Unraveling the mystery of Nod factor signaling by a genomic approach in *Medicago trunactula*

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The availability of reduced nitrogen and phosphate are limiting factors in the productivity of many terrestrial ecosystems. The majority of land plants increase phosphate nutrition by developing intimate associations with beneficial mycorrhizal fungi. Legumes are unusual among plants because they also establish a symbiosis with nitrogen-fixing bacteria, known generally as rhizobia. A high level of host specificity characterizes symbiotic nitrogen fixation, but such specificity is not observed in mycorrhizal associations. Nevertheless, genetic analyses suggest that a common signaling pathway underlies rhizobial and mycorrhizal associations (1, 2). Until recently, the molecular nature of the plant symbiosis signaling pathway was unknown, but a spate of articles, including the report by Mitra *et al.* (3) in this issue of PNAS, have begun to unravel this mystery. The *dmi3* gene of *Medicago truncatula* described by Mitra *et al.* is necessary for both rhizobial and mycorrhizal interactions and is predicted to encode a calcium- and calmodulin-dependent protein kinase. The homology of DMI3 to calcium-regulated proteins is particularly intriguing, because oscillations in intracellular calcium are a well characterized and specific response to the Nod factor ligand produced by symbiotic rhizobia (4). By analogy to mammalian CaM kinase II (5), DMI3 may function to interpret and transduce intracellular calcium oscillations to pathways for symbiotic development. But the article by Mitra *et al.* is equally important for another reason, namely, the means by which *dmi3* was identified. *dmi3* was uncovered in a transcriptional profiling experiment wherein the candidate gene was revealed as an expression-level polymorphism between mutant and wild-type plants. Mitra *et al.* demonstrate the application of transcript-based cloning to large and complex plant genomes by characterizing expression-level polymorphisms associated with the *rar1-2* mutant of barley. The barley genome is nearly twice the size of the human genome and ≈10 times the size of the *M. truncatula* genome, suggesting that transcript-based cloning may be a powerful tool for functional genomics in a range of biological systems.

A Long-Standing Question

One of the classic conundrums in plant biology has been the molecular basis of host specificity in the *Rhizobium*–legume symbiosis. This subterranean rendezvous between otherwise saprophytic bacteria and the roots of legume plants results in the dramatic transformation of each partner. The end product is a unique plant organ that provides the context for a fine-tuned metabolic collaboration, wherein atmospheric dinitrogen is converted into biologically useful organic nitrogen. Beyond its importance as a major source of reduced nitrogen in both agricultural and natural ecosystems, the *Rhizobium*–legume symbiosis involves a cross-kingdom molecular dialogue that has fascinated plant biologists for decades (6). The chemical players in this dialogue have been described since the early 1990s. Root-derived exudates (e.g., isoflavonoids) are perceived by the bacterium, where they trigger synthesis of a bacterially derived oligosaccharide signal that is in turn perceived by the plant. The core structure of the bacterial ligand, an N-acylated *N*-acetyl-glucosamine oligomer known as "Nod factor" (7), is well conserved among symbiotic rhizobia, whereas further genotype-specific decorations (e.g., sulfation, acetylation, fucosylation) are necessary for biological activity (8). These structural features of the Nod factor ligand are correlated with the host range of the particular bacterial strain; for example, rhizobia that nodulate alfalfa will not nodulate pea, and vice versa. Both genetic and molecular studies demonstrate that host-specificity is encoded in the fine structure of the Nod factor ligand (6, 8), implying the existence of a plant-encoded system of cognate receptors and signal transduction proteins.

***dmi3* is necessary for rhizobial and mycorrhizal interactions and encodes a calcium- and calmodulin-dependent kinase.**

Defining the Nod Factor Perception and Signal Transduction Pathway

Among the earliest responses to Nod factor is the periodic oscillation of intracellular calcium concentrations—termed "calcium spiking" (4, 9). Several plant genes required for early symbiotic development are also required for Nod factor-induced calcium spiking (10–12), including a family of genes that are strong candidates for the long sought after Nod factor receptor (13–15). The deduced proteins are putative receptor-like kinases, with an extracellular LysM domain that is predicted to bind the *N*-acetyl-glucosamine backbone of Nod factor. Loss of function mutations in these putative Nod factor receptors lead to a complete lack of Nod factor responsiveness. Additional genes required for calcium spiking include a putative cation channel identified in *M. truncatula* (*dmi1*) (16), and a leucine-rich repeat (LRR) receptor-like kinase (Mt*dmi2* and its orthologs) (17, 18). By contrast, mutants of *dmi3* retain Nod factor-induced calcium spiking, but lack downstream Nod factor responses such as plant gene expression and the induction of organ development. Interestingly, *dmi1*, *dmi2*, and *dmi3* are also required for mycorrhizal association (2), indicating that the Nod factor signaling pathway shares genetic components with the more ancient fungal symbiosis. This overlap is also suggestive of specific plant receptors for mycorrhizal ligands that are yet to be identified (19).

A Possible Molecular Function for DMI3

It seems likely that Nod factor-induced calcium spiking encodes biological information, as has been demonstrated in other eukaryotic signaling systems (e.g., ref. 22). In the case of Nod factor perception, the gaseous plant hormone ethylene can modulate the frequency of calcium spiking as well as the sensitivity of calcium spiking to Nod factor (20). Moreover, ethylene-insensitive plant mutants display increased susceptibility to

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rhizobial infection (21), consistent with the possibility that these attributes of calcium spiking affect the degree of symbiotic development. Presumably plants, and legumes in particular, possess mechanisms that can perceive and transduce calcium oscillations into biological responses. DMI3 is an excellent candidate to fulfill such a role, not only because of its genetic position downstream of spiking, but also because of its homology to calcium- and calmodulin-dependent protein kinases in plants and to neuronal CaM kinase II in animals. Neuronal CaM kinase II is able to translate the frequency information in calcium spikes into discrete amounts of kinase activity (5, 23). This capacity is due to a self-reinforcing loop, where calmodulin binds to and stimulates CaM kinase activity. High concentrations of calcium-bound calmodulin simultaneously increase the affinity of multimeric CaM kinase for calmodulin and increase the level and stability of kinase activity. The end result is that the action of CaM kinase II on downstream targets is directly correlated with the frequency of calcium spikes. Although the analogy of DMI3 to neuronal CaM kinase II may be instructive, it is unlikely to be adequate, as DMI3 and its close plant homologs possess an additional domain that can interact directly with calcium, leading to autophosphorylation and consequent increased affinity for calmodulin (24). Thus, these plant specific calcium- and calmodulin-dependent protein kinases can interact with calcium in at least two forms, free Ca^{2+} and calcium-bound calmodulin, suggesting additional layers to the complexity of these unusual plant proteins.

Riding the Genomics Wave

The recent cloning of several genes in the Nod factor perception pathway has been greatly facilitated by the advance of genomics in the model legumes *M. truncatula* and *Lotus japonicus*. Both genomes are the subject of whole genome sequencing projects, and *Medicago* in particular has also been subject to extensive sequencing and analysis of ESTs. The bioinformatic analysis of ESTs, in particular the identification of a unigene set, has enabled the development of microarray resources such as the oligonucleotide arrays used in the accompanying study by Mitra *et al.* (3). A particularly unique feature of this study is the use of expression-level polymorphisms to identify candidate genes without the aid of extensive genetic analysis. Interestingly, the *dmi3* locus was simultaneously cloned by Lévy *et al.* (25) by using a more traditional genetic approach. A requirement of the Mitra method is that mutations must lead to a measurable decrease in transcript levels. The authors demonstrate the general feasibility of this approach by identifying expression-level polymorphisms in mutant alleles of *M. truncatula dmi2* and barley *rar1-2*. The result with barley is particularly exciting, as it demonstrates the application of transcript-based cloning to large and complex genomes, where gene isolation based on the more traditional approach of map-based cloning is difficult at best. In prior studies, transcriptional profiling has been combined with traditional genetic analysis to unravel the genetic control of gene transcription in yeast (26) and mice (27). Among the important findings from these later studies is the fact that some transcriptional patterns segregate as simple Mendelian traits, allowing the mapping of both cis- and trans-acting loci. By extension, the analysis of Mitra *et al.* holds promise not only for identifying cis-acting mutations, as demonstrated for *dmi3*, but also to mine transcriptional pathways where single gene mutations act in trans on suites of transcripts.

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