounds the vascular bundle and the vascular endodermis. In alfalfa, the nodule parenchyma consists of 4–5 cell layers. Transcripts encoding the early nodulin ENOD2 have been localized to cells of the nodule parenchyma in indeterminate nodules (van de Wiel *et al.*, 1990*a*, *b*; Allen, Raja & Dunn, 1991). Like the ENOD12 series of nodulins, ENOD2 is proline rich and presumably a cell wall protein. On the basis of its cellular localization between the nodule and vascular endodermises, ENOD2 has been implicated in limiting O_2 diffusion. An extracellular glycoprotein of 95 kDa has recently been identified in cell walls and intercellular spaces of nodule parenchyma cells in pea nodules (Rae *et al.*, 1991). Its location suggests that it may be related to ENOD2. However, the 95 kDa glycoprotein is also found within the infection thread matrix (VandenBosch *et al.*, 1989), a site where ENOD2 transcripts have not been detected.

3. *Determinate nodules*

Legumes with spherical nodules include French or common bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* L. Merr.), mungbean (*Vigna radiata* L.), and bird's foot trefoil (*Lotus* sp.), among others. Like the indeterminate nodule, the determinate

nodule can be broadly subdivided into central and peripheral tissues. The peripheral tissues consist of nodule parenchyma and nodule cortex separated from each other by the nodule endodermis. Vascular bundles are embedded in the nodule parenchyma. The nodule cortex, which is to the outside of the nodule endodermis, is derived from the root cortical cells that surround the nodule primordium.

The first cell divisions that occur in response to rhizobial infection are anticlinal and hypodermal (Newcomb *et al.*, 1979; Rolfe & Gresshoff, 1988). Later, cell divisions occur in the pericycle and inner cortex, several cells removed from the initial divisions. Eventually, the two meristematically active regions coalesce and give rise to the incipient nodule. The derivatives of the hypodermal cells form the central tissue of the nodule, which is composed of cytoplasmically-rich cells. The highly vacuolate derivatives of the inner cortex and pericycle develop into the nodule parenchyma which surrounds the central tissue. Vascular strands differentiate from small, cytoplasmically dense cells in the nodule parenchyma. The early nodulin gene GmENOD40 is expressed in the pericycle of the vascular tissue of soybean nodules, while transcripts for the early nodulin genes GmENOD2 and GmENOD13 are detected in nodule parenchyma cells (van de Wiel *et al.*, 1990 *b*; see references in Franssen *et al.*, 1992). GmENOD13 is 50% homologous with GmENOD2 and like ENOD2, GmENOD13 is probably a cell wall component (see references in Franssen *et al.*, 1992).

In soybean nodules, it has been suggested that cells of the nodule parenchyma hinder O_2 diffusion into the central tissues of the nodule (Tjepkema & Yocum, 1974; Witty *et al.*, 1986). Tjepkema & Yocum (1974) demonstrated that p_{O_2} dropped abruptly across the nodule parenchyma zone and into the central zone. This region is characterized by several layers of cells that are separated from one another by very small intercellular spaces. Early nodulins like GmENOD2 and GmENOD13 may be involved with limiting O_2 diffusion into the central tissue. Not only the site, but also the timing of expression of the ENOD2 gene, bolsters this hypothesis. The ENOD2 gene is expressed prior to the onset of N_2 fixation (4–8 d after inoculation depending on the plant). In addition, ENOD2 is likely to be a cell wall-based hydroxyproline-rich glycoprotein (HRGP) and thus may contribute to the formation of an O_2 barrier layer.

Much of the cell division activity of the central region of the nodule ceases 12–18 d after inoculation (Newcomb *et al.*, 1979). Some cells of the central tissue become invaded by infection threads. These cells are large and dense due to the presence of released bacteria and differentiating bacteroids. Interspersed among the infected cells are small, highly vacuolated, uninfected cells. In soybean, these interstitial cells, which contain a nodule-specific form of uricase, outnumber the infected cells by a ratio of approximately 3:2 (VandenBosch & Newcomb, 1986). Immunolocalization studies also show that these cells have about one-fourth the level of Lb as found in infected cells (VandenBosch & Newcomb, 1988).

The innermost cells of the nodule parenchyma are also likely to participate in ureide production. Like the interstitial cells in the central tissue of the nodule, the three innermost layers of the nodule parenchyma contain enlarged peroxisomes and large amounts of tubular endoplasmic reticulum (E. H. Newcomb, Kaneko & VandenBosch, 1989). Nodule-specific uricase has also been detected by immunolocalization in these cells. Thus, the nodule parenchyma in determinate nodules may have multiple functions.

IV. THE SECOND SIGNAL FOR NODULE MORPHOGENESIS: ROLE FOR THE PLANT HORMONES?

Since Thimann (1936) first hypothesized that auxin was involved in pea root nodulation, the plant hormones have been presumed to play a major role in nodule development. Could these endogenous growth regulators function as a 'second signal' for morphogenesis after the recognition of a *Rhizobium*-derived signal? As we have seen, interaction of *Rhizobium* cells or *Rhizobium* Nod factors with receptive legume root hair cells leads to root hair deformation and cortical cell division. Due to the size and complexity of NodRm-1, it seems unlikely that this sulphated glycolipid or any similar molecule could diffuse across plant membranes in an intact form. Furthermore, the ability of certain genotypes of alfalfa to form nodules spontaneously (Truchet *et al.*, 1989) suggests that a second signal, independent of Nod factor, is transmitted to internal cortical cells which are then stimulated to divide and form a bacteria-free indeterminate nodule (Joshi *et al.*, 1991). Transcripts of MsENOD2 are detected in the spontaneously developed nodules (Truchet *et al.*, 1989), and are localized to nodule parenchyma-like cells adjacent to the nodule endodermis and surrounding the vascular bundles (Hirsch, McKhann & Löbler, 1992). Recently, Caetano-Anollés, Joshi & Gresshoff (1992) have described the Nar^+ (*N*odulation in the *A*bsence of R*hizobium*) phenotype in detail. Nar is heritable, dominant, and likely to be caused by an alfalfa gene that, although normally inducible by interaction with *R. meliloti*, becomes active. The product of this gene could be involved in any one of the steps of the signal transduction chain that starts with the perception of the Nod factor and culminates in nodule development.

In *Rhizobium*-infected roots, the morphogenetic signal is propagated from the root hair to the cortical

cells which will undergo an anticlinal cell division. This signal initially could involve changes in membrane potential and Ca^{2+} levels. Experiments by Ehrhardt, Atkinson & Long (1992) indicate that root hair membranes depolarize rapidly after addition of Nod factor. Allen, Ehrhardt & Long (1991) found that vacuoles change their shape and that cytoskeletal rearrangements occur within root hair cells soon after alfalfa roots were perfused with extracts containing Nod factors. Many plant developmental processes are mediated by plant hormones, small molecules that are widespread in the plant and which can rapidly diffuse across membranes. Some plant hormones such as ethylene influence microtubule orientation (Lang, Eisinger & Green, 1982), while others (gibberellins) elicit changes in Ca^{2+} levels (Bush, Biswas & Jones, 1989). What role(s) do the hormones play in nodule development?

Endogenous levels of plant hormones have been measured in root nodules, but no firm conclusions can be reached because of differences in measurement techniques and the limited numbers of species examined. Furthermore, measurements of hormone amounts in mature nodules do not indicate a definitive role for the plant hormones in early nodule development. Auxins and cytokinins are most commonly found in nodule extracts, but other plant hormones including gibberellins (Dobert, Rood & Blevins, 1992) have been shown to be present. Some of these measurements are listed in Torrey's review of lateral roots (Torrey, 1986).

The best way to analyze the role of plant hormones in nodulation would be to study gene expression or protein patterns relative to the hormone biosynthetic pathways. In the absence of detailed knowledge of these pathways, particularly for auxin and cytokinin biosynthesis, a number of studies have been performed whereby roots are treated with exogenous plant hormones. Many of these investigations are described in Libbenga & Bogers (1974) and Dart (1977). Torrey (1961) found that adding kinetin to pea root explants induces cortical cell divisions, while Bauer *et al.* (1985) reported that the cytokinin benzyladenine induces cortical cell divisions in soybean, cowpea, and alfalfa. Libbenga *et al.* (1973) determined that inner cortical cells opposite the xylem poles were stimulated to divide in devascularized cortical explants after treatment with auxin and cytokinin. They also found that an extract from the stele could replace exogenous cytokinin in promoting cell division, suggesting that the stele extract contains a compound with cytokinin-like activity.

When roots of the actinorhizal plant *Alnus* were treated with exogenous cytokinin, pseudonodules were formed (Rodriguez-Barrueco & Bermudez De Castro, 1973). An involvement of cytokinin in the elicitation of bacteria-free nodules on alfalfa roots has also been shown by Long & Cooper (1988), who introduced the *tzs* (*trans*-zeatin synthesis or secretion) gene from *Agrobacterium tumefaciens* into a *nodA*::Tn*5* mutant of *R. meliloti*. These structures were similar to nodules elicited by NodRm-1 on alfalfa and contained transcripts for MsENOD2 (J. Cooper, personal communication). Dehio & de Bruijn (1992) have recently found that after *Sesbania rostrata* roots were treated with zeatin for 48 h, the SrENOD2 gene was expressed. They also determined that tumours generated on *Sesbania rostrata* stems by wild-type *A. tumefaciens* as well as by agrobacteria with mutations in the auxin biosynthetic genes contained SrENOD2 transcripts. However, agrobacteria with a mutant cytokinin biosynthetic gene 4 did not induce SrENOD2 gene expression in *Sesbania* tumour tissue.

Rhizobia can produce auxins, gibberellins, and cytokinins (see references in Torrey, 1986). IAA production by *R. meliloti* is stimulated by the addition of flavonoids (Prinsen *et al.*, 1991). Taller & Sturtevant (1991) found that there was a difference in the type of cytokinins produced by *R. meliloti* after treatment with *nod*-gene inducing flavonoids, implying that cytokinin production may be NodD-regulated. Nevertheless, *B. japonicum* with mutated *nodA*, *nodB*, *nodC*, or *nodD* genes still produce cytokinins. Genes involved in auxin biosynthesis have been cloned from *B. japonicum* (Sekine, Watanabe & Syono, 1989) and *R. meliloti* (Kittell, Helinski & Ditta, 1989). However, the effects of mutations in these genes on establishing the symbiosis have not been studied. Thus, the role of *Rhizobium*-produced plant hormone-like compounds in nodulation remains obscure. Furthermore, it is likely that plant-produced hormones rather than those generated by rhizobia play the major role in nodule development. However, this hypothesis is difficult to test without having a complete knowledge of the biosynthetic pathways of plant hormones.

Other hormones, besides those that are generally considered growth-promoting like auxin and cytokinin, have been implicated in nodulation, namely, ethylene. The Tsr response of *Vicia* roots requires *nodABC* and is stimulated by gene products that are flavonoid-inducible (van Brussel *et al.*, 1986). The Tsr response appears to be related to ethylene. If ethylene (as ethephon) is applied to *Vicia* roots, the Tsr phenotype is mimicked, but root hair deformation does not take place (Zaat *et al.*, 1989). On the other hand, when vetch roots are treated with AVG (aminoethoxyvinylglycine), an inhibitor of ethylene biosynthesis, the Tsr phenotype is suppressed, but root hair deformation still takes place (Zaat *et al.*, 1989), suggesting that Tsr and Had are brought about by different factors.

Ethylene may also be involved in nitrate inhibition of nodule formation. Ligero *et al.* (1991) treated nitrate-grown, *Rhizobium*-inoculated alfalfa roots with AVG and found that the plants nodulated. This

strongly suggests that the inhibitory effect of nitrate is mediated through ethylene.

Certain plant mutant phenotypes can be restored to wild type by adding inhibitors of ethylene biosynthesis or action. Fearn & LaRue (1991*b*) have described the *sym5* pea mutant, which nodulates poorly at 20 °C but forms more nodules at 12 °C. Although rhizobia invade the roots at the higher temperature, half of the infection threads abort either in the epidermis or in the root cortex (Guinel & LaRue, 1991). When *sym5* peas are grown at 12 °C or treated with AVG or Ag^+, which block ethylene action, the number of infections and N_2-fixing nodules that develop are increased.

Other investigators have studied the effects of inhibitors of hormone action or transport on legume roots. Torrey applied substances known to block auxin transport in stems, namely, NPA and related compounds, to clover roots, and found that nodule-like structures were formed (cited in Torrey, 1986). These 'pseudonodules' were bacteria-free, did not fix N_2, and histologically resembled modified lateral roots. Allen, Allen & Newman (1953) had found previously that pea, soybean, and cowpea formed pseudonodules in response to 2-bromo-3,5-dichlorobenzoic acid. Hirsch *et al.* (1989) extended these observations and found that the early nodulin genes ENOD2 and Nms 30 were expressed in alfalfa pseudonodules formed in response to treatment with NPA or TIBA (2,3,5-triidobenzoic acid).

Hirsch *et al.* (1989) proposed that treating alfalfa roots with auxin transport inhibitors could lead to an endogenous hormone imbalance which is manifested by cell divisions, the formation of pseudonodules, and the expression of early nodulin genes. Recently, we have found that, in addition to ENOD2 transcripts, mRNAs of the early nodulin PsENOD12 are detected in NPA-induced pseudonodules formed on roots of pea cv. Afghanistan (B. Scheres, H. I. McKhann, A. Zalensky, M. Löbler, T. Bisseling & A. M. Hirsch, unpublished results). Transcripts of both PsENOD2 and PsENOD12 were localized by *in situ* hybridization to tissues corresponding to nodule parenchyma or nodule primordium cells in *Rhizobium*-induced nodules. Although the pseudonodules differ from *Rhizobium*-induced nodules in several ways (a central vascular bundle is present instead of peripheral vascular bundles, and a defined apical meristem is absent in the NPA-induced pseudonodules), the fact that these genes were expressed argues for developmental rather than symbiotic control of early nodulin gene expression. Furthermore, it indicates that some of the early nodulins may be diagnostic for an altered hormone balance in certain tissues.

Together, the experiments described above suggest that plant hormones are involved in eliciting early nodulin gene expression. However, they demonstrate neither a direct effect nor a role for any one plant hormone. Although TIBA and NPA are presumed to block auxin transport in roots just as they do in stems, confirmatory experiments have not been rigorously performed on root tissue. Furthermore, even if such a block in auxin transport occurs in roots, it is not known whether the block results in higher or lower concentrations of auxin relative to the levels of the other endogenous hormones in the pseudonodule-forming root segment.

It is known, however, that some of the responses to NPA occur as quickly as those that take place after Nod factor application. Transcripts of PsENOD12 can be detected in pea root hairs 48 h after the addition of NPA (B. Scheres, H. I. McKhann, A. Zalensky, M. Löbler, T. Bisseling & A. M. Hirsch, unpublished results). These kinetics of PsENOD12 gene expression are the same as with *Rhizobium* inoculation or treatment with Nod factor. If NPA is indeed disturbing the endogenous hormone balance of the root, it is reasonable to assume that a similar phenomenon occurs in response to *Rhizobium* infection or treatment with Nod factor. Is this change in plant hormone balance and/or sensitivity of root hair cells a direct response to *Rhizobium*? Or is the change in endogenous hormone levels removed from the perception of the initial *Rhizobium*-generated signal so that an intermediate step is required?

As the previous compilation of experiments with plant hormones shows, the evidence for either a direct or indirect effect of the plant hormones on nodulation is meager. However, suggestions have been recently made that an early response to *Rhizobium* inoculation is a change in the internal flavonoid pool of the root. *Vicia sativa* roots react to Nod factor application by showing the so-called Ini response, an *i*ncrease in n*od* gene-*i*nducing flavonoids secreted by the root (van Brussel *et al.*, 1990). Flavonoids are synthesized by the enzymes of the phenylpropanoid pathway. Recently, Recourt *et al.* (1991) have determined that some of these enzymes increase in activity soon after *Rhizobium* inoculation. Soybean seedlings have also been examined for changes in flavonoid profile before and after inoculation. The isoflavone daidzein is extractable from the tips, but not the rest of the root of uninoculated plants. Nine to twelve hours after inoculation, a flavonoid different from daidzein is detected in the root hair zone (R. Kosslak, personal communication). As additional support for the hypothesis that an early response to *Rhizobium* inoculation is an increase in flavonoid synthesis, Estabrook & Sengupta-Gopalan (1990) have found symbiosis-enhanced members of the PAL (phenylammonia lyase) and CHS (chalcone synthase) gene families that are specifically induced in response to a symbiotic interaction with *B. japonicum*. Inoculation with heterologous *Bradyrhizobium* strains induces a different set of PAL and CHS genes, which are

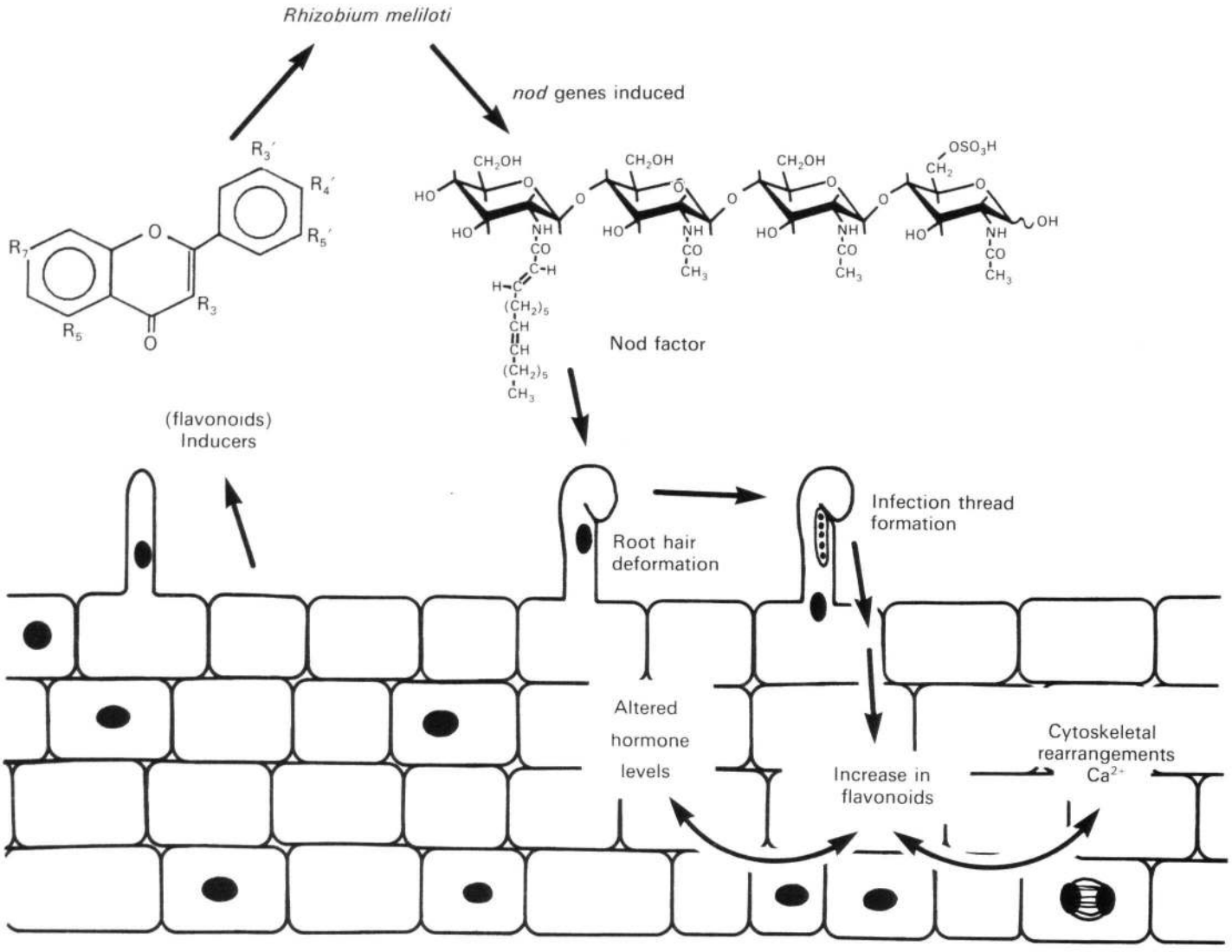


Figure 5. The Second Signal Model. Legume roots secrete flavonoids that induce rhizobial *nod* genes. Nod factors are produced and these elicit root hair curling. The level or type of flavonoids is proposed to increase in the root cells, causing a change in plant hormone concentrations or altering cytoskeletal components or intracellular Ca^{2+}. These changes lead to cell divisions which produce the nodule primordium.

probably related to stress-induced genes. Similarly, symbiosis-enhanced cDNAs of CHS have been detected in alfalfa (H. I. McKhann & A. M. Hirsch, unpublished results). These members of the CHS family appear to be expressed in inoculated roots shortly after *R. meliloti* inoculation.

What role do the flavonoids play in the root after inoculation? One possibility, as suggested by van Brussel *et al.* (1990), is the new *nod* gene-inducing flavonoids are secreted into the root's environment. These additional inducers may be required to induce *nod* gene expression in rhizobia in the rhizosphere or after infection; *nod* genes are expressed even after *Rhizobium* cells have invaded the nodule (Sharma & Signer, 1990). However, as of yet, few legumes have been tested for the Ini response. Preliminary results with soybean indicate that although the profile of internal flavonoids changes (as discussed above), the flavonoids that are secreted into the root exudate do not (R. Kosslak, personal communication). It will be important to see if the Ini response is common to infected legumes and to see if legumes having determinate or indeterminate nodules respond in the same way or in different ways.

Another possibility is that the root responds to *Rhizobium* inoculation as an infected plant would to an invading pathogen. Several reviews have discussed the apparently controlled pathogenesis that *Rhizobium* exhibits (Vance, 1983; Djordevic, Gabriel & Rolfe, 1987). As described earlier, responses similar to a hypersensitive response (HR) occur after infection with *R. meliloti exo* mutants or when infection threads abort in developing alfalfa nodules. However, Estabrook & Sengupta-Gopalan (1990) found that a distinct set of PAL and CHS genes is induced after inoculation with heterologous *Rhizobium* compared to inoculation with the homologous *Rhizobium* strain. This finding indicates that the homologous *Rhizobium* triggers a separate set of genes, presumably unrelated to those genes induced by stress or pathogen attack.

A third possibility is that flavonoids disrupt the normal endogenous hormone levels of the root by functioning as endogenous auxin transport inhibitors (Jacobs & Rubery, 1988). By interacting with an as yet uncharacterized receptor, flavonoids like quercetin, apigenin, and kaempferol could block the normal basipetal movement of auxin, thereby up-

setting the endogenous hormone balance. This may in turn render the cortical cells susceptible to infection thread penetration and/or ready for cell division.

Although none of the three possibilities described above are mutually exclusive, I have presented a model based on the third possibility, that flavonoids mediate the second signal for morphogenesis by disrupting the normal endogenous hormone balance of the root (Fig. 5). Briefly, the model states that upon perception of the *Rhizobium*-derived primary signal, NodRm-1 (the synthesis of which is stimulated by flavonoids secreted by the host), genes for specific members of the phenylpropanoid biosynthetic pathway are induced in the root. Particular flavonoids are synthesized and these accumulate in discrete root cells which are presumed to carry receptors for the flavonoids. Flavonoid binding to the receptors causes a localized block in auxin transport, shifting the endogenous hormone balance to change the levels of auxin relative to cytokinin. Whether or not all root cells have such receptors is unknown. Cells in a lateral gradient from the vascular system to the root hair cell, as visualized by Libbenga *et al.* (1973), could exhibit differential sensitivity to plant hormones. Alternatively, a change in hormone balance could occur in response to the flavonoids in specific root cortical cells. In any case, in indeterminate nodules, the inner cortical cells divide while the outer cells reorganize their cytoplasms to form the bridge through which the infection thread passes (see Kijne, 1992). In determinate nodules, a different lateral gradient must exist because cell divisions which give rise to the central portion of the nodule occur in the outer cortex.

V. LATERAL ROOT DEVELOPMENT

Lateral roots, like nodules, have an endogenous origin and, like nodules, lateral roots are complex structures made up of cells that are organized in a defined pattern. In angiosperms, lateral roots usually develop from periclinal divisions of cells of the pericycle – the lateral meristem of the root. However, other tissues may be involved as well (Peterson & Peterson, 1986). Generally, these cell divisions take place opposite a protoxylem pole. As cell divisions proceed and give rise to the lateral root primordium, the endodermal and cortical cells of the parent root also divide, keeping pace with the expanding lateral root for a time. When the lateral root emerges, the endodermis and cells of the parent root cortex become mechanically or enzymatically disrupted. Distinct tissues then become visible within the lateral root primordium. A root cap is differentiated, the root meristem is established, and the central stele is laid down. In some plants, a quiescent centre is not detectable in the subterminal cells of the root until the lateral root emerges. However, in *Pistia* and *Eichhornia*, two aquatic plants, the quiescent centre is formed before lateral root emergence (see references in Peterson & Peterson, 1986).

Lateral roots are regularly positioned along the primary root, but the factors that control this distribution as well as lateral root origin are unknown. If the primary root is decapitated, lateral roots are initiated and expand, probably due to a release of root apical dominance. Several factors, including plant hormones, nucleotides, vitamins and micronutrients, stimulate lateral root formation in isolated root segments grown in culture (see references in Torrey, 1986). Blakely *et al.* (1982) determined that IAA treatment of radish root segments led to an increase in lateral root formation from the pericycle. Auxins, have been shown to be essential for promoting lateral root formation in a number of species, while cytokinins, abscisic acid, and ethylene inhibit lateral root development.

Further evidence for the involvement of plant hormones in lateral root formation is the phenotype of the tomato mutant *diageotropica* (*dgt*). Not only does the plant exhibit an agravitropic behavior, but lateral root development is also inhibited. If the roots are excised from the shoot and grown *in vitro*, some lateral roots expand, suggesting that the inhibitory influence on lateral root development comes from the shoot. Hicks, Rayle & Lomax (1989) have found that *dgt* tomato shoots lack proteins that bind azido-labelled IAA, suggesting a role for auxin in lateral root development. Moreover, *dgt* is auxin non-responsive. In addition to *dgt*, other root mutants are listed in Schiefelbein & Benfey (1991). Some of these mutants should be useful for studying lateral root development.

Although auxins may be important for initiating cell divisions from the pericycle, cytokinesis is not a prerequisite for lateral root formation. Foard, Haber & Fishman (1965) treated wheat seedlings with colchicine and found that cells of the pericycle enlarged to form 'primordiamorphs'. If colchicine was removed, the primordiamorphs developed into normal lateral roots. This experiment indicates that lateral root position is mediated by other factors, their identities unknown. Recently, genes that are specifically expressed during lateral root development have been identified (Keller & Lamb, 1989; N. Kerk & I. Sussex, personal communication). It will be interesting to see whether determining the identities of any of these genes will help us come to a better understanding of lateral root formation or whether any of them are also expressed during nodule development.

VI. ARE NODULES MODIFIED LATERAL ROOTS?

In contrast to tumours elicited by pathogenic *Agrobacterium tumefaciens* or to the galls formed by

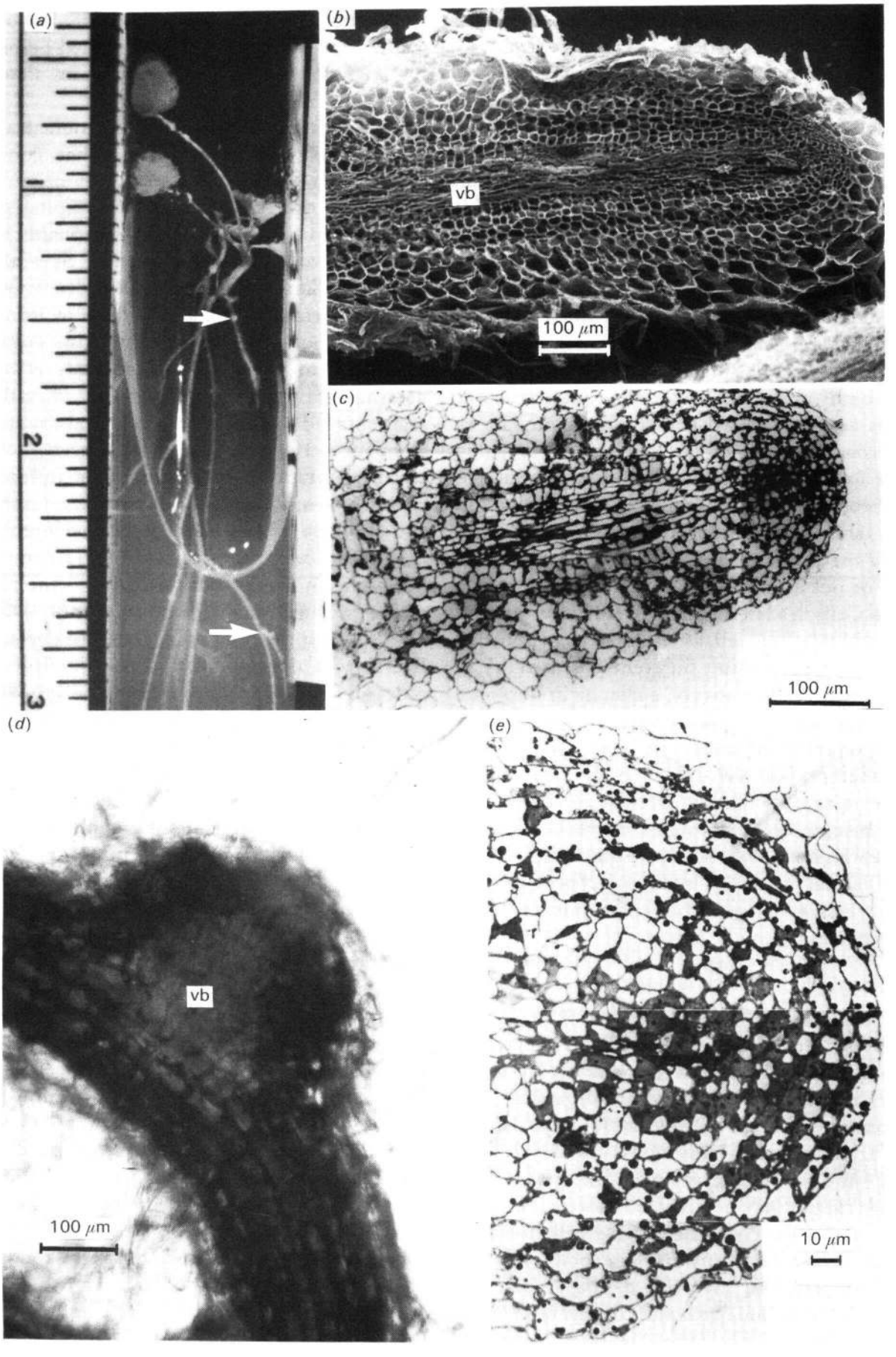


Figure 6. (*a*) *Trifolium repens* plant inoculated with pTi-cured *Agrobacterium tumefaciens* carrying the common *nod* genes of *Rhizobium meliloti*. Arrows indicate nodule-like protrusion. (*b*) Scanning electron micrograph of a longitudinally cut protrusion induced on a clover root. Like a lateral root, there is a centrally positioned vascular bundle (vb). (*c*) Light micrograph of another nodule-like protrusion, showing the central vascular bundle. (*d*) Nodule-like protrusion with a central vascular bundle and rounded distal end without a root cap. (*e*) Enlargement of (*c*). There is no root cap.

Pseudomonas savastonoi pv. *syringae* or by nematodes on certain hosts, root nodules are highly organized structures composed of specialized tissues. Although legume nodules are structurally and developmentally distinct from lateral roots, actinorhizal nodules and nodules formed on the roots of *Parasponia* are considered to be modified lateral roots. Non-legume nodules originate in the cortex as prenodules, but these divisions soon subside. The pericycle divides and takes over as the initiator of the nodule (Lancelle & Torrey, 1985). Both non-legume nodules and lateral roots are characterized by a single vascular bundle that traverses the nodule, dividing the infected cell zone into two sectors in longitudinal view. Also, many actinorhizal nodules have nodule roots emerging from the nodule meristem.

Legume nodules differ from lateral roots in site of origin, level of DNA endoreduplication, and final structure. However, peanut nodules are reported to have a pericyclic origin in contrast to other legumes (Allen & Allen, 1940). Like lateral roots, however, nodules frequently arise opposite protoxylem points (see references in Libbenga & Bogers, 1974), but they also originate opposite the phloem or between the xylem and phloem. According to Nutman (see references in Libbenga & Bogers, 1974), legume nodules form only at zones with growing root hairs and at points of incipient meristematic activity, i.e. at foci of lateral root formation. Nutman noted that there seems to be a physiological relationship between lateral roots and nodules. For example, nodulated clover seedlings have fewer lateral roots than uninoculated plants. Also, the total number of nodules and lateral roots on a plant with either effective or ineffective nodules remains the same: if a plant has many ineffective nodules, it has few lateral roots, and vice versa. In addition, excising the main root tip enhances both nodule and lateral root number. According to Libbenga & Bogers (1974), Nutman also noted that nodules frequently arise in the axils of lateral roots, suggesting that these nodules were derived from lateral root primordia.

Libbenga & Bogers (1974) offer a number of arguments against Nutman's hypothesis. In addition to site of origin, legume nodules differ from roots in being devoid of root caps and exhibiting a peripheral rather than central arrangement of vascular bundles. Libbenga & Harkes (1973) also showed that pea lateral root primordia arise closer to the root apical meristem than do nodule primordia.

However, structural intermediates between roots and nodules have been described. Nodules exposed to high temperatures (35 °C), then returned to 20 °C, differentiated roots from tissue at the termini of the vascular bundles. Older nodules of the perennial plants *Sesbania grandiflora* and *Caragana arborescens* have been reported to change their pattern of growth and form roots (see references in Dart, 1977). *A. tumefaciens* transconjugants, carrying *R. meliloti* *nod* gene sequences, induced structures on clover roots that superficially resemble nodules (Fig. 6). Detailed microscopic examination showed that these structures lacked a root cap and had swollen cortical tissue, but contained no bacteria (Hirsch *et al.*, 1985; Truchet *et al.*, 1985). However, like a lateral root, the vascular bundle was centrally located. Dudley, Jacobs & Long (1987) also described structures elicited by *R. meliloti* mutants that appeared to resemble roots more closely than nodules. The lateral outgrowths were elongated and possessed a central vascular bundle.

In summary, whether or not the legume nodule is a highly modified lateral root or a completely new plant organ cannot be judged without a more thorough understanding of the signals and genes involved in lateral root development. It is likely that many of the genes that are expressed during the initiation of each structure are the same. However, a difference in developmental signalling exists in that the cues for lateral root formation are generated internally, while those for nodulation are externally derived. Moreover, it is possible that both signals could be similar types of molecules, i.e. oligosaccharides or glycolipids, that somehow bind to receptors provoking signal transduction chains and triggering changes in the internal conditions of the responding cell.

VII. CONCLUSIONS AND FUTURE PROSPECTS

Our understanding of the *Rhizobium*–legume symbiosis has expanded immensely in the last ten years, particularly with respect to our knowledge of the genes and chemical signals perceived and produced by the bacterial partner. Although the plant side of the symbiosis has been more difficult to analyze, it seems likely that it too will be opened up to investigation in the next few years. Already, investigators have shown that a large number of genes are involved in the formation of a nodule. Thus, endeavors to manipulate agronomically important nonlegumes by transferring a package or 'cassette' of genes that are expressed specifically during nodule development will probably fail. It may be more desirable to modify lateral root development in nonlegume plants so that some well chosen diazotrophs can associate with host tissues and fix N_2. Already, some experiments have shown that rhizobia and other N_2-fixing bacteria form nodule-like structures on roots of rice, rapeseed, barley or wheat (Al-Mallah, Davey & Cocking, 1989, 1990; Jing, Zhang & Shan, 1990; Rolfe & Bender, 1991). Many of these nodule-like structures look like stunted lateral roots with bacteria enclosed in intercellular spaces. However, before lateral roots or any other root structure can be manipulated to form an N_2-fixing association with rhizobia or other diazotrophs, we must have a clearer understanding of what provokes lateral root

formation. These developmental triggers are virtually unknown. Furthermore, these modified lateral roots must contain some sort of a micro-aerobic environment for protection of the O_2-sensitive nitrogenase. For example, the plant could be engineered so that the intercellular space becomes filled with polysaccharides or other O_2-excluding material upon infection.

In the future, greater emphasis needs to be placed on analyzing specific legume mutants and thoroughly defining those mutations that result in interrupted nodule development. Currently, the connection between a particular mutation and a known feature, such as a defect in a transcription factor or receptor protein, has not been made. The nexus between genetics, molecular biology, and development must be strengthened. Without these linkages, the study of the plant contribution to symbiosis will continue to lag behind that of *Rhizobium*. Besides studying plant mutants, the study of cloned genes in transgenic plants provides a powerful method to investigate the genes involved in nodule development. This approach has already been undertaken by several investigators and has yielded useful data (Stougaard-Jensen *et al.*, 1986; de Bruijn, Szabados & Schell, 1990). Recently, there has been a call to focus research efforts on legumes with small genomes (Barker *et al.*, 1990; J. Stougaard, personal communication). The goal is to bridge the gap between genetics and molecular biology. However, the need to study legumes with small genomes may not be as critical in the future once the techniques of chromosome walking, transposon tagging, artificial chromosomes, etc. become routinely applied to plants. Even plants with large genomes, like pea with its wealth of genetic mutants, may become manageable. In addition, our understanding of how the plant responds to *Rhizobium* inoculation will be greatly aided by learning more about basic mechanisms in plants. We need to identify membrane and cytosolic receptors, not only for *Rhizobium* but also for the endogenous plant hormones. We must learn more about the mechanism of plant cell wall deposition and understand in greater detail how cytoskeletal arrangement and orientation affects the planes of cell division. We need to find out more about how signals are communicated across cellular boundaries and how meristems are established and perpetuated. With so many unknowns, it would seem that developmental biology remains the final frontier we need to cross in order to achieve a complete understanding of the *Rhizobium*–legume symbiosis.

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