Chapter 11
Use of Hairy Root System to Study Signaling Pathways During Nodule Formation

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Abstract Nodule formation by a specialized group of plants is one of the most beneficial plant-environment interactions, where atmospheric nitrogen is biologically fixed into ammonia, which is subsequently converted to nitrates and amino acids. The molecular basis of nodule formation has been studied in detail, and work done in the past few years has led to seminal discoveries, connecting the initial signal perception by the root hairs to the downstream signaling events and finally to cellular and developmental changes that result in organogenesis and nodule formation. Although the physiology of biological nitrogen fixation has been well known for many years, the exquisite molecular details of nodule formation have been made possible, mostly, by using the transgenic hairy roots on composite plants. Development of hairy roots by *Agrobacterium rhizogenes* (*A. rhizogenes*) infection provides an excellent experimental system to rapidly and efficiently evaluate the effect of changes in the expression of specific genes or gene families on a range of root phenotypes. By using this system, the Nod factor receptor-mediated signaling has been linked to the infection thread formation and nodule organogenesis, two critical events of nodulation. The use of hairy root system has made it possible to uncover the details of signaling and developmental events using molecular genetics, genomics, proteomics, and cell biological approaches, making the nodulation signaling pathway one of the best understood in leguminous plants. This article provides an overview of multiple rhizobium-legume interaction studies that utilized the hairy root system to uncover the signaling pathways and offers perspectives on its future uses in the context of the development of novel gene-editing capabilities in plants.

Keywords Biological nitrogen fixation · Hairy roots · Legumes · Nodulation · Rhizobia · Symbiosis

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11.1 Biological Nitrogen Fixation

Nitrogen is an essential element for plant survival. It is a key constituent of amino acids, the building blocks of proteins, as well as of chlorophyll, a pigment required for photosynthesis. Increasing nitrogen content biologically in the soil is an effective strategy to produce higher crop yields while reducing chemical nitrogen fertilization and, subsequently, environmental pollution. Among plants, legumes (family Fabaceae) and few nonlegumes (some member of Cannabaceae) are able to fix atmospheric nitrogen in soil through symbiotic association with rhizobia, by a process known as biological nitrogen fixation (BNF). In agricultural systems, BNF is an environmentally sound alternative to chemical fertilizers and economically beneficial for crop production.

Only a subset of bacteria can convert atmospheric nitrogen to ammonia for BNF in the host plants, due to the catalytic activity of nitrogenase. These important nitrogen-fixing bacteria are called diazotrophs which include gram-negative *Rhizobia* sp. and gram-positive *Frankia* sp. Rhizobia are responsible for the most efficient nitrogen fixation processes by the formation of root nodules on legumes and few nonlegumes (Oldroyd and Downie 2008; Desbrosses and Stougaard 2011). Different genera of rhizobia including *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* are capable to form a symbiotic association with different legumes depending on their genetic compatibility. Symbiotic interaction between diazotrophs and legumes is responsible for the majority of BNF, although minor contribution from certain actinomycete members such as *Frankia* sp. which can form symbiotic relationship either by root hair infection or intercellularly with a broad spectrum of plant families belonging to Betulaceae, Casuarinaceae, Myricaceae, Rosaceae, Elaeagnaceae, Rhamnaceae, Datiscaceae, and Coriariaceae also exists. In addition, some diazotrophs including *Azospirillum* sp. and *Azoarcus* sp. form endophytic relationships with a wide variety of cereal roots. Finally, certain cyanobacteria, mainly *Nostoc* sp., can fix atmospheric nitrogen to colonize different plant organs (Santi et al. 2013; Pawlowski and Bisseling 1996).

11.2 Nodule Formation in Legumes

In this chapter, our main focus is on the underlying signaling mechanisms of root nodule symbiosis in legumes via rhizobia and how the use of hairy roots has helped uncover the exquisite details of these pathways (Fig. 11.1). The legume family is the third largest family of flowering plants and includes plants varying from annual herbs to large trees with many agronomically and economically important crops. Research on legumes is driven, to a large extent, by their biological nitrogen-fixing capacity. The formation of nodules leading to nitrogen fixation is quite complex and tightly regulated but also inadequately understood at the molecular level. However, recent advances in genome sequencing and development of hairy root

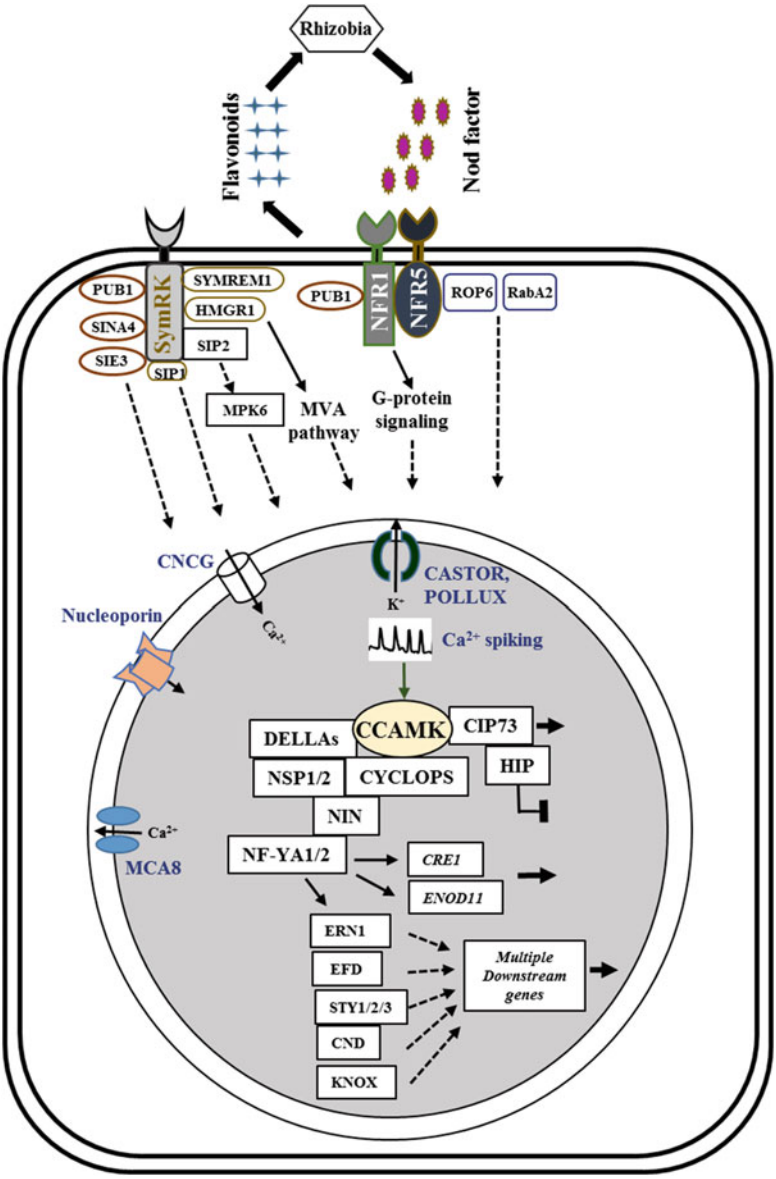

Fig. 11.1 Schematics of nodulation signaling as established using hairy root transformation system. Infection starts by secretion of flavonoids from roots, which trigger the production of bacterial Nod factors (NF). NF perception involves plant receptor-like kinases comprising lysin motifs NFR1 and NFR5 and a leucine-rich repeat SYMRK receptor. Receptors propagate signal from the plasma membrane to cytosol via heterotrimeric and monomeric (ROP6, RabA2) G-proteins, MAP kinase pathway (SIP2), HMGR1-dependent mevalonate (MVA) pathway, ubiquitination (PUB1, SINA4, SIE3), and other proteins (SYMREM1, SIP1) to generate unidentified secondary messengers, which are essential for calcium flux and calcium spiking in

transformation methods of different legumes have helped tremendously our understanding of the signaling mechanisms during nodulation.

Generally, two major types of nodules are formed on legumes: (a) determinate nodules that are characterized by a spherical shape and the lack of a persistent nodule meristem, producing ureide products, e.g., in *Lotus japonicus* and *Glycine max*, and (b) indeterminate nodules that are characterized by a cylindrical shape and the presence of a persistent nodule meristem, producing amide products, e.g., in *Medicago truncatula* and *Pisum sativum* (Sprent 2007). While determinate nodule initials arise from the outer or mid-cortical cells of the root, indeterminate nodule initials arise from the inner cortical cells. Two essential steps are needed for both types of nodule development: one an early infection phase and two a late developmental phase or organogenesis. The infection phase is started by the secretion of flavonoids from the legume roots, which trigger the production of lipochitin oligosaccharide known as Nod factors (NFs) from bacteria. NFs are sensed by Nod factor receptors (NFRs) present in the roots, and a number of morphological, biochemical, and cellular changes begin which allow the rhizobia to invade the host root cells. The most common entry strategy for rhizobia is by epidermal root hair curling and infection thread (IT) formation, observed in *L. japonicus*, *M. truncatula*, *G. max*, and *Phaseolus vulgaris* (Oldroyd and Downie 2008). Another nonclassical rhizobial invasion strategy is known as crack invasion. In this case, the rhizobia access the cortical cells through epidermal cracks, a point of epidermal damage, which is generally caused by the emergence of lateral roots. This is commonly observed in the Aeschynomeneae tribe of legumes, e.g., *Arachis hypogea* and *Sesbania rostrata* (Sprent 2007; Oldroyd and Downie 2008). After access to the host cell, most rhizobia invade the cytoplasmic space of the host cells via an endocytosis-like process and stimulate the root cells to proliferate by cortical cell division. Subsequently, the rhizobia in the infected plant cells are enclosed within membrane-bounded structures that develop into symbiosomes, where they differentiate into a nitrogen-fixing form called bacteroids. This symbiosome membrane maintains the exchange of substrate and signal molecules between host plant cell and the bacteroids (Verma and Hong 1996). Finally, the cortical cells of symbiosomes continue to divide and fuse together to form the nodule (Oldroyd and Downie 2008; Desbrosses and Stougaard 2011).

The progress in understanding the molecular details of nodulation signaling has been greatly improved by the use of two model plant species, *M. truncatula* and

Fig. 11.1 (continued) the nucleus. Several potassium and calcium channels, calcium pump, and nucleoporins modulate the calcium flux at the nuclear membrane that may activate the calcium/calmodulin-dependent protein kinase (CCaMK) by triggering the calcium spiking inside the nucleus. CCaMK, the master regulator, interacts with other proteins and transcription factors to activate transcriptional programs, leading to stimulation of multiple downstream genes. Dotted arrows indicate proposed signaling routes, whereas solid arrows indicate established signaling pathway. Inside the nucleus arrowheads and blunt heads represent positive and negative regulators, respectively

L. japonicus, although the studies in soybean, peas, peanuts, and few other leguminous species have also been helpful. Both these model species are diploid, have sequenced genome with modest genome sizes, are important representatives of indeterminate and determinate nodules, respectively, and are amenable to genetic manipulation by hairy root transformation as well as by tissue culture-based transgenic plant development (Boisson-Dernier et al. 2001; Limpens et al. 2004; Stiller et al. 1997). Besides, the availability of genetic variants of these two plants from different resources makes it easier for further studies (https://medicago-mutant.noble.org/mutant) (Sandal et al. 2006; Cheng et al. 2014). In addition, the genome-wide synteny between these two plants and other legumes suggests that the study of these model legumes will provide important insight into the important biological questions related to nodulation in other plants as well.

11.3 Hairy Root Transformation: A Strategy for Functional Analysis of Genes

Efficient plant transformation by *Agrobacterium tumefaciens* has been described only in few model legumes (Iantcheva et al. 2013). This procedure is plagued by lengthy shoot regeneration period to analyze the transgenic constructs, and the transformation frequency is relatively poor. To avoid these complications encountered with *A. tumefaciens* transformation techniques, it was important to find a convenient way to allow more rapid evaluation of gene function in the model and other legumes. To address this problem, *Agrobacterium rhizogenes*-mediated hairy root transformation technique was developed. It is a versatile and adaptable model system for a wide variety of plants. Recently, hairy root transformation system has been extensively used to rapidly generate transgenic roots for genetic and molecular analysis.

Hairy roots originate from plants due to the *A. rhizogenes*-mediated transformation. The genetic determinant of hairy root infection is a *rol* gene cluster located on the *A. rhizogenes* root-inducing Ri plasmid (White et al. 1985). This powerful and simple transformation tool facilitates the integration of novel genes into the infected host plant. During this transformation process, the infection occurs within a host plant with a compatible *A. rhizogenes* strain which results in the formation of chimeric transgenic plants, consisting of untransformed shoots with multiple transgenic hairy roots (Lin et al. 2011). In addition to its speed and efficiency, this system offers multiple advantages: (i) the transgenic roots are stably transformed in contrast to transient transformations achieved by biolistic methods, so the results obtained from such studies are more physiologically relevant; (ii) the vectors typically have a GFP marker that allows easy identification of transgenic roots; (iii) the roots remain responsive to various biological treatments, so the effects of transgenes on root biology and physiology can be easily evaluated in an approximately natural environment; (iv) the system provides an efficient way of evaluating multiple constructs

for expression and functionality in a relevant genetic background; (v) the constructs used with *A. rhizogenes* can be used with *A. tumefaciens* without the need for any alteration, so the same constructs can be used for the generation of stable transgenic plants. The system therefore becomes useful for the evaluation of plant-microbe interactions, plant-fungus interactions, plant-nematode interaction, secondary metabolite production, host-parasitic plant interaction, etc. (Boisson-Dernier et al. 2001; Limpens et al. 2004; Chandra and Chandra 2011).

The root nodule (RN) symbiosis has been actively studied for the last three decades using hairy root transformation. The first hairy root transformation by *A. rhizogenes* was reported for *Lotus corniculatus* (Jensen et al. 1986). Subsequently it has been extended to other legumes for nodulation studies (Table 11.1), for example, *Trifolium repens* (Diaz et al. 1989), *Vigna aconitifolia* (Lee et al. 1993), *G. max* (Cheon et al. 1993), *Vicia hirsuta* (Quandt et al. 1993), *L. japonicus* (Stiller et al. 1997; Kumagai and Kouchi 2003), *Trifolium pratense* (Diaz et al. 2000), *M. truncatula* (Boisson-Dernier et al. 2001), *P. sativum* (Clemow et al. 2011), *A. hypogea* (Sinharoy et al. 2009), *S. rostrata*, and *Phaseolus* spp. (Estrada-Navarrete et al. 2007). In the following sections, we will the signaling circuit of nodulation, which has been uncovered based on research using hairy root transformation.

11.4 Role of Hairy Roots in Establishing Flavonoids as a Host-Derived Early Signal for Activation of Bacterial Nod Factors

Flavonoids are one of the largest classes of phenylpropanoid-derived plant secondary metabolites with different functions in plants. More than 10,000 different flavonoids have been identified and are classified into two major groups: 2-phenylchromans (flavonoids) and 3-phenylchromans (isoflavonoids). These secondary metabolites are involved in multiple physiological processes including plant structural integrity, protection against ultraviolet (UV) radiation and phytopathogens, auxin transport, coloration of flowers, and importantly nodulation signaling process (Ferreyra et al. 2012). During nodulation, legume roots release specific flavonoids into the surrounding soil to attract the rhizobia. Flavonoids also act as auxin transport inhibitors inside the plant roots to change its direction and accumulate auxin at specific sites to allow cortical cell division for nodule formation. To assess the functional role of flavonoids during nodulation genetically, hairy root transformation was used in *M. truncatula*. RNAi-mediated knockdown of *chalcone synthase* gene, which catalyzes the first committed step in the flavonoid biosynthesis pathway, significantly inhibited flavonoid production in transgenic hairy roots. These flavonoid-deficient transgenic roots were unable to initiate nodules, although the auxin transport remains unaffected in this root (Wasson et al. 2006). To investigate further details of the role of individual flavonoids, different biosynthetic enzymes of the flavonoid pathway including *isoflavone synthase*, *chalcone*

Table 11.1 Examples of successful hairy root transformation in different legumes

Plant common name	Scientific name	Family	*Agrobacterium rhizogenes* strain	*Rhizobia species*	Protocol
Barrel clover	*Medicago truncatula*	Fabaceae	*Agrobacterium rhizogenes* ARqua1	*Sinorhizobium meliloti* strain RCR2011	Boisson-Dernier et al. (2001)
Bird's-foot trefoil	*Lotus japonicus*	Fabaceae	*Agrobacterium rhizogenes* LBA1334	*Mesorhizobium loti* Tono	Kumagai and Kouchi (2003)
Soybean	*Glycine max*	Fabaceae	*Agrobacterium rhizogenes* K599	*Bradyrhizobium japonicum* (61A76)	Cheon et al. (1993)
Common bean	*Phaseolus vulgaris*	Fabaceae	*Agrobacterium rhizogenes* K599	*Rhizobium tropici* strain CIAT899	Estrada-Navarrete et al. (2007)
Pea	*Pisum sativum*	Fabaceae	*Agrobacterium rhizogenes* AR12 and AR1193	*Rhizobium leguminosarum* bv. viciae 128C53K	Clemow et al. (2011)
Peanut	*Arachis hypogea*	Fabaceae	*Agrobacterium rhizogenes* R1000	*Bradyrhizobium* sp. (Arachis) NC92	Sinharoy et al. (2009)
Moth bean or Turkish gram	*Vigna aconitifolia*	Fabaceae	*Agrobacterium rhizogenes* A4	*Bradyrhizobium* sp. *cowpea strain 3456*	Lee et al. (1993)
Hairy tare or tiny vetch	*Vicia hirsuta*	Fabaceae	*Agrobacterium rhizogenes* 15834, C58C1, AR12, R1000, ARqua1, ARqua2	*Rhizobium leguminosarum* bv. viciae	Quandt et al. (1993)
White clover	*Trifolium repens*	Fabaceae	*Agrobacterium rhizogenes* LBA9402	*Rhizobium leguminosarum* bv. viciae	Diaz et al. (1989)
Red clover	*Trifolium pratense*	Fabaceae	*Agrobacterium rhizogenes* LBA 1334	*Rhizobium leguminosarum* bv. trifolii ANU843, *Rhizobium leguminosarum* bv. viciae 248, *Mesorhizobium loti* E1R, M. loti E1R, *Sinorhizobium meliloti* 2011 pMP604	Diaz et al. (2000)

reductase, *flavone synthase*, and *chalcone synthase* were silenced by hairy root transformation in *M. truncatula*. These experiments revealed that the activation of rhizobial Nod operon and sustained induction of NF biosynthesis during infection thread development were indeed facilitated by flavone, whereas auxin transport was modulated by flavonols. Conversely, no significant role was assigned to isoflavonoids during nodulation signaling (Zhang et al. 2009). Overall, these data confirmed that legumes use different flavonoid compounds to activate the rhizobial nod operon and auxin transport modulation in roots during nodulation, underpinning a link between RN symbioses and auxin signaling through flavonoids.

11.5 The Plasma Membrane-Localized Components of Nodulation Signaling and Role of Hairy Roots in Their Discovery

Flavonoids stimulate the transcription of bacterial nodulation genes leading to the production of a lipochitin oligosaccharide signal, the Nod factors (NFs). NFs are perceived by a pair of membrane-bound LysM receptors which lead to multiple cellular responses including deformation and curling of root hairs for eventual invasion of rhizobia and cortical and pericycle cell divisions (Oldroyd and Downie 2008; Oldroyd et al. 2011; Wais et al. 2002). LjNFR1 and LjNFR5 in *L. japonicus*, MtLYK3 and MtNFP in *M. truncatula*, and GmNFR1α and GmNFR1β and GmNFR5α and GmNFR5β in *G. max* are the LysM-RLKs (LysM-receptor-like kinases) crucial for NF perception and activation of symbiotic signaling (Oldroyd et al. 2011). Both LysM receptors (NFR1 and NFR5) interact with each other to form a heterodimer, which can initiate downstream signaling. NFR1 contains an active kinase domain, whereas NFR5 lacks several conserved kinase subdomains and acts as a co-receptor. Mutants of these genes show complete impairment of nodule formation due to the lack of NF perception (Radutoiu et al. 2003; Limpens et al. 2003; Madsen et al. 2003;Smit et al. 2007). Functional roles of these receptors were established by the use of transgenic hairy roots. For example, overexpression or complementation of *NFR1* in *nfr1* mutant (*nod49*) or *NFR5* in *nfr5* mutant (*nod133*) background by strong constitutive or native promoter results in nodule formation after rhizobial infection in soybean (Indrasumunar et al. 2011; Indrasumunar et al. 2010; Lin et al. 2011; Roy Choudhury and Pandey 2015). Similarly, functional complementation of *nfr* mutants using the *A. rhizogenes* hairy root transformation revealed that NFR1 kinase activity is essential for the in vivo function of NFR1, and NFR1 can activate the NFR5 by phosphorylation (Madsen et al. 2011). These data led to an important question: How does the signal perception by membrane-bound NFRs connect with the downstream signaling in the nucleus? Again, hairy root transformation-based research helped elucidate many nuclear and cytoplasmic components of this signaling pathway.

In addition to the LysM receptors, another leucine-rich repeat receptor-like kinase (LRR-RLK) proteins play a significant role in nodulation by infection initiation as well as for the internalization of bacteria in cortex cells during symbiosome formation (Endre et al. 2002). This LRR-RLK commonly known as symbiosis receptor kinase (SymRK) in *L. japonicus*, DOES NOT MAKE INFECTIONS 2 (DMI2) in *M. truncatula*, NORK (nodulation receptor kinase) in *G. max*, and SYM19 (Symbiosis 19) in *P. sativum* contains three LRR domains, a transmembrane domain, and an intracellular kinase domain. Several studies revealed that SymRK interacts with and functions downstream of the NFR1/LYK3 and NFR5/NFP receptors (Endre et al. 2002; Stracke et al. 2002; Markmann et al. 2008; Oldroyd and Downie 2008). Although the activation mechanisms of SymRK are unclear, it is proposed that these receptor-like proteins form a complex with NFR proteins. It is also possible that SymRK accepts yet unknown extracellular signals by its LRR domain (Stracke et al. 2002). Recently the autophosphorylation of a tyrosine residue of SymRK was determined to be important for regulating its symbiotic activity (Saha et al. 2016). *RNAi*-mediated knockdown of *SymRK* by hairy root transformation in *M. truncatula* and *S. rostrata* established that the protein is crucial not only for early infection stage but also for symbiosome formation during nodule development (Capoen et al. 2005; Limpens et al. 2005).

11.6 The Cytosolic Components of Nodulation Signaling and Role of Transgenic Hairy Roots in Their Identification and Characterization

Relatively few cytoplasmic components involved in regulation of nodule formation immediately following the NF perception are known to date. To identify potential interacting proteins of the receptors, a yeast two-hybrid-based cDNA library screen was performed using LYK3 of *M. truncatula* (Andriankaja et al. 2007). This study identified PUB1, a UND-PUB-ARM protein or U-box (PUB) E3 ubiquitin ligase protein, as an interactor of LYK3. PUB1 is strongly induced by NFs, specifically in the roots during nodulation. Additionally, PUB1 is phosphorylated by LYK3 in vitro. To address the question of the physiological role of PUB1, both knockdown and overexpression approaches were used in hairy root transformation system. A strong increase in the number of nodules was observed by suppressing *PUB1* levels, whereas its overexpression caused a delay in nodulation. This study established that a possible receptor-mediated, phosphorylation-based mechanism modulates PUB1 (or E3 ubiquitin ligases in general) in controlling plant-rhizobial interactions by functioning as a negative regulator of LYK3 signaling pathway (Mbengue et al. 2010).

A similar yeast-based library screening was performed by using kinase domain of NFR5 in *L. japonicus*. This screen identified Rho-like GTPase (ROP6) protein, which interacts with NFR5 in a GTP-binding-dependent manner. Again, to establish

the role of ROP6 in regulating nodulation, transgenic hairy roots were generated by RNAi-mediated silencing of *ROP6*. A detailed study of transgenic hairy roots at different developmental stages confirmed that rhizobium entry was not influenced by ROP6, but ROP6 is most likely responsible for the establishment of infection thread (IT) growth through the root cortex. Suppression of *ROP6* resulted in fewer nodules, whereas its overexpression or expression of a constitutively active version of ROP6 (ROP6-CA) using hairy root transformation exhibited extensive root hair deformation after rhizobium (*Mesorhizobium loti*) infection, resulting in an increase of infection threads and nodule number (Yuan et al. 2012). Further study on ROP6 has led to a model of clathrin-mediated endocytosis by clathrin triskelion (CHC1), as an interactor of ROP6. The potential role of CHC1 was also ascertained by reduction of nodule number in transgenic hairy roots after overexpression of inactive domain of CHC1 or silencing of CHC1 during hairy root transformation (Wang et al. 2015). Taken together these data suggest a possibility of endocytosis of NFRs by the potential link between NFR5 and clathrin via ROP6 GTPase during nodulation.

A suppressive subtractive approach in response to infection with *Rhizobium etli* strains in *P. vulgaris* found a GTPases of the Rab subfamily, RabA2, which is responsible for the polar growth of root hair. Interestingly, nodulation was impaired in *RabA2* RNAi-silenced hairy roots indicating nodulation in *RabA2* RNAi plants is most likely the consequence of a compromised vesicle trafficking, which is required for deposition of cell wall material for the infection thread formation (Blanco et al. 2009). These data suggest the involvement of GTPases in signaling during nodulation.

One of the most well-defined membrane-bound signaling systems present in all eukaryotes is the heterotrimeric G-protein complex, consisting of Gα, Gβ, and Gγ proteins. Earlier pharmacological evidences provided the evidence for the involvement of heterotrimeric G-proteins in atmospheric nitrogen-fixing nodulation process in leguminous plants (Kelly and Irving 2003; Sun et al. 2007). Different downstream components of the heterotrimeric G-protein signaling, including phospholipase C and D, phosphatidic acid, diacylglycerol pyrophosphate, monomeric G-proteins, and MAP kinases, have been proved to involve in the regulation of nodulation (Pingret et al. 1998; Sun et al. 2007; Kelly and Irving 2003; Peleg-Grossman et al. 2007; Oldroyd et al. 2011; Tirichine et al. 2006). To directly establish the involvement of heterotrimeric G-protein during nodulation signaling, specific subunits of this signaling complex were knocked down or overexpressed in soybean hairy root system. Detailed analyses of the transgenic root phenotypes revealed that the Gβ and Gγ subunits act as positive regulators of nodule development, whereas the Gα subunits act as a negative regulator (Roy Choudhury and Pandey 2013). To establish the direct role of G-protein signaling per se in regulation of nodulation process in soybean, additional members of the G-protein complex were evaluated. A regulator of G-protein signaling, a GTPase activity-accelerating protein (GAP), which deactivates the G-protein cycle, positively regulated nodule development as revealed by gene silencing and overexpression approaches using hairy root transformation (Roy Choudhury and Pandey 2015). To probe if the heterotrimeric G-proteins are directly interacting with the NFRs, an interaction screen was performed. Both the Gα

proteins and RGS proteins interacted with the NFR1 protein of soybean. Furthermore, NFR1 was able to phosphorylate the RGS proteins, and the phosphorylation led to an increase in its activity. This model suggested that at least one of the functions of the NFRs after activation is to phosphorylate the RGS proteins, which deactivates the Gα protein. Because the Gα protein is a negative regulator of nodule formation, its deactivation led to successful nodulation. To further validate this model, it was hypothesized that if one of the roles of the NFRs is to phosphorylate RGS proteins, then introduction of a phosphomimic mutant of RGS protein in a mutant lacking the receptor should be able to restore nodulation, at least partially. To confirm this hypothesis, a phosphomimic version of RGS protein was introduced in the *nod49* (NFR1) mutant of soybean by hairy root transformation. Partial restoration of nodule formation was observed, validating the hypothesis that the heterotrimeric G-protein cycle is acting directly downstream of the NFRs to control nodule formation in plants (Roy Choudhury and Pandey 2015, 2016).

The proteins functioning downstream of the SymRK complex and the signaling pathways that follow are also beginning to be explored (Stracke et al. 2002). In search of the potential interacting partner of SymRK, a yeast-based library screening was performed by using intracellular kinase domain of *L. japonicus* SymRK as bait. An AT-rich interaction domain (ARID) containing SymRK-interacting protein 1 (SIP1) was identified as an interacting partner of SymRK (Zhu et al. 2008). Silencing or overexpression of *SIP1* using transgenic hairy roots led to reduced or increased nodule numbers, respectively, suggesting a positive role of SIP1 during nodulation (Wang et al. 2013). Yeast-based library screening also identified SymRK-interacting protein 2 (SIP2) as another potential interacting partner of SymRK in *L. japonicus*. SIP2 belongs to the plant MAPKK family, and in vitro analysis revealed that SymRK has a specific inhibitory effect on the kinase activity of SIP2 toward its substrate MPK6 (Chen et al. 2012). To establish the functional role of SIP2, hairy root transformation was used to knock down its expression. Suppression of *SIP2* reduced infection thread formation and nodule organogenesis, indicating a positive role of SIP2 in nodulation similar to SIP1. Overall, these data suggest that the route of signal transmission from SymRK to downstream components is likely via the MAPK-based signaling module.

Several E3 ubiquitin ligases were also identified as potential interactors of SymRK in yeast-based screening. Similar to NFR1, SymRK can interact with and phosphorylate PUB1, an E3 ubiquitin ligase in *M. truncatula* (Vernie et al. 2016; Mbengue et al. 2010). Additional genetic analysis revealed that PUB1, via its ubiquitination activity, is essential for rhizobial infection and nodulation. Another E3 ubiquitin ligase, SEVEN IN ABSENTIA (SINA4), interacts with the kinase domain of SymRK in *L. japonicus*. Ectopic expression of *SINA4* negatively influenced SymRK protein levels for its ubiquitination activity resulting in the impairment of infection thread formation and a strong reduction in bacteroid abundance. Additionally, promoter analysis of *SymRK* and *SINA4* after hairy root transformation has shown partially overlapping expression patterns of these genes during rhizobial infection and early nodule development (Den Herder et al. 2012). Furthermore, another SymRK-interacting E3 ubiquitin ligase, SIE3, has been shown to bind

with and use SymRK as a substrate for ubiquitination in *L. japonicus*. Silencing of *SIE3* transcripts via RNAi in hairy roots inhibited infection thread development and nodule organogenesis, whereas overexpression resulted in increased nodule numbers (Yuan et al. 2012). Overall, these data imply that the modulation of protein turnover of membrane-bound receptors like NFR and SymRK by ubiquitination is a key regulatory strategy during RN symbiosis.

In addition to the ubiquitin ligases, yeast-based library screening also identified 3-hydroxy-3-methylglutaryl-CoA reductase1 (HMGR1), a key enzyme regulating the mevalonate (MVA) pathway, as a specific interactor of the SymRK or DMI2 kinase domain in *M. truncatula* (Kevei et al. 2007). The N-terminal of HMGR1 catalytic region is sufficient and specific for binding to DMI2. RNAi silencing of *HMGR1* by hairy root transformation indicated a requirement of HMGR1 activity in the infection process during nodulation. It was also predicted that the active DMI2-HMGR1 complex at early root hair infection induced an invagination of the plasma membrane to initiate infection thread growth when rhizobia were entrapped in a root hair curls. These data also revealed that the Nod factor signaling recruits specific isoprenoid biosynthesis pathways via DMI2-HMGR1 for the production of cytokinins and steroids to modulate the cell division, which is essential for nodule organogenesis. In addition, an analysis of epidermal cells of *HMGR1-RNAi* transgenic hairy roots after application of NFs exhibits altered Ca^{2+} spiking and *ENOD11* (a key transcription factor of nodulation) expression in *M. truncatula*, indicating a role for the mevalonate pathway in early RN symbiotic signaling (Venkateshwaran et al. 2015).

Another example of a potential interactor in *M. truncatula* is the symbiotic remorin 1 (SYMREM1) protein, which is usually required for plant-bacteria interactions. This SYMREM1 can specifically interact with the symbiotic RLKs including LYK3/NFR1, NFR5/NFP, and DMI2/SymRK. The study of hairy roots in transgenic RNAi lines suggested that SYMREM1 functions as a scaffolding protein, and it might be required at the preinfection stage through the regulation of receptor proteins for the perception of bacterial signaling molecules (Lefebvre et al. 2010).

11.7 The Nuclear Components of Nod Factor Signaling and Their Identification via Hairy Root Transformation

11.7.1 Ion Channels and Nucleoporins

Calcium ions are key secondary intracellular messengers for a multitude of processes, relaying precise information by their ability to produce a wide variety of molecular signatures in both animal and plant cell signaling. Calcium signals are generated by a number of channels and pumps. In response to NFs, two different

calcium responses have been observed in legume roots, calcium flux and calcium oscillations. Calcium influx arises rapidly after receiving bacterial NFs, and subsequently a wave of calcium influx begins at the root hair tips and moves along the length of the root hair cell toward the nuclear membrane for membrane depolarization. Calcium oscillations or calcium spiking is observed approximately 10 minutes after the initial signal within the nucleus (Wais et al. 2002).

The mechanisms underlying calcium spiking during RN symbioses in the nucleus of root cells, which function downstream of the receptor and the cytosolic signaling, were enigmatic. A major breakthrough was attained by the characterization of genetic mutants, providing crucial information for understanding the nodulation signaling pathways. The two mutants of *L. japonicus*, *castor* and *pollux* genes, retained Ca^{2+} influx at the root hair tip but were impaired in the perinuclear Ca^{2+} spiking, which was required for establishing symbiotic relationships. The electrophysiological, yeast complementation and localization studies suggested that CASTOR and POLLUX are potassium-permeable cation channels. Homologs of these genes were identified in *M. truncatula* where DMI1 (DOES NOT MAKE INFECTIONS1) was characterized as a putative ortholog of POLLUX and in *P. sativum* where SYM8 (SYMBIOSIS8) was characterized as a putative paralogs of CASTOR (Edwards et al. 2007; Matzke et al. 2009). CASTOR and POLLUX share similarity with the NAD-binding TrkA domain of bacterial K^+ channels (Ane et al. 2004; Imaizumi-Anraku et al. 2005; Chen et al. 2009). Although CASTOR and POLLUX were reported to be localized in plastids, later investigation unveiled that DMI1 (POLLUX ortholog in *M. truncatula*) is restricted to the nucleus periphery and has a direct role in conducting ions in the nuclear compartment (Riely et al. 2007). In order to test the biological function of CASTOR and POLLUX, hairy root transformation was performed by complementing two mutants, namely, *castor-12* and *pollux-5*, respectively, with native genes expressed with a constitutive promoter. The results confirmed a positive role of CASTOR and POLLUX in mediating perinuclear Ca^{2+} spiking by the release of calcium from the nuclear envelope to modulate the nodulation signaling (Charpentier et al. 2008). A series of cross-species complementation experiments by transgenic hairy root transformation revealed that both DMI1 in *M. truncatula* and SYM8 in pea also have the capacity to compensate for the loss of CASTOR and POLLUX in *L. japonicus*, uncovering an unexpected twist in the evolution of ancestral and essential symbiotic proteins. An additional complementation assays using hairy roots revealed that a single amino acid change in DMI1 (serine to alanine substitution in the filter) is responsible for the improvement of DMI1 by enhancing the Ca^{2+}-induced Ca^{2+} release and reducing potassium conductance (Venkateshwaran et al. 2012). These data provide novel insights into the mechanism of DMI1 or CASTOR and POLLUX as calcium ion channels and underline its importance during rhizobial infection.

Considering that calcium changes also occur in the cytoplasm, an additional component, preferably a calcium pump, would be required at the nuclear membrane for efficient reuptake of the nuclear calcium. In an attempt to elucidate such components, MCA8, a nuclear-localized SERCA-type calcium ATPase, was

identified in *M. truncatula*. MCA8 localization was confirmed in transgenic hairy root cells by immunogold labeling. Unlike DMI1, MCA8 is present on both inner and outer nuclear membranes and at the ER to modulate the nuclear calcium oscillations by capturing the released calcium into the nuclear-associated cytoplasm. Furthermore, silencing of *MCA8* by hairy root transformation diminished NF-induced calcium oscillations, confirming its role as a calcium pump (Capoen et al. 2011).

Recently, for the identification of additional calcium channel proteins, hairy root transformation-based gene silencing approaches were used to assess the roles of different members of the cyclic nucleotide-gated channel (CNGC) gene family. RNAi-mediated silencing of *CNGC15a*, *CNGC15b*, and/or *CNGC15c* correlated with the defects in symbiotic associations. Localization studies confirmed that CNGC15 proteins are present in the nuclear envelope and are permeable to Ca^{2+}. Moreover, hairy root transformation revealed that variants of CNGC15 members complemented their respective mutant phenotypes establishing their roles in nuclear Ca^{2+} oscillations and in the symbiotic signaling pathway (Charpentier et al. 2016).

Characterization of another nodulation-deficient mutant *nup133* in *L. japonicus* by genetic and physical mapping, followed by sequencing of the mutant alleles, identified nucleoporins as regulators of nodulation signaling, especially by working at the junction of nuclear and intracellular plastid organelle membranes. To ascertain functionality of NUP133, *in planta* complementation of mutant alleles was performed using the hairy root transformation. *NUP133* gene successfully restored the mutant phenotype confirming its role in a rapid nuclear-cytoplasmic communication after host-plant recognition of symbiotic microbes (Kanamori et al. 2006). Later, another putative nucleoporin gene, *NUP85*, was identified through positional cloning and phenotypic analysis of a mutant from *L. japonicus*. Complementation of the *nup85* mutant with the putative nucleoporin-like gene demonstrated that *NUP85* is a prerequisite for nodule formation (Saito et al. 2007). Overall, multiple biochemical and genetic results suggest that nucleoporins such as NUP133 and NUP85 likely modulate the permeability of the nuclear pores to calcium ions, thereby facilitating the calcium spiking. Alternatively, nucleoporins might facilitate transport of POLLUX or CASTOR through the nuclear pore to the inner nuclear membrane (Matzke et al. 2009).

Major components of the nodulation signaling pathway including DMI2/SYMRK/Sym19, DMI1/POLLUX, NUP85, NUP133, and CASTOR are responsible for the establishment of both nodulation and mycorrhization. All these proteins are directly or indirectly involved to facilitate the calcium spiking for following a common symbiotic pathway. A genetic screen of a mutant related to arbuscular mycorrhizal (AM) symbiosis identified a WD40 repeat protein related to the nucleoporins, known as NENA. To test the functionality and localization of NENA during nodulation, hairy root transformation-based assays were performed. Complementation of *nena-1* mutant with the corresponding gene under native or constitutive promoter restored the nodule formation capacity. Interestingly NENA is localized at the nuclear rim by its interaction with NUP85 (Groth et al. 2010), implying an additional role of a nucleoporin in the control of symbiotic associations.

11.7.2 The CCaMK Complex

The LysM receptor kinase mutants (*nfr1* and *nfr5*) lacked both calcium influx and calcium spiking, whereas five other mutants including *SymRK* (LRR receptor kinase), *castor*, *pollux* (cation channels), *nup133*, and *nup85* (nucleoporins) were defective for calcium spiking but retained a calcium flux suggesting that these two steps can be delineated (Shaw and Long 2003; Miwa et al. 2006).

In *M. truncatula*, *dmi1* (*pollux*) and *dmi2* (*SymRK*) mutants were defective for calcium spiking, whereas *dmi3* mutants showed steady calcium spiking in response to NFs, suggesting that calcium spiking acts downstream of both DMI1 and DMI2 but upstream of DMI3. *DMI3* codes for CCaMK, a calcium/calmodulin-dependent serine-threonine protein kinase. *dmi3* mutants form no nodules, but this phenotype can be fully complemented by the introduction of the wild-type genomic sequence of *DMI3* gene by hairy root transformation (Levy et al. 2004). Furthermore, hairy root transformation of *snf1* (spontaneous nodule formation) mutant of *L. japonicus* with a candidate *CCaMK* gene resulted in the formation of spontaneous nodules, independent of the NFs, suggesting a central regulatory position of CCaMK upstream of all components required for cell cycle activation (Tirichine et al. 2006). Hairy root-based complementation analysis of another *CCaMK* mutant in *L. japonicus* (*ccamk-3*) by wild-type and gain-of-function variant of CCaMK ($CCaMK^{T265D}$) revealed that the protein is specific for nodule development (Shimoda et al. 2012) and works downstream of the common symbiotic pathway which is shared by nodulation and mycorrhization. This is different from DMI1 and DMI2 proteins, which are a part of the common symbiotic pathway.

Given the central importance of CCaMK, several methodologies have been used to identify its interacting partners in the last few years. A yeast-based approach identified a novel protein named IPD3 (interacting protein of DMI3) from *M. truncatula* as an interacting partner of CCaMK. Localization studies and promoter analysis by hairy root transformation revealed that IPD3 expresses primarily in the root vasculature and co-localizes with DMI3 to the nucleus (Messinese et al. 2007). Further characterization of *ipd3* mutants in *M. truncatula* confirmed that IPD3 function is partially redundant, i.e., nodulation (and mycorrhization) was initiated but then aborted (Horvath et al. 2011). PsSYM33 is an ortholog of IPD3 in *P. sativum* (Ovchinnikova et al. 2011), which also has a role in nodule development in pea. The IPD3 homolog in *L. japonicus* turned out to be the CYCLOPS gene. CYCLOPS is a phosphorylation target of CCaMK. The involvement CYCLOPS in rhizobial infection during symbiotic signaling was further confirmed after hairy root transformation-based complementation of *cyclops* mutant by the corresponding gene. Since *cyclops* mutants retained the ability to initiate cortical cell division during nodule organogenesis, it suggests that CYCLOPS is dispensable for nodule organogenesis (Yano et al. 2008; Limpens and Bisseling 2014). Moreover,

cyclops mutants cannot be complemented by either CCaMK gain-of-function mutant ($CCaMK^{T265D}$) or wild-type CCaMK indicating that CYCLOPS is positioned downstream of CCaMK in the symbiotic pathway (Hayashi et al. 2010). Later, hairy root transformations in different mutants of *L. japonicus* confirmed that CYCLOPS, a DNA-binding transcriptional activator, activates the *NODULE INCEPTION (NIN)* genes in a phosphorylation-dependent manner to regulate the symbiotic signaling (Singh et al. 2014).

Similar to CYCLOPS, CIP73, which belongs to the large ubiquitin superfamily, has been found to be a direct interacting partner and phosphorylation target of CCaMK. RNAi-mediated silencing of *CIP73* in *L. japonicus* hairy roots resulted in fewer nodules, suggesting that CIP73 is a positive regulator of nodulation (Kang et al. 2011). Further yeast-based experimental analysis recognized a cochaperone protein, HIP (HSC/HSP70 interacting protein), as an interacting partner of CIP73. Unlike CIP73, the suppression of HIP expression in hairy roots led to increased nodule numbers, indicating that HIP is a negative regulator of nodulation (Kang et al. 2015).

CCaMK-CYCLOPS complex initiates nodule organogenesis following calcium oscillations in the host nucleus. Further investigation of downstream signaling identified DELLA protein in *M. truncatula*, which are the central regulators of gibberellic acid (GA) signaling. These DELLAs increased the phosphorylation of CYCLOPS by forming a complex with CCaMK-CYCLOPS. To assess whether DELLA proteins have a role during symbiosis, hairy root transformation was used to decrease the expression of *DELLAs* by RNAi approaches. Knocking down *DELLAs* caused a decrease in nodule number in the hairy roots demonstrating their positive regulatory roles in RN symbiosis (Jin et al. 2016). Furthermore, DELLAs can form a protein complex with transcription factors NSP1-NSP2 (nodulation signaling pathway 1 and 2) and are able to form a connection between CYCLOPS and NSP2 (Jin et al. 2016) indicating their role in GA-mediated RN symbiosis.

11.7.3 Transcription Factors Involved in RN Symbiosis

Both *NSP1* and *NSP2* of *M. truncatula* encode genes with similarity to members of the GRAS family of putative transcriptional regulators or transcription factors. *SYM7* of *P. sativum* is a possible ortholog of *NSP2*. In addition to the classical genetic analysis, hairy root transformation was used to decipher the function of NSP1 and NSP2 by complementation and subcellular localization. Complementation of *nsp1 and nsp2* mutants by native genes and subcellular localization using native promoter-driven *NSP1/2-GFP* established that both these proteins co-localize with CCaMK in the nucleus, and CCaMK acts directly upstream of NSP1 and NSP2 (Catoira et al. 2000; Kalo et al. 2005; Smit et al. 2005). NSP1-NSP2 heteropolymerization is essential for nodulation signaling (Hirsch et al. 2009). *NSP1* binds to the promoter of the NF-inducible genes, namely, *ENOD11*, *ERN1*,

and *NIN*. To assess the functional role of NSPs, *nsp2* mutants were complemented with the wild-type gene as well as the domain-swapped *NSP2* or variant *NSP2* which can no longer dimerize with NSP1. Termination of nodule formation in domain-swapped NSP2 and a decreased nitrogen fixation activity in variant *NSP2* transgenic hairy roots confirmed their role as functional heterodimers.

To identify additional regulatory components of nodulation, a transposon-tagged *L. japonicus* mutant *nin* (nodule inception) was identified, which produces no nodules (Schauser et al. 1999). NIN is an essential transcription factor responsible for initiating nodulation-specific symbiotic processes, and it acts downstream of the *NSP* genes. *Sym35* gene required for root nodule development in *P. sativum* is an ortholog of *NIN* (Borisov et al. 2003). Hairy root transformation confirmed the functional complementation of *nin-1* mutants by *NIN1*. Additionally, the overexpression of *NIN* in *M. truncatula* induced cortical cell divisions leading to spontaneous nodule development in the transgenic roots in the absence of rhizobia, suggesting that NIN functions downstream of CCaMK (Soyano et al. 2013; Marsh et al. 2007). Transcriptional profiling and promoter analysis revealed that *NIN* restricts the *ENOD11* expression through competitive inhibition of *ERN1* (Vernie et al. 2015).

To investigate the downstream targets of NIN, two transcriptional targets, namely, *NF-YA1* and *NF-YB1*, were identified by a screen for suppressors of the *L. japonicus har1-1* hypernodulation phenotype. These NF-Ys (A, B, C subunits) are heterotrimeric CCAAT box-binding transcription factors. HAP2 and HAP3 in *M. truncatula* are the possible orthologs of LjNF-YA1 and LjNF-YB1. Interestingly, *RNAi*-mediated silencing of *NF-YA1* in *L. japonicus* hairy roots did not inhibit the epidermal responses and led infection thread formation and growth but prevented cortical cell division required for the development of nodules. Similar response was exhibited by the loss of function of *NIN*. Conversely, overexpression of *NIN* and *NF-Y* genes in *L. japonicus* enhanced cell division during nodule formation, implying that NIN is a key player in root nodule organogenesis and NF-Y subunits function downstream of NIN (Soyano et al. 2013; Combier et al. 2006). Additionally, gene expression analysis in the hairy roots of *NF-YA* RNAi lines suggested that *NF-YA1/2* acts upstream of *ERN1* and *ENOD11* in the nodulation signaling pathway (Laloum et al. 2014). Recently, three more members of SHI/STY (SHORT INTERNODES/STYLISH) transcription factor gene families, namely, STY1, STY2, and STY3, have been identified as direct targets of *NF-YA1* (Hossain et al. 2016). A cytokinin receptor CRE1 (cytokinin response element 1) is essential for nodule organogenesis (Plet et al. 2011; Gonzalez-Rizzo et al. 2006), and *CRE1* promoter-driven expression of GUS in *M. truncatula* hairy roots was significantly reduced in the *nin-1* mutant compared to the wild type. This suggests that NIN binds to the *CRE1* promoter and activates *CRE1* expression in the root cortex (Vernie et al. 2015; Soyano et al. 2014).

A genetic screen in a population of fast neutron-mutagenized *M. truncatula* plants identified a gene, *BIT1* (*branching infection threads 1*), necessary for the infection thread formation. Overexpression of auto-activated CCaMK in *bit1-1* mutants by hairy root transformation did not produce any spontaneous nodules, demonstrating

that *BIT1* functions downstream of CCaMK for the activation of nodule organogenesis. Overexpression of *ENOD11-GUS* in *bit1-1* mutants showed severely reduced *ENOD11-GUS* induction after NFs application, confirming BIT1's role in nodulation pathway. Furthermore, an ethylene response factor (ERF) required for nodulation, ERN (ERF required for nodulation), complemented the *bit1-1* mutant phenotype and confirmed that ERN is necessary for NF signaling and functions by activation of *ENOD11* (Middleton et al. 2007; Andriankaja et al. 2007). Silencing and overexpression of *EFD* (ethylene response factor required for nodule differentiation), another ERF transcription factor, by hairy root transformation affected the nodule development by regulation of the cytokinin pathway genes (Vernie et al. 2008). These data provide a new connection between ethylene and cytokinin pathway transcription factors during nodulation signaling.

RNAi-mediated silencing and overexpression studies in hairy root system also demonstrated the role of KNOX transcription factors in nodule development in *M. truncatula* (Di Giacomo et al. 2017). Similarly, gene silencing also revealed that a Myb transcription factor, *control of nodule development* (*CND*), is also involved in regulation of soybean nodulation (Libault et al. 2009).

11.7.4 Downstream Regulatory Genes Involved in Nodule Development

Genetic studies and transcriptome analysis have identified a number of downstream genes essential in NF signaling. To understand the molecular mechanisms of these genes, hairy root transformation became a suitable tool to assess them by complementation analysis, *RNAi*-mediated gene silencing, overexpression, and promoter analysis. Table 11.2 lists a number of genes, which were identified as potential regulators of nodule formation by using hairy root approaches. Further targeted analysis will pinpoint how these genes are connected to the established modules of nodulation signaling.

11.8 Long-Distance Control of Nodulation

Nodulation is an extremely energy-requiring process, and plants control both the timing and number of nodules formed by a shoot-derived protein which encodes a putative leucine-rich, serine-threonine receptor kinase with homology to *Arabidopsis* CLAVATA1 (CLV1). This receptor-like kinase is activated from root-derived CLE peptides. The lack of CLV1 protein due to gene disruption causes hyper- or supernodulation in legume roots due to a defect in the systemic negative feedback mechanism called autoregulation of nodulation (AON). AON is initiated during nodule development by the synthesis of a root-derived signal named "Q" or

Table 11.2 Examples of downstream genes responsible for nodule formation as confirmed by hairy root transformation

Gene name	Scientific name (Plant)	Gene silencing (RNAi)	Gene silencing (Micro RNA)	Over-expression	References
ENOD40 (*ENOD40-1* and *ENOD40-2*)	*Medicago truncatula*	+	–	–	Kumagai et al. (2006), Wan et al. (2007)
CDC16 (*CELL DIVISION CYCLE16*)	*Medicago truncatula*	+	–	–	Kuppusamy et al. (2009)
RbohA (*NADPH oxidase*)	*Medicago truncatula*	+	–	–	Marino et al. (2011)
nsRING (an RING-H2 finger domain protein)	*Lotus japonicus*	+	–	–	Shimomura et al. (2006)
GS52, an *ecto-apyrases*	*Glycine max*	+	–	–	Govindarajulu et al. (2009)
FWL1 (*FW2-2-like1*)	*Glycine max*	+	–	–	Libault et al. (2010)
EXPB2, an *expansin* gene	*Glycine max*	+	–	+	Li et al. (2015)
SGF14c/SGF14l, an G-box factor	*Glycine max*	+	–	–	Radwan et al. (2012)
PT5, a phosphate transporter	*Glycine max*	+	–	+	Qin et al. (2012)
UPS1 (*ureide permease 1*)	*Glycine max*	+	–	–	Collier and Tegeder (2012)
ACP, an acyl carrier protein	*Glycine max*	+	–	–	Wang et al. (2014)
S6 kinase 1	*Glycine max*	+	–	–	Um et al. (2013)
GH3, *GRETCHEN HAGEN 3*	*Glycine max*	–	+	–	Damodaran et al. (2017)
NMHC5, a sucrose regulatory MADS-box transcription factor	*Glycine max*	–	–	+	Liu et al. (2015)
Early nodulin 93 (*ENOD93*)	*Glycine max*	–	+	–	Yan et al. (2015)
Mannosyl-oligosaccharide 1, *2-alpha-mannosidase* (*MNS*)	*Glycine max*	–	+	–	Yan et al. (2016)
Rhizobium-induced peroxidase 1(*RIP1*)	*Glycine max*	–	+	–	Yan et al. (2016)
Rbohb (NADPH oxidase)	*Phaseolus vulgaris*	+	–	–	Montiel et al. (2012)
IFR1, an isoflavone reductase gene family	*Phaseolus vulgaris*	+	–	–	Ripodas et al. (2013)

(continued)

Table 11.2 (continued)

Gene name	Scientific name (Plant)	Gene silencing (RNAi)	Gene silencing (Micro RNA)	Over-expression	References
RACK1, a receptor for activated C kinase	*Phaseolus vulgaris*	+	–	–	Islas-Flores et al. (2011)
TOR, a protein kinase gene, rapamycin	*Phaseolus vulgaris*	+	–	–	Nanjareddy et al. (2016)
TPS9, a class II trehalose-6-phosphate synthase	*Phaseolus vulgaris*	+	–	–	Barraza et al. (2016)
HK1, a cytokinin receptor histidine kinase	*Arachis hypogaea*	+	–	–	Kundu and DasGupta (2017)

CLE peptide. CLE peptides move from the roots to shoots through xylem after inoculation with rhizobia and are perceived by CLV1. In *L. japonicus*, CLE Root Signal 1 (CLE-RS1) and CLE Root Signal 2 (CLE-RS2) are representative members of CLV3-like peptides and are strong candidates for the root-derived signal, which modulate nodulation by following CLV signaling pathway. *CLE12* and *CLE13* are two representative CLE peptide genes in *M. truncatula*, which potentially bind to CLV1. A hairy root transformation study in *M. truncatula* showed that overexpressing both these peptide genes inhibited nodulation systemically, and knockdown of *CLE12* and *CLE13* resulted in an increase in nodule number (Okamoto et al. 2009; Mortier et al. 2012). Additionally, the *CLE-RS1/2* of *L. japonicus* can directly bind to CLV1 or HAR1, and the suppression of nodule numbers due to the overexpression of *CLE-RS1/2* depends on CLV1/HAR1 (Okamoto et al. 2013). Similarly, three candidates of CLE peptide-encoding genes, *RIC1*, *RIC2*, and *NIC1*, have been identified in soybean. Overexpression of these peptides in wild-type plants inhibits nodulation, whereas their overexpression in *clv1* or *nark* mutants had no effect on the nodule number, confirming that nodule number inhibition by CLE peptide is CLV1/NARK1 (nodulation autoregulation receptor kinase) dependent (Reid et al. 2011; Lim et al. 2011).

A screen for supernodulating mutants, defective in AON, identified loss-of-function alleles of several genes. For example, the *rdn1* mutant of *M. truncatula* and a *nod3* mutant of pea exhibit increased nodulation and reduced root growth. In *M. truncatula*, this mutant phenotype was rescued by expressing *RDN1* (*ROOT DETERMINED NODULATION1*) by hairy root transformation (Schnabel et al. 2011) suggesting that it may have a role in the production or transport of CLE peptides (Li et al. 2009).

These CLE peptides activated CLAVATA1 leucine-rich serine-threonine receptor kinase protein which is essential for shoot-controlled regulation of root growth, nodule number, and nitrate sensitivity of symbiotic development. The supernodulation phenotype is caused due to a mutation in a *CLV1*gene known as

SUNN1 (super numeric nodules) in *M. truncatula* and *HAR1* in *L. japonicus* (Nishimura et al. 2002; Schnabel et al. 2005). In pea and soybean, the orthologs of this gene are named *SYM29* and *NARK*, respectively (Krusell et al. 2002; Searle et al. 2003). Additional proteins, corresponding to CLAVATA2, which is known to work together with CLV1, have also been identified in *L. japonicus* (*CLV2*) and *P. sativum* (*sym28*) (Krusell et al. 2011). Another LRR-RLK kinase, KLAVIER (KLV), identified from *L. japonicus* is also involved in shoot regulation of nodulation (Miyazawa et al. 2010). Some of the phenotypes of *klavier* mutants are similar to the *clv1/har1* mutant phenotype suggesting that KLAVIER is likely involved in the CLV signaling pathway.

Two kinase-associated protein phosphatases (KAPP1 and KAPP2) interact with the phosphorylated kinase domains of NARK or CLV1. Both KAPP1 and KAPP2 are transphosphorylated by NARK, and, in turn, the PP2C domain of the KAPP1 and KAPP2 dephosphorylates NARK receptor to relay the signal generated by the formation of shoot-derived inhibitor (SDI). The SDIs enter the phloem and move down to the roots to prevent further nodule development (Miyahara et al. 2008; Lin et al. 2010).

TML (TOO MUCH LOVE) encodes a kelch repeat-containing F-box protein, which has a role in AON signaling. Gene silencing and overexpression approaches by hairy root transformation revealed that TML, HAR1, and CLE-RS1/RS2 negatively regulate nodule organogenesis in the same genetic pathway. Furthermore, TML might suppress the nodulation signaling downstream of the HAR1 and CLE peptides and might function in the long-distance regulation of the legume-rhizobium symbiosis (Takahara et al. 2013).

11.9 Conclusions and Future Work

As is evident from the examples listed in the previous sections, the use of hairy roots has been transformative in studying and deciphering almost every aspect of nodulation signaling. It was especially useful as early on, most legumes were considered recalcitrant to tissue culture-based transformation and regeneration. Recent advances in the genome-editing technologies are going to make it even more useful, as constructs can be evaluated using the hairy root system before investing in stable transformation and genetic manipulation of important leguminous crops. There are already studies demonstrating its feasibility (Wang et al. 2017). This could be especially useful in case of polyploid legumes where multiple genes can be edited simultaneously to achieve desired phenotypes and potentially improved nitrogen use efficiency in crops.

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