MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs

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Legumes and many nonleguminous plants enter symbiotic interactions with microbes, and it is poorly understood how host plants respond to promote beneficial, symbiotic microbial interactions while suppressing those that are deleterious or pathogenic. Trans-acting siRNAs (tasiRNAs) negatively regulate target transcripts and are characterized by siRNAs spaced in 21-nucleotide (nt) “phased” intervals, a pattern formed by DICER-LIKE 4 (DCL4) processing. A search for phased siRNAs (phasiRNAs) found at least 114 Medicago loci, the majority of which were defense-related NB-LRR-encoding genes. We identified three highly abundant 22-nt microRNA (miRNA) families that target conserved domains in these NB-LRRs and trigger the production of trans-acting siRNAs. High levels of small RNAs were matched to >60% of all ~540 encoded Medicago NB-LRRs; in the potato, a model for mycorrhizal interactions, phasiRNAs were also produced from NB-LRRs. DCL2 and SGS3 transcripts were also cleaved by these 22-nt miRNAs, generating phasiRNAs, suggesting synchronization between silencing and pathogen defense pathways. In addition, a new example of apparent “two-hit” phasiRNA processing was identified. Our data reveal complex tasiRNA-based regulation of NB-LRRs that potentially evolved to facilitate symbiotic interactions and demonstrate miRNAs as master regulators of a large gene family via the targeting of highly conserved, protein-coding motifs, a new paradigm for miRNA function.

[Keywords: microRNA; tasiRNA; NB-LRR; symbiosis; Medicago]

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Legume species are agronomically important crops that are rich sources of human dietary protein that also develop unique nitrogen-fixing nodules through a symbiotic relationship with microbes. Root nodules house symbiotic bacteria [rhizobia] that convert atmospheric dinitrogen to ammonia using the energy of the host’s photosynthate. During establishment of the symbiosis, rhizobia invade the legume root via a plant-derived infection thread and are ultimately released into the host cytoplasm as membrane-bound facultative organelles called “bacteroids,” which are the differentiated nitrogen-fixing form or rhizobia. While beneficial interactions with mycorrhiza are known for most plant species [with exceptions that include Arabidopsis], the symbiotic relationships that legume species have with bacteria are highly evolved and require many components related to plant pathogenesis (Deakin and Broughton 2009). Thus, nodulation may require the suppression of host defenses to prevent immune responses; for example, the classically defined, allelic Rj2 and Rfg1 loci from soybeans restrict nodulation with specific rhizobial strains and encode a TIR-NB-LRR [TNL] protein (Yang et al. 2010).
The only known function of plant NB-LRR proteins is in microbial recognition as activators of defense responses (Eitas and Dangl 2010). The hundreds of diverse NB-LRRs encoded in plant genomes comprise an innate immune system that allows recognition of many pathogens (Meyers et al. 2005).

In the last 10 years, the functions of small RNAs in plants have been extensively explored. These molecules in their mature form are generally 20–24 nucleotides (nt) and are produced by several genetically separable pathways. Plant microRNAs (miRNAs) are typically 21 or 22 nt and function in a post-transcriptional manner by down-regulating target gene products involved in a variety of cellular processes (Bartel 2004; Jones-Rhoades et al. 2006; Mallory and Vaucheret 2006). Another major class of small RNAs are heterochromatic siRNAs (hc-siRNAs) that suppress the activities of transposable elements and maintain genome stability via DNA methylation and chromatin modifications (Vaucheret 2006). Trans-acting siRNAs (tasiRNAs) are a third class of plant small RNAs. Their formation is dependent on miRNA triggers (Allen et al. 2005; Yoshikawa et al. 2005) and requires either the so-called “two-hit” model of dual miRNA target sites in the noncoding RNA precursor (Axtell et al. 2006) or “one-hit” [single-target site] by 22-nt miRNAs [Chen et al. 2010; Cuperus et al. 2010]. Four families comprising eight tasiRNA loci have been described in Arabidopsis, while hundreds of noncoding loci of unknown function generate phased small RNA in grasses (Johnson et al. 2009; The International Brachypodium Initiative 2010). The noncoding TAS3 gene is broadly conserved in seed plants (Axtell et al. 2006). Here we report that the model legume Medicago encodes and produces a much more expansive number of phased siRNA [phasiRNAs] and that these are predominantly associated with regulation of numerous and diverse members of the NB-LRR class of disease resistance genes. We demonstrate a substantial network of miRNAs and resulting phasiRNAs that target NB-LRR genes, and we propose that the suppression of the small RNA silencing system and disease resistance machinery may play a role in plant–microbe interactions. While most of these findings were made in Medicago, we present evidence indicating that parallels exist in other legumes and nonleguminous plant species. These data demonstrate that a small number of miRNAs function as master regulators of arguably the largest gene families found in plant genomes.

Results

MiRNA identification in Medicago truncatula and soybeans

Although numerous miRNAs have been identified from legume species, the availability of complete genome sequences provides an opportunity for identification of poorly conserved or other novel miRNAs. In a recent release of miRBase [version 16], a total of 383 miRNA genes have been annotated in M. truncatula, 203 have been annotated in Glycine max (soybean), and many fewer have been annotated in Arachis hypogaea (peanut) and Phaseolus vulgaris (common bean). We believed that miRNA identification is not yet saturated in these species. Therefore, we used a larger set of libraries and tissues, combined with the more complete genome sequences of M. truncatula and soybeans, comparative genomics methods, a powerful new miRNA prediction pipeline, and large-scale validation of target cleavage to identify new legume miRNAs, phased or trans-acting-like small RNAs, and hc-siRNAs. Twenty-one small RNA libraries were made from tissues of four legumes, including M. truncatula, soybeans, peanuts, and common beans (Supplemental Table S1). These libraries included ~62 million small RNA reads. Given the absence of peanut and common bean genomes, we used those species’ data for comparative analysis and focused on the M. truncatula and soybean data.

Using the M. truncatula libraries, we applied aninformatics pipeline for filtering plant miRNAs from the complete set of small RNAs. Our input to this pipeline was 9,282,720 distinct small RNAs sequenced from eight M. truncatula libraries (Supplemental Fig. S1); among these sequences were 98 different, known, mature M. truncatula miRNA sequences of the 383 annotated in miRBase version 16. The other 285 annotated miRNAs not sequenced may be weakly expressed in the tissues we sampled or are not real miRNAs. After passing the data through the pipeline [see the Supplemental Material], 90 M. truncatula miRNA candidates remained, generated from 137 precursors, which were compared against miRBase version 16 to identify high-similarity homologs. Excluding 26 M. truncatula miRNAs that were previously annotated, 22 sequences were found with >85% similarity to known plant miRNAs, leaving 42 new miRNA candidates from 51 precursors [Supplemental Table S2]. A similar strategy for the soybean genome and small RNA libraries started from 6,135,687 distinct small RNAs. Our input to this pipeline was 9,282,720 distinct small RNA reads. Given the absence of peanut and common bean genomes, we used those species’ data for comparative analysis and focused on the M. truncatula and soybean data.

We noticed that many M. truncatula and soybean miRNA sequences were 22 nt in length, some of which were quite abundant. Recent reports show that 22-nt miRNAs are necessary and sufficient to trigger phasing at tasiRNA loci [Chen et al. 2010; Cuperus et al. 2010]. In our M. truncatula data, the 22-nt miR1507, miR1509, miR2109, miR2118, and miR2597 were highly abundant [Supplemental Table S2]. Several of these mature miRNAs previously were annotated with sizes other than 22 nt, and, based on our libraries, we believe those earlier size annotations are incorrect. For example, in miRBase, miR2109 is annotated as 20 nt, miR2597 is annotated as 21 nt, and miR1509 is annotated as 21 nt. In M. truncatula, we predicted eight new 22-nt miRNAs [Supplemental Table S2C], plus we found 22-nt variants of previously described 21-nt miRNAs (miR156 and miR169), and we found 22-mers that passed our miRNA filters, corresponding to the miRNA* positions of conserved miRNAs [two copies of miR169*, plus miR398*] [Supplemental Table S2B]. This abundance of 22-nt miRNAs is in

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contrast to Arabidopsis, which has few 22-nt mature miRNAs annotated in miRBase, most of which occur at low abundances: miR173 [targeting TAS1 and TAS2], miR393, miR472, and miR828 [targeting TAS4]. The soybean genome included at least 28 loci producing 22-nt mature miRNAs [Supplemental Table S3]. miR1507, miR1509, and miR2118 are highly abundant 22-nt miRNAs in both M. truncatula and soybeans [Supplemental Tables S4,S5]. Three differences of abundant 22-nt miRNAs were observed in the M. truncatula–soybean comparison: miR2597 was abundant in M. truncatula and not found in soybeans, miR1512 was abundant in soybeans but not found in M. truncatula, and miR2109 was abundant in both species but was 22 nt in M. truncatula and predominantly 21 nt in soybeans. The conservation and expression of these 22-nt miRNAs in peanuts and common beans are described below. We concluded that 22-nt miRNAs are both numerous and expressed abundantly in legumes.

Identification and validation of M. truncatula miRNA targets

We predicted potential miRNA targets and integrated the matches with empirical cleaved miRNA data to identify valid miRNA targets. The 90 unique M. truncatula miRNA candidate sequences from 137 precursors that passed our filters were searched against both genome and cDNA sequences. Predicted matches with penalty scores ≤5 [>50,000 predicted miRNA–target pairs in the genome, and >15,000 pairs from cDNAs] were combined with a PARE [parallel analysis of RNA ends] library [German et al. 2008] made from M. truncatula flower tissue [Supplemental Table S1]. The observation of several conserved miRNA–target pairs [miR156–SPL, miR172–AP2, miR167–ARF8, and miR390–TAS3] suggested that library quality was sufficient for validation of miRNA targets; these targets exhibited precise, high-abundance cleavage products at the predicted target sites [Supplemental Fig. S2].

To detect novel miRNA targets in M. truncatula, we used several stringent filters for the PARE data [see the Materials and Methods]. We confirmed 144 cleavage sites from 89 genes and 30 intergenic regions, targeted by 46 different miRNAs [Supplemental Table S4A]. These targets include a broad set of genes not previously known to be targets of miRNAs. While most target genes were cleaved by only one miRNA at a single recognition site, we identified two target sites for miR1509 in Medtr7g012810; this is significant because in the Arabidopsis TAS3 gene, two “hits” by the 21-nt miR390 are required to trigger the production of tasiRNAs [Axtell et al. 2006]. The combination of the stringent filters to identify the miRNA candidates and the conservative methods of experimental validation with the PARE data allowed us to confirm 49 functionally validated M. truncatula miRNAs in flower tissue, and thus these were added to miRBase. We also propose to correct several M. truncatula miRNAs that we believe were annotated previously with incorrect sizes or mature sequences.

Identification of tasiRNA-like phasiRNA loci in M. truncatula and soybeans

The identification of two target sites for miR1509 in Medtr7g012810 led us to ask how many M. truncatula miRNAs might trigger tasiRNAs. The eight Arabidopsis "TAS" genes generate miRNA-triggered secondary siRNAs in a 21-nt “phased” pattern (Howell et al. 2007). We previously refined and applied a computational approach to evaluate the phasing pattern of small RNAs [De Paoli et al. 2009]. The small RNAs identified from this algorithm are phased but do not necessarily function in trans [or even in cis], therefore, we call these phased siRNAs “phasiRNAs.” Since tasiRNA-generating loci are TAS genes, we propose that phasiRNA-generating loci are PHAS genes.

We applied this same algorithm to the M. truncatula and soybean genomes for our libraries. Using Arabidopsis as a control, all loci with a phasing score ≥15 in any one library are considered above background [summarized in Supplemental Table S5]. PhasiRNAs with only 21-nt intervals were identified from a large number of loci [after removing false positives] [see the Materials and Methods]. This set included 112 genes and two intergenic regions in M. truncatula [examples in Fig. 1; Supplemental Table S6] and 26 genes and 15 intergenic regions in soybeans [Supplemental Table S7]. MtTAS3 is the only M. truncatula phased locus that has been described previously [Jagadeeswaran et al. 2009], and the remainder are novel PHAS loci that have not been previously described in other species.

We integrated our miRNA lists, target prediction, and the PARE data to identify the triggers for the M. truncatula PHAS loci. We were able to identify the miRNA triggers for most PHAS loci [summarized in Table 1, Supplemental Table S6]. At least 77 of the 114 M. truncatula PHAS loci (~68%) are triggered via single cleavage of a 22-nt miRNA trigger, we call this “1,2,” for a single-target, 22-nt miRNA trigger event. The majority of these PHAS loci were triggered by a few high-abundance 22-nt miRNAs [miR1507, miR1509, miR2109, and miR2118a/b/c]. There were just a few exceptions to the predominance of 1,2 PHAS loci. We also identified a novel two-hit [2,1] PHAS locus [the second known example, in addition to TAS3], an AP2-like gene [Medtr2g093060]. Consistent with the two-hit model, Medtr2g093060 includes a conserved, cleaved miR172 target site [Aukerman and Sakai 2003], plus a predicted noncleaving and highly degenerate miR156 target site [Fig. 2A]. While the miR156 target site has extensive mismatches and a poor score, no other target sites were identified in the upstream region for any other miRNA. The miR172 upstream direction of the phasiRNAs and the validation of the cleaved site in the PARE data resemble TAS3, consistent with the two-hit model of phasiRNA biogenesis from this transcript. Two other related miR172 targets identified in the M. truncatula genome [Medtr7g100590 and Medtr4g061200] do not have this miR156 site and thus showed no evidence of phasiRNAs. Soybean orthologs of Medtr2g093060 [Glyma13g40470 and Glyma15g04930, two due to genome duplications]
Figure 1. Twenty-two-nucleotide miRNAs trigger phased siRNA production in *M. truncatula*. Above are alignments of well-conserved miRNAs and their targets; in the alignment, vertical lines indicate matches, missing lines indicate mismatches, and GU wobble pairs are indicated with a circle. Black arrowheads above the target sequence indicate the cleavage site in the target, and the numbers above separated by the backslash indicate the number of PARE reads in the small window \( W_s \) (first number) and the number of reads in the large window \( W_l \) (second number), as described in the Supplemental Material. Below the alignments are small RNA abundances and phasing score distributions for the regions indicated by the gray trapezoids; abundances are normalized in TPM (transcripts per million). Below this are images from our Web site showing the *M. truncatula* flower small RNA data at each PHAS locus, with the red arrowhead pointing to the miRNA cleavage site. Colored spots are small RNAs with abundances indicated on the Y-axis; light-blue spots indicate 21-nt sRNAs, green spots are 22-nt sRNAs, orange spots are 24-nt sRNAs, and other colors are other sizes. Blue boxes on the bottom strand or red boxes on the top strand are annotated exons. Purple lines indicate a k-mer frequency for repeats; yellow, pink, or orange shading indicates DNA transposons, retrotransposons, or inverted repeats. (A) The new miRNA mtr-miR5754 targets a gene encoding a protein kinase; both the miRNA trigger and the phasiRNAs are specific to the flower library. (B) miR2118 targets a gene encoding a TNL. (C) miR1507 targets a gene encoding a CNL. (D) miR2109 targets a gene encoding a TNL.
both have conserved miR172 target sequences but lack any conservation of the untranslated region [UTR] that includes the miR156 site and showed no evidence of phasiRNA production, the miR156 target sequence was also not conserved in *Lotus japonicas* [data not shown]. This unique example of an AP2-like *M. truncatula* PHAS gene suggests that the spontaneous acquisition of a new miR156 or other yet-to-be-identified 5’ noncleaving target site is a recent evolutionary event. Another exception to the 122 PHAS loci that predominate was a 222 PHAS gene demonstrating double cleavage by a 22-nt miRNA [Fig. 2B; Medtr7g012810] is targeted by miR1509 at two cleaved sites [Supplemental Tables S6, S8]. Nearly all small RNAs were found between the two cleaved target sites, with a near absence of small RNAs 3’ of the poly-A-proximal target site [Fig. 2C]. Small RNAs generated immediately downstream from the 5’ cleavage site, as well as those immediately upstream of the 3’ cleavage site, were perfectly in phase with their respective cleavage sites, consistent with bidirectional processing of the central portion of this 222 PHAS locus. In summary, the *M. truncatula* small RNA and PARE data identified many 122 loci, two 221 loci, and one 223 PHAS locus.

Two genes known to be involved in small RNA biogenesis [DICER-LIKE [DCL2] and SUPPRESSOR OF GENE SILENCING3 [SGS3]] were also identified as PHAS genes [Supplemental Fig. S3]. The DCL2 trigger is predicted to be miR1507 in *M. truncatula* but miR1515 in soybeans [validated by Li et al. 2010], with the phasiRNAs initiating from different sites in the orthologs, consistent with different miRNA target sites [Supplemental Fig. S3A]. PhasiRNAs were identified from the soybean ortholog of SGS3 [Glyma05g33260 [GmSGS3]], but not the three paralogs of SGS3 in *M. truncatula* [data not shown]; GmSGS3 was previously validated as a target of a miR2118 family member [Song et al. 2011]. The recruitment of genes involved in phasiRNA biogenesis as sources of phasiRNAs suggests a feedback mechanism reminiscent of the regulation of *Arabidopsis AGO1* and *DCL1* by miR168 and miR162, respectively [Xie et al. 2003; Vaucheret et al. 2004, 2006].

We identified a novel, potentially noncoding, 122 PHAS locus in *M. truncatula* of particular interest because the phasiRNAs were the most abundant in our data set [10-fold higher than TAS3, the most abundant conserved TAS locus], the PARE reads on both strands, and the phasing score was extremely high [Supplemental Table S6; Supplemental Fig. S4]. This intergenic region on Chr. 2 has very weak similarity to the PPR family of genes, suggestive of the *Arabidopsis TAS2* locus targets PRP genes [Montgomery et al. 2008b]. TAS2 is triggered by miR173, but the trigger of this *M. truncatula* PHAS gene is a novel 22-nt miRNA candidate at moderate abundances in all of our *M. truncatula* libraries but with no known genomic origin [perhaps due to gaps in the genome]. The noncoding precursor and tasiRNAs that initiate cleavage on numerous targets [Supplemental Table S7] are reminiscent of TAS genes; we named this locus PHAS_IGR1. The abundant PARE reads from both strands [Supplemental Fig. S4] are not predicted for RDR6 products, since PARE reads are derived from poly-A mRNA. Another unusual aspect of this locus is the evidence of cleavage by siRNAs acting in cis, also previously reported at TAS3 in which the −D2 siRNA cleaves the primary TAS3 transcript out of phase and without producing secondary siRNAs [Allen et al. 2005; Jagadeeswaran et al. 2009]. The cis-targeting siRNAs might serve as a negative feedback loop in overall phasiRNA production.

To systematically determine whether the *M. truncatula* phasiRNAs function in cleavage, we again examined the PARE data. By definition, tasiRNAs should function to direct cleavage [or silencing] at second sites [e.g., in trans], although data suggest some may function in cis [Allen et al. 2005]. Using the top five phasiRNAs by abundance from each of the 114 PHAS loci and integrating genomic target predictions and our PARE data, a stringent cutoff [see the Materials and Methods] identified ~2000 sites whose cleavage is guided by these 570 phasiRNAs, including numerous cases of cis regulation [Supplemental Table S8]. Therefore, we identified many verifiable trans- and cis-acting siRNAs produced from the large number of legume PHAS loci.

**Table 1.** *M. truncatula* PHAS loci triggered by legume miRNAs

| miRNA     | Length [nt] | Targeted gene family | Number of PHAS loci in *Medicago* |
|-----------|-------------|----------------------|-----------------------------------|
| miR390    | 21          | TAS3                 | 1                                 |
| miR172    | 21          | AP2-like             | 1                                 |
| miR5754   | 22          | Predicted protein kinase | 1                           |
| miR2109   | 22          | NB-LRRs               | 9                                 |
| miR2118a/b/c | 22          | NB-LRRs, Gma-SGS3, etc. | 32                           |
| miR1507   | 22          | NB-LRRs, Mrx-DCL2    | 14                                |
| miR1509   | 22          | Predicted transcription factor | 4                           |
| miR1515   | 22          | Gma-DCL2             | —                                 |

Twenty-two-nucleotide miRNAs as master regulators of legume NB-LRR-encoding genes and generators of phasiRNAs

A feature of the legume phasiRNA loci was the preponderance of NB-LRR-encoding genes, including 79 of 112 *M. truncatula* phasiRNA loci. We call these genes phasi-NB-LRRs, or pNLs. We found that just three 22-nt miRNA families [miR1507, miR2109, and miR2118] are responsible for the initiation of the phasiRNAs at 79 of the 79 pNLs [Fig. 3A; Supplemental Table S6]. miR1507 “specializes” in targeting *CC-NB-LRR* (CNL) genes, with strong complementarity to the encoded kinase-2 motif, centered near a highly conserved tryptophan [W] [Fig. 3A]. miR2109 targets the *TNL* class, matching the encoded TIR-1 motif of the TIR domain [described in Meyers et al. 1999]. The three-member miR2118 family [miR2118a/b/c; miR2118c is renamed from miR2089] targets sequences encoding the most well-conserved NB-LRR motif, the P-loop [Fig. 3A; Meyers et al. 1999; miR2118a and miR2118c preferentially target *TNL* genes, while miR2118b almost
exclusively targets CNL genes (Supplemental Table S6). Thus, a specialized group of miRNAs targets conserved domains of NB-LRRs in legumes.

Next, we asked whether the pNLs represent a single, distinct clade within the broader phylogenetic group of NB-LRRs. We mapped the pNLs and their miRNA triggers onto cladograms of the TNL and CNL groups from *M. truncatula* (Fig. 3B, Supplemental Fig. S5). pNLs were found to be widely distributed across both the CNL and TNL groups. The triggers for the pNLs were similarly distributed, with no apparent pattern or grouping in the tree; this was especially evident in the TNL group (Fig. 3B). With many small RNAs (>50 TPM [transcripts per million]) matched to >60% of the ~540 NB-LRRs in the
assembled Medicago genome (Fig. 3B; Supplemental Fig. S5A; Supplemental Table S9), there may be many more pNLs. An analysis of all NB-LRR-associated small RNAs demonstrated that they were predominantly 21-mers, and the majority of NB-LRRs have more 21-nt small RNAs than any other size class (Supplemental Fig. S5B,C). This indicates that the silencing of NB-LRRs by phased small RNAs is a phenomenon that occurs broadly across the gene family via a small number of miRNA triggers, each of which is effective against distantly related members of the family. These unique attributes are the result of the targeting of sequences encoding a conserved protein motif by a miRNA, an activity that has not been previously described but may provide flexibility yet rigidity to target a large number of diverse yet related genes.

We next searched for evidence of soybean pNLs, identifying 13 pNL loci. Only 13 of 41 soybean PHAS loci are pNLs (Supplemental Table S7). Glyma16g33780 produces limited phased 21-nt siRNAs; this is the Rj2/Rfg1 allelic pair, a TNL of known function that defines rhizobial host specificity (Yang et al. 2010). PhasiRNAs from this gene and many pNLs match more than one gene; those from Rj2/Rfg1 averaged more than four matching genomic loci. This interconnectivity between pNLs would facilitate an extensive and coordinated regulatory network. In the absence of soybean PARE data, we assessed whether the soybean pNL triggers were related to the M. truncatula pNL triggers. The highly expressed M. truncatula miR1507 is well conserved in soybeans and is predicted to initiate pNLs (data not shown). The M. truncatula 22-nt miR2109 is predominantly 21 nt in soybeans, suggesting that it may not initiate soybean pNLs (vs. triggering ≥14 pNLs in M. truncatula), consistent with fewer pNLs in soybeans. In soybeans, the miR2118 family is slightly more complicated, because this miRNA is multicopy and is the star sequence to miR482 (J Zhai and BC Meyers, unpubl.). We checked to see whether the M. truncatula and soybean pNLs are found in syntenic locations indicative of an origin early in legume evolution; only two pairs were found in syntenic blocks (Mt-TAS3:Gma-TAS3a and Mt-TAS3:Gma-TAS3b) and no pNLs were syntenic (Supplemental Table S10). This finding and the broad phylogenetic distribution of miRNA targets and pNLs suggest a dynamic nature to the subset of NB-LRRs that are pNLs.

Figure 3. miRNAs trigger phasiRNAs from diverse NB-LRR genes by targeting conserved motifs. (A) Three 22-nt miRNA families, as indicated in the key, trigger the production of phasiRNAs from NB-LRR-encoding genes; miR2118a/b/c are relatively dissimilar members of one family, so all three are shown. On the left and right are illustrations of TNL and CNL proteins, with domains and motifs as indicated in the key at the bottom. Example alignments of target sites for specific miRNAs and motif-encoding regions are indicated in the gray boxes. Red amino acids indicate the core of the conserved motif. (B) An unrooted cladogram was constructed of TNL protein sequences from Medicago. A similar cladogram for the CNLs is found in Supplemental Figure S5. If the gene encoding that protein produces phased small RNAs, the identifying name in the tree was left intact (e.g., Medtr4g050410), the identifier for genes not generating phased small RNAs was replaced by a small integer (e.g., 115). Red dots denote genes with small RNAs exceeding 50 TPM in their summed, hit-normalized abundances. Gray dots denote NB-LRRs from the unassembled Medicago genomic contigs; these were not used in our small RNA analysis. Targets of miRNAs described in the text are marked using symbols as indicated in the key. The miR2118 family is similar enough that it is possible that more than one of these miRNAs targets the same gene, possibly confounding some of our predictions of which miR2118 family member is the trigger. Curly braces group genes targeted by the same miRNA.
Evolutionary conservation of 22-nt miRNA families from legumes

We examined sequence conservation among 30 diverse and agronomically relevant plant species, including lower plants and basal angiosperms, for homologs of the six most-abundant and PHAS targeting 22-nt legume miRNA families: miR1507, miR1509, miR1510, miR1515, miR2109, and miR2118. The common bean and peanut data demonstrated that most of the 22-nt miRNAs were highly abundant within legumes (Fig. 4; Supplemental Fig. S6). Comparisons across the larger set of species showed these miRNAs present at low abundances (<10 TPM) (yellow in Fig. 4) in many species; those levels are low enough that the precursors and expression will need to be verified when the genomes become available. Analysis of the grape, maize, and potato genomes showed that some but not all of these small RNAs come from predicted miRNA-like hairpins (data not shown). At a more robust threshold of abundance of 11–100 TPM, we found that miR1507, miR1509, miR1515, and miR2118 are present in nonleguminous species (Fig. 4). miR1507 is highly abundant in grapes and avocados, and miR2118 was broadly represented and relatively abundant (≥100 TPM) outside of legumes, including moderate signals in all four libraries of the Ginko (GBI in Fig. 4) and Norway spruce (PAB in Fig. 4). This presence in two gymnosperms that date back >250 million years suggests that these phased siRNAs may represent an ancient regulatory mechanism.

miR2118 was also abundant in potatoes (Fig. 4), and the potato genome was recently sequenced (Xu et al. 2011), so we examined whether this genome also contains many phasiRNAs or even pNLs. At the same cutoff used previously (≥15), the three potato small RNA libraries identified 36, 33, and 43 PHAS loci (Supplemental Table S5). Examination of a subset of these phased loci identified numerous pNLs (Supplemental Fig. S7A). Many of these phasiRNAs also matched to pNLs in grapes (http://www.intl-pag.org/19/abstracts/W84_PAGXIX_S25.html), these data suggest that 22-nt pNL-targeting miRNAs evolved early in plants and that pNLs are found in nonleguminous species.

Discussion

We identified a large number of novel miRNAs, and the analysis of these miRNAs and their targets has substantially expanded our understanding of small RNA biology in plants. The extensive network of phasiRNAs is apparently absent in Arabidopsis, yet we believe that the implications of our data go beyond legumes, as we also demonstrated the presence of many PHAS loci and their miRNA triggers in other plants.

Legume miRNAs have evolved in unique ways

We characterized novel miRNAs in legumes and identified and validated novel legume targets. By integrating PARE data with small RNA data and novel bioinformatics analyses, we identified 42 new miRNA candidates from 51 precursors in M. truncatula and 40 new miRNA candidates from 45 precursors for soybeans. Our analysis demonstrated that both the M. truncatula and soybean genomes encode a larger set of 22-nt miRNAs than any plant genome described to date. This size class of miRNAs has an innate ability to trigger phased small RNA cascades...
in plants (Chen et al. 2010; Cuperus et al. 2010). The 22-nt miRNAs are produced from at least 21 loci in the M. truncatula genome and 28 loci in the soybean genome, whereas Arabidopsis generates just a few known 22-nt mature miRNAs (Cuperus et al. 2010), most of which are weakly expressed. Many of these legume 22-nt miRNAs are highly abundant in the tissues that we characterized. Many of the new 22-nt miRNAs we identified have no or few validated target sites in this set; it is possible that these function to trigger phased miRNAs in tissues we did not examine. The profusion in these legume genomes of 22-nt miRNAs and the phased miRNAs that they initiate suggests that the tasiRNA pathway is more broadly useful as a genetic regulatory circuit, raising the question of whether the relatively low number of phased loci in Arabidopsis is a general feature of plant genomes or is exceptional. This can be addressed by the analysis of phased small RNAs across the increasingly large number of sequenced plant genomes.

Legume phasiRNAs and the general ‘rules’ for their biogenesis in plants

We identified a large number of loci that fit with the original two-hit (21-nt trigger, or “211”) or single-hit (22-nt trigger, or “121”) models for tasiRNA biogenesis (for review, see Allen and Howell 2010); the diversity of phased miRNAs described in our study has implications for understanding the general properties of their biogenesis. For example, we identified a new “two-hit” plant PHAS locus (Fig. 2A), demonstrating that this pathway is not unique to TAS3, a well-conserved but heretofore unique plant developmental regulatory circuit. In fact, evidence of phased siRNAs generated from multiple target sites in a transcript have been reported for the PPR-encoding targets of TAS2 siRNAs and miR161 (Howell et al. 2007; Chen et al. 2010). In our data, the paired configuration of two 21-nt miRNA target sites (cleaving [miR172] and noncleaving [miR156]) is similar to TAS3. Like TAS3, the mRNA fragment 5’ of the miRNA cleavage site in the AP2-like transcript is converted into phased small RNAs, confirming the distinct 5’ directonality of processing under the two-hit model (Axtell et al. 2006). Like miR390, miR172 falls into the minority of conserved plant miRNAs that lack a 5’ U, since the 5’-terminal nucleotide is important for sorting miRNAs and loading onto different Argonaute proteins [Mi et al. 2008], it is possible that this characteristic is functionally important for the two-hit triggers. Montgomery et al. (2008a) demonstrated that miR390 is selectively bound by AGO7, and this AGO7–miR390 complex is required at the noncleaving TAS3 target site for tasiRNA biogenesis; they inferred that AGO7 binding may be a requirement for the two-hit tasiRNA pathway. In contrast, miR156, the noncleaving trigger in the M. truncatula AP2-like RNA, is known to be bound by AGO1 in Arabidopsis, suggesting that AGO7 loading may not be necessary for two-hit biogenesis of tasiRNAs or that miR156 is bound by AGO7 in M. truncatula.

Our work has expanded our understanding of tasiRNA triggers. This diversity of phasiRNA loci suggests a need for a better organizational scheme to describe these secondary siRNAs. Here we propose to use the term “phasiRNA,” as we believe that a tasiRNA cannot be called such without evidence of targeting activity in trans. There is evidence of cis activity in TAS3, and this functional self-targeting small RNA is conserved in Arabidopsis and M. truncatula (Allen et al. 2005; Jagadeeswaran et al. 2009); phasiRNAs targeting their source locus like this could be “casiRNAs.” We summarized these classes of phasiRNAs and their precursors in Figure 5A.

In both the two-hit model and a single-hit model for tasiRNA biogenesis, cleavage occurs at only one site; the two “hits” of the two-hit model include one uncleaved target site [Axtell et al. 2006; for review, see Allen and Howell 2010]. In our study, a new class was represented by a 222 PHAS gene. PhasiRNAs at Medr7g012810 are triggered after double cleavage by a 22-nt miRNA. Because 22-nt miRNAs trigger phasiRNA production in the poly-A-proximal fragment of PHAS transcripts, and because we observed high-abundance small RNAs between the two target sites [Fig. 2B,C] yet in phase with both the 5’ cleavage site (downstream phased) and the 3’ cleavage site (upstream phased), we infer a synergistic effect of having two adjacent 22-nt target sites. This suggests that a 222 cleavage product is processed inwardly from the two cleavage sites in both the cap- and poly-A-proximal directions, consistent with the directionality of processing for both the single- and two-hit models [Allen and Howell 2010]. One alternative explanation for the phasiRNAs at this locus is that the two target sites function independently, whereby we might have expected similar levels of small RNA 3’ of both target sites; instead, almost no small RNAs were found 3’ of the 3’-most target site [brown box in Fig. 2C], suggesting that this 22-nt target site does not function as an independent 121 site. Based on the conservation of mir1509 across legumes, 222-based PHAS genes are likely moderately conserved.

Taken together, these results allow us to refine earlier models for phasiRNA biogenesis. Prior work demonstrated that phasiRNA production is dependent on either 221 or 122 targeting, resulting in either cap- or poly-A-proximal processing (Fig. 5B; Allen et al. 2005; Axtell et al. 2006; Montgomery et al. 2008b; Chen et al. 2010; Cuperus et al. 2010). We summarized the loci that exemplify these cases in Figure 5C. Experiments have demonstrated that the noncleaving site both is conserved in flowering plants [Axtell et al. 2006] and cannot be replaced by a cleaving site in the 221 model [Montgomery et al. 2008a; Felippes and Weigel 2009], although double cleavage has been observed in mosses (Axtell et al. 2006). We showed that 222 loci are processed into phasiRNAs almost exclusively between two cleaved target sites [Fig. 2B,C]. These data suggest two points: First, the two-hit 222 pathway is epistatic to the hypostatic single-hit 122 mode of processing; and second, double cleavage can strictly delimit the boundaries of phasiRNA production. Although we do not know the directionality of the processing, if the 22-nt miRNAs trigger 222 phasiRNAs, as in the 122 PHAS transcripts, processing may occur from both cleaved ends toward the center of the double-cleaved transcript.
from at least 79 *M. truncatula* pNL genes. The 22-mer pNL trigger miR2109 in *M. truncatula* is mostly 21 nt in soybeans; size diversification by altering the proportion of 21-nt versus 22-nt variants of a miRNA may allow flexibility in the degree of silencing of target genes due to differences in their ability to trigger phased miRNAs that amplify post-transcriptional silencing. The larger family of plant NB-LRR targeting miRNAs includes the 22-nt miR472, which was first identified in poplar (S Lu et al. 2007) and later found in Arabidopsis (called miR772 at that time) (Lu et al. 2006), in which it was demonstrated to target and cleave NB-LRR transcripts at P-loop-encoding regions. Based on a simple sequence comparison, both miR472 and miR1510* are closely related to miR482 and miR2118. The 21-nt miR1510 annotated in soybeans and abundant in our *M. truncatula* libraries is also predicted to target NB-LRR-encoding transcripts (Valdes-Lopez et al. 2010). Most plant miRNAs target many fewer genes than the number of targets of the pNL triggers; this suggests that the pNL-triggering phased miRNAs are also quite unusual because they target conserved motifs, regulating an extensive gene family. In the 1990s, degenerate oligos were widely used to amplify large sets of NB-LRRs (Michelmore 1996); it now appears that nature beat scientists to the punch, taking advantage of the “degeneracy” of miRNA–target interactions to broadly interact with the NB-LRR gene family.

pNL triggering may evolve rapidly. The pNLs are distributed throughout the CNL and TNL families of *M. truncatula* and are well represented in soybeans. We found no evidence for synteny among pNLs in these two legume genomes. One interpretation of the lack of synteny between the *M. truncatula* and soybean pNLs is that miRNA target sites may be gained or lost with relative ease by substitutions in the few nucleotides for which changes would not disrupt the protein motif but would disrupt the miRNA–mRNA interaction. While deeper sequencing may yet identify paralogous legume pNLs, the pNL subset of NB-LRRs may evolve relatively quickly depending on microbial selection pressures.

Is this phenomenon of pNLs specific to the legumes? In Arabidopsis, only one NB-LRR, a TNL, was identified as generating phased miRNAs (Howell et al. 2007), our reanalysis of this locus with much deeper data did not confirm this, yet there are many 21-nt siRNAs at this locus (J Zhai and BC Meyers, unpubl.) reported to be RDR6-dependent (Howell et al. 2007). Klevebring et al. (2009) described phased miRNAs associated with a small number of NB-LRRs in poplars, but the triggers were not identified. Our cross-species analysis of the pNL-triggering phased miRNAs demonstrated that at least the miR2118 family (including miR472, miR482, and miR2089) is well conserved in many plant species. miR2118 is particularly interesting because it also triggers phased miRNAs from intergenic regions in rice panicles (Johnson et al. 2009); these are noncoding loci that have no apparent relationship to the miR2118 pNL targets in legumes that we have described. pNLs potentially function to coregulate en masse the NB-LRR family in legumes. Thus, the small set of pNL triggers could function as master regulators of genes that are the first line of plant defense against many pathogens.

**NB-LRRs are targets of an extensive small RNA regulatory network**

Our study demonstrated that NB-LRRs are targeted by multiple, independent miRNA families, and each of these miRNAs targets a region encoding highly conserved protein motifs. At least three miRNA families in legumes are predicted to target transcripts from hundreds of NB-LRR-encoding genes, and phased small RNAs are generated from at least 79 *M. truncatula* pNL genes. The 22-mer pNL trigger miR2109 in *M. truncatula* is mostly 21 nt in soybeans; size diversification by altering the proportion of 21-nt versus 22-nt variants of a miRNA may allow flexibility in the degree of silencing of target genes due to differences in their ability to trigger phased miRNAs that amplify post-transcriptional silencing. The larger family of plant NB-LRR targeting miRNAs includes the 22-nt miR472, which was first identified in poplar (S Lu et al. 2007) and later found in Arabidopsis (called miR772 at that time) (Lu et al. 2006), in which it was demonstrated to target and cleave NB-LRR transcripts at P-loop-encoding regions. Based on a simple sequence comparison, both miR472 and miR1510* are closely related to miR482 and miR2118. The 21-nt miR1510 annotated in soybeans and abundant in our *M. truncatula* libraries is also predicted to target NB-LRR-encoding transcripts (Valdes-Lopez et al. 2010). Most plant miRNAs target many fewer genes than the number of targets of the pNL triggers; this suggests that the pNL-triggering phased miRNAs are also quite unusual because they target conserved motifs, regulating an extensive gene family. In the 1990s, degenerate oligos were widely used to amplify large sets of NB-LRRs (Michelmore 1996); it now appears that nature beat scientists to the punch, taking advantage of the “degeneracy” of miRNA–target interactions to broadly interact with the NB-LRR gene family.

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The extent of this system in legumes makes it tempting to speculate that this is a critical regulatory circuit that is important for symbiosis. There are data to support this idea; for example, overexpression of the 22-nt miR482 [a member of the miR2118 family] leads to hypernodulation in soybeans (Li et al. 2010). Li et al. (2010) also demonstrated that miR482 is up-regulated 6 d after inoculation, and the pNL trigger miR1507 is up-regulated in a hypernodulating soybean mutant. Differences in the utilization of pNLs between M. truncatula and soybeans could potentially reflect well-described differences in their nodulation processes. However, the finding that some of these pNL targeting miRNAs are conserved outside of legumes suggests hypotheses other than a role specific to nodulation; one related possibility would be in the global regulation of NB-LRR genes to promote mycorrhizal colonization, a symbiotic interaction in which Arabidopsis does not participate but that is common among legumes and nonleguminous plants and is particularly well studied in potatoes (Hata et al. 2010). This pNL mechanism could have been refined in legumes for rhizobial symbiosis.

Howell et al. (2007) described the role of phasiRNAs in a gene family (PPR-P) that shares some features with the NB-LRR gene family: It is large and dynamic, targeted by more than one miRNA, and may benefit evolutionarily from diversity in the gene family. Howell et al. (2007) suggest that the phasiRNAs could minimize the number of active PPR copies to suppress gene dosage. pNLs may be part of a similar regulatory system to constrain the number of active NB-LRRs. It has been known for many years that the TNL class of NB-LRRs is absent from grass genomes but is found in lower plants (Meyers et al. 1999). We believe that we have identified a phenomenon distinct from the phasiRNA-generating PPR genes because the pNL siRNAs are generated by direct targeting of large numbers of diverse genes by just a few miRNAs that target several distinct, highly conserved, protein motif-encoding sequences. One enduring question has been how an entire clade of genes could be lost or driven out of a genome. Curiously, the miR2118 family is conserved as a phasiRNA trigger between grasses and legumes, but in legumes it targets NB-LRRs and in grasses it targets noncoding RNAs found in intergenic regions that form clusters (Johnson et al. 2009). Perhaps the reason for the absence of the TNL class from grass genomes is because of phasiRNA suppression of the gene family followed by pseudogenization and, ultimately, neofunctionalization as regulatory noncoding RNAs in grasses, although no function is yet known for the grass phasiRNAs. Extending this hypothesis, perhaps the extensive set of noncoding phasiRNA precursors found in grass genomes is derived from the protein-coding TNL family as pseudogenized, extinct PHAS loci that are vestiges of the extant clusters of pNLs observed in the potato genome, for example (Supplemental Fig. S7), which resembles the clustered distribution of those noncoding phasiRNAs loci in grass, such a scenario has been described for the Xist noncoding RNA that functions in X-chromosome inactivation in animals (Duret et al. 2006).

Diverse pathways in legumes are subject to regulation by phased small RNAs

In addition to the pNL genes, we identified a number of protein-coding genes and miRNA triggers that generate phasiRNAs (Table 1). Among the more unusual is an AP2-like gene with both a miR156 and a conserved miR172 site; miR156 and miR172 are intimately involved in juvenile-to-adult-phase transition in Arabidopsis (Wang et al. 2009; Wu et al. 2009). However, in Arabidopsis, miR156 targets the SPL family upstream of the miR172-targeted AP2-like genes, and the levels of miR156 and miR172 are inversely proportional during maturation of the plant (Wu et al. 2009). The co-occurrence of these miRNA target sites in an AP2-like gene suggests a model in which the AP2 phasiRNAs functions in cis to positively regulate silencing of the AP2 gene (as casiRNAs), while also functioning in trans to target other AP2-like genes.

We also characterized phasiRNA biogenesis from genes important for small RNA biogenesis and pathogen defense. GmSGS3 is targeted by miR2118, the same miRNAs that target many NB-LRR genes. SGS3 has an important role in juvenile development (Peragine et al. 2004) and is critical in tasiRNA production (Elmayan et al. 2009). The silencing of both NB-LRR genes and SGS3 by miR2118 suggests a coupling of these regulatory events. DCL2 genes in both M. truncatula and soybeans independently acquired 22-nt miRNA target sites, resulting in phasiRNAs from this gene in both species (Supplemental Fig. S3A); interestingly, overexpression of miR1515, the soybean DCL2 trigger, was demonstrated to lead to hypernodulation (Li et al. 2010). Target analysis of the M. truncatula DCL2 suggests that the phasiRNA trigger is miR1507, the CNL-specific pNL trigger. Because DCL2 is important for small RNA biogenesis and SGS3 is important for tasiRNA biogenesis, these results are reminiscent of the DCL1–miR162 [Xie et al. 2003] or the AGO1–miR168 feedback loops [Vaucheret et al. 2004, 2006]. Since DCL2 and SGS3 are involved in both small RNA silencing and viral resistance (Mourrain et al. 2000), and we demonstrated a substantial network of miRNAs and resulting phasiRNAs that target NB-LRR genes, we propose that the suppression of the small RNA silencing system and disease resistance machinery may play a role in plant–microbe interactions. Therefore, our data are indicative of an extensive regulatory network controlling the transcript levels in these interactions. While most of these findings were made in M. truncatula, we have evidence indicating that parallels exist in other legumes and nonleguminous species, suggesting a small number of miRNAs function as master regulators of the largest gene families found in plant genomes.

Materials and methods

Plant materials

M. truncatula A17 (femalongo) For developing seeds, flowers from greenhouse-grown plants were date-tagged at full bloom, and pods were selected for seed collection at 20 d after anthesis. For seedlings, seeds were surface-sterilized and sown on water agar in the dark as in Catalano et al. [2004], with whole seedlings
Sequencing of small RNAs from legumes

Total RNA was isolated using Trizol reagents or plant RNA reagent, both from Invitrogen. *M. truncatula* small RNA libraries (except MTR01) were constructed and sequenced at Illumina; other libraries were constructed as previously described (C Lu et al. 2007) and sequenced on an Illumina GAIIx instrument at the Delaware Biotechnology Institute.

Twenty-one small RNA libraries were made from the materials described above, representing four legume species, including eight libraries from *Medicago*, seven from *G. max* (soybean), two from *A. hypogaea* (peanut), and four from *P. vulgaris* (common bean). Approximately 62 million small RNA sequences were obtained after removing adapters and low-quality reads, with trimmed lengths between 18 and 34 nt. After excluding small RNAs matching structural RNAs (tRNA and rRNA loci), 12.1 million and 12.6 million reads were mapped to the *Medicago* genome (*Mt3.5*) and soybean (*Gmax101*) genomes, we performed pairwise alignments with BLASTN with default parameters. Pairs of sequences with high similarity (identity $>60\%$ and aligned coverage $>500$ bp) were selected to check for synteny between *Medicago* and soybeans by comparing their locations to syntenic blocks between *Medicago* and soybeans detected by SyMAP (Soderlund et al. 2011). Because of the possibility that some small regions of colinearity may have been masked by a larger region, MUMMer (Kurtz et al. 2004) was used to align each pair of 1-Mb extended regions (500 kb on each side of each PHAS locus) in order to further identify sequences that are in the region of colinearity.

Phylogenetic analysis of the family of NB-LRR proteins encoded in the *M. truncatula* genome

We extracted predicted protein sequences for 312 TNLs and 385 CNLs from the *Medicago* genome sequence, including unassembled contigs. The unassembled contigs were not integrated into the genome, and thus were not used for phased siRNA or any small RNA analysis. A total of 542 NB-LRR proteins were encoded in the assembled *Medicago* genome and used for small RNA analysis; the subset of these NB-LRRs with a high level of small RNAs (≥50 TPM) is denoted in the cladograms with red dots. The remaining 155 NB-LRRs presumably map to gaps in the assembled genome sequence; these are denoted in the cladograms by gray dots.

The cladograms were constructed using maximum parsimony based on only the nucleotide-binding site of the NB-LRR proteins [the conserved NB-ARC domain]. Separate TNL class and CNL class trees were rooted with the nearest neighbor in the other class determined from a joint tree (data not shown).

**GenBank accession numbers**

The GenBank Gene Expression Omnibus (GEO) accession numbers for these data are GSE28755 for the small RNA data.
from 30 diverse plants (also found at http://smallrna.udel.edu) and GSE31061 for the legume small RNAs and PARE sequencing results. The legume data are also available at http://mpss.udel.edu/mt_sbs and http://mpss.udel.edu/soy_sbs.

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