J Plant Growth Regul (2000) 19:155–166
DOI: 10.1007/s003440000023

J P G R
Journal of Plant Growth Regulation

Molecular Mechanisms in Root Nodule Development

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Abstract

Under nitrogen-limiting conditions, bacteria from the family Rhizobiaceae establish a symbiosis with leguminous plants to form nitrogen-fixing root nodules. These organs require a coordinated control of the spatiotemporal expression of plant and bacterial genes during morphogenesis. Both plant and bacterial signals are involved in this regulation in the plant host. Plant genes induced during nodule development, the so-called nodulin genes, have been extensively characterized. Products of several of these genes show homologies to known regulators of signal transduction pathways in other plant or animal systems. Initial functional analysis of the molecular mechanisms implicated in nodulation have been undertaken using model legumes. Insertion mutagenesis and transgenic technologies to modify nodulin gene expression, as well as pharmacologic approaches, have been used to analyze molecular mechanisms involved in morphologic responses induced by the bacterial symbiont in the plant. G protein–mediated transduction mechanisms have been implicated, and the *nin* transcription factor appears to be required for early steps in nodule development. *ENOD40,* a gene coding for an RNA that contains only short ORFs, seems to be closely tied to nodule primordium formation. In addition, a vascular-associated Krüppel-like transcription factor and small *Rab* type G-proteins affect bacteroid differentiation and the function of the nitrogen-fixing zone. These initial results presage a wealth of information that will be obtained from the application of genomic approaches to legumes.

Key words: Symbiosis; Nodulins; Nod factors; Transduction pathways; Functions of regulatory genes

Introduction

Plants from the family Fabaceae when grown under nitrogen-limiting conditions, can enter into a symbiotic relationship with bacteria from the family Rhizobiaceae to form a new root-borne organ, the nodule. In this organ, the bacteria, differentiated into bacteroids, fix nitrogen for the plant host, and in turn, the plant provides carbon to the rhizobia. In this review, we focus on the plant responses to the bacterial symbiont and various plant genes that appear to be involved in nodule organogenesis.

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The Symbiotic Interaction

Members of Rhizobiaceae are gram-negative bacteria present in the soil. Their symbiotic interaction with different legumes shows a high level of host-specificity. The interaction starts when host-specific rhizobial strains attach to growing root hairs, inducing curling and branching. In certain cases, complete entrapment of the bacteria in a "shepherd's crook" leads to infection (Dénarié and others 1996; Schultze and Kondorosi 1998). Simultaneously, pericycle and cortical cells are activated for division, usually in front of a xylem pole, close to the infection point. The cortical cells actively divide to form the nodule primordium wherein large amounts of

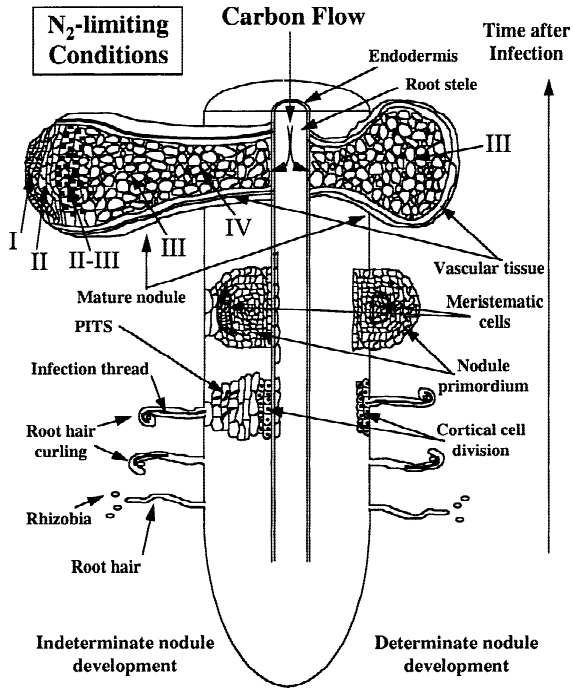

Figure 1. Indeterminate vs determinate nodule development. Rhizobia interact with root hairs inducing deformation and curling. An infection thread allows penetration of bacteria into root tissues. Simultaneously, pericycle and inner (indeterminate) or outer (determinate) cortical cells are activated and divide to form the nodule primordium that contains large amounts of amyloplasts. Carbon flow through the stele is required starting with the initial steps of nodule development. A group of cells from the primordium forms a meristem, allowing indeterminate nodule growth. In determinate nodules, cell expansion of a transient meristematic region determines nodule growth. The indeterminate mature symbiotic organ possesses different regions along a differentiation gradient (I to IV). Nitrogen-limiting conditions are required for nodule formation. *PITS,* preinfection threads.

amyloplasts accumulate (Figure 1). At the root surface, rhizobia caught in the root hairs locally degrade the plant cell walls, and the infection thread develops around the growing bacterium. These threads, containing dividing bacteria, grow toward the root cortex. However, in most cases, infection thread progression aborts in the cortex (Vasse and others 1993). Only very few (around 1–5%) infection threads reach the nodule primordium cells. The infection threads penetrate and ramify into primordium cells traversing their walls; they then enter cortical cells, initiating a differentiation process that is heralded by cell enlargement. Bacteria, differentiated into bacteroids, are released into the cytoplasm of the plant cells and become surrounded by the peribacteroid membrane (Hirsch 1992). The differentiation process of the bacterial symbiont into nitrogen-fixing bacteroids is accompanied by significant morphologic changes. Bacteroids of rapidly growing species such as *Sinorhizobium meliloti* stop dividing and increase their size up to 30 times, whereas slow-growing rhizobia such as *Bradyrhizobium japonicum* retain their initial volume and division capacity. Differentiated bacteroids present important physiologic adaptations with respect to their enzymatic capacity, notably the production of nitrogenase. This enzyme converts atmospheric nitrogen into ammonium, which is then exported to the plant cell cytosol.

Nodule Types

Nodules fall into two different types: indeterminate or determinate (Hirsch 1992). During development of indeterminate nodules, usually formed on roots of temperate legumes (pea, alfalfa, vetch), the nodule primordium starts in the root inner cortex. Cells of the outer cortex are also activated, as detected by microtubular reorientations that occur in these cells. These outer cortical cells form a preinfection structure, named the preinfection thread or PIT, which allows for passage of the infection thread (Timmers and others 1999; van Brussel and others 1992). Mature indeterminate nodules are characterized by the presence of a persistent apical meristem responsible for nodule growth. Such nodules are divided into several regions along a differentiation gradient (Vasse and others 1990): (1) zone I or the meristem; (2) zone II or the invasion zone, where bacteria are released into the plant cytosol; (3) interzone II–III, characterized by an accumulation of amyloplasts, a zone where the bacteria differentiate into bacteroids; (4) zone III or the nitrogen-fixing zone, composed of both bacteroid-containing plant cells and small bacteroid-free plant cells where the fixed nitrogen is assimilated; and (5) zone IV or the senescing zone, where both the bacteroids and plant cells degenerate. These zones are enveloped by peripheral cell layers, known as the outer and the inner cortex. Indeterminate nodules export fixed nitrogen predominantly as asparagine and glutamine, which are the most abundant nitrogen species found in the phloem tissues of these legumes. A scheme for indeterminate nodule formation is presented in Figure 1.

Determinate nodules are usually formed on tropical and subtropical legume plants (for example, soybean, bean). Nodule primordia are formed in the root outer cortex, and their meristematic activity disappears very early after nodule initiation. Accordingly, nodule growth takes place by cell expansion

rather than by cell division and shows only a temporal differentiation pattern. Like indeterminate nodules, determinate nodules are also surrounded by outer and inner cortex but unlike indeterminate nodules, they export mainly ureides (Vance and Gantt 1992).

Certain alfalfa cultivars develop nodules spontaneously under nitrogen starvation conditions. These indeterminate nodules are devoid of the bacterial symbiont *Sinorhizobium meliloti* (NAR^+ phenotype; Caetano-Anollés and others 1992; Truchet and others 1989). These structures are histologically similar to *S. meliloti*–induced nodules, showing a meristem, a differentiation region, and a central zone, the cells of which are filled with starch. Based on this phenotype, nodule initiation and development are likely to be controlled by the plant partner.

The evolutionary origin of nodules remains unclear. Two main hypotheses have been proposed; nodules could be highly modified pre-existing organs such as stems, lateral roots, or carbon storage organs; or alternatively, nodules could be novel, *sui generis* organs evolved from the interaction between a plant and another organism (reviewed in Hirsch and LaRue 1997).

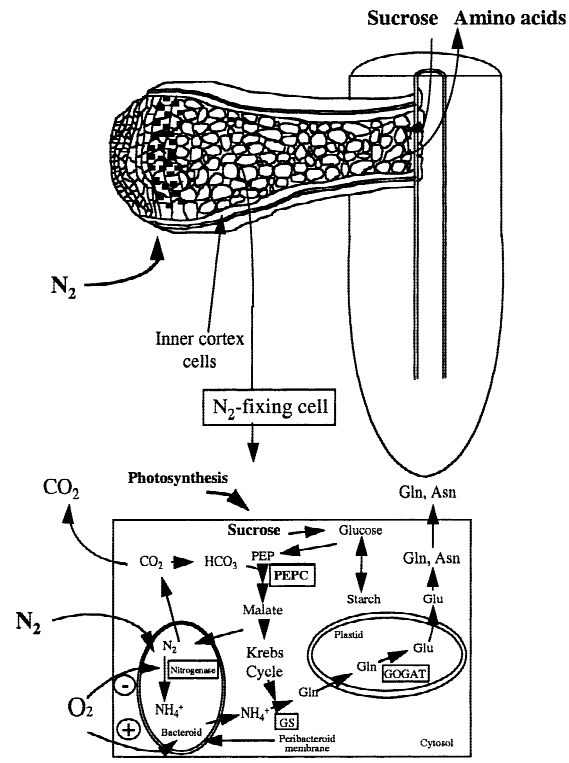

Figure 2. The mature indeterminate nodule and the symbiotic fixation process. Sucrose transported from aerial tissues feed the symbiotic organ that exports amino acids. Oxygen concentration is regulated inside the nodule by a diffusion barrier present in the inner cortex cells. Nitrogen-fixing cells transform sucrose into malate to furnish dicarboxylic acids both for bacteroid respiration and plant ammonia assimilation into amino acids. The bacteroid is isolated from the plant cytoplasm by the peribacteroid membrane. Oxygen is required for bacteroid metabolism, although it is a potent inhibitor of nitrogenase.

Symbiotic Fixation of Nitrogen

Nitrogen is fixed into ammonium, a reduced form that is very toxic for the plant cell. Thus, the plant cell must rapidly assimilate the ammonium released by the bacteroid into amino acids through the concerted action of several enzymes, notably glutamine synthetase (GS) and glutamate synthase (GOGAT) (Vance and Gantt 1992). The GS/GOGAT pathway is the major nitrogen assimilation route in plants. In addition, a continuous supply of carbon skeletons, mainly intermediates of the Krebs cycle, is required both for active bacteroid respiration and for assimilation of fixed nitrogen. Phosphoenol pyruvate carboxylase (PEPC) is responsible for the anaplerotic replenishment of the Krebs cycle, allowing the use of dicarboxylic acids to assimilate ammonium and to feed the bacteroids. PEPC is thus an important link for coupling nitrogen and carbon metabolisms in the nodule.

Nitrogen fixation is based on a paradox: nitrogenase activity requires both reducing power and energy that are furnished by bacterial respiration, but at the same time, it is rapidly inactivated by oxygen. To provide a microaerobic environment without adversely affecting nitrogen fixation, the plant partner controls the oxygen concentration inside the nodule. The presence of leghemoglobin, an abundant nodule protein, ensures the transport of oxygen into the bacteroid, whereas a physical barrier in the inner cortex at the nodule periphery limits oxygen diffusion into the nodule (Hunt and Layzell 1993). Mechanisms involving changes in osmotic potential of the inner cortex cells (Drevon and others 1995), secretion of extracellular glycoproteins (Wycoff and others 1998), and glutamine concentration in the phloem (Neo and Layzell 1997) may be involved in controlling oxygen permeability in nodules. A scheme showing the different interactions between carbon and nitrogen metabolic pathways and the control of oxygen diffusion is presented in Figure 2.

Plant Genes Acting in Nodule Organogenesis

Identification of molecular mechanisms involved in organogenesis requires multidisciplinary approaches to analyze the genes involved and the fate and ac-

tion of their encoded products. During nodule development, many plant genes, the so-called nodulin genes (van Kammen 1984), need to be coordinately induced in the different steps of the process. Two classes were distinguished according to their temporal expression pattern, *e*arly *nod*ulin (or *ENOD*) genes whose transcripts were detected before nitrogen fixation starts, and late nodulin genes, whose expression is induced during or after the nitrogen fixation process. Several strategies have been used to identify differentially expressed genes in nodules and roots. Initially, differential screening of cDNA libraries of mRNA populations from root hairs activated by rhizobia, Nod factors, or nodules at different steps of development were used. Later, differential display, subtractive and cold-plaque screenings were used, yielding a large diversity of molecular markers associated with different steps of nodule development in several legumes (for example, in *Medicago* species, Cook and others 1995; de Carvalho Niebel and others 1998; Frugier and others 1998; Gamas and others 1996). Roles for these genes were mainly deduced from their sequences and from their spatiotemporal expression patterns during nodule differentiation. A large number of them code for proteins putatively associated to cell walls, supporting the major role of wall modifications in infection and organogenesis (for example, proline-rich proteins such as *ENOD2, ENOD5, ENOD10, ENOD12, ENOD13, PRP-4,* Didi-2; glycine-rich proteins; extensins and the peroxidase *Mtrip 1;* reviewed in Bladergroen and Spaink 1998). Other nodulins are homologous to lectins (Bauchrowitz and others 1996), enzymes of the phenylpropanoid pathway or pathogenesis proteins (for instance, chalcone synthase and chalcone isomerase genes (Mathesius and others 1998; McKhann and Hirsch 1994) PR10 homologues (Gamas and others 1998) or chitinases (Goormachtig and others 1998). Apart from these nodulin genes, others related to nitrogen fixation and assimilation have been detected, such as sucrose synthase, GS, GOGAT, PEPC, carbonic anhydrase, and aspartate aminotransferase (Shi and others 1997; Vance and Gantt 1992). These latter genes are mainly expressed in the symbiotic zone and induced late in nodule development except for carbonic anhydrase whose transcripts accumulate specifically in the inner cortex cells (Coba de la Pena and others 1997), the same cells involved in controlling oxygen permeability. Other nodulins are leghemoglobins, constituents of the peribacteroid membrane (for example, Nod 26), peptide transporters, and a cytochrome P450 (Szczyglowski and others 1997). Recently, systematic sequencing of either root hair or nodule cDNA libraries allowed the identification of a large number of genes (Covitz and others 1998; Gyorgyey and others 2000). Ongoing development of functional genomic approaches in model legumes will yield a precise description of temporal expression patterns of large gene collections during nodulation (Cook and others 1997). These molecular markers are also excellent tools for understanding genetic control of nodule organogenesis.

Another approach for identifying genes involved in nodule organogenesis is mutagenesis. Several symbiotic mutants have been described in various leguminous species (Caetano Annollés and Gresshoff 1991; Kneen and LaRue 1988; Penmetsa and Cook 1997; Sagan and others 1995). Identified symbiotic mutants are either perturbed in their capacity to form nodules (absent or very few nodules per plant) or show increased numbers of nodules (supernodulators). However, except for the *nin* gene (see later), the molecular nature of these mutations is unknown. Moreover, most of the agriculturally important legumes have large genomes and cloning of the mutated genes can be very difficult. This has led to the development of model legumes such as *Medicago truncatula* (Barker and others 1990) and *Lotus japonicus* (Handberg and Stougaard 1992) for indeterminate and determinate nodules, respectively. These diploid autogamous plants possess small genomes three to four times larger than *Arabidopsis thaliana*) and insertional mutagenesis approaches based on T-DNA or transposable elements are being developed (Cook and others 1997; Schauser and others 1998). To date cloning of one tagged Nod$^-$ mutant (by an AC transposon), *nin,* has been reported in *Lotus japonicus* (Schauser and others 1999). Nevertheless, other legumes such as pea and sweetclover are also useful models because of ease of mutagenesis and a long history of study (Hirsch and others 2000; Schneider and others 1999).

Originally, nodulins were defined as genes exclusively expressed in nodules (van Kammen 1984). However, expression pattern analysis of inducible nodulation genes has shown that most of them are also expressed in nonsymbiotic tissues. Nodulin gene homologues even exist in nonlegumes (Arredondo-Peters and others 1998; Kouchi and others 1999). These results suggest that several functions required for nodulation have been recruited from pre-existing genes that are present in both legumes and nonlegumes (Bladergroen and Spaink 1998).

Plant and Bacterial Signals Involved in Nodulation

Nodule development requires signal exchange during the different steps of the process (for example,

infection thread growth, activation of cell division and bacterial invasion) to ensure coordinated regulation of the spatiotemporal expression of genes from both partners (reviewed in Denarié and others 1996; Hirsch 1992; Schultze and Kondorosi 1998; Spaink 1996).

Nod factors are the key molecules determining specific recognition by the plant host of the bacterial partner. These signals are lipochitooligosaccharides consisting of a skeleton of three to five *N*-acetyl D-glucosamine residues linked to a lipid moiety on the nonreducing end. These molecules present variable substitutions involved in recognition specificity and are able to induce various cellular responses related to nodule initiation in plant hosts: deformation of root hairs, formation of PITs, and division of cortical cells (Ardourel and others 1994; Schultze and Kondorosi 1998; Truchet and others 1991; van Brussel and others 1992). However, these phenotypes are not induced in all legumes and may vary considerably even between closely related plant hosts. The formation of spontaneous nodules in alfalfa (Truchet and others 1989) suggests that Nod factors trigger, under nitrogen-limiting conditions, a preexisting genetic program of the plant host. Nod factor-induced events related to infection (such as PIT formation) required highly specific substituents, whereas other events, such as deformation of root hairs, activation of cortical cells, or induction of nodulin genes, are less stringent for Nod factor substituents. This has led to the hypothesis that two receptors may exist in the host. The first one may be involved in signaling processes showing less stringency for Nod factor structure and a second one, highly specific, may be required for allowing the penetration of the symbiont into root tissues (Ardourel and others 1994). Alternatively, it has been proposed that the different effects of Nod factors could be explained by the presence of a single receptor showing different binding affinities (Hirsch 1992).

Bacterial surface polysaccharides are also compounds important for symbiotic nodule development, namely for the infection process (see Hirsch and McFall-Ngai, this volume). Mutants affected in their synthesis are unable to penetrate cortical cells in indeterminate nodules, and thus bacteria-free nodules form (Hirsch 1992; Niehaus and others 1993). During root infection by these mutants, plant defense gene expression is induced in the plant (Niehaus and others 1993; Perotto and others 1994); this might be responsible for infection arrest. These surface compounds may protect the symbiotic bacterium against defense responses by the plant host and might act also as signals to suppress those host responses. An alternative hypothesis is that the exopolysaccharide (EPS) may itself act as a symbiotic signal during infection (González and others 1996).

Plants exert a complex physiologic control on the determination of competence for organogenesis through the action of plant hormones and metabolic factors linked to appropriate environmental conditions. Thirty years ago, it was proposed that susceptibility of the root cortical cell to respond to rhizobia may depend on several opposing gradients of morphogenetic signals (Libbenga and others 1973). A role for cytokinins in nodule initiation has been suggested because a cytokinin-synthesizing gene elicits a partial morphogenetic rescue of Nod$^-$ mutant strains (Cooper and Long 1994). Moreover, cytokinin addition induced pseudonodules in several species (including certain non-legumes, Hirsch and others 1997), as well as similar responses as those induced by Nod factors in leguminous roots, including cortical cell division, amyloplast accumulation, and early nodulin gene expression (Dehio and de Bruijn 1992; Fang and Hirsch 1998; Hirsch and others 1997). On the other hand, treatment with auxin transport inhibitors resulted in the formation of pseudonodules on the roots of certain legumes (Hirsch and others 1989) even though these structures exhibited a central vascular bundle and also developed in the presence of nitrogen. Local addition of auxin transport inhibitors to white clover roots using a microtargeting device (Mathesius and others 1998) provoked a local and transient modification of the expression of an auxin-sensitive promoter. This response is also induced by local application of Nod factors to these roots. These data suggest that Nod-factor–induced cortical cell divisions are the consequence of local perturbations of auxin and cytokinin gradients at the point of infection. Ethylene has also been linked to nodulation because it is a potent inhibitor of this process (reviewed in Hirsch and LaRue 1997). Several mutants with perturbations in ethylene responses showed disturbed nodulation patterns (the *sym5* mutant or the *sickle* mutant of *M. truncatula;* Fearn and LaRue 1991; Penmetsa and Cook 1997). Finally, another plant factor likely to be involved is uridine (or the "stele factor"), which activates cell proliferation of root cortical explants of pea at pico- to nanomolar concentrations (Libbenga and others 1973; Smit and others 1995). Thus, a basoapical gradient of cytokinins, an apical-basal gradient of auxins, and transversal gradients of ethylene and uridine coming from the stele probably determine a specific spatial position where cortical cells might be able to respond to bacterial Nod factors (Hirsch and others 1997; Schultze and Kondorosi 1998).

The host plant regulates the total number of nodules that will develop on its root system, as well as the size of the nodules, a phenomenon known as autoregulation of nodulation (Caetano Anollés and Gresshoff 1991). The number of nodules must be strictly adapted to the photosynthetic capacity of the plant to produce carbon skeletons, because nitrogen fixation is energetically very expensive (between 12 and 17 g of carbohydrate per gram of nitrogen fixed; Vance and Gantt 1992). Once appropriate numbers of primordia are induced, autoregulation blocks further infection at different steps that vary in the different leguminous plants (Caetano Anollés and Gresshoff 1991). Mutants affected in autoregulation have been described as supernodulators and, using grafting techniques, it has been shown that this regulation depends on a shoot factor (likely derived from leaves) that travels to the root system in a systemic way. This autoregulatory factor may be the link between nodule initiation and plant photosynthetic activity. Heterologous grafting indicates that the autoregulatory signal might be common between legumes (Sheng and Harper 1997).

Nodulation Signal Transduction: Nod Factor Perception

Nod factors elicit biologic responses at very low concentrations, suggesting that specific receptors recognize these signals in the plant host. Several perception and transduction pathways may exist in different cell types, because structural requirements for Nod factor activity differ between target tissues (such as root hairs, outer or inner cortex) or between nodulin gene responses (Bladergroen and Spaink 1998; Schultze and Kondorosi 1998).

Binding sites for Nod factors have been identified in cell suspensions of *M. sativa* and root extracts of *M. truncatula:* a low affinity site (NFBS1, for *N*od *F*actor *B*inding *S*ite 1) and a high affinity site NFBS2 (Bono and others 1995; Gressent and others 1999). However, these sites do not discriminate Nod factor sulfation, an essential substitution for nodulation on *Medicago* (Niebel and others 1997). Lectins have also been suggested as candidates that are involved in host specificity. Ectopic expression of a soybean lectin in white clover (Diaz and others 1989) or in *Lotus coniculatus* (van Rhijn and others 1998) allowed changes in the host-specificity of nodulation. However, it is not yet clear which bacterial compound(s) (for example, surface polysaccharides) is (are) recognized by the soybean lectin although EPS may be involved (van Rhijn and others 1998). Recently, a lectin from *Dolichos biflorus* having apyrase activity was shown to bind Nod factors from the bacterial partner of this legume (Etzler and others 1999). Moreover, this binding activated the apyrase *in vitro.*

An alternative approach to identify Nod factor receptors is to analyze non-nodulating mutants whose phenotype is restricted to rhizobial strains producing certain Nod factors. However, at present, there is no definitive proof for any of these candidates to be *bona fide* Nod factor receptors.

G-Proteins

Very few genes have been identified that could be potentially linked to signal transduction pathways controlling nodulation. Nevertheless, participation of proteins from the GTPase superfamily (or G-proteins) in Nod factor transduction have been demonstrated by showing that agonists and antagonists are able to elicit or block *ENOD12* expression in alfalfa root hairs, respectively (Pingret and others 1998). Recently, 33 small G-proteins from *L. japonicus* belonging to the Ypt, Rab, Rho, and Rac families have been isolated and their expression during nodule development studied. Only five of them were induced in nodules (Borg and others 1997). Previously, small G-proteins of the *Rab* type (*Rab1* and *Rab7*) induced in mature nodules were characterized. Antisense expression of these genes driven by the nodule-specific leghemoglobin promoter in transgenic hairy root blocked vesicle transport into the peribacteroid membrane (Cheon and others 1993). For *Rab1,* a decrease in nodule size, bacteroid number per cell, and nitrogen fixation activity was observed.

Ionic Fluxes and Second Messengers in Nod Factor Transduction

A very early response induced by Nod factors in root hairs is the transient activation of ionic fluxes. In a few seconds, Ca^{2+} influx followed by Cl^{-} efflux provoke a transient depolarization of the plasmalemma compensated for by extrusion of K^{+} (reviewed in Downie and Walker 1999; Schultze and Kondorosi 1998). Pharmacologic approaches have also revealed that inhibitors of Ca^{2+} influx or phospholipase C antagonists block Nod factor–induced *ENOD12* gene expression (Pingret and others 1998). A later response concerns oscillations in the concentration of intracellular calcium in root hairs treated with Nod factors (Ehrhardt and others 1996). The frequency, amplitude, and timing of these oscillations may be important for decoding Nod factor stimulation. Thus Ca^{2+} is likely to be a second mes-

Table 1. Summary of the Identified Transcription Factors Related to Nodulation

Transcription factor	Domain	Promoter target	Function	Reference
GmNAT2		Leghemoglobin	?	Jensen and others 1988
VsENBP1	GRP, Cys-rich	ENOD12	Repressor	Christiansen and others 1996 Hansen and others 1999
Msnmh7	MADS box	?	?	Heard and Dunn 1995
Msnmh5				Heard and others 1997
GmNdx	Homeobox	?	?	Jorgensen and others 1999
	His-binding	?	?	Covitz and others 1998
	Leu-zippers	?		Gyorgyey and others 2000
Mszpt2-1	Krüppel-like Zn-finger	?	Nodule organogenesis	Frugier and others 2000
LjPZF	RING Zn-finger	?	?	Schauser and others 1995
GmPZF				
Ljnin	Leu-rich	?	Nodule initiation	Schauser and others 1999

senger involved in root hair deformation, perhaps by enabling exocytosis of new wall material into the root hair apex (Kropf and others 1998). Interestingly, the gene *MtAnn1* corresponding to an annexin, proteins binding both Ca^{2+} and phospholipids, was shown to be induced early after infection (De Carvalho-Niebel and others 1998).

Protein Kinases, Phosphatases, and Transcription Factors

Protein kinase and phosphatase genes (for example, alfalfa MAP kinases [Hirt 1997], a soybean protein kinase [Feng and others 1993], a protein phosphatase 2C induced in mature *L. japonicus* nodules [Szcyglowski and others 1997]) have been isolated from nodules by different techniques, including systematic sequencing of cDNA libraries (for example, Gyorgyey and others 2000). Previously, nodule-specific protein kinase activities, Ca^{2+}-dependent, and phosphorylating nuclear factors were detected in soybean nuclear extracts (Suzuki and Verma 1989). In addition, a novel type of protein kinase induced in young and spontaneous nodules has been cloned in alfalfa (*Mspk1;* Frugier and others 1998). However, the involvement of these proteins in nodulation is still under investigation.

Transcription factors involved in nodulation were investigated using promoter *cis* elements controlling nodulin expression, to clone *trans*-acting factors. Alternatively, cDNAs coding for putative transcription factors such as MADS box-containing genes, *Ndx* homeobox genes, homeodomain and zinc-finger proteins, have been isolated from root and nodule libraries and shown to be induced during nodule development. Table 1 shows a summary of the identified transcription factors related to nodulation.

Functional analysis of the Krüppel-like zinc finger, *Mtzpt2-1,* was carried out in *M. truncatula.* Antisense plants exhibited a Fix^- phenotype caused by an arrest of bacteroid development (Frugier and others 2000). *Mtzpt2-1* transcripts were detected in vascular tissues from roots and nodules, suggesting that this gene plays a role in a noncell-autonomous process acting in the nitrogen-fixing region. Finally, the *nin* gene was recently cloned using gene tagging (Schauser and others 1999). It codes for a putative transcription factor induced in nodule primordium cells. The Nod^-*nin* mutant did not form infection threads or nodule primordia, but its root hairs reacted to Nod factors, placing *nin* downstream of the early signal exchange between symbionts. Nodule inception (NIN) contains membrane-spanning helices and a nuclear localization signal, suggesting that, similar to Notch and SREBEP regulators from animals, a membrane-bound NIN in target cells may be proteolytically cleaved to release a fragment able to relocalize in the cell nucleus after bacterial infection (Schauser and others 1999).

Target Genes: Cell cycle and Nodulin Genes

It is still very difficult to link the steps between Nod factor perception and induction of target genes, such as cell cycle or nodulin genes. The early nodulin genes, the targets of transduction pathways, may, in turn, participate in the activation of downstream genes. Nevertheless, few data are available on the role of early nodulins in nodule development. The

absence of *ENOD12* genes in a diploid subspecies of *M. sativa* did not evoke any detectable phenotype on development or symbiotic fixation of these plants (Csanadi and others 1994), indicating that this gene is not essential for nodulation. Another early nodulin gene that has been extensively studied is *ENOD40*. The *ENOD40* genes code for RNAs (around 700 base pairs) that contain only short ORFs in their sequences (10–37 amino acids). Modeling predicted that these genes code for highly structured RNAs, suggesting that they may act as riboregulators or regulatory RNAs (Crespi and others 1994). In addition, Asad and others (1994) showed that *ENOD40* mRNA did not bind to polysomes. On the other hand, van de Sande and others (1996) presented data suggesting that a small peptide encoded in a small ORF yielding a primary translation product of 10 to 13 amino acids is the active gene product. However, not enough experimental data have accrued to support either hypothesis definitively. Thus, the mechanism of action of this gene is still unclear.

ENOD40 is rapidly induced on inoculation in the pericycle and within inner (Kouchi and Hata 1993; Yang and others 1993), and outer cortical cells ahead of infection threads (Fang and Hirsch 1998). In mature nodules, expression is detected in vascular bundles of roots and nodules and, in addition, in indeterminate nodules, in the nodule meristem, as well as in differentiating cells. Expression was also detected in stem vascular tissues at specific stages of development, lateral roots and flowers from legumes and nonlegumes such as rice and tobacco (Asad and others 1994; Fang and Hirsch 1998; Kouchi and others 1999; van de Sande and others 1996) indicating that *ENOD40* function is not exclusively associated with nodulation. Overexpression of this gene either stably or transiently resulted in the induction of spontaneous cortical cell division in the root inner cortex in *Medicago* spp. (Charon and others 1997). In addition, *ENOD40*-overexpressing plants showed accelerated nodulation accompanied by extensive proliferation of cortical cells. Among these transgenic plants, two lines showing cosuppression of this gene formed few nodules with arrested meristems, strongly suggesting that *ENOD40* function is required for nodule morphogenesis (Charon and others 1999).

Cell cycle genes are also targets of Nod factor signaling. Transcription of genes associated with the S and M phases of the cell cycle, such as *cdc2,* histones, and mitotic cyclins (Yang and others 1994) was induced in inner cortex cells by application of Nod factors and rhizobia. However, in outer cortical cells, only induction of histone *H4* could be detected after inoculation, indicating that these reactivated cells remain blocked in G2. This arrest might be required for construction of the PITs (van Brussel and others 1992). However, susceptibility to rhizobial infection does not correlate with a specific blockage of the cell cycle as previously proposed (Yang and others 1994). This means that other mechanisms might exert spatial control of cell division during nodulation (Cohn and others 1998). Another cell cycle gene that may be involved in nodulation is *ccs52* (for *c*ell *c*ycle *s*witch), which is activated at later stages of development (Cebolla and others 1999). This gene seems to be involved in the control of endoreduplication, a process occurring during differentiation of the central symbiotic zone, because it codes for a WD40 cell cycle regulator involved in mitotic cyclin degradation.

We are only beginning to understand the molecular mechanisms governing nodule differentiation. A summary of the function of selected plant genes in different steps of nodule development is presented in Figure 3. The isolation of Nod factor receptors will allow identification of elements implicated in Nod factor signal transduction pathways (for example, their putative interaction with G-proteins). Great effort is also being dedicated to establish changes in global patterns of gene expression occurring in both symbiotic partners during nodulation in model *Rhizobium*-legume interactions by using microarray technologies; the goal is to identify key genes controlling symbiosis. Concerning the plant partner, several kinds of experimental approaches are being used in parallel to identify genes involved in nodule development. First, random sequencing programs of BAC clones containing identified nodulation molecular markers are providing a growing amount of genetic information on the plant partner. Second, several insertion mutagenesis strategies (transposon tagging, T-DNA transformation) are being used to generate mutant plant pools for screening symbiotic nodulation phenotypes. This will allow a rapid identification of the affected gene. Finally, several already identified regulatory plant genes (for example, *nin, Mszpt2-1*) have homologs in *Arabidopsis thaliana*. Thus, molecular mechanisms in which they participate can be analyzed taking advantage of the knowledge already available on this model plant. Such information might then be useful to determine the molecular elements implicated in nodule development in legume plants.

The multiple approaches devoted to research on

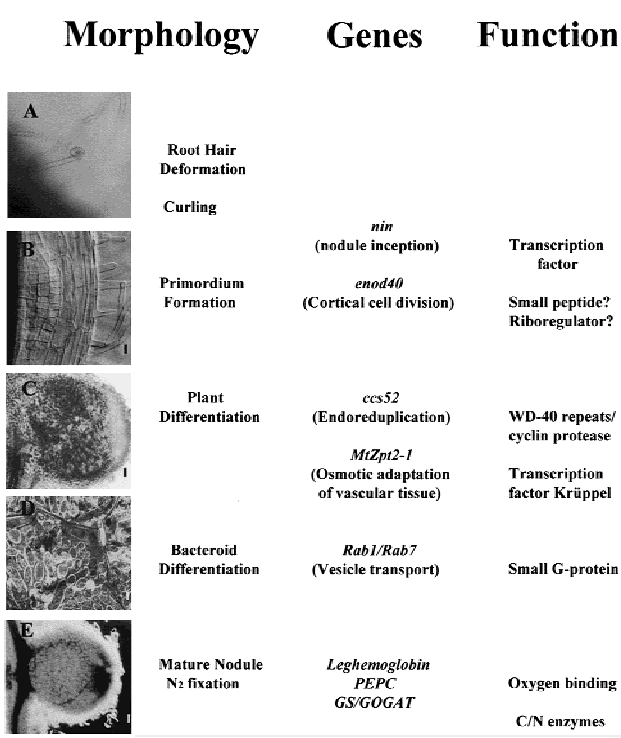

Figure 3. Function of selected genes involved in different steps of nodule development. The different morphologic steps involved in nodule development are illustrated. **(A)** Root hairs are deformed and curled. Bar = 40 µm. **(B)** As root hairs curl, cortical cells actively divide to form the nodule primordium. The *nin* gene, encoding a transcription factor containing a transmembrane domain, is required for nodule inception. Division of cortical cells involves a process depending on *ENOD40.* Bar = 20 µm. **(C)** Plant differentiation requires cell enlargement accompanied by endoreduplication, a process controlled in part by *ccs52,* a WD40-repeat cell cycle regulator. Expression of *Mtzpt2-1* in the vascular tissue is required to allow proper bacteroid development. Bar = 0.4 cm. **(D)** Differentiated bacteroids are surrounded by a peribacteroid membrane whose growth requires vesicle transport mediated by *Rab1/Rab7* genes encoding small G-proteins. Bar = 2 µm. **(E)** In the mature nodule, nitrogen fixation takes place and carbon/nitrogen metabolism enzymes are fully active. Oxygen transport into the bacteroids is mediated by leghemoglobins. Bar = 0.4 cm.

nodule symbiosis predict a rapid growth of this field in the near future. Dissection of molecular mechanisms involved in root nodulation will provide exciting perspectives for understanding plant organogenesis and differentiation.

ACKNOWLEDGMENTS

We are grateful to Drs. Adam Kondorosi, Michael Hodges, and Ann Hirsch for critical reading of the manuscript. We are indebted to Nathalie Mansion for excellent photographic assistance. We apologize to authors whose work has not been cited because of space constraints. SG was supported by the European Union Biotechnology program FIXNET N° PL962319.

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