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REVIEW PAPER

The role of microRNAs in the legume–*Rhizobium* nitrogen-fixing symbiosis

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Abstract

Under nitrogen starvation, most legume plants form a nitrogen-fixing symbiosis with *Rhizobium* bacteria. The bacteria induce the formation of a novel organ called the nodule in which rhizobia reside as intracellular symbionts and convert atmospheric nitrogen into ammonia. During this symbiosis, miRNAs are essential for coordinating the various plant processes required for nodule formation and function. miRNAs are non-coding, endogenous RNA molecules, typically 20–24 nucleotides long, that negatively regulate the expression of their target mRNAs. Some miRNAs can move systemically within plant tissues through the vascular system, which mediates, for example, communication between the stem/leaf tissues and the roots. In this review, we summarize the growing number of miRNAs that function during legume nodulation focusing on two model legumes, *Lotus japonicus* and *Medicago truncatula*, and two important legume crops, soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*). This regulation impacts a variety of physiological processes including hormone signaling and spatial regulation of gene expression. The role of mobile miRNAs in regulating legume nodule number is also highlighted.

Keywords: Common bean, legume, *Lotus japonicas*, microRNAs, *Medicago truncatula*, nodulation, rhizobia, soybean, symbiosis.

Introduction

Small non-coding RNAs, which include miRNAs and siRNAs, are well established as major regulators of gene expression in eukaryotic organisms. Legume plants, in order to enter a beneficial symbiosis with nitrogen-fixing rhizobia, undergo massive cellular reprogramming. Hence, it is no surprise that a number of miRNAs have been implicated in regulating the formation of the symbiosis.

Legume–Rhizobium *symbiosis*

Most legume plants form a mutualistic relationship with nitrogen-fixing rhizobia. The symbiosis results in biological nitrogen fixation (BNF), in which atmospheric N_2 is converted into NH_3, a form that can be used by the plant host. This unique ability makes legume species not only important ecologically, but also key crops for sustainable agricultural production. Annually, legumes provide ~21 Mt of nitrogen to agriculture on a global scale (Foyer *et al.*, 2016). In return, legumes provide the symbionts with a steady supply of photosynthetically derived carbon. Rhizobia induce the formation of a novel organ, the root nodule, to create a microaerobic compartment protecting their oxygen-sensitive nitrogenase enzyme complex (Stacey *et al.*, 2006; Oldroyd *et al.*, 2011).

In order to initiate the symbiosis, legumes release flavonoids in root exudates to attract rhizobia (Oldroyd *et al.*, 2011). In turn, rhizobia respond by activating nodulation genes to

synthesize nodulation factors (Nod factors; NFs). NFs are perceived by the host plant and trigger a symbiotic signaling cascade necessary for symbiotic development (Oldroyd *et al.*, 2011). NFs are lipo-chitooligosaccharides (LCOs) (Denarie *et al.*, 1996; D'Haeze and Holsters, 2002). Recognition of NFs by Nod Factor Receptor 1 (NFR1) and Nod Factor Receptor 5 (NFR5) [i.e. the nomenclature used in *Lotus japonicus* and in soybean (*Glycine max*)] triggers many of the early events in the root hair infection process (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003, 2007; Geurts *et al.*, 2005; Indrasumunar *et al.*, 2010, 2011).

Following signal exchange, rhizobia enter legume roots primarily by root hairs or by crack entry on the root surface (Oldroyd, 2013). Application of purified NFs to *Medicago truncatula* root hairs can induce root hair tip growth reorientation and partial root hair curling (Esseling, 2003). Similarly, application of NFs to soybean roots can induce cortical cell division similar to that occurring during nodule primordium formation (Cohn *et al.*, 1999).

Successful formation of nitrogen-fixing nodules is controlled by two parallel processes, bacterial infection in root epidermal cells and nodule primordia initiation in root cortical cells (Madsen *et al.*, 2010; Yoro *et al.*, 2014). NF-induced curling of root hairs entraps the bacteria, eventually leading to invagination of the root hair cell membrane to form an infection thread (Stacey *et al.*, 2006; Gage, 2004). This infection thread grows into the root cortex allowing the bacteria to find and infect specific cortical cells. In advance of the penetrating infection cell, root cortical cells are de-differentiated and re-activate their cell cycle with rapid cell division to trigger the formation of the nodule primordium, which develops into a nodule. The nodule is a true organ with clear tissue differentiation; for example, the nodule has clearly delineated infected and uninfected cells that work coordinately to fix nitrogen, incorporate it, and transport it out of the nodule. Within the nodule-infected cells, rhizobia reside in an organelle-like structure called the symbiosome (Roth *et al.*, 1988; Oldroyd, 2013).

Introduction to miRNAs

The complexity of the legume nodulation process, from infection to development and subsequent nodule maintenance, requires complex, coordinated regulatory processes. Among the key regulators involved are miRNAs. miRNAs are non-coding, endogenous RNA molecules that vary between 20 and 24 nucleotides in length. miRNAs find their target genes through Watson–Crick base pairing complementarity and down-regulate gene expression by either translational repression or mRNA cleavage (Treiber *et al.*, 2019). In both plants and animals, miRNAs are involved in a variety of biological and metabolic processes including, but not limited to, defense against viruses, gene expression regulation during development, organ development, and stem cell differentiation (Carrington and Ambros, 2003; Zhang *et al.*, 2007). Especially in plants, miRNAs are crucial in controlling tissue differentiation and development, signal transduction, vegetative to reproductive growth transition, and response towards biotic and abiotic stress (Zhang *et al.*, 2008; Djami-Tchatchou *et al.*, 2017).

Unlike human miRNAs, most plant miRNA-encoding genes (Yu *et al.*, 2017) are located inside intergenic regions between two adjacent genes, and are transcriptionally regulated by their own promoters and terminators (Tang, 2010). miRNA-encoding genes are transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II in the nucleus (Zhang *et al.*, 2007). Subsequently, the transcripts may be capped, polyadenylated, and sometimes contain introns. Approximately 1–5% of the total protein-coding genes in genomes encode miRNA genes (Lai *et al.*, 2003; Lewis *et al.*, 2003; Lim *et al.*, 2003). In plants, pri-miRNAs are cleaved by Dicer-Like 1 (DCL1) enzymes into miRNA precursors (pre-miRNAs) that typically have a hairpin-like structure. Subsequently, the pre-miRNA is cleaved again by DCL1 enzymes to produce the miRNA/miRNA* duplex, which is a short double-stranded RNA (dsRNA) molecule with a characteristic two nucleotide 3' overhang (Yu *et al.*, 2005; Turner *et al.*, 2012). miRNA/miRNA* duplexes are methylated on the hydroxyl group of the last nucleotide (3' end) by the methyltransferase protein HUA ENHANCER1 (HEN1) in the nucleus, and are believed to be translocated to the cytoplasm by a protein called Hasty (HST) (Yu *et al.*, 2017). The methyl group in plant miRNAs is thought to protect miRNAs from cleavage by exonuclease enzymes that target the 3' end of miRNAs (Chen, 2005; Yu *et al.*, 2005). In the cytoplasm, miRNA* is often considered to be non-functional and degraded. The mature miRNA strand is usually more abundant than the miRNA*, and is incorporated into a RNA-induced silencing complex (RISC), where it interacts with the target mRNA. RISC consists of different proteins including the catalytic protein ARGONAUTE (AGO). This complex regulates target gene expression either by inhibiting translation or by cleaving the complementary target mRNAs (Treiber *et al.*, 2019). Inhibition of mRNA translation happens when there is low complementarity between the miRNA and its target mRNA, while mRNA cleavage requires high complementarity between the two (Iwakawa and Tomari, 2013).

In plants, miRNAs can move between neighboring cells through plasmodesmata (Wang *et al.*, 2017) and can also be transported long distances via phloem (Kehr and Buhtz, 2008; Gursanscky *et al.*, 2011). Small RNAs, 18–25 nucleotide long, have been found in phloem sap of cucurbit, castor bean, lupine, and yucca plants (Yoo *et al.*, 2004). These small RNAs (sRNAs) were confirmed to be authentic regulatory RNAs, including siRNA and miRNA. Furthermore, in pumpkin (*Cucurbita maxima*), miRNA trafficking in phloem was facilitated by a protein called PHLOEM SMALL RNA BINDING PROTEIN1 (CmPSRP1) (Yoo *et al.*, 2004). The transport of small regulatory RNAs through the phloem was also documented in transgenic yellow crook-neck squash (*Cucurbita pepo*) plants expressing viral coat protein (Yoo *et al.*, 2004). Related to this, several heterografting studies were used to demonstrate the movement of miRNAs between different plant organs through phloem-aided long-distance transport [e.g. in pumpkin, tomato, Arabidopsis, and potato (Ruiz-Medrano *et al.*, 1999; Kim *et al.*, 2001; Haywood *et al.*, 2005; Banerjee *et al.*, 2006)].

Since their discovery, miRNAs have been shown to regulate various growth and development processes in both plants and

animals. In this review, we highlight the current knowledge of miRNA participation in various aspects of legume–*Rhizobium* symbiosis.

miRNA and miRNA target discovery in the legume–*Rhizobium* symbioses

miRNA genes and their target mRNAs have been implicated in the legume nodulation process using two main approaches, bioinformatic/computational prediction followed by experimental validation, and sequencing of sRNA libraries followed by miRNA–target functional validation.

Bioinformatic/computational prediction

Bioinformatic and computational prediction tools predict evolutionarily conserved miRNAs between different species based on sequence homology and miRNA secondary structure comparison (Jones-Rhoades *et al.*, 2006; Simon *et al.*, 2009). miRNAs are defined as conserved if they share the same hairpin structure and mature sequence with no more than three mismatches when aligned with an annotated miRNA (Ambros *et al.*, 2003; Meyers *et al.*, 2008). Bioinformatic and computational predictions help to identify potentially conserved miRNAs quickly, whereas they are not practical for non-conserved miRNAs (Simon *et al.*, 2009). A great resource for plant miRNA sequences and annotation is the miRBase database (http://mirbase.org). As of July 2019, there were 790 *M. truncatula*, 756 soybean, 365 *L. japonicus*, and 10 common bean (*Phaseolus vulgaris*) mature miRNAs available at miRBase (Kozomara *et al.*, 2019). In addition, the Plant Non-coding RNA Database (http://structuralbiology.cau.edu.cn/PNRD/index.php) and Rfam (http://xfam.org) are two other databases for miRNAs in plants, such as soybean, *Arabidopsis thaliana*, and wheat (Yi *et al.*, 2015; Kalvari *et al.*, 2018). *De novo* miRNA prediction is available at miRCat (http://srna-workbench.cmp.uea.ac.uk) (Paicu *et al.*, 2017).

For miRNA target prediction, computational tools were developed based on four main criteria, namely seed sequence complementarity, sequence conservation, Gibbs free energy, and site accessibility (Peterson *et al.*, 2014). The first criterion, 'seed' sequence complementarity, refers to the Watson–Crick matching between miRNA and its corresponding target transcript at the second to eighth position of the mature miRNA sequence, counting from the 5' end. The seed sequence matching is critical for miRNA–target function. The second criterion is sequence conservation, considering both gaps and mismatches (Jones-Rhoades *et al.*, 2006). The third criterion, Gibbs free energy, is a measure of the stability of miRNA–target binding. The last criterion, site accessibility, predicts how structure may facilitate or inhibit the accessibility of target sites and affect miRNA functioning. In addition, many prediction tools also incorporate other criteria such as machine learning approaches, target site abundance, and local AU content (the concentration of A and U nucleotides flanking the seed/functional region of a miRNA) (Peterson *et al.*, 2014). For legume species, there are a number of online prediction tools available such as (i) the Plant Non-coding RNA Database (http://structuralbiology.cau.edu.cn/PNRD/index.php) (Yi *et al.*, 2015); (ii) psRNATarget (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011); and (iii) the UEA sRNA toolkit (http://srna-workbench.cmp.uea.ac.uk/) (Stocks *et al.*, 2018).

Sequencing of small RNA libraries

A popular approach to identify miRNAs in legumes and nitrogen-fixing nodules is sRNA library sequencing (Table 1). In this method, pooled, enriched sRNAs are used as templates for cDNA synthesis, usually as concatenated strings, and subsequently sequenced (Lu *et al.*, 2007). A number of studies have successfully applied this approach (Song *et al.*, 2011; Turner *et al.*, 2012; Holt *et al.*, 2015; Yan *et al.*, 2015, 2016). To confirm that the predicted miRNAs are genuine, one can perform real-time PCR (RT-PCR) to check miRNA expression. However, because of the small size, the quantification of miRNA expression levels using RT-PCR can be potentially misleading. Therefore, northern blotting is a more reliable method (He and Green, 2013; Li and Zamore, 2018). One of the earliest miRNAs identified as having a regulatory role in the legume–*Rhizobium* symbiosis is miR169. El Yahyaoui *et al.* (2004) identified MtHAP2-1, a CCAAT-binding transcription factor, as important for the early stages of nodulation in *M. truncatula*. In 2006, the same group showed that MtHAP2-1 was highly expressed in the nodule meristematic zone and regulated by miR169. MtHAP2-1 RNAi and miR169 ectopic overexpression both impaired the nitrogen-fixing ability of nodules (Combier *et al.*, 2006).

Construction of parallel analysis of RNA ends (PARE) libraries (or also referred to as degradome sequencing) is very common in studies of the legume–*Rhizobium* symbiosis as a means to predict cleaved mRNA targets of specific miRNAs (German *et al.*, 2008, 2009; Zhai *et al.*, 2014). This technique allows high-throughput identification of mRNA targets using a modified 5′-RACE method that captures the 3' cleavage products of miRNAs (German *et al.*, 2008; Zhai *et al.*, 2014). Several research groups have successfully applied this method (Song *et al.*, 2011; Yan *et al.*, 2015, 2016; Formey *et al.*, 2016) (Table 1).

Experimental validation is a necessary step to confirm that a specific mRNA is targeted by a miRNA. The expression levels of a miRNA and its target(s) mRNA are usually inversely correlated. Therefore, checking the expression level of both the miRNA and the putative target gene is a very informative first step in narrowing down physiologically relevant miRNA target genes (Li *et al.*, 2010). Similarly, ectopic overexpression of the miRNA in transgenic tissues should result in a concomitant down-regulation of the mRNA target (Guo *et al.*, 2010; Thomson *et al.*, 2011). Using differential gene expression to identify miRNA targets is informative to filter potential miRNA target genes. However, this method cannot distinguish between miRNA direct, physiological targets and the downstream regulatory effects (Thomson *et al.*, 2011). An important method for experimental validation is to observe *in planta* miRNA-dependent cleavage of mRNA using *Agrobacterium tumefaciens* infiltration in *Nicotiana benthamiana* leaves to co-express both the miRNA and its target (Li, 2011).

Table 1. miRNAs identified by sequencing of small RNA libraries

Time point and tissue types	miRNAs identified	Novel miRNAs	Number of conserved miRNAs	Legume-specific miRNAs	Types of abundant miRNAs	Reference
Soybean						
Roots at 3 hpi	55 families	35 novel families	20 conserved miRNA families	N/A	20–24 nucleotides	Subramanian *et al.* (2008)
Nodules at 28 dpi	32 miRNAs identified belonging to 11 families	4 novel miRNAs	8 miRNAs are conserved across plant species	20 soybean-specific miRNAs	21 nucleotides	Wang *et al.* (2009)
Roots of 3-week-old seedlings, seeds harvested at 20 dpi, flowers collected at –2 d to 2 d after anthesis, and nodules at 7, 14, and 21 dpi	129 miRNAs	87 novel miRNAs	42 conserved miRNAs between soybean and other species	N/A	21, 22, and 24 nucleotides	Joshi *et al.* (2010)
B. japonicum-inoculated roots	120 miRNA genes	5 novel miRNA families			21 nucleotides	Turner *et al.* (2012)
Nodules at 10, 15, 20, 25, and 30 dpi	284 miRNAs in which 139 miRNAs were significantly regulated during nodule development	178 novel soybean miRNAs	4 conserved miRNAs were highly regulated during nodule development	1 legume-specific miRNA was highly regulated during nodule development	N/A	Yan *et al.* (2015)
Root hairs at 12, 18, 24, and 48 hpi	114 miRNAs	22 novel miRNAs	17 miRNA families are conserved across plant species	52 miRNAs within 41 miRNA families that might be soybean specific	21, 22, and 24 nucleotides	Yan *et al.* (2016)
Medicago truncatula						
Root tips treated with NaCl for 1 h and nodules at 21–30 dpi		100 novel miRNAs	73 miRNAs corresponded to 24 miRNA families across plant species	23 legume-specific miRNAs	21 and 24 nucleotides	Lelandais-Briere et al. (2009)
Lotus japonicus						
Roots at 3 hpi and 3 dpi; nodules at 3 wpi			45 conserved miRNA families across plant species	32 *L. japonicas*-specific miRNAs and 8 miRNA families that are highly expressed in mature nodules	24 nucleotides	De Luis *et al.* (2012)
Roots at 3 dpi	232 miRNAs	219 novel miRNAs from 114 newly assigned families	65 conserved miRNAs	76 infection-responsive sRNAs	24 nucleotides	Holt *et al.* (2015)
Common bean						
Root hairs harvested from roots at 6 hpi	132 mature miRNAs	63 novel miRNAs, in which one miRNA, miR-RH82, was differentially expressed during Nod factor induction	47 conserved miRNAs	miR-RH82 is a common bean-specific miRNA	20–24 nucleotides	Formey *et al.* (2016)

For example, in soybean, Gma-miR171q and Gma-miR171o target NSP2.1 and SCARECROW-LIKE6 (SCL6) transcription factors that are localized in the nucleus. When co-infiltrating Gma-miR171o/q and either NSP2.1–green fluorescent protein (GFP) or SCL6–GFP, the GFP signal expression in the nucleus was reduced because of the miRNA's effect (Hossain *et al.*, 2019). In addition, 5'-RACE following sequencing is a useful method to detect miRNA-mediated cleavage *in vivo* (Jones-Rhoades *et al.*, 2006; Yeku and Frohman, 2011).

miRNAs regulate different stages of the legume–*Rhizobium* symbiosis

miRNAs modulate early stages of nodulation

During the initiation of the symbiosis, rhizobia enter the plant root through a preferred entry point, the root hair. The single-cell root hairs curl and trap rhizobia, which subsequently leads to the formation of the infection thread, a tubular structure of plant origin which delivers the bacteria into the underlying,

dividing cortical cells, forming the nodule primordium (Oldroyd, 2013). Although the root has thousands of root hairs, only a small fraction is successfully infected leading to nodule formation. Hence, there is a great potential for non-responding tissues to dilute any physiological signal arising from those few root cells directly responding to rhizobial inoculation. For this reason, our group developed a process to isolate soybean root hair cells in bulk as a means to enrich for the type of cell that first responds to rhizobial infection signals (Libault *et al.*, 2010; Hossain *et al.*, 2015). In this section, we will review our results using the root hair single-cell system as well as other work, with a focus on early rhizobial infection events, such as bacterial infection (rhizobial attachment, root hair curling, infection thread formation, and elongation), and early development events, such as cortical cell division and nodule primordium emergence (Fig. 1).

Subramanian *et al.* (2008) sequenced a sRNA library derived from whole soybean roots harvested at 3 hpi (hours post-inoculation) with *Bradyrhizobium japonicum* to identify miRNAs regulated during the early stages of the symbiotic interaction. They found 20 conserved miRNA families, 35 novel miRNAs, and verified 14 novel miRNA families by northern blot analysis (Subramanian *et al.*, 2008). With a similar goal, our laboratory sequenced miRNA libraries derived from isolated soybean root hairs at 12, 18, 24, and 48 hpi with *B. japonicum*, and compared the results with those in the roots after the root hairs were removed (i.e. stripped roots), as well as uninoculated control roots (Yan *et al.*, 2016). The use of isolated root hairs resulted in the identification of 114 miRNAs, including 22 novel miRNAs, with 66 miRNAs differentially expressed between root hairs and stripped roots, and 48 miRNAs differentially regulated between inoculated and uninoculated root hairs. This same study also sequenced a degradome library, resulting in the identification of 405 putative miRNA target mRNAs. In order to provide further verification, Yan *et al.* (2016) explored the functional relevance of four miRNAs (Gma-miR2606b and miR1514 that appeared to be legume specific and TAG2382310 and Gma-miR4416 that seemed to be soybean specific). All four miRNAs were transformed into soybean hairy roots and constitutively expressed under the strong CvMV (*Cassava vein mosaic virus*) promoter. Expression of TAG2382310 and miR1514 had no apparent effect on nodulation. The expression levels of both Gma-miR2606b and Gma-miR4416 were down-regulated upon rhizobial treatment. However, constitutive expression of Gma-miR2606b in transgenic roots led to significantly increased nodule numbers, while constitutive expression of Gma-miR4416 resulted in reduced nodule

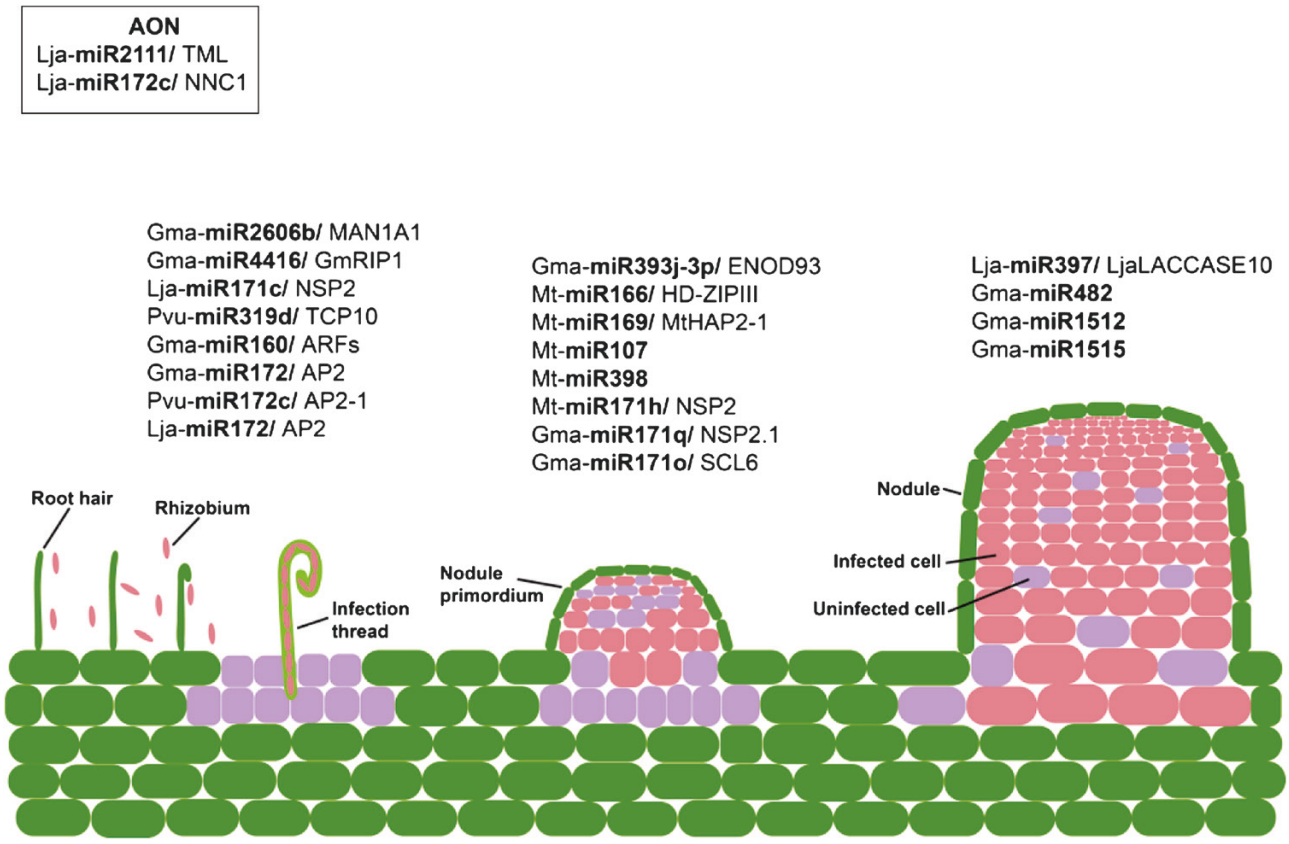

Fig. 1. miRNAs validated to regulate different stages of the legume–*Rhizobium* symbiosis. miRNAs and their corresponding targets are classified into three groups based on the stage of their activity, namely early rhizobial infection events (rhizobial attachment, root hair curling, infection thread formation, and elongation); early development events (cortical cell division and nodule primordium emergence); and nodule development and functioning. A two (Mt) or three (Gma, Lja, and Pvu) letter designation is used to identify from which plant species each miRNA was detected. The box in the upper left corner indicates miRNAs that are involved in the autoregulation of nodulation (AON) pathway. See text for specific information and references for the various miRNAs listed.

numbers (Yan *et al.*, 2016). From the degradome library, six putative target mRNAs for Gma-miR4416 were identified. One of the target genes, *Rhizobium Induced Peroxidase* (*RIP*), was found to share significant sequence similarity to *MtRIP1*. Expression of *MtRIP1* was shown to be rapidly and transiently induced in *M. truncatula* upon inoculation with *Sinorhizobium meliloti* and the transcript was localized to epidermal cells, which were subsequently infected with rhizobia (Cook *et al.*, 1995). The inverse relationship of *GmRIP1* and Gma-miR4416 expression, their early induction, and spatial co-expression suggested that *GmRIP1* was a likely physiological target for miRNA silencing. It is known that rhizobial perception elicits rapid, but transient, plant immune responses, including the production of reactive oxygen species (ROS) (Tóth and Stacey, 2015). The production of ROS has also been associated with the loosening of the cell wall, which might be important for the infection process (Fry, 1998; Müller *et al.*, 2009). In addition to GmRIP1, Yan *et al.* (2016) identified a mannosyl-oligosaccharide 1,2-α-mannosidase gene as the likely, physiological target of Gma-miR2606b silencing. This enzyme is probably involved in modification of the plant cell wall (Yan *et al.*, 2016).

De Luis *et al.* (2012) generated sRNA libraries from *L. japonicus* roots harvested at 3 hpi, 3 dpi (days post-inoculation), and 3 wpi (weeks post-inoculation) with *Mesorhizobium loti*. They used these data to search for miRNAs with altered abundance when comparing mock and rhizobial-inoculated plants (De Luis *et al.*, 2012). The 3 hpi samples were tested since this timing correlates with the observations of Ca^{2+} oscillations and spiking, as well as extracellular space alkalinization, that are among the first observable responses to rhizobial treatment. The 3 dpi samples were chosen since this time correlates with the observation of infection thread formation and cortical cell division after rhizobial inoculation. Surprisingly, no specific miRNAs were found to be differentially regulated at either of these time points. Given the results found in soybean, it seems likely that the use of whole roots at these early time points resulted in a dilution of infection-specific responses due to the preponderance of non-responding tissues. On the other hand, the authors found multiple miRNAs with altered abundance in their 3 wpi samples, probably due to the fact that isolated nodules were used to construct the sRNA libraries (De Luis *et al.*, 2012). For the results of the 3 wpi libraries, they chose two miRNAs, miR397 and miR171c, for further study. The authors examined the expression of these miRNAs in wild-type plants, but also in two plant mutants, which can produce spontaneous nodules without rhizobial infection; specifically, *snf1* (*spontaneous nodule formation1*, a gain-of-function mutant of CCaMK, a Ca^{2+}/calmodulin-dependent protein kinase which acts downstream of Ca^{2+} spiking) and *snf2* (*spontaneous nodule formation2*, a gain-of-function mutant of LHK1, a histidine kinase) (Tirichine *et al.*, 2006, 2007). The expression of Lja-miR171c was up-regulated in *M. loti*-infected *snf1/2* nodules but not in the uninfected, spontaneous nodules. This suggested that Lja-miR171c was associated with bacterial infection but not nodule organogenesis (De Luis *et al.*, 2012). It was found that Lja-miR171c targets the *L. japonicus* GRAS transcription factor, Nodulation Signaling Pathway 2 (NSP2), an important transcription factor that acts downstream of Ca^{2+} spiking and CCaMK in the Nod factor signaling pathway (Kaló *et al.*, 2005; Murakami *et al.*, 2006; Oldroyd, 2013). Complementation of *nsp2* mutant plants using a *NSP2* gene in which the sites of miR171c cleavage were modified restored the wild-type phenotype of infection threads, nitrogen-fixing nodules, and nodule morphology in *nsp2* mutant plants. This implied that miR171c might not be required for NSP2 functioning during rhizobial symbiosis (De Luis *et al.*, 2012). On the other hand, another member of the miR171 family, miR171h, was found in *M. truncatula* to also target *NSP2* (Hofferek *et al.*, 2014). Therefore, in retrospect, one cannot exclude that miRNA silencing of NSP2 is indeed critical for its regulatory role in nodulation. Members of the GRAS family of transcription factors appear to be a favorite for miRNA silencing; for example, soybean miR171o and miR171q also target this gene family (Hofferek *et al.*, 2014; Hossain *et al.*, 2019). In both *M. truncatula* and soybean, quantitative RT-PCR and promoter–β-glucuronidase (GUS) histochemical studies showed an inverse correlation between the expression of Mt-miR171h and MtNSP2 and Gma-miR171q and GmNSP2.1, respectively (Hofferek *et al.*, 2014; Hossain *et al.*, 2019). In soybean, our group investigated the function of Gma-miR171q and Gma-miR171o, which have identical mature miRNA sequences but originate from diverged stem–loop sequences. As mentioned earlier, Gma-miR171q targets NSP2, while Gma-miR171o targets another member of the GRAS family, SCL6, which had not previously been implicated in the symbiosis (Hossain *et al.*, 2019). Constitutive expression of miR171q/o led to reduced nodule numbers, while constitutive expression of an miR171o-resistant mutated form of SCL6 led to significantly increased nodule numbers. Similar results were obtained using a miRNA-resistant, mutated form of NSP2. Gma-miR171o/q and their target genes exhibit distinct, spatio-temporal expression patterns, which probably explains their unique functions during nodule development. Analysis of the expression of downstream components of the early symbiotic signaling found that NIN, ENOD40, and ERN1 were down-regulated in transgenic soybean roots constitutively expressing miR171o/q in comparison with roots carrying the control vector (Hossain *et al.*, 2019).

Transcription factors are common targets for miRNA silencing (Lelandais-Brière *et al.*, 2016). Another example is the TEOSINTE-BRANCHED1/CYCLOIDEA/PCF (TCP) family, which are characterized by the presence of the conserved basic helix–loop–helix (bHLH)-containing DNA-binding motif, also known as the TCP domain (Li, 2015). Members of this family were shown to be silencing targets of miR319 in *M. truncatula,* common bean (Martín-Rodríguez *et al.*, 2018; Wang *et al.*, 2018*b*), and soybean (NTH and GS, unpublished data). In each of these species, ectopic overexpression of miR319 led to reduced nodule numbers on transgenic roots (Martín-Rodríguez *et al.*, 2018; Wang *et al.*, 2018*b*). The predicted target of miRNA319d silencing in common bean was TCP10 and, indeed, the expression of this transcription factor was reduced during nodulation. Ectopic overexpression of miR319d in common bean resulted in increased numbers of deformed root hairs and infection threads, with a concomitant reduction

in the expression level of TCP10 (Martín-Rodríguez *et al.*, 2018). These phenotypes suggest a role for miR319d in the bacterial infection process. However, lacking in these studies was confirmation that silencing of TCP10 was causal for the phenotypes seen.

A number of miRNAs target further components of the symbiotic signaling pathway in order to regulate nodule formation. For example, members of the miR172 family are positive regulators of nodulation (Lelandais-Brière *et al.*, 2016). There are three Lja-miR172, four Pvu-miR172, four Mt-miR172, and 12 Gma-miR172 in *L. japonicus*, common bean, *M. truncatula* and soybean, respectively (miRBase) (Nova-Franco *et al.*, 2015). Ectopic expression of miR172 in soybean increased nodule numbers, nitrogenase activity, and the expression of both symbiotic leghemoglobin and non-symbiotic hemoglobin (Yan *et al.*, 2013). Similar results were observed in common bean (Nova-Franco *et al.*, 2015). In *L. japonicus*, the miR172a promoter is induced in root hairs of infected roots and nodule primordia (Holt *et al.*, 2015). These nodulation effects were mediated through miR172 silencing of the APETALA2 (AP2) transcription factor (Yan *et al.*, 2013; Wang *et al.*, 2014; Holt *et al.*, 2015; Nova-Franco *et al.*, 2015). In the case of soybean, the targeted AP2 transcription factor was shown to be *Nodule Number Control1* (*NNC1*). The NNC1 protein directly binds to the promoter of the early nodulin gene ENOD40, which appears to play a role in nodule primordium formation (Wang *et al.*, 2014) (Fig. 1).

miRNAs regulate nodule formation and development

In this section, we discuss the role of miRNAs in controlling the later stages of the symbiosis, from nodule primordium emergence, bacterial release into nodule cells, through nodule development from the young nodule, to the mature nodule, and, finally, nodule senescence (Fig. 1). Soybean and *L. japonicus* develop determinate, round-shaped nodules with the nodule primordium arising from cell division of both inner and outer cortical cells (Kijne, 1992). In 2009, Wang and colleagues sequenced sRNA libraries from 28-day-old soybean nodules after inoculation with *B. japonicum* to identify miRNAs that function in nitrogen-fixing nodules (Wang *et al.*, 2009). They identified 32 small miRNA sequences with eight conserved miRNAs (belonging to the families of miR167, miR172, miR396, and miR399) and 20 soybean-specific miRNAs (within the families of Gma-miR1507, Gma-miR1508, Gma-miR1509, and Gma-miR1510). They predicted miRNA targets using a computational approach to search the soybean UniGene database for sequences that were complementary to the query miRNAs. The prediction showed that miRNA targeted genes involved in auxin response, defense-related proteins, and nitrate transporters (Wang *et al.*, 2009). In 2015, our group also sequenced sRNA libraries derived from soybean nodules at different stages of nodule development (Yan *et al.*, 2015). In total, we sequenced 15 soybean sRNA libraries derived from young, mature, and senescent nodules post-infection with rhizobium (10, 15, 20, 25, and 30-day-old soybean nodules) (Yan *et al.*, 2015). We also sequenced degradome libraries to more accurately identify the mRNA targets for the miRNAs identified. Sequencing identified 284 miRNAs, including 178 novel soybean miRNAs. Of these, 139 miRNAs were differentially regulated during nodule development, including 12 miRNAs whose expression changed >10-fold. Examples of miRNAs that were >10-fold up-regulated during soybean nodule development include TAG-107, TAG-12, Gma-miR390a-5p, TAG-103, TAG-38, Gma-miR319d, TAG-138, Gma-miR397a, Gma-miR2119, TAG-73, TAG-56, and Gma-miR398c. Sequencing of the degradome libraries identified 533 miRNA targets, including three nodulation-related genes (NSP2 targeted by miR171, CYCLOPS targeted by miR167, and ENOD93 targeted by miR393j-3p) (Yan *et al.*, 2015). Subsequent experiments confirmed that ENOD93 was the physiological target of miR393j-3p, which negatively regulates soybean nodule formation (Yan *et al.*, 2015).

In contrast to soybean and *L. japonicus*, *M. truncatula* and pea (*Pisum sativum*) develop indeterminate nodules. Indeterminate nodules derive primarily from cell divisions originating in the inner cortex, and have an elongated shape with a persistent meristematic apex. The attractive feature of indeterminate nodules is that all stages of the infection process can be visualized from tip to base; that is, the apical meristematic zone, the pre-infection zone, the infection zone, the differentiation zone, the nitrogen fixation zone, and the basal senescent zone (Gage, 2004). Using *in situ* hybridization, specific miRNAs were found to be spatially enriched in the different functional zones of *M. truncatula* nodules (Lelandais-Brière *et al.*, 2009). In this assay, specific miRNAs, including Mtr-MIR2586, Mtr-sRNA107, miR167, miR398, miR172, miR399, and miR160, were expressed within the apical meristematic zone of the nodules. Moreover, miR167, which targets auxin response factors, accumulated at the differentiating peripheral vascular bundles, while miR172 and miR398 localized in the differentiation zone, and miR399 was localized to the nitrogen-fixing zone (Lelandais-Brière *et al.*, 2009). These data suggest a possible role for miRNA in determining the spatial distribution of specific transcripts in indeterminate nodules.

In *M. truncatula*, miR166, which is processed from a tandem MtMIR166a precursor, regulates vascular bundle patterning in lateral roots and nodules. miR166 targets a conserved class-III homeodomain-leucine zipper (HD-ZIPIII) transcription factor family as validated by 5'-RACE PCR (Boualem *et al.*, 2008). HD-ZIPIII is well known to control lateral root development in Arabidopsis (Hawker and Bowman, 2004). miR166 and its target, HD-ZIPIII, are co-expressed in vascular bundles and distal regions of roots and nodules, as shown by *in situ* hybridization. Ectopic overexpression of miR166 negatively regulates vascular bundle organization, lateral root density, and nodule number in *M. truncatula* (Boualem *et al.*, 2008).

Following up on the earlier identification of six novel miRNA families in soybean, Subramanian *et al.* (2008) demonstrated that three of these miRNAs—miR482, miR1512, and miR1515—function during soybean nodulation (Li *et al.*, 2010). Constitutive expression of miR482 and miR1515 resulted in a dramatic increase in the number of mature nodules compared with transgenic control vector roots upon *B. japonicum* inoculation (Li *et al.*, 2010). In contrast, similar constitutive expression of miR1512 had no apparent effect

on nodule numbers. To avoid the pleiotropic effects of constitutively overexpressing miRNAs, the *Rhizobium*-responsive soybean promoter ENOD40 was used to drive the expression of miR482, miR1512, and miR1515 for further analysis. As a result, roots expressing miR482 and miR1512 under the ENOD40-driven promoter produced significantly higher nodule numbers. In contrast, roots expressing miR1515 from the ENOD40 promoter did not affect nodule numbers relative to controls. The study suggests that miR482, miR1512, and miR1515 regulate soybean nodule number in different ways and point to the importance of the relative expression levels of miRNA expression as an important parameter in assessing the associated phenotypes (Li *et al.*, 2010).

De Luis and colleagues investigated the levels of miR171c and miR397 expression in young (7–14 dpi) and mature (21–28 dpi) *L. japonicus* nodules. They found that miR171c abundance was steady over the studied time-course, while miR397 abundance was strongly up-regulated in nodules at 28 dpi in comparison with younger nodules, which might suggest a role in mature nodule maintenance or its contribution to nodule senescence (De Luis *et al.*, 2012). Furthermore, when the authors searched for possible miR397 targets, they found Cu^{2+}-containing LACCAse-like genes as likely candidates. They also found that miR397 abundance correlated with available Cu^{2+} levels, suggesting a link between copper nutrition/homeostasis and the symbiosis.

miRNAs modulate hormone homeostasis in the legume–*Rhizobium* symbiosis

Phytohormones are signaling molecules synthesized by plants at extremely low concentrations to fine-tune cellular activities, embryonic development, pathogen and stress response, as well as vegetative and reproductive development (Santner *et al.*, 2009). The link between phytohormones and the legume–*Rhizobium* symbiosis is well established where disruption of synthesis/activity of virtually any phytohormone affects nodulation (Liu *et al.*, 2018). Classical hormones including cytokinin, auxin, and the recently characterized strigolactones positively regulate nodule development. Ethylene, jasmonic acid, abscisic acid, brassinosteroids, and gibberellic acid are negative regulators of infection thread formation and nodule development. Salicylic acid can either enhance or inhibit nodule formation in indeterminate and determinate nodules (Liu *et al.*, 2018). Unfortunately, only a few studies have studied the link between miRNA regulation and phytohormone action in the legume–*Rhizobium* symbiosis. These examples are discussed below.

miRNAs modulate auxin homeostasis during nodulation

Auxin is critical for infection thread formation and nodule development during nodulation. In a study by Libbenga *et al.* (1973) using 7-day-old pea explants, addition of both auxin and cytokinin to the nutrient medium resulted in ~80% of explants with increased cell division. More importantly, these patterns of cell division resembled the initial proliferative stages in root nodule formation in regular pea roots. Thus, the authors proposed that auxin and cytokinin may be essential for the induction of cell divisions during nodule initiation (Libbenga *et al.*, 1973). In *M. truncatula*, application of the auxin influx inhibitors 1-naphthoxyacetic acid (1-NOA) and 2-NOA, which can block auxin from entering plant cells, resulted in decreased nodule numbers and density. Meanwhile, induction of the symbiotic marker gene *ENOD11* and NF-induced calcium spiking were not affected by addition of either 1-NOA or 2-NOA. These results suggest that inhibition of auxin influx directly regulates nodule development without affecting NF signaling (Roy *et al.*, 2017).

The first study to investigate the link between miRNA, hormone, and the legume symbiosis demonstrated that miR164 plays a role in regulation of MtNAC1 from the NAC family [NO APICAL MERISTEM (NAM), the Arabidopsis transcription factor 1 (ATAF1), and CUP-SHAPED COTYLEDON2 (CUC2)], which shows induced expression by auxin. Ectopic overexpression of MtmiRNA164 resulted in a reduction of nodule numbers at 7 and 15 dpi. However, the nodulation phenotype of miR164 is not caused by inactivation of MtNAC1 since overexpression, silencing, or mutation of MtNAC1 showed no effect on nodulation (D'haeseleer *et al.*, 2011).

In Arabidopsis, miR393 expression is the only miRNA found to be induced and regulated by high nitrate treatment in roots, as demonstrated by sequence-based screening for nitrate-responsive sRNAs (Vidal *et al.*, 2010). The auxin receptor AFB3 mRNA is a target of miR393 silencing and its expression is also regulated by nitrate in roots. AFB3 expression is induced in roots, peaked at 1 h after nitrate treatment, and gradually decreased after that, while miR393 expression was induced in roots at 2 h after nitrate treatment. The miR393 silencing of AFB3 is regulated by nitrate and controls primary and lateral root growth (Vidal *et al.*, 2010). On the other hand, as mentioned above, in soybean, miR393j-3p targets the early nodulin gene, ENOD93, to regulate nodule numbers in soybean. Overexpression of miR393j-3p and RNAi silencing of ENOD93 both strongly restricted nodule formation. This suggests a role for miR393j-3p in regulating nodule cell meristematic activity and nitrogen use efficiency during the early stages of nodule development (Yan *et al.*, 2015). However, a clear connection between ENOD93 and phytohormone activity was not shown.

In Arabidopsis, miR160 targets three of the 23 *AUXIN RESPONSE FACTOR* (*ARF*) genes, ARF10, ARF16, and ARF17. Specifically, ARF17 silencing by miR160 regulates the expression of auxin-inducible GH3-like mRNAs and controls various developmental processes, including embryo and leaf symmetry, leaf shape, and root growth (Mallory *et al.*, 2005). In soybean, Turner *et al.* (2013) ectopically overexpressed miR160 in order to target a set of repressors involved in auxin-related signaling, which led to auxin hypersensitivity in transgenic roots. These roots were not aberrant in their response to rhizobia in the epidermal layer, but they did show altered root hair responses. These same plants displayed significantly reduced primordia formation, suggesting that hypersensitivity to auxin impairs nodule development (Turner *et al.*, 2013).

Silencing of soybean GmARF8 expression by miR167c was shown to function in auxin-mediated nodule and lateral root formation (Wang *et al.*, 2015). miR167c is the closest homolog of Arabidopsis miR167a, which targets ARF8 to mediate lateral root development and cell-specific nitrogen responses (Gifford *et al.*, 2008). Strong expression of miR167 was detected in mature soybean root nodules (Wang *et al.*, 2009; De Luis *et al.*, 2012; Wang *et al.*, 2015). MiR167c expression in soybean roots was induced upon 2,4-D treatment. miR167 was found to control lateral root architecture and nodule number in soybean upon *B. japonicum* inoculation. Ectopic overexpression of miR167c significantly increased nodule numbers at 28 dpi. *GmARF6* and *GmARF8* are targets of Gm-miR167c as validated by 5'-RACE PCR and expression levels. Moreover, the authors showed that miR167c regulates nodule numbers by targeting *GmARF8/6* (Wang *et al.*, 2015).

miRNAs modulate cytokinin homeostasis during nodulation

Since its discovery in the 1950s, cytokinin has been implicated in the regulation of various aspects of plant growth and development, notably including cell division, *de novo* organ formation, biotic and abiotic stress, and inhibition of leaf senescence (Kieber and Schaller, 2014). Cytokinin has a dual function in nodulation. Cytokinin positively regulates nodule primordium development but also plays a role in the negative control of nodulation during autoregulation of nodulation (AON). Exogenous application of cytokinin can induce *de novo* nodule primordia formation in various nodulation-defective plant mutants, including *nfr1*, *nfr5*, *symRK*, *nup133*, *nup85*, *castor*, *pollux*, and *ccamk* (Heckmann *et al.*, 2011). Further data suggest that cytokinin is essential for root nodule organogenesis and for induction of *NIN* (Murray *et al.*, 2007). The gain-of-function mutant, *snf2*, was mapped to the *L. japonicus* cytokinin receptor *LjLHK1*. In the absence of inoculation, *snf2* mutant roots develop white, rhizobia-free root nodules (Tirichine *et al.*, 2007).

Silencing of Mt*NSP2* expression by miR171h was shown to be essential for nodule primordium initiation in *M. truncatula*. Strikingly, regulation is tightly controlled by the *Medicago* homolog of LjLHK1, cytokinin-dependent CRE1 (CYTOKININ RESPONSE 1) pathway (Ariel *et al.*, 2012). According to this study, NSP2 was among those transcription factors found to bind to a consensus sequence within the cytokinin signaling component, MtRR1 (*M. truncatula* Response Regulator 1). In addition, the expression levels of Mtr-miR171h and its target MtNSP2 were inversely affected by the addition of exogenous cytokinin (Ariel *et al.*, 2012).

In soybean, ectopic overexpression of miR160 (miR160ox) led to auxin hypersensitivity and cytokinin hyposensitivity (Turner *et al.*, 2013). The expression levels of the cytokinin-responsive Nodule Inception (NIN), NSP1, and HAP2 transcription factors was much lower at 8 dpi in miR160-overexpressing transgenic roots in comparison with vector control plants, confirming that overexpression of miR160 leads to cytokinin hyposensitivity (Turner *et al.*, 2013).

The role of miRNAs in the autoregulation of nodulation

BNF is beneficial but costly for legumes due to its high consumption of energy in the form of photosynthetically fixed carbon (Valentine *et al.*, 2010). Therefore, legumes tightly control the number of nodules during symbiosis by systemic AON (Ferguson *et al.*, 2019). AON is characterized by long-distance signaling between root and shoot to regulate early nodulation events and prevent future nodule formation (Ferguson *et al.*, 2019). In legumes, the CLAVATA3/ESR-related (CLE) peptide acts as a signaling molecule that is transported from the root to shoot via xylem after rhizobial inoculation (Ferguson *et al.*, 2019). In soybean, the two root-derived peptides are rhizobia-induced CLV3/ESR-related peptides 1 and 2 (RIC1 and RIC2) (Reid *et al.*, 2011). In *M. truncatula*, the equivalent function is performed by CLE12 and CLE13. In *L. japonicus* there are three CLE peptides (CLE-RS1–CLE-RS3) involved in AON, and their expression is regulated by a key transcription factor, NIN, which is essential for cortical cell division (Soyano *et al.*, 2014). Subsequently in the shoot, CLE is recognized by homologous CLAVATA-like leucine-rich-repeat receptor like kinases (RLKs), namely NODULE AUTOREGULATION RECEPTOR KINASE (NARK) in soybean (Searle *et al.*, 2003), HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1) in *L. japonicus* (Wopereis *et al.*, 2000), and SUPER NUMERIC NODULES (SUNN) in *M. truncatula* (Penmetsa *et al.*, 2003). Mutants in these RLKs display a supernodulation and nitrate-tolerant nodulation phenotype (Wang *et al.*, 2018*a*). Perception of CLE peptides in shoots induces the production of a shoot-derived inhibitor (SDI), which is later transported back to roots to inhibit further nodulation (Wang *et al.*, 2018*a*).

It is now clear that the SDI is a mobile miRNA, miR2111 (Tsikou *et al.*, 2018). The data indicate that in *L. japonicus*, and probably other legumes, miR2111 is synthesized in the leaves and travels from the shoots to the roots. Stable transgenic *L. japonicus* lines expressing a pMIR2111:GUS construct showed promoter activity only in the leaf phloem, but not in the root tissue. Furthermore, shootless roots showed a marked reduction in miR2111 levels consistent with a shoot-derived origin for miR2111. The arrival of miR2111 in the roots results in targeting of the transcript of *TOO MUCH LOVE* (*TML*), a Kelch-repeat F-box protein that is a negative regulator of nodulation (Takahara *et al.*, 2013). The loss of TML through mutation or ectopic miR2111 overexpression results in a hyperinfection phenotype, which resembles the hypernodulation phenotype shown by *har1* mutants in *L. japonicus* (Wopereis *et al.*, 2000; Tsikou *et al.*, 2018). The expression levels of miR2111 in the cytokinin receptor *lhk1-1* mutant are maintained in both roots and shoots after rhizobia infection, in contrast to the reduction of miR2111 levels found in wild-type plants. This suggests that miR2111 accumulation is dependent on LHK1 cytokinin signaling, which is known to regulate AON (Sasaki *et al.*, 2014; Tsikou *et al.*, 2018). Current results are consistent with a model by which miR2111 silencing of TML expression maintains the root in an infection-susceptible default mode that operates in uninfected roots.

This regulation works downstream of the cytokinin receptor LHK1 and HAR1, as an important shoot component of AON (Tsikou *et al.*, 2018). The findings from studies of miR2111 in *L. japonicus* suggest a model by which rhizobial infection triggers cytokinin signaling, resulting in the synthesis of the CLE-like peptides that travel from the roots to the shoots, down-regulating miR2111 expression, which ultimately leads to higher levels of TML and inhibition of nodulation.

miR172c also appears to play a role in AON. Ectopic overexpression of miR172c positively regulates soybean nodulation, including root hair deformation, number of infection foci, number of nodule primordia, and nodule numbers following *B. japonicum* inoculation (Wang *et al.*, 2014). These effects are the result of miR172c targeting *NNC1* expression. In turn, NNC1 directly targets the promoters of the early nodulin genes *ENOD40*-1/2 to repress their transcriptional activity. Moreover, expression of miR172 was shown to be dependent on the NF receptors, NFR1 and NFR5. Notably, miR172c expression is negatively regulated by NARK, the AON receptor, based on several lines of evidence: (i) the expression level of miR172c is induced in the *nark* receptor mutant during early stages of nodulation; (ii) the increased level of miR172c in the background of the *nark* mutant, *nts1116*, intensifies the supernodulation phenotype of *nt1116*; and (iii) reduction of miR172c in *nts1116* mutant plants significantly reduces nodule numbers (Wang *et al.*, 2014).

A more recent publication validated the role of miR172c in soybean AON (Wang *et al.*, 2019). In that paper, the authors demonstrate that NNC1 functions as an upstream regulator of GmRIC1 and GmRIC2, the two rhizobial-induced CLE peptides in soybean. Modulating the expression levels of either miR172c or NNC1 had a corresponding effect on the expression of these two peptides. NNC1 directly targets GmRIC1 and GmRIC2. Interestingly, NNC1 interacts with the soybean NIN (GmNINa), in order to repress the expression of *GmRIC1* and *GmRIC2*. GmNINa can activate the transcription of miR172c, while NNC1 can inhibit the transcription of its regulatory miR172c (Wang *et al.*, 2019), indicative of a complex feedback regulatory loop.

Conclusions and future perspectives

Legumes are rich in nutritional value for human and livestock consumption, supplying an important source of protein, carbohydrates, fiber, and minerals (de Jager *et al.*, 2019). The ability of legumes to establish a nitrogen-fixing symbiosis contributes to their ecological and agricultural importance. A long-term goal of symbiotic nitrogen fixation research is to gain a sufficient understanding so that this capability can be transferred to other, non-leguminous crop plants, such as maize (Mus *et al.*, 2016). However, as our knowledge increases, the biological complexity of the nodulation process appears more and more daunting. Among the important regulators of the nodulation process are miRNAs that target key steps at all stages of nodule ontogeny. Although not discussed here, many of these same miRNAs probably also play important roles in regulating the arbuscular mycorrhizal symbiosis. For example, miR171 regulation of NSP2 was shown to significantly impact formation of the arbuscular mycorrhizal symbiosis, in addition to impacting nodulation (Lelandais-Brière *et al.*, 2016).

In this review, we highlighted studies that document that miRNAs regulate nodulation initiation and formation, hormone homeostasis, and the autoregulation of nodulation. The information presented focused exclusively on the miRNAs and their targets while we have excluded information on the effects of mutations that affect miRNA processing. For example, expression of Argonaute 5 (AGO5), which is involved in miRNA synthesis, was shown to be induced in common bean roots at 1 hpi with *Rhizobium*. Silencing of *AGO5* in both common bean and soybean transgenic roots resulted in reduced root hair curling and nodule numbers. Consistent with these results, the expression of NIN, FLOT2, and NF-YB was affected (Reyero-Saavedra *et al.*, 2017). These results suggest that a fruitful area for future research is to explore how miRNA synthesis impacts the symbiosis. Another area of interest is the interface between the nitrogen-fixing symbiosis and pathogen responses. For example, miR482 was shown to play a role in both the nitrogen-fixing symbiosis and the pathogen defense response (Li *et al.*, 2010).

In this review, we discussed the function of plant host-derived miRNAs in the legume–*Rhizobium* symbiosis. However, recently, rhizobial tRNA-derived sRNAs were shown to regulate nodulation by taking advantage of the host RNAi machinery (Ren *et al.*, 2019). Thus, this points to cross-kingdom sRNA-mediated regulation of the legume nitrogen-fixing symbiosis.

The ability of sRNAs to move within the vascular tissue of the plant presents another layer of complexity. The recent discovery that the mobile miRNA, miR2111, regulates TML in the AON pathway is a notable example (Tsikou *et al.*, 2018). A more thorough understanding of the molecular components of miRNA transport between different legume plant tissues would open up a whole new chapter in studies of the nitrogen-fixing symbiosis.

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