Giving the Nod to Bacterial Symbionts: The Nod Signal Transduction Pathway

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Abstract

This in-depth review summarises recent advances in the elucidation of the Nod factor-induced signal transduction pathway. It discusses the discovery and characterisation of three proteins, DMI3, CIP73 and IPD3. DMI3 is a CCaMK-like kinase which acts as a master switch for both rhizobial and mycorrhizal symbioses. IPD3 is a downstream phosphorylation target of DMI3, specific to the nodulation pathway. Its function is not fully elucidated, however is required for correct infection thread formation, and development of functional nodules. CIP73 is also a downstream target of DMI3, however its function is not known. *Cip73* mutants show impaired nodule development, and reduced rhizobial infection, indicating that CIP73 is required for nodule organogenesis. The discovery of these three proteins represents a significant advance in the elucidation of the molecular mechanisms of rhizobial nodulation, and furthers the goal of harnessing this sustainable source of fixed nitrogen for agricultural purposes.

Introduction

Legumes have long been known to enter into symbiotic associations with specific rhizobial bacteria, for the purpose of obtaining symbiont-generated nitrogen sources. The interaction between the symbiont and host is highly specific, and involves a complex bidirectional signalling pathway (Lerouge et al. 1990). Flavonoids and isoflavonoids are released by the roots of legumes, which are detected by specific rhizobia. In reply, these release lipochitooligosaccharide molecules termed Nod factors. These Nod factors are perceived by legumes using both nodulation-specific (Nod) and the pathway common to both mycorrhization and nodulation, general symbiosis (Sym), signal transduction pathways, effecting the morphological, biochemical and transcriptomic changes required to allow symbiosis (Ané et al. 2002; Catoira et al. 2000; Lerouge et al. 1990). The upstream elements of these pathways is shown graphically in Figure 1

below. The precise elucidation of these signal transduction pathways is crucial to understanding the mechanisms of nodulation, and to any possible future application of this knowledge to crop science and biotechnology. This essay will summarise a number of important studies which have identified elements of these pathways.

Figure 1: Upstream components of the sym and nod pathways.

The pathways described here are still putative. While the relative ordering of regulatory elements has been at least partially elucidated, no full signalling pathway has been published.

The DMI3 Ca²⁺ and Camodulin-dependent protein Kinase

A study by Gleason *et al.* (2006) investigated the structure and function of *M. truncatula* DMI3, key regulator of the Nod and Sym signal transduction pathways. The DMI3 protein is a Ca²⁺/Camodulin-dependent protein Kinase (CCaMK), which is activated by Ca²⁺ and/or camodulin (CaM) binding and phosphorylates specific substrates including itself, signalling the reception of Nod factors to downstream regulatory proteins (Lévy *et al.* 2004). DMI3 is

a 523aa protein, which consists of (from N- to C-terminus) a serine/threonine protein kinase, overlapping CaM binding and autophosphorylation domains and three EF hand Ca^{2+} binding domains.

Gleason and colleagues created a series of mutants which lacked various domains of the DMI3 protein, and point mutations which altered the autophosphorylation site. Deletion mutants lacking the autoinhibition domain cannot bind CaM, and constitutively phosphorylate substrates and were termed autoactivated. Autoactivated mutants could not complement *dmi3* mutants to allow establishment of symbionts. Point mutations in the autophosphorylation site showed similar phenotypes to autoactivated mutants, however these point mutations complemented *dmi3* mutants, and allowed establishment of symbionts.

Gleason and colleagues also investigated the ability of autoactivated mutants of DMI3 to complement other Nod and Sym pathway mutants. Autoactivated DMI3 does not complement GRAS transcription factor mutants *nsp2-1* and *nsp1-2*, indicating that NSP2 and NSP1 probably function downstream of DMI3, and supporting previous studies (Catoira et al. 2000; Kalo 2005; Smit et al. 2005). These authors also observed that autoactivated mutants of DMI3 can complement loss of function mutants in the putative receptor-like kinase DMI2 and the putative cation channel DMI1, indicating that these proteins must function upstream of the DMI3 protein, as suggested by previous results (Catoira et al. 2000; Endre et al. 2002; Ané et al. 2004). Additionally, these authors have shown that expression of Nod factor-induced (NOD) genes becomes constitutive in autoactivated mutants. Expression of a Nod factor-induced GUS expression construct was used to evaluate NOD gene expression; mutants that showed constitutive substrate phosphorylation also showed constitutive GUS expression. This result lends further evidence to the propositions that DMI3 is the master regulator of the nodulation signalling pathway.

Gleason et al. (2006) illustrate the role of each domain in the function of DMI3, and shed light on the regulatory mechanism of this crucial step in the Nod and Sym signal transduction pathways. In wild type *M. truncatula*, Ca^{2+} -induced autophosphorylation is thought to allow binding of CaM to the CaM binding domain, alleviating autoinhibition and allowing substrate phosphorylation. Gleason et al. (2006) demonstrate that removal of the autoinhibition domain causes spontaneous nodule formation in the absence of rhizobia, indicating that this domain negatively regulates DMI3 activity and nodule formation. Gleason et al. (2006) interpret their results in the context of the partially elucidated Nod and Sym signal transduction pathways. These authors propose that DMI3 functions downstream of the DMI1 and DMI2 proteins, and regulates expression of NOD genes, indicating it is an upstream regulator of these genes and ultimately

of nodulation. This functional characterisation of the DMI3 upstream regulator allowed subsequent studies to elucidate the existence and function of elements downstream of DMI3.

Recent advances in elucidation of the Sym and Nod signal transduction pathways

Identification of the interacting protein of DMI3 (IPD3) protein

A study by Messinese et al. (2007) has identified a novel protein which interacts with *DMI3* in *M. truncatula*. Using a yeast two-hybrid (Y2H) system, these authors identified a novel protein, which interacts with *DMI3* *in vitro* and *in planta*. The IPD3 protein is reported by these authors as functionally redundant for the formation of rhizobial nodules.

Messinese et al. (2007) used a Y2H system to identify the IPD3 protein from a cDNA library from *M. truncatula* roots. A full coding sequence of the gene was obtained, and putative domains were identified. The *IPD3* protein was identified as a coiled-coil containing protein, and was mapped to a BAC corresponding to the short arm of *M. truncatula* chromosome 5. Putative orthologs were identified in several monocots and dicots, including *L. japonicus* but not in *Arabidopsis*, indicating it is a conserved locus.

A quantitative PCR (qPCR) experiment showed preferential expression of IPD3 and DMI3 to root tissue, and split YFP bimolecular fluorescence complementation studies showed co-localisation of DMI3 and IPD3 to the nuclei of these cells. These results confirm those previously obtained for DMI3 (Gleason et al. 2006). A subsequent mutagenic study of *L. japonicus* by Yano et al. (2008) identified the CYCLOPS protein, an ortholog of IPD3. These authors show that CYCLOPS is highly structurally and sequentially similar to IPD3, and that it is expressed and localised in the nucleus, similarly to DMI3 and IPD3.

RNAi knock-down caused significant reductions in the qPCR abundance of *IPD3* transcripts in transgenic plants, however did not cause any significant change in nodule morphogenesis or rhizobial infection phenotypes. Messinese et al. (2007) suggest that this indicates that either the IPD3 protein is specific to the arbuscule formation pathway (which was not investigated), or that the IPD3 protein is functionally redundant, despite finding no candidate homologs, as nodulation still occurs at low *IPD3* transcript levels. These statements appear to have been disproven by a subsequent study by Horváth et al. (2011), discussed

in detail below. Additionally, CYCLOPS mutants had phenotypes deficient in formation of functional arbuscules and nodules, but not nodule organogenesis itself (Yano et al. 2008).

Messinese et al. (2007) identify and partially characterise a novel protein that interacts *in vivo* with the DMI3 protein of *M. truncatula*. This investigation has made important headway in elucidating the signal transduction pathway downstream of CCaMK/DMI3 in *M. truncatula*. However, this paper does not fully characterise the IPD3 protein, nor does this investigation record comprehensive phenotypes of IPD3 mutants, although this is listed as a future priority of the authors.

Further characterisation of the interacting protein of DMI3 (IPD3) protein

A recent investigation by Horváth et al. (2011) characterises the IPD3 protein identified by Messinese et al. (2007) in a more rigorous manner. These authors identified *IPD3* from a transposon-tagged insertion mutagenised *M. truncatula* population, and characterise this mutant, thus inferring the function of IPD3. They find that the primary function of IPD3 is in correct infection thread (IT) formation, and confirm previous mapping, localisation and structural studies and the action of IPD3 as a downstream phosphorylation target of DMI3 (Messinese et al. 2007).

Horváth et al. (2011) report novel results regarding the *in planta* function of IPD3. Two IPD3 mutants, *ipd3-1* and *ipd3-2*, showed IT formation defective to various degrees; nodules which formed in the presence of rhizobia did not contain bacteroids, and showed no nitrogenase activity. Differences in cellular structure of ITs were also observed. These mutants were complemented with *IPD3*, both using native and constitutive promoters. Constitutive *IPD3* expression did not complement *ipd3* mutants, forming non-functional nodules. Native *IPD3* expression fully complemented both *ipd3* mutants. *Ip3* mutants were also fully complemented with the *L. japonicas* CYCLOPS ortholog under its native promoter (Yano et al. 2008). The phenotype of IPD3 mutants with regard to arbuscule formation was also investigated, showing that these mutants were defective in the formation of arbuscules. These results also indicate that native temporal specificity in the expression of IPD3 is required for correct function

Ip3 mutants did not show Nod factor-inducible expression of NOD genes, indicating that IPD3 is upstream of such genes. A Ca^{2+} spiking signal identical to that of wild type plants indicates that the *IPD3* mutant is downstream of this signal. In the context of previous work on IPD3 (Messinese et al. 2007), this

places the IPD3 protein between DMI3 and the expression of Nod factor-induced genes. The observation that both arbuscule and nodule formation are affected by *ipd3* mutants places IPD3 in the Sym pathway common to both symbioses.

Horváth et al. (2011) communicate a study which characterises the IPD3 protein. These authors find that IPD3 is a regulatory element downstream of DMI3, but upstream of NOD genes. IPD3 is observed to be crucial to the correct formation of ITs and arbuscules, placing it in the Sym pathway common to both rhizobial and mycorrhizal symbioses.

The CIP73 protein, a novel CCaMK phosphorylation target

Kang et al. (2011) present a study that identified and characterised a novel protein, which acts as a phosphorylation target for the CCaMK protein of *Lotus japonicus*. These authors identified a protein that interacted with DMI3 in Y2H studies, and conducted several further analyses. They determined that CIP73 acts downstream of the DMI3 protein and is part of the Nod pathway.

These authors conducted a Y2H experiment to determine protein-protein interactions of the serine/threonine protein kinase domain and proteins encoded by an *L. japonicus* root cDNA library, as a screen for proteins which interact with the kinase domain of CCaMK. Further protein-protein interaction studies showed that *in vivo* and *in planta* full length CIP73 interacts with full length CCaMK. The full cDNA sequence was obtained and bioinformatic studies revealed that the protein contained an N-terminal domain with similarity to *Xenopus* Scythe ubiquitin, and a C-terminal nuclear localisation signal. Orthologous genes were discovered in several plant genomes, including *A. thaliana* and *M. truncatula*.

Kang and colleagues also investigated the expression and localisation of CIP73. Using qualitative RT-PCR, CIP73 was found to be expressed at higher levels in roots compared to all other tissues. CIP73-GFP gene fusions were found to be localised to the nucleus of epidermal cells of *L. japonicus*. *In vivo* studies used to characterise the interaction between CIP73 and CCaMK found that the N-terminus of CIP73 is phosphorylated by CCaMK in yeast. Autophosphorylation of CCaMK was increased by addition of Ca^{2+} , however phosphorylation of CIP73 was only increased in the presence of CaM, a phosphorylation pattern which matches that of CYCLOPS (Yano et al. 2008).

Using RNAi, Kang and colleagues show that the CIP73 protein is required for correct *Rhizobium*-induced nodule formation, but is not required for arbuscule formation. On inoculation of CIP73 RNAi knockdown roots with *Mesorhizobium loti*, significantly fewer nodules formed, and observation of nodules stained for β -galactosidase activity showed that significantly fewer rhizobia reached nodule

cells. Kang and colleagues interpret this observation to indicate the CIP73 protein controls nodule organogenesis, not rhizobial infection. A similar investigation of arbuscule formation after inoculation of CIP73 RNAi transformants with the mycorrhizal fungus *Glomus intraradices* found typical arbuscule formation, indicating that CIP73 is specific to the Nod signal transduction pathway.

Kang and colleagues present a comprehensive study, which identifies and characterises a novel protein that is a phosphorylation target for CCaMK in *L. japonicus*. CIP73 showed expression, localisation and *DMI3* interaction characteristics similar to those described in other CCaMK phosphorylation targets (Horváth et al. 2011; Messinese et al. 2007). The CIP73 protein was determined to act downstream of the *DMI3* protein. RNAi mutants demonstrate that CIP73 is required for *Rhizobium*-induced nodule formation, but is not required for arbuscule formation, and therefore is part of the Nod pathway. However, these authors do not propose any mechanism of action for this novel protein.

Conclusion

This essay has discussed three recent investigations that elucidate and characterise two novel regulatory proteins downstream of the master regulator of the Sym and Nod pathways. IPD3 and its orthologs are regulators crucial to the establishment of symbioses with rhizobia and arbuscular mycorrhizae, but not to organogenesis. The CIP73 protein is required for correct nodule formation, but not for arbuscule formation. While these results were obtained in *M. truncatula* and *L. japonicus*, previously observed similarity between *L. japonicus*, *M. truncatula* and other legumes in several elements of Sym and Nod pathway (Horváth et al. 2011; Sieberer et al. 2009; Yano et al. 2008) suggests that these results may be generalised to most legumes.

Through these and other similar studies we have the beginnings of an outline of regulatory networks involved in nodule formation and rhizobial symbiosis. However, we are far from fully elucidating the full Nod and Sym pathways; nor have we fully characterised the molecular function of discovered elements of these pathways. Given the high cost of nitrogenous fertilisers in a world facing increasing energy costs and food demand, there is keen interest in the application of these discoveries to non-legumes, aiming to impart the symbiont-generated nitrogen sources to these crops. To be able to put knowledge of rhizobial symbiosis obtained thus far to practical use in these areas, a clearer understanding of the regulation of nodulation is required. Work on elucidation of these pathways is ongoing, and represents an important line of future investigation in plant and crop science.

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