Misexpression of miR482, miR1512, and miR1515 Increases Soybean Nodulation[1][W][OA]

Hui Li, Ying Deng, Tianlong Wu, Senthil Subramanian, and Oliver Yu*

Shanghai JiaoTong University, School of Agriculture and Biology, Shanghai 200240, China (H.L., T.W.); Donald Danforth Plant Science Center, St. Louis, Missouri 63132 (Y.D., S.S., O.Y.); and Plant Science Department, South Dakota State University, Brookings, South Dakota 57007 (S.S.)

MicroRNAs (miRNAs) are important regulators of plant growth and development. Previously, we identified a group of conserved and novel miRNA families from soybean (*Glycine max*) roots. Many of these miRNAs are specifically induced during soybean-*Bradyrhizobium japonicum* interactions. Here, we examined the gene expression levels of six families of novel miRNAs and investigated their functions in nodule development. We used northern-blot analyses to study the tissue specificity and time course of miRNA expression. Transgenic expression of miR482, miR1512, and miR1515 led to significant increases of nodule numbers, while root length, lateral root density, and the number of nodule primordia were not altered in all tested miRNA lines. We also found differential expression of these miRNAs in nonnodulating and supernodulating soybean mutants. The expression levels of 22 predicted target genes regulated by six novel miRNAs were studied by real-time polymerase chain reaction and quantitative real-time polymerase chain reaction. These results suggested that miRNAs play important roles in soybean nodule development.

Small regulatory RNAs (20–31 nucleotides in size) play important roles in the development of higher eukaryotic organisms. MicroRNAs (miRNAs), in particular, have been shown to regulate several essential biological processes in higher plants. miRNAs are 21 to 24 nucleotides in length and are synthesized from 70- to 300-bp primary miRNA transcripts (pri-miRNAs). The pri-miRNA is then processed by DCL1, a Dicer-like protein known to assist in miRNA maturation, in two or more steps to generate a miRNA:miRNA* duplex, resulting in mature miRNA and its near complementary strand (Bartel, 2004; Chen, 2009). This duplex is unwound into single strands by a helicase, and the mature miRNAs are selectively loaded to the RNA-induced silencing complex. Complementarity between miRNA and its mRNA target results in either cleavage of target mRNA or inhibition of translation. The former mechanism is most common in plants, whereas the latter mechanism is most common in animals (Mallory and Bouche, 2008).

miRNAs have been discovered through genetic and direct cloning approaches (Reinhart et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). In plants, miRNAs were identified by direct cloning after isolation of small RNAs (Reinhart et al., 2002; Sunkar et al., 2005; Subramanian et al., 2008) or computational prediction followed by experimental validation (Wen et al., 2008; Zhou et al., 2009). In higher plants, at least 27 miRNA families in 71 different plant species have been identified (miRBase version 14.0; Griffiths-Jones, 2006). The rate of miRNA discovery has increased dramatically with the advent of novel high-throughput sequencing methods and improved computational and experimental methods to validate genuine miRNA genes. Examples include Arabidopsis (*Arabidopsis thaliana*; Xie et al., 2005; Lu et al., 2006; Rajagopalan et al., 2006), rice (*Oryza sativa*; Sunkar et al., 2005), wheat (*Triticum aestivum*; Yao et al., 2007), *Medicago truncatula* (Jagadeeswaran et al., 2009; Lelandais-Briere et al., 2009), and soybean (*Glycine max*; Subramanian et al., 2008; Joshi et al., 2010).

The plethora of plant miRNAs target a large number of genes that function in plant development, including auxin signaling (Wang and Li, 2007; Meng et al., 2009), boundary formation/organ separation (Berger et al., 2009; Chitwood et al., 2009), floral development and vegetative phase change (Willmann and Poethig, 2005; Zhang et al., 2007), leaf organ morphogenesis and polarity (Nogueira et al., 2006; Husbands et al., 2009), as well as shoot and root development (Laporte et al., 2007; Chuck et al., 2009). However, the functions of a large majority of these novel miRNAs remain to be

[1] This work was supported by the National Science Foundation (grant no. MCB0519634 to O.Y.) and the U.S. Department of Agriculture (grant no. NRI2005–05190 to O.Y.), by Shanghai JiaoTong University (graduate student fellowship no. T250660301 to H.L.), and by the National Ministry of Science and Technology of China (grant no. 2008zx08004–003 to T.W.).

* Corresponding author; e-mail oyu@danforthcenter.org.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Oliver Yu (oyu@danforthcenter.org).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.110.156950

elucidated. All miRNA functions identified so far came from analyses of a handful of miRNA families (Bartel, 2009).

Recent studies have discovered novel and species-specific miRNAs from leguminous plants. In *M. truncatula*, 25 conserved miRNA families and 100 novel miRNA candidates were identified using high-throughput sequencing and computational analysis (Szittya et al., 2008; Jagadeeswaran et al., 2009; Lelandais-Briere et al., 2009; Zhou et al., 2009). In *Phaseolus vulgaris*, 16 conserved miRNA families and six novel miRNAs in response to stress were identified (Arenas-Huertero et al., 2009). We and others also reported at least 42 conserved and 87 novel miRNAs from soybean (Subramanian et al., 2008; Wang et al., 2009; Joshi et al., 2010). Additional large-scale sequencing targeting soybean and *M. truncatula* small RNAs have been carried out (L.O. Vodkin and B.C. Meyers, personal communications). The conserved legume miRNAs, such as miR399 from *P. vulgaris*, was shown to negatively regulate the ubiquitin E2 conjugase during phosphorus starvation responses (Valdes-Lopez et al., 2008). This function was consistent with miR399's function reported in Arabidopsis. However, none of the novel miRNAs has been investigated for its functions other than tissue-specific expression analysis (Jagadeeswaran et al., 2009; Joshi et al., 2010).

One important area of legume miRNA research is to decipher their roles in legume-*Rhizobium* symbiosis. Legume roots infected by Rhizobiaceae bacteria develop a specialized symbiotic organ known as nodules that are capable of fixing atmospheric nitrogen (Crespi and Frugier, 2008; Markmann and Parniske, 2009). Two conserved miRNAs have been studied in *M. truncatula* for their functions during nodulation. miR169 was shown to regulate MtHAP2-1, which altered nodule development (Combier et al., 2006), while overexpression of miR166 reduced the number of symbiotic nodules and lateral roots (Boualem et al., 2008).

Previously, we generated two libraries of small RNAs from *Bradyrhizobium japonicum*-inoculated and uninoculated soybean roots and obtained approximately 350,000 tags using high-throughput 454 sequencing (Subramanian et al., 2008). We identified 55 families of miRNAs in these two libraries: 20 families of conserved miRNAs that exist in other higher plants, and 35 families of "novel" miRNAs that had not been reported before. Out of these novel families, we classified soybean miR482 as a novel miRNA based on pre-miRNA divergence from known miR482 genes. However, the "miR482" name was assigned by the miRBase based on the homology criteria of the 22-nucleotide miRNA sequence. In this report, we sought to examine the role of a selected group of miRNAs during nodule development in soybean. We studied their tissue-specific expression levels and expression during nodulation, identified their putative target genes, and more importantly examined the effect of their misexpression on nodulation and root development. The results revealed that some of the miRNAs and their target genes played important roles in nodule development. It is possible that soybean evolved to utilize these miRNAs to regulate the symbiotic process.

RESULTS

Genome Organization of Selected miRNAs

We had identified a set of 35 novel miRNA families from soybean (Subramanian et al., 2008). We selected 17 moderately abundant miRNA families (at least 10 sequence reads in our high-throughput sequencing libraries) for detailed northern-blot analysis. Among these, only six had detectable expression in the roots: miR482, miR1507, miR1511, miR1512, miR1515, and miR1521 (Table I; Fig. 1; Supplemental Fig. S1). The other 11 miRNAs were most likely expressed at levels below the limit of detection by northern-blot analysis.

We examined the soybean genome (http://www.phytozome.com/soybean) to identify genes encoding these miRNAs (Table I). Among these six miRNAs, four are encoded by two genes (Table I). However, both genes are predicted to produce identical mature miRNAs except for miR1512. The two different genes encoded two different mature forms of miR1512 (Table I). Interestingly, none of these miRNAs have been identified in Arabidopsis, and a BLAST search of the Arabidopsis genome with these miRNA sequences did not yield any significant matches (data not shown). miR1507 might be a legume-specific miRNA family. Members of this gene family have been identified only in legume species thus far (Jagadeeswaran et al., 2009; Lelandais-Briere et al., 2009; Joshi et al., 2010). Additionally, miR1512, miR1515, and miR1521 have not been reported in species other than soybean (as of miRBase version 14). Therefore, we examined the expression levels of these miRNAs in different soybean organs with the larger goal of identifying and understanding their roles in root development and nodulation.

Tissue-Specific Expression of Selected Novel miRNAs

We first examined the general expression levels of these six miRNAs in different organs of soybean (cv Williams 82) by northern-blot analysis: root, stem, mature leaf, fully opened flowers, young pods, and developing seeds at stages R5 and R6. Differential expression of these miRNAs was observed in various organs. miR1507 was expressed in the root, stem, leaf, and mature green seed (R6) but was absent in flowers, pods, and younger seeds (R5; Fig. 1). miR482, miR1511, miR1515, and miR1521 were expressed in all organs tested, but their expression levels varied significantly (Fig. 1). miR482 and miR1511 had the highest expression level in stem (Fig. 1); miR1521 had the highest expression level in leaf; and miR1515 had a

Table I. *miRNA family with high levels of root expression during nodulation*

Sequence No.	miRNA Name	Sequence	Genome Location
1	miR482a	5′-UCUUCCCAAUUCCGCCCAUUCCUA-3′	Chromosome 2
	miR482b	5′-UCUUCCCAAUUCCGCCCAUUCCUA-3′	Chromosome 18
2	miR1507a	5′-UCUCAUUCCAUACAUCGUCUGA-3′	Chromosome 13
	miR1507b	5′-UCUCAUUCCAUACAUCGUCUGA-3′	Chromosome 17
3	miR1511	5′-AACCAGGCUCUGAUACCAUG-3′	Chromosome 18
4	miR1512a	5′-UAACUGAAAAUUCUUAAAGUAU-3′	Chromosome 18
	miR1512b	5′-UAACUGGAAAUUCUUAAAGCAU-3′	Chromosome 2
5	miR1515a	5′-UCAUUUUGCGUGCAAUGAUCUG-3′	Chromosome 1
	miR1515b	5′-UCAUUUUGCGUGCAAUGAUCUG-3′	Chromosome 11
6	miR1521	5′-CUGUUAAUGGAAAAUGUUGA-3′	Chromosome 11

constant expression level in all tissues (Fig. 1). Interestingly, miR1512 was only expressed in root and was undetectable in all other tissues (Fig. 1).

Northern-Blot Analysis of miRNA Expression in Nodulating Roots

We then examined the expression of these miRNAs in soybean roots inoculated with *B. japonicum* at 0, 1, 3, 6, 9, and 12 h after inoculation (hpi) to identify "early" expression patterns and at 1, 3, 6, and 14 d after inoculation (dpi) to examine "late" expression patterns. These time points represent important events during the development of nodules in soybean roots (Patriarca et al., 2004).

Based on the expression patterns (Fig. 2), we were able to group the miRNAs into three classes: (1) those that had a gradual increase over the period of nodule development, (2) those that oscillated during nodule development, and (3) those that were unaffected. miR482 and miR1521 fall into the first category. The expression of miR482 increased over time, reaching a peak at 6 dpi, and the expression of miR1521 increased gradually up to about 12 hpi and reached a plateau. miR1511 and miR1512 were "oscillators," whose expression showed two different transient increases over the period of assay: miR1511 at 1 hpi and 1 dpi and miR1512 at 3 hpi and 14 dpi. The expression of miR1507 and miR1515 was relatively unaltered over the period of nodule development. These different expression patterns suggested that these miRNAs might play different regulatory roles at early and late stages of nodule development.

Northern Analysis of miRNA Expression in Nodulation Mutants

We then examined the expression levels of these miRNAs in the nonnodulating soybean mutant NOD49, the supernodulating soybean mutant NTS382, and the corresponding wild-type cv Bragg. NOD49 has a mutation in one of the Nod factor receptor genes, NFR1 (Wan et al., 2008). Soybean NFR1 is the dominant LysM receptor kinase; thus, NOD49 is impaired in Nod factor perception, including early responses. NTS382 is a supernodulating mutant with a defect in the nodule autoregulation receptor kinase (NARK; Searle et al., 2003; Kinkema and Gresshoff, 2008). NARK activates a shoot-derived inhibition mechanism that significantly reduces additional nodulation after initial interactions with rhizobia. In the absence of NARK-induced autoregulation, there is significantly increased nodulation in the NTS382 mutant.

We examined the expression of these miRNAs at 0, 6, and 12 hpi as well as 2 and 6 dpi in nodulation mutants. These time points were the ones when we observed significant changes in miRNA expression in wild-type roots. Equal amounts of RNA from all three genotypes (five time points each) were resolved on the

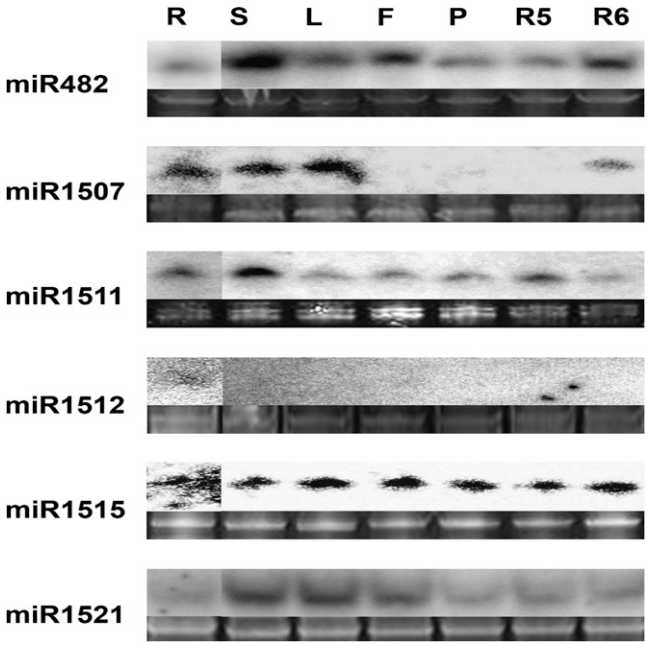

Figure 1. Expression levels of selected miRNAs in different tissues. Total RNA was isolated from soybean (Williams 82) tissues and subjected to northern-blot analysis. R, Root; S, stem; L, leaf; F, flower; P, young pod, R5, R5-stage seed; R6, R6-stage seed. Corresponding rRNA fragments stained by ethidium bromide are shown below each northern-blot panel to indicate equal loading in all lanes.

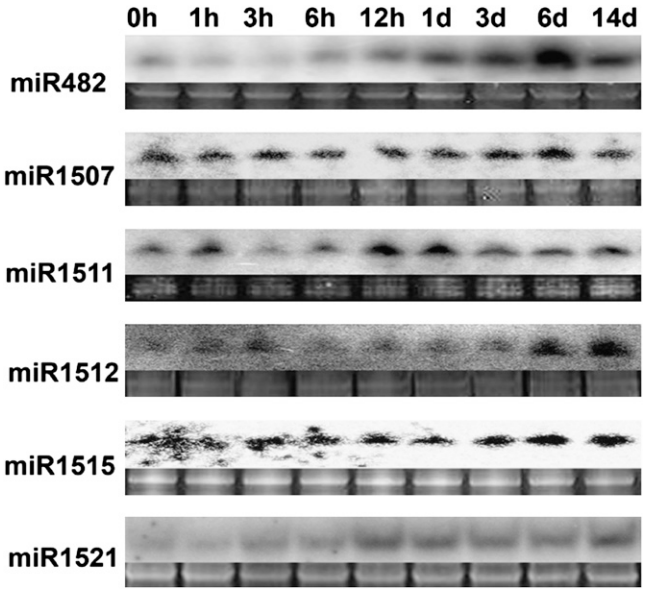

Figure 2. *Bradyrhizobium*-induced expression of selected miRNAs in soybean roots. Total RNA was isolated from soybean (Williams 82) roots after inoculation with *B. japonicum* and subjected to northern-blot analysis. The control tissue was 0-hpi root (same as in Fig. 1, lane R). Additional time points examined were 1, 3, 6, and 12 hpi and 1, 3, 6, and 14 dpi. Corresponding rRNA fragments stained by ethidium bromide are shown below each northern-blot panel to indicate equal loading in all lanes.

same gel and probed on the same blot to minimize variations and enable direct comparison. The time course of miRNA expression in Bragg roots inoculated with *B. japonicum* was strikingly similar to what was observed in Williams 82 (Fig. 2). For example, the expression of miR482 and miR1521 increased gradually over the time course (Fig. 3), as observed in Williams 82 (Fig. 2). In NOD49 mutant roots, the basal expression of miR482 and miR1521 was noticeably higher than in wild-type roots (Fig. 3). The expression of these miRNAs increased in response to *B. japonicum* inoculation in NOD49 roots similar to wild-type roots (Fig. 3). It is likely that *B. japonicum*-induced expression of these miRNAs is not dependent on the function of GmNFR1. The basal levels of miR482 and miR1521 in NTS382 were significantly lower than in wild-type roots. However, their expression levels increased after inoculation similar to what was observed in wild-type roots (Fig. 3). Since *B. japonicum*-responsive expression of miR482 and miR1521 was not affected in both NOD49 and NTS382 mutant roots, we concluded that the Nod factor perception and Nod autoregulation had little effect on the *B. japonicum*-inducible expression of miR482 and miR1512. Nevertheless, it must be noted that the basal levels of these miRNAs in soybean roots seem to be influenced by these mutations.

In contrast to the above miRNAs, *B. japonicum*-induced expression of miR1511 and miR1512 showed strikingly opposite expression levels between wild-type and NOD49 roots. Their expression levels transiently increased approximately 6 hpi in wild-type roots but decreased in response to *B. japonicum* inoculation in NOD49 roots. This suggested that *B. japonicum*-responsive expression of miR1511 and miR1512 is regulated by NFR1. The transient induction of miR1511 and miR1512 in response to *B. japonicum* was not observed in NTS382, suggesting that their induction might be governed by the Nod autoregulation pathway as well.

Similarly, the expression of miR1515, which remained unaffected in wild-type roots and NTS382 roots, increased significantly in response to *B. japonicum* inoculation in NOD49 roots. On the other hand, the expression of miR1507 was not influenced by *B. japonicum* inoculation in NOD49 as in wild-type roots. However, the expression of miR1507 increased upon rhizobia infection in NTS382 mutant roots. This suggested that miR1507 (and its targets) might be influenced 0by the Nod autoregulation pathway. Any disturbance in autoregulation led to activation of

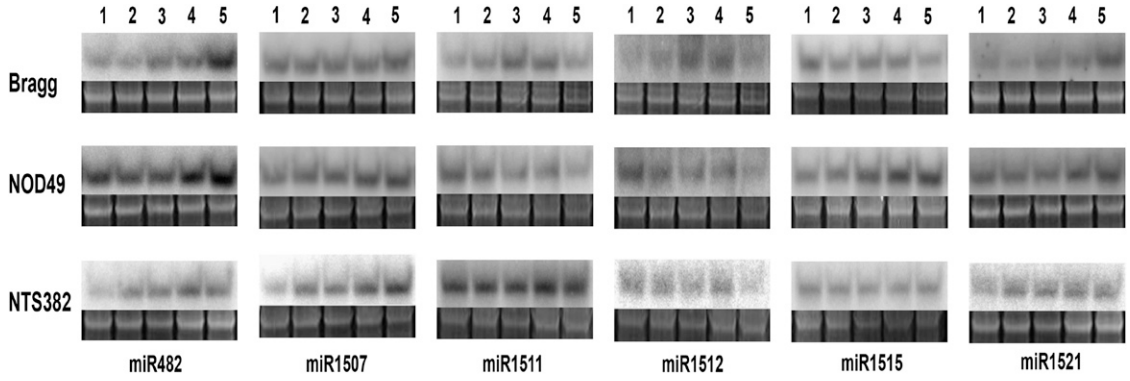

Figure 3. *Bradyrhizobium*-induced expression of selected miRNAs in Bragg (wild-type), NOD49 (nonnodulation mutant), and NTS382 (supernodulation mutant) roots. Total RNA was extracted from soybean roots at various time points (lane 1, 0 hpi; lane 2, 6 hpi; lane 3, 12 hpi; lane 4, 2 dpi; lane 5, 6 dpi) after inoculation with *B. japonicum*. Northern-blot analysis was performed using six probes as indicated (miR482, miR1507, miR1511, miR1512, miR1515, and miR1521).

miR1507 and presumably silencing of the corresponding target genes.

In summary, NFR1 and GmNARK1 signaling might not govern *B. japonicum*-induced expression of miR482 and miR1521, while both these pathways influence miR1511 and miR1512. Uniquely, the expression of miR1507 in response to *B. japonicum* was influenced only by GmNARK1 and not by GmNFR1. It was the opposite for miR1515, whose expression was influenced by GmNFR1 but not by GmNARK1 (Table II).

Prediction of miRNA Targets in the Soybean Genome

We used a custom Perl script to predict the targets of these miRNAs using the criteria and methods outlined previously (Allen et al., 2005; Schwab et al., 2005). We used conserved miRNAs as positive controls to validate our script. We searched the soybean genome database (predicted coding sequences, Glyma no.) using the six miRNA sequences. Gaps were not allowed in the homology algorithm, and G:U and other noncanonical pairs were treated as mismatches. We identified a total of 22 potential targets for the six miRNAs analyzed (Table III). Targets of miR482 were primarily resistance (R) gene receptor kinases, with two out of three targets being defense-related kinases. miR1511 and miR1512 targeted genes encoding protein phosphatase 2C and Copine I-like calmodulin-binding proteins, respectively. The putative legume-specific miR family, miR1507, targeted a sorbitol transporter-like protein and others. miR1521 might regulate a putative peptidase gene (Table III).

Expression of Putative Targets in the Wild Type and Nodulation Mutants

For expression analysis of the target genes, RNAs from wild-type Williams 82 were isolated as described above for northern-blot analysis. The 22 putative targets were analyzed by qualitative reverse transcription-PCR. The expression of only 13 genes could be detected in roots (Table III). The expression of these 13 genes was assayed by quantitative real-time (qRT)-PCR. The same sets of tissues used for northern analysis of miRNA expression in response to *B. japonicum* inoculation were used to examine the expression of target mRNAs by qRT-PCR.

We compared the expression patterns of miRNAs and their respective targets to identify if any of the miRNA-target pairs had inverse expression patterns. Five target genes and corresponding miRNAs, Gm12g28730 and Gm17g04060 (miR482), Gm06g42720 (miR1511), Gm04g05920 (miR1512), and Gm08g12340 (miR1521), showed very good correlations in all three soybean lines (Supplemental Fig. S2).

For example, miR482 and its target Gm12g28730 gene showed strong inverse expression patterns (Fig. 4). In wild-type Bragg roots, the expression of the target gene was significantly reduced at 12 h and 2 d, coinciding with the increased expression of miR482. Remarkably, in the NOD49 mutant roots where the expression of miR482 was decreased in response to *B. japonicum* inoculation, expression of Gm12g28730 was very high at the corresponding time points, suggesting that the expression of Gm12g28730 was indeed down-regulated significantly by miR482 during nodulation. In the NTS382 mutant, miR482 expression was first increased and then decreased, while Gm12g28730 was first suppressed and later expressed at higher levels, matching the miR482 expression levels perfectly.

However, the remaining eight predicted targets did not clearly match the expression levels of their corresponding miRNAs. As shown in Supplemental Figure S2, these putative target genes mostly had higher expression levels at 12 hpi, when most of the miRNAs had very high expression levels. It should be noted that we examined expression levels of miRNAs and their targets using RNA isolated from whole roots. It is possible that miRNA regulation of these targets occurs in specific root tissues to fine-tune gene expression. Such regulation can only be detected by in situ expression assays.

We then experimentally validated the predicted targets of miRNAs using 5′ RACE analysis. Cleavage products were amplified using nested gene-specific primers and sequenced. We selected targets of miR482, miR1512, and miR1515 for 5′ RACE analysis. The targets were selected based on their ability to alter nodule number (see below). We detected cleavage products for all examined targets at the expected site of miR binding except for the target of miR1512 (Table IV). These results suggested that our target gene prediction was reliable. For the target genes that did not have reverse expression patterns with their corresponding miRNAs, some other regulation mechanisms existed to control their gene expression levels.

Constitutive Overexpression of miR482 and miR1515 Increased Nodule Numbers in Transgenic Soybean Hairy Roots

Next, we examined the effect of overexpression of miR482, miR1507, miR1512, and miR1515 on the nodulation of soybean roots. These were selected based on the expression of their targets during nodule develop-

Table II. *miRNA had different expression patterns in soybean nodulation mutants*

↑, Up-regulated; ↓, down-regulated; =, unchanged; ↑↑, strongly up-regulated; ↓↓, strongly repressed.

Plant	miR393	miR482	miR1507	miR1511	miR1512	miR1515	miR1521
Wild type	↑	↑↑	=	↑↓	↑↓	=	↑
NOD49	↑	↑↑	=	↓	↓	↑	↑
NTS382	↓	↑	↑	=	↓	=	↑

Table III. *Predicted targets of selected miRNAs*

Tissue of expression in the last column is as follows: S, stem; L, leaf; F, flower; P, young pods; R, roots; 6, 6 dpi of *B. japonicum*; 14, 14 dpi of *B. japonicum*. Tissues in which expression was detected are marked by an x. Gene annotations were based on Phytozome Soybean Genome version 5.0. Gene names in boldface indicate expression in roots during nodulation.

miRNA Family	Predicted Target (Glyma1 Gene Name)	Annotation	Expression during Nodulation						
			S	L	F	P	R	6	14
miR482	**Glyma12g28730.1**	GSK-3-like protein MsK4	x	x	x		x	x	x
	Glyma17g04060.1	Unknown protein	x	x	x	x	x		x
	Glyma01g31520.1	TIR disease-resistant protein		x			x		
miR1507	**Glyma03g29370**	Unknown protein	x	x	x			x	x
	Glyma04g01550	Sorbitol-like transporter	x	x		x	x	x	x
	Glyma08g04230	Dynein light chain type 1 family protein		x		x	x	x	x
	Glyma15g21140	Disease resistance protein		x		x			
miR1511	Glyma18g43950.1	Protein phosphatase 2C, putative							
	Glyma06g02710.1	Unknown protein	x	x	x		x	x	x
	Glyma09g41720.1	Protein phosphatase 2C, putative					x	x	x
	Glyma06g42720	Unknown protein							
	Glyma09g16310	Epoxide hydrolase							
	Glyma12g19490	Putative gag-pol polyprotein-related				x	x	x	x
	Glyma17g24430	α-Carboxyltransferase precursor							
	Glyma18g48190	Unknown protein							
miR1512	**Glyma08g17790**	Ser/Thr protein kinase		x		x	x	x	x
	Glyma06g05910	Calmodulin-binding region myosin head				x			
	Glyma04g05920	Calmodulin-binding region					x	x	
miR1515	**Glyma09g27690**	β-Glucosidase, putative	x	x	x		x	x	x
	Glyma09g02920	Putative Dicer-like protein				x	x	x	
miR1521	**Glyma08g12340**	Peptidase C1A, papain		x			x	x	x
	Glyma03g39500.1	ATPase; 26S proteasome subunit P45	x	x	x	x	x	x	x

ment. The expression levels of the targets of miR1511 and miR1521 were deemed too low for downstream analysis, so these were not included in the transgenic experiments (Supplemental Fig. S1). We cloned the pri-miRNAs of the above four miRNAs by PCR, cloned them into a binary vector (pCAMGFP-CsVMV:GWOX; see "Materials and Methods"), and generated hairy root composite plants using *Agrobacterium rhizogenes*. The vector has a GFP cassette for the identification of transgenic roots. The promoter that controlled miRNA expression was a strong constitutive promoter from the *Cassava vein mosaic virus* (CsVMV). The empty vector-transformed roots were used as controls. Hairy root composite plants were examined for root growth characteristics (root length, no. of lateral roots) and nodulation by *B. japonicum*.

First, we verified overexpression of miRNAs in transgenic roots by northern-blot analysis. Total RNAs were isolated from GFP-positive roots 4 weeks after *Agrobacterium* inoculation. The results confirmed that all four lines had significantly higher miRNA expression (Fig. 5). Under our growing conditions, overexpression of these miRNAs did not cause any significant changes in root growth characteristics examined. Compared with vector-transformed controls, the four transgenic lines showed no obvious differences in root length or lateral root density (data not shown).

We then examined the nodule primordia numbers in the transgenic hairy roots approximately 10 d after rhizobia inoculation. The GFP-positive roots were collected, the tissues were cleared, and the roots were stained with methylene blue. We found no statistical difference among all four miRNA-overexpressed lines and vector-transformed controls. We observed miR482, miR1507, miR1512, miR1515, and vector control to have 0.295 ± 0.107, 0.641 ± 0.105, 0.348 ± 0.190, 0.383 ± 0.220, and 0.456 ± 0.210 primordia per milligram of dry root (average ± SD), respectively.

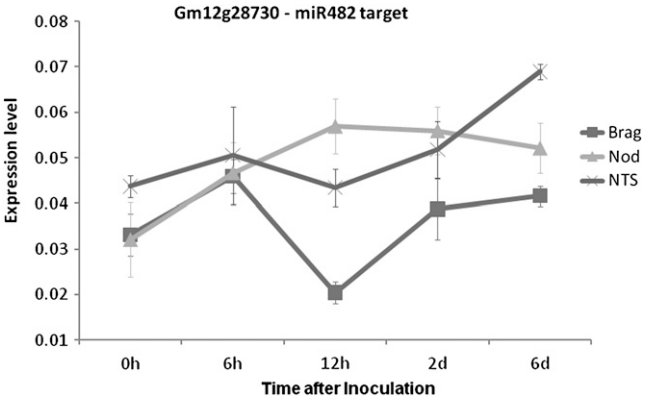

Figure 4. The expression pattern of Gm12g28730, a target of miR482, in wild-type and nodulation mutant roots. Total RNA was extracted from soybean roots at various time points after inoculated with *B. japonicum* (0 hpi, 6 hpi, 12 hpi, 2 dpi, and 6 dpi). After cDNA synthesis, the transcript levels were measured with RT-PCR. The expression levels are presented as fold actin levels. Three biological repeats were averaged. Error bars indicate SD. Brag, Wild-type Bragg; Nod, nonnodulating mutant NOD49; NTS, supernodulating mutant NTS382.

Table IV. *The RNA processing sites of the target genes based on 5′ RACE*
The predicted base pairing of miRNA and target mRNA is shown in Supplemental Figure S4.

miRNA	Target Gene	Cleavage Site	Frequency of Positive Clones
miR482	Gm12g28730	5′-GTTGAATGGGTG↓GAATTGGAAATC-3′	3 out of 21
miR1515	Gm09g02920	5′-TGGTCATTGC↓ACACAAAATGA-3′	13 out of 15

However, the nodule number per transgenic root varied significantly. Transgenic roots overexpressing miR482 exhibited a dramatic increase in the formation of mature nodules compared with control vector roots (Table V). Likewise, transgenic roots overexpressing miR1515 had a 1.5-fold higher nodule count compared with vector controls, which is statistically significant ($P < 0.05$). In comparison, overexpression of miR1512 and miR1507 did not alter nodule numbers significantly. These results provided genetic evidence that miR482 and miR1515 might regulate nodulation in soybean roots.

We also examined the expression levels of the putative target genes that have been confirmed by 5′ RACE (Table IV; Supplemental Fig. S3). In miR482-overexpressing lines, one pf the target genes, Gm12g28730, showed significant reduction in levels compared with vector-transformed controls. The other putative target, Gm17g04060.1, was not significantly changed. In the miR1515-overexpressing lines, Gm09g02920 also showed significant reduction in expression. This result further confirmed that Gm12g28730 (a GSK3-like kinase) and Gm09g02920 (a putative Dicer-like protein) are the targets of their corresponding miRNAs and might play a role in the nodulation process.

Nodulation-Induced Expression of miRNA Genes Increased the Nodule Numbers in Transgenic Hairy Roots

To reduce the pleiotropic effects of constitutively overexpressing miRNAs, the above four miRNAs (miR482, miR1507, miR1512, and miR1515) were expressed in roots using the *Rhizobium*-responsive soybean promoter ENOD40. The ENOD40 promoter is expressed at very low levels in lateral roots in uninoculated roots. Its expression increases significantly in response to rhizobial inoculation and is very high in nodule tissues (Yang et al., 1993). We examined the expression of an ENOD40:GUS construct in soybean hairy root composite plants and observed an expression level consistent with previous reports (data not shown).

The root phenotypes of these transgenic lines were characterized as above after generating hairy root composite plants. There was no significant difference in the number or density of lateral roots. However, the nodule number per transgenic root varied significantly between different constructs (Fig. 6; Table V). Roots expressing miR482 in a *Rhizobium*-inducible manner exhibited a dramatic increase (2-fold higher) in the number of mature nodules compared with control vector roots, consistent with what was ob-

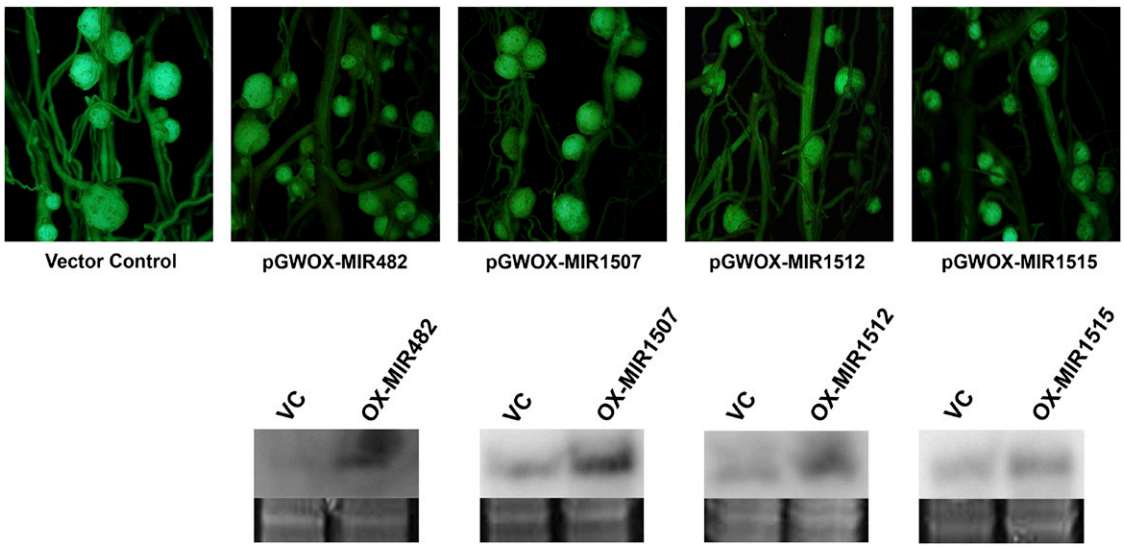

Figure 5. Constitutive overexpression of miRNAs in soybean hairy root composite plants led to altered nodule numbers. The miRNAs were driven by a constitutive CsVMV promoter in soybean composite plant roots. The top panels show GFP-expressing root nodules 4 weeks after inoculation with *B. japonicum*, including empty vector-transformed controls (VC) and roots overexpressing mi482, miR1507, miR1512, and miR1515. The bottom panels show northern-blot analyses of the same roots 4 weeks post inoculation, showing the increased abundance of mature miRNAs in transgenic lines.

Table V. *Overexpression of selected miRNAs altered nodule numbers in transgenic hairy root composite plants*

The expression of transgenic miRNA was under the transcriptional control of a constitutive promoter (CsVMV) or a nodulation-induced promoter (soybean ENOD40). The nodule numbers were counted 4 weeks after *B. japonicum* inoculation. The nodule numbers are converted to fold increase over one vector control, overexpressing a nonrelated bacterial *ccdB* gene that was used as the Gateway vector selection. For the nodulation-induced promoter control, the same *ccdB* gene in reverse orientation was transformed into soybean hairy root composite plants and compared with the previous control. The values shown are averages of three biological repeats ± SD. For each sample in these repeats, at least 24 transgenic plants, representing at least 100 individual transgenic roots, were counted. Student's *t* test was performed, and the statistically significant treatments ($P < 0.05$) are marked with asterisks.

miRNA	Nodule No.	
	Constitutive Promoter (CsVMV)	Nodulation-Induced Promoter (ENOD40)
Control	1.00 ± 0.02	0.97 ± 0.02
miR482	2.15 ± 0.13*	1.71 ± 0.22*
miR1507	1.08 ± 0.04	1.32 ± 0.23
miR1512	1.25 ± 0.01	1.66 ± 0.35*
miR1515	1.65 ± 0.03*	1.49 ± 0.49

served in roots constitutively overexpressing miR482. This observation suggested that the proper regulation of miR482 expression (and perhaps its targets) in nodule tissues might be crucial for the proper regulation of nodule numbers. Either overexpression or nodule-specific misexpression of miR482 led to an increase in nodulation. Further examination of miR482 during nodule development by in situ hybridization and expression of miR-resistant targets of miR482 in soybean roots might provide further insights into the role of miR482 during nodulation.

In contrast to miR482, ENOD40-driven expression of miR1515 did not significantly alter the number of nodules, whereas constitutive overexpression of miR1515 resulted in a significant increase in the number of nodules. This indicated that miR1515 might not play a significant role in nodule tissues. It is possible that the increased nodule numbers observed in miR1515-overexpressing roots was due to pleiotropic effects on the roots. We cannot exclude the possibility that miR1515 might play a role in nodule-associated tissues where ENOD40 is not expressed. Neither overexpression of miR1507 by CsVMV promoter nor misexpression by the ENOD40 promoter significantly altered nodule numbers, suggesting that it might not play a crucial role in the regulation of nodulation in soybean.

Interestingly, the expression of miR1512 under the control of the ENOD40 promoter resulted in a significant increase in nodule numbers, whereas its constitutive overexpression under the control of the CsVMV promoter did not significantly alter nodule numbers when compared with control vector roots. It is possible that ENOD40-driven expression of miR1512 in nodule tissues was significantly higher than CsVMV expression of the miRNA, resulting in a more significant silencing of targets. A more interesting possibility is that differences in expression gradients of miR1512 between nodule tissues and surrounding tissues might be crucial for the regulation of nodule numbers. The results suggested that miR482, miR1512, and miR1515 might play specific and important functions during soybean nodulation. Additional experiments, such as examination of tissue-specific expression of miRNAs and their targets, expression of miR-resistant targets, and misexpression and overexpression of these miRNAs in supernodulating mutant roots, might provide additional insights into the function of these miRNAs in nodulation.

DISCUSSION

B. japonicum-Induced miRNA Expression in Soybean Roots

We had earlier reported the regulation of a number of miRNAs in response to *B. japonicum* inoculation in soybean roots (Subramanian et al., 2008). Further analysis of a selected set of miRNAs in this study revealed interesting patterns of miRNA expression during the course of nodulation. A set of miRNAs (miR482 and miR1521) were induced in response to *B. japonicum* but were independent of the putative Nod factor receptor NFR1. While Nod factors are major signaling molecules during rhizobial symbiosis, a number of genes are induced independent of Nod

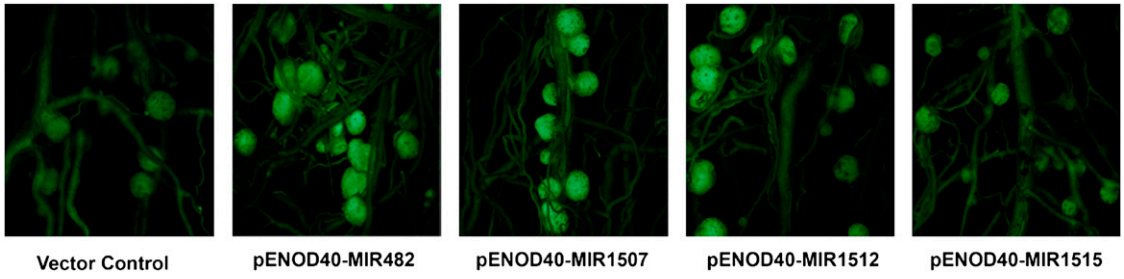

Figure 6. Nodulation-induced expression of miRNAs in soybean hairy root composite plants led to altered nodule numbers. The miRNAs were driven by nodulation-induced ENOD40 promoter in soybean composite plant roots. Four weeks after inoculation with *B. japonicum*, the GFP-expressing root nodules are shown, including empty vector-transformed controls and roots overexpressing mi482, miR1507, miR1512, and miR1515.

factor perception during nodulation. For example, in *M. truncatula hcl-1* (null allele of LYK3, the ortholog of the Nod factor receptor NFR1) mutant roots, the induction of only about 50% of the genes is impaired (Catoira et al., 2001; Mitra and Long, 2004; Smit et al., 2007). It is likely that these genes are induced in a nonspecific manner or through signaling by other *Rhizobium*-associated molecular signals such as exopolysaccharides. In some legume species (e.g. alfalfa [*Medicago sativa*]), these exopolysaccharides help evade plant resistance mechanisms (Cosme et al., 2008). It is interesting that miR482 regulates resistance-associated R genes. It is possible that modulation of plant defense mechanisms occurs to accommodate symbiosis and nodule development.

B. japonicum-regulated expression of miR1507, miR1511, and miR1512 was influenced at least in part by the Nod factor receptor NFR1 or the nodulation autoregulation kinase NARK1. These miRNAs target putative signal transduction components such as protein phosphatase 2Cs, Copine-like calmodulin-binding proteins, and small GTPase. Additional experiments to examine the functional significance of these targets in nodule development are necessary. These might be novel signaling elements in the Nod factor signaling pathway, the nodulation autoregulation pathway, or might play a role in nodule development.

Novel miRNAs That Play a Role in Nodulation

In addition to discovering the regulation of a number of novel miRNAs during nodulation, we also identified miRNAs that might play a significant role in the process. Previously, only two miRNAs were shown to affect nodule development. Both of them are conserved miRNAs. The target of miR169, the CCAAT-binding transcription factor MtHAP2-1, affects nodule development in *M. truncatula* (Combier et al., 2006); and overexpression of miR166 (which targets HDZIPIII transcription factors) inhibited nodule development in *Medicago* (Boualem et al., 2008). In our report here, misexpression of miR482 using either a constitutive promoter or a *Rhizobium*-responsive promoter resulted in an approximately 2-fold increase in nodule number (Table V). Some targets of miR482 are R genes involved in plant disease resistance (Table III). Indeed, we experimentally validated miR482-directed cleavage of two of these targets in soybean roots (Table IV). As mentioned above and suggested by Simon et al. (2009), it is possible that modulation of plant defense responses might be crucial to the establishment of symbiosis. Misexpression of miR482 might have resulted in enhanced degradation of targeted R genes, resulting in increased nodulation. Although its pri-mRNA is highly divergent, miR482 has been reported in poplar (*Populus trichocarpa*; Lu et al., 2005, 2008), where it is mostly expressed in leaf and xylem tissues (the root expression was not shown). Interestingly, the poplar miR482 also targets putative disease-resistant proteins. There are also four copies of miR482 from pine (*Pinus taeda*) in the miRBase without detailed functional analysis (Lu et al., 2007).

Overexpression of miR1515 that regulates a Dicer-like protein (the closest Arabidopsis homolog is DCL2) and nodule-specific expression of miR1512 that regulates Copine-like calmodulin-binding proteins also increased the number of nodules (Table V). miR1512 was a root-specific miRNA, while miR1515 was expressed evenly in all tested tissues (Fig. 1). miR1512 was an oscillator during the nodulation process. It had two highly induced periods, 3 hpi and 6 dpi (Fig. 2). ENOD40-induced expression may perturb either one of these expressions. Interestingly, Copine-like protein was implicated in cell signaling and membrane trafficking (Damer et al., 2005; Omerovic and Prior, 2009). Unfortunately, since the only tissue overexpressing miRNAs are developing nodules, northern-blot analysis and qRT-PCR on whole roots will not be suitable for transgene or target gene expression analysis in ENOD40-induced miR1512 lines. We still need further confirmation from more complex experiments.

The target of miR1515, the Dicer-like protein, may serve as a major regulator of double-stranded RNA processing primarily in viral defense responses (Xie et al., 2004; Allen et al., 2005). The 5′ RACE-confirmed target aligns well with the Arabidopsis DCL2 gene. Unlike Arabidopsis DCL1, which showed drastic developmental phenotypes when mutagenized, DCL2 knockout mutants do not show major developmental defects. It has been shown to function in controlling RNA virus and transitive silencing (Diaz-Pendon et al., 2007; Moissiard et al., 2007). However, the exact functions of this protein during the symbiotic process are largely unknown. Our results identified some novel target genes and suggested novel roles of miRNAs during nodulation. Further characterization of these miRNAs and their targets would provide interesting insights on miRNA-regulated processes during nodulation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Soybean (*Glycine max*) cv Williams 82, the reference accession for genome sequencing, was used as the wild-type control for miRNA identification, cloning of miRNAs, northern-blot analysis, and functional assays. For experiments involving nodulation mutants (nonnodulating NOD49 and supernodulating NTS382), we used the corresponding wild-type accession Bragg as a control (kind gifts from Dr. Peter Gresshoff, University of Queensland). Seeds were surface sterilized by treating with 8% Clorox bleach for 4 min, followed by 70% ethanol for 4 min. The seeds were then rinsed three times with sterile deionized water. Sterilized seeds were planted in a 4-inch pot filled with a 1:3 (v/v) mixture of sterilized vermiculite and perlite (Hummer International). These pots were prewetted with nitrogen-free plant nutrient solution (N-PNS; Subramanian et al., 2004) and put into a growth chamber (26°C, 200 μmol m^{-2} s^{-1} light, 60% relative humidity, 16/8-h day/night photoperiod). Seeds were watered every other day, alternating water with N-PNS until sample collection. For rhizobia inoculation, *Bradyrhizobium japonicum* cells (USDA110) were grown in Vincent's rich medium (Vincent and Humphrey, 1970) containing chloramphenicol at 20 μg mL^{-1} and resuspended in N-PNS to a final concentration of optical density at 600 nm = 0.08. Ten-day-old seedlings were flood inoculated with the above *B. japonicum* suspension (15 mL per pot).

For RNA extraction, seedlings were uprooted and rinsed briefly in sterile deionized water to remove vermiculite and perlite particles. Roots were blot dried gently and quickly with sterile paper towels, immediately frozen in liquid N_2, and stored at −80°C until further processing. For time-course analyses, Williams 82 roots were harvested at 1, 3, 6, and 12 h, and at 1, 3, 6, and 14 d, after *B. japonicum* inoculation. Bragg and the two nodulation mutants were harvested at 6 h, 12 h, 2 d, and 6 d after inoculation. For mock inoculation, seedlings were watered with N-PNS without rhizobia.

RNA Extraction and Northern-Blot Analysis

RNA isolation and northern-blot analysis were performed essentially as described (Subramanian et al., 2008). Total RNA was extracted from soybean roots inoculated with *B. japonicum* at different time points using Trizol reagent (Invitrogen). The concentration of RNA was quantified by the A_{260}. Approximately 20 μg of total RNA was separated on a Criterion 15% TBE-Urea gel (Bio-Rad Laboratories), transferred to positively charged nylon membranes (Roche) using a Criterion wet transfer blot apparatus (Bio-Rad), and fixed on the membranes using a UV cross-linker (Stratagene). For the detection of miRNAs, oligonucleotide probes complementary to miRNAs were end labeled with [γ-^{32}P]dATP (Perkin-Elmer) using the miRNA StarFire DNA polymerase kit (Integrated DNA Technologies) and purified using a nucleotide removal column (Qiagen). RNA blots were prehybridized for about 1 to 1.5 h with Perfect Hyb Plus hybridization buffer (Sigma-Aldrich) at 38°C and hybridized with the labeled probes for 16 to 24 h at 38°C. After hybridization, blots were washed twice (20 min each) with 2× SSC plus 0.2% SDS at 40.7°C, then rinsed twice with 2× SSC plus 0.2% SDS at room temperature. The membranes were exposed to phosphorimager screens (Amersham) for at least 3 h, and the exposures were scanned using a Typhoon phosphorimage analyzer (GE Healthcare).

Qualitative PCR and qRT-PCR Assays

Total RNA was treated with DNase (Invitrogen) to remove genomic DNA contamination. cDNAs were synthesized from 2 μg of total RNA by Moloney murine leukemia virus reverse transcriptase (New England Biolabs) using oligo(dT) primers according to the manufacturer's instructions. Qualitative PCR and qRT-PCR assays were performed using the cDNAs as template and gene-specific primers for the genes analyzed (Supplemental Table S1).

qRT-PCR of miRNA targets was analyzed using SYBR Advantage qPCR Premix (Clontech) on a StepOnePlus Real-Time PCR System (Applied Biosystems). For comparable quantification among different sets of primers, PCR efficiency was determined by a series of 10-fold dilutions of cDNAs (Schmittgen et al., 2004). Estimates of initial transcript amount were performed using the comparative threshold cycle method. Relative expression levels were normalized using actin as an internal control (Subramanian et al., 2006). The primers were designed across the miRNA target sites and are listed in Supplemental Table S1.

Prediction of miRNA Targets

Putative targets of miRNAs were predicted using criteria outlined by previous studies (Xie et al., 2004; Allen et al., 2005; Schwab et al., 2005) using a custom Perl script. Briefly, we used the script to search for complementary matches primarily in the "seed" region of miRNA (second to 12th nucleotides), not allowing more than two consecutive mismatches and checking for at least 70% minimum free energy compared with the perfect complement of the miRNA sequence. The script will be presented as part of a Web site to predict soybean miRNA targets and is available on request from the authors (senthil.subramanian@sdstate.edu).

5′ RACE Mapping of miRNA Target Cleavage Sites

Total RNA was isolated from roots of 4-week-old soybean using the Plant RNA reagent (Invitrogen) according to the manufacturer's recommended protocol. The GeneRacer Kit (Invitrogen) was used to process the total RNA and map the 5′ terminus of the primary transcript. Exactly 2 μg of mRNA isolated from wild-type Bragg (12 h after rhizobia treatment) was directly ligated to the RNA oligonucleotide and reverse transcribed with SuperScript III reverse transcriptase using oligo(dT) primer (as provided in the kit). The cDNA samples were amplified with nested PCR according to the manufacturer's protocols. The gene-specific primers (Supplemental Table S1) were designed using the PrimerSelect program (DNAStar). After amplification, 5′ RACE products were gel purified and cloned to TA vector (Invitrogen), and at least 10 independent clones were sequenced for each PCR product.

Vector Construction

miRNA overexpression constructs were made for nodulation assays. The respective pre-miRNA fragments were amplified by PCR from Williams 82 genomic DNA using miRNA-specific primers as given in Supplemental Table S1 and cloned to the pGEM-T Easy vector (Promega). The amplified miRNA fragments were gel purified (Qiagen) and subcloned into Gateway vector pMH-ENTR40 (Yu et al., 2006) to produce a set of Gateway entry vectors. The entry vectors were then swapped with the pCAMGFP-GWOX destination vector, which contains a CsVMV promoter-driven expression cassette for constitutive expression, and the pCAMGFP-ENOD40-GWOX destination vector, which contains a soybean ENOD40 promoter-driven cassette for nodulation-inducible expression. The clones were verified by enzyme digestion and sequencing. These binary vectors were electroporated into *Agrobacterium rhizogenes* strain K599.

Composite Plant Transformation and Analyses

Soybean transformation to generate hairy root composite plants was performed as described previously (Collier et al., 2005). Briefly, soybean seeds were surface sterilized and grown in a greenhouse for 10 d. *A. rhizogenes* cultures were grown in Luria-Bertani broth with kanamycin (50 μg mL^{-1}) at 28°C overnight. Bacterial cells were then spun down and resuspended in N-PNS to an optical density at 600 nm = 0.3. Sterilized FibrGro cubes (16 cm^3; Hummert International) were inoculated with 6 mL of *A. rhizogenes* suspension. Apical stem sections were excised from soybean seedlings and inserted into the inoculated cubes. These plants were placed in a growth chamber for 10 d (22°C, 15 μmol m^{-2} s^{-1} light, 30% relative humidity, 16/8-h day/night photoperiod), transferred to individual 4-inch pots containing sterile sand, and watered regularly with N-PNS. After 1 week in the growth chamber, *B. japonicum* bacterial suspension (10 mL) was inoculated onto each composite plant. Four weeks after rhizobia inoculation, roots were harvested and screened based on their GFP epifluorescence using a Zeiss Stemi SV11 microscope outfitted with a 480-nm excitation/515-nm emission fluorescein isothiocyanate filter. At least 24 individual plants were scored for GFP roots, representing at least 100 individually transformed roots in each treatment. For statistical analysis, the entire experiment was repeated three times over the period of 4 months under the same transformation and culture conditions.

To count nodule primordia in the transgenic roots, the roots were harvested 2 weeks after inoculation and frozen at −70°C until analysis. The roots were later fixed with ethanol:glacial acetic acid (3:1) for 30 min and cleared by immersing the roots in sodium hypochlorite 6.5% active chlorine solution for 20 min. The roots were then stained with 0.01% methylene blue (Subramanian et al., 2004). The stained roots were observed with a dissecting microscope. Nodule primordia were counted from approximately 500 mg of root at 60× magnification.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Predicted hairpin structures of pri-miRNA transcripts.

Supplemental Figure S2. Expression of predicted miRNA targets during nodulation in the wild type and nodulation mutants.

Supplemental Figure S3. Expression of selected miRNA target genes in transgenic roots overexpressing corresponding miRNAs.

Supplemental Figure S4. Cleavage sites of selected targets of miR482 and miR1515 mapped by 5′ RACE assay.

Supplemental Table S1. Sequences of PCR primers used in the study.

ACKNOWLEDGMENTS

We thank Dr. Guohong Mao, Andrew Williams, and Rui Zhong for their technical support. Andrew Williams was supported by the Students and Teachers as Research Scientists (St. Louis) program in 2008.

Received March 28, 2010; accepted May 20, 2010; published May 27, 2010.

LITERATURE CITED

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell **121:** 207–221

Arenas-Huertero C, Perez B, Rabanal F, Blanco-Melo D, De la Rosa C, Estrada-Navarrete G, Sanchez F, Covarrubias AA, Reyes JL (2009) Conserved and novel miRNAs in the legume Phaseolus vulgaris in response to stress. Plant Mol Biol **70:** 385–401

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116:** 281–297

Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell **136:** 215–233

Berger Y, Harpaz-Saad S, Brand A, Melnik H, Sirding N, Alvarez JP, Zinder M, Samach A, Eshed Y, Ori N (2009) The NAC-domain transcription factor GOBLET specifies leaflet boundaries in compound tomato leaves. Development **136:** 823–832

Boualem A, Laporte P, Jovanovic M, Laffont C, Plet J, Combier JP, Niebel A, Crespi M, Frugier F (2008) MicroRNA166 controls root and nodule development in Medicago truncatula. Plant J **54:** 876–887

Catoira R, Timmers AC, Maillet F, Galera C, Penmetsa RV, Cook D, Denarie J, Gough C (2001) The HCL gene of Medicago truncatula controls Rhizobium-induced root hair curling. Development **128:** 1507–1518

Chen X (2009) Small RNAs and their roles in plant development. Annu Rev Cell Dev Biol **25:** 21–44

Chitwood DH, Nogueira FT, Howell MD, Montgomery TA, Carrington JC, Timmermans MC (2009) Pattern formation via small RNA mobility. Genes Dev **23:** 549–554

Chuck G, Candela H, Hake S (2009) Big impacts by small RNAs in plant development. Curr Opin Plant Biol **12:** 81–86

Collier R, Fuchs B, Walter N, Kevin Lutke W, Taylor CG (2005) Ex vitro composite plants: an inexpensive, rapid method for root biology. Plant J **43:** 449–457

Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernie T, Ott T, Gamas P, Crespi M, et al (2006) MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in Medicago truncatula. Genes Dev **20:** 3084–3088

Cosme AM, Becker A, Santos MR, Sharypova LA, Santos PM, Moreira LM (2008) The outer membrane protein TolC from Sinorhizobium meliloti affects protein secretion, polysaccharide biosynthesis, antimicrobial resistance, and symbiosis. Mol Plant Microbe Interact **21:** 947–957

Crespi M, Frugier F (2008) De novo organ formation from differentiated cells: root nodule organogenesis. Sci Signal **1:** re11

Damer CK, Bayeva M, Hahn ES, Rivera J, Socec CI (2005) Copine A, a calcium-dependent membrane-binding protein, transiently localizes to the plasma membrane and intracellular vacuoles in Dictyostelium. BMC Cell Biol **6:** 46

Diaz-Pendon JA, Li F, Li WX, Ding SW (2007) Suppression of antiviral silencing by cucumber mosaic virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. Plant Cell **19:** 2053–2063

Griffiths-Jones S (2006) miRBase: the microRNA sequence database. Methods Mol Biol **342:** 129–138

Husbands AY, Chitwood DH, Plavskin Y, Timmermans MC (2009) Signals and prepatterns: new insights into organ polarity in plants. Genes Dev **23:** 1986–1997

Jagadeeswaran G, Zheng Y, Li YF, Shukla LI, Matts J, Hoyt P, Macmil SL, Wiley GB, Roe BA, Zhang W, et al (2009) Cloning and characterization of small RNAs from Medicago truncatula reveals four novel legume-specific microRNA families. New Phytol **184:** 85–98

Joshi T, Yan Z, Libault M, Jeong DH, Park S, Green PJ, Sherrier DJ, Farmer A, May G, Meyers BC, et al (2010) Prediction of novel miRNAs and associated target genes in Glycine max. BMC Bioinformatics **11:** S14

Kinkema M, Gresshoff PM (2008) Investigation of downstream signals of the soybean autoregulation of nodulation receptor kinase GmNARK. Mol Plant Microbe Interact **21:** 1337–1348

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science **294:** 853–858

Laporte P, Merchan F, Amor BB, Wirth S, Crespi M (2007) Riboregulators in plant development. Biochem Soc Trans **35:** 1638–1642

Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science **294:** 858–862

Lee RC, Ambros V (2001) An extensive class of small RNAs in Caenorhabditis elegans. Science **294:** 862–864

Lelandais-Briere C, Naya L, Sallet E, Calenge F, Frugier F, Hartmann C, Gouzy J, Crespi M (2009) Genome-wide *Medicago truncatula* small RNA analysis revealed novel microRNAs and isoforms differentially regulated in roots and nodules. Plant Cell **21:** 2780–2796

Lu C, Kulkarni K, Souret FF, MuthuValliappan R, Tej SS, Poethig RS, Henderson IR, Jacobsen SE, Wang W, Green PJ, et al (2006) MicroRNAs and other small RNAs enriched in the Arabidopsis RNA-dependent RNA polymerase-2 mutant. Genome Res **16:** 1276–1288

Lu S, Sun YH, Amerson H, Chiang VL (2007) MicroRNAs in loblolly pine (Pinus taeda L.) and their association with fusiform rust gall development. Plant J **51:** 1077–1098

Lu S, Sun YH, Chiang VL (2008) Stress-responsive microRNAs in Populus. Plant J **55:** 131–151

Lu S, Sun YH, Shi R, Clark C, Li L, Chiang VL (2005) Novel and mechanical stress-responsive microRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*. Plant Cell **17:** 2186–2203

Mallory AC, Bouche N (2008) MicroRNA-directed regulation: to cleave or not to cleave. Trends Plant Sci **13:** 359–367

Markmann K, Parniske M (2009) Evolution of root endosymbiosis with bacteria: how novel are nodules? Trends Plant Sci **14:** 77–86

Meng Y, Huang F, Shi Q, Cao J, Chen D, Zhang J, Ni J, Wu P, Chen M (2009) Genome-wide survey of rice microRNAs and microRNA-target pairs in the root of a novel auxin-resistant mutant. Planta **230:** 883–898

Mitra RM, Long SR (2004) Plant and bacterial symbiotic mutants define three transcriptionally distinct stages in the development of the *Medicago truncatula/Sinorhizobium meliloti* symbiosis. Plant Physiol **134:** 595–604

Moissiard G, Parizotto EA, Himber C, Voinnet O (2007) Transitivity in Arabidopsis can be primed, requires the redundant action of the antiviral Dicer-like 4 and Dicer-like 2, and is compromised by viral-encoded suppressor proteins. RNA **13:** 1268–1278

Nogueira FT, Sarkar AK, Chitwood DH, Timmermans MC (2006) Organ polarity in plants is specified through the opposing activity of two distinct small regulatory RNAs. Cold Spring Harb Symp Quant Biol **71:** 157–164

Omerovic J, Prior IA (2009) Compartmentalized signalling: Ras proteins and signalling nanoclusters. FEBS J **276:** 1817–1825

Patriarca EJ, Tate R, Ferraioli S, Iaccarino M (2004) Organogenesis of legume root nodules. Int Rev Cytol **234:** 201–262

Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana. Genes Dev **20:** 3407–3425

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature **403:** 901–906

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev **16:** 1616–1626

Schmittgen TD, Jiang J, Liu Q, Yang L (2004) A high-throughput method to monitor the expression of microRNA precursors. Nucleic Acids Res **32:** e43

Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific effects of microRNAs on the plant transcriptome. Dev Cell **8:** 517–527

Searle IR, Men AE, Laniya TS, Buzas DM, Iturbe-Ormaetxe I, Carroll BJ, Gresshoff PM (2003) Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. Science **299:** 109–112

Simon SA, Meyers BC, Sherrier DJ (2009) MicroRNAs in the rhizobia legume symbiosis. Plant Physiol **151:** 1002–1008

Smit P, Limpens E, Geurts R, Fedorova E, Dolgikh E, Gough C, Bisseling T (2007) *Medicago* LYK3, an entry receptor in rhizobial nodulation factor signaling. Plant Physiol **145:** 183–191

Subramanian S, Fu Y, Sunkar R, Barbazuk WB, Zhu JK, Yu O (2008) Novel and nodulation-regulated microRNAs in soybean roots. BMC Genomics **9:** 160

Subramanian S, Hu X, Lu G, Odelland JT, Yu O (2004) The promoters of two isoflavone synthase genes respond differentially to nodulation and defense signals in transgenic soybean roots. Plant Mol Biol **54:** 623–639

Subramanian S, Stacey G, Yu O (2006) Endogenous isoflavones are

essential for the establishment of symbiosis between soybean and Bradyrhizobium japonicum. Plant J **48:** 261–273

Sunkar R, Girke T, Jain PK, Zhu JK (2005) Cloning and characterization of microRNAs from rice. Plant Cell **17:** 1397–1411

Szittya G, Moxon S, Santos DM, Jing R, Fevereiro MP, Moulton V, Dalmay T (2008) High-throughput sequencing of Medicago truncatula short RNAs identifies eight new miRNA families. BMC Genomics **9:** 593

Valdes-Lopez O, Arenas-Huertero C, Ramirez M, Girard L, Sanchez F, Vance CP, Luis Reyes J, Hernandez G (2008) Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signalling in common bean roots. Plant Cell Environ **31:** 1834–1843

Vincent JM, Humphrey B (1970) Taxonomically significant group antigens in Rhizobium. J Gen Microbiol **63:** 379–382

Wan J, Zhang XC, Neece D, Ramonell KM, Clough S, Kim SY, Stacey MG, Stacey G (2008) A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. Plant Cell **20:** 471–481

Wang QL, Li ZH (2007) The functions of microRNAs in plants. Front Biosci **12:** 3975–3982

Wang Y, Li P, Cao X, Wang X, Zhang A, Li X (2009) Identification and expression analysis of miRNAs from nitrogen-fixing soybean nodules. Biochem Biophys Res Commun **378:** 799–803

Wen J, Frickey T, Weiller GF (2008) Computational prediction of candidate miRNAs and their targets from Medicago truncatula non-protein-coding transcripts. In Silico Biol **8:** 291–306

Willmann MR, Poethig RS (2005) Time to grow up: the temporal role of small RNAs in plants. Curr Opin Plant Biol **8:** 548–552

Xie Z, Allen E, Fahlgren N, Calamar A, Givan SA, Carrington JC (2005) Expression of Arabidopsis MIRNA genes. Plant Physiol **138:** 2145–2154

Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. PLoS Biol **2:** E104

Yang WC, Katinakis P, Hendriks P, Smolders A, de Vries F, Spee J, van Kammen A, Bisseling T, Franssen H (1993) Characterization of GmENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development. Plant J **3:** 573–585

Yao Y, Guo G, Ni Z, Sunkar R, Du J, Zhu JK, Sun Q (2007) Cloning and characterization of microRNAs from wheat (Triticum aestivum L.). Genome Biol **8:** R96

Yu O, Matsuno M, Subramanian S (2006) Flavonoids in flowers: genetics and biochemistry. *In* JAT de Silva, ed, Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues Vol 1. Global Science Books, Middlesex, UK, pp 283–293

Zhang B, Wang Q, Pan X (2007) MicroRNAs and their regulatory roles in animals and plants. J Cell Physiol **210:** 279–289

Zhou X, Sunkar R, Jin H, Zhu JK, Zhang W (2009) Genome-wide identification and analysis of small RNAs originated from natural antisense transcripts in Oryza sativa. Genome Res **19:** 70–78