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MicroRNA Metabolism in Plants

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Abstract

MicroRNAs (miRNAs) are 21- to 24-nucleotide (nt) RNAs that are the final products of nonprotein-coding genes. miRNAs are processed from single-stranded precursors that form hairpin structures, with the miRNAs residing in one arm of the stems. miRNAs were first isolated and recognized as regulators of protein-coding genes through forward genetic screens in *Caenorhabditis elegans*, but were not recognized as universal regulators of gene expression in animals until three landmark studies in year 2001 demonstrated the widespread existence of miRNAs in animals. Soon after, studies from a few groups identified a number of miRNAs from *Arabidopsis*, providing the first evidence for the existence of these regulatory molecules in plants. Since then, numerous miRNAs from a number of land plants ranging from mosses to flowering plants were identified, and functional studies in *Arabidopsis* established a framework of understanding of miRNA biogenesis and function. This chapter summarizes the current knowledge as well as gaps in our understanding of plant miRNA biogenesis and function.

1 MicroRNA Discovery in Plants

The discovery of microRNAs (miRNAs) in plants is still an ongoing process. Although much effort has been directed toward miRNA identification in *Arabidopsis* and rice, many economically or evolutionarily important species have yet to be examined, and more miRNAs likely remain to be discovered even in the species that have been extensively studied. One limiting factor in the miRNA discovery process is the availability of a sequenced genome, without which a comprehensive analysis of the potential precursor structures of cloned small RNAs is not possible. During the initial cloning of small RNAs from *Arabidopsis* and rice, it became clear that plants are extremely rich in endogenous small RNAs and that only a small portion of cloned small RNAs corresponds to miRNAs (Llave et al. 2002a; Mette et al. 2002). The majority of endogenous small RNA species represents small interfering RNAs (siRNAs). The only difference between miRNAs and siRNAs lies in their biogenesis. While an miRNA and its miRNA* are the main, if not only, sequences resulting from the processing of a single-stranded precursor that forms hairpin structures, siRNAs are generated from long double-stranded RNAs (dsRNAs) or single-stranded RNAs that form hairpin structures. In the cases where siRNAs are derived from a single-stranded hairpin precursor, usually multiple species from the same precursor are produced. Therefore, it is crucial that sequences that flank a cloned small RNA be available to allow the analysis of the potential precursor structure. It is also important that enough small RNAs have been found from a particular locus to distinguish an miRNA-generating locus from an siRNA-generating locus.

In *Arabidopsis*, three major methods have been used for miRNA discovery: forward genetics, direct cloning and sequencing, and bioinformatic prediction. Although forward genetic studies have only resulted in the identification of a few miRNAs, this method provides hints to the

functions of these miRNAs in addition to their isolation. For example, an early flowering mutant was isolated and the early flowering phenotype was caused by the overexpression of miR172 (Aukerman and Sakai 2003). Leaf wrinkling in a mutant was caused by the overexpression of miRJaw (Palatnik et al. 2003). In a genetic screen for floral patterning mutants, a mutant that produces extra petals in early flowers was isolated and the mutation was in one of the three members of the miR164 family (Baker et al. 2005). This loss-of-function mutant in one member of a small miRNA gene family demonstrates that the members of the gene family are not completely functionally redundant. A mutant that had extremely large and disorganized meristems was found to be due to the over-expression of miR166g (Williams et al. 2005). While a loss-of-function mutation in a *MIR* gene clearly indicates the function of this miRNA in the developmental process, the gain-of-function mutants resulting from overexpression of the miRNAs do not necessarily suggest a function of these miRNAs in the developmental processes but do indicate that proper control of the miRNA levels is important for the developmental processes.

A second approach to miRNA discovery was bioinformatic prediction. While a number of published studies employed several different algorithms to predict miRNAs, the features that the algorithms search for in the genomic sequences are based on our current knowledge of plant miRNAs and are largely similar among the studies (Bonnet et al. 2004; Jones-Rhoades and Bartel 2004; Wang et al. 2004; Adai et al. 2005). These features include the intergenic location of the *MIR* genes, the high degree of sequence complementarity of miRNAs to their mRNA targets, the hairpin structures of the precursors, and the conservation of some miRNAs between two species (*Arabidopsis* and rice). Most algorithms begin by extracting intergenic sequences, and then apply other filters such as conservation between rice and *Arabidopsis*, or complementarity to target mRNAs to further refine the prediction. Others start by extracting all sequence segments that have the potential to form hairpin RNAs, and then apply other filters such as conservation between rice and *Arabidopsis* or complementarity to mRNAs to further refine the search. One obvious drawback of the bioinformatic approach is that either the initial prediction phase or the subsequent refinement phase relies on sequence conservation, which makes it difficult to predict species-specific miRNAs.

A third, and perhaps the most effective, method for miRNA discovery was direct cloning and sequencing. In particular, deep sequencing of cloned small RNA libraries using massively parallel signature sequencing (MPSS) or pyrosequencing allowed the identification of numerous small RNAs from *Arabidopsis* and provided a picture of the genomic landscape of small RNAs (Lu et al. 2005, 2006; Axtell et al. 2006; Henderson et al. 2006; Rajagopalan et al. 2006; Fahlgren et al. 2007; Kasschau et al. 2007; Zhang et al. 2007). One added advantage of this approach is that most small RNA species from a particular genomic loci are exhibited, which helps discern whether the locus gives rise to an miRNA or multiple siRNAs. If multiple small RNAs mapping to both strands of a locus are present, these small RNAs are most likely siRNAs. Another advantage of deep sequencing is that the antisense strands to the small RNAs (also known as miRNA* or siRNA*) that are also released from the precursor during small RNA biogenesis are often detected, although at much lower frequency compared to the sense strands. This also helps identify the miRNAs from the small RNA populations since, by definition, the miRNA and the miRNA* should map to the same strand and should be separated by tens to hundreds of nucleotides.

2 MicroRNA Genes in Plants: Organization, Conservation, and Origin

As of June 2007, the miRBase (<http://microrna.sanger.ac.uk/sequences/index.shtml>) version 9.2 contains a total of 959 *MIR* genes from 10 plant species including moss, dicots, and monocots. Several features of plant *MIR* genes can be readily discerned from the current sets of miRNAs. First, plant *MIR* genes often have paralogs such that the 184 *Arabidopsis* miRNAs

in miRBase version 9.2 represent approximately 100 families of related miRNAs. *MIR* gene families arose from the process of gene duplication and diversification that also drives the evolution of protein-coding gene families (Maher et al. 2006). Second, each species has an evolutionarily fluid set of miRNAs. Some miRNA families, such as miR156, miR160, miR319, and miR390, appear to be of ancient origin such that they are conserved from mosses to flowering plants (Arazi et al. 2005; Axtell and Bartel 2005). Since the complete genome of a nonflowering land plant is not currently available, it is not possible to determine how many miRNA families are conserved among land plants through homology searches. Some miRNA families evolved after mosses and flowering plants diverged but before the divergence of monocots and dicots. Intriguingly, a large set of miRNA families is not shared among two of the three sequenced angiosperm genomes (*Arabidopsis*, poplar, and rice), suggesting that these miRNAs are evolutionarily “young” miRNAs. Among the known families of miRNAs in *Arabidopsis*, 4 are conserved down to mosses, 20 are shared between *Arabidopsis* and rice, while 22 are conserved between *Arabidopsis* and poplar. The remaining families are so far unique to *Arabidopsis*, but as the genomes of species closely related to *Arabidopsis* become available, some of these families may be found to be common to these related species. Consistent with the notion that the nonconserved miRNAs represent evolutionarily “young” miRNAs, these miRNAs are predominantly found at single loci in the genome. Third, the great majority of plant miRNA genes are located in intergenic regions, which is in contrast to animal miRNA genes that tend to be localized in introns or exons of protein-coding genes (reviewed in Kim 2005). Finally, unlike animal miRNA genes that are often found in clusters and that are transcribed into a polycistronic RNA (reviewed in Kim 2005), plant miRNA genes are usually not arranged in tandem in the genome or co-expressed. In the current set of *Arabidopsis* miRNAs, only three pairs of *MIR* genes (*MIR169i* and *MIR169j*; *MIR169k* and *MIR169l*; *MIR169m* and *MIR169n*) are arranged such that the two miRNAs are in the same orientation and are within 500 bp of each other. It is possible that each gene pair is co-transcribed.

The fact that *Arabidopsis* has many *MIR* gene families that are not found in poplar or rice suggests that *MIR* genes continue to be generated during the evolution of land plants. Some of these “young” *Arabidopsis* miRNAs (such as miR161, miR163, miR826, miR841, miR842, and miR846) revealed one potential mechanism by which miRNA genes originate in evolution (Allen et al. 2004; Rajagopalan et al. 2006; Fahlgren et al. 2007). The precursors to these miRNAs show extensive sequence similarity to their target genes, which led to the model that de novo generation of miRNA genes results from an inverted duplication event of the target genes. Transcription through the inverted repeats, which are likely to diverge in sequence after the initial duplication event, would result in an RNA with an imperfect hairpin structure reminiscent of miRNA precursors.

Plants and animals were thought to have evolved miRNAs independently since they do not share common miRNAs. However, a recent study identified an *Arabidopsis* miRNA, miR854, that has potential homologs in four examined animal (including human) genomes, and these homologs only differ from miR854 by one nucleotide (Arteaga-Vazquez et al. 2006). Human and mice miR854 were detectable by RNA filter hybridization. *Arabidopsis* miR854 has multiple binding sites in the 3' untranslated region (UTR) of the *UBP1* gene that encodes an hnRNP protein and causes translation inhibition of *UBP1* expression. The animal miR854 is also complementary to a site in the 3' UTR of the *UBP1* homologs. If the animal miR854 is to be confirmed as a regulator of its predicted target in animals in the future, miR854 and its target gene will be the first example of a conserved miRNA/target pair between plant and animal kingdoms.

3 MicroRNA Biogenesis in *Arabidopsis*

3.1 Transcription

All *Arabidopsis* miRNAs analyzed so far have their own transcriptional units such that each *MIR* gene is transcribed into a primary precursor known as pri-miRNA. This was first suggested by the intergenic location of *MIR* genes and later confirmed by the presence of expressed sequence tags (ESTs) corresponding to miRNA precursor transcripts. The first indications that plant *MIR* genes are transcribed by RNA polymerase II were that pri-miRNAs can be found to correspond to ESTs representing polyadenylated transcripts and that some pri-miRNAs that have been characterized contain introns. The most conclusive study showed that 5' capped transcripts were detected for 52 of the 99 tested *MIR* genes (Xie et al. 2005a). The great majority of the pri-miRNAs begin with an adenosine, which is located within 40 nt downstream of a conserved TATA box-like sequence. A bioinformatic analysis of 800-nt regions upstream of the mapped transcription start sites in these genes identified binding motifs for a number of known transcription factors (Megraw et al. 2006).

3.2 Dicer Processing

Plant miRNAs, like their animal counterparts, are released from pri-miRNAs through at least two sequential processing steps by RNase III enzymes. In animals, pri-miRNAs are first processed by an RNase III enzyme Drosha to the hairpin RNAs, known as pre-miRNAs, the immediate precursors to miRNAs (reviewed in Kim 2005). This processing step occurs in the nucleus and is assisted by the dsRNA-binding protein DGCR8. The pre-miRNAs are then exported to the cytoplasm by exportin 5 and the pre-miRNAs are further processed by another RNase III enzyme, Dicer, to a duplex of the miRNAs and the antisense strands (miRNA*s). Plants do not have Drosha homologs, and the two sequential processing steps are both carried out by Dicer-like 1 (DCL1), a Dicer homolog.

The requirement for DCL1 in miRNA processing was revealed by the fact that partial loss-of-function mutants in *DCL1* have greatly reduced accumulation of miRNAs and consequently exhibit pleiotropic developmental defects (Ray et al. 1996; Jacobsen et al. 1999; Park et al. 2002; Reinhardt et al. 2002). Null mutations in *DCL1* lead to embryonic lethality (Schwartz et al. 1994; McElver et al. 2001). Immunoprecipitated DCL1 from *Arabidopsis* was shown to be able to produce 21-nt small RNAs from long dsRNAs (Qi et al. 2005). This study, however, did not test whether DCL1 can release miRNAs from a pri- or pre-miRNA. That DCL1 converts pri-miRNAs to pre-miRNAs is inferred from the fact that pre-miRNAs are reduced in abundance while pri-miRNAs accumulate to higher levels in *dcl1* mutants (Kurihara and Watanabe 2004; Kurihara et al. 2006). The processing of pri-miRNAs to pre-miRNAs by DCL1 also requires two other proteins, HYPONASTIC LEAVES1 (HYL1) and SERRATE (SE). HYL1 belongs to a family of dsRNA-binding proteins in *Arabidopsis* (Lu and Fedoroff 2000; Hiraguri et al. 2005). Loss-of-function mutations in *HYL1* result in reduced accumulation of many miRNAs and elevated expression of miRNA target genes (Han et al. 2004; Vazquez et al. 2004a). The increased accumulation of pri-miRNAs and decreased levels of pre-miRNAs in *hyl1* mutants suggests that HYL1 is required for the processing of pri-miRNAs to pre-miRNAs (Kurihara et al. 2006). In fact, HYL1 has been found to interact with DCL1 in vitro and in a transient expression assay in *Nicotiana benthamiana* (Hiraguri et al. 2005; Kurihara et al. 2006). SE encodes a C2H2 zinc finger protein that was initially found to specify leaf polarity through promoting the accumulation of miR165/166 (Grigg et al. 2005). Later, it was demonstrated that SE plays a general role in the biogenesis of many miRNAs (Lobbes et al. 2006; Yang et al. 2006a). Since *se* mutants lead to increased accumulation of pri-miRNAs, SE likely acts in the processing of pri-miRNAs to pre-miRNAs (Lobbes et al. 2006; Yang et al. 2006a). This is consistent with the finding that SE interacts with HYL1 (Lobbes et al. 2006; Yang et al. 2006a). Although there is so far no direct genetic or biochemical evidence that

DCL1 also processes pre-miRNAs, the fact that mutations in other *Arabidopsis* Dicer homologs (*DCL2*, *DCL3*, and *DCL4*) do not affect the accumulation of the great majority of miRNAs (Xie et al. 2004; Gascioli et al. 2005; Xie et al. 2005b; Yoshikawa et al. 2005) suggests that this is most likely the case. Recently, a study showed that HYL1 immunoprecipitate from *Arabidopsis* was able to process pre-miRNAs in vitro (Wu et al. 2007). RNAi-mediated knockdown of *DCL1* in rice led to reduced accumulation of miRNAs and pleiotropic developmental phenotypes, suggesting that the function of DCL1 in miRNA biogenesis is conserved between monocots and dicots (Liu et al. 2005).

While DCL2, DCL3, and DCL4 are mainly responsible for processing long dsRNAs into siRNAs, a recent study showed that DCL4 also plays a minor role in the biogenesis of a small number of miRNAs in *Arabidopsis*. In particular, two “young” miRNAs, miR822 and miR839, still accumulate in *dcl1* mutants but fail to accumulate in *dcl4* mutants (Rajagopalan et al. 2006). Interestingly, the precursors of these two miRNAs are hairpins of relatively long double-stranded regions. The extensive double-strandedness of the precursors perhaps reflects a short evolutionary course after the initial duplication events that gave rise to the miRNA genes. miR822 was previously annotated as an siRNA due to its independence on DCL1 for biogenesis (Allen et al. 2004). However, deep sequencing revealed that this miRNA comes from a single-stranded hairpin RNA rather than a long dsRNA. In addition, the accumulation of this miRNA does not depend on RDR2 or RDR6, which are responsible for generating long dsRNAs in the biogenesis of siRNAs (Boutet et al. 2003; Vazquez et al. 2004b; Xie et al. 2004; Borsani et al. 2005; Yoshikawa et al. 2005; Katiyar-Agarwal et al. 2006). We may hypothesize that during the evolution of *MIR* genes, the inverted duplication of a locus initially gives rise to a hairpin RNA with perfect or near perfect double-strandedness and this hairpin is initially recognized by DCL4 (or may be DCL3 or DCL2). As the inverted repeat sequences diverge and the hairpin accumulates more and more bulges and mismatches, DCL4 fails to recognize the RNA and DCL1 gains access to the RNA.

It should also be noted that DCL1 is not limited to the production of miRNAs. DCL1 is required for the biogenesis of one class of siRNAs known as nat-siRNAs (Borsani et al. 2005; Katiyar-Agarwal et al. 2006), although the biochemical mechanism of DCL1 in this process is unknown.

3.3 Methylation

3.3.1 HEN1 Is a MicroRNA Methyltransferase—The biogenesis of plant miRNAs differs from that of animal miRNAs in that methylation of miRNAs occurs after Dicer processing. We discovered this additional step in plant miRNA biogenesis from our studies on a gene named *HEN1*, which was first isolated as a gene important in flower development (Chen et al. 2002). The fact that *hen1* mutants share similar developmental defects with partial loss-of-function *dcl1* mutants prompted us to test whether HEN1 is a general miRNA biogenesis factor. Indeed, we found that most miRNAs are reduced in abundance in *hen1* mutants, which confirmed a general role of HEN1 in miRNA biogenesis (Park et al. 2002). The clue that HEN1 may be a methyltransferase came from position-iterated basic local alignment search tool (PSI-BLAST) searches that showed that the C-terminal domain (~200 amino acid) of HEN1 resembles methyltransferases. In particular, a highly conserved S-adenosyl methionine (SAM)-binding site is found in this region of HEN1. The known *hen1* mutant alleles that carry point mutations, such as *hen1-1*, *hen1-2*, and *hen1-4*, all contain mutations in the methyltransferase region (Chen et al. 2002; Bonnet et al. 2004). Knowing that HEN1 serves as a general factor in miRNA biogenesis and that HEN1 has a potential methyltransferase domain, we postulated that HEN1 is an miRNA methyltransferase.

We tested our hypothesis by purifying glutathione S-transferase (GST)-HEN1 protein from *E. coli* and carrying out methyltransferase assays using various molecules in miRNA biogenesis as substrates. The substrates tested included pre-miRNAs, miRNAs, miRNA*, and miRNA/

miRNA* duplexes. The assay was set up such that the substrates were each incubated with GST-HEN1 or GST alone, C¹⁴-SAM as the methyl donor, and buffer. After the reactions, the RNAs were extracted and analyzed by gel electrophoresis followed by autoradiography. We found that among the various intermediates in miRNA biogenesis tested as substrates, only miRNA/miRNA* duplexes were methylated by GST-HEN 1 (Yu et al. 2005). A number of different miRNA/miRNA* duplexes were substrates of HEN1, indicating that HEN1 is a general, sequence-independent miRNA methyltransferase.

3.3.2 HEN1 Deposits a Methyl Group on the 2' OH of the 3' Terminal Nucleotide

—We determined the features that HEN1 recognizes in its substrates. We found that HEN1 has a strict requirement for the 2 nt 3' overhang in the miRNA/miRNA* duplex, a characteristic of Dicer products. Although blunt ends can be methylated at greatly reduced efficiency, miRNA/miRNA* duplexes with 1 nt, 3 nt, 4 nt, or 5 nt overhangs fail to be methylated (Yang et al. 2006b). We tested whether other features of Dicer products, such as 5' P and 3' OH, are recognized by HEN1. We found that the 3' OH but not the 5' P is a necessary feature of the HEN1 substrates (Yu et al. 2005). The 2' OH of the 3' terminal nucleotide is also necessary for HEN1-mediated methylation (Yu et al. 2005). Another feature of Dicer products is the specific size; *Arabidopsis* DCL proteins generate 21–24 nt small RNAs (Gasparioli et al. 2005; Xie et al. 2005b). We found that HEN1 is able to “measure” the size of the miRNA/miRNA* duplexes. While duplexes ranging from 19 nt to 27 nt in size can be methylated in vitro by HEN1, 21- to 24-nt duplexes are methylated with the best efficiency (Yang et al. 2006b). The strict requirement for the 2-nt overhang and the 3' OH, and the preference for 21- to 24-nt duplexes, probably ensure that only Dicer products are methylated in vivo. miRNA/miRNA* duplexes often contain mismatches or bulges whereas siRNA/siRNA* duplexes do not. We found that HEN1 can methylate both miRNA/miRNA* and siRNA/siRNA* duplexes in vitro.

We determined where the methyl group(s) is deposited onto the miRNA after the in vitro reaction. The requirement for both the 2' and 3' OH on the ribose of the 3' terminal nucleotide for methylation prompted us to test whether the methyl group(s) is deposited on one of the OH groups on the 3' terminal nucleotide. We first tested whether the OH groups are blocked after the HEN1-mediated reaction. The status of the OH groups can be assayed with chemical reactions (periodate treatment followed by β elimination) that require the presence of both OH groups on the ribose of the 3' terminal nucleotide. These chemical reactions lead to the elimination of the 3' terminal nucleotide to result in an RNA that is shorter (by 1 nt) and contains a 3' P (Alefelder et al. 1998). The resulting RNA can be distinguished from the original RNA by high-resolution gel electrophoresis. We assayed the miRNAs after the HEN-mediated in vitro reaction and found that the miRNAs are resistant to periodate/ β elimination reactions, indicating that the methyl group(s) is on one of the OH groups on the 3' terminal nucleotide (Yu et al. 2005). To determine whether methylation occurs on the 2' OH, 3' OH, or both, we established conditions that allowed us to separate 2'-O-methyl cytidine and 3'-O-methyl cytidine by HPLC. Analysis of the nucleosides from miRNAs methylated by HEN1 in vitro demonstrated that HEN1 deposits a methyl group exclusively onto the 2' OH of the 3' terminal nucleotide (Yang et al. 2006b).

3.3.3 Plant MicroRNAs and siRNAs Carry a Methyl Group—

The fact that miRNAs are reduced in abundance in *hen1* mutants and that HEN1 possesses miRNA methyltransferase activity in vitro suggests that plant miRNAs carry a methyl group in vivo. This is indeed true. We first demonstrated that plant miRNAs from the wildtype but not the *hen1-1* genotype are resistant to periodate/ β elimination reactions, suggesting that at least one of the OH groups on the 3' terminal nucleotides of plant miRNAs is blocked through a process requiring HEN1 (Yu et al. 2005). Next we isolated miR173 from *Arabidopsis* through affinity purification and measured the mass of this miRNA. The molecular mass of this miRNA from *Arabidopsis* is

14 Da larger than that of an in vitro synthesized unmodified miR173, consistent with the presence of a methyl group in miR173 from plants (Yu et al. 2005).

We also examined all types of currently known siRNAs except nat-siRNAs with the periodate/ β elimination assay. siRNAs from sense transgenes and inverted-repeat transgenes, *trans*-acting siRNAs, and heterochromatic siRNAs were all resistant to the chemical reactions in a HEN1-dependent manner, suggesting that all siRNAs are methylated in vivo by HEN1 (Li et al. 2005). An independent study also demonstrated the methylation of siRNAs (Ebhardt et al. 2005). Interestingly, not all siRNAs require HEN1 for accumulation despite the fact that they are all methylated by HEN1 (Boutet et al. 2003; Xie et al. 2004).

3.3.4 Lack of Methylation Results in Uridylation and Degradation of MicroRNAs

—We uncovered a novel uridylation activity that targets unmethylated miRNAs. We found that miRNAs become heterogeneous in size in *hen1* mutants such that a ladder of bands with 1-nt increments (mostly larger than the miRNA in wildtype plants) is present in *hen1* for any particular miRNA when examined by RNA filter hybridization. Primer extension showed that the heterogeneous species have the same 5' end and that the total abundance of the heterogeneous species is reduced as compared to wildtype (Li et al. 2005). This suggests that the heterogeneous species differ in their 3' ends and that miRNAs in *hen1* contain additional nucleotides at their 3' ends. Indeed, sequence analysis of miR173 and miR167 from wildtype and *hen1-1* plants confirmed the presence of 3' additional nucleotides in *hen1-1*. These nucleotides do not correspond to those 3' to the miRNAs in the pre-miRNAs, indicating that these nucleotides are added after the processing of the miRNAs from the precursors. These nucleotides are predominantly, but not exclusively, U residues. This led us to conclude that a novel polymerase activity, which we refer to as the uridylation activity, adds the additional nucleotides to miRNAs in the *hen1* mutant (Li et al. 2005).

The sequence analysis of miR167 and miR173 also revealed that unmethylated miRNAs are more susceptible to a 3'-to-5' exonuclease activity. 3' truncated miRNAs containing U tails were frequently found in *hen1-1* but were also present at a low frequency in wildtype (Li et al. 2005). We suspect that uridylation attracts an exonuclease to degrade the miRNA from the 3' end and that the exonuclease is not highly processive so that it only truncates a few nucleotides at a time. The truncated miRNAs are again uridylated. However, it is also possible that uridylation and exonucleolytic degradation of miRNAs are two independent events.

3.4 RISC Assembly

A methylated miRNA/miRNA* duplex next undergoes RNA-induced silencing complex (RISC) assembly, a process in which the miRNA strand is incorporated into a protein complex whose major protein component is an Argonaute (AGO) protein. AGO proteins contain PAZ domains that bind RNA and piwi domains that assume a folded structure that resembles RNase H (Ma et al. 2004, 2005; Parker et al. 2004, 2005; Song et al. 2004). Some AGO proteins possess key catalytic residues in the piwi domain and cleave the target mRNA in the middle of the complementary region between the mRNA and the siRNA or miRNA (Liu et al. 2004; Meister et al. 2004; Miyoshi et al. 2005). Other AGO proteins lack the key catalytic residues and cannot cause mRNA cleavage. For human and *Drosophila* AGO2, which possesses the endonucleolytic, or slicer, activity, it has been shown that the slicer activity is crucial for RISC loading (Matranga et al. 2005; Miyoshi et al. 2005). The passenger strand (miRNA*) is cleaved by AGO2 and the cleaved fragments are released to result in the formation of RISC with one miRNA strand. The determination of which strand of the duplex ends up in RISC is largely based on the thermodynamic properties of the two ends of the duplex. The strand in which the 5' end is less thermodynamically stable in the duplex becomes preferentially incorporated into RISC (Khvorova et al. 2003; Schwarz et al. 2003).

Plant miRNAs and siRNAs appear to follow this asymmetry rule in RISC loading. In fact, most plant miRNAs begin with a U residue such that the 5' end of the miRNAs tend to be engaged in A–U, rather than G–C, hydrogen bonding (Reinhart et al. 2002; Rajagopalan et al. 2006). There are 10 AGO proteins in *Arabidopsis* and most of these contain the key catalytic residues critical for slicer activity (reviewed in Herr 2005). *Arabidopsis* AGO1 has been demonstrated to be associated with most miRNAs and contains the slicer activity that cleaves miRNA targets (Baumberger and Baulcombe 2005; Qi et al. 2005; Qi et al. 2006). AGO1 is also associated with some siRNAs such as transgene siRNAs, *trans-acting* siRNAs, and viral siRNAs (Baumberger and Baulcombe 2005; Zhang et al. 2006). *ago1* mutants have severe developmental defects (Bohmert et al. 1998), suggesting that AGO1 probably mediates the functions of many miRNAs. AGO4 is required for heterochromatin formation and for the accumulation of repeat associated siRNAs (Zilberman et al. 2003). AGO4 also has the slicer activity and this activity is required for heterochromatin formation at some but not all loci (Qi et al. 2006). Intriguingly, while AGO4 binds 24-nt endogenous siRNAs, it is also found to bind some 21-nt miRNAs (Qi et al. 2006). Immunopurified AGO4 from *Arabidopsis* can lead to the cleavage of some miRNA targets.

3.5 Export

The production of miRNA/miRNA* duplexes occurs in the nucleus since DCL1 is a nuclear protein (Papp et al. 2003). Two processes that act on miRNA/miRNA* duplexes are methylation and incorporation of the miRNA strand into a protein complex named RISC. Since miRISCs are known to cause mRNA cleavage, miRISCs should be present in the cytoplasm where mRNAs are located. One plant miRNA has also been shown to trigger DNA methylation at the target genomic locus (Bao et al. 2004); therefore, some miRNAs should also be present in the nucleus. The nuclear generation of miRNAs and the functionality of miRNAs in the cytoplasm require an export step in miRNA biogenesis.

HASTY, the *Arabidopsis* homolog of exportin 5, plays a role in miRNA nuclear export (Bollman et al. 2003; Park et al. 2005). *hasty* mutants have pleiotropic developmental defects and reduced accumulation of most miRNAs. However, it is not known whether the cargo of HASTY is miRNA/miRNA* duplexes or miRISCs. *hasty* mutants show reduced levels of miRNAs in both nuclear and cytoplasmic fractions. In addition, in both cellular compartments, miRNA abundance is higher than that of the miRNA*, suggesting that detectable miRNAs are present in miRISCs in both compartments. Either RISC loading occurs in the nucleus followed by export of miRISC into the cytoplasm or RISC loading occurs in the cytoplasm following the export of miRNA/miRNA* duplexes. Perhaps some miRISCs can then be imported back into the nucleus. A recent study showed that a sequence motif in an animal miRNA specifies nuclear import of the miRNA (Hwang et al. 2007). If RISC loading occurs in the nucleus, then the methylation of the miRNA/miRNA* duplex should also occur in the nucleus because the singled-stranded miRNA in RISC cannot be methylated by HEN1. In the scenario whereby RISC loading occurs in the cytoplasm, methylation can theoretically occur in either the nucleus or the cytoplasm. The subcellular location of miRNA methylation is currently unknown.

4 Regulation of MicroRNA Biogenesis

4.1 Feedback Regulation

miRNA biogenesis is under feedback regulation such that two key players in miRNA biogenesis and function are themselves regulated by miRNAs. The *DCL1* gene may be regulated by the status of miRNA biogenesis by two different mechanisms. First, *DCL1* mRNA has a binding site for miR162, which leads to the cleavage of *DCL1* mRNA (Xie et al. 2003). Consistent with this, *DCL1* mRNA levels are elevated in the *hen1-1* mutant, in which the abundance of miR162 is reduced. Second, the 14th intron of the *DCL1* gene appears to harbor

the precursor to miR838. Although not proved, it is possible that the precursor is not transcribed independently but is released from the 14th intron of *DCL1* pre-mRNA through DCL1-mediated processing. If this is the case, it is possible that miRNA biogenesis competes with the splicing of *DCL1* pre-mRNA. In fact, truncated forms of *DCL1* mRNAs were found (Xie et al. 2003), some of which may correspond to the fragments generated through the process that releases the pre-miR838 from the 14th intron (Rajagopalan et al. 2006).

The *AGO1* gene encoding the main miRISC component is also under the regulation by an miRNA. miR168 has a binding site in *AGO1* mRNA and leads to AGO1-mediated cleavage of *AGO1* mRNA (Vaucheret et al. 2004). Therefore, the amount of functional miR168 bound by AGO1 determines the levels of *AGO1* mRNA. In addition, *MIR168* and *AGO1* genes are transcribed in a similar pattern, which probably ensures that *AGO1* is under the regulation of miR168 at all times and in all the cells that express *AGO1*. Among all miRNAs tested so far, miR168 appears to be preferentially stabilized by AGO1 such that miR168 levels are the least sensitive to reduced DCL1 activity but are sensitive to changes in the levels of AGO1 protein. This probably ensures that *AGO1* mRNA levels are under tight control by the levels of functional AGO1 protein.

4.2 Viral RNA Silencing Suppressors Affect MicroRNA Metabolism

A number of virally encoded proteins that suppress RNA silencing also negatively affect miRNA metabolism (reviewed in Voinnet 2005). The viral proteins can affect miRNA metabolism or function at a number of steps in the miRNA pathway. Some of these proteins, such as P1/HcPro from *Turnip mosaic virus*, p19 from the *Tomato bushy stunt virus*, and p21 from the *Beet yellows virus*, when expressed in plants from transgenes, cause the plants to show developmental phenotypes reminiscent of those exhibited by weak *dcl1* mutants. HcPro, p19, and p21 bind to miRNA/miRNA* (Chapman et al. 2004; Dunoyer et al. 2004; Lakatos et al. 2006), which are products of DCL1, and prevent subsequent steps in miRNA biogenesis. For example, methylation by HEN1 is reduced in transgenic plants expressing these viral proteins (Yu et al. 2006). RISC assembly is presumably also affected because the relative ratio of miRNA*s to miRNAs is much higher in plants expressing these viral proteins (Chapman et al. 2004; Dunoyer et al. 2004). Despite the accumulation of miRNAs in the transgenic plants expressing the viral proteins, sometimes to higher levels than in wildtype, miRNA targets overaccumulate, suggesting that miRNAs are not RISC bound or are not functional. Another viral protein, Cucumber mosaic virus 2b, has been recently shown to interact with AGO1 and inhibit its slicer activity (Zhang et al. 2006).

5 Mode of Action of Plant MicroRNAs

Plant miRNAs have a high degree of sequence complementarity to their target mRNAs and direct the slicing of the target mRNAs in the middle of the complementary regions (Llave et al. 2002b; Tang et al. 2003). This has been demonstrated by the detection of 3' cleavage products that have 5' ends that start at the middle of the complementary regions. While this is probably mediated by AGO1 (Baumberger and Baulcombe 2005; Qi et al. 2005), other AGO proteins may also play a role in this process. For example, immunopurified AGO4 is able to cleave some miRNA targets in vitro (Qi et al. 2006).

The mRNA cleavage products are then further degraded by other mechanisms. The 3' cleavage products of some miRNA targets are degraded by the 5' to 3' exonuclease XRN4 such that they accumulate in *xrn4* mutants (Souret et al. 2004). The 3' cleavage products of other miRNA targets must be degraded by an XRN4-independent mechanism because they do not accumulate in *xrn4* mutants. The 5' cleavage products are usually undetectable by RNA filter hybridization but can be detected by sensitive, PCR-based methods. It was found that the 5' cleavage products tend to acquire an oligo U tail, and the presence of the U tail correlates with shortening of the

RNA from the 5' ends, which leads to the conclusion that the oligo U tail causes 5' to 3' exonucleolytic degradation of the 5' cleavage products (Shen and Goodman 2004). Intriguingly, this mechanism is conserved in the unicellular alga *Chlamydomonas reinhardtii*. The 5' products of RISC-cleaved transcripts in *Chlamydomonas* tend to acquire an oligo A tail, which also correlates with the degradation of the 5' cleavage products (Ibrahim et al. 2006). The enzyme that adds the oligo A tail, MUT68, belongs to the broad polyA polymerase family. Other members of the family also have small RNA-related functions. For example, *C. elegans* rde-3 is necessary for RNAi and the Cid 12 protein from *Schizosaccharomyces pombe* is required for the accumulation of heterochromatic siRNAs and heterochromatin formation (Motamedi et al. 2004; Chen et al. 2005).

Translation inhibition is also a mechanism of regulation by plant miRNAs. Overexpression of miR172 does not lead to decreased accumulation of the target *AP2* mRNA but does lead to the reduction of *AP2* protein levels as well as phenotypes that imply compromised *AP2* function (Aukerman and Sakai 2003; Chen 2004). This suggests that miR172 inhibits the translation of its target mRNAs. It was also found that miR172 can lead to the cleavage of their target mRNAs including *AP2* mRNA (Aukerman and Sakai 2003; Schwab et al. 2005). The fact that miR172-mediated cleavage of *AP2* mRNA does not result in reduced *AP2* mRNA accumulation was likely due to feedback regulation such that *AP2* transcription is increased to compensate for the loss of *AP2* mRNA. Alternatively, the cleavage that was detected by the sensitive rapid amplification of cDNA ends (RACE)-PCR approach is not sufficient to result in a gross reduction in target mRNA levels. Regardless of the molecular mechanisms underlying the stable levels of *AP2* mRNA, it is clear that miR172 has to exert its effect on *AP2* mRNA at a level other than mRNA cleavage; otherwise, the reduction in *AP2* protein levels by miR172 is hard to explain. Recently, it was also observed that miR156/157 and miR854 lead to reduced protein but not mRNA levels of their target genes (Arteaga-Vazquez et al. 2006; Gandikota et al. 2007).

6 Outstanding Questions

Despite the rapid progress in the field of miRNA research in plants, many outstanding questions remain to be addressed.

6.1 The Dynamics of MicroRNA Evolution

The fact that over half of the known miRNA families in *Arabidopsis* are not conserved in poplar or rice suggests that miRNA genes evolve rapidly in plants. It also suggests that homology-based miRNA gene discovery will result in gross underestimation of miRNA gene numbers. Deep sequencing of small RNAs under normal and various stress conditions will be necessary to uncover the full complement of miRNA genes in any plant species. To understand the evolutionary history of miRNAs in any species, it will also be necessary to obtain the full complements of miRNA genes in a number of species closely related to the species in question. Finally, uncovering miRNAs from key representative species spanning the entire evolutionary distance from unicellular plants to angiosperms will be necessary to provide a comprehensive picture of miRNA evolution in plants. It is worth noting that miRNAs have recently been identified from the unicellular green alga *C. reinhardtii* (Molnar et al. 2007; Zhao et al. 2007).

6.2 Regulation of MicroRNA Biogenesis

Other than negative feedback regulation of miRNA biogenesis, little is known about how miRNA biogenesis is regulated in plants. Viruses have evolved proteins that can repress miRNA biogenesis or function at multiple steps of the pathway. It is also possible that endogenous proteins regulate various steps in miRNA biogenesis or function in a negative

manner. However, no such proteins have been identified so far. One of the challenges in the future would be to continue to identify the components of the miRNA pathway, especially genes that act negatively to regulate miRNA biogenesis.

Another obvious gap in our knowledge is that nothing is known about how miRNAs are turned over. Presumably miRNAs are subject to degradation by nucleases that, together with positive factors in miRNA biogenesis, determine the steady-state abundance of miRNAs. The identity of these nucleases needs to be revealed.

6.3 Mode of Action of Plant MicroRNAs

Based on the fact that most plant miRNAs lead to characteristic RISC-mediated cleavage of target mRNAs, it has been widely assumed that plant miRNAs primarily cause target mRNA cleavage. This assumption, however, is unfounded since the levels of target proteins have not been examined for all plant miRNAs except for miR156/157, miR172, and miR854, for which a role in translational regulation has been established (Aukerman and Sakai 2003; Chen 2004; Arteaga-Vazquez et al. 2006; Gandikota et al. 2007). It is possible that most plant miRNAs also have a role in translational control. It is important that the protein levels of miRNA target genes be examined for multiple miRNAs.

6.4 Function of MicroRNAs

Most of the evolutionarily conserved miRNAs belong to gene families with multiple members. Studies on the miR164 family suggest that members of a gene family can have distinct and partially overlapping functions (Baker et al. 2005; Guo et al. 2005; Sieber et al. 2007). One challenge in the future is to uncover the functional relationship among members of miRNA families.

Most miRNAs also have multiple target genes. What is the regulatory relationship between multiple members of an miRNA gene family and the multiple target genes? Does each miRNA regulate multiple targets? This can only be addressed when a member of the miRNA family can be specifically knocked out to allow an examination of the consequence of the loss of a particular miRNA gene on the expression of each target gene. Although the currently available T-DNA insertion collections of mutant *Arabidopsis* are a valuable resource to find knockouts in specific *MIR* genes, T-DNA insertions at a *MIR* locus may not lead to a complete knockout of the miRNA if the T-DNA insertions are not within the pre-miRNA. Other approaches to obtain a clean deletion of the miRNA sequence or a specific blockade of miRNA function will be complementary approaches that have yet to be developed. To specifically knock out an miRNA gene is the best way to evaluate the biological function of this miRNA gene as opposed to the most commonly adopted method currently, which involves expressing miRNA-resistant versions of a target mRNA. This current approach only reveals the consequence of loss of miRNA-mediated regulation of one of a number of target genes. It also fails to reveal the functional distinction among miRNA family members.

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