

# MicroRNAs in the Rhizobia Legume Symbiosis<sup>1</sup>

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### LEGUME SYMBIOSES

Legumes are agronomically valuable crops for food and fodder production worldwide because they are rich in protein, oil, fiber, and micronutrients. In addition, legumes require less chemical fertilizer than other major crop plants since they can assimilate some nutrients through symbiotic interactions with soil microbes. These relationships are mutually beneficial for the partners because the plant provides carbon-based energy to the microbe in exchange for essential nutrients. In permissive environmental conditions, legumes can establish symbiotic interactions with rhizobial bacteria and with arbuscular mycorrhizal (AM) fungi. The rhizobia-legume symbiosis leads to formation of root nodules, the site of bacterial nitrogen fixation, and nitrogen uptake by the host plant, whereas the AM symbiosis results in the formation of arbuscules, the site for phosphorous nutrient exchange. Notably, a wide range of plant hosts can form a mycorrhizal symbioses, as opposed to the rhizobial symbiosis that is nearly exclusive to legumes. Formation of a successful symbiosis is contingent upon a nutritional insufficiency of the plant. In the case of the rhizobia-legume symbiosis, the plants form symbiotic root nodules when grown in nitrogen-limiting conditions, but do not initiate the symbiosis when nitrogen levels are adequate. Nitrogen-stressed plants begin a molecular dialogue with rhizosphere microbes, initiating the early stages of symbiosis.

Ultimately, changes in plant and bacterial gene expression facilitate microbial infection of the plant root, the development of symbiotic root structures, and the maintenance of nitrogen-fixing root nodules. Plants secrete flavonoids and related compounds from the actively growing region of the root. These compounds promote the expression of nod genes in compatible rhizobial species, and nod-gene products synthesize nod factor, bacterial lipochito-oligosaccharide signaling molecules. Plant perception of nod factors by

LysM receptor-like kinase potentiates immediate sub-cellular changes in the root epidermis and later changes in the root cortex. Root epidermal cells respond by depolarization of the root hair plasma membrane, and cytoplasmic Ca<sup>2+</sup> spiking in the root hair initiates a signaling pathway involving a calcium and calmodulin-dependant protein kinase (CaMK), two GRAS family proteins Nodulation Signaling Pathway1 (NSP1) and NSP2, and an ethylene response factor transcription factor, ERN (for ethylene response factor required for nodulation). Cortical cells respond to nod factor signals by reinitiating the cell cycle, forming a nodule meristem. Subsequent to plant responses to nod factor, bacteria invade the plant body (see Ding and Oldroyd, 2009 for a summary of early signaling). Select epidermal cells form infection threads that are plasma membrane-lined conduits of extracellular matrix. Rhizobia invade root through the infection thread by a combination of cell division and tumbling within the thread matrix (Fournier et al., 2008). The threads branch and form through cortex cells, ultimately uniting with cells in the nascent nodule where bacteria are released into the cell cytoplasm in a process resembling endocytosis. Within the host-plant cytoplasm, the bacteria differentiate into nitrogen-fixing bacteroids that exchange bioavailable nitrogen for carbon energy from the host plant.

The molecular determinants and regulatory networks involved in symbiotic nodule development and function have not been completely elucidated, although recently there has been remarkable progress. The relatively recent discovery of small RNAs (sRNAs) as important components of plant gene regulation and plant development has initiated questions about their role in the legume symbioses. To date, the functions of sRNAs in legume symbioses have been largely unexplored. However, this area of research is advancing rapidly. The aim of this *Update* is to summarize the current state of knowledge about legume sRNAs, specifically microRNAs (miRNAs), in the rhizobia-legume symbiosis. sRNA regulation in complimentary research areas will also be discussed, including the roles of miRNAs in nutrient balance, plant development, plant microbe interactions, since all of these are essential to the rhizobia-legume symbiosis.

### SRNAs IN LEGUMES

Plant sRNAs are short, noncoding RNAs between 20 and 24 nucleotides (nt) in length. The two predomi-

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nant and prevalent sizes are 21 and 24 nt. In general (but not exclusively), miRNAs comprise the 21-nt class of sRNAs and short-interfering RNAs comprise the 24-nt class of sRNAs. Both of these molecules have been found across a broad set of eukaryotic species including legumes, but some of the more recent research in legumes has focused mainly on miRNAs since they regulate a number of developmental processes at the posttranscriptional level in other species (Bartel, 2004; Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006). miRNAs are derived from imperfectly matched stem-loop structures that are formed from single-stranded RNA precursors. miRNAs direct cleavage of a specific messenger RNA (mRNA) based on sequence homology between the miRNA and target mRNA. The miRNA is initially expressed as part of the transcript called the primary miRNA (pri-miRNA). The pri-miRNA is transcribed by RNA polymerase II and the miRNA transcript forms a hairpin-like secondary structure that is subsequently processed by the RNase III enzyme Dicer-Like1 and associated proteins. The hairpin is cleaved from the flanking regions, generating the precursor miRNA. The precursor miRNA is processed further into the miRNA/miRNA\* duplex, a paired set of sRNAs that are modified with a 3' methylation, and the set is exported from the nucleus to the cytoplasm by HASTY (Bollman et al., 2003; Han et al., 2004; Chen, 2005; Jones-Rhoades et al., 2006). One of these two molecules, the mature, methylated miRNA, is incorporated into the RNA-induced silencing complex containing ARGONAUTE1, which directs cleavage of the target mRNA.

MiRNAs can be identified with computational approaches that couple prediction of secondary structures (i.e. hairpin-forming pri-miRNA precursor sequences) with the presence of a conserved, mature miRNA sequence. A miRNA can be considered conserved if both the hairpin structure and the mature miRNA sequence are preserved across lineages; typically, a conserved miRNA has fewer than three mismatches when aligned with an annotated miRNA (Ambros et al., 2003; Meyers et al., 2008). Sunkar and Jagadeeswaran (2008) identified 682 miRNAs in 155 diverse plant species with the use of publicly available nt databases. In the legumes *Medicago truncatula*, *Lotus japonicus*, and soybean (*Glycine max*), they identified 19 conserved miRNA families. Since that study, the number of legume miRNAs recorded in the miRBase Sequence Database (release 13.0; March, 2009) has increased to 136, two, and 78 sequences from *M. truncatula*, *L. japonicus*, and soybean, respectively (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008). A benefit to using computational versus experimental approaches to identify miRNAs is that computational approaches are not dependent on the abundance, time, or place of expression of the miRNA. A drawback to the computational approach is that the nonconserved miRNAs are not easily identified. Nonconserved or novel miRNAs lack the sequence conservation across

species, so detailed analyses of their sequence, secondary structure, biogenesis, expression pattern, and/or silencing function are important to validate that these are miRNAs and to eliminate false positives. Annotation of novel miRNAs has been described (Griffiths-Jones et al., 2006, 2008; Meyers et al., 2008). In brief, novel miRNAs are assigned a number in an ordered manner and sometimes also a letter (as in miR156a) if several, paralogous miRNA-producing loci give rise to other identical or highly similar mature miRNAs. The letters are provided as sequential alphabetical suffixes (i.e. miR156a, miR156b, etc.). Finally, the computational approaches are also greatly hindered when there is a lack of substantial genomic sequence data or if the sequences that are available are not well documented. To date, this has been the case for legumes.

Another method for identifying miRNAs uses experimental protocols, such as the cloning and sequencing of sRNA libraries. In this approach, a cDNA library is created from a size-fractionated sample of sRNAs and the library is sequenced (Lu et al., 2007a). This approach has been used successfully in legumes and in the study of nitrogen-fixing nodules. sRNA libraries created from *M. truncatula* leaves identified 25 conserved miRNA families with the highest representation from the highly conserved plant miRNAs miR156, 159, and 166 (Szittyá et al., 2008). The set of miRNAs conserved across higher plants also tend to be the most abundantly expressed miRNAs. The mRNA targets for miR156, 159, and 166 encode transcription factors such as *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*, or members of the MYB and homeodomain-Leu zipper (HD-ZIP) families, respectively. A large proportion of miRNA targets encode transcription factors and many of these are involved in plant development (Kidner and Martienssen, 2005). MiRNA targets in plants including legumes are likely not limited to transcription factors, as two from this study were predicted to target disease resistance genes; in the case of the conserved miR395, it is predicted to target a sulfur transporter (Szittyá et al., 2008). The same authors also identified 26 novel miRNA candidates, of which eight were supported by sequencing data and confirmed by northern-blot analysis (Szittyá et al., 2008). The eight new miRNAs have not been previously described in other plant species so they likely represent either legume-specific miRNAs or miRNAs that have evolved recently. Finally, Arenas-Huertero and coworkers identified miRNAs in common bean (*Phaseolus vulgaris*) whose abundance changed in response to cold treatment, salt treatment, drought (water deprivation), abscisic acid (ABA) treatment, and in the stage of rhizobial infection of common bean. A total of 2,000 sequences were identified from these libraries, including 16 conserved miRNA families. The main point in highlighting this study is that it demonstrates the ability to discover conserved and novel sRNAs whose abundance is dynamic in response to specific abiotic and biotic challenge (Arenas-Huertero et al., 2009).

## MIRNAS REGULATE NUTRITIONAL BALANCE IN PLANTS

Formation of root nodules is dependent upon nitrogen stress of the host plant, but at this time, the role of miRNAs in nitrogen homeostasis in legumes is unknown. Results from other plant models demonstrate that plants utilize miRNA-mediated cleavage of target genes to coordinate the regulation of complex plant processes including the maintenance of nutrient homeostasis. One of the earliest examples of a miRNA involved in nutrient sensing was miR395, whose expression increased with sulfate starvation (Jones-Rhoades and Bartel, 2004). MiR395 was implicated in regulating sulfate homeostasis because it targets ATP sulfurylases and a low-affinity sulfate transporter (Jones-Rhoades and Bartel, 2004; Allen et al., 2005). Notably, miR395 and its target mRNA, *SULTR2;1*, were shown to be expressed in different cell types under the same condition (Kawashima et al., 2009). This finding suggests it may be important to determine the exact expression pattern of the miRNA and its target, as this may reveal insights into the process of nutrient delivery for development. Individual cell types may differ in their response to environmental stimuli, and the study presented by Gifford et al. (2008) partly focused on determining the link between cell-specific nitrogen responses and miRNA regulation using cellular profiling of *Arabidopsis thaliana* root cell types. It was previously determined that miR167 targets *AUXIN RESPONSE FACTOR8* (*ARF8*), which has developmental and phenotypic roles in the shoot and root, respectively (Tian et al., 2004; Wu et al., 2006). Gifford et al. (2008) tested *ARF8* as a regulator of lateral root growth in response to nitrogen and found *ARF8* to be induced in the pericycle and lateral root cap. Interestingly, the authors observed miR167 to be expressed in the pericycle and lateral root cap with *ARF8* but repressed in these regions in response to nitrogen. There are also examples that have linked phosphorous responses and miRNA regulation. Another study in *Arabidopsis* showed that miR399 is predicted to target a phosphate transporter and a putative ubiquitin-conjugating enzyme, UBC24, that negatively regulates the expression of phosphate transporters via protein degradation (Sunkar and Zhu, 2004). MiR399 expression is suppressed when there is a sufficient amount of phosphorous available, and the target UBC24 is expressed. MiR399 appears to be a key player in maintaining phosphorous homeostasis. In common bean, miR399 was implicated in phosphorous regulation responses as it was shown to increase dramatically during phosphorous starvation. PvmiR399 (the common bean miR399) was found to regulate the MYB transcription factor PvPHR1 in phosphorous-limiting conditions (Valdes-Lopez et al., 2008). PvPHR1 plays a key role in phosphorus signaling by regulating the expression of target genes involved in phosphorous transport,

remobilization, and homeostasis (Valdes-Lopez et al., 2008). A more recent study in *Arabidopsis* utilized real-time PCR profiling in combination with sRNA sequencing to detect phosphorous or nitrogen-responsive pri-miRNA transcripts (Pant et al., 2009). Using PCR primers specific to pri-miRNA sequences that were obtained from the miRBase database (<http://microrna.sanger.ac.uk/>), this group identified 20 pri-miRNAs that showed differential expression in phosphorous- or nitrogen-limiting conditions (Pant et al., 2009). MiRNAs that had not previously been implicated in nutrient status responses were regulated differentially during nutritional studies. The following was observed: pri-miR447c, 778, 827, 169m, and 169n increased during phosphorous limitation, whereas pri-miR398a decreased during phosphorous limitation and pri-miR169h through 169n decreased during nitrogen limitation, while pri-miR156e, 156g, and 157d increased during nitrogen limitation. Unexpectedly, the sRNA sequencing results from their study also identified a high abundance of miRNA\* strands, and in some cases their abundance exceeded the corresponding miRNA. The high abundance of the miRNA\* strands suggests a possible biological function for this molecule, but this has yet to be determined.

## MIRNAS IN PLANT RESPONSE TO MICROBIAL CHALLENGE

Some endogenous sRNAs in plants are regulated in response to interaction with microbes, even if the microbe is a potential symbiotic partner. In the case of pathogen attack, the regulation of miRNAs leads to gene expression reprogramming as the sRNAs subsequently regulate the expression of genes involved in the defense response (Jin, 2008). The *Arabidopsis* miR393 was the first miRNA shown to play an important role in plant immune responses. The flagellin-derived bacterial pathogen-associated molecular pattern, flg22, induced miR393 expression, which in turn decreased the expression of its target, the transcript encoding the auxin receptor known as Transport Inhibitor Response1 and the two functional paralogs AFB2 and AFB3 (Navarro et al., 2006). *Arabidopsis* miR772 also was shown to target two transcripts encoding putative disease resistance proteins (Lu et al., 2006). In loblolly pine (*Pinus taeda*), where the rust fungus *Cronartium quercuum* induces gall formation, 10 out of 11 miRNA families were down-regulated, including seven that were pine specific (Lu et al., 2007b). Based on miRNA target prediction, many of the targets were growth and disease-related transcripts, which suggests that the miRNA regulation created a tempered response that allowed for miRNA-mediated repression of both positive and negative defense regulators to restrict organ development and pathogen growth.

## MIRNAS IN NODULATION

To identify potential regulators of the earliest stages of nodule development Subramanian et al. inoculated soybean roots with *Bradyrhizobium japonicum* and identified miRNAs 3 h postinoculation (hpi; Subramanian et al., 2008). They found 20 conserved miRNA families and 35 novel miRNAs, and a subset of these sRNAs were differentially regulated in the first 12 hpi prior to nodule morphogenesis (Subramanian et al., 2008). For example, miR168 and miR172 were up-regulated at 1 or 3 hpi but returned to basal levels by 12 hpi; miR159 and miR393 were up-regulated by 3 hpi and continued to maintain this level to 12 hpi; miR160 and miR169 were down-regulated in response to rhizobia, and there were some that were not perturbed at all by infection with rhizobia. It is an intriguing observation that there is an overlap in the miRNAs involved in plant-pathogen interactions with those identified in the early stages of symbiosis. For example, miR393 and miR160 both promote plant basal immunity by the suppression of negative regulators of this defense pathway. Notably, Subramanian et al. also observed a coordinated expression pattern among a subset of miRNAs implicated in auxin signal transduction and proposed a role for these miRNAs as endogenous regulators that modulate auxin signaling and/or homeostasis during early nodulation (Subramanian et al., 2008). In many cases these targets were auxin response activators and repressors.

Based on work from Comber et al. (2006) that showed that miR169 regulates the expression of transcription factor *MtHAP2-1*, Subramanian et al. (2008) proposed additional studies that included the functional analysis of some of the more interesting miRNAs. These include miR172, which regulates a putative *Apetala2*-like transcription factor and miR169, which has two predicted targets in soybean that are nearly identical to the *HAP2-1* transcriptional regulator of symbiotic nodule development found in *M. truncatula* (Comber et al., 2006; Subramanian et al., 2008). The transcription factor *MtHAP2-1* was identified in a transcriptome study that showed *MtHAP2-1* to be strongly up-regulated during symbiotic interactions (Comber et al., 2006). They also used RNAi to show that a reduction in *MtHAP2-1* expression could significantly alter nodule development. The onset of nodulation was delayed and nodule growth was arrested approximately 8 to 10 d postinoculation with rhizobia. The arrested growth was also associated with the inability to fix N<sub>2</sub>. Based on earlier work that described *HAP2* transcription factors as targets of miR169 in *Arabidopsis* (Jones-Rhoades and Bartel, 2004), Comber et al. (2006) performed overexpression experiments using a *M. truncatula* miR169 precursor to investigate the functionality of *MtHAP2-1*. The nodulation phenotype was similar to that observed with the RNAi experiment, and this coincides with a reduction in *MtHAP2-1* transcript levels and miR169-mediated cleavage of *MtHAP2-1* mRNA, the latter confirmed

with 5'-RACE PCR. Expression of *MtHAP2-1* was confined to the nodule meristematic zone, suggesting miR169 is responsible for this spatial regulation during nodule differentiation into a nitrogen-fixing cell. Spatial regulation by a miRNA was also demonstrated with miR166 in *M. truncatula*; Boualem et al. (2008) showed that miR166 has a similar spatial expression pattern to its target genes, which encode class III HD-ZIP transcription factors. MiR166 appeared to affect the stability of the class III HD-ZIP transcripts in root vascular and apical regions because overexpression of the miR166a precursor led to alterations in vascular bundle patterning in the roots and a decrease in lateral root and nodule formation (Boualem et al., 2008).

MiRNAs were also shown to play a role in mature root nodules. Lelandais-Brière et al. (2009) constructed sRNA libraries from *M. truncatula* root tips and mature nodules to provide a global view of sRNAs from these tissues. In this study, the group identified 36 conserved miRNAs and 100 novel RNAs from both libraries. In situ analysis of select miRNAs demonstrated that novel miRNAs miR2586 and miR107 accumulated in the nodule meristem, as opposed to miR396 that accumulated in the root tips. The specificity of tissue distribution allowed the authors to speculate that these sRNAs function in stem cell renewal. MiR172 and miR398 were enriched in the nodule invasion zone, suggesting a function in cell differentiation or bacterial release into the plant cytoplasm. In a related study, Wang et al. (2009) created a sRNA library from functional nodules harvested 28 d postinoculation with *B. japonicum*. As previously mentioned, miRNAs can be regulated in a spatial or temporal manner so examining miRNAs at a later stage in nodule development may reveal insights into the regulatory networks that permit fixation of N<sub>2</sub> in mature nodules. Wang et al. (2009) showed that the 21-nt class of miRNAs is the most abundant class compared to other size classes of small noncoding RNAs, and they identified 32 miRNA sequences that belong to 11 miRNA families; eight of these were conserved across species and belong to miRNA families miR167, miR172, miR396, and miR399. From the sequences identified, 20 of the miRNAs constitute four miRNA families previously identified in early nodule development (Subramanian et al., 2008). They also cloned four sRNA sequences that belong to three novel miRNA families that were not identified by Subramanian et al. (2008). It is worth mentioning that the depth of sequencing performed in these studies is far from saturation so it can be expected that additional miRNAs will be discovered in future studies. The expression analysis performed on cloned miRNAs from leaf, stem, root, and nodule tissues revealed differential expression of the miRNAs (Wang et al., 2009). The differential expression may reflect a synchronized regulation of miRNAs involved in early and late nodulation. Consistent with many of the studies previously described, some of the putative targets identified by Wang et al. (2009) are transcription factors,

**Table 1.** Interaction between miRNAs and hormones in plant development

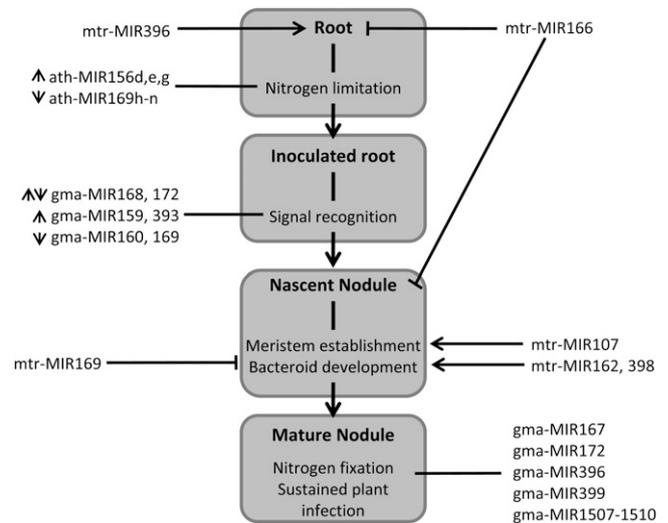
This table was adapted and modified from Liu and Chen (2009). JA, Jasmonic acid. Note: The developmental phenotype may not correspond to each hormone interactor.

miRNA	Hormone Signaling Involving a miRNA	Plant Developmental Process Mediated by miRNA
miR159 miR160	ABA, ethylene, GA Auxin	Germination, flower development Determination of leaf shape, hypocotyl elongation, flower development, and reproduction (fertility)
miR164 miR167	Auxin ABA and auxin	Root development Reproduction (fertility)
miR172	Cytokinin	Flower development
miR319 miR393	ABA, cytokinin, GA, JA ABA, auxin	Determination of leaf shape Root development

genes involved in plant defense responses, and genes involved in hormonal signaling pathways.

There is strong evidence that fluxes in hormone concentrations are critical for nodule formation and development, and this data has been compiled in an excellent review (Ding and Oldroyd, 2009). The stress hormones ethylene, salicylic acid, and jasmonic acid and ABA function in the earliest stages of nodule formation during nod factor signal transduction, whereas auxin, ABA, auxin, and cytokinin play a role in root architecture and nodule position and number. From the experiments described above and in other miRNA studies, there is a connection between miRNA regulation and hormone responses. Plant hormones are regulators of growth and development, and some miRNAs facilitate hormone-induced responses (Table 1). One of the earliest reports that linked miRNAs and hormones was from a study in Arabidopsis describing a mutation in a double-stranded RNA-binding protein, HYL1, that caused decreased sensitivity to cytokinin, as well as an altered response to ABA and auxin (Lu and Fedoroff, 2000). Some miRNAs implicated in regulation of auxin signaling target transcripts encoding ARFs. For example, miR167 in Arabidopsis and in cultured rice (*Oryza sativa*) cells targets ARFs, as does miR160 in Arabidopsis (Liu and Chen, 2009). Regulation of ARF expression has been shown to affect reproduction and cause striking morphological changes. In addition to ARFs, auxin receptors are targets of miRNA regulation. For example, miR393 targets transcripts encoding F-box proteins that in response to auxin target for degradation the protein repressors of ARF transcriptional activators (Dharmasiri et al., 2005; Jones-Rhoades et al., 2006). MiR164 is up-regulated by auxin, leading to a decrease in the levels of the transcriptional activator NAC1, which facilitates lateral root initiation; therefore, this miRNA regulates auxin signaling to reduce lateral root production (Guo et al., 2005). Other hormone responses are also mediated by miRNAs; miRNA159 has been implicated in regulation of gibberellin (GA) signaling, affecting flower development, floral meristem identity, and anther development (Achard et al., 2004). Several miRNAs are regulated

by more than one plant hormone, and this may reflect a convergence of regulatory pathways or cross talk between pathways. Notably, there are pathogens that can mimic hormones to promote their success and interfere with plant processes. For example, *Pseudomonas syringae* makes a form of jasmonic acid that can prevent salicylic acid-mediated defense responses, and *Gibberella fujikuroi*, a fungi, produces GAs that can cause the plant to grow very quickly, which makes them spindly and prone to breakage, and pathogens that produce cytokinin can inhibit senescence in tissues (Jones and Dangel, 2006).



**Figure 1.** Model of miRNA interactions in symbiotic root nodule development. The interactions are inferred from the current knowledge of the distribution and abundance of miRNA in libraries of developing nodules. Lines indicate points of potential miRNA control of nodulation in soybean (gma) and *M. truncatula* (mtr), and the potential role of miRNAs in nitrogen homeostasis are extrapolated from Arabidopsis (ath) miRNAs; arrows indicate promotion of stage, blunted line indicate repression of stage; plain line indicates that the miRNA is present, but basic roles are uncharacterized. Small arrowheads indicate changes in levels of miRNA levels in response to specific events. Vertical arrows connecting boxed areas indicate the developmental progression from root to mature root nodule.

## CONCLUSION

Gene expression regulation by miRNAs facilitates numerous plant processes involved in symbiotic root nodule development in legumes (Fig. 1). The perception of nutrient availability and modulation of gene expression in response to low-nitrogen status precludes nodule initiation, and miRNAs function in nutrient management. A clear connection of sRNAs has been demonstrated in phosphorous status in legumes (important for AM symbiosis), and data from other plant model systems suggest that this will also be true for nitrogen status in legumes. Interestingly, some legume mutants nodulate in adequate nitrogen conditions, and it is now possible to test if a global regulatory mechanism, like the coordinated modulation of gene expression by miRNAs is responsible for uncoupling nutrient status from nodule initiation. It is also quite intriguing that some miRNAs are up-regulated during both symbiotic nodule initiation and pathogenesis. Further experiments to identify and validate downstream targets of these regulatory molecules have the potential to define how plant defense mechanisms are modulated to accommodate symbiosis. Though perhaps initially counterintuitive, it is likely that up-regulation and fine tuning of disease resistance pathways may be essential for nodule formation. These sRNAs involved in early stages of symbiosis may also function in the regulation of nodule numbers and in later nodule senescence, although this has yet to be shown.

The identification of miRNAs from legumes and the discovery of miRNA-mediated gene regulation in legume symbiosis has helped us understand that there is another level to regulation of plant gene expression and orchestration of complex developmental processes such as root nodule formation. miRNAs have the ability to fine tune transcript levels in a manner that reflects a systematic response. This change in the transcript level of a single target, resulting from the expression of a 21-nt RNA, can have a huge impact on the plant phenotype. As we continue to develop expression profiles for sRNAs in a variety of plant species, including different tissues, diverse conditions, and perhaps ultimately cell types, we may start to unravel the full extent of pathways that are modulated by miRNAs. Many important questions still remain. What are the targets of all of these miRNAs and how are they linked to phenotypic changes? If miRNAs have such an important role in nodulation, then do other small noncoding RNAs like short-interfering RNAs also affect gene expression during nodulation? Furthermore, do small noncoding RNAs from bacteria modulate gene expression of the host plant, or vice versa, in a mechanism similar to miRNAs? Finally, how are the genes encoding miRNAs regulated during root nodule development? Legume scientists now face the awesome challenge of elucidating global pathways regulating symbiosis by linking the sRNA gene regulation with downstream cellular

and biochemical events. Important insights and some as-yet-unappreciated roles of sRNAs are likely to emerge.

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