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Small RNA metabolism in *Arabidopsis*

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Abstract

The *Arabidopsis* genome encodes two major classes of riboregulators ranging from 20 to 24 nucleotides in length: microRNAs (miRNAs) and small interfering RNAs (siRNAs). These small RNAs act as sequence-specific repressors of target gene expression either at the transcriptional level through DNA and/or histone methylation or at the posttranscriptional level through transcript cleavage or translational inhibition. Small RNAs are processed from precursor RNAs by one or more of the four DICER-LIKE (DCL) RNase III enzymes. Small RNAs are then modified with a 2'-O-methyl group on their 3' terminal ribose by HUA ENHANCER 1 (HEN1), a small RNA methyl transferase. In the absence of methylation, small RNAs become oligouridylated and/or degraded. The function of oligouridylation of small RNAs is currently unknown, but it is likely to cause small RNA degradation. Here, we review the biogenesis of small RNAs with an emphasis on the methylation and uridylation of small RNAs. We also discuss the major outstanding questions in small RNA metabolism.

Introduction

In eukaryotes small non-protein-coding RNAs of 20–30 nucleotides (nt) have emerged as key guide molecules to regulate various biological processes including developmental transition and patterning, responses to environment, maintaining genome stability, and defense against viruses and bacteria. One common theme underlying the actions of small RNAs is that an effector protein belonging to the argonaute (AGO) family binds a small RNA and confers the regulatory functions on target genes (reviewed in¹). Argonaute proteins are classified into two phylogenetic subfamilies, the argonaute subfamily and the piwi subfamily. Whereas animals have members of both subfamilies, plant AGO proteins all belong to the argonaute subfamily. Concomitantly, plants appear to lack a class of RNAs known as piRNAs that are bound by piwi subfamily of argonaute proteins in animals (glossary).

microRNAs (glossary) were first identified through forward genetic screens as regulators of developmental timing in *C. elegans*^{2, 3} and later found to be nearly universally present in eukaryotes^{3–8}. The fact that some miRNAs are conserved in sequence over long evolutionary distances^{9, 10} and the presence of miRNAs in the unicellular green alga *Chlamydomonas reinhardtii*^{11, 12} suggest that miRNA-based gene regulation is an ancient and evolutionarily conserved mechanism. miRNAs are processed from precursor RNAs that form hairpin structures by RNase III enzymes (Drosha and Dicer in animals and DCL1 in plants) and are bound by an AGO subfamily member and inhibit their target genes by causing translational inhibition or RNA degradation (reviewed in¹³).

siRNAs (glossary) were first found in plants undergoing post-transcriptional gene silencing¹⁴ and later found to be universal effectors of RNA silencing in plants, fungi, and animals. However, siRNAs are not always directed towards transgenes or invading viruses.

Large numbers of endogenous siRNAs have been discovered in *C. elegans*, rice and *Arabidopsis*^{15–19}. In *Arabidopsis*, several distinct classes of endogenous siRNAs have been uncovered and found to regulate gene expression at the transcriptional and post-transcriptional levels. The regulatory functions of these endogenous siRNAs range from genome stability, responses to stress conditions, to developmental patterning.

Major players in miRNA biogenesis and function

The *Arabidopsis* genome contains hundreds of miRNA encoding regions designated as *MIR* genes. Unlike *MIR* loci in animals that tend to contain clusters of miRNAs and that are transcribed into polycistronic precursors (reviewed in²⁰), plant *MIR* genes are usually not clustered and each gene is transcribed into a precursor that gives rise to a single miRNA species. Like protein coding genes, *MIR* genes are transcribed by RNA polymerase II (Pol II) and the primary transcripts (pri-miRNAs) are 5'-capped and 3'-polyadenylated²¹. A pri-miRNA contains a local hairpin structure in which the miRNA is embedded. The processes that generate the miRNAs from the pri-miRNAs are similar between plants and animals.

In animals, miRNAs are generated in two successive endonucleolytic steps (reviewed in²⁰): an initial nuclear step converting pri-miRNAs into pre-miRNAs by Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) protein, a double-stranded RNA (dsRNA) binding protein. This is followed by processing of pre-miRNAs into miRNA/miRNA* duplexes by Dicer in association with another dsRNA-binding protein, human immunodeficiency virus trans-activation responsive RNA-binding protein (TRBP) in the cytoplasm. In between, Exportin-5 transports the pre-miRNAs from the nucleus to the cytoplasm (reviewed in²⁰).

In plants, miRNAs are generated from pri-miRNAs in a two- or sometimes multi-step process involving mainly DCL1 to yield miRNA-miRNA* duplexes^{8, 22, 23} (Figure 1). These processes are assisted by HYPONASTY LEAVES1 (HYL1), a dsRNA binding protein^{24, 25}. Mutations in either gene result in reduced accumulation of miRNAs and a concomitant increase in pri-miRNA levels²⁶. DCL1 and HYL1 interact with each other *in vitro* and co-localize in the cell, suggesting that they form a complex to process pri-miRNAs^{26–29}. SERRATE (SE), a zinc finger protein, interacts with DCL1 and HYL1 and also plays a role in the processing of pri-miRNAs to miRNAs since miRNAs are reduced in abundance while their pri-miRNAs accumulate to higher levels in *serrate* mutants^{28, 30, 31}. After the miRNA/miRNA* duplex is released from the pre-miRNA, the miRNA strand is selectively incorporated into the so-called RNA-induced silencing complex (RISC), whose major, if not only, protein component in *Arabidopsis* is ARGONAUTE 1 (AGO1)^{32, 33} (Figure 1). AGO1 has endonucleolytic activity that cleaves the target mRNA in the middle of the mRNA-miRNA duplex^{32, 33}. HASTY, an Exportin-5 homolog, is thought to be involved in the transport of miRISCs or miRNA/miRNA* duplexes from the nucleus to the cytoplasm³⁴ (Figure 1).

Although the biogenesis of the great majority of *Arabidopsis* miRNAs depends solely on DCL1 among the four DCLs, a few exceptions exist. miR822 and miR839 are DCL4- rather than DCL1-dependent³⁵. This is likely due to the extensive sequence complementarity in the stem-loop precursors, which makes these precursors better substrates for DCL4.

Feed-back autoregulation

miRNA biogenesis is under feedback regulation, a mechanism that helps ensure proper overall levels of miRNAs. The *DCL1* gene encoding the predominant miRNA Dicer is itself a target of miR162. miR162-directed cleavage product of *DCL1* mRNA is detected, indicating that *DCL1* expression is under negative regulation by miR162³⁶. In addition, one of the *DCL1* introns hosts a miRNA, miR838³⁵. This is a rare example where a *MIR* gene is located in the intron of a protein coding gene in *Arabidopsis*. Intronic miRNAs are very frequent in animals

and the processing of these miRNAs does not appear to interfere with the splicing of the host RNAs³⁷. In case of *DCL1*, however, the processing of *DCL1* pre-mRNA to release pre-miR838 probably generates two truncated transcripts that accumulate but that cannot give rise to *DCL1* mRNA^{35, 36}. Therefore miR838 may also limit *DCL1* expression. *AGO1*, which encodes a slicer that mediates the functions of miRNAs, is negatively regulated by miR168³⁸. miR403 targets *AGO2*, however, the biological significance of *AGO2* in small RNA metabolism remains unknown.

Major siRNA biogenesis pathways

siRNAs are derived from long dsRNAs, which are generated through the transcription of inverted repeats or converted from single-stranded RNAs by RNA-dependent RNA polymerases (RDR). The dsRNAs are then processed into multiple siRNAs through the action of one or more of the four DCL proteins in *Arabidopsis*. Cloning followed by deep sequencing of small RNAs from *Arabidopsis* and rice show that numerous genomic loci give rise to siRNAs^{16–18, 39, 40}. The biogenesis of three classes of endogenous siRNAs is best understood: RDR2-dependent siRNAs, *trans*-acting siRNAs (ta-siRNAs), and natural antisense siRNAs (nat-siRNAs).

RDR2-dependent siRNAs represent the largest and most diverse set of siRNAs in *Arabidopsis*. Deep sequencing of siRNAs from wild type and an *rdr2* mutant shows that these siRNAs can be mapped to loci throughout the five chromosomes but that pericentromeric regions have the highest density of distinct siRNAs as well as the highest number of siRNAs¹⁶. RDR2-dependent siRNAs are preferentially associated with transposon and retroelement loci and repetitive DNA, and tend to correspond to regions containing DNA methylation^{16, 17, 40}. At some loci, it has been shown that these siRNAs lead to transcriptional gene silencing by causing DNA methylation or histone H3 lysine 9 methylation^{41, 42}. The biogenesis of these siRNAs requires RDR2⁴², which presumably converts a transcript from an siRNA generating locus into dsRNA. DCL3 then processes the dsRNA into 24 nt siRNAs⁴². In the absence of DCL3, the other three DCLs can also process the dsRNAs into 21 nt or 22 nt siRNAs^{16, 39, 42}. 24 nt siRNAs are bound by AGO4, and perhaps AGO6, effectors that are required for DNA methylation and histone methylation at the target loci^{42–45}. The biogenesis of these siRNAs also requires one isoform of DNA-dependent RNA polymerase IV, pol IVa, and to some extent, another isoform pol IVb^{46–50}. Pol IVa probably acts in the generation of dsRNA precursors to the siRNAs, while Pol IVb physically interacts with AGO4 and is thought to somehow facilitate siRNA-directed DNA or histone methylation^{51–53}.

Trans-acting siRNAs (ta-siRNAs) are a class of endogenous siRNAs that act *in trans*, i.e. they regulate target genes that are different from their originating loci (reviewed in⁵⁴). In *Arabidopsis* four *TAS* family genes (*TAS1* to 4) encode non-protein coding transcripts that are themselves targets of miRNAs. *TAS1* (*a*, *b* and *c*) and *TAS2* are targets of miR173, *TAS3* is targeted by miR390 and *TAS4* is a target of miR834^{35, 55–57}. The *TAS* transcripts are cleaved at specific positions by the corresponding miRISCs^{35, 55–57}. The cleavage fragments are probably stabilized by SGS3 and converted into dsRNAs by RDR6^{56, 57}. DCL4 then processes the dsRNAs into 21 nt ta-siRNAs from one end of the dsRNA defined by the cleavage event^{55, 58–60}. Therefore, specific miRNA-programmed cleavage of the *TAS* transcripts triggers the production of ta-siRNAs and sets the registry for ta-siRNA production at 21 nt increments from the cleavage site⁵⁵. A dsRNA binding protein (DRB4) that is associated with DCL4, and SDE5, a putative homolog of a human mRNA export factor, also participate in ta-siRNA biogenesis^{61–63}. ta-siRNAs act through either AGO1 or AGO7 to regulate their target genes at the post-transcriptional level^{32, 61}. Notable ta-siRNA targets include *ARF3* and

ARF4, transcription factor genes that modulate auxin responses and plant development^{55, 61, 64}.

Nat-siRNAs are produced from loci with overlapping bidirectional transcripts. The two best studied nat-siRNAs are induced upon abiotic or biotic stresses and act in the stress response by down-regulating one of the gene pair that gives rise to the nat-siRNAs^{65, 66}. The biogenesis of the nat-siRNAs requires RDR6 and one or two of the DCL proteins.

Subcellular locations of small RNA biogenesis

miRNA processing may occur in subnuclear bodies. When fused to fluorescent proteins and expressed in *Arabidopsis* or *N. benthamiana*, DCL1 and HYL1 are co-localized in nuclear bodies, termed Dicing bodies or D-bodies, in addition to diffuse nucleoplasmic localization^{27–29}. The D-bodies do not co-localize with nucleolin, a nucleolus marker or Atcoilin, a marker for Cajal bodies, and are still present in an Atcoilin mutant lacking Cajal bodies, suggesting that D-bodies are not Cajal bodies^{27–29}. D-bodies may also include SmD2 and SmD3 proteins that are also found in Cajal bodies²⁸. Two pri-miRNAs have been found to co-localize with DCL1 and HYL1 in D-bodies, suggesting that D-bodies are miRNA processing centers or storage sites of the processing machinery or substrates^{27, 28}. The functional importance of D-bodies in miRNA biogenesis cannot be confirmed without the identification of mutations or conditions that disrupt D-bodies.

The processing of RDR2-dependent siRNAs may also occur in subnuclear bodies. A number of siRNAs and an siRNA precursor are concentrated in a subnucleolar body. A number of proteins in the biogenesis pathway, such as RDR2, DCL3, AGO4, and pol IVb, are co-localized with siRNAs in this body^{52, 53}. The co-localization of AGO4 with Cajal body markers identifies this site as the Cajal body⁵². It is thought that siRNA precursors are transported to Cajal bodies, where they are processed to siRNAs and where siRNAs are bound by AGO4 and pol IVb. The functional importance of Cajal bodies in siRNA processing and RISC assembly still awaits further interrogation of mutants lacking Cajal bodies.

Small RNA modification by HEN1

In plants, small RNA biogenesis requires an additional step apart from the endonucleolytic RNase III cleavage steps. After miRNA/miRNA* or siRNA/siRNA* duplexes are released from their precursors by the DCL proteins, the duplexes are methylated on the 2' OH of the 3' terminal ribose by HEN1, a small RNA methyltransferase^{67, 68}. *In vitro* biochemical studies using purified recombinant HEN1 protein show that HEN1 methylates miRNA and siRNA duplexes in a sequence-independent manner. HEN1 prefers 21 to 24 nt RNA duplexes with 2-nt overhangs at their 3' ends, typical features of DICER products. miRNAs and siRNAs in wild-type plants but not *hen1* mutants are resistant to chemical reactions that target the 2' and 3' OH in the 3' terminal nucleotide, suggesting that at least one of the OH groups on the last ribose of small RNAs is modified *in vivo*⁶⁸. Mass spectrometry analysis of affinity purified miR173 from plants revealed the presence of a single methyl group⁶⁸. These studies demonstrate that small RNAs in *Arabidopsis* are present in 2'-O-methylated forms.

HEN1 homologs are present in various invertebrate and vertebrate animals⁸. Although animal miRNAs are not methylated, piRNAs are 2'-O-methylated by HEN1 homologs^{69–71}.

2'-O-methylation protects small RNAs from degradation and uridylation

The protective role of the 2'-O-methyl group is recognized from analyses of miRNAs in *hen1* mutants that lack small RNA methylation⁷². In *hen1* mutants, miRNAs fail to accumulate or accumulate to lower levels. When they are detectable by Northern blotting, miRNAs appear

as a ladder of bands rather than as a single species⁷². miRNA cloning and sequencing revealed the presence of a number of U residues at the 3' end in *hen1* mutants, indicating that unmethylated miRNAs are prone to an unknown enzymatic activity that leads to oligouridylation⁷². Also, clones representing 3'-to-5' shortening of miRNAs were found, implicating exonucleolytic degradation in the absence of methylation⁷². Therefore, methylation protects small RNAs from uridylation and degradation. siRNAs, such as ta-siRNAs, RDR2-dependent siRNAs, and transgene-derived siRNAs all become heterogeneous in size in *hen1* mutants, suggesting that oligouridylation also happens to siRNAs lacking methylation⁷².

Uridylation of RNAs has been found in other circumstances and has been correlated with RNA decay. The 5' fragment of an mRNA cleaved by an miRISC becomes uridylated at its 3' end and the uridylation is accompanied by 5'-to-3' degradation of the fragment⁷³. In humans, histone mRNAs lack a polyA tail and their degradation is triggered by 3' uridylation⁷⁴. In case of miRNAs, it is possible that uridylation recruits an exonuclease to degrade the miRNAs.

Outstanding questions in small RNA metabolism

The last few years saw rapid progress in our understanding of small RNA metabolism in *Arabidopsis* such that the main framework of small RNA biogenesis is established. However, key outstanding questions remain to be addressed. First and the foremost is how small RNAs are turned over. Currently we know nothing about the stability of small RNAs or the enzymes that lead to their degradation. Uridylation and exonucleolytic degradation are implicated in miRNA degradation, but the enzymes catalyzing these reactions remain to be uncovered and the functions of these processes in miRNA metabolism await genetic confirmation by analysis of mutants in the corresponding genes. Another fundamental question is how some loci give rise to endogenous siRNAs while others do not. siRNAs tend to be associated with repetitive loci, but this is not true for all siRNAs. What are the defining features that predispose siRNA production? Finally, how small RNAs are loaded onto specific RISC complexes is completely unknown. There are 10 AGO proteins in *Arabidopsis* and there is clear division of labor among the ones that have been studied. For example, AGO1 mediates the actions of miRNAs and transgene siRNAs, AGO4 and AGO6 mediate the functions of RDR2-dependent siRNAs, and AGO7 acts with ta-siRNAs from the *TAS3* locus. However, how different small RNAs are channeled to different AGO proteins is unknown. Perhaps subcellular localization of small RNA biogenesis plays a role in the specificity of RISC loading. The functions of the remaining AGO proteins have yet to be elucidated.

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Glossary

Dicer

is an RNase III enzyme that cleaves double-stranded RNAs or single-stranded RNAs with imperfectly double-stranded hairpin regions to release 20–25 nt small RNAs

miRNAs

are 21–24 nt RNAs as the final products of non-protein coding genes. miRNAs are processed from longer, single-stranded precursor RNAs that form hairpin structures by Dicer and guide a silencing effector complex to target mRNAs through sequence complementarity

siRNAs

are 21–24 nt RNAs that are processed by Dicer from long double-stranded RNAs or single-stranded RNAs with extensive, perfect or near perfect hairpins. siRNAs can be derived from endogenous loci, transgenes, or viruses. Usually more than one siRNA species accumulate from a single precursor RNA. siRNAs guide a silencing effector complex to homologous DNA loci to trigger transcriptional gene silencing or to target mRNAs to cause transcript cleavage

Piwi-interacting RNAs

or piRNAs, are 25–30 nt RNAs found in the germline of invertebrate and vertebrate animals. They are generated in a Dicer-independent manner and act in transposon silencing and genome stability

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Figure 1.

Schematic illustration of miRNA metabolism in *Arabidopsis*. (A) Major steps in miRNA biogenesis. Primary miRNA transcripts (pri-miRNAs) are processed into precursor miRNAs (pre-miRNA) and then to miRNA/miRNA* duplexes by the coordinated activities of DCL1, HYL1 and SE within the nucleus. miRNA and miRNA* strands are represented in red and green, respectively. Both strands of the miRNA/miRNA* duplex are 2'-*O*-methylated on the 3' terminal ribose by HEN1, a small RNA methyltransferase (The 2'-*O*-methyl groups are represented by blue squares). The miRNA strand gets incorporated into an AGO1 containing RNA-induced silencing complex (miRISC) and directs either mRNA cleavage or translational inhibition. miRISCs or an intermediate, such as miRNA/miRNA* or methylated miRNA/miRNA*, in the biogenesis pathway is exported from the nucleus (represented by the oval) to the cytoplasm by the Exportin-5 homolog HASTY. (B) The position of the methyl group in miRNAs. In wild-type plants, HEN1 adds a methyl group (CH₃, shaded box) at the 2' position of the last ribose. In *hen1* mutants, miRNAs are not methylated and have a 2' hydroxyl group (shaded box). (C) A diagram depicting the abundance and modification of miRNAs in wild-type plants and in *hen1* mutants. The abundance and size of miRNAs are represented by the numbers and length of the lines, respectively. In wild-type plants, miRNAs are methylated and accumulate as full-length molecules. In *hen1* mutants, miRNAs are not methylated and accumulate at much lower levels as modified molecules. The modification includes 3' truncation, 3' uridylation, or 3' truncation plus 3' uridylation.

Figure 2.