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Mechanisms of microRNA turnover

Maryam Sanei[1] and **Xuemei Chen**[1,2,*]

[1]Department of Botany and Plant Sciences, Institute of Integrative Genome Biology, University of California, Riverside, CA

[2]Howard Hughes Medical Institute, University of California, Riverside, CA

Abstract

MicroRNAs (miRNAs) are 20–24 nucleotide (nt) RNAs that regulate gene expression by guiding Argonaute (AGO) proteins to specific target RNAs to cause their degradation or translational repression. The abundance of miRNAs is strictly controlled at the transcriptional or post-transcriptional levels. miRNA turnover is presumably a necessary means to regulate miRNA levels in response to physiological, developmental, and environmental changes. miRNA 3' end methylation, 3' end nucleotide addition, AGO and complementary target transcripts are known or probable processes/factors that affect miRNA stability and turnover. Here we discuss the mechanisms that control miRNA turnover in plants and, where applicable, make references to similarities and differences in these mechanisms between plants and animals.

The biogenesis of miRNAs

Regulation of gene expression by miRNAs helps to achieve the proper levels as well as the spatial and temporal patterns necessary for the biological functions of target genes. As regulatory molecules, miRNAs are known to impact plant and animal growth, development, metabolism, defense, and stress responses. Aberrant expression of miRNAs has been linked to many diseases in humans [1–3] and severe developmental abnormalities in *Arabidopsis* [4], and mice lacking miRNAs can not survive [5]. In plants, RNA polymerase II (Pol II) transcribes miRNA genes (*MIR*) to result in pri-miRNAs that are 5' capped and 3' polyadenylated (miRNA biogenesis is reviewed in [6–8]). A primiRNA is cleaved at least twice by DICER-LIKE1 (DCL1) to form first the pre-miRNA and then the miRNA/miRNA* duplex. The duplex is methylated on the 3' ends by HUA ENHANCER1 (HEN1). The miRNA strand is selectively loaded into ARGONAUTE1 (AGO1) to form the miRNA-Induced Silencing Complex (miRISC), and the passenger strand (miRNA*) is usually degraded. In plants, an miRISC targets specific mRNAs for cleavage or translational repression (Fig 1).

*Corresponding author: xuemei.chen@ucr.edu.

The widespread roles of miRNAs in gene regulation suggest that miRNAs themselves need to be tightly regulated. The steady-state levels of miRNAs can be controlled at multiple levels, such as transcription of *MIR* genes, processing of precursors, and turnover of mature miRNAs. Transcriptional regulation of *MIR* genes, like that of protein coding genes, relies on transcription factors and promoter activity. The TATA box [9] and many transcription factor binding motifs are present in the promoters of *MIR* genes in *Arabidopsis* [10–12]. Several transcription factors have been implicated or shown to influence *MIR* expression through binding sites in *MIR* promoters [10,11,13–15]. In plants, conserved miRNAs tend to belong to gene families with more than one member such that identical or highly similar miRNAs can be produced from different *MIR* genes. *MIR* family members can differ considerably in gene structure and regulatory sequences and undergo transcriptional regulation differently in response to environmental changes or developmental cues [9,11,13,15–19].

The biogenesis of miRNAs is regulated at the global level through feedback on *DCL1* and *AGO1*, critical players in miRNA biogenesis. The expression of *DCL1* and *AGO1* is repressed by two miRNAs, miR162 and miR168, respectively [20,21]. Feedback control by miR168 is necessary as *Arabidopsis* expressing miR168-resistant *AGO1* exhibits developmental defects [22].

Knowing that miRNAs are on average ten times more stable than mRNAs [23] and that there may be needs for rapid changes in miRNA levels in biological settings makes the turnover of mature miRNAs a potentially important way of miRNA regulation. In this review, we summarize recent progress in the research on miRNA stability and turnover.

3’ methylation protects miRNAs from degradation

5′ cap structures and 3′ polyA tails are added to most protein-coding Pol II transcripts to protect them from degradation. Unprotected 5′ or 3′ ends leave mRNAs vulnerable to exonucleolytic decay pathways. While pri-miRNAs are capped and polyadenylated, mature miRNAs lack any 3’ poly A or 5’ cap structures. 3’ methylation protects nearly all miRNAs in plants from degradation. HEN1 proteins methylate the 3’ ends of miRNAs and siRNAs in plants [24,25] and some siRNAs and nearly all PIWI-interacting RNAs (piRNAs) in animals [26–31]. The 2’-*O*-methyl mark protects small RNAs from 3’ end uridylation and 3’->5’ exonucleolytic degradation [32]. The HEN1 protein in *Arabidopsis* consists of five structural domains, four of which directly interact with the small RNA substrate: a methyltransferase domain (MTase), two double-stranded RNA binding domains (dsRBD1 and dsRBD2) and a La-motif containing domain (LCD) that binds specifically to the 3’ protruding end of the small RNA duplex. *Arabidopsis* HEN1 prefers to act on short double-stranded RNAs (19–25 nt) with 2 nt 3’ overhangs [33]. Substrate length recognized by HEN1 is determined by the distance between the active site of the MTase domain and the 5′ end-capping site in the LCD [33].

Arabidopsis HEN1 methylates double-stranded small RNAs before their loading into AGO proteins [24], but HEN1 homologues in animals lack any dsRBDs [34] and methylate single-stranded small RNAs bound by AGO or PIWI (a clade of AGO proteins) [26,29].

This is consistent with the fact that nearly all small RNAs are 2′-*O*-methylated in plants, regardless of the AGO proteins to which they bind, but in animals small RNA methylation depends on which AGO protein HEN1 interacts with. siRNAs associated with AGO2 in *Drosophila* [35] and piRNAs loaded into PIWI in nearly all animals are methylated [26–30,36]. In *Caenorhabditis elegans,* in addition to piRNAs, small RNAs loaded to ERGO-1 (a clade of AGO proteins), named 26G RNAs, are methylated as well [37]. In *Tetrahymena,* small RNAs that are ~28–29 nt in length and interact with the PIWI protein Twi1p, known as scnRNAs, are methylated by HEN1 [31].

HEN1 promotes miRNA accumulation in plants - miRNAs in *hen1* mutant *Arabidopsis* and rice plants are less abundant than in wild type [32,38–40]. The reduced miRNA accumulation in *hen1* mutants is accompanied by 3’ truncation and 3’ non-templated nucleotide addition (with uridine being the predominant nucleotide added) [32]. Loss of function in *HEN1* in *Drosophila* [26,29], zebra fish [30], *C. elegans* [37], *Tetrahymena* [31], and mouse [34] also results in loss of small RNA methylation and increased 3’ truncation and/or tailing. Although *Drosophila* miRNAs are generally loaded into AGO1 and are not methylated, a small fraction of miRNAs is loaded into AGO2 and is methylated by HEN1. Loss of 3’ methylation of these miRNAs in *Drosophila* leads to their tailing and trimming, indicating that methylation, although not occurring to many miRNAs, does have a stabilization effect on some animal miRNAs as it does on plant miRNAs [29,35,41].

In *Arabidopsis*, miRNA 3’ methylation by HEN1 occurs widely to nearly all miRNAs, with the exception of miR158 and perhaps miR319. The incomplete methylation of these miRNAs results in their 3’ truncation and tailing in wild type [42,43]. miRNA methylation in *Drosophila* occurs in a developmentally regulated manner, with the methylation status and abundance of some miRNAs increasing during aging. Lack of miRNA methylation results in accelerated neurodegeneration and shorter life span [41]. These observations suggest that small RNA methylation is regulated by as yet unknown mechanisms.

Non-templated nucleotide addition to miRNAs

3’ non-templated nucleotide addition to miRNAs (or miRNA tailing) is a common phenomenon in plants and animals. The two most common types of miRNA tailing are adenylation and uridylation, and are carried out by nucleotidyl transferases such as non-canonical PolyA Polymerases (PAPs) and Terminal Uridylyl Transferases (TUTases) [44]. Non-templated nucleotide addition can impact miRNA gene regulatory networks through the control of miRNA biogenesis or stability, or by affecting miRNA activity. In human cell lines, specific miRNAs undergo changes in their 3’ nucleotide addition during differentiation, indicating that 3’ nucleotide addition is a biologically regulated process [45].

miRNA adenylation

Adenylation seems to stabilize miRNAs. In cotton wood, a significant portion of miRNAs was found to contain one or a few post-transcriptionally added adenylic acid residues at their 3’ ends, and adenylated miRNAs were degraded slower in plant extracts *in vitro* [46]. Similarly, the addition of a single adenine to some miRNAs in animals appears to stabilize the miRNAs [47,48]. GLD2 (also known as TUT2, PAPD4 or HS1 in other organisms) was

found to adenylate miRNAs and its depletion caused a reduction in miRNA accumulation [47,48]. 3' adenylation can also affect miRNA activity without affecting their stability. In humans, a small fraction of miRNAs sometimes undergoes adenylation after DICER processing but before RISC assembly. This adenylation is believed to interfere with RISC assembly and modulate miRNA targeting effectiveness [49,50].

miRNA uridylation

Mature miRNAs can be uridylated in different species. In *Chlamydomonas*, MUT68 uridylates the 3′ termini of miRNAs and stimulates their degradation. *MUT68* loss of function results in the accumulation of miRNAs *in vivo* [51]. In *Arabidopsis*a MUT68 homologue HEN1 SUPPRESSOR1 (HESO1) is a terminal nucleotidyl transferase that adds non-templated uridine to the 3′ end of unmethylated miRNAs. HESO1 overexpression in a *hen1* background causes reduced miRNA accumulation and more severe morphological defects [52]. Mutations in *heso1* (including a null allele) reduce miRNA uridylation in *hen1* mutant backgrounds, increase miRNA accumulation, and partially rescue the morphological phenotypes of *hen1* [43,52]. This indicates that HESO1-mediated uridylation leads to miRNA degradation. The residual miRNA uridylation and incomplete rescue of *hen1*'s morphological phenotypes by loss of function in *HESO1* are attributable to another nucleotidyl transferase URT1 [53–55]. Both HESO1 and URT1 act on unmethylated miRNAs - their activities are completely inhibited by 3' methylation on the substrate miRNA [43,52,54,55]. Therefore, they may not play a role in miRNA turnover in the wild-type background in which most miRNAs are methylated. It remains to be determined whether other nucleotidyl transferases (there are a total of ten such potential enzymes in *Arabidopsis*) affect the turnover of methylated miRNAs. In humans, uridylation also has a general, negative effect on miRNA abundance by causing miRNA degradation [56]. Selective miRNA uridylation has been documented to play a role in development [57].

Uridylation may also affect the activities of miRNAs. In human cancer cells, terminal uridylation of miR26 by Zcchc11 appears to decrease its ability to repress its target genes without affecting its abundance [58]. In an *Arabidopsis hen1* background, uridylation of miR158 by URT1 also affects the target repression activity of this miRNA without affecting its abundance [55]. In addition, uridylation of AGO1-bound miR165/6 by URT1 *in vitro* greatly reduces the slicing activity of miR165/6-AGO1 on its target RNA [55]. Intriguingly, monouridylation of miR171 in *Arabidopsis hen1* backgrounds to result in a 22 nt miRNA endows this miRNA the ability to trigger the biogenesis of secondary, phased siRNAs (also known as phasiRNAs) from its target mRNA [42]. Although 22 nt miRNAs tend to trigger the production of phasiRNAs [59,60], miR171 is the exceptional miRNA that gains this ability in *hen1* backgrounds, despite the fact that many miRNAs have 22 nt forms in *hen1* backgrounds.

Nucleases involved in miRNA degradation

Several exonucleases have been found to participate in miRNA degradation. SDN (SMALL RNA DEGRADING NUCLEASE) is a family of 3'->5' exonucleases that degrades mature miRNAs in *Arabidopsis*. Knockdown of three *SDN* family members (*SDN1, 2*, and *3*) results in elevated levels of endogenous miRNAs and developmental defects [61]. SDN1 prefers

single-stranded, non-uridylated miRNA substrates and its activity is partially inhibited by miRNA methylation [61]. As knocking down three *SDN* family members in the wild-type background, in which miRNAs are mostly methylated, results in higher levels of miRNAs, the SDN proteins must be able to degrade methylated miRNAs *in vivo*, as it does *in vitro* [61]. In *C. elegans*, the 5'->3' exonuclease XRN-2 degrades mature miRNAs *in vitro* and *xrn-2* mutant worms over-accumulate some but not all miRNAs [62]. The *Arabidopsis* genome encodes three XRN enzymes. XRN4 degrades the 3′ fragments of miRISC-cleaved mRNAs and functions in general mRNA turnover, by means of degrading de-capped mRNAs [63,64]. XRN2 and XRN3 act in the process of miRNA biogenesis and digest the loops resulting from miRNA precursor processing [65]. *xrn3* and *xrn2* mutants exhibit strong developmental defects and accumulate high levels of miRNA loops [65]. There is as yet no evidence for a 5'->3' exonuclease activity acting on mature miRNAs in plants.

The fact that uridylation promotes miRNA degradation implicates the existence of an enzyme that prefers uridylated miRNAs over non-uridylated miRNAs. This enzyme is unlikely SDN1 in *Arabidopsis*, as SDN1 is unable to degrade uridylated miRNAs *in vitro* [61]. In mammals, *DIS3-like exonuclease 2* (DIS3L2), a 3'->5' exonuclease belonging to the RNase II family, degrades oligouridylated pre-let-7 *in vivo* [66,67]. DIS3L2 also acts in the decay of mRNAs in mammals and yeast [68–70]. It exhibits tighter binding and higher activity on uridylated than non-uridylated substrates [68]. This suggests that DIS3L2 is a general RNA 3'->5' exonuclease that degrades uridylated RNAs. In *Chlamydomonas*, Ribosomal RNA-Processing protein 6 (RRP6), an exosome subunit, degrades uridylated small RNAs *in vitro* and *RRP6* knockdown leads to increased accumulation of small RNAs *in vivo* [51]. In *Arabidopsis*, *Suppressor Of Varicose* (*SOV*), an orthologue of DIS3L2 in mammals, and *RRP6* (there are three *RRP6* genes) are candidates for the degradation of uridylated miRNAs.

AGO1 influences miRNA 3' modifications and stability

AGO proteins are key players in miRNA biogenesis and function. Besides serving as an effector of miRNAs' activities, they protect and stabilize miRNAs from degradation in plants and animals [22,71]. In mutants in *AGO1*, the major miRNA effector in *Arabidopsis*, many miRNAs are reduced in abundance [22]. Loss of function in mammalian AGO2 significantly reduces miRNA stability, while overexpression of AGO2 prevents the degradation of miRNAs and increases miRNA half-life [72]. AGO's function in miRNA stability is independent of its endonuclease activity, as slicing-deficient mutants in mammalian AGO2 can still increase miRNA abundance [71]. A fraction of miR165/166 in plants is bound by AGO10, but AGO10 loss of function surprisingly increases the abundance of miR165/miR166 [73], which indicates that not all AGO proteins stabilize their associated miRNAs.

AGO proteins consist of four distinct domains: the N-terminal, PAZ, MID and PIWI (reviewed in [74]). The PAZ domain contains a specific binding pocket for the 3' end of a small RNA and the MID domain binds the 5' phosphate of a small RNA. Thus, AGO presumably protects both ends from degradation by making the ends inaccessible to nucleases. After the loading of miRNA/miRNA* to AGO, miRNA* is selectively degraded

while the guide miRNA strand is bound by AGO. The disparate levels of miRNA and miRNA* strands *in vivo* support the protective role of AGO towards its associated small RNAs and indicates the presence of an efficient small RNA decay system that degrades unprotected small RNA strands [75].

Given the protective role of *Arabidopsis* AGO1 towards its associated miRNAs, it is counter-intuitive that miRNA 3' truncation and 3' tailing require AGO1 *in vivo*. This was suggested by the observation that the partial loss of function *ago1* allele, *ago1-11*, suppresses the 3' truncation and 3' tailing of miRNAs in a *hen1* background [42]. Reciprocal co-immunoprecipitation experiments show that HESO1 interacts with AGO1 in an RNA-independent manner [76]. URT1 also interacts with AGO1 *in vivo* [54], and both HESO1 and URT1 can uridylate AGO1-bound miRNAs *in vitro* [55,76]. The interactions between AGO1 and HESO1 or URT1 probably serve two purposes. First, AGO1 probably recruits HESO1 to miRNA target transcripts to uridylate the 5' mRNA fragments generated by miRISC-mediated cleavage; this uridylation triggers the further degradation of these 5' mRNA fragments [76]. Second, AGO1 recruits HESO1 and URT1 to probably ensure the degradation of damaged or even intact miRNAs that need to be eliminated. In *hen1 heso1 urt1* or *heso1 urt1*3' truncated miRNAs accumulate to higher levels, suggesting that HESO1 and URT1 eliminate shortened miRNAs or antagonize an exonulcease that trims miRNAs [54].

Target transcripts may influence miRNA stability

Target mimicry was first found in *Arabidopsis* to have a regulatory effect on miRNA activity. A non-protein-coding transcript from the *IPS1* (*INDUCED BY PHOSPHATE STARVATION1*) gene in *Arabidopsis* has a binding site for miR399. A three-nucleotide bulge between the 10th and 11th position of the miRNA in *IPS1* RNA prevents miR399-guided cleavage such that *IPS1* RNA serves as a target mimic (TM) to sequester miR-399 from its endogenous target RNA [77]. Bioinformatics analyses suggest that many miRNAs in plants could potentially be regulated by endogenous TMs [78,79]. Endogenous TM RNAs are predicted to originate not only from noncoding regions but also from annotated genes and intragenic sequences [78,79]. TM RNAs with varied expression in different tissues may contribute to miRNA regulation in spatially or temporally specific manners [78].

Artificial TMs have become a powerful tool for regulating miRNA activities *in vivo* [79,80]. In transgenic lines expressing various artificial TMs, it was noticed that the levels of the cognate miRNAs were reduced [78–80], suggesting that the TMs led to miRNA degradation. Short Tandem Target Mimic (STTM) is an effective TM strategy that employs an artificial, non-coding STTM transcript that harbors two miRNA binding sites separated by a linker [81,82]. Intriguingly, the accumulation of an STTM transcript *in vivo* correlates with reduced levels of the cognate miRNA, suggesting that STTM induces miRNA degradation. STTM-induced degradation of miR165/6 was shown to require SDN1 and SDN2 [81]. The observations that TMs lead to lower miRNA levels raise many questions – How do TMs trigger miRNA degradation? Do natural target transcripts that can be cleaved by miRISC trigger miRNA degradation?

The effects of TM transcripts on miRNA stability may result from miRISC structural changes induced by TM RNAs. Structural studies with *Thermus thermophilus* AGO and its guide DNA and target RNA [83] revealed that a target RNA with extensive complementarity to the guide induces the release of the 3' end of the guide from the AGO PAZ domain, whereas a target RNA with a short stretch of sequence complementarity to the guide does not do so. If these observations can be extrapolated to eukaryotic AGO-miRNA-target interactions, it would suggest that a target transcript with a high degree of sequence complementarity to the miRNA could condition the accessibility of the miRNA 3' end to exonucleases or nucleotidyl transferases and thus influence miRNA stability. miRNAs in animals recognize target mRNAs through seed pairing [84]. Extensive 3' pairing of miRNAs to artificial target RNAs triggers miRNA 3'->5' truncation, 3' tailing and decay in *Drosophila* [35] and mammalian cells [85,86] (Fig 2). By contrast, plant miRNAs are nearly perfectly complementary to their target RNAs [7]. Recognition of target transcripts by plant miRISCs could dislodge miRNA 3' ends from the PAZ domain and render miRNAs susceptible to 3' truncation and tailing (Fig 2) - this perhaps necessitates the methylation of plant miRNAs to protect them from degradation. TM transcripts could accumulate to high levels *in vivo* as they cannot be cleaved by miRISCs and therefore are more effective in rendering the degradation of miRNAs than natural target transcripts. Note that TMs can induce miRNA degradation in the WT background in which miRNAs are mostly methylated. This suggests that mechanisms are in place to degrade methylated miRNAs. While there could be an unidentified enzyme that removes the methyl group, the 3' truncation by an exonuclease such as SDN1 could also effectively result in an unmethylated miRNA, which would be susceptible to 3' tailing by nucleotidyl transferases (Fig 2). It remains to be determined whether STTM-induced miRNA degradation requires nucleotidyl transferases such as HESO1 and URT1.

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Highlights

- miRNA degradation in both plants and animals involves 3’ truncation and 3’ tailing
- 3’ uridylation by HESO1 and URT1 is associated with miRNA degradation
- 3’ truncation and 3’ tailing probably occur to Argonaute-bound miRNAs in vivo
- Target mimic RNAs influence miRNA stability

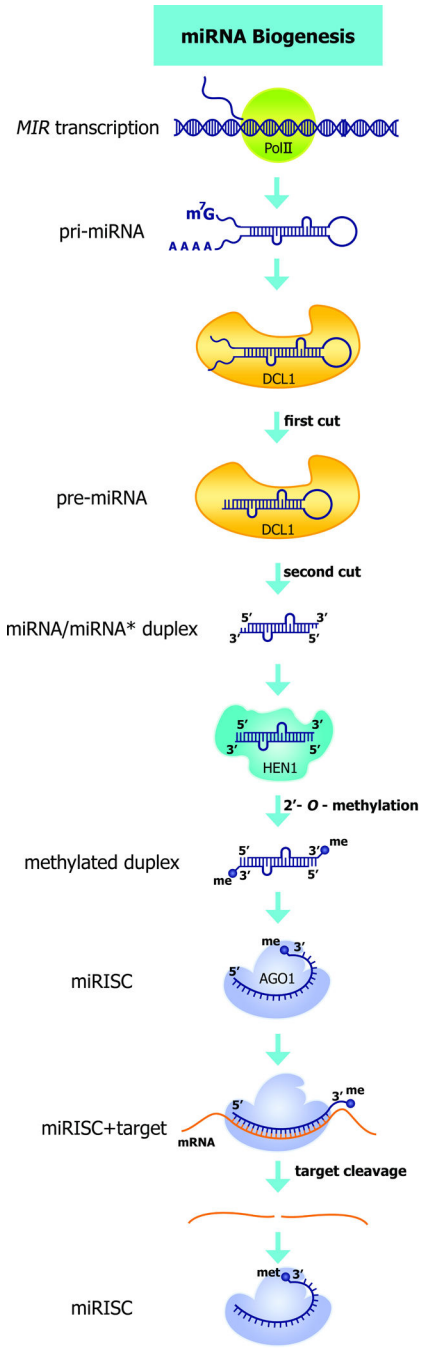

Figure 1.

The miRNA biogenesis pathway in *Arabidopsis*. *MIR* genes are transcribed by Pol II to produce a long primary miRNA transcript (pri-miRNA). DCL1 processes the primiRNA to a miRNA/miRNA* duplex with a pre-miRNA intermediate. HEN1 stabilizes the duplex via *2'-O-* methylation of the 3' ends. The miRNA strand is loaded selectively into AGO1, forming a miRISC that regulates miRNA target gene expression.

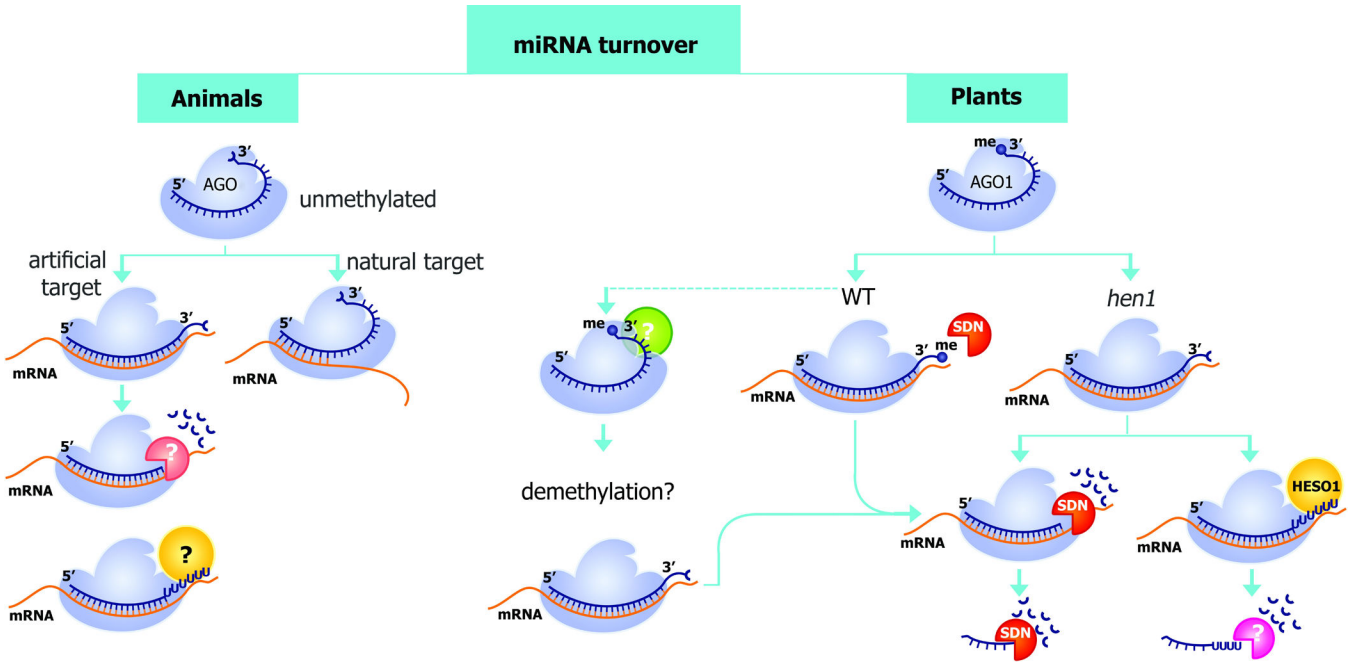

Figure 2.

Target complementarity probably affects miRNA degradation in animals and plants. In animals, miRNA pairs with its target mRNA through the 5’ seed region. The limited complementarity probably leaves the unmethylated miRNA 3’ end protected inside the AGO protein. An artificial target RNA with extensive pairing to an miRNA leads to miRNA 3’ trimming and 3’ tailing by unknown enzymes, perhaps by dislodging the miRNA 3’ end from AGO. In plants, miRNAs are nearly fully complementary to their targets and miRNA/target pairing may expose the miRNA 3’ end. In the absence of HEN1 activity, an unmethylated miRNA is subjected to 3’ trimming and tailing by exonucleases (perhaps SDN1 being one of them) and nucleotidyl transferases (HESO1 and URT1), respectively. Enzymes involved in degrading tailed miRNAs are unknown. Although the 2’-*O*-methyl mark stabilizes plant miRNAs, methylated miRNAs can be degraded. The degradation of methylated miRNAs may entail demethylation by an unknown enzyme, or may be initiated by exonucleolytic trimming, which results in a 3’ truncated and unmethylated miRNA.