Small RNA Biogenesis and Degradation in Plants

Qiming Yu, Yaling Liu, Mu Li, and Bin Yu

Abstract Small RNAs (sRNAs), ~20–25 nucleotide (nt) in size, regulate various biological processes in plants through directing sequence-specific gene silencing. sRNAs are derived from either single- or double-stranded precursor RNAs. Proper levels of sRNAs are crucial for plant growth, development, genomic stability, and adaptation to abiotic and biotic stresses. Studies have identified the machineries controlling sRNA levels through biogenesis and degradation. This chapter covers recent progresses related to mechanisms governing small RNA biogenesis and degradation.

Keywords Plants • miRNAs • ta-siRNAs • pha-siRNAs • nat-siRNAs • ra-siRNAs • Biogenesis • Degradation

Contents

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1 Introduction

Small RNAs (sRNAs) are repressors of gene expression and play essential roles in various biological processes (Baulcombe 2004; Bologna and Voinnet 2014; Borges and Martienssen 2015). Upon production, sRNAs are sorted into their effector protein called ARGONUATE (AGO) and guide it to recognize target RNAs or DNA loci through sequence complementarity. AGO then silences gene expression at transcriptional levels through directing DNA methylation or histone modification and/or at post-transcriptional levels through target RNA cleavage or translational inhibition. microRNAs (miRNAs) and small interfering RNAs (siRNAs) are two major classes of sRNAs. They are chemically identical, but disguisable at their origin and biogenesis (Chen 2009). miRNAs are derived from primary miRNA transcripts (pri-miRNAs), which contain one or more miRNA-residing imperfect step-loops, while siRNAs are produced from long perfect double-stranded RNAs (dsRNAs) (Chen 2009). Depending on their origin, biogenesis and acting model, endogenous siRNAs can be further divided into several classes: trans-acting siRNAs (ta-siRNAs), phased siRNAs (pha-siRNAs), repeated DNA-derived siRNAs (ra-siRNAs), and natural *cis*-antisense siRNAs (nat-siRNAs) (Baulcombe 2004; Bologna and Voinnet 2014; Borges and Martienssen 2015). sRNAs are also subject to degradation (Xie et al. 2015). Turnover is also critical for proper function of sRNAs because excess amount of sRNA impairs plant development. Here, we review our current knowledge related to sRNA biogenesis and degradation.

2 miRNA Biogenesis in Plants

Most pri-miRNA-encoding genes (*MIR*) are localized at intergenic regions and transcribed as independent units by the DNA-dependent RNA polymerase II (Pol II) (Coruh et al. 2014; Nozawa et al. 2012; Xie et al. 2005). However, some *MIRs* are co-transcribed with host genes, some of which are transposons, as intronic or exonic sequences (Piriyapongsa and Jordan 2008; Yang et al. 2012).Upon transcription, pri-miRNAs are cut by the RNAseIII enzyme called DICER-LIKE 1 (DCL1) at least two times to release the miRNA/miRNA* (passenger strand) duplexes in the nucleus (Fig. 1). HUA1 ENHANCER 1 (HEN1), a small RNA methylase, then deposits a methyl group at the 3′ end of the miRNA duplex to stabilize them (Fig. 1) (Xie et al. 2015; Yu et al. 2005).

Many additional factors contribute to miRNA biogenesis by modulating pri-miRNA transcription, processing, and/or stability. Similar to mRNA-coding

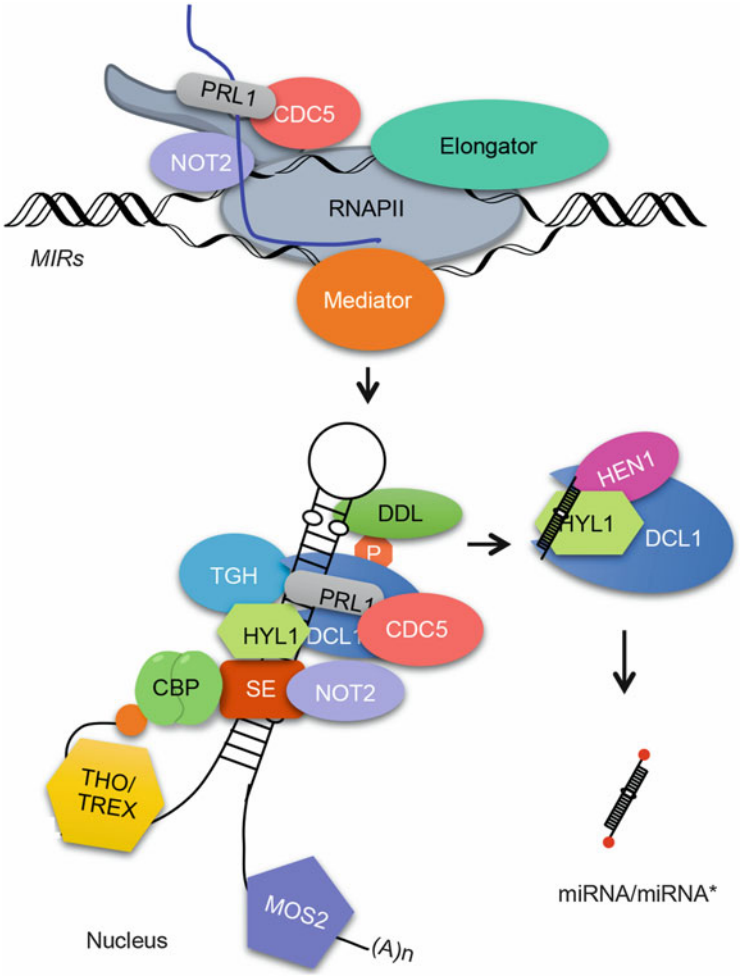

Fig. 1 The miRNA biogenesis pathway. Most *MIRs* are transcribed by Pol II to produce pri-miRNAs. CDC5, NOT2, and Mediator interact with Pol II and *MIR* promoters and are required for *MIR* transcription. After transcription, DDL-binding and PRL1-binding stabilize pri-miRNAs. Pri-miRNAs are co-transcriptionally processed, which requires the elongator complex. SE and HYL1 form a complex with DCL1 to precisely and efficiently process pri-miRNAs in the nucleus. Additional factors such as TGH, CDC5, PRL1, NOT2, CBP20/80, and DDL associate with the DCL1 complex to facilitate its activity. NOT2, MOS2, and TGH also promote the recruitment of pri-miRNAs to the DCL1 complex. After processing, HEN1, which interacts with HYL1 and DCL1, methylates the miRNA duplex

genes, *MIR* transcription requires general and specific transcription factors. NOT2a and its homolog NOT2b, which are core subunits of the conserved CARBON CATABOLITE REPRESSION4 (CCR4)-NOT complex, CDC5, which is an atypical MYB transcription factor, and Mediator (a transcription factor) interact with

Pol II and *MIR* promoters, and positively regulate transcription of many *MIR* genes (Fig. 1) (Kim et al. 2011; Wang et al. 2013; Zhang et al. 2013b). Besides them, the cycling DOF transcription factor (CDF2) binds a subset of *MIR* promoters to promote or repress their activities (Sun et al. 2015). In addition, the ATP-dependent SWR1 chromatin-remodeling complex (SWR1-C) also positively contributes to the expression of *MIRs* through changings of the nucleosome dynamics (Choi et al. 2016). Notably, transcription of some *MIRs* is temporally and spatially regulated. For instance, the transcription factors SCARECROW (SCR) and SHORT ROOT activate the expression of *MIR166* in root endodermis (Carlsbecker et al. 2010). After transcription, a 5′ 7-methylguanosine cap and a 3′ polyadenylated tail (poly-A) are added to pri-miRNAs (Jones-Rhoades and Bartel 2004; Xie et al. 2005). 5′ cap likely stabilizes pri-miRNAs since defection in 5′ capping reduces pri-miRNA accumulation (Hajheidari et al. 2012). Besides 5′ cap, two proteins, DAWDLE (DDL) and PLEIOTROPIC REGULATORY LOCUS 1 (PRL1), bind and stabilize pri-miRNAs following transcription (Fig. 1) (Yu et al. 2008; Zhang et al. 2014, 2015).

Pri-miRNAs are co-transcriptionally processed by DCL1, which is evidenced by the involvement of the elongator complex, which is required for the elongation of Pol II-dependent transcripts, in miRNA biogenesis (Fig. 1) (Fang et al. 2015a). Elongator interacts with DCL1 and is required for the association of DCL1 with *MIR* loci. This observation suggests that DCL1 may be recruited to the nascent pri-miRNAs during transcript elongation (Fang et al. 2015a). The efficient cleavage of miRNA/miRNA duplex from pri-miRNAs by DCL1 requires HYL1 (and sRNA-binding protein), TOUGH (TGH; an RNA-binding protein), and SERRATE (SE; a zinc-finger protein) (Fig. 1) (Dong et al. 2008; Fang and Spector 2007; Fujioka et al. 2007; Ren et al. 2012b; Ren and Yu 2012). HYL1 and SE are also required for precise cleavage of miRNA/miRNA* from pri-miRNAs (Dong et al. 2008), while TGH also modulates the interaction between pri-miRNAs and the processing complex (Ren et al. 2012b). It has been proposed that DCL1, SE, and HYL1 form a Dicing body (D-body) (Fang and Spector 2007), whose formation requires MOS2, an RNA-binding protein (Wu et al. 2013). In addition, NOT2, CDC5, PRL1, and CDF2 also interact with DCL1 and SE to promote pri-miRNA processing (Fig.1) (Sun et al. 2015; Wang et al. 2013; Zhang et al. 2014, 2013b). As these proteins also associate with Pol II, it is possible that they play a role in the co-transcriptional recruitment of DCL1 to pri-miRNAs. Interestingly, the CAP-binding proteins 80 (CBP 80) and CBP20 also associate with the DCL1 complex and are required for miRNA accumulation (Fig. 1) (Gregory et al. 2008; Kim et al. 2008; Laubinger et al. 2008). The recruitment of pri-miRNA to the DCL1 complex involves the THO/TREX complex that functions in the transport of nascent mRNAs from the nucleus towards the cytoplasm (Fig. 1) (Francisco-Mangilet et al. 2015). Furthermore, several additional proteins participate in miRNA biogenesis through the interaction with the accessory factors of DCL1. RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1) that serves as a scaffold for protein bindings interacts with SE to promote pri-miRNA processing (Speth et al. 2013), whereas SICKLE (SIC), a proline-rich protein, co-localizes with HYL1

and is required for the accumulation of a subset of miRNAs (Zhan et al. 2012). Interestingly, GRP7, a homology of human hnRNP A1 involved in splicing, binds a subset of pri-miRNAs to repress their processing (Koster et al. 2014). Notably, REGULATOR OF CBF GENE EXPRESSION 3 (RCF3, also known as HOS5 and SHI1) can bind a subset of pri-miRNAs to regulate their processing in a tissue-specific manner (Chen et al. 2015; Karlsson et al. 2015).

The miRNA biogenesis machinery itself is regulated at both transcriptional and post-transcription levels. The histone acetyltransferase GCN5 promotes the transcription of both *DCL1* and *HYL1* (Kim et al. 2009). Optimal *DCL*1 transcription also requires the STA1, a splicing factor, and CAM33/XAP CIRCADIAN TIMEKEEPER (XCT, a nuclear localized protein) (Ben Chaabane et al. 2013; Fang et al. 2015b). Notably, SE and DCL1 are targets of miR863 and miR162, respectively (Niu et al. 2016; Rajagopalan et al. 2006). This suggests that *DCL1* and *SE* transcripts subject to feedback regulation. Furthermore, both DCL1 and HYL1 activities are regulated by protein phosphorylation. HYL1 is phosphorylated by the MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3), which inhibits miRNA biogenesis (Raghuram et al. 2015). To counteract MPK3 activity, C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1 and 2 (CPL1 and CPL2) dephosphorylate HYL1 to enhance miRNA biogenesis in an SE-dependent manner (Manavella et al. 2012). Besides HYL1, CPL1 and CPL2 also recognize RCF3 to positively impact its function in miRNA biogenesis (Chen et al. 2015; Karlsson et al. 2015). DCL1 interacts with the forkhead domain (FHA) of DDL, which mediates protein–protein interactions by targeting phospho-threonine containing motifs (Machida and Yuan 2013). The phospho-threonine binding cleft of FHA interacts with the helicase domain of DCL1 that contains potential phospho-threonine motifs, suggesting that DCL1 may be phosphorylated for its optimal activity (Machida and Yuan 2013). Interestingly, ubiquitination also plays a role in miRNA biogenesis. For instance, Constitutive Photomorphogenic 1 (COP1), an E3 ubiquitin ligase, has been recently shown to block an activity degrading HYL1 in light via unknown mechanism (Cho et al. 2014).

3 The Biogenesis of ta-siRNAs and pha-siRNAs

ta-siRNAs refer to a class siRNAs that act on targets other than the genes that derive ta-siRNAs (Allen et al. 2005; Peragine et al. 2004). This distinguishes ta-siRNAs from other endogenous siRNAs, which mostly silence genes that are the same as or homologs to the genes from which they derive. The production of ta-siRNAs requires miRNA-directed cleavage of primary ta-siRNA transcripts (*TASs*) that have the same structures as mRNAs (Fig. 2a) (Allen et al. 2005; Axtell et al. 2006; Yoshikawa et al. 2005). Two models for ta-siRNA production have been proposed. In the one-hit model, a 22-nt miRNA first directs AGO to cleave *TASs* (Fig. 2a) (Axtell et al. 2006). The 3′ cleavage products are then used as a template to synthesize dsRNAs by RDR6 (Fig. 2a). In the two-hit model, two 21-nt miRNAs

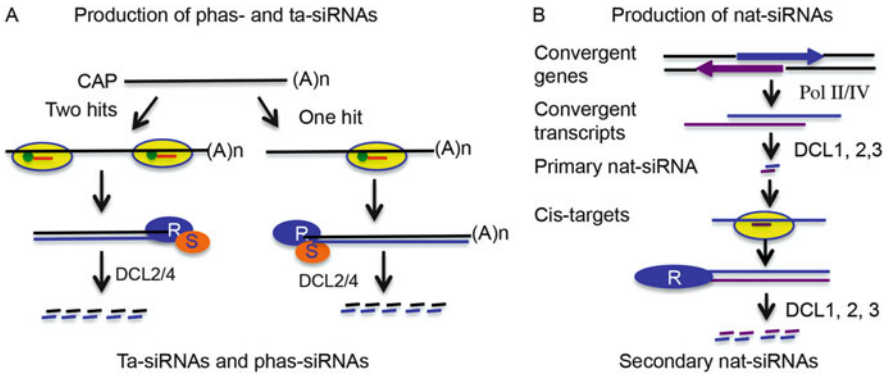

Fig. 2 The biogenesis pathways for ta-/phas-siRNAs and nat-siRNAs. (**a**) Proposed biogenesis pathways for phas- and ta-siRNAs. The precursor RNAs of ta- and phas-siRNAs are first targeted by miRNAs through the one-hit or two-hit model, which triggers the production of dsRNAs by RDR6 (R) with the assistance of SGS3 (S). The resulting dsRNAs will be cleaved by DCLs to produce phased siRNAs. *Yellow oval* indicates AGOs associated with miRNAs. (**b**) Proposed biogenesis pathways for nat-siRNA production. Pol II- or Pol IV-dependent transcription of convergent genes results in dsRNAs. The dsRNAs will be processed by one or more DCLs to produce primary nat-siRNAs. Primary nat-siRNAs will be loaded into AGO (*Yellow oval*) to recognize one of original transcripts. This leads to the production of RDR-dependent dsRNAs, which will be further processed by DCLs to generate secondary nat-siRNAs

recognize *TASs* at independent target sites along the transcript (Fig. 2a) (Axtell et al. 2006). RDR6 is then recruited to the cleavage products to generate dsRNAs. In both scenarios, the dsRNAs are cleaved by DCL4 or DCL2 every 21 or 22 nt from the initial miRNA cleavage point, resulting in a phased production of secondary siRNAs (Fig. 2a) (Fei et al. 2013; Ronemus et al. 2006). ta-siRNA biogenesis requires the assistance of SGS3 and DRB4 (Fig. 2a). DRB4 is a HYL1 homolog and interacts with DCL4 (Adenot et al. 2006), while SGS3 binds dsRNAs with 5′ overhang and partners with RDR6 (Fukunaga and Doudna 2009). In Arabidopsis, miR173 and miR828, 22 nt in size, induce the production of ta-siRNAs from *TAS1*/*TAS2* and *TAS4*, respectively, whereas miR390, 21 nt in size binds AGO7 to target TAS3 (Allen et al. 2005; Axtell et al. 2006; Yoshikawa et al. 2005). The miR390-AGO7-TAS3 combination appears to be conserved among moss, rice, maize, and gymnosperms, suggesting that the two-hit model may be ancestral to the one-hit model (Fei et al. 2013).

Unlike the Brassica plants that only encode few *TAS* loci, most non-brassica plants contain larger number of loci that produce pha-siRNAs (Arikit et al. 2014; Johnson et al. 2009; Shivaprasad et al. 2012; Zhai et al. 2015b). These pha-siRNAs are derived from many mRNAs and long noncoding RNAs called *PHAS* ncRNAs that are transcribed by Pol II, capped and polyadenylated, resembling mRNAs. However, some pha-siRNAs act in *cis* rather in *trans*. The production of pha-siRNAs resembles that of ta-siRNAs and is triggered by miRNA-directed cleavage (Fig. 2a). It has been proposed that RDR6 together with SGS3 use the 3′ cleavage fragments of *PHAS* transcripts as templates to synthesize dsRNA from the

poly-A tail to the cleavage site (Fig. 2a) (Song et al. 2012b). DCL4 and DCL5/DCL3b subsequently process the dsRNAs to generate 21- and 24-nucleotide pha-siRNAs, respectively (Song et al. 2012a). In dicots, there is a conserved miR2118-482 superfamily that triggers the production of pha-siRNAs from transcripts encoding nucleotide-binding/leucine-rich repeat (NB-LRR) proteins (Arikit et al. 2014; Shivaprasad et al. 2012; Zhai et al. 2011). NB-LRR-derived pha-siRNAs act both in *cis* and in *trans* and thereby regulate additional members of NB-LRR family (Arikit et al. 2014; Shivaprasad et al. 2012; Zhai et al. 2011). This pha-siRNAs seem to benefit the plant resistance to bacterial infection, as NB-LRRs play essential role in plant immunity. However, NB-LRR pha-siRNAs are lost in grass genomes, which possess anther-specific pha-siRNAs instead (Arikit et al. 2014; Shivaprasad et al. 2012; Zhai et al. 2011).

4 The Biogenesis of Natural *cis*-antisense siRNAs (nat-siRNAs)

nat-siRNAs are derived from dsRNAs that are formed by convergent bidirectional transcripts generated from two partially overlapping genes (Fig. 2b) (Borsani et al. 2005; Katiyar-Agarwal et al. 2006; Ron et al. 2010; Zhang et al. 2012; Zubko and Meyer 2007). These *cis*-antisense transcripts are common in plant genome. Notably, nat-siRNAs are often induced by various stresses or at specific developmental stage or tissues and appear to be required for plant immunity and development (Borsani et al. 2005; Katiyar-Agarwal et al. 2006; Ron et al. 2010; Zhang et al. 2012; Zubko and Meyer 2007). DCL1, DCL2, and/or DCL3 cleave the dsRNA formed by the *cis*-antisense transcripts to initiate the production of primary nat-siRNAs (Fig. 2b) (Borsani et al. 2005; Katiyar-Agarwal et al. 2006; Ron et al. 2010; Zhang et al. 2012; Zubko and Meyer 2007). The primary nat-siRNAs will then guide the cleavage of the complementary transcripts (Fig. 2b). The resulting cleavage products will be used as templates for RDRs to produce dsRNAs, which will be further processed by DCLs into secondary siRNAs, leading to the reinforcement phase (Fig. 2b) (Borsani et al. 2005; Katiyar-Agarwal et al. 2006; Ron et al. 2010; Zhang et al. 2012; Zubko and Meyer 2007). The second phase resembles the production of pha-siRNAs involving RDRs and SGS3. The production of DCL3-dependent nat-siRNAs also requires the RNA-dependent RNA polymerase 2 (RDR2) and plant-specific RNA polymerase IV (Pol IV) (Zhang et al. 2012). In contrast, not all DCL1-dependent nat-siRNAs require RDR6/RDR2 and Pol IV for production (Zhang et al. 2012).

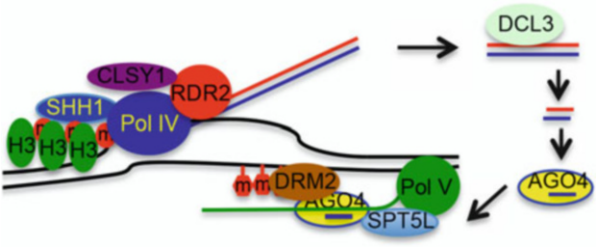

Fig. 3 The biogenesis of canonical ra-siRNAs. SHH1 recognizes the K9 dimethylation (m in *red circle*) of H3 (shown in *green oval*) and recruits Pol IV-RDR2 to the RdDM loci, leading to the production of P4R2 transcripts that are converted to dsRNAs by RdR2. CLSY1 helps the correct localization of Pol IV and RDR2. The resulting dsRNAs are processed by DCL3 to produce 24-nt siRNAs, which are loaded into AGO4 (*Yellow oval*). The AGO4-ra-siRNAs are recruited to chromatin by the Pol V–AGO 4 interaction and the base-pairing between ra-siRNAs and Pol V-dependent transcripts flanking the RdDM loci. AGO4 recruits DRM2 to catalyze the de novo methylation (*Red hexagon*) of RdDM loci

5 The Production of sRNAs Involved in RNA–Direct DNA Methylation (RdDM)

RdDM is a conserved process to silence transposable elements, to direct gene imprinting, and to maintain genome stability in plants and many metazoans (Castel and Martienssen 2013; Law and Jacobsen 2010; Matzke and Mosher 2014). Studies have revealed the presence of both canonical and non-canonical RdDM mechanisms in plants. In canonical RdDM, 24-nt siRNAs derived from repeated DNAs or heterochromatic regions (ra-siRNAs) direct the DNA de novo methyl transferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) to recognize RdDM target loci and to catalyze the methylation of previously unmodified cytosine in any sequence context (Fig. 3) (Castel and Martienssen 2013; Law and Jacobsen 2010; Matzke and Mosher 2014). In contrast, in non-canonical RdDM, miRNAs, ta-siRNAs, and 21-nt siRNAs partner with AGO to direct DNA methylation (Xie and Yu 2015). The mechanisms leading to the production of sRNAs involved in canonical and non-canonical RdDM have been proposed.

5.1 The Biogenesis of Canonical ra-siRNAs

The biogenesis of ra-siRNAs starts with Pol IV-dependent transcription from the RdDM loci (Herr et al. 2005; Onodera et al. 2005). Pol IV is a plant-specific DNA-dependent RNA polymerase. It composes of twelve subunits, which are either identical or paralog to subunit of Pol II (Haag et al. 2014; Ream et al. 2009). Pol IV interacts with RDR2 (Haag et al. 2012; Law et al. 2011), which is also required for the production of Pol IV-dependent transcripts (Fig. 3) (P4R2 RNAs). After

transcription, RDR2 converts the Pol IV-dependent transcripts into dsRNAs in the nucleus (Fig. 3). DCL3 then cuts the dsRNAs into 24-nt siRNAs (Fig. 3). When DCL3 is defective, DCL2 and DCL4 can process the dsRNAs into 22-nt and 21-nt siRNAs, respectively. 24-nt siRNAs are then loaded into AGO4 or AGO6 and recruited to the RdDM loci through base-pairing with Pol V-dependent transcripts and the interaction between AGO4 and Pol V (Fig. 3). AGO4/AGO6 subsequently recruits DRM2 to catalyze de novo DNA methylation (Fig. 3). Consequently, DNA methylation causes histone deacetylation, histone H3 lysine 9 methylation, and histone H3 lysine 4 demethylation, which repress gene transcription and cause heterochromatin formation.

The nature of Pol IV-dependent transcripts (P4R2 RNAs) has been mystery since the discovery of Pol IV. Recently, several groups identified P4R2 RNAs through sequencing RNAs in the mutant deficient in DCL3 (Blevins et al. 2015; Li et al. 2015; Yang et al. 2016; Ye et al. 2016; Zhai et al. 2015a). The sizes of P4R2 RNAs are ranging from ~26 to 45 nt although long P4R2 RNAs may also exist (Li et al. 2015). Unlike Pol II transcripts, P4R2 RNAs are not polyadenylated and capped (Blevins et al. 2015; Li et al. 2015; Yang et al. 2016; Ye et al. 2016; Zhai et al. 2015a). They often start with a purine (A or G) at their 5′ ends and contain one or two untemplated nucleotide (s) at the 3′ end. Notably, P4R2 RNAs can guide DNA methylation without being diced into siRNAs (Yang et al. 2016; Ye et al. 2016), raising the possibility that ra-siRNAs may not be required for RdDM. Pol IV activity may not require the promoters since Pol IV-associated regions do not contain consensus sequences (Law et al. 2013). Instead, Pol IV needs chromatin marks to define the transcript sites (Blevins et al. 2014; Law et al. 2013; Law et al. 2011; Zhang et al. 2013a). Histone deacetylation, maintenance DNA methylation, and histone H3K9 dimethylation (H3K9me2) have been shown to recruit Pol IV to the chromatin (Blevins et al. 2014; Law et al. 2011, 2013; Zhang et al. 2013a). Consistent with these observations, SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1), which interacts with Pol IV and binds H3K9me2 and unmethylated H3K4 through its unique tandem Tudor-like fold, is required for the recruitment of Pol IV to the chromatin (Fig. 3) (Law et al. 2011; Zhang et al. 2013a). In addition, CLSY1, a putative chromatin-remodeling factor, is required for Pol IV-RDR2 localization and activity (Fig. 3) (Smith et al. 2007). Interestingly, Pol II also assists the recruitment of Pol IV to chromatin at some RdDM loci presumably through its transcription activity, which suggest the interplay among different polymerase may be required for ra-siRNA production (Zheng et al. 2009).

5.2 The Biogenesis of Non-canonical sRNAs Involved in RdDM

Plants also use non-canonical RdDM pathways to defend transposons. In non-canonical RdDM pathways, sRNAs are produced differently from those in

canonical pathways. Some Pol II-derived hairpin transcripts and pri-miRNAs can be processed by DCL3 to generate 24-nt siRNAs or miRNAs (Chellappan et al. 2010; Dunoyer et al. 2010; Khraiwesh et al. 2010; Slotkin et al. 2005; Wu et al. 2010). These 24-nt siRNAs and miRNAs are then fed into the canonical RdDM pathway to direct DNA methylation either in *trans* or in *cis*. RDR6 also converts TE mRNAs into dsRNAs if these TEs are highly expressed (Gasciolli et al. 2005; Mari-Ordonez et al. 2013). When DCL2 and DCL4 become saturated, DCL3 cuts TE-derived, RDR6-dependent dsRNAs, which are not typically DCL3 substrates, into 24-nt siRNAs (Gasciolli et al. 2005; Mari-Ordonez et al. 2013). This pathway relies on the hierarchical activity of DCLs. Interestingly, RDR6-dependent 21- and 22-nt siRNAs can also participate in RdDM, which is evidenced by the fact that ta-siRNAs can be loaded into AGO4 or AGO6 to direct DNA methylation at *TAS* loci (McCue et al. 2015; Wu et al. 2012). Notably, this pathway is independent of Pol IV, RDR2, and DCL3 although it requires Pol V and DRM2 (Wu et al. 2012). Subsequent studies show that similar mechanisms are employed to initiate and establish the silencing of transcriptionally active TEs, which are long and autonomous (Nuthikattu et al. 2013). Like ta-siRNAs, the production of TE-derived 21-/22-nt siRNAs depends on Pol II, SGS3, DCL2, and DCL4 (McCue et al. 2015; Wu et al. 2012). Recent studies also uncovered a non-canonical RdDM pathway that targets a subset of non-conserved genomic loci (Garcia et al. 2012; Pontier et al. 2012). This pathway depends on Pol IV, Pol V, AGO2, and a protein named Needed for RDR2-independent DNA methylation (NERD), which contains GW repeats and a PHD finger domain and interacts with Pol V and AGO2 (Garcia et al. 2012; Pontier et al. 2012). The biogenesis pathway of siRNAs that act in NERD-dependent RdDM is not well established. However, it seems that siRNA production in this pathway requires the combination of canonical and non-canonical RdDM proteins since siRNA accumulation is reduced in *rdr1*, *rdr6*, *sde3*, *dcl2*, *dcl3,* and *nrpd1a* (a Pol IV mutant) (Garcia et al. 2012; Pontier et al. 2012).

6 Methylation Stabilizes miRNAs and siRNAs

In plants, the 3′ termini of miRNAs and siRNAs harbor a 2′-*O*-methyl group (Fig. 1) (Yu et al. 2005). This modification is added by HEN1 following the release of the miRNA or siRNA duplexes (Fig. 1) (Yang et al. 2006; Yu et al. 2005). HEN1 exists in both eukaryotes and prokaryotes (Huang et al. 2009). Plant HEN1 contains several protein domains, including two dsRNA-binding domains (dsRBD1 and dsRBD2), a La-motif containing domain (LCD), and a methyltransferase domain (MTase) (Huang et al. 2009). Among these protein domains, the dsRBD1 and dsRBD2 domains enable HEN1 to recognize dsRNAs, while the distance between the LCD domain and the MTase domain helps HEN1 to determine its substrate length (Huang et al. 2009). As a result, plant HEN1 specifically deposits a methyl group to the 2′ OH position of the 3′ end in each strand of 21–24 base-pair (bp) dsRNAs with 2-nt overhangs (Huang et al. 2009; Yang et al. 2006), which

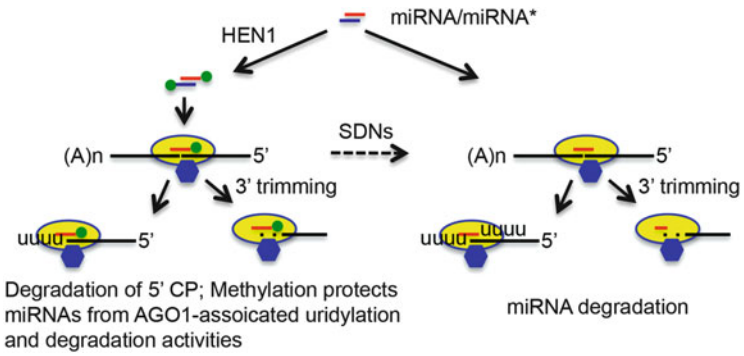

Fig. 4 Proposed model for miRNA methylation and degradation. After production, the miRNA/miRNA* duplexes are methylated (*Green* cycle) by HEN1. Methylated miRNAs are then loaded into AGO1 (*Yellow oval*) and direct AGO1 to cleave targets. The resulted 5′ cleavage products (5′ CP) are degraded through HESO1/URT1 (*Blue hexagon*)-mediated uridylation or 3′ trimming. The 2′-*O*-methylation protects miRNAs from uridylation and 3′ trimming. When methylation is lacking, miRNAs will be attacked by the AGO1-associated uridylation and 3′ trimming activities, leading to degradation. SDN1 may target AGO1-bound miRNAs with the 2′-*O*-methyl group and lead to their uridylation and degradation

are typical features of the miRNA and siRNA duplexes. HEN1 interacts with HYL1 and DCL1 (Fig. 1) (Baranauske et al. 2015), suggesting that miRNA production and methylation are a coupled process. In contrast, HEN1s from metazoans and bacteria lack the dsRNA-binding domain, and therefore, act on ssRNAs (Chan et al. 2009a; Horwich et al. 2007; Kirino and Mourelatos 2007). Consistent with this, metazoan HEN1 recognizes AGO-bound sRNAs (Ohara et al. 2007; Saito et al. 2007), whereas bacterial HEN1 modifies transfer RNAs (tRNAs) (Chan et al. 2009b). Interestingly, fly HEN1 interacts with PIWI (an AGO protein) (Ohara et al. 2007; Saito et al. 2007), indicating a potential role of AGO in determining substrate specificity for HEN1 in metazoans.

In plant *hen1*, sRNAs are reduced in abundance and become heterogeneity in size that is caused by untemplated uridine addition at 3′ termini (uridylation) and 3′-to-5′ exonucleolytic trimming activity (Fig. 4) (Abe et al. 2010; Li et al. 2005; Zhai et al. 2013). Consistent with the observation in plant *hen1*, sRNAs are also subjected to 3′-to-5′ trimming and uridylation in metazoan *hen1* (Billi et al. 2012; Horwich et al. 2007; Kamminga et al. 2010; Kamminga et al. 2012; Montgomery et al. 2012). These results demonstrate that methylation is a conserved mechanism to protect sRNAs from degradation and uridylation.

7 Uridylation Triggers the Degradation of siRNAs and miRNAs

In plants, sRNAs become uridylated globally when HEN1 is lacking (Ren et al. 2014a). In Arabidopsis, a terminal uridyl transferase (TUTase) named HEN1 SUPPRESSOR1 (HESO1) catalyzes the uridylation of miRNAs and siRNAs (Ren et al. 2012a; Zhao et al. 2012). HESO1 acts progressively on RNAs *in vitro*, but its activity is blocked by 2′-*O*-methylation (Fig. 4) (Ren et al. 2012a; Zhao et al. 2012). Besides HESO1, UTP: RNA uridylyltransferase (URT1), which has been shown to uridylate some mRNAs, also act on miRNAs (Fig. 4) (Tu et al. 2015; Wang et al. 2015). However, unlike HESO1, URT1 does not recognize ra-siRNAs, likely due to its exclusive localization in the cytoplasm (Wang et al. 2015). In addition, URT1 appears to add short U-tail to miRNAs in vivo and seems to have a different preference to 3′ end nucleotides with HESO1 (Tu et al. 2015).

In *hen1*, *heso1* reduces the U-tail length, resulting in increased abundance of most normal-sized, 3′ trimmed, and/or short-tailed sRNAs (Ren et al. 2012a; Zhao et al. 2012), whereas *urt1* only affects a few miRNAs (Tu et al. 2015; Wang et al. 2015). However, when both HESO1 and URT1 are lacking, miRNA uridylation is globally abolished in *hen1*, resulting in elevated abundance of miRNAs and an extensive increase of 3′-to-5′ trimming (Wang et al. 2015). Furthermore, overexpression of HESO1 in *hen1* further reduced the accumulation of normal-sized and 3′ trimmed miRNAs (Ren et al. 2012a). Taken together, these results reveal that HESO1 and URT1 synergistically and independently act on miRNAs and that uridylation triggers miRNA degradation and competes with the 3′-to-5′ trimming activity for substrates (Fig. 4) (Tu et al. 2015; Wang et al. 2015). In Chlamydomonas, the MUT68 nucleotidyltransferase uridylates miRNAs and siRNAs to trigger their degradation by the exosome components (Ibrahim et al. 2010). Bedside triggering degradation, uridylation may also block miRNA activity, which is evidenced by the fact that tailing of AGO1-bound miRNA165/6 greatly reduced its cleavage activity on their targets (Tu et al. 2015). Intriguingly, a single U addition in miR171 in *hen1 urt1* enables miR171 to trigger the production of secondary siRNAs from its targets (Tu et al. 2015), suggesting that U addition may also alter miRNA activity. sRNA uridylation also exist in metazoans (Burroughs et al. 2010; Wyman et al. 2011). Like in plants, uridylation affects stability and function of metazoan sRNAs. Interestingly, many TUTases act on sRNAs and some of them act on miRNAs in a sequence-specific manner in metazoans (Burroughs et al. 2010; Wyman et al. 2011).

In addition to miRNAs, HESO1 and URT1 also uridylates the 5′ RNA fragments (5′ CP) generated by AGO1 cleavage of target RNAs (Fig. 4) (Ren et al. 2014b; Wang et al. 2015). Uridylation of 5′ CP is also a conserved process in both metazoans and plants (Shen and Goodman 2004). Similar to its effect on sRNAs, uridylation triggers degradation of 5′ CP, but competes with 3′-to-5′ exonucleolytic trimming activity (Fig. 4) (Ren et al. 2014b). Interestingly, MUT68 from Chlamydomonas also acts on 5′ CP (Ibrahim et al. 2006). These results suggest

the presence of a common mechanism, by which TUTase recognize both sRNAs and 5′ CP. Indeed, both HESO1 and URT1 interact with AGO1 and add U-tails to AGO1-bound miRNAs (Ren et al. 2014b; Tu et al. 2015; Wang et al. 2015). Furthermore, defection of AGO1 abolishes uridylation of miRNAs in HEN1 (Ren et al. 2014b; Zhai et al. 2013). Thus, it is likely that TUTases recognizes its substrates in the AGO1 complex. These results also answer the question why plant miRNAs, but metazoan miRNAs, require methylation for stability (Ren et al. 2014a). In plants, miRNAs majorly direct target cleavage. The cleavage products need to be further eliminated. Otherwise, they cause lethality of plants. To ensure the rapid degradation of 5′ CP, uridylation and other degradation activities are associated with the AGO1 complex (Fig. 4). However, base-pairing plant miRNAs and their targets may expose miRNA 3′ end to these AGO1-associated activities due to the extensive complementarity (Ren et al. 2014a). Thus, plant miRNAs may need methylation to protect them from such activities. In contrast, metazoan miRNAs are less complementary to their targets and majorly inhibit translation. They consequently may not be exposed to uridylation/degradation activities when meeting with their targets. Consistent with this notion, the high complementarity between aritificial target RNAs and endogenous miRNAs. triggers miRNA tailing and trimming in metazoans (Ameres et al. 2010, 2011).

Similar to uridylation, a common mechanism may exist to degrade both uridylated miRNAs and 5′ CPs, which transiently associate with AGO. Indeed, the exosome has been shown to degrade both miRNAs and 5′ CP in Chlamydomonas (Ibrahim et al. 2006, 2010). However, such enzymes remain to be identified in higher plants. In metazoans, Dis3l2, a paralog of RRP44 that is a core component of the exosome, degrades uridylated precursor of let-7 miRNA (pre-let-7) (Chang et al. 2013; Ustianenko et al. 2013). By analog, plant homologs of Dis3l2 such as SUPPRESSOR OF VARICOSE and RRP44A may act on uridylated sRNAs and 5′ CPs (Ren et al. 2014a). Alternatively, U-tail may disassociate miRNAs and 5′ CPs from the AGO1 complex, causing their rapid degradation. This is supported by the observation that long tails can be added to AGO1-bound miRNAs in vitro (Ren et al. 2014b), but no long-tailed miRNAs/5′ CP can be detected in vivo (Tu et al. 2015; Wang et al. 2015). It is also possible that some unusual exoribonucleases may recognize the 3′ U-overhang of miRNA-5′ CP duplex to trigger their degradation.

8 Exoribonucleases Degrading sRNAs in Plants

In *Arabidopsis*, a family of 3′-to-5′ exoribonucleases including SMALL RNA DEGRADING NUCLEASE 1 (SDN1), 2, and 3 have been shown to degrade mature miRNAs (Ramachandran and Chen 2008). SDNs appear to act on short single-stranded RNAs, but not sRNA duplexes or pre-miRNAs (Ramachandran and Chen 2008). Furthermore, SDNs act on methylated, but not uridylated miRNAs (Ramachandran and Chen 2008), suggesting that SDNs may function coordinately

with HESO1/URT1 to regulate miRNA abundance (Fig. 4). Lack of SDNs increases the abundance of miRNAs and causes pleiotropic development defects, demonstrating that turnover is an essential mechanism to maintain proper miRNA activities (Ramachandran and Chen 2008). Recent studies show that target mimicry, which blocks miRNA-mediate target cleavage, can induce the degradation of miRNAs by SDNs (Yan et al. 2012). This result further reinforces that the function of SDNs is to eliminate the unnecessary miRNAs. AGO10 has been shown to decoy miR165/166 from AGO1, and AGO10 binding also seems to trigger degradation of miR165/166 (Zhu et al. 2011). It is possible that SDNs may play a role in miR165/166 degradation caused by AGO10-decoy.

Unmethylated miRNAs also subjects to 3′-to-5′ trimming activity in higher plants. However, the enzyme remains to be identified. In Chlamydomonas, the RRP6 that possesses 3′-to-5′ exoribonuclease activity can degrade miRNAs (Ibrahim et al. 2006; Ibrahim et al. 2010). Arabidopsis encode three RRP6-LIKE (RRP6L) proteins (Lange et al. 2008). It is possible that RRP6L can act on unmethylated miRNAs in higher plants. In fly, a 3′-to-5′ exoribonuclease named Nibbler, binds AGO to trim miRNAs (Han et al. 2011; Liu et al. 2011). It has two homologs in Arabidopsis (Xie et al. 2015). It is reasonable to speculate that these two enzymes may act on unmethylated miRNAs in Arabidopsis.

9 Perspective

In the past decades, the framework for sRNA biogenesis has been established and progresses have been made towards to sRNA degradation. In addition, factors which play regulatory roles in miRNA biogenesis also have been isolated. These advances have resulted in a better understanding of biological processes involving sRNAs and improved our ability to apply related technologies. However, challenges still exist. Plants employ multiple mechanisms to regulate gene expression and genome stability. How sRNAs are coordinated with other regulatory mechanisms in various biological processes is not well known. In addition, the functional mechanisms of accessory proteins involved in sRNA biogenesis are mostly not well defined. Notably, many of these protein factors also function in transcription, RNA processing, splicing, and RNA decay. Thus, sRNA biogenesis may have interconnections with other RNA metabolisms. Further elucidation of these interconnections is still an obstacle to our understanding of various sRNA pathways. miRNA biogenesis is regulated through transcription, processing, and stability. It remains poorly understood how plants coordinate these processes to ensure proper miRNA levels in response to development and physiological signals. In higher plants, the enzymes degrading modified and unmodified sRNAs are largely unknown, which have greatly limited our understanding of sRNA turn over. Furthermore, the factors regulating siRNA production are largely unknown. Consequently, it is not clear how related biological processes such as DNA methylation are regulated at various developmental stages and in response to biotic and abiotic stresses. Finally, a

practical challenge is the optimization of sRNA-based technology and related application used to improve agricultural trait of crops.

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