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Martin Crespi and Florian Frugier (9 December 2008)
Science Signaling **1** (49), re11. [DOI: 10.1126/scisignal.149re11]

DEVELOPMENTAL BIOLOGY

De Novo Organ Formation from Differentiated Cells: Root Nodule Organogenesis

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Published 9 December 2008; Volume 1 Issue 49 re11

The symbiotic interaction between *Rhizobium* bacteria and legume plants leads to the induction of a new developmental program: the formation of nitrogen-fixing root nodules. Nodulation is triggered by specific bacterial signals, the Nod factors, and integrates plant developmental regulatory pathways to reactivate differentiated cortical cells. This results in the formation of a de novo meristem, corresponding to a plant stem cell niche. Recent data have shown a crucial function of the phytohormone cytokinin and its signaling pathway in nodule initiation. Activation of either cytokinin or components of the Nod factor signaling pathway leads to spontaneous induction of the nodule organogenesis program. These genetic analyses have been complemented with genomic studies of transcriptional networks activated during early nodulation. Transcriptional and posttranscriptional regulation, notably involving transcription factors and microRNAs, fine-tune the dynamic equilibrium between proliferating meristematic and differentiated nitrogen-fixing cells. The recent identification of these regulatory mechanisms has helped elucidate nodule organogenesis and the agriculturally relevant process of symbiotic nitrogen fixation and extended our understanding of how differentiated root cells acquire developmental plasticity to form a new organ.

Introduction

A major difference between plant and animal development is that positional information, rather than cell lineage, determines cell fate in plants (*1*). Plant cells are characterized by a high degree of totipotency, such that differentiated cells can dedifferentiate, proliferate, and redifferentiate with a new identity. Postembryonic plant development is driven by stem cells in the apical regions of shoots and roots, which are called apical meristems. This allows plants, which are sessile organisms, to adapt their morphology and development to prevailing environmental conditions. For example, the root system and its spatial configuration (number and length of lateral organs), the so-called root architecture, vary greatly depending on the plant species, soil composition, and particularly on the availability of water and mineral nutrients (*2*, *3*).

In legumes, soil conditions and interactions with symbiotic microorganisms are major determinants of root architecture. Legumes can develop two types of secondary root organs: lateral roots and nitrogen-fixing nodules. The latter develop in response to symbiotic interactions with soil bacteria known as rhizobia, which, after internalization inside the root nodule, differentiate into a nitrogen-fixing form, the bacteroids, that is able to convert atmospheric nitrogen into ammonia (*4*). These nitrogen-fixing nodules allow legumes to grow without the addition of nitrogen-containing fertilizers to the soil and thus represent a major contribution of legumes to sustainable agriculture.

Formation of the symbiotic nodule (nodulation) in legumes provides a model system for studying cell dedifferentiation and acquisition of a new identity. The presence of symbiotic rhizobia at the root surface leads to nodule organogenesis at regions where growing root hairs develop. Nodule organogenesis and symbiont colonization result from a molecular dialog involving flavonoid signals, produced by the plant host and found in root exudates, and lipochitooligosaccharidic signaling molecules called Nod factors, which are synthesized and secreted by specific soil rhizobia. Perception of these bacterial signaling factors by the plant triggers a series of morphological and physiological changes in root hairs, including depolarization of the plasma membrane; generation of an oscillatory calcium signal (calcium spiking); remodeling of actin filaments near the root hair tip; induction of gene expression; and root hair deformation, curling, and branching (*4*, *5*). These changes in the root hairs enable the bacterial colonization of the host plant. Nod factor perception by the host plant and the process of bacterial infection have recently been reviewed (*6*, *7*); here, we focus mainly on regulatory mechanisms that control the formation and differentiation of the root nodule.

Concomitant with rhizobial infection of root hairs, Nod factors stimulate distant cells of the root pericycle layer to undergo cytoskeletal rearrangement and transient proliferation (*8*). Cortical cells near the infection point and close to a specific location in the vascular bundles (a protoxylem pole) divide to establish the nodule primordium, a mass of rapidly proliferating undifferentiated cells (Fig. 1). In *Medicago truncatula* and other temperate legumes, inner cortical cells dedifferentiate and proliferate, whereas in *Lotus japonicus* and other tropical legumes outer cortical cells are recruited for this process (*4*). Simultaneously, bacteria penetrate the root tissues and progress toward the primordium inside channels called infection threads, whose formation is primarily host-driven (*9*). In tropical-type nodules, the meristematic activity of the nodule occurs only at early stages of organogenesis, leading to round-shaped nodules with determinate growth. The meristem is transient, and all of the primordia cells differentiate into mature nitrogen-fixing nodule cells. In temperate legumes, however, the growing primordium develops a new stem cell niche or meristem at its tip, and this leads to indeterminate growth. Consequently, continuous differentiation of cells derived from persistent meristematic activity allows coexistence of various developmental zones inside mature nodules: an invasion zone (zone II) below the meristematic apical zone (zone I), followed by a nitrogen fixation zone (zone III) and a senescence zone (zone IV).

De Novo Formation of the Root Nodule Organ: An Interplay Between Bacterial and Plant Signals

Nod factor perception by the host root initiates epidermal infection and stimulates the cortical cell divisions that give rise to the first cells of the new root-derived organ. Thus, application of Nod factors mimics the initial steps of this interaction and

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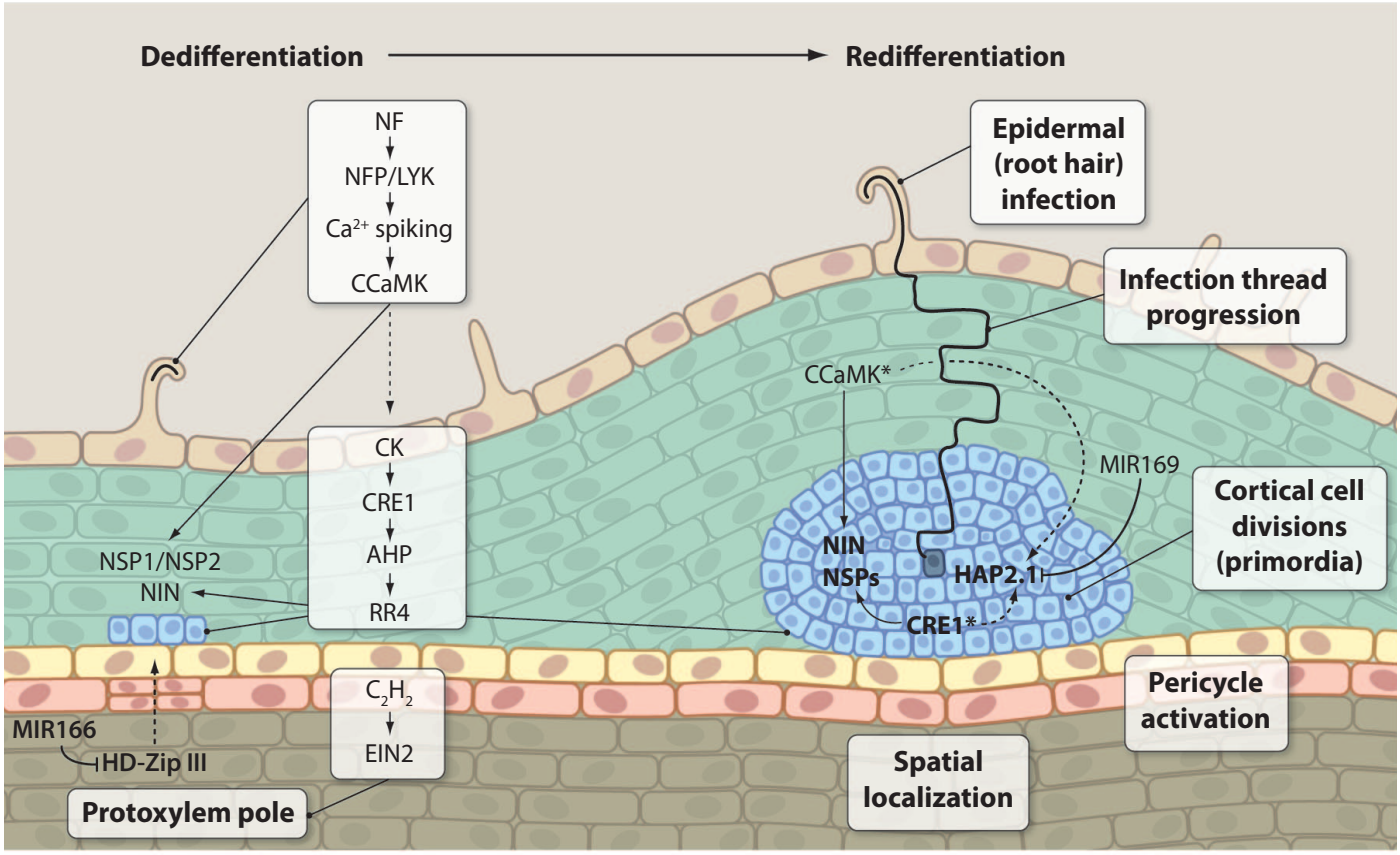
Fig. 1. Regulatory pathways associated with nodule organogenesis. Dedifferentiation and redifferentiation of root cortical cells leads to formation of a nodule primordium as schematized here for a *M. truncatula* indeterminate nodule. Root cell layers from top to bottom are epidermis (brown), including root hair cells, cortex (green), endodermis (yellow), pericycle (red), and stele, including vascular bundles (dark brown). Cells that form the nodule primordium are depicted in blue. Root cortical cells close to the bacterial infection site (curled root hair) and to a specific position of the vascular bundles (a protoxylem pole) dedifferentiate (left). Rhizobial infection then occurs through curled root hairs, and bacteria progress across root cell layers inside infection threads (in black) to finally infect plant cells in the growing primordium (right scheme). Redifferentiation of the meristematic cells takes place to form a functional nitrogen-fixing nodule. Nod factor (NF) perception through LysM kinase complexes (NFP/LYK) in the epidermis (and likely during the infection process) lead to nodule initiation. Nod factor signaling involves calcium spiking, which may be decoded by the kinase CCaMK, thereby activating the NSP1/NSP2 and NIN transcription factors. Perception of cytokinin (CK) phytohormones by the CRE1 histidine kinase receptor, and subsequent signaling, likely through a multistep phosphorelay involving AHPs (aspartate-histidine phosphotransfer proteins), lead to A-type response regulator (such as RR4) and NIN activation. Both pathways determine dedifferentiation of specific root cortical cells to form the initial cells that give rise to the nodule. In addition, vascular tissues express HD-Zip III transcription factors which are regulated by MIR166 at posttranscriptional level, and activate ethylene (C_2H_2) signaling through the EIN2 regulator. These pathways control nodule localization close to protoxylem poles and nodule number. Signaling downstream of both cytokinins and Nod factors leads to spontaneous nodulation as shown by dominant alleles of CCaMK and CRE1 kinases (asterisks, right). Activation of downstream transcriptional networks participating in the redifferentiation of the dividing cortical cells into nodule cell types involves NIN, NSPs, and HAP2.1. The regulation of the meristem-specific HAP2.1 transcription factor by MIR169 contributes to the differentiation of the nodule primordia cells. Arrows indicate functional links, primarily based on genetic data; lines indicate in which tissue the pathway acts; and dashed lines with arrows indicate hypothetical relationships.



elicits the formation of nodulelike structures in some legumes (*4*, *7*). However, other bacterial surface components, such as exopolysaccharides or lipopolysaccharides, are required for the elongation of infection threads and further stages of nodulation (*6*). Bacteria lacking *nodABC* genes, which are required for Nod factor synthesis, can develop a successful symbiotic interaction with some legumes that leads to nodule organogenesis. Indeed, two photosynthetic *Bradyrhizobia* species use an alternative pathway, which may involve purine derivatives, to establish nodules on the stems of the sensitive joint vetch (*Aeschynomene sensitive*) (*10*).

The identification of mutants with defects in nodulation has enabled the dissection of the Nod factor signaling pathway (*4*, *6*, *7*, *11*); here, we review only selected key genes that may be involved in cortical events leading to nodule organogenesis (Table 1). Further, we focus on genetic studies carried out in the two model legumes *M. truncatula* and *L. japonicus*.

Early responses to Nod factor, such as root hair curling and deformation and cal-

cium spiking, are defective in the *L. japonicus Nod factor receptor* mutants *nfr1* and *nfr5* and in *M. truncatula Nod factor perception* (*nfp*) mutants (*12*–*14*). The genes associated with these mutants encode transmembrane LysM-type serine/threonine receptor kinases (LYKs), which may act as heterodimeric Nod factor–binding complexes. The expression patterns of LYK genes suggest that different LYK complexes located in distinct root cell layers may play diverse roles during the symbiotic interaction (*14*, *15*) from initial infection thread development to bacterial release in plant cells inside the differentiated nodule. Other components of the signaling pathways that act downstream of Nod factor recognition include the genes implicated in the *M. truncatula doesn't make infection* mutants (*dmi1*, *dmi2*, and *dmi3*). The *MtDMI1* and *MtDMI2* genes (and their orthologs in *L. japonicus*, CASTOR/POLLUX and SYMRK, for symbiosis receptor kinase) respectively encode a putative cation channel and a leucine-rich-repeats receptor-like kinase (called NORK in alfalfa, for nodulation receptor kinase) and are required for calcium spiking and nodulation (*16*–*19*). The *M. truncatula dmi3* mutant, which carries a loss-of-function mutation in a gene that encodes a calcium- and calmodulin-dependent protein kinase (CCaMK) (*20*, *21*) located in the nucleus (*22*), responds to Nod factors with calcium spikes but shows defects at later stages of the symbiotic interaction. The possible activation of MtDMI3 by both calcium and calmodulin suggests that CCaMK may integrate calcium spiking into the Nod factor signaling pathway by decoding spiking amplitude and frequency, thereby leading to nodule organogenesis (Fig. 1). The role of the *L. japonicus* and *M. truncatula DMI* genes is not restricted to nodulation; mutants are also defective in establishing mycorrhizal interactions (*16*, *17*, *19*, *20*, *23*, *24*), MtDMI2/NORK has been linked to the epidermal response to "touch" stimulation (*25*), and MtDMI3 to growth-promoting bacterial interactions (*Pseudomonas fluorescens*) (*26*).

Evidence that nodule organogenesis is controlled by the plant rather than the bacterial symbiont comes from the identification of mutants producing nodule organs in the absence of *Rhizobium* (NAR phenotype) or spontaneous nodules (*27*). In *M. sativa* (alfalfa) plants, these spontaneous nodules possess all the histological features characteristic of indeterminate nodules: an apical meristem, peripheral vascular bundles connected to the root vascular system, endodermis, and surrounding layers of

Nodulation phenotype	Mutant name	Plant species	Mutated gene	Gene expression	Protein localization	Additional phenotypes	Selected references
nod-	*nfr1*	*Lj*	LysM RK	Roots			(*14*)
	nfr5	*Lj*	LysM RK				(*13*, *14*)
	nfp	*Mt*	LysM RK	Roots and infection zone of nodules			(*12*)
nod-	*dmi2*	*Mt*	LRR RK	Roots and infection zone of nodules	Plasma membrane, infection thread membrane	*myc-* Enhanced root hair response to touch	(*25*)
	symrk	*Lj*	LRR RK				(*19*)
	nork	*Ms*	LRR RK				(*18*)
nod-	*dmi1*	*Mt*	Ion-channel protein	Roots and infection zone of nodules	Nuclear envelope	*myc-*	(*16*)
	pollux	*Lj*	Ion-channel protein	Various organs; stronger in nodules	Plastid localization		(*17*)
	castor	*Lj*	Ion-channel protein	Various organs	Plastid localization		(*17*)
nod-	*dmi3*	*Mt*	CCaMK	Roots and nodules	Nucleus	*myc-*	(*20*)
						Pseudomonas fluorescens defective perception	(*26*)
snf	*snf1 (gof)*	*Lj*	CCaMK	Roots and nodules			(*29*)
nod-	*nsp1*	*Mt*	GRAS-family TF	Preferentially in roots	Nucleus		(*55*)
	nsp1	*Lj*	GRAS-family TF	Roots and nodules			(*57*)
	nsp2	*Mt*	GRAS-family TF	Various organs and induced by Rhizobia	Nuclear envelope, endoplasmic reticulum		(*22*)
	nsp2	*Lj*	GRAS-family TF	Roots and nodules			(*57*)
nod-	*hit1*	*Lj*	Cytokinin receptor LHK1	Roots, nodules, and shoots			(*36*)
snf	*snf2 (gof)*		Cytokinin receptor LHK1	Roots, nodules, and shoots			(*31*)
nod-	*nin*	*Lj*	Putative TF	Nodules			(*58*)
	Nin	*Mt*	Putative TF	Nodules			(*59*)
nod+/–	*bit1/pdl*	*Mt*	AP2/ERF family TF (ERN1)	Induced by Rhizobia		Aberrant or aborted infection threads	(*60*)
nod++	*sunn*	*Mt*	CLAVATA1-like RK			Nitrate tolerant, shortened primary root	(*41*)
	har1	*Lj*	CLAVATA1-like RK	Ubiquitous		Nitrate tolerant, increased density of lateral root, shorter primary root	(*42*, *43*)
	nark	*Gm*	CLAVATA1-like RK			Nitrate tolerant, increased density of lateral roots	(*44*)
	sickle	*Mt*	EIN2			Increased in root length, delayed petal and leaf senescence, ethylene insensitivity	(*39*, *40*)
	astray	*Lj*	bZIP TF (HY5)			Pleiotropic effects on photomorphogenesis	(*62*)

Table 1. Mutants affected in nodule development. *bit*, branching infection threads; *Gm*, glycine max; *har*, hypernodulation and aberrant root; *hit*, hyperinfected; HY, long HYpocotyl; *Lj*, *Lotus japonicus*; *Ms*, *Medicago sativa*; *Mt*, *Medicago truncatula*; *nark*, nodule autoregulation receptor kinase; *nod-*, defective in nodulation; *nod+/-*, nodules initiate but rapidly abort; *nod++*, hypernodulation; *nork*, nodulation receptor kinase; RK, receptor kinase; *skl*, sickle; *sunn*, super numeric nodules; *symrk*, symbiosis receptor kinase; TF, transcription factor.

cortex. Like *Rhizobium*-induced nodules, these structures express early nodulation markers (such as *MsEnod2* and *MsEnod40*). Spontaneous nodules have also been identified in *L. japonicus* through a genetic screen (*28*). As in *M. sativa*, these *spontaneous nodules formed* (*snf*) mutants show ontogeny and histology characteristic of *Rhizobium*-induced nodules, including induction of early nodulation markers and similar metabolic regulation (such as nitrate inhibition). Interestingly, the first *snf* mutant identified in *L. japonicus*, *snf1*, carries a mutation in a gene that corresponds to the previously mentioned Nod factor signaling component DMI3, leading to production of a CCaMK protein with an alteration in the autophosphorylation site of the kinase domain (*29*). Consistent with this, modification of the MtDMI3 autoinhibitory domain induces formation of spontaneous nodules in *M. truncatula* (*30*). Activation of this proposed integrator of Nod factor signaling is therefore sufficient to dedifferentiate cortical cells and trigger the initial stages of nodule organogenesis (Fig. 1), linking specific components of this pathway to cortical events.

Further insight into the endogenous pathways regulating nodulation emerged with identification of the genetic lesion in the *snf2* mutant, a gain-of-function mutation of the *LHK1* (*lotus histidine kinase 1*) gene, which is closely related to the *Arabidopsis* gene encoding the AHK4/CRE1 (authentic histidine kinase 4/cytokinin response 1) cytokinin receptor (*31*). This indicates that cytokinins are necessary and sufficient to initiate nodule organogenesis (Fig. 1). Physiological studies showing that exogenous application of cytokinins can induce cortical cell division or even nodulation under certain conditions suggested that these hormones were involved in nodule organogenesis (*32*). Accordingly, manipulation of the cytokinin pool through overexpression of a catabolic enzyme (a cytokinin oxidase) reduces nodulation (*33*). Furthermore, a cytokinin signaling pathway that involves a histidine kinase receptor similar to *AHK4*/*CRE1* and specific cytokinin primary responsive genes, the A-type response regulators (RR), is activated during nodulation both in *L. japonicus* and *M. truncatula* (*33*–*35*). Based on RNA interference (RNAi) analysis of the three *M. truncatula* cytokinin receptors, only the MtCRE1 histidine kinase (orthologous to LHK1) could be linked to the control of root sensitivity to cytokinins and nodulation ability (*35*). Early stages of the symbiotic interaction, such as growth of infection threads in the epidermis and induction of cortical cell division, are blocked by RNAi specifically directed against root MtCRE1. The *L. japonicus hit1* (*hyperinfected 1*) mutant, which has a loss-of-function mutation in the LHK1 gene, also shows inhibited nodulation; however, the *L. japonicus* phenotype is additionally associated with increased growth of infection threads (*36*). Crosstalk between Nod factors and cytokinin signaling pathways has been identified: Cytokinin regulation of early nodulation markers (such as *NIN*; see below) depends on MtCRE1/LHK1 (*31*, *35*, *36*), and, conversely, rhizobial induction of certain cytokinin signaling response regulator (*MtRR*) genes depends on Nod factors (*35*) (Fig. 1).

Collectively, these data suggest that, similar to the constitutively active CCaMK *snf1* allele, constitutive activation of LHK1/MtCRE1-dependent cytokinin signaling is sufficient to trigger cortical cell activation and nodule organogenesis. A genetic cross between the *snf1* and *snf2* mutants revealed an additive effect on spontaneous nodulation, whereas a cross of the *snf2* gain-of-function allele with the loss-of-function *ccamk* allele still allows spontaneous nodulation to occur. This indicates either that a CCaMK-dependent Nod factor pathway activates LHK1-dependent cytokinin signaling or that crosstalk between both signaling pathways exists downstream of CCaMK and LHK1. A critical question concerns how Nod factors regulate distant effects on pericycle and cortical cells. In contrast to epidermal events associated with the infection process, which are likely directly activated by these bacterial signals, an intermediate messenger may be necessary to drive inner cortical cell activation, and it is tempting to speculate that cytokinins may provide such a signal. Differences in plant hormonal controls in tropical and temperate legumes may also explain the activation of outer versus inner cortical cells or determinate versus indeterminate nodule growth. Similar to the reported broad role of DMI genes in root responses to the environment, reducing cytokinin content or signaling mediated by MtCRE1 also lead to phenotypes associated with processes unrelated to symbiosis. Such phenotypes may be developmental, such as increased lateral root formation (*35*), or related to environmental interactions, such as reduced ability to form nematode-induced root galls (*33*). The fact that cytokinins promote nodulation but repress lateral root formation suggests that this signaling pathway may integrate the regulation of different root-derived meristems to adapt root architecture to environmental conditions (*37*). In this context, it is worth noting that stimulatory effects of Nod factors on lateral root formation have also been reported (*23*), a phenotype that has been linked to changes in root phytohormonal imbalances occurring in response to these symbiotic signals. The existence of these single mutations leading to the formation of spontaneous nodules suggests that minor changes in gene regulation could be sufficient to trigger a complex organogenesis in plants.

A crucial step in the regulation of nodule organogenesis has been linked to the control of nodule number by the plant host. Indeed, only mutations in plant (but not bacterial) genes have been identified as causing a supernodulation phenotype, suggesting that the plant host controls nodule number (*38*). The ethylene-insensitive *M. truncatula sickle* mutant, in which the ethylene regulator EIN2 (ethylene insensitive 2) ortholog is mutated, is hyperinfected by its rhizobial symbiont and shows increased nodulation (*39*, *40*). Hence, ethylene inhibits rhizobial infection in *M. truncatula* and also determines the spatial localization of nodule primordia (Fig. 1). In addition to this local ethylene effect, other mechanisms control nodule number through long-distance communication between shoots and roots (*38*). The *L. japonicus har1* (*hypernodulation and aberrant root 1*) and *M. truncatula sunn* (*super numerous nodules*) mutants show increased nodulation and reduced root growth, phenotypes that grafting experiments showed to depend on the shoot genotype (*41*–*44*). The genetic lesions in these supernodulation mutants occur in a receptor kinase called nodule autoregulation receptor kinase (NARK) (*44*) expressed mainly in leaf tissues, suggesting that perception of a mobile signal transported through vascular tissues from roots to shoots is likely involved in nodule autoregulation. This receptor kinase is closely related to *CLAVATA 1*, which regulates the size of the shoot apical meristem in *Arabidopsis* and recognizes the *CLAVATA 3* signaling peptide (*45*). This suggests that signaling peptides may be involved in the long-distance regulation of nodule meristem size and number. However, the ligand for the NARKs remains to be identified. Long-distance auxin transport from shoot to root was shown to be increased in the *sunn* mutant (*46*), possibly leading to greater auxin accumulation in roots and hypernodulation. In addition, certain ethylene effects on nodulation might involve the

local regulation of polar auxin transport in roots (*47*). Plant flavonoids have been proposed to act as secondary signals that could mediate the action of Nod factors on polar auxin transport (*48*). Indeed, RNAi silencing in *M. truncatula* and soybean of genes involved in the flavonoid pathway led to roots deficient in flavonoid content, exhibiting reduced nodulation and increased auxin transport compared to control roots (*49*, *50*). However, in soybean, the ability of flavonoids to modulate auxin transport does not seem essential for nodulation (*50*).

Nodule initiation and regulation of nodule number may also involve short-distance cell-to-cell communication. This communication is mediated by plasmodesmata (PD), channels that span cell walls and connect the cytoplasm of neighboring plant cells (*51*). Regulation of PD-mediated trafficking through vascular tissues seems to be pivotal to communication between all of the plant meristems (*52*). PD-mediated communication between the phloem and nodule primordia has been analyzed in *Medicago* species (*53*) by using fluorescent tracers and specific expression of green fluorescent protein (GFP) in phloem tissues (*54*). After *Rhizobium* inoculation, GFP could be detected in the initial dividing cortical cells that give rise to the nodule primordia. GFP fluorescence preceded cortical cell division and was concomitant with a rearrangement of the PD network in these initial cells. Macromolecular PD-mediated communication between the Nod factor–stimulated cells and the rest of the plant through the vascular tissues may be crucial for this de novo organogenesis (*53*).

Transcriptional Regulatory Networks Determining Root Nodule Differentiation

The existence of mutations that lead to spontaneous nodule formation suggests that a single change in a component of the nodulation signaling pathways (such as activation of a specific kinase) can recruit a preexisting developmental program to form a new root-derived legume-specific organ. In this case, mapping the transcriptional networks downstream of Nod factors and cytokinins would provide crucial insights into the mechanisms involved in nodule organogenesis. Components of such networks have been identified by characterizing *M. truncatula* and *L. japonicus* nodulation mutants with mutations in genes that encode transcription factors (Table 1).

The *M. truncatula nsp1* and *nsp2* mutants (*nodulation signaling pathway 1* and *2*) show wild-type calcium spiking but inhibition of infection thread growth, reduced expression of early nodulation markers, and no initiation of nodule primordia (*22*, *55*) (Fig. 1). Genetic analyses carried out with *snf1* and *snf2* mutants indicated that the NSP proteins act downstream of CCAMK and CRE1/LHK1 (*29–31*). The *M. truncatula MtNSP1* and *MtNSP2* genes respectively expressed preferentially in roots or in all vegetative organs, encode putative GRAS-family transcription factors (named for the founding members GAI, RGA, and SCARECROW) (*22*, *55*). Expression of *LjNSP1*, *LjNSP2*, and *MtNSP2* is induced after rhizobial inoculation or Nod factor treatment (*22*, *55–57*), in contrast to *MtNSP1*. MtNSP2 is predominantly localized to the nuclear envelope and endoplasmic reticulum and translocates to the nucleus after such treatments, whereas MtNSP1 and LjNSP2 are located in the nucleus.

Another gene that probably encodes a transcriptional regulator is *NIN* (for *nodule inception*) (*58*, *59*); indeed, NIN contains membrane-spanning helices and a nuclear localization signal, similar to Notch and SREBP (sterol responsive element binding protein) transcription factors in animals. The exact function of NIN and how it is activated, however, remain unknown. The *nin* mutants show excessive root hair curling, abortive infection, and no cortical cell division or formation of nodule primordia. *LjNIN* expression is up-regulated during nodulation, and its transcripts are found in dividing cells of nodule primordia as well as in different tissues of the mature nodule (vascular bundles and the nitrogen-fixing zone) (*58*). Genetic analyses in *L. japonicus* placed *LjNIN* downstream of CCaMK and LHK1 (*29*, *31*). Accordingly, *LjNIN* and *MtNIN* are up-regulated by cytokinins and Nod factors (*35*, *36*), implicating this gene in the regulation of cortical events (Fig. 1). Like NSPs, NIN may mediate crosstalk between Nod factors and cytokinin signals. Unlike *nsp* mutants, however, *nin* mutants show epidermal activation in response to Nod factors, suggesting that NIN may be a positive regulator of cortical responses (*7*). Abortive infection could therefore be a secondary effect of the lack of cortical cell division and nodule organogenesis. This indicates that cortical cell division and primordium formation may be necessary to achieve rhizobial infection.

The *bit1/pdl* alleles (*branching infection threads 1/poodle*) carry a loss-of-function mutation in the *ERN* (for *ERF required for nodulation*) gene, which encodes MtERN1, a transcription factor in the AP2/ERF family (apetala 2/ethylene response element) (*60*) that acts downstream of CCaMK-dependent Nod factor signaling. Formation of infection threads and Nod factor–induced gene expression are blocked in the *bit1* mutant, but nodule initiation takes place. Hence, this transcription factor seems to be specifically associated with Nod factor–dependent epidermal infection rather than cortical cell division and nodule organogenesis. A parallel study showed that MtERN1, 2, and 3 bind the Nod factor responsive regulatory unit (the NF box) present in the promoter of the early nodulation marker *MtENOD11* (*61*). Another symbiosis mutant affected in a transcription factor, *astray*, shows additional pleiotropic shoot and root phenotypes (*62*). The corresponding mutated gene, *LjBzf*, is closely related to the *Arabidopsis HY5* gene and acts not only as an early negative regulator of nodule development but also affects responses to light and gravity, notably in roots. However, no information concerning its relationship with Nod factor and cytokinin signals is available.

Most of the downstream Nod factor signaling pathway components identified as transcriptional regulators are up-regulated by Nod factors, cytokinins, or both. This means that genetic and transcriptomic approaches can be complementary for the identification of genes involved in nodule organogenesis. Nodulation requires the precise spatiotemporal expression of specific plant host genes called nodulin genes, which were historically defined as differentially expressed between (non–rhizobial-infected) roots and nodules. Several strategies have been used in diverse legumes to identify differentially expressed genes in infected root hairs or in nodules at various stages of their organogenesis, yielding a large number of molecular markers with specific temporal and spatial expression patterns. Recently, transcriptomic approaches such as serial analysis of gene expression (SAGE) (*63*), microarray profiling (*34*, *64*, *65*), and substractive hybridization (*66*) have been developed in the model legumes *L. japonicus* and *M. truncatula* and an ATLAS of gene expression for *M. truncatula* has been developed with data from Affymetrix chips (http://bioinfo.noble.org/gene-atlas/) (*67*). Transcription factors differentially expressed during

nodulation have been found with each of these approaches, but only a few [for instance, MtZPT2-1 (*68*), MtHAP2.1 (*69*), and LjERF1 (*70*)] have been functionally characterized by reverse-genetic approaches (such as antisense or RNAi). Additional transcription factors have been identified as potentially involved in nodulation based on their ability to interact with known cis elements from nodule marker promoters [(*61*) and reviewed in (*71*)], but a clear role for these factors in nodulation remains to be established. Comparative genomic approaches involving transcription factor mutants could contribute to dissecting the complex regulatory networks controlled by these genes and to identifying target promoters. At present, characterizing transcription factor complexes, target promoters, and biological activities to establish reliable gene networks involved in nodule organogenesis remains a challenge.

The dynamics of the transcriptome depends not only on gene transcription but also on mRNA stability. This latter process plays an important role in the differentiation of eukaryotic cells and is often regulated by small noncoding RNAs called microRNAs (miRNAs). In plants, miRNAs regulate various targets, including transcription factors (*72*), posttranscriptionally by affecting their stability, their translation, or both (*73*). Transcription factors and miRNAs are the major trans-acting regulators that determine the dynamic equilibrium of transcriptional networks at each developmental stage (*74*). In animals, miRNAs tend to have highly cell-type specific expression profiles that appear late in development, and it has therefore been proposed that miRNA action may be biased toward controlling the terminal differentiation of individual cell types (*75*). miRNAs and targets expressed in mutually exclusive domains may confer robustness to developmental programs, whereas coexpression of miRNAs and their targets may buffer fluctuations in target expression, thereby contributing to the coordinated flow of developmental processes (or canalization) (*75*).

M. truncatula HAP2.1 is among the very few genes expressed in the nodule meristematic cells known to be down-regulated once differentiation starts. This gene encodes a transcription factor showing a tight gradient of down-regulation along the differentiation zone of the nodule (*69*). *MtHAP2.1* expression is spatially controlled by MIR169 and decreasing its expression using either RNAi or overexpression of MIR169 similarly blocks nodule differentiation (*69*). Abolishment of MIR169-mediated posttranscriptional regulation (using a miRNA-resistant version of *MtHAP2.1* mRNA) leads to delayed nodule development, likely caused by misregulated activity of the nodule meristem. In this case, MIR169 may confine the expression of the *HAP2.1* gene to the meristematic cells, and rapid posttranscriptional degradation may allow their differentiation (Fig. 1).

More recently, MIR166 has been shown to posttranscriptionally regulate a family of HD-Zip III (homeodomain-leucine zipper class III) transcriptional factors expressed in vascular tissues and associated with nodule formation (*76*). Overexpression of MIR166 down-regulates expression of at least three members of this family during nodule differentiation and, concomitantly, affects root vascular tissue patterning. Both initiation of symbiotic nodules and lateral roots are impaired in these MIR166-overexpressing roots, possibly because of the mispatterning of vascular bundles [including protoxylem poles (Fig. 1)]. MIR166 also regulates vascular development in *Arabidopsis* shoots (*77*) and may have a general role in regulating formation of secondary organs. Hence, miRNAs contribute to the spatial and temporal regulation of transcription factor action in nodule organogenesis during the dedifferentiation (nodule initiation) and reacquisition (transition from meristem to differentiated cells) of cellular identities. The diversity of these small RNA regulatory molecules suggests that novel roles for miRNA-mediated posttranscriptional regulation of nodule organogenesis may be identified in the future, including roles for legume-specific miRNAs.

Concluding Remarks

Root nodule organogenesis results in the formation of a de novo meristem from differentiated cortical cells. Dissection of the molecular mechanisms involved implicates specific perception of bacterial Nod factors and plant host signals such as cytokinin phytohormones and activation of specific signaling genes, including kinases and transcription factors. These transcriptional networks may be fine-tuned by miRNA-mediated posttranscriptional regulation. Interestingly, similar sequences to most of the regulatory genes required for nodulation have been identified in nonlegume plants that are able to interact with mycorrhizal fungi, a symbiotic interaction very common across the plant kingdom that has been shown to control lateral root formation and phytohormone action. These functions may have thus been recruited from preexisting developmental programs into the nodule organogenesis process (*78*). Thus, reorganization of regulatory patterns controlling root architecture, rather than the generation of new proteins by domain shuffling, may have been critical for the evolution of nodule organogenesis in legume roots. Interactions between endogenous and bacterial signals in different root cell layers may define the complex transcriptomes of each developmental transition: dedifferentiation of cortical cells, formation of a new meristem, and redifferentiation into nodule cell types. These gene networks assure the coordinated expression of specific metabolic and effector genes required for the formation of a functional nitrogen-fixing organ.

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10.1126/scisignal.149re11

Citation: M. Crespi, F. Frugier, De novo organ formation from differentiated cells: Root nodule organogenesis. *Sci. Signal.* **1**, re11 (2008).