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Genetics and functional genomics of legume nodulation

Gary Stacey[1], Marc Libault[1], Laurent Brechenmacher[1], Jinrong Wan[1] and Gregory D May[2]

Gram-negative soil bacteria (rhizobia) within the Rhizobiaceae phylogenetic family (α-proteobacteria) have the unique ability to infect and establish a nitrogen-fixing symbiosis on the roots of leguminous plants. This symbiosis is of agronomic importance, reducing the need for nitrogen fertilizer for agriculturally important plants (e.g. soybean and alfalfa). The establishment of the symbiosis involves a complex interplay between host and symbiont, resulting in the formation of a novel organ, the nodule, which the bacteria colonize as intracellular symbionts. This review focuses on the most recent discoveries relating to how this symbiosis is established. Two general developments have contributed to the recent explosion of research progress in this area: first, the adoption of two genetic model legumes, *Medicago truncatula* and *Lotus japonicus*, and second, the application of modern methods in functional genomics (e.g. transcriptomic, proteomic and metabolomic analyses).

Addresses
[1] National Center for Soybean Biotechnology, Divisions of Plant Science and Biochemistry, University of Missouri, Columbia, Missouri 65211, USA
[2] Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, Oklahoma 73401, USA

Corresponding author: Stacey, Gary (staceyg@missouri.edu)

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Introduction

Nodulation is a highly host-specific interaction in which, with few exceptions, specific rhizobial strains infect a limited range of plant hosts. Plants secrete (iso)flavonoids that are recognized by the compatible bacteria, resulting in the induction of nodulation genes. These nodulation genes encode enzymes that synthesize a specific lipochitin nodulation signal (Nod signal), which activates many of the early events in the root hair infection process [1–5]. During the infection process, the bacteria enter the plant via the root epidermis and induce the reprogramming of root cortical cell development and the formation of a nodule. In the most well-studied cases, infection occurs through root hairs. The first observable event in the infection process is the curling of the root hair, which likely occurs through the gradual and constant reorientation of the direction of root hair growth. The bacteria become enclosed within the root hair curl where the plant cell wall is degraded, the cell membrane is invaginated and an intracellular tubular structure (i.e. the infection thread) is initiated. It is within this infection thread that the bacteria enter the root hair cell and eventually ramify into the root cortex. Before the infection thread reaches the base of the root hair cell, the root cortical cells are induced to de-differentiate, activating their cell cycle and causing them to divide to form the nodule primordium. In addition to the cortical cells, pericycle cells are also activated and undergo some cell divisions. When the infection thread reaches the cells of the developing primordium, the bacteria are released into cells via endocytosis. Inside a plant cell, the bacteria are enclosed in vacuole-like structures (symbiosomes) in which they differentiate into bacteroids. It is within these symbiosomes that the bacteria convert N_2 to NH_3. The nodule is a true organ in which there is cellular specialization. For example, in addition to infected plant cells, uninfected plant cells also carry out the function of nitrogen assimilation and a well-developed symplastic transport system allows the exchange of nutrients between the nodule and peripheral vascular tissue.

Large gaps remain in our understanding of root hair infection by rhizobia owing, in part, to the considerable amount of attention focused only on the pre-infection signaling events. Even here, current understanding is largely observational with relatively little information available on molecular mechanisms (c.f. [1]). More recently, significant advances have been made through the analysis of a variety of plant mutants in which early infection is blocked.

Mutants in which nodulation is defective have begun to reveal the pathway of Nod signal recognition

The establishment of *Lotus japonicus* and *Medicago truncatula* as legume genetic model systems has greatly expedited discoveries concerning the nodulation process [6,7]. Analysis of nodulation-defective plant mutants (Table 1) has resulted in the development of a rudimentary pathway for Nod signal recognition (Figure 1).

The *Nod*- (defective in nodule formation) mutants cannot form nodules. Perhaps the most interesting are the *L. japonicus nfr1* and *nfr5* mutants, formerly known as *sym1*

Table 1

Mutants affected at different stages of early nodule development.

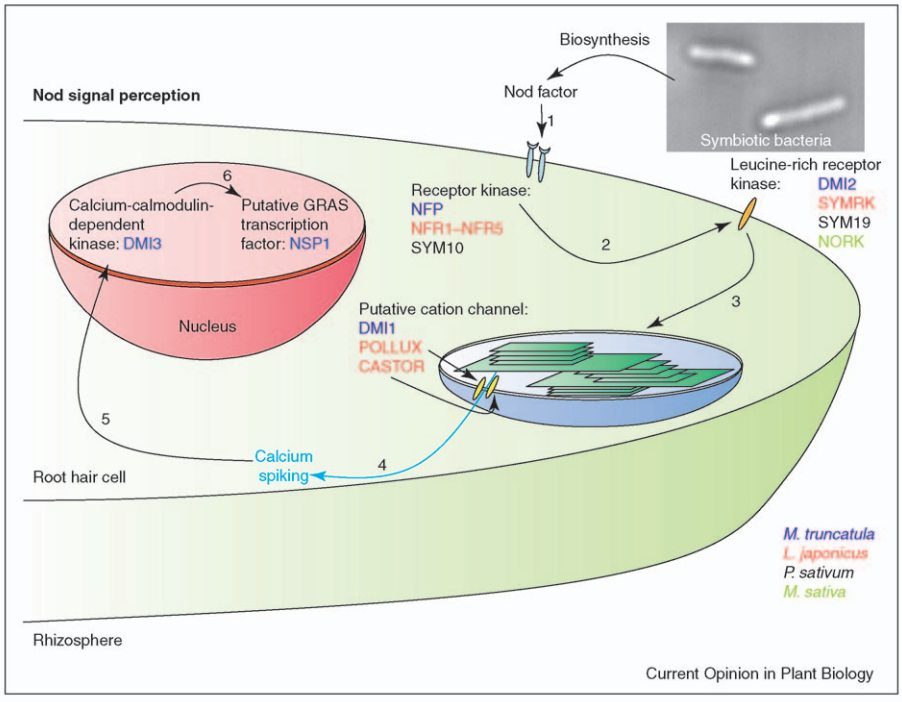
Mutant blocked	Mutants	Genes mutated	Plant species	Reference(s)
At Nod factor perception	*nfr1*, *nfr5*	LysM RK	*L. japonicus*	[8,9••]
	nfp	LysM RK	*M. truncatula*	[10]
	sym10	LysM RK	*P. sativum*	[8]
After calcium flux, root-hair deformation	*dmi2*	LRR RK	*M. truncatula*	[17]
	symrk	LRR RK	*L. japonicus*	[19]
	nork	LRR RK	*M. sativa*	[17]
	sym19	LRR RK	*P. sativum*	[21]
	dmi1	Ion-channel protein	*M. truncatula*	[13]
	pollux	Ion-channel protein	*L. japonicus*	[22••]
	castor	Ion-channel protein	*L. japonicus*	[22••]
After calcium spiking	*dmi3*	CCaMK	*M. truncatula*	[14••]
	nsp1	GRAS-family TF	*M. truncatula*	[28]
	nsp2	GRAS-family TF	*M. truncatula*	[27]
	nin	Putative TF	*L. japonicus*	[29]
	sym35	Putative TF	*P. sativum*	[30]
	hcl	Unknown	*M. truncatula*	[25]
After root hair curling, infection thread and nodule-like formation	*lin*	Unknown	*M. truncatula*	[32]
	crinkle	Unknown	*L. japonicus*	[33]
	alb1	Unknown	*L. japonicus*	[34]
At controlling of nodule number	*har1*	CLAVATA1-like RK	*L. japonicus*	[35]
	sym29	CLAVATA1-like RK	*P. sativum*	[35]
	nark	CLAVATA1-like RK	*G. max*	[37]
	sunn	CLAVATA1-like RK	*M. truncatula*	[38]
	sickle	Unknown	*M. truncatula*	[40]
	klavier	Unknown	*L. japonicus*	[42]
	astray	bZIP TF	*L. japonicus*	[43]

Abbreviations: *alb1*, *aberrant localization of bacteria inside the nodule1*; CCaMK, calcium-calmodulin-dependent protein kinase; *dmi*, *doesn't make infections*; *ers1*, *ethylene receptor sensitive1*; *har1*, *hypernodulation aberrant root 1*; *hcl*, *hair curling*; *lin*, *lumpy infections*; *nark*, *nodule autoregulation receptor kinase*; *nfp*, *nod factor perception*; *nfr*, *nod-factor receptor kinase gene*; *nin*, *nodule inception*; *nsp*, *nodulation signaling pathway*; RK, receptor kinase; *sunn*, *super numeric nodules*; *sym*, *symbiotic*; TF, transcription factor.

and *sym5*, which appear devoid of early Nod signal perception events. The *NFR1* and *NFR5* genes were cloned and shown to encode transmembrane LysM-type serine/threonine receptor kinases (LysM RLKs) [8,9••]. The LysM domain is the peptidoglycan-binding motif found in many bacterial peptidoglycan-binding proteins (Figure 2). Peptidoglycan is a linear chain of β 1→4 N-acetylmuramic acid and N-acetylglucosamine and, therefore, is structurally related to chitin (a β-1→4 linked polymer of N-acetylglucosamine). Therefore, although biochemical proof is still lacking, the general consensus is that NFR1 and NFR5 are the long-sought receptors that interact directly with the lipo-chitin Nod signal. Because NFR5 lacks a kinase activation motif (Figure 2), Radutoiu *et al.* [9••] proposed that the NFR1 and NFR5 proteins might form a heteromeric Nod-signal receptor. On the basis of sequence comparison, NFR1 and NFR5 also identify two unique clades of LysM RLKs (Figure 2). Similar mutants, *M. truncatula nfp* [10] and pea (*Pisum sativum*) *sym10* [8], which also block early Nod signaling, were identified and shown to encode LysM RLKs. The pea *sym2* allele from *cv. Afghanistan* has a phenotype that is interesting because it controls the recognition of a Nod signal that has a specific chemical modification (i.e. acetylation). Therefore, this allele was suspected early on of encoding a protein that is directly involved in Nod signal recognition. Indeed, the corresponding *SYM2* locus in *M. truncatula* was recently cloned and found to contain a cluster of LysM RLK genes, of which two (*LYK3* and *LYK4*) seem to be involved in nodulation [11]. Unlike *nfr*, *nfp*, and *sym10*, however, the *sym2* mutant shows some early Nod-signal-induced responses (e.g. calcium spiking). The suggestion was made, therefore, that LYK3 and LYK4 may function in Nod signal induction of infection thread formation, perhaps in Nod signal induction. These results suggest that Nod signal recognition is more complex than previously thought, involving a cascade of receptors that mediate early signaling events and then subsequent infection, which is mediated by the infection thread.

It is now clear, from the study of plant mutants that are defective in nodulation, that nodulation and mycorrhizal infection are related mechanistically. Kosuta *et al.* [12] reported a diffusible 'Myc signal' that could induce *MtENOD11* expression, which can also be induced by the lipo-chitin Nod signal. Thus, signals that are involved in both nodulation and mycorrhization, if not structurally

Figure 1



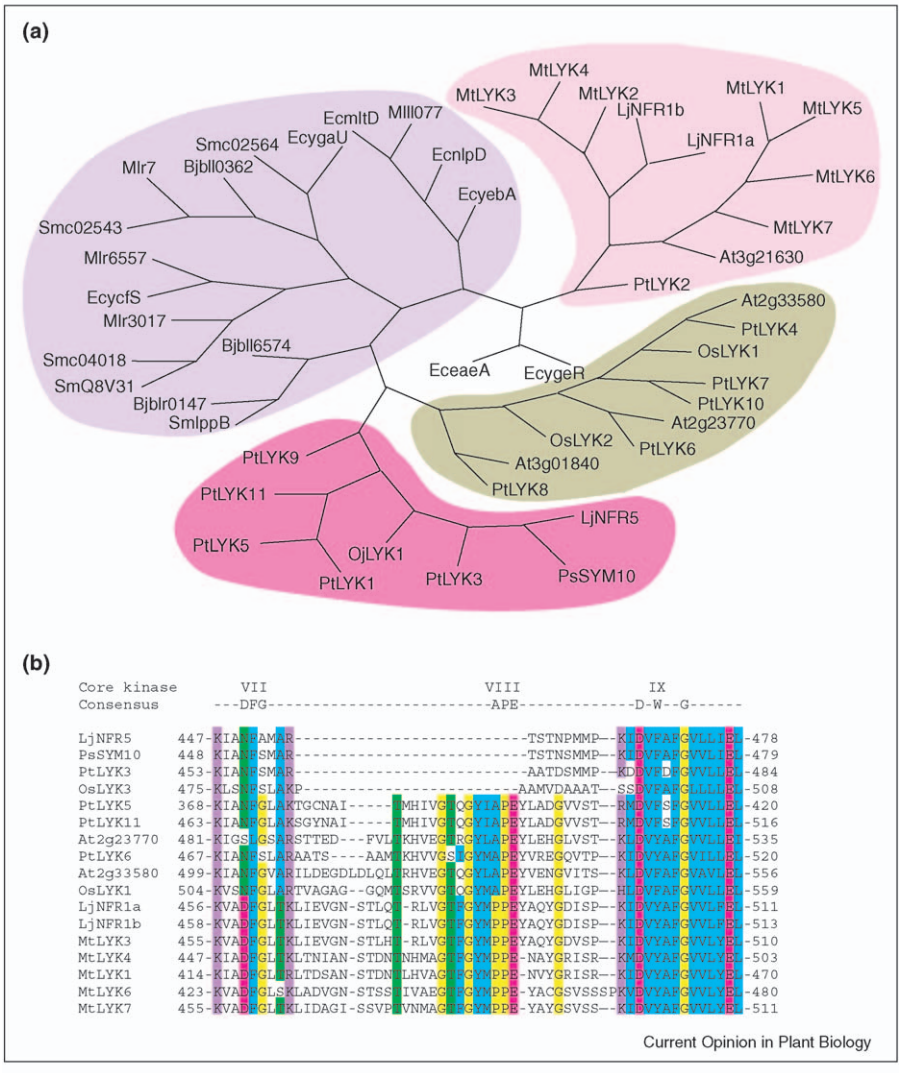
The postulated position and interaction between elements of the Nod signal recognition pathway. Colors indicate the various species from which the specific gene product was identified.

similar, might be functionally similar. Indeed, there appear to be initial branches of the infection pathway that are specific to either nodulation or mycorrhization. Other mutants appear to mark a central common infection pathway, that then diverges to pathways that are specific to either symbiosis. Examples of mutants that identify these common components are the *doesn't make infections* mutants (*dmi1*, *dmi2* and *dmi3*) of *M. truncatula* and related mutants in *L. japonicus* and pea [13,14••]. The response of *M. truncatula* plants to a diffusible 'Myc signal', produced by mycorrhizal fungi, was blocked in *dmi1*, *dmi2* and *dmi3* mutants [15,16]. The *dmi1* and *dmi2* genes were recently cloned and shown to encode a putative cation channel [13] and a leucine-rich repeat receptor kinase [17], respectively. Since calcium flux and root hair deformation still occur in the *dmi1* and *dmi2* mutants, but not in the *nfp* mutant, we know that DMI1 and DMI2 act downstream of NFP in the NF signaling pathway (Figure 1). Recently, DMI2 was shown to be located in the plasma membrane and in the infection thread membrane [18]. Indeed, *dmi2* mutants were shown to be defective in the formation of symbiosomes, indicating that the *dmi2* gene has a role beyond initial infection and Nod signal recognition. This work, as well as that suggesting a role for LYK3 and LYK4 in infection thread development, suggests that Nod signal recognition remains pertinent to the infection process beyond the initial penetration of the root by rhizobia.

Receptor kinases that are orthologous to DMI2 were also identified from *L. japonicus* (symbiosis receptor-like kinase [SYMRK]; [19,20]), *M. sativa* (nodulation receptor kinase [NORK]; [17]), and *P. sativum* (SYM19; [21]). When originally cloned, because of their phenotype, these leucine-rich repeat receptor-like kinases (LRR-RLKs) were suggested to be directly involved in Nod signal recognition. However, with the discovery of the LysM RLKs, it is now thought that they function downstream, perhaps via a branch pathway that is activated by the LysM RLKs (Figure 1), but this remains conjecture.

DMI1 orthologous proteins (e.g. POLLUX) were also identified from *L. japonicus* [20] and shown to act downstream of NFR1 and NFR5 and upstream of intracellular calcium spiking; as the branching and deformation of the root hairs still occur in the *pollux* mutant, but the hair curling, infection thread formation and calcium spiking are abolished [22••]. Another mutant, called *castor*, which has the same phenotype as *pollux*, was also identified in *L. japonicus*. Like POLLUX, the CASTOR protein has a plastid localization signal sequence. Therefore, Imaizumi-Anraku *et al.* [22••] proposed that CASTOR and POLLUX, both of which are predicted to encode ion-channel proteins, could form heteromeric complexes that are responsible for calcium ion flux between the plastids and cytosol (Figure 1).

Figure 2



Sequence analyses of plant LysM RLKs. **(a)** Phylogenetic analysis of the LysM domains of plant LysM RLKs. Protein sequences were extracted from the public databases and put through the Pfam web server to query the LysM domains. The derived LysM domain sequences were aligned using ClustalX (1.83) [73] before generating a parsimony tree using PAUP* version 4.0 Beta [74]. Plant LysM RLKs form two clades, which are represented by LjNFR1 and LjNFR5, and one undefined clade whose members might have a function other than nodulation. Species abbreviations are At, *Arabidopsis thaliana*; Bj, *Bradyrhizobium japonicum*; Ec, *Escherichia coli*; Lj, *Lotus japonicus*; Ml, *Mesorhizobium loti*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Ps, *Pisum sativum*; Pt, *Populus trichocarpa*. Sm, *Sinorhizobium meliloti*. **(b)** ClustalX alignment of kinase domains of plant LysM RLKs illustrating the absence of activation loop (motif VIII) in NFR5 and its homologs.

The *DMI3* gene was also cloned recently and predicted to encode a calcium-calmodulin-dependent protein kinase (CCaMK) [14••,23]. Unlike *dmi1* and *dmi2*, *dmi3* mutants are not defective in calcium spiking but are still blocked in nodulation. A similar phenotype is exhibited by *nodulation signaling pathway1* (*nsp1*) and *nsp2* mutants. Thus, the gene products encoded by the *NSP* genes are predicted to act downstream of NFR1/NFR5, DMI1 and DMI2 [14••]. Furthermore, genetic analyses placed *NSP1* and *NSP2* downstream of *DMI3* [24–26]. Very recently, the *NSP1* and *NSP2* genes were cloned and predicted to encode putative GRAS-family transcription factors [27,28]. Both NSP1 and DMI3 were shown to localize in the nucleus of epidermal and cortical root cells [28], whereas NSP2 was localized in the nuclear envelope and endoplasmic reticulum [27]. DMI3 was proposed to be responsible for transducing the Nod-signal-induced calcium spiking signal [23] and for controlling the expression (presumably through protein phosphorylation) of NSP1 [28]. Recent microarray work using RNA from these mutants demonstrates their crucial role in Nod-signal-induced gene expression.

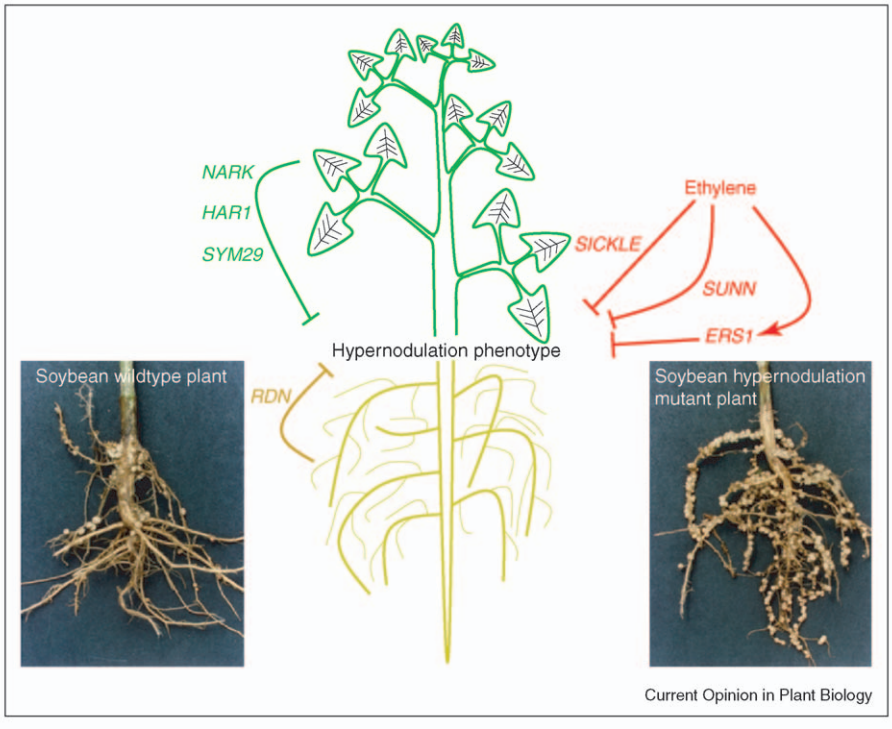
Other mutants that cannot form nodules have been identified. For example, the *nodule inception* (*nin*) mutant is able to perceive the Nod signal but is unable to form infection threads, nodule primordia or first cell divisions in the outer cortex. Therefore, this mutant is defective in nodule development or initial signal transduction [29]. The *NIN* gene is a putative transcription factor [29]. An apparent *NIN* gene ortholog is encoded by the *SYM35* locus in *P. sativum* [30]. The *M. truncatula hair curling* (*hcl*) mutant has a phenotype similar to that of the *nin* mutant. Although it shows root hair deformation in response to *Sinorhizobium meliloti* [31] and displays calcium spiking [25], *hcl* is unable to form infection threads or to generate nodule primordia [25] because of a reduction in cortical cell divisions [31]. The *hcl* mutant is defective in microtubule organization and, therefore, the HCL protein might control microtubule organization and the induction of root hair curling [31].

The *Histogenesis* (*Hist-*) mutants can perceive the lipochitin Nod signal and produce nodule-like structures, but these structures are defective in tissue differentiation. The *M. truncatula lumpy infections* (*lin*) mutant is defective in infection-thread formation in root cortical cells despite the fact that the early nodulation responses still occur in this mutant [32]. Therefore, LIN probably acts downstream of the first events in the Nod signaling pathway [32]. The *crinkle* mutant (*Ljsym79*) from *L. japonicus* is defective in plant development (i.e. trichome, seed pod, and root hair development) and exhibits abnormal nodulation because of an arrested infection thread in the epidermis [33]. The *aberrant localization of bacteria inside the nodule* (*alb1*) mutant (*Ljsym74*) is characterized by ineffective nodules that have enlarged infection threads full of bacteria and incomplete vascular bundles [34]. This phenotype suggests that the *ALB1* gene is involved in infection thread development, in the release of the bacteria from the infection thread and in the differentiation of vascular bundles in nodules [34].

Hypernodulation plant mutants

The hypernodulation mutants are capable of forming an excessive number of nodules when compared with wild-type plants (Figure 3). For example, the *L. japonicus sym78* mutant *hypernodulation aberrant root1-1* (*har1-1*) is a hypernodulation mutant [35]. The *HAR1* gene encodes a LRR-RLK that shares high homology with CLAVATA1, a protein that controls shoot apical meristem identity [36]. Grafting experiments between HAR1-defective and wildtype plants showed that hypernodulation is shoot controlled and led to the postulation of a shoot-localized ligand that controls nodule number [35]. Indeed, hypernodulation appears to be generally shoot-controlled in mutants of this type. However, the *M.*

Figure 3



The involvement of various gene products and other factors in the negative and positive regulation of legume hypernodulation. This phenotype is primarily shoot controlled but root-controlled hypernodulation was reported recently.

truncatula root-determined nodulation (*rdn*) hypernodulating phenotype was recently found to be under the control of the root (J Frugoli, pers. comm.).

HAR1 homologous proteins in soybean (nodule autoregulation receptor kinase [NARK]) [37], in pea (SYM29) [35] and in *M. truncatula* (super numeric nodules [SUNN]) [38] are also required for the shoot-controlled regulation of nodule number. The conservation of this communication between the shoot and the root via CLAVATA1-like proteins suggests that plant growth and development directly affect symbiosis.

Ethylene is a known inhibitor of nodulation and, in addition to showing hypernodulation, the *M. truncatula SUNN* and *SICKLE* mutants are insensitive to this hormone (reviewed in [39]). Analysis of a *sunn skl* double mutant suggested that these two genes act in separate pathways [40]. Ethylene appears to control the spatial success of infections and the formation of the nodule primordium. Expression of the negative-dominant melon ERS1 ethylene receptor in transgenic *L. japonicus* conferred ethylene insensitivity with a concomitant increase in infection thread formation [41•]. According to expression analysis, ethylene perception by ERS1 affects the expression of *NIN* but not of *SYMRK* [41•].

The *klavier* mutant of *L. japonicus* is hypernodulated but also shows a variety of other phenotypes (e.g. changes in leaf morphology, delayed flowering, and dwarfism) [42]. Another *L. japonicus* mutant, called *astray* (*sym77*), which can form twice the number of nodules formed by the wildtype, is also affected in non-symbiotic processes, such as gravitropism and photomorphogenesis. The recent cloning of *ASTRAY* [43] revealed it to be an ortholog of the *Arabidopsis* gene *HY5*, which encodes a bZIP transcription factor that is involved in photomorphogenesis. As more genes that generally affect nodulation are cloned, it is likely that additional functions will be revealed that affect both nodule formation and general plant developmental processes.

Did the Nod-factor signaling pathway evolve from a more ancient chitin signaling pathway?

The base structure of the Nod signal is a short chitin polymer (of 3–6 residues). It is therefore similar to the chitin polymers that can be hydrolytically released from the cell walls of plant pathogenic fungi. Such chitin polymers are well characterized as potent elicitors of plant defense responses in a wide variety of plants, and chitin perception appears to be evolutionarily well conserved in flowering plants. Indeed, Day *et al.* [44] showed that the Nod signal from *Bradyrhizobium japonicum* (the symbiont of soybean) is a weak elicitor of plant defense responses in soybean. Given this similarity, how does the lipo-chitin Nod signal induce a beneficial nodulation response, whereas chitin induces a strong defense response that is detrimental to plant–microbe infection?

The structural similarity between the Nod signal and chitin oligomers clearly suggests a possible relationship between the plant perception mechanisms for these signals. Indeed, like the early events in the nodulation process, chitin oligomer elicitors induce transient membrane depolarization, ion and calcium flux, reactive oxygen generation, and downstream gene expression (reviewed in [45]). Could the chitin recognition systems of non-legumes recognize the lipo-chitin Nod signal? This might indeed be the case as the promoter of the early nodulin genes (e.g. *ENOD12*) can be induced by exogenously added Nod signal and chitin oligomers in both legumes and non-legumes (e.g. [46]).

As discussed above, two putative GRAS transcription factors, NSP1 and NSP2, were recently shown to be directly involved in the regulation of gene expression in response to the Nod signal [27,28]. Similarly, two rice GRAS genes, *CHITIN-INDUCIBLE GIBBERELLIN-RESPONSIVE1* (*CIGR1*) and *CIGR2*, were also found to be induced directly by chitin oligomers and the rice blast fungus but not by phytopathogenic bacteria [47]. Like NSP1 and NSP2, CIGR1 and CIGR2 are localized in the nucleus. The data suggest that these two rice GRAS proteins act to regulate gene expression in the chitin signaling pathway. It will be interesting to see if more parallels arise as the signaling pathways for the Nod signal and chitin elicitors are elucidated. It is possible that the more ancient chitin signaling pathway gave rise to the Nod signaling pathway, perhaps by gene duplication and divergence. If so, then one would predict that the chitin oligomer receptor, which is involved in plant defense, will be a member of the LysM RLK family. Indeed, LysM RLKs that have high sequence similarity to the putative Nod signal receptor are found throughout the plant kingdom, often in plant species that are not known to interact with rhizobia (Figure 2). It is also likely that the postulated receptor that recognizes the diffusible 'Myc factor' also derives from this same origin.

Functional characterization of nodule biology through -omics approaches

The application of DNA microarray and transcript profiling studies to nodulation is relatively recent compared with the various profiling studies conducted on *Arabidopsis*. However, the large numbers of expressed sequence tags (ESTs) available for legume plants, in addition to cDNA, oligonucleotide and Affymetrix microarrays, have made large-scale transcriptomic studies on nodulation possible. Second-generation high-density arrays also contain probe features that allow the parallel expression of both host and symbiont genes. Simultaneous expression profiling of large numbers of genes facilitates the identification of novel candidate genes that might be implicated

in nodulation [23,48••,49••,50,51•]. Several nodule transcription profiling studies identified numerous genes whose relative transcript abundance levels are modulated during the interaction between the roots of various legume species and their corresponding rhizobia. A significant number of resources are available to support functional genomic studies of various legumes (Table 2).

The genes identified in these studies include not only various nodulin genes previously shown to be regulated in the nodulation process but also many new genes [49••,52–54]. For example, genes encoding proteins that are involved in various metabolic pathways, such as nitrogen and carbon metabolism, were found to be upregulated in *L. japonicus*, *M. truncatula* and *G. max* nodules [48••,49••,50,52–54,55•]. Numerous genes coding for transporters (sugar, peptide, nitrate and H^+-ATPase transporters) were also shown to be upregulated in *M. truncatula* and *L. japonicus* nodules [48••,49••,50,52,53,55•], suggesting an important role for these transporters in the exchange of carbon sources and nitrogenous compounds between legumes and rhizobia. In addition, a large number of genes that are involved in signal transduction (e.g. genes encoding receptor kinases, calmodulins, kinases, and phosphatases), as well as genes coding for transcription factors (such as Myb transcription factors and zinc-finger proteins), are upregulated in *L. japonicus* and in *M. truncatula* nodules [49••,52,53]. Various defense-related genes were also regulated during the nodulation process; for example, genes coding for enzymes of the phytoalexin biosynthesis pathway (i.e. phenylalanine ammonia lyase [PAL] and chalcone reduc-

Table 2

Web based genomic resources for legumes.

Database (URL)	Data resource	Represented organism(s)
The Legume Information System (http://comparative-legumes.org/)	EST, genome, QTL and comparative maps	*Glycine*, *Medicago*, *Lotus* and *Phaseolus*
The Institute for Genomics Research (www.tigr.org)	EST, genome, repeat sequence and pathways	*Glycine*, *Medicago* and *Lotus*
NCBI (www.ncbi.nlm.nih.gov)	EST, genome and expression	*Glycine*, *G. soja*, *Medicago*, *M. sativa*, *Lotus* and *Phaseolus*
MtDB–CCGB (www.medicago.org/MtDB/)	EST and genome	*Medicago*
Medicago EST Navigation System (MENS) (http://medicago.toulouse.inra.fr/Mt/EST/)	EST and pathways	*Medicago*
OpenSputnik Comparative genomics platform (http://sputnik.btk.fi/ests)	EST, BLAST and SNP	*Glycine*, *Medicago*, *Lotus* and *Phaseolus*
PlantGDB (www.plantgdb.org)	EST and BLAST	*Glycine*, *Medicago*, *M. sativa*, *Pisum*, *Arachis* and *Phaseolus*
SoyBase (http://soybase.ncgr.org)	EST, genome, QTL and genetic maps, pathways, germplasm and literature	*Glycine*
Sequencing *M. truncatula*, University of Oklahoma (http://www.genome.ou.edu/medicago.html)	Genome and BLAST	*M. truncatula*
Medicago Genome Database (http://mips.gsf.de/projects/medicago)	MIPS genome	*M. truncatula*
SIU Soybean Genome (http://soybeangenome.siu.edu/)	EST, QTL, physical map, FPC contigs and markers	*Glycine*
Kazusa *Lotus japonicus* (www.kazusa.or.jp/lotus/)	EST, genome and genetic map	*Lotus*
M. truncatula Consortium (www.medicago.org/genome/)	Linkage maps, BAC overlap and clone/marker data	*M. truncatula*
Soybean Functional Genomics (Vodkin) (http://soybeangenomics.cropsci.uiuc.edu/)	Transcriptomics	*Glycine*
Soybean Genomics and Microarray Database (http://psi081.ba.ars.usda.gov/SGMD/Default.htm)	Transcriptomics	*Glycine*
Noble Foundation (Sumner) (www.noble.org/2DPage/Search.asp)	Proteomics	*Medicago*
M. truncatula Functional Genomics and Bioinformatics (http://medicago.vbi.vt.edu/)	Transcriptomics, proteomics, metabolomics, pathways and literature	*Medicago*
Mt Proteomics (http://www.mtproteomics.fr.st/)	Proteomics	*Medicago* and *Sinorhizobium meliloti*
Australian National University 2D-PAGE Database (http://semele.anu.edu.au/)	Proteomics	*Medicago*
AlfaGenes (http://ukcrop.net/perl/ace/search/AlfaGenes)	EST, genetic map and pathways	*M. sativa*
BeanGenes (http://beangenes.cws.ndsu.nodak.edu/)	Genetic map, gene classification, pathology and cultivar data	*Phaseolus* and *Vigna*
CoolGenes (http://ukcrop.net/perl/ace/search/CoolGenes)	Genetic map	*Cicer* and *Lens*

Abbreviations: BAC, bacterial artificial chromosome; FPC, fingerprint contig; QTL, quantitative trait loci ; SNP, single nucleotide polymorphism; SSR, single sequence repeat.

tase), genes coding for proteins that are involved in cell wall modifications (β-1,3-glucanase and peroxidase) and pathogenesis-related (PR) genes coding for PR10 and a chitinase. Interestingly, these defense-related genes were upregulated during the early stage of nodulation and then decreased in nodules [23,50,52,53], suggesting that the invading rhizobia suppress the host's defense strategies to successfully colonize roots and so form nodules. However, a large set of genes encoding nodule-specific cysteine-rich proteins, which have a putative role as antimicrobial defensins, was upregulated in *M. truncatula* nodules [48••,56], but their specific metabolic function is unknown.

Relatively few studies have used DNA microarray analysis to examine the gene expression responses in rhizobial mutants that are blocked at various stages of nodulation. As might be expected, Mitra *et al.* [23] found no transcriptional response of *M. truncatula* Nod⁻ mutants (i.e. *nfp*, *dmi1*, *dmi2*, *dmi3*, *nsp1* and *nsp2*) to inoculation by *S. meliloti*. Consistent with its phenotype, the *hcl* mutant showed a reduced response to inoculation. However, the Affymetrix array used for these experiments contained only 9935 *M. truncatula* genes and, therefore, a full genome array might yield different results. (A version of the Affymetrix *Medicago* GeneChip genome array featuring over 50 000 *M. truncatula* EST/mRNA-based and gene-prediction-based probe sets is currently available.) Suganuma *et al.* [51•] utilized a macroarray representing 18 432 non-redundant gene clones to analyze the response of *L. japonicus* to a *M. loti sen1* mutant, which forms non-nitrogen-fixing nodules. The results revealed 18 genes that were significantly upregulated in the mutant nodules. This set of genes was enriched in genes encoding hydrolase enzymes, which might be involved in the senescence of the nodule tissue. Another 30 genes were significantly downregulated in the mutant nodules. Among these were genes encoding several known nodulins and enzymes in carbon and nitrogen metabolism, perhaps reflecting the differing energy and nitrogen environment in non-fixing nodules.

In addition to DNA-microarray-based transcriptomic studies, a recent serial analysis of gene expression (SAGE) study on *L. japonicus* revealed that 11 antisense tags were induced during nodulation, suggesting that post-transcriptional gene regulation might also play a role in the nodulation process [55•].

As already discussed above, the rhizobial and mycorrhizal symbioses with legumes share common crucial components in their pathways. Interestingly, with the exception of a few genes that encode nodulins and proteins involved in metabolism, cell wall modifications, signal transduction, and host defense, microarray comparisons revealed only limited overlap in gene regulation between the symbioses in *M. truncatula* and *L. japonicus* [20,57–59].

Given that many biological phenomena lack the requirement for *de novo* gene transcription, proteomic approaches facilitate the investigation of changes in steady-state protein levels and posttranslational modifications that may occur during developmental processes. This lack of a transcriptional requirement can often lead to discrepancies between transcript and protein profiling results for the same gene and gene products. For example, Becker *et al.* [60] reported that fluctuations in the transcript levels in bacteroid and free-living forms of *S. meliloti* did not parallel changes in the abundance of the corresponding proteins. Proteomic investigations into nodule biology complement parallel transcription-profiling studies by revealing subtleties that might reflect differential levels of gene regulation.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has been used in protein profiling for the past 20 years. Current 2-D PAGE technology is capable of resolving 2000–10 000 proteins. Combined with 2-D PAGE, recent advances in mass spectrometry (MS) techniques and the establishment of protein databases have greatly facilitated protein identification.

Soybean, *Lotus* and *Medicago* have been the subject of numerous proteomic studies [61–63,64•]. These studies were targeted to various tissues during development or under biotic and abiotic elicitation. Detailed analyses of the sub-cellular proteome of *M. truncatula* root microsomal fraction identified 96 of 440 highly resolved proteins using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS [65]. Eighty-three percent of the proteins identified were obtained through comparisons with clustered *M. truncatula* EST sequences. Since the root hair is the site of initial rhizobial infection, Wan *et al.* [61] developed a method to isolate soybean root hairs in sufficient quantity for proteomic analysis. Their data identified 16 root hair proteins whose abundance increased within 12 h of inoculation by *Bradyrhizobium japonicum*. The accumulation of 11 proteins appeared to require the lipo-chitin Nod signal as they did not respond to inoculation with a *B. japonicum nodC* mutant, which is defective in Nod signal production.

Several recent studies focused proteomics methods on examining the changes in host and symbiont protein profiles during nodule development in *L. japonicus*, *M. truncatula*, and *Melilotus alba* [66–71]. Using nano-scale liquid chromatography, Wienkoop and Saalbach [66] analyzed proteins that are affiliated with the symbiosome membrane, which surrounds rhizobia in *L. japonicus* nodule cells, and identified nutrient-associated transporters, signaling proteins and proteins that are implicated in symbiosome biogenesis.

Studies to generate a proteome map of roots of five-day-old *M. truncatula* seedlings resolved approximately 2950

proteins [67]. Mass fingerprinting of peptides was performed on 485 of the most abundant, of which 179 were identified by comparison with EST databases. A majority of the proteins identified, of which PR10-class PR proteins were the most abundant, were metabolic enzymes, including proteins that are associated with flavanoid biosynthesis, or proteins associated with stress conditions. Approximately 44% of the proteins identified were isoforms and were not predicted on the basis of DNA sequence composition alone [67].

Attempts to establish a proteome map of *M. alba* nodules resolved 1700 proteins using silver-stained gels [71]. Of the 250 proteins upregulated in nodules, 180 were of bacterial origin. Approximately 100 nodule, bacterial, and bacteroid proteins were identified using amino-terminal amino-acid sequencing and MALDI-TOF MS. Seventy of the identified proteins were novel to nodule tissues. In this study, 20 root proteins were found to be downregulated upon nodule formation. Proteome comparisons between cultured bacteria and the bacteroid form were also made. Steady-state levels of abundance of approximately 350 proteins were lower in the bacteroid form than in cultured cells. Many of the downregulated proteins were associated with nitrogen acquisition, such as glutamine synthase and urease, whereas the relative abundance of 130 proteins was increased in the bacteroid form.

Metabolite profiling is essential to gaining a better fundamental understanding of how changes at the levels of transcription and translation affect cellular function. Metabolomic studies, unlike proteomics, require a variety of analytical techniques to profile the low-molecular-weight metabolites of the cell. Metabolic changes in both the host and symbiont occur during symbiotic nitrogen fixation. Until recently, legume metabolite studies were, in essence, targeted metabolite analyses that focused upon predetermined families of compounds, such as amino acids, sugars, and phenolics, with little emphasis placed upon the characterization of unknown compounds. A recent report by Desbrosses *et al.* [72] details protocols for the measurement and analysis of hundreds of *L. japonicus* metabolites using gas-chromatography-coupled MS. Mass spectral tag libraries that represent known and unknown metabolites were created for the flowers, leaves, roots and nodules of symbiotic plants. 'Marker metabolites' were identified for various *L. japonicus* plant organs, including nodules, using principle component analysis and hierarchical cluster analysis [72]. Nodule-enriched metabolites identified in this study include specific amino and organic acids, polyols, phosphates and nitrogen-containing compounds. Structural determination of unknown compounds will aid in unraveling novel biosynthetic pathways and reveal new opportunities in nodule biology.

One of the first studies to incorporate metabolic profiling with transcriptomics in the investigation of nodule development revealed several metabolic pathways that are coordinately upregulated in *L. japonicus* nodules [49••]. These pathways include glycolysis, carbon fixation, amino-acid biosynthesis, and purine and redox metabolism. Modulation of the transcript abundance of genes, and their regulators, that are associated with hypoxia, phosphate-limitation and osmotic stresses also revealed changes in the physiological status within nodules.

Conclusions

The adoption of model legumes for genetic analysis of nodulation has led to major advances in our understanding of the initial steps in Nod signal recognition and subsequent signaling. However, much remains to be elucidated concerning downstream events and the details of cellular processes that construct the infection thread and specialized nodule structures. We are in the early stages of integrating transcript, protein and metabolite data for the study of plant–microbe interactions. Advances in techniques such as laser-capture microdissection and improved micro-scale analytical methods will enable us to gain insight into cell-type specific changes at the single-cell level. There is still a need to complete the development of informatics tools for the processing, visualization and integration of -omics data, including genome sequence, across legume species (www.comparative-legumes.org). As evident from studies combining transcriptomics and metabolomics in *L. japonicus* [49••], these tools will provide correlative insight into global molecular changes in cellular physiology and will enhance our understanding of nodule biology. The ultimate goal is a detailed, system-wide understanding of the fascinating and complex events leading to legume nodule formation.

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