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## Abstract

- Small RNAs 20–30 nucleotides in length are sequence-specific regulatory RNAs that guide nucleic acid-based processes in eukaryotic organisms.
- In plants, small RNAs are classified as microRNAs (miRNAs) or small interfering RNAs (siRNAs) based on differences in their precursors and biogenesis.
- miRNAs are generated from precursors with an imperfect intramolecular hairpin structure and silence their target genes at the posttranscriptional level through mRNA cleavage or translational inhibition.
- Endogenous siRNAs are processed from long double-stranded RNAs with perfect base-pair complementarity and are classified as heterochromatic siRNAs (hc-siRNAs) or *trans*-acting siRNAs (ta-siRNAs).
- Heterochromatic siRNAs (hc-siRNAs) are endogenous siRNAs generated from transposons and repeats and guide cytosine methylation, which induces heterochromatin formation and transcriptional gene silencing.
- *Trans*-acting siRNAs (ta-siRNAs) are endogenous siRNAs whose biogenesis is triggered by specific miRNAs. Like miRNAs, ta-siRNAs repress their targets *in trans* at the posttranscriptional level and are important for plant development.
- Exogenous siRNAs are generated in both virus-infected plants and transgenic plants.
- The steady-state levels of small RNAs are precisely regulated through their biogenesis and turnover.
- A specific modification of small RNAs protects them from uridylation and truncation, processes associated with small RNA turnover.

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## Introduction

Ribonucleic acid (RNA) is a major macromolecule that executes biological events in living organisms. According to the central dogma of gene expression, RNA serves as an intermediate in the flow of genetic information from DNA to protein in the form of messenger RNA (mRNA). Additionally, there are different types of non-protein-coding RNAs (ncRNAs), whose classification is based primarily on their molecular functions but also reflects differences in size and accumulation. Several classes of ncRNAs are abundant and perform housekeeping duties: ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are involved in protein synthesis, small nuclear RNAs (snRNAs) are involved in pre-mRNA splicing, and small nucleolar RNAs (snoRNAs) guide the modification of other RNAs. The length of

ncRNAs is another important criterion for their classification. Long noncoding RNAs (lncRNAs) refer to ncRNAs that are longer than the arbitrary size of 200 nucleotides (nt). While it remains unclear whether the vast number of intergenic lncRNAs detected in microarray or RNA-seq experiments are transcriptional noise or functional RNAs, there has been increasing evidence of their regulatory functions in gene expression, especially in animals. Small RNAs 20–30 nt in length, the focus of this chapter, have come to be recognized as an important class within the broad spectrum of ncRNAs because their regulatory functions are critical for biological processes.

Small RNAs are a central component of RNA-mediated silencing in all eukaryotes and regulate genes in a sequence-specific manner through the recognition of complementary nucleic acid sequences. Small RNAs are known to act via two main mechanisms. In posttranscriptional gene silencing (PTGS), small RNAs guide the cleavage or translational inhibition of their target mRNAs. In transcriptional gene silencing (TGS), small RNAs guide DNA or histone methylation, resulting in heterochromatin formation.

In plants, small RNAs are classified as microRNAs (miRNAs) or small interfering RNAs (siRNAs) based on their precursors and biogenesis. miRNAs derive from longer RNA precursors containing a stem-loop or hairpin structure with imperfect base-pairing in the stem region. While the mature miRNA is the single most abundant species generated from a precursor, the passenger strand (miRNA\*) is the second most abundant species and may also be found *in vivo*. In contrast, siRNAs derive from longer double-stranded RNAs (dsRNAs) that exhibit nearly perfect sequence complementarity. Typically, multiple siRNA species are generated from a single precursor. Despite the differences in precursors and biogenesis that distinguish the different classes of small RNAs, however, it is important to emphasize that all small RNAs function as sequence-specific guides in target regulation.

Research over the past two decades has significantly improved the understanding of small RNAs and their regulatory mechanisms. Since the initial discovery of an miRNA, *lin-4*, from genetic screens of the nematode *Caenorhabditis elegans* in 1993, hundreds of thousands of small RNAs have been identified, particularly with the aid of next-generation sequencing technology. Along with the improved understanding of its critical regulatory functions for numerous biological processes, small RNA-mediated gene silencing is also recognized as a powerful research tool in biology. The use of small RNAs to knock down selected genes permits the dissection of the molecular functions of those genes and related pathways. Small RNA-based gene silencing has also been used for crop improvement and fighting human diseases. The awarding of the 2006 Nobel Prize in Physiology and Medicine for the discovery of RNA interference (RNAi), a homology-based gene silencing phenomenon conferred by small RNAs, further exemplifies the significance of small RNA-mediated gene regulation.

In this chapter, small RNAs in the model plant *Arabidopsis thaliana* will be discussed in terms of their biogenesis, their molecular mechanisms for target repression, and their biological functions. Major differences with animal small

RNAs will also be introduced. Finally, mechanisms that contribute to small RNA homeostasis such as degradation will be discussed.

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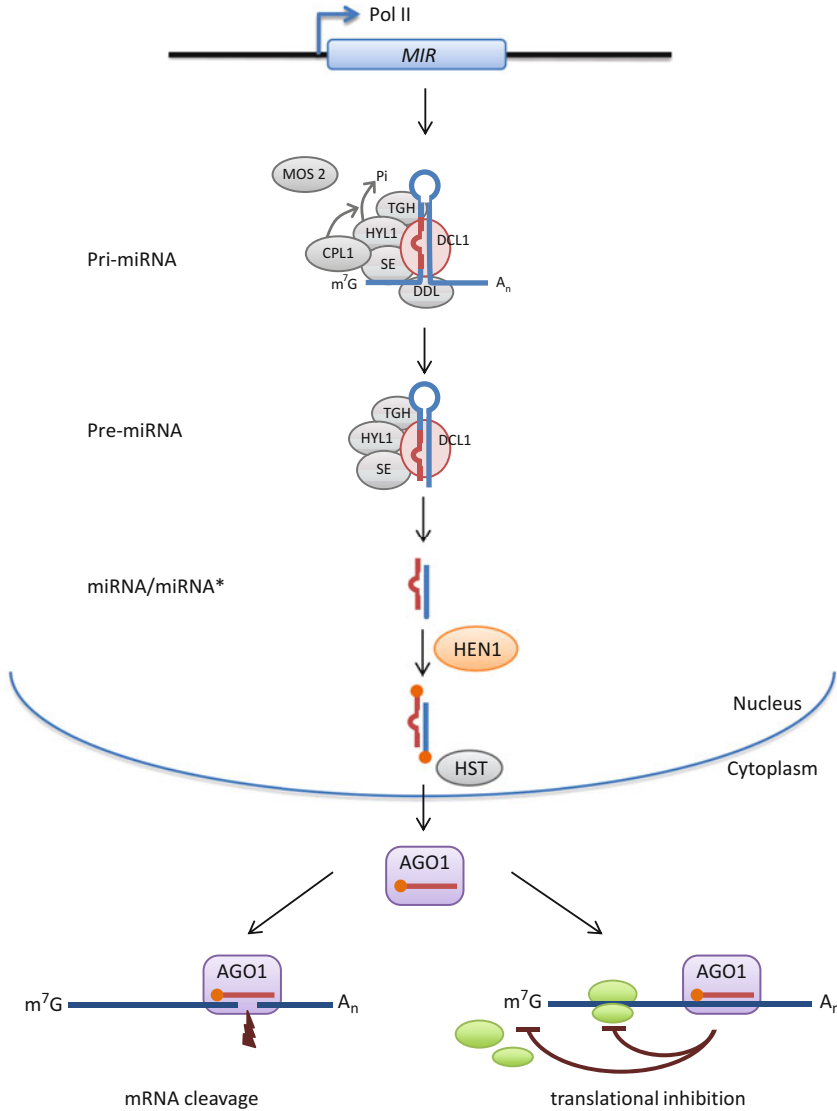
## miRNAs

### Biogenesis

miRNAs are small regulatory RNAs 20–22 nt in length that act in a sequence-specific manner primarily through PTGS (reviewed in Chen 2009). Their biogenesis involves the following steps: transcription of a *MIR* gene to produce the miRNA precursor, cleavage to yield the mature precursor, stabilization by methylation, nuclear export, and incorporation into effector proteins (Fig. 1).

miRNA precursors that give rise to mature miRNAs are encoded by *MIR* genes, which are located in intergenic regions. *MIR* genes are individual gene units with their own promoters and terminators and are transcribed by RNA polymerase II (Pol II) (Fig. 1). Accordingly, *MIR* gene promoters harbor *cis*-acting elements for transcription by Pol II. As with protein-coding genes, the expression of *MIR* genes is subject to regulation, with Pol II transcription affected by spatiotemporal inputs specific to particular developmental stages and organs. In addition to these endogenous signals, exogenous cues from the environment, such as biotic and abiotic stresses, also affect transcription. Thus, the transcription of *MIR* genes and, ultimately, miRNA abundance are governed by regulatory frameworks that respond to various signals. Mediator, a multi-protein complex, serves as a general transcription factor and is thought to integrate various signals to promote the recruitment of Pol II to promoters. Mediator is required for the transcription of *MIR* genes in *Arabidopsis*. After and/or during transcription, miRNA precursors are capped and polyadenylated at their 5' and 3' ends, respectively, and introns are spliced out in a manner similar to the processing of Pol II-transcribed pre-mRNA. These *MIR* gene transcripts, or primary miRNAs (pri-miRNAs), form hairpin structures with imperfect base-pairing in the stem regions and are subsequently processed by small RNA biogenesis enzymes.

Through the successive action of Dicer-like (DCL) RNase III enzymes in the nucleus, a pri-miRNA is processed into a precursor miRNA (pre-miRNA), which is in turn processed into a mature miRNA/miRNA\* duplex (Fig. 1). This duplex contains both the guide strand, the functional miRNA species that promotes PTGS, and the miRNA\* passenger strand, which is eventually degraded. DCL1, an RNase III enzyme that specifically cleaves dsRNA, is responsible for the processing of most miRNAs. However, the *Arabidopsis* genome encodes four DCL genes and several evolutionarily young miRNAs are processed by DCL4 instead of DCL1. The first dicing step generates the pre-miRNA from the pri-miRNA: DCL1 cleaves the stem approximately 15 nt away from the base of the stem and generates a 2-nt 3' overhang. The second dicing step by DCL1 cleaves the newly formed pre-miRNA at a position closer to the terminal loop, generating a 20–22-nt miRNA/miRNA\* duplex with 2-nt 3' overhangs. During this process, the



**Fig. 1 miRNA biogenesis and silencing mechanism.** Pri-miRNA transcripts are generated from *MIR* gene loci by RNA polymerase II and processed into pre-miRNAs by DCL1 with the assistance of HYL1, SE, DDL, CPL1, MOS2, and TGH. Pre-miRNAs are further processed into miRNA/miRNA\* duplexes by DCL1. Both strands of the miRNA/miRNA\* duplex are methylated by HEN1 either before or after HST-mediated transport to the cytoplasm. Mature miRNAs are loaded into AGO1 and guide target repression via mRNA cleavage or translational inhibition

DCL1 enzyme is aided by DAWDLE (DDL), a forkhead-associated (FHA) domain protein; SERRATE (SE), a zinc finger protein; and HYPOPLASTIC LEAVES1 (HYL1), an RNA binding protein. It has been proposed that DDL facilitates the

recognition of pri-miRNAs by DCL1, while SE and HYL1 may improve the accuracy and efficiency of the dicing activity of DCL1.

Following the release of the miRNA/miRNA\* duplex from the pre-miRNA, HUA ENHANCER 1 (HEN1) methylates both ends of the duplex (Fig. 1). Specifically, HEN1 deposits a single methyl group at the 2'-OH position of the 3' terminal ribose. As discussed in section “[Small RNA Turnover](#)” below, HEN1-mediated methylation enhances the stability of miRNAs. That miRNAs are generated in the nucleus while miRNA-directed PTGS occurs in the cytoplasm indicates that miRNAs are exported from the nucleus to the cytoplasm. HASTY (HST), an Exportin-5 (Exp-5) homolog, has been implicated in the nuclear export of miRNAs, but it is unknown whether HEN1-mediated methylation precedes or follows nuclear export.

In the cytoplasm, miRNAs are loaded into ARGONAUTE (AGO) effector proteins and small RNA-mediated repression reflects the functions of these two key players: guidance by small RNAs and the catalytic activity of AGO-containing protein complexes (Fig. 1). The *Arabidopsis* genome encodes ten AGO homologs. Among these, AGO1 functions as the major effector protein for miRNA-mediated PTGS and binds most miRNAs. AGO7 specifically binds miR390, while AGO10 exhibits a binding preference for miR166/165 over other miRNA species.

## Molecular Mechanism

miRNAs repress the expression of targets via PTGS, which is associated with two modes of repressive action: mRNA cleavage and translational inhibition. In miRNA-pathway-compromised mutants, these changes can be assessed through the detection of the mRNA transcript levels or protein abundance of miRNA targets. In the case of mRNA cleavage, target mRNAs are sliced at the center of the sequence bound by the miRNA. The cleaved products, particularly the 3' fragments, can be detected in wild-type plants. When miRNAs that regulate their targets via mRNA cleavage are disrupted, the levels of both target mRNAs and the corresponding protein products increase. In contrast, there is a disproportionate accumulation of target protein relative to that of target mRNAs when miRNAs that regulate their targets by translational inhibition are disrupted. mRNA cleavage and translational inhibition may occur in parallel. For instance, a fraction of the mRNA pool targeted by a single miRNA may be repressed by cleavage while the remaining fraction is regulated by translational inhibition.

In plants, miRNA-guided cleavage has been observed for most miRNAs and is considered to be a widespread regulatory mechanism of plant miRNAs. The endonucleolytic activity of AGO1 cleaves (or slices) the phosphodiester bond linking two nucleotides in the target mRNA that correspond to the 10th and 11th nucleotides from the 5' terminal end of the miRNA. The newly exposed 5' and 3' fragments are subsequently degraded by the exosome with 3'-5' exonuclease activity and EXONUCLEASE4 (XRN4) with 5'-3' exonuclease activity, respectively. The degradation of the 5' fragment is further accelerated by template-independent oligo-

uridylation. Uridine tails are attached at the 3' end of the 5' fragment, which promotes decapping activity at the 5' end. The 5' fragment is thus rendered susceptible to 5'–3' degradation, and translation of the cleavage fragment is prevented.

miRNA-directed translational inhibition is less commonly observed in plants than transcript cleavage, and there are two main explanations for this. While miRNAs in animals require perfect base pairing with the target mRNA only in the seed region, which corresponds to the 2nd to 7th nucleotides from the 5' end of the miRNA, plant miRNAs require much more extensive complementarity with the target mRNA for PTGS. This difference may underlie the predominance of distinct repressive mechanisms in the two kingdoms, i.e., translational inhibition in animals and transcript cleavage in plants. Alternatively, the perceived predominance of miRNA-directed transcript cleavage in plants may reflect technical limitations. Although monitoring the effects of miRNAs on their targets is facilitated by their sequence complementarity, high-quality antibodies for the proteins corresponding to the targeted mRNAs are necessary to assess the occurrence or extent of translational inhibition. Thus, the technical challenge of producing high-quality antibodies may contribute to the less frequent observation of translational inhibition in plants.

The earliest reports of miRNA-directed translational inhibition in plants were the findings that *APETALA2* (*AP2*) and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3* (*SPL3*) are translationally repressed by miR172 and miR156/157, respectively. Subsequent studies identified additional miRNAs that regulate their target mRNAs via translational inhibition. Moreover, several players in miRNA-directed translational inhibition have been identified from forward genetic screens in *Arabidopsis*. Mutations disrupting the microtubule-severing enzyme *KATANIN1* (*KTN1*), the P body component *VARICOSE* (*VCS*)/*Ge-1*, the GW-repeat protein *SUO*, and the ER membrane protein *ALTERED MERISTEM PROGRAM1* (*AMP1*) compromise miRNA-mediated translational inhibition of exogenous reporter constructs and endogenous miRNA targets. Notably, these studies show that miRNAs, such as miR156/157, miR164, miR165/166, miR172, and miR398, which are known to guide target transcript cleavage, also inhibit the translation of target mRNAs. The fact that genetic mutations (in *KTN1*, *VCS*, *SUO*, and *AMP1*) can uncouple the transcript cleavage and translational inhibition activities of these miRNAs suggests that the two repressive modes of action are independent and occur in parallel to regulate target transcripts. The molecular mechanism of translational inhibition and the events that follow remain unclear. However, two studies in zebra fish and fruit fly suggest that translational inhibition primarily affects the initiation step rather than elongation or termination and that the subsequent stimulation of mRNA deadenylation and decay occurs in an miRNA-cleavage-independent manner (Bazzini et al. 2012; Djuranovic et al. 2012).

## Biological Function

Because miRNAs repress or silence their target mRNAs, studies of the targets of miRNAs have also been critical for understanding miRNA function. According to

the miRBase miRNA database ([www.mirbase.org](http://www.mirbase.org), Release 19) (Kozomara and Griffiths-Jones 2011), the numbers of mature miRNAs and precursors in *Arabidopsis* are 338 and 299, respectively. Among known miRNA targets, transcription factors are the most highly represented functional group. By recognizing *cis*-acting elements at the promoters of their target genes, transcription factors can systematically activate or repress genes belonging to a particular regulatory or functional network. The regulation of the transcription of downstream genes by transcription factors is affected by both endogenous and exogenous signals. miRNAs regulating transcription factors therefore provide an additional layer of regulation for specific biological processes.

Although miRNA-mediated PTGS affects a wide variety of biological phenomena, its role in development is particularly well established, and a large number of miRNA-targeted transcription factors are implicated in developmental processes (reviewed in Chen 2009). Interactions between miRNAs and their targets have been reported in a wide range of developmental contexts, such as embryogenesis, cell differentiation, pattern formation, phase transition, and hormone signaling. Loss- or gain-of-function mutations in *MIR* genes or their targets often result in specific developmental phenotypes that are informative about their functions. Expressing miRNA-resistant targets under their endogenous promoters also affects plant morphology, demonstrating that miRNA-mediated PTGS is a critical regulatory component of developmental programs. Mutations disrupting miRNA biogenesis genes and *AGO1* consistently result in pleiotropic developmental defects. For example, the null *dcl1* allele is embryonic lethal, and the morphological phenotype of hypomorphic *dcl1* is similar to those of null *hyl1*, *hen1*, and *hst* alleles. Mutations disrupting *AGO1* also yield phenotypes similar to those of mutants with disrupted *DCL* function. Taken together, the developmental defects of miRNA pathway mutants further establish the vital functions of miRNAs in development.

Other miRNAs affect the gene regulatory networks that govern responses to environmental cues. Although less is known about miRNAs involved in stress responses compared to miRNAs involved in developmental processes, deep sequencing of small RNA populations has increased the number of identified miRNAs that are specifically expressed under certain environmental conditions, such as biotic and abiotic stresses. For example, stress-related hormones such as abscisic acid can activate or repress the expression of certain miRNAs. In turn, stress-responsive miRNAs may target genes involved in detoxification or enhancing resistance. Because stress response signals also impact the developmental network, these changes may be critical for the ability of plants to alter their developmental program under harsh external conditions and to resume the normal program when the stress condition is removed.

## Autoregulation of the miRNA Pathway

Several self-feedback mechanisms are known to regulate the miRNA pathway. Two critical components of the miRNA pathway, *DCL1* and *AGO1*, are themselves



targets of miRNA-mediated repression. Whereas DCL1 protein catalyzes miRNA biogenesis, there are three possible fates for *DCL1* transcripts: they may be translated into DCL1 protein, recognized as a cleavage target of miRNA-mediated PTGS, or processed as a pri-miRNA. When miRNA levels are high, miR162-directed cleavage of *DCL1* transcripts by an AGO1-containing complex is likely favored. Alternatively, the foldback RNA structure within the *DCL1* transcript may recruit the miRNA biogenesis machinery and be diced by DCL1 to generate miR838. Thus, high miRNA levels or high DCL1 protein levels lead to a decrease in *DCL1* mRNA abundance and consequently reduced *DCL1* expression and activity. *AGO1* mRNA contains a miR168 binding site, which similarly permits feedback regulation of *AGO1*. In this manner, the autoregulation of critical enzymes ensures the balanced dynamics of the miRNA pathway.

## miRNAs in Animals

As in plants, miRNAs in animals represent an essential regulatory module of gene expression (reviewed in Kim et al. 2009; Krol et al. 2010). Although miRNAs and miRNA targets are not well conserved between the two kingdoms, the general principles of miRNA biogenesis and function are held in common: stem-loop-containing precursors are processed into mature miRNAs, and target repression is accomplished by miRNA-directed AGO function. Nevertheless, there are specific characteristics of animal miRNAs that are not observed in plants. First, a large fraction of miRNA genes in animals are clustered together and generate polycistronic precursors. Moreover, miRNA genes may reside within the transcriptional units of other genes and consequently depend on the transcription of these genes for their own expression. Following the transcription of miRNA genes in animals, the processing of the precursors involves two RNase III enzymes, Drosha and Dicer. These enzymes perform two independent dicing events, in contrast to DCL1 in plants, which performs both dicing steps. Animal pri-miRNAs are initially processed into pre-miRNAs by Drosha within the nucleus, and the pre-miRNAs are subsequently transported to the cytoplasm by Exp-5. Further processing of pre-miRNAs to generate the miRNA/miRNA\* is performed by Dicer in the cytoplasm. In contrast to the methylation of plant miRNAs by HEN1, the miRNA/miRNA\* duplex is not methylated in animals. Stable incorporation of the mature miRNA into an AGO protein subsequently directs target repression.

In animals, miRNA-directed target repression generally occurs through translational inhibition and RNA decay. Transcript cleavage is not widely observed for animal miRNAs, which may reflect the relatively low complementarity between animal miRNAs and their targets. miRNA binding sites are typically located in the 3' untranslated region (3' UTR) of target transcripts, with single mRNA molecules bound by multiple miRNAs for silencing. As previously described, miRNA-mediated PTGS in plants requires high sequence complementarity at target binding sites, which are generally found within coding sequences. In animals, perfect complementarity of the miRNA and its target within the seed region alone (the

2nd to 7th nucleotides from the 5' end of the miRNA) is sufficient for target recognition. As a result, a single miRNA generally has a large number of mRNA targets. Thus, miRNA-mediated silencing in animals involves a less stringent hybridization requirement between miRNAs and their targets, and target transcript cleavage is uncommon in animals.

## Evolution of *MIR* Genes

High-throughput deep sequencing of small RNA populations has led to the discovery of many miRNA species. The miRNA database ([www.mirbase.org](http://www.mirbase.org), Release 19) (Kozomara and Griffiths-Jones 2011) currently lists 338 mature miRNAs in *A. thaliana*, 2,042 in humans, and 368 in *C. elegans* in addition to the thousands of mature miRNAs from 190 other species. Despite the large number of identified miRNAs in both plants and animals, the poor conservation of miRNA sequences between the two kingdoms suggests that miRNA families in plants and animals evolved independently or that miRNAs were present in the common ancestor but the genes evolved so fast that no sequence similarities could be detected in plants and animals.

There are two major hypotheses regarding the evolution of miRNAs. The first proposes that *MIR* genes evolved from inverted duplications of miRNA targets. In this model, the duplication of protein-coding genes in a head-to-head or tail-to-tail orientation yields stem-loop structures whose stem regions exhibit extensive base-pair complementarity and are processed by the siRNA pathway rather than the miRNA pathway. Small RNAs from these young *MIR* genes regulate their homologous targets. Over evolutionary time, both *MIR* genes and their targets may undergo duplication and accumulate mutations. Some of the double-stranded precursors eventually acquire the characteristic hairpin structure of miRNA precursors and are processed by the miRNA pathway rather than the siRNA pathway. Over time, the targets come to be regulated by a limited number of specific small RNAs. According to this model, recently evolved *MIR* genes are expected to have a higher degree of sequence similarity with their targets, which has been observed for *MIR161* and *MIR163*.

Because the precursors of many young miRNAs do not match any other sequences in the genome, the duplication hypothesis cannot explain the genesis of all of the young miRNAs in plants. The random hairpin theory was proposed in part to address this shortcoming. Organisms produce a large number of hairpin structures that could potentially generate foldback precursors of small RNAs. For example, the *A. thaliana* genome contains more than 130,000 imperfect inverted repeats. The random hairpin theory proposes that *MIR* genes can evolve when the following conditions are met: a DNA segment that generates a foldback structure retains a transcriptional unit; by chance, a small RNA produced from the structure targets a protein-coding gene; and the resulting regulatory relationship confers an evolutionary advantage.

The classification of an miRNA as old or young is based on its degree of conservation among different species. While ancient miRNAs are conserved

among animal or plant species of great evolutionary distance, young miRNAs are specific to a species or genus. miRNA genes may be duplicated as a result of gene duplication, whole segment duplication, or genome duplication, thereby giving rise to an miRNA family. The miRNAs produced by these families are considered old miRNAs, and their abundance is high probably because they are encoded by multiple genes. The processing of the precursors of old miRNAs by the DCL1-mediated biogenesis pathway is more precise than the processing of young miRNA precursors. Having multiple *MIR* genes within each conserved family may result in a complex relationship among the individual miRNA family members and their targets. In contrast, non-conserved miRNAs tend to be encoded by a single locus and are characterized by their short evolution times. In addition to being weakly expressed, young miRNAs typically regulate few, if any, genes, and the processing of their precursors is less precise.

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## Heterochromatic siRNAs

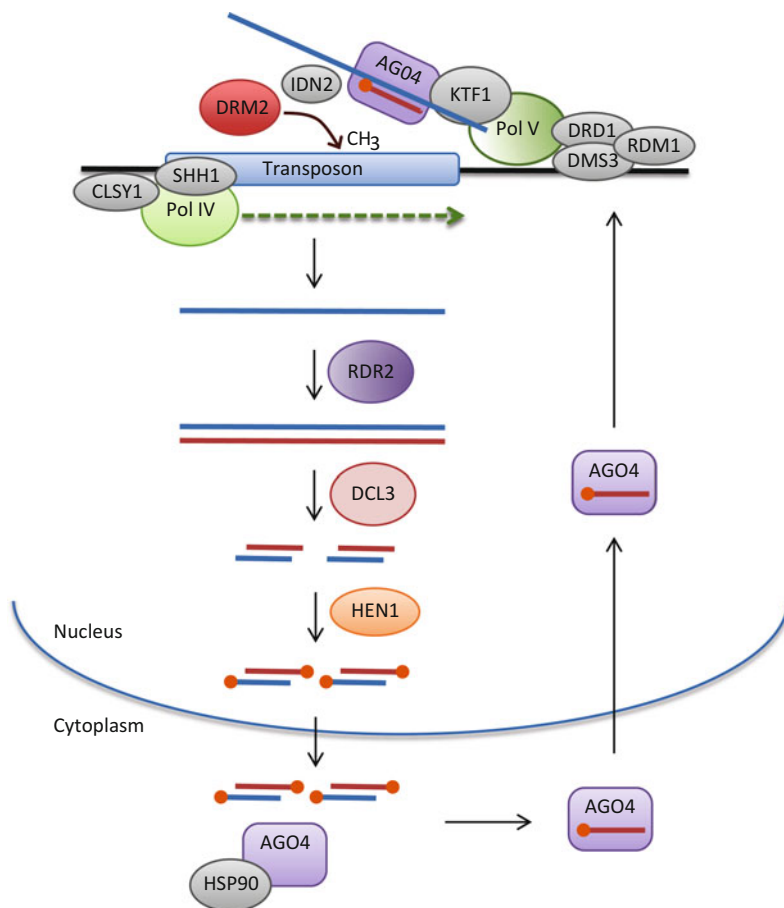
siRNAs are small RNAs 21–24 nt in length that are generated from long dsRNAs or single-stranded RNAs (ssRNAs) that produce longer and more perfect hairpin structures compared to miRNA precursors. While only one miRNA is produced from a single precursor, siRNA precursors generate multiple siRNAs. siRNAs trigger TGS or PTGS and direct the enzymatic action of their effector proteins through sequence-specific interactions with their targets.

Plants have two major families of endogenous siRNAs: heterochromatic siRNAs (hc-siRNAs) and *trans*-acting siRNAs (ta-siRNAs). siRNAs may also derive from exogenous sources, such as viruses and transgenes.

## Biogenesis

Hc-siRNAs are small RNAs 21–24 nt in length that derive from heterochromatic regions, including repeats, transposons, and intergenic regions (reviewed in Law and Jacobsen 2010; Castel and Martienssen 2013). They comprise the most abundant and diverse small RNA family: approximately 80 % of all small RNAs are hc-siRNAs, with tens of thousands of unique hc-siRNAs present in wild-type *Arabidopsis* (Zhang et al. 2007; Mosher et al. 2008). Hc-siRNAs mediate TGS of heterochromatin by guiding DNA methylation and histone modification in a sequence-specific manner. In *Arabidopsis*, the biogenesis of hc-siRNAs involves the transcription of primary precursors, the conversion of the precursors to dsRNAs, further maturation by dicing, methylation, and association with AGO proteins (Fig. 2).

That the sequences of hc-siRNAs often map to repeats and transposons implies that hc-siRNA precursors are transcribed from these regions. In *Arabidopsis*, this transcription is probably performed by the plant-specific DNA-dependent RNA polymerase Pol IV (reviewed in Haag and Pikaard 2011). Although Pol IV is evolutionarily derived from Pol II, the largest subunit of Pol IV, NUCLEAR



**Fig. 2 Hc-siRNA biogenesis and silencing mechanism.** RNA polymerase IV generates long noncoding RNAs (lncRNAs) from target regions with the aid of CLSY1 and SHH1. These lncRNAs are made double stranded by RDR2. DCL3 dices the double-stranded RNAs into 24-nt siRNAs, which are subsequently methylated by HEN1 and transported to the cytoplasm. siRNAs are loaded into AGO4 with the assistance of HSP90 then transported into the nucleus. AGO4-loaded siRNAs recognize the nascent transcripts generated by RNA polymerase V, and the DRM2 methyltransferase is recruited to the target. These interactions confer sequence-specific target methylation

RNA POLYMERASE D1 (NRPD1), is distinct from that of Pol II and confers the specific catalytic activity of Pol IV. Pol IV-dependent transcripts have not been detected experimentally, but more than 90 % of hc-siRNAs are lost in the *nprd1* mutant. The current model suggests that Pol IV produces long ssRNAs from regions that spawn siRNAs (Fig. 2). The chromatin remodeling factor CLASSY1 (CLSY1) may promote hc-siRNA biogenesis through Pol IV, while the homeodomain protein SAWADEE HOMEODOMAIN HOMOLOG1 (SHH1) has been implicated in the recruitment of Pol IV to regions that produce hc-siRNAs.

siRNAs are typically generated from the cleavage of long dsRNAs into smaller fragments of a precise length. Hc-siRNAs are produced in this manner, following the conversion of Pol IV-dependent ssRNA transcripts into dsRNAs (Fig. 2). Of the six RNA-dependent RNA polymerase (RDR) homologs encoded in the *Arabidopsis* genome, RDR2 is responsible for this conversion. RDR2 physically interacts with Pol IV and converts Pol IV-transcribed ssRNAs into long dsRNAs with perfect sequence complementarity. Thereafter, the siRNA precursors are diced into 24-nt small RNAs with 2-nt overhangs by DCL3 and methylated at the 2'-OH group of the 3' terminal nucleotide by HEN1. A mutation disrupting DCL3 function can be compensated by other DCL homologs: in *dcl3*, DCL2 and DCL4 generate hc-siRNAs 22 nt and 21 nt in length, respectively. As previously mentioned, the siRNA precursors processed by DCL3 yield multiple siRNAs rather than a single species from a locus.

After processing, mature hc-siRNAs are loaded into AGO effector proteins belonging to the AGO4 clade (Fig. 2). Although hc-siRNAs are synthesized in the nucleus, their abundance is ten times greater in the cytoplasm than in the nucleus. It has been reported that hc-siRNAs are incorporated into AGO4 in the cytoplasm with the assistance of HEAT-SHOCK PROTEIN90 (HSP90) and then transported to the nucleus. Four of the ten AGO homologs in *Arabidopsis*, AGO4, AGO6, AGO8, and AGO9, belong to the AGO4 clade. All of these except AGO8 function in hc-siRNA-mediated TGS, and AGO4 is the major binding partner of hc-siRNAs. AGO6 and AGO9 have been reported to function as hc-siRNA effector proteins in specific cell types and organs, while *AGO8* may be a pseudogene.

## Molecular Mechanism

As the name implies, hc-siRNAs are generated from heterochromatin, and they guide cytosine methylation at the site of their transcription. In *Arabidopsis*, hc-siRNAs play a major role in determining the methylated targets of the RNA-directed DNA methylation (RdDM) pathway (reviewed in Law and Jacobsen 2010; Castel and Martienssen 2013). In RdDM, two classes of ncRNAs and a methyl transferase enzyme are required for target selection and catalytic activity, respectively. A number of subsidiary players have also been implicated in RdDM.

In addition to hc-siRNAs, another type of ncRNA is also generated from RdDM target regions and helps direct cytosine methylation (Fig. 2). The biogenesis of these long ncRNAs requires a second plant-specific RNA polymerase, Pol V (reviewed in Haag and Pikaard 2011). Like Pol IV, Pol V evolved from Pol II, and its largest subunit, NUCLEAR RNA POLYMERASE E1 (NRPE1), is distinct from those of Pol II and Pol IV. The recruitment of Pol V to its targets in the genome is facilitated by the DDR complex, whose major components are DEFECTIVE IN MERISTEM SILENCING3 (DMS3), a structural maintenance of chromosome (SMC) domain protein; DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1 (DRD1), an SNF2-like chromatin remodeling protein; and REQUIRED FOR DNA METHYLATION1 (RDM1), a single-stranded methyl

DNA-binding protein. At a subset of the methylated targets of RdDM, Pol II is responsible for synthesizing the long ncRNA rather than Pol V.

AGO4-loaded hc-siRNAs are recruited to their targets through two distinct interactions: physical contact between AGO4 and WG/GW motif-containing proteins and the binding of hc-siRNAs to Pol V-dependent ncRNAs. The WG/GW motif is an AGO hook motif found in AGO4-binding proteins, and the interaction between AGO4 and WG/GW motif-containing proteins governs downstream molecular events (reviewed in Azevedo et al. 2011). It has been proposed that hc-siRNAs recognize the nascent Pol V-dependent ncRNA through base-pair complementarity and guide silencing of the target DNA in a sequence-specific manner. NRPE1 contains a WG/GW motif in its C-terminal region and is known to physically interact with AGO4. Thus, an hc-siRNA-containing AGO4 protein may be shuttled to the target region through the interaction with NRPE1 while the hc-siRNA recognizes the nascent, long ncRNA generated by Pol V. Another WG/GW motif-containing protein, SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L)/KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR1 (KTF1), interacts with both Pol V-dependent ncRNA and AGO4. Thus, SPT5L/KTF1 may stabilize the hybridization of AGO4-loaded hc-siRNAs and Pol V-dependent ncRNAs by bridging AGO4 and Pol V-dependent ncRNAs.

These complex interactions among proteins and ncRNAs must ultimately provide a stable foundation for the recruitment of a methyl transferase enzyme to the hc-siRNA targets. It has been proposed that the binding of AGO4-loaded hc-siRNAs to Pol V-dependent ncRNAs is followed by the release of AGO4. The INVOLVED IN DE NOVO2 (IDN2)/REQUIRED FOR DNA METHYLATION12 (RDM12)-containing complex is critical for the consolidation and integration of factors required for the downstream methylation event. In addition to IDN2/RDM12, the complex contains an IDN2/RDM12 paralog, either FACTOR OF DNA METHYLATION1 (FDM1)/IDN2-LIKE1 (IDNL1)/IDN2 PARALOG1 (IDP1) or FDM2/IDNL2/IDP2. The protein domains of IDN2/RDM12 and its two paralogs in *Arabidopsis* occur in the following order from the N-terminus: a zinc finger for RNA and/or DNA binding, an XS domain for dsRNA recognition, and a coiled-coil domain and XH domain for protein dimerization. The zinc finger domain may bind the methylated target DNA or function as a second RNA binding motif alongside the XS domain. XS domains bind RNAs with 5' overhangs; in the context of RdDM, the XS domain may stabilize the duplex formed by hc-siRNAs and Pol V-dependent ncRNAs. IDN2/RDM12 dimerizes with FDM1/IDNL1/IDP1 or FDM2/IDNL2/IDP2 through the coiled-coil and XH domains in an antiparallel manner, permitting the recruitment of two distinct hc-siRNAs in a single IDN2/RDM12-containing complex. The IDN2/RDM12-containing complex may anchor the dsRNA duplex formed by hc-siRNAs and long ncRNAs to the target DNA. While the recruitment of DOMAIN REARRANGED METHYLTRANSFERASE2 (DRM2) to the target remains unclear, the RdDM effector protein RDM1 has been proposed to mediate this recruitment based on its ability to physically interact with AGO4 and DRM2. Methylation by DRM2 involves the deposition of a methyl group to the fifth carbon of cytosine residues. Considering these complex

interactions in aggregate, hc-siRNAs ultimately guide DRM2, which is responsible for the methylation of the target regions.

After de novo methylation of the siRNA target regions by RdDM, cytosine methylation is maintained by different methyl transferase enzymes based on the sequence context of the methylated cytosines (reviewed in Chan et al. 2005; Law and Jacobsen 2010). The three possible sequence contexts of cytosine are the symmetric CG and CHG contexts and the asymmetric CHH context, where H stands for A, C, or T. Following DNA replication, fully methylated symmetric cytosines are hemi-methylated: the template DNA strand maintains the methylated cytosine, while the newly synthesized DNA strand is unmethylated. Using the methylated cytosine in the template as a methylation cue, CG and CHG cytosines in the nascent DNA are methylated by METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLTRANSFERASE3 (CMT3), respectively. The enzymatic activity of MET1 is facilitated by the chromatin remodeling factor DECREASED IN DNA METHYLATION1 (DDM1), and there is some degree of cross talk between CMT3 and another repressive chromatin modifier, SUPPRESSOR OF VARIATION3-9 HOMOLOGUE4/KRYPTONITE (SUVH4/KYP), which mediates histone H3K9 methylation. In contrast to CG and CHG residues, CHH residues require hc-siRNAs for methylation maintenance after DNA replication.

## Biological Function

Hc-siRNAs and the RdDM pathway determine the methylation landscape in the genome. Genome-wide analyses in *Arabidopsis* have revealed a high degree of overlap among regions containing transposons and repeats, cytosine methylation, and hc-siRNAs in terms of their distribution and abundance. By guiding methylation at repeats and transposons, hc-siRNAs are critical for the maintenance of genome integrity and gene expression regulation.

Transposons and repeats occupy large portions of the *Arabidopsis* genome (reviewed in Slotkin and Martienssen 2007; Lisch 2009). Some of these elements have the ability to jump to other regions or to amplify themselves, which may disrupt functional genes or be detrimental to the organization of the host genome. However, there are several defense mechanisms that protect the genome from the movement or amplification of transposons and repeats. For example, epigenetic modifications such as cytosine methylation help silence and immobilize repeats and transposons. Reduced cytosine methylation and derepression of the expression of transposons and repeats have been observed in loss-of-function RdDM pathway mutants. Similarly, loss of MET1 and DDM1 function leads to a reduction in cytosine methylation and induces amplification and mobilization of some transposons.

Some repeats and transposons are located in intergenic regions, particularly in the promoters of protein-coding genes, and generate 24-nt small RNAs. These mobile elements are regulated by RdDM, and their methylation level affects the expression of nearby genes. As one example, two tandemly arranged repeats are



found within the promoter of the gene encoding the homeobox transcription factor FLOWERING WAGENINGEN (*FWA*) and are highly methylated in most tissue types in wild-type *Arabidopsis*. Accordingly, *FWA* is transcriptionally silenced in these tissues. However, *FWA* is actively transcribed in the endosperm, where an active demethylation mechanism removes methylated cytosines from the repeats of the maternal *FWA* promoter. In one epigenetic *fwa* mutant, the repeats in the promoter region are hypomethylated, resulting in the transcriptional activation of *FWA* and late flowering.

## Hc-siRNAs and Reproductive Growth

During the reproductive growth stage of *Arabidopsis*, the embryo and endosperm are characterized by opposing hc-siRNA-mediated DNA methylation programs. This difference in DNA methylation is also observed between the gametes and their supporting cells (reviewed in Law and Jacobsen 2010; Castel and Martienssen 2013). The female gametophyte contains the egg cell, the central cell, and five accessory cells, while the male gametophyte contains two sperm cells and one enlarged vegetative cell. The process of double fertilization in angiosperms involves the fertilization of both the egg cell and the central cell by the two sperm cells, thereby producing the embryo (zygote) and the endosperm, respectively. As companion cells, the central cell and the vegetative cell support the development of their adjacent cells, the egg cell and the sperm cells, respectively. Similarly, the endosperm supports the development of the zygote. The gametes and zygote exhibit a sharp contrast with their respective companion cells and the endosperm in terms of hc-siRNA biogenesis and DNA methylation. While the nursing cells lose CG DNA methylation and exhibit increased expression of transposons and siRNAs, the gametes and zygote maintain their CG methylation and other repressive marks at repeats and transposons. It has been proposed that the decrease in CG methylation in companion cells and the increased transcription from transposons and transcribed RNAs enlarge the siRNA pools. Subsequently, transposon-specific siRNAs may be transported from the companion cells and endosperm to the gametes and zygote to enhance transposon silencing through cytosine methylation. Whereas a germ line is established during the early stages of animal embryogenesis, the differentiation of germ cells from somatic stem cells occurs late in the plant life cycle. In effect, the changes in DNA methylation in the companion cells, whose genetic material is not transferred to the next generation, may help overcome problems resulting from the delayed establishment of the germ cells in plants and ensure the integrity of the parental genomes transferred to the offspring. DDM1 and MET1 are repressed in the vegetative cell and the central cell, respectively, resulting in a global decrease in cytosine methylation. In the companion cells of both gametes, DEMETER (DME), an active demethylase enzyme, further reduces the level of methylation through the demethylation of methylated cytosines. Transcripts are generated from the demethylated transposons and made double stranded. The resulting precursor dsRNAs are further processed into 21- and



24-nt small RNAs in the vegetative and central cells, respectively. Expression of a GFP transgene and an artificial transgene-targeting miRNA in the sperm and the vegetative cell, respectively, leads to the suppression of the GFP expressed in the sperm. A similar outcome is also observed in the egg cell and central cell, indicating that siRNAs produced in one cell can move into an adjacent cell and induce silencing during reproductive growth in *Arabidopsis*.

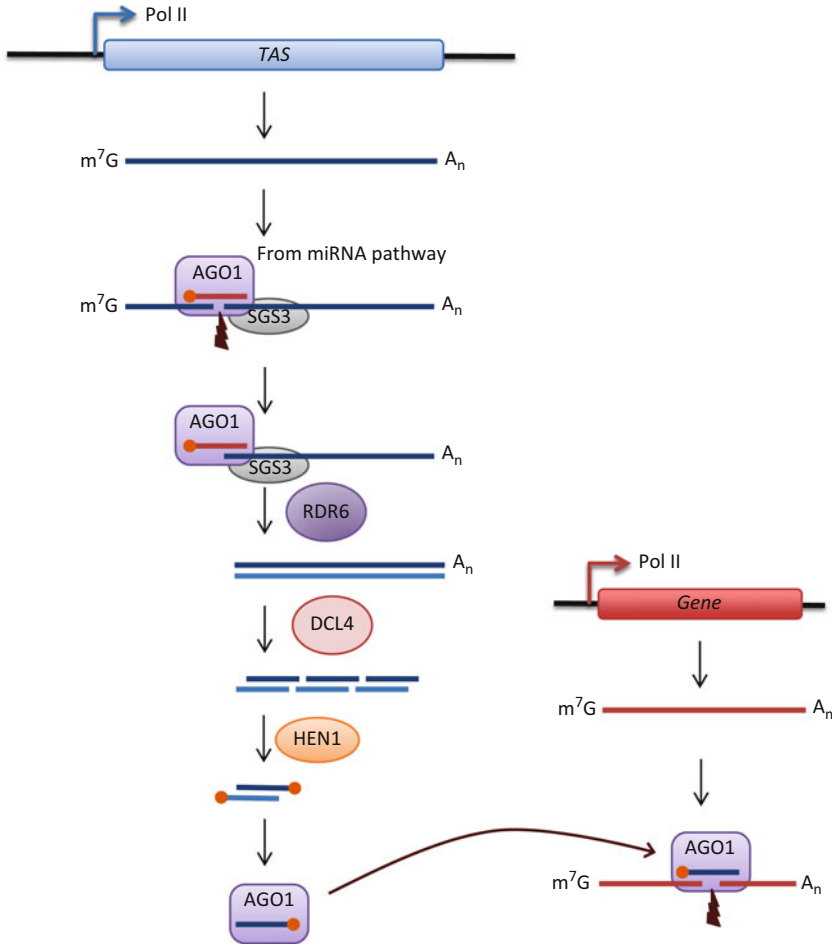
## piRNAs in Animals

A specialized class of small RNAs known as piRNAs is enriched in animal germ line cells and is also present in somatic cells (reviewed in Ishizu et al. 2012; Castel and Martienssen 2013). piRNAs are 25–30 nt in length and are incorporated into an AGO protein belonging to the PIWI clade. These small RNAs guide heterochromatin formation in a manner similar to hc-siRNAs. Unlike small RNAs in plants, however, piRNA biogenesis is Dicer independent and entails a “ping-pong” mechanism of primary biogenesis and amplification; whether one or both of these two processes occur depends on the cell type. *Drosophila* germ cells require three members of the PIWI protein subfamily for piRNA-mediated genome protection: AGO3, Aubergine (AUB), and PIWI. Transposon fragments or relics aggregate into large clusters that generate piRNAs, and the transcription of these piRNA clusters generates long ssRNAs that are antisense to the transposons. During primary processing, these transcripts are processed into antisense piRNAs. These antisense piRNAs exhibit a uridine bias at their 5' ends and bind AUB or PIWI. During the amplification phase, sense RNAs from transcribed transposons are recognized and cleaved by AUB-loaded antisense piRNAs, generating sense piRNAs. Transposon-specific sense piRNAs exhibit an adenosine bias at the 10th nucleotide from the 5' terminus and are incorporated into AGO3. Finally, AGO3-loaded sense piRNAs trigger the biogenesis of antisense piRNAs from long piRNA cluster transcripts, and the ping-pong cycle is reiterated. As sense RNAs from transposons are consumed during the ping-pong cycle, the ping-pong pathway promotes the posttranscriptional silencing of targeted transposons. piRNA-guided slicing requires the endonucleolytic activity of the PIWI-family proteins. Furthermore, piRNAs are stabilized by HEN1-mediated methylation. In addition to the repression of transposons at the posttranscriptional level via transcript slicing, piRNAs also guide the deposition of repressive epigenetic marks at homologous chromatin to induce transcriptional silencing.

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## ta-siRNAs

siRNAs such as hc-siRNAs function at their origin or at homologous regions. Thus, hc-siRNAs act *in cis*, as their sources coincide with their targets. In contrast, *trans*-acting siRNAs (ta-siRNAs) function at loci distinct from the site of their biogenesis. As their name indicates, ta-siRNAs act *in trans*, and their regulatory mechanism is similar to that of miRNAs.



**Fig. 3 ta-siRNA biogenesis and silencing mechanism.** *TAS* transcripts are generated by Pol II and cleaved by miRNA-associated AGO1. The cleaved 3' fragments are protected from degradation by miRNA-containing complexes, RISC and SGS3, and then made double stranded by RDR6. The double-stranded RNAs are processed into 21-nt siRNAs by DCL4 and methylated by HEN1. ta-siRNAs are loaded into AGO1 and regulate their targets in the same manner as miRNA-mediated target repression

## Biogenesis

*Arabidopsis* ta-siRNAs are small regulatory RNAs 21 nt in length whose precursors are generated from *TAS* loci (reviewed in Allen and Howell 2010). Their biogenesis is clearly distinct from that of other small RNAs: transcripts generated from *TAS* loci are targets of miRNA-directed cleavage, and the cleavage products serve as the sources for the biogenesis of secondary siRNAs (Fig. 3). The biogenesis of the

secondary siRNAs is similar to that of hc-siRNAs, following the conversion of the single-stranded cleavage products into dsRNA precursors.

There are four groups of *TAS* genes in *Arabidopsis*. *TAS1* and *TAS3* are each encoded by three isoforms: *TAS1a*, *TAS1b*, and *TAS1c* for *TAS1* and *TAS3a*, *TAS3b*, and *TAS3c* for *TAS3*. *TAS2* and *TAS4* are transcribed from single loci. *TAS* transcripts are non-protein-coding RNAs and contain RNA sequences that are recognized and regulated by miRNAs. miR173 targets both *TAS1* and *TAS2*, and miR390 and miR828 target *TAS3* and *TAS4*, respectively. *TAS* transcripts are subject to miRNA-directed cleavage carried out by miRNA-bound AGO1 or AGO7 (Fig. 3). The stabilization of the cleavage products involves SUPPRESSOR OF GENE SILENCING3 (SGS3), and the ssRNA products are made double stranded by RDR6. The resulting long dsRNA precursors are processed into 21-nt small RNAs by DCL4 in a precisely phased manner: DCL4 successively cleaves the dsRNA precursor beginning at one end and generating multiple small RNAs at 21-nt intervals. Although diverse small RNAs are generated from a single dsRNA precursor, only some of the small RNAs are stable and incorporated into AGO1. Like miRNAs, ta-siRNAs are methylated by HEN1, and their downstream function is similar to that of miRNAs.

Although hundreds of miRNAs have been identified in *Arabidopsis*, only three miRNAs (miR173, miR390, and miR828) are known to initiate the biogenesis of ta-siRNAs. Several studies have revealed specific factors that influence the initiation of secondary siRNA biogenesis: a specialized AGO protein, the length of the miRNAs, the structure of the miRNA/miRNA\* duplex, the position of the miRNA binding site within the target RNA, and the degree of sequence complementarity between the miRNAs and their targets. Among the ten AGO homologs in *Arabidopsis*, AGO7 is the only family member that can generate ta-siRNAs from *TAS3*. AGO7 exclusively binds miR390, which has two target sites in the *TAS3* transcript. The 3' miRNA-target site has nearly perfect complementarity with miR390, and AGO7-mediated cleavage at this site triggers ta-siRNA biogenesis. However, ta-siRNA biogenesis at the *TAS3* locus also requires the 5' target site of miR390 in the *TAS3* transcript, as described by the two-hit trigger model. Although the 5' miR390 target site is resistant to AGO7 cleavage, AGO7 must be recruited to both the 5' and 3' target sites to initiate ta-siRNA biogenesis from *TAS3* transcripts.

miRNA length is another determinant of ta-siRNA biogenesis. While the majority of miRNAs are 21 nt in length, ta-siRNA-generating miRNAs are 22 nt in length with the exception of miR390, which is 21 nt in length. In a transient expression study in *Nicotiana benthamiana*, artificially engineered miRNAs (miR173, miR472, and miR828) 21 or 22 nt in length were tested for their ability to trigger secondary siRNA biogenesis from a co-infiltrated target construct. Only the 22-nt forms of the miRNAs successfully triggered secondary siRNA biogenesis.

The asymmetric structure of the miRNA/miRNA\* duplex has also been found to affect the initiation of ta-siRNA biogenesis. In a transient system similar to that described in the preceding paragraph, four artificial miR173/miR173\* duplexes were examined: 22/21-, 21/22-, and 21/21-nt duplexes with asymmetric bulges along with a symmetric 21/21-nt duplex. The experiment showed that duplexes

with asymmetric bulges could generate secondary siRNAs, regardless of their length. High-throughput sequencing techniques have identified more pairs of miRNAs and mRNAs that generate secondary siRNAs. When miRNAs were found to induce the production of secondary siRNAs from target mRNAs, the miRNA/miRNA\* duplexes were found to contain a 22-nt strand and a 21-nt strand (i.e., either 21/22- or 22/21-nt duplexes). Additionally, the miRNA/miRNA\* duplexes tended to be asymmetrically bulged in terms of their structure. It has been proposed that the AGO1-containing RNA-induced silencing complex (RISC) induces either target repression or secondary small RNA biogenesis based on the structure of the miRNA/miRNA\* duplex.

Two additional factors that influence the initiation of ta-siRNA biogenesis are the location of the miRNA binding site in the target transcript and the degree of sequence complementarity between the miRNA and its target site. In a study in which ta-siRNAs were generated in plants from a synthetic GFP reporter by miR173, the efficiency of ta-siRNA generation was maximal when the miR173-binding site was located immediately after the stop codon. When premature stop codons were introduced further upstream of the miR173-binding site, the abundance of ta-siRNAs decreased while the abundance of longer transcripts from the transgene increased. These observations suggest a link between translation by ribosomes and *TAS* precursor processing. Finally, reduced complementarity at the 3' end of synthetic miR173 has been shown to abolish the generation of ta-siRNAs, while reduced complementarity at the 5' end has a less detrimental effect.

## Molecular Mechanism and Biological Function

As observed for miRNAs, ta-siRNAs regulate the expression of their target genes *in trans*. Furthermore, ta-siRNAs are 21 nt in length, associate with AGO1, and direct PTGS of their targets. *TAS1* ta-siRNAs target several uncharacterized genes and multiple mRNAs encoding pentatricopeptide repeat (PPR) proteins. *TAS2* ta-siRNAs similarly target *PPR* mRNAs. *PPR* genes are commonly found in eukaryotes and appear to have undergone a rapid expansion in plants; the *Arabidopsis* genome, for example, encodes over four hundred *PPR* genes. Some *PPR* proteins bind RNA and are predicted to regulate gene expression through RNA processing, editing, stability, and translation in mitochondria and chloroplasts. Although *PPR* genes are targeted by *TAS1* and *TAS2* ta-siRNAs, the biological relevance of these regulatory interactions remains unclear. Interestingly, *PPR* genes are targeted by both ta-siRNAs and miRNAs, and some transcripts contain multiple small RNA binding sites. Considering the large number of *PPR* genes in plants, ta-siRNAs and small RNAs may serve to dampen the detrimental effects caused by the rapid expansion of gene families.

*TAS3* ta-siRNAs modulate auxin signaling networks by targeting *AUXIN RESPONSE FACTOR2* (*ARF2*), *ARF3*, and *ARF4* and are thus referred to as tasiR-ARF. Auxin is a major plant hormone and is involved in every phase of plant development. Although auxin may affect growth and development through

numerous mechanisms, the mechanism that is best understood is auxin-mediated regulation of gene expression through the ARF and Aux/IAA proteins. In the basal condition, ARF proteins are bound and repressed by Aux/IAA proteins, and auxin-responsive genes are not expressed. When the level of auxin is increased, Aux/IAA is degraded by the ubiquitin-mediated proteasome pathway, and ARF proteins are released from Aux/IAA repression. ARFs recognize auxin-responsive elements in the promoters of downstream genes and activate their expression. Two of the diverse developmental processes affected by tasiR-ARF are phase transition and leaf pattern formation. When the tasiR-ARF binding site in *ARF3* is mutated to make *ARF3* resistant to tasiR-ARF, juvenile plants enter the adult phase prematurely, which is similarly observed in ta-siRNA biogenesis mutants such as *ago7*, *sgs3*, and *rdr6*. Thus, tasiR-ARF suppresses the juvenile-to-adult phase transition. In terms of leaf development, tasiR-ARF is expressed in the adaxial (upper) leaf region, and its movement to the abaxial (lower) region generates a concentration gradient of tasiR-ARF. *ARF3* is expressed throughout the leaf primordia, and *ARF4RNA* is detected in abaxial leaf tissue. Due to the higher concentration of tasiR-ARF in the adaxial region, ARF activity is higher in or restricted to the abaxial leaf region. Thus, the pattern of ARF activity across the adaxial and abaxial regions contrasts that of tasiR-ARF accumulation, and these distinct gradients are critical for polarized leaf pattern formation.

Lastly, *TAS4* ta-siRNAs are predicted to repress genes encoding MYB transcription factors. However, the *TAS4* ta-siRNAs were the last to be identified, likely owing to their low abundance, and their function is currently unknown.

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## Exogenous siRNAs

In addition to endogenously produced small RNAs, plants also contain small RNAs that derive from exogenous sources. In fact, small RNAs were first detected in plants that were infected with viruses and plants harboring transgenes. This pioneering discovery revealed the first clues that small RNAs play an important role in the repression of viruses and transgenes and revolutionized the field of RNA silencing.

## Viral siRNAs (viRNAs)

Plants have adopted a small RNA-mediated repression mechanism to combat viral infection (reviewed in Ding and Voinnet 2007). After infection, plant DCL enzymes generate primary viRNAs from viral dsRNAs, which are produced by viral RDR during replication, by intramolecular hybridization, or by convergent transcription. Primary viRNAs elicit the biogenesis of secondary viRNAs in a manner similar to that of ta-siRNA biogenesis: viral target RNAs are cleaved, the cleavage products are made double stranded by plant RDRs, and DCL enzymes cleave the newly generated double-stranded precursors. Amplified viRNAs are

incorporated into AGO proteins and repress the virus through PTGS. Multiple DCLs, RDRs, and AGOs in host plants have redundant functions, work in tandem, and/or perform a specialized function to defend plants against viral infection. The activities of these proteins also depend on the type of viral infection.

In response to the antiviral defense of the host plant, viruses have also developed mechanisms to counteract the host response. Numerous viruses encode viral suppressors of RNA silencing (VSRs) that oppose the repressive action of viRNA-mediated silencing in the host (reviewed in Burgyán and Havelda 2011). Specifically, VSRs intercept viral dsRNAs or silencing factors generated by the host plant. P19 from *Cymbidium ringspot virus* and P21 from *beet yellows virus* sequester short dsRNAs, effectively disrupting RISC assembly with viRNAs in the host. Other VSRs are capable of binding AGO proteins: 2b from *cucumber mosaic virus*, P0 from *beet polerovirus*, P1 from *sweet potato mild mottle virus*, and P38 from *turnip crinkle virus*. 2b inhibits the slicing activity of AGO1 in preassembled RISC. P0 contains a minimal F-box motif that may induce AGO1 degradation. P1 and P38 contain an AGO-hook GW/WG motif and may therefore compete with endogenous AGO-binding proteins in plants (Azevedo et al. 2011). Other components of the plant silencing machinery are also targeted by viruses (reviewed in Burgyán and Havelda 2011). For example, the binding of V2 from *tomato yellow leaf curl virus* to SGS3 compromises RDR6-mediated secondary viRNA biogenesis. Additionally, HC-Pro from *zucchini yellow mosaic virus* disrupts the methylation of small RNAs by HEN1.

## siRNAs from Transgenes

Early studies in which plants were transformed with sense transgenes revealed the suppression of both transgenes and endogenous homologous genes in several transgenic lines, and the silencing phenomenon was termed co-suppression. Subsequent studies revealed that transgene-specific small RNAs accumulate in silenced plants and that proteins required for small RNA biogenesis and action are also involved in transgene silencing. In cases of transgene silencing, the ssRNA transcripts generated from the transgene are recognized by the plant machinery as aberrant and made double stranded by RDR6. The dsRNA subsequently triggers downstream events, including primary and secondary siRNA biogenesis. As a result, both the transgene and endogenous homologous genes are targeted for silencing.

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## Small RNA Turnover

Consistent with the critical roles of small RNAs in diverse biological processes, the abundance of small RNAs is also precisely regulated. Disrupting the homeostasis of small RNAs detrimentally affects developmental and metabolic processes. Because the abundance of small RNAs is affected by both internal and external signals, the

balanced expression of small RNAs requires a precise regulatory mechanism. In plants, small RNA biogenesis and turnover are the critical phases for regulating the dynamics of small RNA populations (reviewed in Ji and Chen 2012).

The methylation of small RNAs during biogenesis is crucial for their stabilization. Small RNAs in *Arabidopsis* are methylated at the 2'-OH of the 3' terminal ribose by HEN1. In *hen1* mutants, the abundance of small RNAs is dramatically reduced, and the residual small RNAs are tailed or trimmed. High-throughput sequencing of the small RNA population in *hen1* further revealed that the residual small RNAs are identical at their 5' ends but heterogeneous at their 3' ends. Specifically, the small RNAs were found to have oligonucleotide tails 1–7 nt in length, with a predominant enrichment of uridine among the four nucleotides. Furthermore, truncation from the 3' terminus was observed for both intact and uridylylated small RNAs in the *hen1* mutant. Thus, HEN1-mediated methylation at the 3' end of small RNAs ultimately inhibits their degradation.

In *Arabidopsis*, the SMALL RNA-DEGRADING NUCLEASE (SDN) family of 3'–5' exonucleases is responsible for small RNA degradation. When multiple SDN genes are simultaneously knocked down, increased miRNA levels and pleiotropic developmental defects are observed. SDN1 specifically degrades 17–27-nt single-stranded small RNAs, and its activity is partially inhibited by 2'-O-methylation at the 3' end of small RNAs. Based on these observations, the removal of the 3' most nucleotide by SDNs may be rate limiting and probably requires other assistant proteins or the combined activity of multiple SDNs.

From both forward and reverse genetic studies of the *hen1* mutant, HEN1 SUPPRESSOR1 (HESO1) was found to poly-uridylylate small RNAs in *hen1* mutants. In contrast to the protective function of methylation, uridine tails at the 3' end of small RNAs make miRNAs more susceptible to 3'–5' exonuclease activity. Consistent with the hypothesis that a defect in uridylation activity should rescue the loss of methylation in *hen1* (i.e., unmethylated small RNAs that do not undergo uridylation should be less susceptible to 3'–5' exonuclease activity), a mutation in *HESO1* suppresses the morphological defects of *hen1* mutants. Compared to *hen1*, miRNA levels are increased in *hen1 heso1*. However, tailed and trimmed miRNAs are still observed in the double mutant. Based on in vitro analysis, HESO1 has terminal nucleotidyl transferase activity with a preference for uridine substrates, and HESO1 function is completely inhibited by 2'-O-methylation at the 3' end of small RNA substrates. High-throughput small RNA data for the *hen1 heso1* double mutant reveal shorter uridine tails in the double mutant compared to *hen1*, which further suggests that HESO1 is partially responsible for uridylation in the *hen1* mutant.

In addition to HEN1, SDN exonucleases, and HESO1, long RNA molecules may influence the rate of degradation of specific small RNAs. In a technical analysis of target mimicry, a short tandem target mimic (STTM), composed of two short sequences mimicking small RNA binding sites tandemly arrayed with an optimal spacing between them, was found to reduce the abundance of miRNAs whose binding sites were mimicked by the STTM. Interestingly, the reduction in miRNA abundance was dependent on SDN1 and SDN2 activity. Similarly,

although less effectively, other artificial target mimicry transgenes led to reductions in the levels of cognate miRNAs. This suggests that target transcripts, especially those that cannot be cleaved by miRNAs, impact the stability of miRNAs. This raises the question of whether such targets exist naturally.

In *Arabidopsis*, miR399 recognizes two target RNAs: the mRNA transcript corresponding to the E2 ubiquitin conjugation enzyme PHOSPHATASE (PHO2) and the *INDUCED BY PHOSPHATASE STARVATION1* (*IPS1*) ncRNA. miR399 is induced under phosphate (Pi) starvation conditions and represses the activity of PHO2 through mRNA cleavage as an adaptive response that alters the metabolism of Pi in plants. In general, signaling cascades triggered by a certain event or treatment are eventually attenuated, and the recovery of steady expression levels facilitates the response to a prolonged stimulus. In a similar way, *PHO2* is temporarily silenced by miR399 under Pi starvation conditions but eventually achieves a steady level of activity, which is mediated by target mimicry. Long *IPS1* ncRNAs are also induced by Pi deficiency and sequester miR399 from *PHO2* mRNAs. Unlike *PHO2* mRNA, which is subject to miRNA-directed cleavage, *IPS1* ncRNAs are bound but not sliced by miR399 due to a mismatch at the cleavage site. Although *IPS1* ncRNAs do not alter the in vivo abundance of miR399, they suppress the effect of miR399 on *PHO2*.

A recent study identified many *IPS1*-like intergenic long ncRNAs that can pair with other miRNAs. Overexpression of some of the long ncRNAs led to a decrease in the abundance of the cognate miRNAs, raising the possibility that long ncRNAs regulate the stability of specific miRNAs in vivo.

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## Future Directions

While the overall framework of miRNA biogenesis is relatively well established, many aspects of the regulation of miRNA biogenesis remain unclear. The abundance of mature miRNAs is regulated by Pol II-mediated transcriptional regulation and during the processing of pri-miRNAs to mature miRNAs. However, it is also possible that the processing of miRNA precursors is also directly affected by the endogenous or exogenous signals integrated by Pol II. Furthermore, unique factors may differentially regulate certain miRNA species during the process of miRNA maturation. In terms of the activities directed by miRNAs, the molecular mechanisms of mRNA cleavage and translational inhibition require further study. It has been proposed that the extent of sequence complementarity between miRNAs and their targets dictates the mode of repression by miRNAs. However, this is unlikely because miRNAs with a high degree of sequence complementarity to their targets have also been shown to act via translational repression. In fact, the two modes of action may occur simultaneously for a given miRNA-target pair. The degree of miRNA-target complementarity that is required for translational repression has not been experimentally determined. If less extensive base pairing is sufficient to induce translational inhibition as observed in animals, the current views of the



regulatory networks between miRNAs and their targets would need to be reevaluated. The translational repression activity of plant miRNAs also needs to be dissected at the mechanistic level. For instance, it is unknown how and at what step (e.g., ribosome loading, elongation, or termination) miRNAs inhibit protein synthesis carried out by ribosomes.

Through intensive genetic studies, the key players in hc-siRNA biogenesis and DNA methylation have been identified. Additionally, high-throughput methylome analysis has provided a wealth of information about targets methylated by RdDM at the nucleotide level. Nevertheless, major aspects of hc-siRNA biogenesis and cytosine methylation are not understood or require further experimental evidence. For example, although Pol IV is essential for the biogenesis of hc-siRNAs, Pol IV-dependent transcripts have not yet been detected. How Pol IV recognizes, and is recruited to, the promoters of these transcripts is also not known. The recruitment of DRM2 to target regions is known to be mediated by small RNAs and Pol V-dependent transcripts, but the underlying molecular mechanism remains to be elucidated. Along with cytosine methylation, there are other epigenetic modifications that undoubtedly contribute to TGS, including histone modification, histone variants, chromatin condensation, and higher-order chromatin structures. Future studies will need to establish the relationships between these different types of modification and address how cross talk among them governs the epigenetic landscape.

Although factors that favor ta-siRNA biogenesis have been uncovered, the biological function of ta-siRNAs and their targets remains enigmatic. Particularly, *PPR* genes are abundant in the *Arabidopsis* genome, but the underlying cause of the rapid expansion of this gene family is unclear, as is the functional relevance of the regulation of *PPR* genes by ta-siRNAs.

Mature small RNAs are loaded into AGO effector proteins to direct silencing activity, and the association with AGO proteins may protect small RNAs from harmful enzymatic activity, such as degradation by SDN1 or uridylation by HESO1. The molecular mechanism by which small RNAs are dislodged from AGO proteins and subsequently degraded is not well characterized. Another possibility is that small RNAs may be degraded while they are associated with AGO proteins. Both uridylation and 3' truncation mechanisms that affect small RNAs warrant further study, not only in terms of the underlying molecular events but also with respect to whether and how these mechanisms are orchestrated in tandem. The fact that loss of HESO1 function reduces but does not eliminate the uridylation of miRNAs suggests the existence of other enzymes with overlapping functions. Moreover, there may be regulatory factors that determine the rate of degradation and sequester or degrade specific small RNAs in response to a signal or stress. Lastly, recent findings about small RNA turnover induced by small RNA target mimics challenge the current understanding of SDN exonuclease activity. Further study is required to address how SDN enzymes, which specifically degrade single-stranded small RNAs, may also be involved in the degradation of sequestered or bound small RNAs.

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