Molecular and biochemical analysis of symbiotic plant receptor kinase complexes

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DE-FG02-01ER15200 was a 36-month project, initiated on Sept 1, 2005 and extended with a one-year no cost extension to August 31, 2009. During the project period we published seven manuscripts (2 in review). Including the prior project period (2002-2005) we published 12 manuscripts in journals that include Science, PNAS, The Plant Cell, Plant Journal, Plant Physiology, and MPMI. The text below describes both the recently published work and ongoing studies to characterize Nod factor signaling. Publication information is given at the end of this document.

I. *DMI1* localization

Legumes utilize a common signaling pathway to form symbiotic associations both with rhizobial bacteria and arbuscular mycorrhizal fungi. The perception of microbial signals is believed to take place at the plasma membrane, activating a cascade that converges on the nucleus where transcriptional reprogramming facilitates the symbioses. Forward genetic strategies have identified genes in this signaling pathway, including the *Medicago truncatula* *DMI1* gene, which are required for nodulation.

Previous support from the Department of Energy Biosciences program financed, in part, the cloning of the *DMI1* and its identification as a putative symbiotic ion channel. [This work was published in the journal Science.](#)

We have followed up the identification of *DMI1* by characterizing its expression and localization. RTPCR experiments indicate that *DMI1* is primarily expressed within the roots, a pattern which is consistent with its root-specific, mutant phenotype. RT-PCR also indicates that the overall level of expression within the root appears unchanged following inoculation with rhizobium. However, *DMI1* promoter:*GUS* fusion experiments indicate that *DMI1* expression is enhanced in the apex of root nodules where bacterial release from infection threads into symbiosomes takes place. *DMI1* expression declines in the nitrogen-fixing zone of the nodule and in non-symbiotic regions of the root. This pattern of expression indicates that *DMI1* function is required not only during the initial infection of the root hair, but also later during bacterial release into the developing nodule. These data are also consistent with previous studies that demonstrated that bacteria continue to produce Nod factors up until the point of symbiosome formation. It is likely that for indeterminate nodules, Nod factor signaling begins prior to infection and continues throughout nodule development.

To identify the subcellular localization of *DMI1*, we generated biologically active *DMI1::GFP* fusions both under the control of the native *DMI1* promoter and the constitutive 35S promoter. Confocal microscopy indicates that *DMI1* localizes in the nuclear envelope of *M. truncatula* roots and root nodules. Nuclear localization is mediated by NLS motifs located in the N-terminus and between the transmembrane domains of *DMI1*. [This work has been published in the Plant Journal.](#)

Figure 2. DMI1 (Doesn't Make Infections 1) is localized to the periphery of nuclei and not to plastids. *Medicago truncatula* root epidermal cells (a–c) or root hairs (d–f) expressing (a and d) empty pRedRoot, (b and e) 35S::DMI1::GFP or (c and f) GS2cTP::GFP were visualized on a confocal microscope. Images represent merged data from both the red and green channels. Bars represent 10 μ m. Red fluorescence indicates propidium iodide staining of cell walls.

The observed nuclear localization of DMI1 is consistent with well-characterized molecular and physiological responses to Nod factors. In particular, the Nod factor ligand induces rhythmic oscillations in calcium within the nucleus, known as “calcium spiking”. Consistent with localization to the nuclear envelope, we speculate that DMI1 may be directly or indirectly involved in mobilizing intracellular calcium. We worked with Dale Sanders’ group (University of York) to pursue the activity of DMI1 in a series of complementation experiments using yeast ion channel mutants. The data suggested that DMI1 may perturb intracellular calcium homeostasis in yeast. [A manuscript describing the yeast *dmi1* analysis was published in Plant Physiology.](#)

II. Characterizing symbiotic receptor kinase complexes

One of the primary objectives of this project was to purify and characterize proteins that interact with the symbiotic receptors NFP, DMI2, and LYK3 (and to a lesser extent the non-receptor kinase DMI3).

NFP, DMI2, DMI3 and LYK3 are all required for *M. truncatula* to form effective symbioses with the nitrogen fixing bacterium *S. meliloti*. Mutations in each of these genes abolish the ability of the bacteria to colonize the host and for the host to develop root nodules. In order to elucidate how the symbiotic receptor kinases effect symbiotic responses, we are identifying proteins that physically interact with each of these signaling molecules using two complementary approaches: (1) a yeast two-hybrid screen and (2) co-immunoprecipitation (co-IP) with epitope tagged proteins from *M. truncatula* roots.

We developed a yeast two-hybrid “prey” library using RNA isolated from the *S. meliloti*

inoculated roots of wild type *M. truncatula*, and from the super- nodulating mutants *SUNN*, and *skl*. The soluble, kinase domains for NFP, DMI2, DMI3 and LYK3 were cloned into the pGAL4-BD “bait” vector. Screening of this library with these constructs identified a putative interacting protein only in the case of DMI3. We refer to the corresponding gene as IPD3 (Interacting Protein of DMI3). Chimeric IPD3::GFP localizes to the nucleus of transformed *Medicago truncatula* root cells, while split yellow fluorescent protein assays suggest that IPD3 and DMI3 physically interact in the nuclei of transformed *Nicotiana benthamiana* cells. Similar to DMI3, IPD3 is extremely well conserved among the angiosperms and is absent from Arabidopsis. Despite this high level of conservation, none of the homologous proteins have a demonstrated biological or biochemical function. [This work was published in MPMI.](#)

No interacting proteins were identified in the cases of NFP, DMI2 or LYK3. Unlike DMI3, NFP, DMI2 and LYK3 are transmembrane proteins, whose analysis may not be amenable to yeast two-hybrid approaches. Alternatively the full-length receptor kinase, or perhaps activation of the pathway, may be required to initiate complex formation. We have developed other methods to identify interacting proteins that circumvent these issues (see below). Nevertheless, this yeast two-hybrid library will serve as a useful resource to perform secondary screens once *bona fide* interacting proteins are identified and characterized.

Development of a TAP system for protein purification. Tandem affinity purification (TAP) has proven to be a robust platform for the purification of proteins and has been used successfully to investigate the “interactomes” of diverse eukaryotic species. We therefore constructed NFP, DMI2, and LYK3 genomic clones translationally fused to a TAP tag containing three copies of the HA epitope and one copy of the StrepII epitope (HAST). To faithfully reproduce the endogenous patterns and levels of expression, we included 2kb of DNA upstream of the start codon, the introns, and 500bp of 3’ UTR for each gene. Additionally, we generated translational fusions of each protein to GFP (i.e., NFP-GFP, DMI2-GFP and LYK3-GFP), using the same native regulatory context as in the HAST tagged versions. These GFP fusions serve as negative controls in protein purification experiments and permit us to validate expression patterns *in planta*. We also have constructs in which the promoters of NFP, DMI2 and LYK3 fused to the GUS reporter gene.

Within the context of this project we developed a robust protocol for transforming *M. truncatula* cultivar A17, the reference genome used for the *Medicago truncatula* genome sequencing project and for the forward genetic screens of nodulation mutants. We used this protocol to stably transform both the HAST and GFP tagged constructs into their respective nod-mutant backgrounds. We have recovered both GFP and HAST tagged transgenic lines for DMI2 and LYK3 and are developing similar lines for NFP. A nodulation bio-assay served to confirm complementation by the transgene in the T1 generation, and to monitor segregation of the transgene in the T2 generation. Our complementation and segregation data indicate that we have identified homozygous, nod+, GFP and HAST tagged lines for both DMI2 and LYK3. As DMI2 and LYK3 are expressed both in roots and the apex of root nodules we have also crossed the transgenes into a supernodulating *Sunn* mutant background that yields increased numbers of transgenic nodules (panel B in figure on preceding page). We routinely detect recombinant DMI2 and LYK3 on protein blots with anti-HA antibodies and have successfully isolated both proteins from total root or nodule extracts using both anti-HA agarose (Roche) and Streptactin (IBA) resins. The figure on the preceding page documents our ability to readily detect the transgenic proteins (panel A), to complement endogenous mutant gene forms (panel B), to monitor protein levels in different tissues and over time (panel C), and to purify recombinant protein on Strep tactin resin followed by detection with anti-HA antibodies (panel D).

Identification and validation of interaction proteins. Essentially nothing is known about protein interactions or the nature of receptor complexes for symbiotic initiation. Now, for the first time, the various transgenic lines we have developed provide tools for receptor complex purification (i.e., the LYK3:HAST and DMI2:HAST lines), while also serving as powerful markers for subcellular structure distribution and dynamics of receptor complexes (i.e., the LYK3:GFP and DMI2:GFP lines), and for monitoring transcriptional activation in specific cell types (i.e., the pLyk3:GUS and pDmi2:GUS lines). See adjacent figure for the example of LYK3 protein purification and western blot detection (top panel), promoter GUS expression (middle panel), and protein localization (bottom panel).

In a recent and exciting development, we have obtained evidence that both LYK3 and DMI2 are components of discrete membrane microdomains, also known as sterol-rich “lipid rafts” (see adjacent figure). Relatively little is known about the function of lipid rafts in plants, and thus this work has the additional novelty of contributing to this important area of basis plant science. Working in collaboration with Drs Sharon Long, Cara Haney and David Ehrhardt at Stanford University, we have determined that following Nod factor perception LYK3 protein co-localizes with FLOT4 (a flotillin protein paralog). Co-localization occurs in membrane microdomains that are re-distribute towards the root hair tip following bacterial inoculation ([manuscript in review at the journal *Science*](#)).

DMI2 also localizes to membrane microdomains (see figure, above) and we have recently shown that DMI2 protein co-immuno precipitates with remorin, a class of proteins that are implicated in signal perception. Medicago remorin has recently been shown to also be a component of membrane microdomains. In the adjacent figure: lane 1, control non-tagged transgenic protein (no signal); lanes 2-5, [top row] DMI2 protein is depleted from the supernatant on continued incubation with Strep tactin resin which binds the C-terminus of DMI2:HAST; lane 7, Strep tactin-resin bound

protein: note that DMI2 is largely associated with the resin-bound fraction [row 1, lane 7], and the remorin protein co-immuno precipitates with DMI2 [row 3, lane 7]).

For purposes of identifying interacting proteins, HAST (test) and GFP (control) extracts were immunoprecipitated using anti-HA resin, and the resin was submitted to the UC-Davis proteomics faculty (<http://proteomics.ucdavis.edu/>) for on-bead digest and tandem mass spectrometry (MS/MS) analyses. By way of example, we reproducibly identified LYK3 in *all* LYK3:HAST samples (3-14 unique peptides per sample) but did not identify LYK3 in any of the LYK3:GFP control samples. Importantly, we also identified several *M. truncatula* proteins specifically in the LYK3:HAST samples (see following Table for initial list of candidate proteins). These proteins form the basis of a large-scale effort to identify and functionally test the components of symbiotic receptor complexes.

Exp.	Annotation	Log(e)	%	unique	total	Genbank
1LHA	Beta-tubulin	-23.3	23	5	6	gi 30100923
1LHA	Helicase	-19.7	7.7	1	1	gi 27524276
4LHA	eif5A	-15.9	11	3	3	gi 13596425
2aLHA	Beta-tubulin	-14.7	16	2	2	gi 9669251
2bLHA	enolase	-13.2	15	2	2	gi 30101209
4LHA	catalase	-13.2	8.9	2	2	gi 55443175 g
1LHA	Ubiquitin-ribosomal fusion protein	-13.2	4.6	1	1	gi 11914851
1LHA	cdc48/Spindle disassembly	-12.4	26	7	8	gi 30100277
1LHA	V. faba ENOD18	-11.6	23	5	6	gi 7571993
1LHA	Glycine rich protein	-11	27	2	2	gi 11908740
1LHA	Methionine synthase	-11	14	2	2	gi 27404082
1LHA	Adenosine Kinase	-10.1	7.6	3	3	gi 30100741
1LHA	Plasma membrane polypeptide	-9.3	16	3	5	gi 8747645
4LHA	1,4-benzoquinone reductase-like protein	-8.7	7.8	2	2	gi 13596889
1LHA	Ran GTPase	-7.4	9.6	3	3	gi 30101764
1LHA	Lipoxygenase	-7.4	8.4	2	2	gi 30100104
1LHA	peroxiredoxin-like protein	-7.2	38	5	5	gi 13598331
5LHA	Elongation factor-1	-7.1	5	2	2	gi 30100869
5LHA	ADP-ribosylation factor	-7	4	1	1	gi 11898639
4LHA	Lectin	-6.5	4	1	2	gi 13597637
1LHA	MtN19	-6.2	8.9	4	4	gi 2598588

III. Characterization of *Medicago* RopGTPases and RopGEFs (manuscript in review at Plant Journal).

RopGTPases regulate root hair growth by affecting the root hair's intracellular poise of Ca^{2+} , ROS, and the actin cytoskeleton -- processes that are also modulated by Nod factors. To analyze the role of these proteins in symbiotic and normal root hair development, we cloned and conducted initial functional analysis of the seven RopGTPase and ten RopGEF genes of *Medicago*. All seven Rops and four of ten RopGEFs are expressed in root tissues. Moreover, over-expression of constitutively active forms of all RopGTPases depolarized root hair growth, which reflects their conserved biochemical function but does not clarify their respective biological roles. The entire set of RopGTPases was also tested for interaction with the symbiotic receptor kinases in yeast. We found that constitutively active forms of RopGTPase8 and RopGTPase9 interact with NFP, but not DMI2 or LYK3. This specificity is consistent with the observation that NFP (but not DMI2 or LYK3) is required for the early Nod factor-induced Ca^{2+} and ROS fluxes, and it fits with the established connection between the Rop pathway and

Ca²⁺/ROS production in *Arabidopsis* root hairs. These results also suggest that different molecular mechanisms underlie the output of each symbiotic receptor kinase.

We also examined the role of RopGEFs in non-symbiotic and symbiotic processes by silencing each of the ten RopGEFs using RNAi. Only MtRopGEF2i plants possessed a root hair phenotype, with short, squat root hairs relative to vector transformed control and wild type roots. As expected, RopGEF2 interacted with the dominant negative forms of all RopGTPases in a yeast two-hybrid assay. We also tested the ability of the RopGEFs to interact with each symbiotic receptor kinase and determined that MtRopGEF5, and to a lesser extent MtRopGEF6, interacted with DMI2, but not NFP or LYK3. Kinase-deficient DMI2 lacked such interaction, implying that the biochemical function of DMI2 is required for interaction with a specific subset of RopGEFs. *We have identified a MtRopGEF5 Tnt1 insertion mutant in the M. truncatula Tnt1 database and are propagating this line for subsequent analyses.*

Figure 4. MtRopGEF2 is required for root hair elongation. (e-h) DIC images of full-grown root hairs from transgenic roots expressing either (e) empty pK7WIWG2(II)::DsRED (f) *MtRopGEF2i* (g) empty pKGW-RR or (h) pKGWRR::35S::Rop3(D121A). (i) Quantitative analysis of root hair length in plants expressing RNAi constructs against each of the ten MtRopGEFs. At least 50 root hairs from five different plants were measured for each genotype. Error bars represent standard error. * indicates significantly different, ($p \leq 0.001$ general linear model ANOVA, SAS) (SAS Institute, Cary, NC) (j) sqRT-PCR of MtRopGEF2 and MtRopGEF3 performed on cDNA isolated from either two independent roots expressing an

MtRopGEF2i construct, or two independent roots containing an empty pK7WIWG2(II)::DsRED vector. Scale bars represent 300µm in (a-d) and 50µm in (e-h).

Taken together, these results suggest a model in which the Rop pathway is involved in both normal and symbiotic root hair development. The Nod factor receptor kinases may interact with the Rop pathway at a minimum two points: first, at initial Nod factor perception, where NFP recruitment of an active RopGTPase could explain Nod factor-stimulated ion fluxes, and subsequently during root hair branching and curling, when an interaction between DMI2 and specific RopGEFs could act to reinitiate root hair growth. The model is speculative, but consistent with our recent observations. However, there are numerous unresolved issues and unanswered questions related to the nature of other protein interactions, relevant downstream targets and mechanisms for altered root hair development and bacterial infection, etc. We expect that further defining the symbiotic interactome by TAP tag approaches will yield additional candidates for Rop-related root hair responses, and that detailed cellular and developmental phenotyping will better define the relationship between the Rop pathway and broader symbiotic interactome.

Our current data implicate symbiotic receptors as mediators of the Rop pathways. We suggest that linking the perception of modified chitin oligomers to the root hair development pathway was a key event in the evolution of the bacterial infection of legumes, from more primitive “crack entry” to the more highly evolved root hair infection mechanism [73]. Demonstrating these linkages at a biochemical and genetic level, and perhaps identifying signals of historical selection in legume homologs, would constitute an important contribution to understanding the origin of symbiosis.

IV. Cloning and characterization of an ERF transcription factor required for early nodulation.

Work under our preceding DOE grant (DE-FG03-98ER20296) involved a non-nodulating mutant named *poodle* (*pdl*). The *pdl* mutant forms numbers of infections similar to that of wild type plants, but all infections arrest in the epidermis and cell divisions in the root inner cortex are not activated. Thus, the *pdl* mutant is defective both in infection and nodule morphogenesis. Working in collaboration with the laboratories of Dr. Giles Oldryod (UK) and Dr. Gyorgy Kiss (Hungary), the *pdl* gene has now been cloned along with a second allele named *bit1*. *Poodle* encodes an ERF transcription factor, which we have named “*ERF Required for Nodulation*” (*ERN*), with a highly conserved AP2 DNA binding domain that is necessary for nodulation. *ERN* is necessary for Nod factor induced gene expression and for spontaneous nodulation activated by a calcium and calmodulin dependent protein kinase that is a component of the Nod factor signalling pathway. We propose that *ERN* is a component of Nod factor signal transduction pathway downstream of the calcium activated kinase and activates nodulation gene expression possibly through combined action with two GRAS proteins encoded by *nsp1* and *nsp2*. [A manuscript describing this work was published in the Plant Cell.](#)

V. Cloning and characterization of the ethylene signal transduction gene, required for regulation of Nod factor signaling in *Medicago truncatula*.

The plant hormone ethylene negatively regulates bacterial infection and nodule formation in legumes in response to symbiotic rhizobia, but the molecular mechanism(s) of ethylene action in symbiosis remain obscure. We identified and characterized multiple mutant alleles of the *MtSk11* gene which controls both ethylene-sensitivity and nodule numbers. We demonstrated that this locus encodes the *M.truncatula* ortholog of the *Arabidopsis* ethylene signaling protein EIN2. In addition to the well characterized role of *MtSk11* in rhizobial symbiosis, we have shown that *MtSk11* is involved in regulating early phases of the symbiotic interaction with mycorrhizal fungi, and in mediating root responses to cytokinin. *MtSk11* also functions in the defense against *Rhizoctonia solani* and *Phytophthora medicaginis*, with the latter interaction likely to involve positive feed back amplification of ethylene biosynthesis. Over expression of the C-terminal domain of MtEIN2 is sufficient to block nodulation responses, consistent with previous reports in *Arabidopsis* on the activation of ethylene signaling. This same C-terminal region is uniquely conserved throughout EIN2 homologs of angiosperms, consistent with its role as a higher plant specific innovation, essential to EIN2 function. [This work was published in Plant Journal.](#)

Publications resulting from this DOE award:

1. Cara H. Haney, Brendan K. Riely, David Tricoli, Doug R. Cook, David W. Ehrhardt, Sharon R. Long (201x) Colocalization of a symbiotic receptor and a plant flotillin is regulated during rhizobial symbiosis. In review.

2. Riely, BK, He H, Venkateshwaran M, Sarma B, Schraider J, Ane JM and Cook DR (201x) Identification of legume RopGEF gene families and characterization of a *Medicago truncatula* *RopGEF* mediating polar growth of root hairs. In review.
3. Penmetsa, R.V. Uribe, P., Anderson, J., Lichtenzweig, J., Gish, J-C., Nam, Y-W., Engstrom, E., Xu, K., Siskel, G., Pereira, M., Baek, J-M., Lopez-Meyer, M., Long, SR, Harrison, M.J., Singh, J.B., Kiss, G.B., and Cook, D.R. (2008) The *Medicago truncatula* ortholog of Arabidopsis EIN2, *sickle*, is a negative regulator of symbiotic and pathogenic microbial associations. Plant Journal 55:580-595.
4. Middleton, P., Jakab, J., Penmetsa, R.V., Starker, C., Doll, J., Kalo, P., Prabu, R., Marsh, J., Mitra, R., Kereszt, A., Dudas, B., VandenBosch, K., Long, S., Cook, D., Kiss, G., and Oldroyd, G. (2007) An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction. The Plant Cell 19:1-14.
5. Messinese, E., Mun, J.H., Yeun, L.H., Jayaraman, D., Loughon, G., Rougé, P., Barre, A., Bono, J.J., Cook, D.R., and Ané, J.M. (2007) A novel nuclear protein interacting with the symbiotic DMI3 CCaMK. MPMI 20:912-21.
6. Peiter, E, Sun, J, Heckmann, AB, Venkateshwaran, M, Riley, BK, Otegui, MJ, Edwards, A, Freshour, G, Hahn, MG, Cook, DR, Sanders, D, Oldroyd, GED, Downie, JA, Ané, JM (2007) The *Medicago truncatula* DMI1 Protein Modulates Cytosolic Calcium Signaling. Plant Physiol 145: 192-203.
7. Riely, B.K., Loughon, G., Ané, J.-M., Cook, D.R. (2007) The symbiotic ion channel homolog DMI1 functions in the nuclear membrane of *Medicago truncatula* roots. The Plant Journal 49:208-216.

Publications from prior award:

8. Veereshlingam, H., Haynes, J.G., Sherrier, D.J., Penmetsa, R.V., Cook, D.R., and Rebecca Dickstein (2004) *nip*, a symbiotic *Medicago truncatula* mutant that forms root nodules with aberrant infection threads and exhibits an altered defense response. Plant Physiology 136:3692-3702.
9. Kuppusamy, K.T., Endre, G., Prabhu, R., Penmetsa, R.V., Veereshlingam, H., Cook, D.R., Dickstein, R., and VandenBosch, K.A. (2004) *LIN*, a *Medicago truncatula* gene required for nodule differentiation and persistence of rhizobial infections. Plant Physiology 136:3682-3691.
10. Riely, B., Ane, J-M., Penmetsa, R.V., and Cook, D.R. (2004) Genetic and genomic analysis in model legume systems bring Nod factor signaling to center stage. Current Opinion in Plant Biology 7:408-413.
11. Cook, D.R. (2004) Unraveling the mystery of Nod factor signaling by a genomic approach in *Medicago truncatula*. Proc. Natl. Acad. Sci. USA 101:4339-4340.
12. Ané, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Ayax, C., Lévy, J., Debellé, F., Baek,

J.M., Kalo, P., Roseberg, C., Roe, B.A., Long, S.R., Dénarié, J., and Cook, D.R. (2004). *Medicago truncatula DMII* required for bacterial and fungal symbioses in legumes. *Science* 303:1364-1367.