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The 'how' and 'where' of plant microRNAs

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Summary

MicroRNAs (miRNAs) are small non-coding RNAs, of typically 20–24 nt, that regulate gene expression post-transcriptionally through sequence complementarity. Since the identification of the first miRNA, *lin-4*, in the nematode *Caenorhabditis elegans* in 1993, thousands of miRNAs have been discovered in animals and plants, and their regulatory roles in numerous biological processes have been uncovered. In plants, research efforts have established the major molecular framework of miRNA biogenesis and modes of action, and are beginning to elucidate the mechanisms of miRNA degradation. Studies have implicated restricted and surprising subcellular locations in which miRNA biogenesis or activity takes place. In this article, we summarize the current knowledge on how plant miRNAs are made and degraded, and how they repress target gene expression. We discuss not only the players involved in these processes, but also the subcellular sites in which these processes are known or implicated to take place. We hope to raise awareness that the cell biology of miRNAs holds the key to a full understanding of these enigmatic molecules.

I. MicroRNA biogenesis in plants

MicroRNA (*MIR*) genes encoding microRNAs (miRNAs) are transcribed by RNA polymerase II (Pol II) into primary miRNAs (pri-miRNAs). The stem loop-containing pri-miRNAs are processed by the RNase III family enzyme DICER-LIKE1 (DCL1) into miRNA/miRNA* duplexes. These duplexes are 2′-*O*-methylated at the 3′ ends by the methyltransferase HUA ENHANCER1 (HEN1). One strand from the duplex is incorporated into ARGONAUTE1 (AGO1) to form an active RNA-induced silencing complex (RISC) (reviewed in Rogers & Chen, 2013) (Fig. 1). The following section focuses on complexity and regulation in miRNA biogenesis unveiled by recent studies.

1. *MIR* transcription and transcriptional regulation

Similar to protein coding genes, most *MIR* genes contain the TATA-box motif and transcription factor binding motifs, such as those of Auxin Response Factors (ARFs) and MYC2, in their promoters, indicating that *MIR* transcription is regulated by general and specific transcription factors (Xie *et al.*, 2005a; Megraw *et al.*, 2006).

Mediator, a general transcriptional coactivator, helps recruit Pol II to *MIR* loci (Kim *et al.*, 2011). Other factors promoting general

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MIR transcription include NEGATIVE ON TATA LESS2 (NOT2), the putative MYB domain-containing DNA-binding protein CELL DIVISION CYCLE 5 (CDC5) and the Elongator complex, which is thought to assist transcriptional elongation (Wang *et al.*, 2013; Zhang *et al.*, 2013; Fang *et al.*, 2015). NOT2, CDC5 and Elongator all interact with Pol II and the dicing complex (the plant miRNA precursor processing complex), implying their functions in bridging Pol II transcription and pri-miRNA processing (Wang *et al.*, 2013; Zhang *et al.*, 2013; Fang *et al.*, 2015). Pol II activity in *MIR* transcription is probably subject to phospho-regulation. miRNA levels are significantly reduced in mutants of *CDKF;1* (*CYCLIN-DEPENDENT KINASE F;1*) and *CDKD* (*CYCLIN-DEPENDENT KINASE D*) genes. These mutants also have reduced phosphorylation marks at the Pol II C-terminal domain (CTD) (Hajheidari *et al.*, 2012; reviewed in Hajheidari *et al.*, 2013).

Factors specifically controlling the transcription of certain miRNAs within an miRNA family have also been characterized. For instance, POWERDRESS promotes the transcription of *MIR172a*, *b* and *c* by enhancing Pol II occupancy at their promoters, without affecting *MIR172d* or *e*. Under phosphate starvation, the MYB2 transcription factor binds to the promoter of *MIR399f* to promote its transcription (Baek *et al.*, 2013; reviewed in Rogers & Chen, 2013; Yumul *et al.*, 2013).

2. miRNA precursor processing

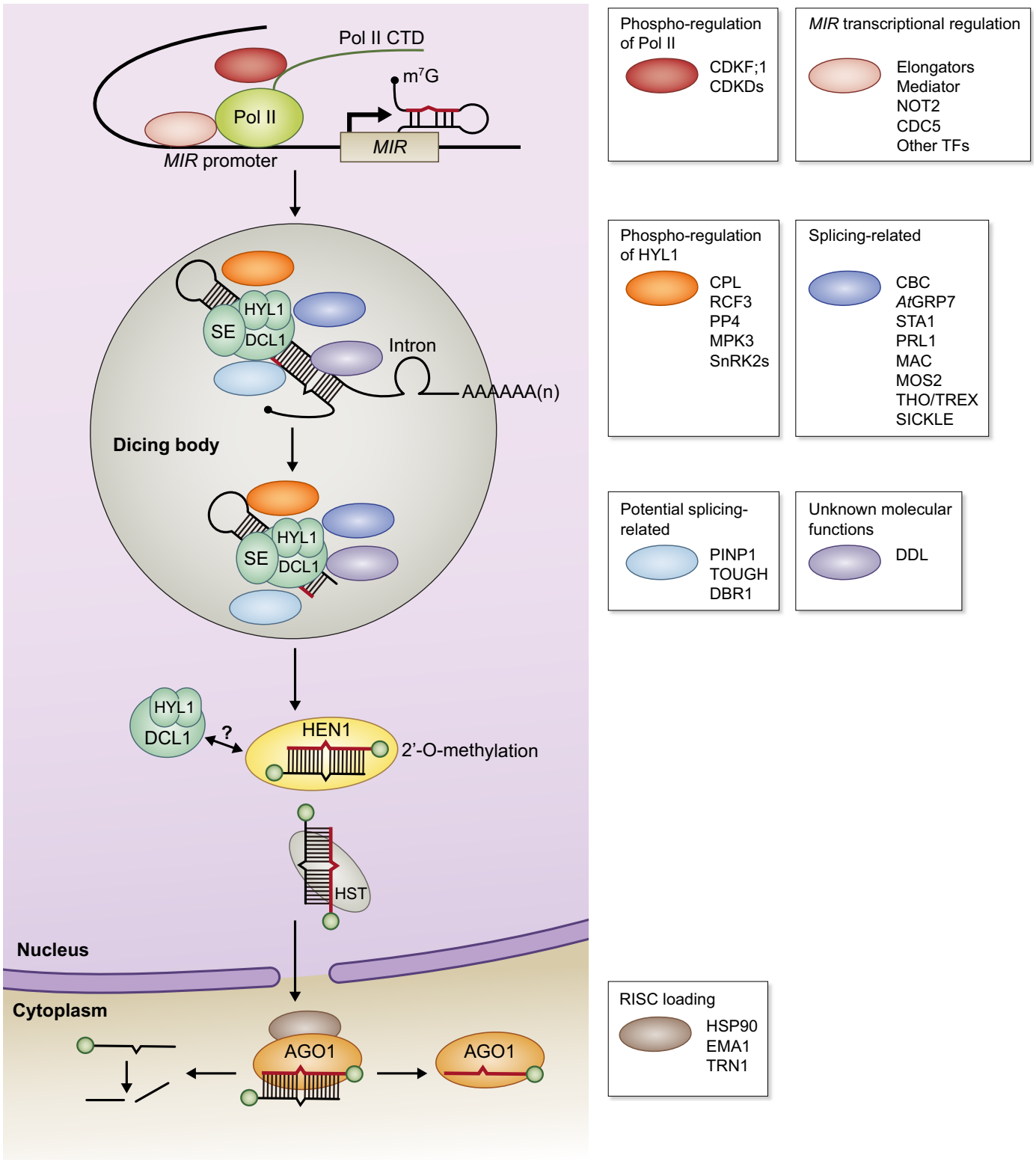
The dicing complex Nascent pri-miRNAs are capped at the 5′ end and polyadenylated at the 3′ end, and intron-containing pri-miRNAs are spliced or alternatively spliced (Xie *et al.*, 2005a; Szarzynska *et al.*, 2009; Zielezinski *et al.*, 2015; reviewed in Stepien *et al.*, 2016). pri-miRNAs are processed by the dicing complex, which contains DICER-LIKE1 (DCL1), HYPONASTIC LEAVES1 (HYL1) and SERRATE (SE) as core components, to yield mature miRNA/miRNA* duplexes (Park *et al.*, 2002; Reinhart *et al.*, 2002; Kurihara & Watanabe, 2004; reviewed in Fukudome & Fukuhara, 2017) (Fig. 1).

Of the four DCL RNase III family endonucleases in *Arabidopsis*, DCL1 is the predominant miRNA precursor processing enzyme (Park *et al.*, 2002; Reinhart *et al.*, 2002). DCL2, DCL3 and DCL4 produce various types of small interfering RNAs (siRNAs), including endogenous siRNAs, as well as viral and transgene siRNAs (Gasciolli *et al.*, 2005; Xie *et al.*, 2005b; Bouche *et al.*, 2006; Mlotshwa *et al.*, 2008; reviewed in Fukudome & Fukuhara, 2017). A notable exception is that several young miRNAs, such as miR822 and miR839, are generated by DCL4 instead of DCL1 (Rajagopalan *et al.*, 2006). DCL proteins appear to function as molecular rulers that measure and cleave small RNA duplexes at a specific length (Macrae *et al.*, 2006). DCL1 mainly processes pri-miRNAs in a base-to-loop manner in two steps. The first cut is 15–17 nt away from the base of the stem or a bulge or unstructured region within the loop-distal stem. The resulting precursor-miRNA (pre-miRNA) is further cleaved by DCL1 to produce a 21-nt miRNA/miRNA* duplex (Song *et al.*, 2010; Liu *et al.*, 2012; Zhu *et al.*, 2013). Alternative processing modes include loop-to-base processing (Bologna *et al.*, 2009).

In a five-member family of DOUBLE-STRANDED RNA-BINDING PROTEINS (DRBs), HYL1/DRB1 is a major miRNA biogenesis factor and DRB2 affects the accumulation of a few miRNAs (Hiraguri *et al.*, 2005; Curtin *et al.*, 2008; Eamens *et al.*, 2012). HYL1 interacts with DCL1 to facilitate efficient and precise miRNA precursor processing (Kurihara *et al.*, 2006; Dong *et al.*, 2008; Manavella *et al.*, 2012a; Yang *et al.*, 2014). Homodimerization of HYL1 is essential for its functions in miRNA precursor processing (Yang *et al.*, 2010, 2014). HYL1 also affects the splicing of some pri-miRNAs and strand selection from miRNA/miRNA* duplexes in AGO1 loading (Szarzynska *et al.*, 2009; Manavella *et al.*, 2012a; Ben Chaabane *et al.*, 2013).

Recent research has uncovered regulatory mechanisms impacting the activity, stability and nuclear localization of HYL1 in miRNA precursor processing (Manavella *et al.*, 2012a; Cho *et al.*, 2014; Karlsson *et al.*, 2015; Raghuram *et al.*, 2015; Zhang *et al.*, 2017a). C-TERMINAL DOMAIN PHOSPHATASE-LIKE (CPL) proteins dephosphorylate HYL1 to facilitate accurate miRNA precursor processing and strand selection during AGO loading (Manavella *et al.*, 2012a). The K homology (KH) domain protein REGULATOR OF CBF GENE EXPRESSION 3 (RCF3) promotes HYL1 dephosphorylation through interaction with CPL proteins (Karlsson *et al.*, 2015). In addition, a PP4 (Protein Phosphatase 4) complex targets HYL1 for dephosphorylation and stabilizes HYTL1 (Su *et al.*, 2017). This dephosphorylation is antagonized by the protein kinases MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) and SNF1-related protein kinase subfamily 2 (SnRK2) (Raghuram *et al.*, 2015; Yan *et al.*, 2017). Phospho-regulation affects not only HYL1 activity, but also its protein stability, for example an *snrk2* mutation leads to reduced levels of HYL1 (Yan *et al.*, 2017). HYL1 protein stability is regulated by the RING-finger E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) through light signaling. Specifically, under light conditions, COP1 shifts to the cytoplasm and suppresses HYL1 cleavage by an unidentified protease, whereas, in darkness, COP1 enters the nucleus thereby releasing the protease that cleaves HYL1 (Cho *et al.*, 2014). KETCH1 (KARYOPHERIN ENABLING THE TRANSPORT OF THE CYTOPLASMIC HYL1), a well-conserved importin-beta protein, transports HYL1 from the cytoplasm to the nucleus to form the dicing complex. *KETCH1* knockdown mutants show similar phenotypes to miRNA biogenesis mutants, including reduced miRNA levels and compromised pri-miRNA processing, indicating the importance of HYL1's nuclear localization in miRNA biogenesis (Zhang *et al.*, 2017a).

In addition to DCL1 and HYL1, SE is also considered as a core member of the miRNA processing complex in *Arabidopsis*. A mutation in *SE* results in reduced levels of mature miRNAs, increased levels of pri-miRNAs and defects in pri-miRNA splicing (Grigg *et al.*, 2005; Lobbes *et al.*, 2006; L. Yang *et al.*, 2006; Laubinger *et al.*, 2008). As a zinc-finger protein with RNA-binding activity, SE also functions outside of miRNA biogenesis. For example, SE interacts with U1 small nuclear ribonucleoprotein (snRNP) components (Knop *et al.*, 2016). Different from DCL1 or HYL1, SE is distributed in a heterogeneous subnuclear pattern, reminiscent of nuclear speckles in which serine/arginine (SR)



splicing factors are enriched (Fang & Spector, 2007). SE affects the alternative splicing of some *Arabidopsis* mRNAs (Laubinger *et al.*, 2008; Raczynska *et al.*, 2014).

Other proteins that influence miRNA precursor processing and/or *MIR* transcription In the past decade, many proteins that influence miRNA precursor processing or *MIR* gene transcription have been identified (Fig. 1). On the one hand, these proteins promote miRNA biogenesis in general, as most miRNAs accumulate to lower levels in mutants of these genes. On the other hand, the functions of these genes are not specific to miRNAs; indeed, many have functions in precursor mRNA (pre-mRNA) splicing. In terms

Fig. 1 Illustrations of major steps in microRNA (miRNA) biogenesis. RNA polymerase II (Pol II)-mediated miRNA gene (*MIR*) transcription is regulated by multiple transcription factors (TFs). Pol II activity itself is also subjected to phospho-regulation at its C-terminal domain (CTD). miRNA precursors are processed at the dicing bodies by the dicing complex, which is mainly composed of DICER-LIKE 1 (DCL1), HYPONASTIC LEAVES 1 (HYL1) and SERRATE (SE). Many other protein factors contribute to miRNA precursor processing through phospho-regulation, RNA splicing and other unknown molecular mechanisms. It remains unclear whether the dicing complex interacts with HUA ENHANCER 1 (HEN1) (question mark) and contributes to miRNA/miRNA* duplex export and RNA-induced silencing complex (RISC) assembly. During RISC loading, one strand of the small RNA duplex is selected as the guide strand (red) and incorporated into ARGONAUTE 1 (AGO1) to form a functional RISC, whereas the other strand (the passenger strand) is removed and degraded. Proteins are color-coded according to their known molecular functions in phospho-regulation of Pol II (red), *MIR* transcription (pink), phospho-regulation of HYL1 (orange), splicing/RNA-binding (dark blue) and potentially splicing/RNA-binding (light blue), and RISC assembly (brown). The core dicing complex components are colored green and protein with unknown molecular functions is colored purple. m^7G, 7-methylguanylate cap at the 5′ end of primary miRNAs; CDKF;1, CYCLIN-DEPENDENT KINASE F;1; CDKDs, CYCLIN-DEPENDENT KINASE D; NOT2, NEGATIVE ON TATA LESS 2; CDC5, CELL DIVISION CYCLE 5; CPL, C-TERMINAL DOMAIN PHOSPHATASE-LIKE; RCF3, REGULATOR OF CBF GENE EXPRESSION 3; PP4, Protein Phosphatase 4 complex; MPK3, MITOGEN-ACTIVATED PROTEIN KINASE 3; SnRK2s, SNF1-related protein kinase subfamily 2; CBC, Cap Binding Complex; *At*GRP7, GLYCINE-RICH RNA-BINDING PROTEIN 7; STA1, STABILIZED 1; PRL1, PROTEIN PLEIOTROPIC REGULATORY LOCUS 1; MAC, MOS4-associated Complex; MOS2, MODIFIER OF SNC1, 2; THO/TREX, suppressor of the Transcription defects of *Hpr1* mutants by Overexpression/TRanscription-EXport complex; PINP1, PSR1-INTERACTING PROTEIN 1; DBR1, LARIAT DEBRANCHING ENZYME 1; DDL, DAWDLE; HST, HASTY; HSP90, HEAT SHOCK PROTEIN 90; EMA1, ENHANCED MIRNA ACTIVITY 1; TRN1, TRANSPORTIN 1.

of miRNA biogenesis, these proteins appear to act before or during pri-miRNA processing, as mutants in these genes have either higher or lower levels of pri-miRNAs. Below, we categorize these proteins into two groups based on their effects on pri-miRNA accumulation and discuss their potential roles in miRNA biogenesis.

A large group of proteins appears to promote pri-miRNA processing, as loss/reduction-of-function mutants in the corresponding genes have reduced levels of mature miRNAs and increased levels of pri-miRNAs. Proteins belonging to this group include CAP-BINDING PROTEIN 80 (CBP80) and CAP-BINDING PROTEIN 20 (CBP20) (Laubinger *et al.*, 2008; reviewed in Gonatopoulos-Pournatzis & Cowling, 2014), STABILIZED1 (STA1) (Ben Chaabane *et al.*, 2013), THO1/HPR1/EMU and THO2 (Furumizu *et al.*, 2010; Francisco-Mangilet *et al.*, 2015), SICKLE (Zhan *et al.*, 2012), TOUGH (Ren *et al.*, 2012b), PSR1-INTERACTING PROTEIN 1 (PINP1) (Qiao *et al.*, 2015) and MODIFIER OF SNC1, 2 (MOS2) (X. Wu *et al.*, 2013). GLYCINE-RICH RNA-BINDING PROTEIN 7 (*At*GRP7) may repress miRNA biogenesis, as its overexpression causes reduced levels of mature miRNAs and increased accumulation of pri-miRNAs (Koster *et al.*, 2014). All of these proteins have demonstrated or predicted ability to associate with or act on RNAs. CBP80 and CBP20 are subunits of the nuclear Cap Binding Complex (CBC), which interacts with SE (Laubinger *et al.*, 2008). The human and yeast homolog of STA1 is the U5 snRNP-associated protein Pre-mRNA Processing Factor 6 (PRPF6), a confirmed splicing factor (reviewed in Will & Luhrmann, 2011; Ben Chaabane *et al.*, 2013). THO1/HPR1/EMU and THO2 are subunits of the THO/TREX (suppressor of the Transcription defects of *Hpr1* mutants by Overexpression/TRanscription-EXport) complex, a conserved multi-subunit complex involved in pre-mRNA co-transcriptional processing and mRNA export in yeast and animals (reviewed in Heath *et al.*, 2016). SICKLE is a plant-specific protein that interacts with many RNA processing proteins (Zhan *et al.*, 2012; Marshall *et al.*, 2016). TOUGH and MOS2 are RNA-binding proteins (Ren *et al.*, 2012b; X. Wu *et al.*, 2013). PINP1 is a putative RNA helicase (Qiao *et al.*, 2015). *At*GRP7 is a heterogeneous nuclear ribonucleoprotein (hnRNP)-like glycine-rich RNA-binding protein (Streitner *et al.*, 2012; Koster *et al.*, 2014). The molecular functions of these proteins in pri-miRNA processing are currently unknown.

A second group of proteins may act differently from the previously discussed group in miRNA biogenesis. Loss/reduction-of-function mutants in genes in this second group have reduced accumulation of both mature miRNAs and pri-miRNAs. Proteins in this group include CDC5, NOT2, Elongator, PRL1 (PROTEIN PLEIOTROPIC REGULATORY LOCUS 1) and DDL (DAWDLE) (Yu *et al.*, 2008; Wang *et al.*, 2013; Zhang *et al.*, 2013, 2014; Fang *et al.*, 2015). Although the reduced pri-miRNA accumulation in the mutants suggests a role of the proteins in *MIR* transcription or pri-miRNA stability, these proteins also seem to affect miRNA precursor processing. For example, mutants in *NOT2* show an increase in the number of dicing bodies, whereas mutants in *MOS2* and Elongator have a reduced number of dicing bodies. CDC5, NOT2, Elongator, PRL1 and DDL were all found to interact with DCL1 and may help to recruit DCL1 to pri-miRNAs (Yu *et al.*, 2008; Wang *et al.*, 2013; Zhang *et al.*, 2013, 2014; Fang *et al.*, 2015). Perhaps the most parsimonious hypothesis is that these proteins promote miRNA biogenesis by recruiting the dicing complex to nascent pri-miRNAs during transcription.

A prominent feature of these two groups of proteins, regardless of their effects on pri-miRNAs, is their demonstrated or predicted roles in splicing. Splicing defects in both pri-miRNAs and pre-mRNAs were detected in *abh1*/*cbp80*, *cbp20*, *sic-1* and *sta1-1* mutants (Laubinger *et al.*, 2008; reviewed in Gonatopoulos-Pournatzis & Cowling, 2014). *AtGRP7* overexpression results in changes in pri-miRNA splicing (Streitner *et al.*, 2012; Koster *et al.*, 2014). MOS2 is required for appropriate splicing of *SNC1* (*SUPPRESSOR OF NPR1-1*, *CONSTITUTIVE 1*), which encodes a Toll Interleukin 1 Receptor Nucleotide Binding Leucine-Rich Repeat (TIR-NB-LRR) class of protein involved in plant defense responses (Zhang *et al.*, 2005; Copeland *et al.*, 2013). Alternatively spliced SR gene transcripts were detected in *tho1* and *tho2* mutants (Furumizu *et al.*, 2010; Francisco-Mangilet *et al.*, 2015). PRL1 belongs to the NineTeen Complex (NTC) or MOS4-associated

complex (MAC), a protein complex with 19 conserved members in yeast, human and plants. MAC is involved in spliceosome assembly and pre-mRNA splicing in all eukaryotic model organisms (Monaghan *et al.*, 2009; reviewed in Johnson *et al.*, 2011 and Koncz *et al.*, 2012). *Arabidopsis* TOUGH colocalizes or interacts with splicing factor SR proteins, indicating a potential role in general pre-mRNA splicing (Calderon-Villalobos *et al.*, 2005; Ren *et al.*, 2012b). Although there is no direct evidence demonstrating PINP1's involvement in pre-mRNA processing in *Arabidopsis*, its yeast homolog, Prp16, is a confirmed splicing factor (Wang *et al.*, 1998).

The large number of proteins acting in both RNA splicing and miRNA biogenesis begs the question of whether or how these two nuclear RNA processing events are related. Some pri-miRNAs harbor introns (Szarzynska *et al.*, 2009), and thus splicing may be an essential step in miRNA biogenesis. However, pri-miRNAs without introns are also affected in mutants in some of the aforementioned genes. For example, the levels of pri-miR159a, which contains no introns, were altered in the mutants of *CBC*, *STA1*, *PRL1*, *MOS2*, *THO2* and *SICKLE* (Laubinger *et al.*, 2008; Szarzynska *et al.*, 2009; Zhan *et al.*, 2012; Ben Chaabane *et al.*, 2013; Copeland *et al.*, 2013; X. Wu *et al.*, 2013; Zhang *et al.*, 2014; Francisco-Mangilet *et al.*, 2015). At least for pri-miRNAs without introns, the aforementioned proteins cannot act in miRNA biogenesis through their functions in RNA splicing. Another formal possibility is that these proteins only act in splicing; in loss/reduction-of-function mutants of these genes, the accumulation of unspliced introns sequesters the dicing complex and thus inhibits miRNA biogenesis. It was found that a mutation in the intron lariat debranching gene *DBR1* (*LARIAT DEBRANCHING ENZYME 1*) results in the over-accumulation of intronic RNAs, which compete with pri-miRNAs for the dicing complex (Z. Li *et al.*, 2016). However, many of the aforementioned proteins interact with the dicing complex or pri-miRNAs, which implies a direct role in miRNA biogenesis. Perhaps many of the proteins act broadly in nuclear RNA metabolism, with RNA splicing and miRNA biogenesis being two independent processes in which they participate.

3. miRNA stabilization and RISC formation

The miRNA/miRNA* duplex is stabilized through 3′-terminal 2′-*O*-methylation by HEN1 (Fig. 1). HEN1 was first discovered in *Arabidopsis* as a methyltransferase that specifically methylates small RNAs (Yu *et al.*, 2005; Yang Z *et al.*, 2006). HEN1 homologs with similar functions were later discovered in other plants, animals and fungi (Kirino & Mourelatos, 2007; Saito *et al.*, 2007; reviewed in Huang, 2012). The crystal structure of an *Arabidopsis* HEN1–small RNA complex suggests that the small RNA duplex is bound by the HEN1 double-strand RNA-binding domains (dsRBD), with one terminus being in the methyltransferase (MTase) active site and methylated in an Mg^{2+}-dependent manner (Huang *et al.*, 2009). A recent study has suggested that HEN1 might interact with DCL1 and HYL1 based on yeast two-hybrid and *in vitro* pull-down assays (Baranauske *et al.*, 2015). However, further *in vivo* analysis is needed to confirm this interaction.

During AGO loading, one strand of the small RNA duplex is selected as the guide strand, whereas the passenger strand is removed (Fig. 1). The current model for *Arabidopsis* RISC loading is as follows. (1) AGO1 and a dimer of HEAT SHOCK PROTEIN 90 (HSP90) form a complex. (2) The binding of adenosine triphosphate (ATP) to HSP90 causes a conformational change of AGO1 that allows the small RNA duplex to be incorporated into the AGO1–HSP90 protein complex. (3) ATP hydrolysis induces AGO1 dissociation from HSP90. (4) The AGO1 conformational change caused by HSP90 dissociation removes the passenger strand and results in a mature RISC (Iki *et al.*, 2010).

Two AGO1-interacting importin-beta family proteins, ENHANCED MIRNA ACTIVITY1 (EMA1) and TRANSPORTIN1 (TRN1), negatively and positively regulate miRNA loading into AGO1, respectively. As importin-beta family proteins, the most intuitive expectation would be that they mediate AGO1's or miRNA's nuclear-cytoplasmic shuttling. However, the nuclear-cytoplasmic partitioning of AGO1 or miRNAs is unchanged in these mutants (Wang *et al.*, 2011; Cui *et al.*, 2016). Nevertheless, the fact that importin-beta family proteins affect the loading of miRNAs into AGO1 suggests that RISC formation occurs at specific subcellular locations.

The selection of miRNA guide strands is not random. In *Arabidopsis*, guide strand selection is known to be affected by miRNA precursor processing factors, the nature of the 5′ end nucleotide and the structure of the small RNA duplex. HYL1 and the HYL1 phosphatase CPL1 facilitate guide strand selection, with *hyl1* and *cpl1* mutants exhibiting accumulated miRNA* strands (Eamens *et al.*, 2009; Manavella *et al.*, 2012a). The nature of the 5′ nucleotides directs AGO loading. Most miRNA guide strands start with a 5′-terminal uridine and are incorporated into AGO1. By contrast, few miRNA star strands have 5′-terminal uridine. miRNA star strands with 5′-terminal adenosine are largely associated with AGO2, whereas those with 5′-terminal cytosine are associated with AGO5 (Mi *et al.*, 2008). The loading of miRNAs into AGO proteins is also affected by the bulges in miRNA/miRNA* duplex structures. AGO2 favors miRNA duplexes without central mismatches, whereas AGO1 prefers duplexes with central mismatches, and the preference of AGO10 for miR165/6 relies on the internal base mismatches of the miRNA166 precursor (Zhu *et al.*, 2011; Ren *et al.*, 2014).

4. The cell biology of miRNA biogenesis: dicing bodies, nuclear export and RISC loading

The nucleus is the site of pri-miRNA processing in *Arabidopsis* (Papp *et al.*, 2003). Live-cell imaging revealed that DCL1 and HYL1 colocalize in round and membrane-less nuclear bodies, namely dicing bodies, which range in number from zero to four in each cell (Han *et al.*, 2004; Fang & Spector, 2007; Song *et al.*, 2007). DCL1 and HYL1 also exhibit diffuse patterns in the nucleoplasm, but are excluded from nucleoli (Fang & Spector, 2007; Z. Li *et al.*, 2016). *In vivo* tracking of a pri-miRNA showed its colocalization with dicing bodies, indicating the role of dicing bodies in pri-miRNA processing (Fang & Spector, 2007). Dicing bodies resemble Cajal bodies in shape, size and number. However,

colocalization analysis demonstrated that dicing bodies and Cajal bodies are distinct structures (Fang & Spector, 2007; Song *et al.*, 2007) (Fig. 1).

Different mechanisms have been proposed for the formation of membrane-less nuclear bodies (e.g. dicing bodies), including stochastic assembly, ordered assembly and seeded assembly (reviewed in Mao *et al.*, 2011). Low-complexity sequences, which are enriched in many RNA- and DNA-binding proteins, contribute to the formation of higher order RNA- and protein-containing structures (Han *et al.*, 2012; Kato *et al.*, 2012). *Arabidopsis* DCL1 contains two dsRBDs. The second dsRBD is truncated in the *dcl1-9* mutant, which exhibits severe miRNA biogenesis defects (Park *et al.*, 2002; reviewed in Schauer *et al.*, 2002); moreover, the truncated DCL1-9 protein fails to localize to dicing bodies (Fang & Spector, 2007). Similarly, the N-terminal dsRBDs of HYL1 are essential for HYL1's localization to dicing bodies (Wu *et al.*, 2007).

Many other miRNA biogenesis factors, such as SE, RCF3 and THO2, largely form splicing speckles and partially colocalize with dicing bodies (Fang & Spector, 2007; Francisco-Mangilet *et al.*, 2015; Karlsson *et al.*, 2015). NOT2, MOS2 and PINP1 show diffuse nucleoplasmic patterns and also partially colocalize with dicing bodies (Wang *et al.*, 2013; X. Wu *et al.*, 2013; Qiao *et al.*, 2015). The subcellular localization patterns of the above factors implicate their roles in both miRNA and mRNA biogenesis. Dicing body formation is affected by several miRNA biogenesis factors. Mutants of *MOS2* and Elongator subunits have a reduced number of dicing bodies, whereas those of *PINP1*, *NOT2* and *DBR1* have more dicing bodies than the wild-type; thus, opposite effects are observed, although all of the above mutants have compromised miRNA levels (Wang *et al.*, 2013; X. Wu *et al.*, 2013; Fang *et al.*, 2015; Qiao *et al.*, 2015; Z. Li *et al.*, 2016).

The export of miRNAs from the nucleus to the cytoplasm is fundamental for miRNA activity (Lund *et al.*, 2004; Park *et al.*, 2005; reviewed in Kohler & Hurt, 2007 and Rogers & Chen, 2013). Exportin 5, a RanGTP-dependent dsRNA-binding protein, mediates the nuclear export of pre-miRNAs in animals (Yi *et al.*, 2003; Bohnsack *et al.*, 2004; Lund *et al.*, 2004). In *Arabidopsis*, miRNA/miRNA* duplexes are probably excised from pre-miRNAs in the nucleus (as DCL1 acts in the nucleus) and are thought to be transported to the cytoplasm by HASTY (Papp *et al.*, 2003; Park *et al.*, 2005) (Fig. 1). In the *hasty* mutant, the nuclear-cytoplasmic partitioning of miRNAs is not altered (Park *et al.*, 2005). Therefore, the functions of HASTY in miRNA nuclear export in *Arabidopsis* still require further investigation. THO/TREX complex components are required for miRNA biogenesis (Furumizu *et al.*, 2010; Francisco-Mangilet *et al.*, 2015). As THO/TREX mediates transcription-coupled mRNA export through interactions with the nuclear pore complex, it is also possible that THO/TREX plays a role in miRNA export (reviewed in Kohler & Hurt, 2007).

It is still unclear whether miRNAs are exported to the cytoplasm before RISC loading or whether RISC loading precedes export to the cytoplasm. However, the involvement of cytoplasmic HSP90 in RISC loading is one line of evidence in favor of RISC loading in the cytoplasm (reviewed in Krishna & Gloor, 2001; Mi *et al.*, 2008).

II. Modes of action of miRNAs

Plant miRNAs regulate target genes at the post-transcriptional level via two major mechanisms: transcript cleavage and translation repression (reviewed in Chen, 2005, Chen, 2009, Voinnet, 2009 and Rogers & Chen, 2013) (Fig. 2). For small RNAs in general, the degree of sequence complementarity between small RNAs and their targets influences the particular mode of action in which the small RNAs can engage, with transcript cleavage requiring a high degree of sequence complementarity (Hutvagner & Zamore, 2002). In plants, miRNAs and their target mRNAs have nearly perfect complementarity, and, because of this, transcript cleavage was thought to be the predominant mode of action of plant miRNAs (reviewed in Chen, 2005, Jones-Rhoades *et al.*, 2006, Chen, 2009 and Voinnet, 2009). However, this is a mis-conception. Although a high degree of sequence complementarity is conducive to RNA cleavage, it is not necessarily refractory to translational repression. Indeed, targets that have been experimentally validated to undergo miRNA-mediated translation inhibition pair with miRNAs with a high degree of sequence complementarity (Brodersen *et al.*, 2008; Yang *et al.*, 2012; Li *et al.*, 2013). Examples are *APETALA 2* (*AP2*), *SCARECROW-LIKE PROTEIN 4* (*SCL4*), *COPPER/ZINC SUPEROXIDE DISMUTASE 2* (*CSD2*) and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* (*SPL3*), targeted by miR172, miR171, miR398 and miR156, respectively (Aukerman & Sakai, 2003; Chen, 2004; Gandikota *et al.*, 2007; Brodersen *et al.*, 2008). Indeed, the same mRNAs also undergo cleavage caused by the same miRNAs (Li *et al.*, 2013; Hou *et al.*, 2016; Yu *et al.*, 2016). Thus, sequence complementarity is not the factor that dictates the mode of action in which plant miRNAs engage. Emerging findings of miRNA target transcripts bound by ribosomes or ribosomes on the endoplasmic reticulum (ER) (Hou *et al.*, 2016; S. Li *et al.*, 2016; Yu *et al.*, 2016) imply that translation inhibition may occur at a larger number of miRNA targets than expected.

1. Transcript cleavage

miRNA-guided RNA cleavage, also known as slicing, occurs at a precise position in the target mRNA (Llave *et al.*, 2002). Genome-wide identification of RNAs with a 5′ monophosphate (the 3′ cleavage fragments have a 5′ monophosphate) found that most plant miRNA targets undergo transcript cleavage (German *et al.*, 2008). Cleavage is accomplished by the PIWI domain of AGO proteins, which forms an RNase H-like fold and exhibits endonuclease activity; this activity has been demonstrated for *Arabidopsis* AGO1, the major miRNA effector, together with AGO2, AGO4, AGO7 and AGO10 (Mi *et al.*, 2008; Montgomery *et al.*, 2008; Takeda *et al.*, 2008; Ji *et al.*, 2011; Maunoury & Vaucheret, 2011; Zhu *et al.*, 2011).

On slicing, the 5′ and 3′ cleavage fragments are subsequently degraded by exonucleases (Fig. 2). In *Arabidopsis*, EXORIBONUCLEASE 4 (XRN4), a 5′-to-3′ exonuclease, is responsible for degrading the 3′ fragments (Souret *et al.*, 2004). Unlike the 3′ fragments, which are usually detectable in wild-type plants, the 5′ fragments are barely detected, probably as a result of rapid degradation. In *Chlamydomonas reinhardtii*, the 5′ fragments are polyadenylated by the nucleotidyl transferase MUT68, followed

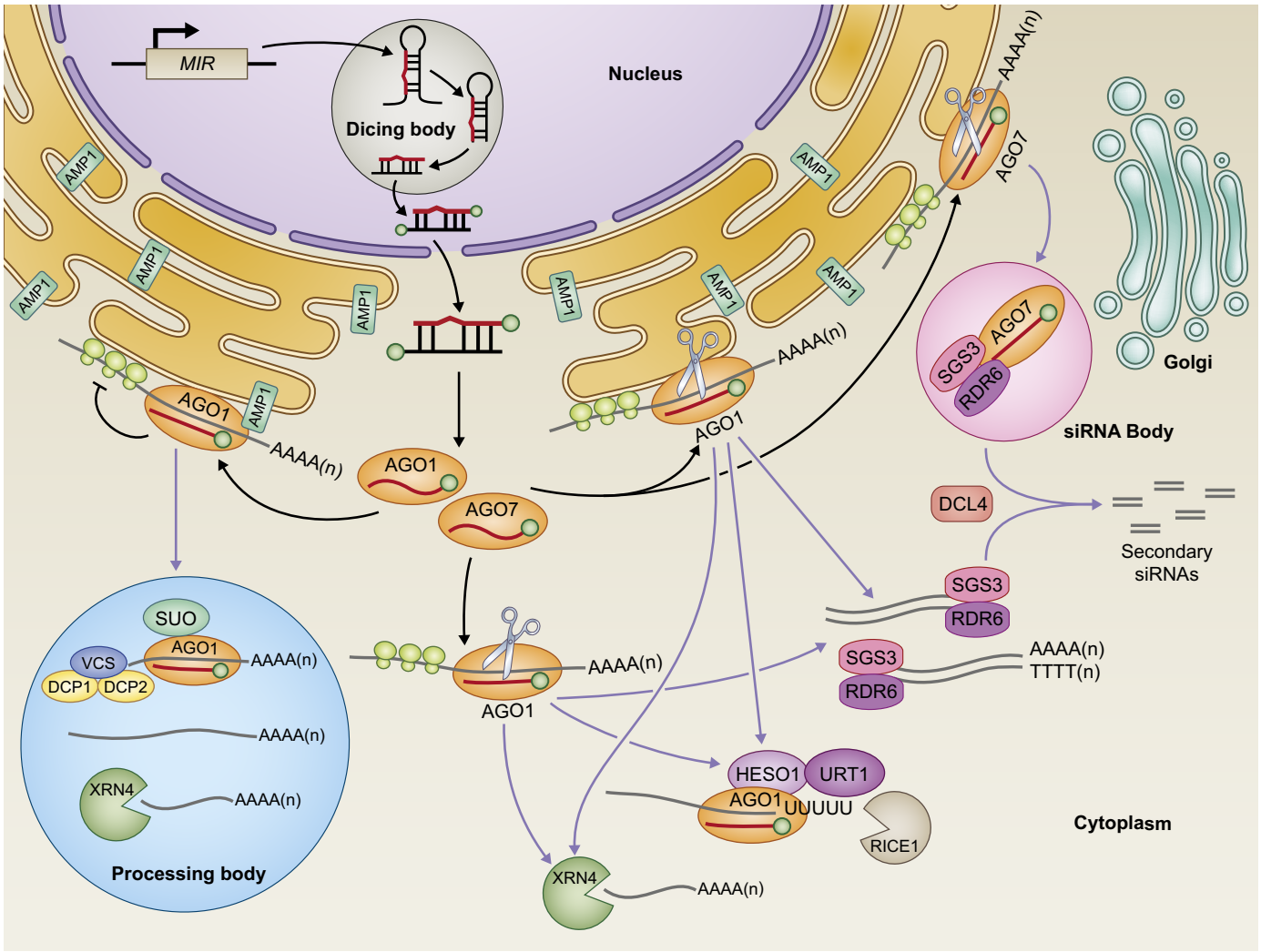


Fig. 2 Overview of microRNA (miRNA) modes of action in plants. Mature miRNAs are incorporated into ARGONAUTE proteins to direct post-transcriptional gene silencing (PTGS) via transcript cleavage and translation repression or trigger the biogenesis of secondary small interfering RNAs (siRNAs). ARGONAUTE 1 (AGO1) mediates miRNA target cleavage followed by degradation of the cleavage fragments. The cytoplasmic location of this event is unclear, but the uridylation and turnover of 5′ cleavage fragments occur on AGO1. Translation repression takes place on membrane-bound polysomes (MBPs), and requires endoplasmic reticulum (ER)-localized ALTERED MERISTEM PROGRAM 1 (AMP1). Components of Processing body (P-body) are also involved in this process, although the function of these factors and their connection to ER remain mysterious. ARGONAUTE 7 (AGO7) cleaves miR390 targets that are associated with MBPs, and forms siRNA bodies together with SUPPRESSOR OF GENE SILENCING 3 (SGS3) and RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) that are adjacent to the *cis*-Golgi. Other *TAS* transcripts that generate phased secondary siRNAs (phasiRNAs) in response to AGO1-mediated cleavage also associate with MBPs. Events are classified with colored lines according to miRNA-mediated actions (dark lines) and subsequent processing (light purple lines) of their targets. DCL4 DICER-LIKE 4; HESO1, HEN1 SUPPRESSOR 1; URT1, UTP:RNA URIDYLYLTRANSFERASE 1; XRN4, EXORIBONUCLEASE 4; SUO, a GW-repeat protein; VCS, VARICOSE; DCP1, DECAPPING 1; DCP2, DECAPPING 2; RICE1, RISC-INTERACTING CLEARING 3′– 5′ EXORIBONUCLEASE 1.

by degradation by the cytoplasmic exosome (Ibrahim *et al.*, 2006). HEN1 SUPPRESSOR 1 (HESO1), an *Arabidopsis* homolog of MUT68, and its paralog UTP:RNA URIDYLYLTRANSFERASE 1 (URT1) polyuridylate the 5′ fragments *in vivo* and *in vitro* (Ren *et al.*, 2014; Wang *et al.*, 2015). RISC-INTERACTING CLEARING 3′– 5′ EXORIBONUCLEASE 1 (RICE1) is responsible for the degradation of uridylated 5′ fragments in *Arabidopsis*, because these uridylated fragments are over-accumulated in plants ectopically expressing a catalytically inactive RICE1 (Zhang *et al.*, 2017b). The cytoplasmic exosome may also play a role, as its cofactor's subunits, SUPERKILLER 2 (SKI2), SKI3 and SKI8, are required for the degradation of RISC-generated 5′ fragments (Branscheid *et al.*, 2015).

2. Translation inhibition

miRNA-mediated translation repression was initially proposed to account for the disproportionate effects of miRNAs on target gene repression at the protein vs mRNA level (Aukerman & Sakai, 2003; Chen, 2004; Gandikota *et al.*, 2007). In plants, translation repression is less frequently observed than transcript cleavage, possibly owing to the universal presence of miRNA-guided cleavage, coupled with difficulty in determining protein levels because of the absence of high-quality antibodies.

Early examples of miRNA-mediated translation inhibition in plants were *AP2* and *SPL3* regulated by miR172 and miR156/7, respectively (Aukerman & Sakai, 2003; Chen, 2004; Gandikota

et al., 2007). When miR172 and miR156/7 accumulated abnormally, *AP2* and *SPL3* transcript levels were comparable with those of the wild-type, but their protein levels were altered (Chen, 2004; Gandikota *et al.*, 2007). Subsequently, similar observations were made for other miRNAs, including miR159 (Alonso-Peral *et al.*, 2010), miR164, miR165/6 (Li *et al.*, 2013), miR171, miR395, miR398 and miR834 (Brodersen *et al.*, 2008). Moreover, the study by Li *et al.* (2013) went beyond observations of effects of miRNAs on target gene expression at the transcript vs protein level by showing that miR398 and miR165/6 inhibit protein synthesis from their target genes *CSD2* and *PHB*, respectively.

Known factors required for miRNA-mediated translation inhibition include the microtubule-severing enzyme KATANIN 1 (KTN1) (Brodersen *et al.*, 2008), the processing body (P body) component VARICOSE (VCS) (Brodersen *et al.*, 2008), the GW-repeat protein SUO (Yang *et al.*, 2012) and the ER membrane protein ALTERED MERISTEM PROGRAM 1 (AMP1) (Li *et al.*, 2013) (Fig. 2). Mutations in these genes selectively interfere with miRNA-guided repression at the protein level, suggesting that transcript cleavage and translation repression are two independent modes of action. Based on the finding that the recruitment of miRNA target transcripts throughout the polysome fractions is enhanced in the *amp1* mutant compared with the wild-type (Li *et al.*, 2013), plant miRNAs may repress translation initiation, but other possibilities exist. Genome-wide analyses of RNA degradation through the profiling of RNAs with 5′ monophosphate in *Arabidopsis* show that co-translational mRNA degradation occurs for most genes, including a large number of miRNA targets (Hou *et al.*, 2016; Yu *et al.*, 2016). The cleavage of presumably ribosome-bound *AP2* and *SPL3* transcripts at the corresponding miRNA binding sites was observed (Yu *et al.*, 2016). An important lesson is that, even for the 'RNA cleavage' mode of action of miRNAs, translating mRNAs are the targets. This is consistent with findings that AGO1 and miRNAs associate with polysomes (Lanet *et al.*, 2009; S. Li *et al.*, 2016). Although the molecular mechanisms underlying miRNA-mediated translation repression are far from clear, an *in vitro* analysis indicated that plant miRNAs could inhibit translation initiation or hinder the movement of ribosomes (Iwakawa & Tomari, 2013). Proposed mechanisms for miRNA-mediated translation repression in animals include the dissociation of translation initiation complexes, recruitment of translational repressors or displacement of polyA binding proteins from mRNAs (reviewed in Iwakawa & Tomari, 2015). The activities of animal miRNAs require a scaffold protein GW182 (reviewed in Iwakawa & Tomari, 2015), which is lacking in plants.

3. Biogenesis of secondary siRNAs

In addition to mRNA cleavage and translation repression, some miRNAs also trigger the production of phased secondary siRNAs (phasiRNAs) from their target transcripts (Fig. 2), and this is a widespread and conserved phenomenon in plants (reviewed in Chen, 2005, Chen, 2009 and Rogers & Chen, 2013). In *Arabidopsis*, after AGO-mediated slicing, either the 5′ or 3′ fragment is stabilized by SUPPRESSOR OF GENE SILENCING 3 (SGS3), which associates with RISC by recognizing specific features of the 22-nt miRNA/target duplex to protect the cleavage fragment from degradation (Yoshikawa *et al.*, 2005, 2013). RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) is recruited to convert the cleavage fragment into dsRNA which is later diced into phasiRNAs at a 21-nt interval (Yoshikawa *et al.*, 2005). This phasing requires AGO1-mediated cleavage: in an *ago1* mutant with defective slicing activity, secondary siRNAs are generated, but the phasing is disrupted (Arribas-Hernandez *et al.*, 2016).

The phasiRNAs generated from four families of non-coding *TAS* genes (*TAS1* to *TAS4*) in *Arabidopsis* were termed tasiRNAs at the time of discovery because of their in-*trans* mode of action similar to miRNAs (Allen *et al.*, 2005; Yoshikawa *et al.*, 2005; Montgomery *et al.*, 2008; Chen *et al.*, 2010; Cuperus *et al.*, 2010). Two mechanisms of tasiRNA production are based on the number of miRNA binding sites within the target transcripts. The predominant mechanism, known as the 'one-hit model', entails one miRNA binding site in the target transcript and a 22-nt miRNA (Allen *et al.*, 2005; Yoshikawa *et al.*, 2005; Chen *et al.*, 2010). The 'two-hit model' requires two miRNA binding sites within the target transcript (Axtell *et al.*, 2006). This is observed for *TAS3* transcripts, which contain two miR390 binding sites. AGO7, instead of AGO1, mediates the cleavage at the 3′ site, but not at the 5′ site (Axtell *et al.*, 2006) (Fig. 2).

In addition to the length of the miRNAs triggering phasiRNA biogenesis, other factors may also be influential. The asymmetric bulge structure within miRNA/miRNA* and the degree of complementarity in miRNA–target pairing affect tasiRNA production (Manavella *et al.*, 2012b; Yoshikawa *et al.*, 2013). The position of the miR173 binding site relative to the short open reading frame in *TAS2* or a transgene containing *TAS1c* sequence was found to be important, as tasiRNA abundance decreased when premature stop codons were introduced further upstream of the miR173 binding site (Zhang *et al.*, 2012; Yoshikawa *et al.*, 2016), suggesting a relationship between translation and tasiRNA biogenesis.

phasiRNAs are not generated from most miRNA target transcripts. Most miRNAs are 21 nt in length and do not trigger phasiRNA biogenesis from their targets. Genome-wide small RNA sequencing and bioinformatic analysis identified a small number of protein coding genes, including immune receptor *NUCLEOTIDE-BINDING LEUCINE-RICH REPEAT* (*NBS-LRR*) and *PENTATRICOPEPTIDE REPEAT* (*PPR*) genes, as targets of 22-nt miRNAs for phasiRNA production in *Arabidopsis* (reviewed in Fei *et al.*, 2013). Monouridylation of miR171 catalyzed by URT1 in the *hen1* mutant leads to a 22-nt miR171 that is able to trigger the production of phasiRNAs (Zhai *et al.*, 2013). A larger number of phasiRNAs, as well as the loci that generate them (*PHAS* loci), have been identified in many non-Brassicaceae plants (reviewed in Fei *et al.*, 2013). The phasiRNAs are derived from transcripts of protein coding genes, such as *NBS-LRR* and *PPR* genes, or long non-coding RNAs (Fei *et al.*, 2013; Zhai *et al.*, 2015; Fan *et al.*, 2016). Although the targets of many phasiRNAs are still unclear, miRNA-triggered production of phasiRNAs is nevertheless hypothesized to act in beneficial microbial interactions or plant defense, or have other long-term evolutionary benefits (reviewed in Fei *et al.*, 2013).

4. Subcellular locations of miRNA activities

Several studies in *Arabidopsis* link the sites of miRNA activity to polysomes (Lanet *et al.*, 2009), the ER membrane (Li *et al.*, 2013) and membrane-bound polysomes (S. Li *et al.*, 2016; Yu *et al.*, 2016) (Fig. 2).

Because AGO1 is the major miRNA effector, the subcellular localization of AGO1 is an important clue for uncovering the sites of miRNA activity. Previous studies have shown that AGO1 is detectable in the cytosol, but excluded from the nucleus, by fluorescence microscopy analysis, and AGO1 is enriched around the nuclear envelope in some cells (Derrien *et al.*, 2012). AGO1 is a peripheral membrane protein, based on fractionation experiments after high-salt or high-pH treatments (Brodersen *et al.*, 2012; Li *et al.*, 2013). The link between AGO1's membrane localization and the rough endoplasmic reticulum (rER) is based on fluorescence microscopy analysis showing that AGO1 accumulates in cytoplasmic granules that colocalize with an ER marker (Li *et al.*, 2013). The association of AGO1 with the rER is further supported by its interaction with AMP1, an integral membrane protein localized to the rER (Li *et al.*, 2013).

Subcellular fractionation detected the association of miRNAs and AGO1 with polysomes (Lanet *et al.*, 2009). Further fractionation revealed the association of AGO1 with membrane-bound polysomes (MBPs) and the preferential association of miRNAs with MBPs rather than polysomes in general (Li *et al.*, 2013; S. Li *et al.*, 2016). AMP1 and its paralog LIKE AMP1 (LAMP1) are both required for miRNA-guided translation repression, but not transcript cleavage (Li *et al.*, 2013). In the *amp1 lamp1* double mutant, miRNA target transcripts are associated with total polysomes as in wild-type plants (Li *et al.*, 2013). However, these transcripts are more enriched on MBPs in *amp1 lamp1* than in the wild-type (Li *et al.*, 2013). Thus, miRNA-mediated translation repression probably occurs on the rER.

How AGO1 associates with the endomembrane is unknown, but it may be independent of AMP1 (Li *et al.*, 2013) or target mRNAs (S. Li *et al.*, 2016). Several *ago1* mutants harboring various point mutations display compromised membrane association, and this association is further reduced by knocking down *HYDROXY METHYLGLUTARYL COA REDUCTASE 1* (*HMG1*), which encodes an isoprenoid biosynthesis enzyme (Brodersen *et al.*, 2012). Thus, aside from AGO1 itself, isoprenoid may influence the membrane association of AGO1. Loss of function in *HMG1* also leads to defective miRNA activity (Brodersen *et al.*, 2012), further suggesting that the membrane association of AGO1 is essential for its role in miRNA-directed activities.

AGO1 also associates with P bodies (reviewed in Xu & Chua, 2011). An *Arabidopsis* P body-localized protein, VCS, was found to play a role in miRNA-guided translation inhibition (Brodersen *et al.*, 2008). VCS is a component of the decapping complex, which is required for 5′-to-3′ exonucleolytic degradation of mRNA. Loss of VCS results in elevated protein levels of several miRNA targets with subtle or no increases in their corresponding mRNA levels (Brodersen *et al.*, 2008). Similar effects were observed for loss of function in *KTN1*, which encodes the P60 subunit of a microtubule-severing enzyme (Brodersen *et al.*, 2008). However, the mechanisms by which VCS and KTN1 influence miRNA-mediated translation repression and the connection of P bodies or microtubules with this process are still unknown.

Unlike translation repression, few reports have directly addressed the site of miRNA-guided transcript cleavage. However, the reduced cleavage efficiency observed in the *hmg1* mutant (Brodersen *et al.*, 2012) and the ER association of AGO1 (Li *et al.*, 2013) suggest that polysomes and the rER are potential sites. In addition, genome-wide analyses of RNA degradation products suggest that miRNA targets undergo cleavage when bound by translating ribosomes (Hou *et al.*, 2016; Yu *et al.*, 2016). Furthermore, 3′ cleavage fragments from a few miRNA targets were detectable in the MBP fraction (S. Li *et al.*, 2016). Therefore, transcripts targeted by miRNAs may undergo co-translational degradation, and at least a fraction of miRNA-guided cleavage may take place on the rER.

The biogenesis of phasiRNAs probably occurs on membrane structures. SGS3 and RDR6, two essential proteins required for phasiRNA biogenesis, form cytoplasmic siRNA bodies that also contain AGO7 (Kumakura *et al.*, 2009; Jouannet *et al.*, 2012). Moreover, both SGS3 and AGO7 are in the microsomal fraction, and AGO7 tends to be adjacent to vesicles decorated by a *cis*-Golgi marker (Jouannet *et al.*, 2012) (Fig. 2). All miRNAs, including 22-nt miRNAs, are enriched on MBPs, and reduced membrane association of 22-nt miRNAs correlates with decreased levels of phasiRNAs (S. Li *et al.*, 2016). *TAS* transcripts are bound by ribosomes (Hou *et al.*, 2016) and MBPs (S. Li *et al.*, 2016). These findings suggest that the initial miRNA-guided cleavage step of phasiRNA biogenesis occurs on MBPs and the subsequent steps occur on certain membrane structures.

III. Turnover of miRNAs

The levels of miRNAs must be precisely and dynamically regulated *in vivo* and miRNA turnover is a mechanism to regulate miRNA levels. Studies of the *hen1* mutant revealed two major mechanisms underlying miRNA degradation in *Arabidopsis*: 3′-to-5′ truncation and 3′ uridylation (Li *et al.*, 2005; Yu *et al.*, 2005; Yang Z *et al.*, 2006) (Fig. 3). A few genes responsible for miRNA degradation via these two mechanisms have been identified (Ramachandran & Chen, 2008; Zhao *et al.*, 2012b; Tu *et al.*, 2015; Wang *et al.*, 2015), but the full picture remains elusive.

1. miRNA stabilization by 3′ methylation

Mature miRNAs are protected by 3′ end methylation catalyzed by HEN1. Loss of function in *HEN1* results in reduced abundance of almost all miRNAs, which are also heterogeneous in size as a result of 3′ truncation and 3′ tailing (predominantly uridylation) (Li *et al.*, 2005; Yu *et al.*, 2005; Yang Z *et al.*, 2006). Similarly, loss of function in *HEN1* homologs in other eukaryotes, including rice (Abe *et al.*, 2010), *Drosophila* (Horwich *et al.*, 2007; Saito *et al.*, 2007), *C. elegans* (Billi *et al.*, 2012), zebra fish (Kamminga *et al.*, 2010) and mouse (Kirino & Mourelatos, 2007), also leads to miRNA or piRNA (piwi-interacting RNA) 3′ truncation and 3′

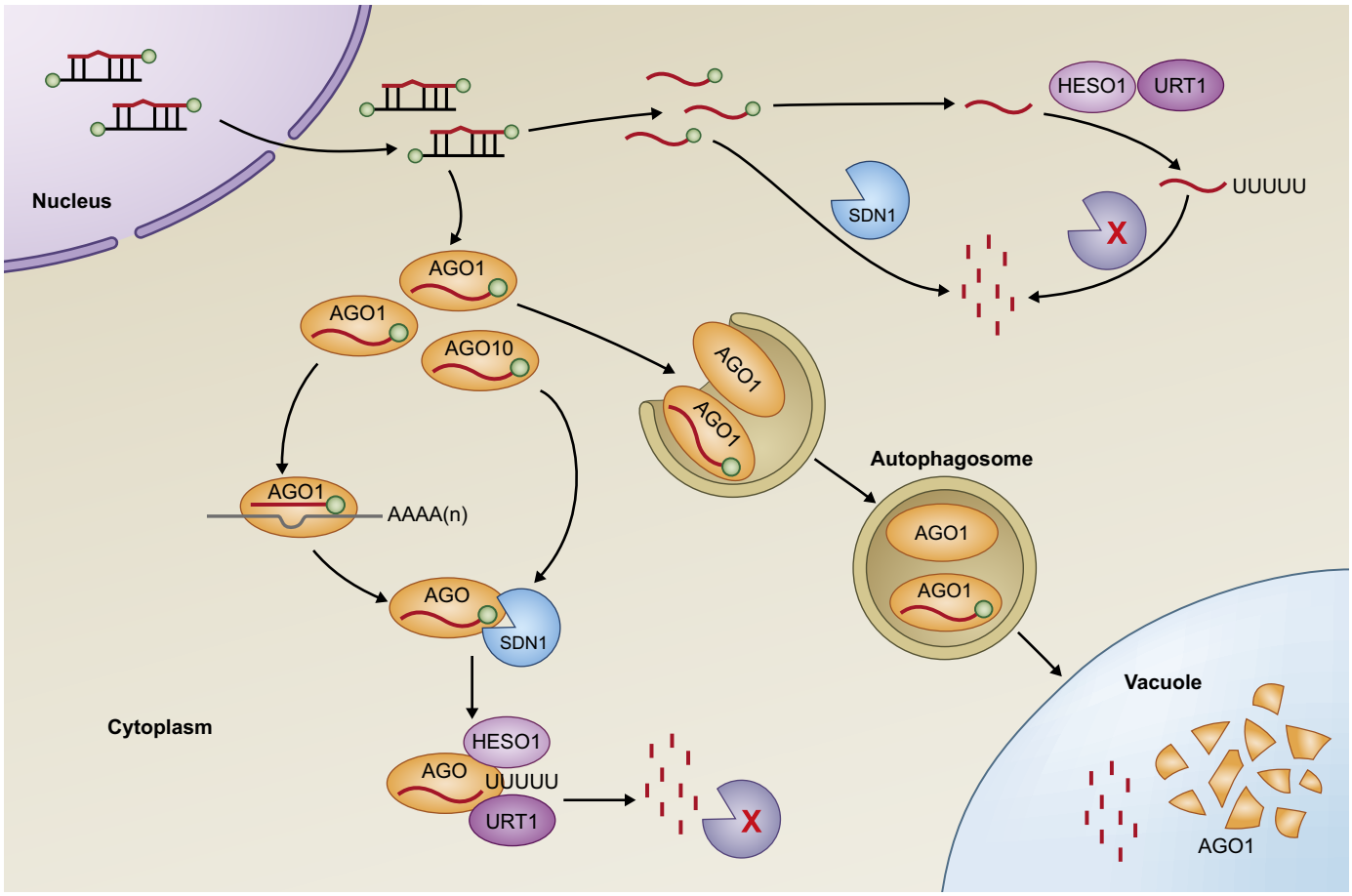


Fig. 3 Mechanisms of plant microRNA (miRNA) turnover. miRNA degradation starts with the removal of the methyl group at the 3′ end by SMALL RNA DEGRADING NUCLEASE 1 (SDN1), which is followed by 3′ uridylation through HEN1 SUPPRESSOR 1 (HESO1) and/or UTP:RNA URIDYLYLTRANSFERASE 1 (URT1). The tailed miRNAs are subsequently degraded by an unknown exonuclease. SDN1 and nucleotidyl transferases (HESO1 and URT1) can act on both ARGONAUTE (AGO)-bound miRNAs and free miRNAs in the cytoplasm. Free miRNAs are also degraded by SDN1 directly. The degradation of AGO1 via autophagy may also contribute to miRNA turnover.

uridylation. Therefore, HEN1-mediated 2′-*O*-methylation plays a general role in protecting the 3′ ends of small RNAs.

2. Exonucleases in miRNA degradation

The *SMALL RNA DEGRADING NUCLEASE* (*SDN*) family encodes four 3′-to-5′ exonucleases that function redundantly in degrading both miRNAs and siRNAs (Ramachandran & Chen, 2008) (Fig. 3). Single and double *sdn* mutants resemble wild-type plants, but knockdown of three *SDN* family members leads to severe pleiotropic developmental defects and elevated miRNA accumulation (Ramachandran & Chen, 2008). *In vitro* enzymatic assays show that SDN1 specifically acts on short single-stranded RNAs, and the exonuclease activity is partially inhibited by the methyl group at the 3′ end of miRNAs (Ramachandran & Chen, 2008).

SDNs are responsible for the 3′ truncation of miRNAs in both *hen1* and wild-type plants (Yu *et al.*, 2017). Comparing miRNA profiles of *hen1* and *hen1 sdn1 sdn2* plants by small RNA-seq showed that the 3′ truncation of some miRNAs is reduced when SDN1 and SDN2 are both absent (Yu *et al.*, 2017). Similar results were observed when comparing the miRNA profiles of wild-type and *sdn1 sdn2* plants, although 3′ truncated miRNAs have very low abundance in the wild-type (Yu *et al.*, 2017). The fact that only a small number of miRNAs are affected by the absence of SDN1 and SDN2 could be a result of the redundant function of other SDN members or of non-SDN exonucleases.

SDN1 is unable to degrade U-tailed miRNAs *in vitro* (Ramachandran & Chen, 2008), and so it does not appear to be responsible for the degradation of uridylated miRNA species. Although it has not been reported in *Arabidopsis*, several exonucleases in other eukaryotes prefer uridylated RNAs as substrates. The 3′-to-5′ exonuclease DIS3-like 2 (DIS3L2) degrades uridylated RNAs in mammals and yeast, including uridylated pre-let-7 in mammals (Chang *et al.*, 2013; Ustianenko *et al.*, 2013). In *Chlamydomonas*, depletion of the exosome subunit *Ribosomal RNA-Processing Protein 6* (*RRP6*) results in elevated accumulation of small RNAs *in vivo*, and RRP6 degrades 3′ uridylated miRNAs rather than non-uridylated miRNAs *in vitro* (Ibrahim *et al.*, 2010). The *Arabidopsis* orthologs of *DIS3L2* and *RRP6* are *SUPPRESSOR*

OF VARICOSE (*SOV*) and three *RRP6-LIKE* (*PPR6L*) genes, respectively, and are therefore the prime candidates for the degradation of uridylated miRNAs.

3. Non-templated tailing of miRNAs

3′ Non-templated tailing is a widespread phenomenon and a common post-transcriptional modification that regulates miRNA biogenesis, stability or activity in diverse model organisms (Wyman *et al.*, 2011). Adenylation and uridylation are the two major types of 3′ tailing and are catalyzed by nucleotidyl transferases including non-canonical PolyA polymerases (PAPs) and terminal uridylyl transferases (TUTases), respectively (reviewed in Martin & Keller, 2007).

In *Chlamydomonas*, uridylation of miRNAs and siRNAs is catalyzed by the nucleotidyl transferase MUT68 (Ibrahim *et al.*, 2010). MUT68 promotes the *in vitro* degradation of uridylated miRNAs through the exosome subunit RRP6 (Ibrahim *et al.*, 2010). MUT68 and RRP6 appear to act only on unmethylated miRNAs, as 2′-*O*-methylated miR912 oligonucleotides failed to be uridylated and degraded *in vitro* (Ibrahim *et al.*, 2010).

3′ Uridylation of miRNAs in *Arabidopsis*, rice and maize is widely observed in *hen1* mutants in which miRNA methylation is abolished (Li *et al.*, 2005; Yu *et al.*, 2005; Yang Z *et al.*, 2006; Abe *et al.*, 2010; Zhai *et al.*, 2013). (Fig. 3). In *Arabidopsis*, HESO1 and URT1 uridylate unmethylated miRNAs in the *hen1* mutant, leading to miRNA degradation (Ren *et al.*, 2012a; Zhao *et al.*, 2012b; Tu *et al.*, 2015; Wang *et al.*, 2015). Loss of function in both *HESO1* and *URT1* rescues the developmental defects of the *hen1* mutant, accompanied by elevated miRNA accumulation and reduced 3′ uridylation (Ren *et al.*, 2012a; Zhao *et al.*, 2012b; Tu *et al.*, 2015; Wang *et al.*, 2015). *In vitro*, both HESO1 and URT1 exhibit nucleotidyl transferase activities on unmethylated RNA oligonucleotides, but not 3′ methylated RNAs (Ren *et al.*, 2012a; Zhao *et al.*, 2012b; Tu *et al.*, 2015; Wang *et al.*, 2015). Although HESO1 and URT1 both prefer U over the other three nucleotides, they have different substrate specificities and cooperatively tail different forms of the same miRNAs *in vivo*. Although HESO1 prefers U-ending miRNAs as substrates, URT1 favors A-ending miRNAs. Given the observation of substantial monouridylated miRNAs in the *hen1 heso1* double mutant (Ren *et al.*, 2012a; Zhao *et al.*, 2012b; Tu *et al.*, 2015; Wang *et al.*, 2015), one possibility is that URT1 first uridylates unmethylated miRNAs to generate monoU-tailed forms, the preferred substrates for HESO1, to produce longer U tails.

3′ Uridylation may also affect miRNA activity. When AGO1-bound miR165/6 was uridylated by URT1 *in vitro*, the slicer activity was reduced (Tu *et al.*, 2015). The monouridylation of miR171a by URT1 in *hen1* makes it capable of triggering the biogenesis of secondary phasiRNAs (Zhai *et al.*, 2013).

In *Populus trichocarpa* (black cottonwood), a few miRNAs undergo 3′ adenylation, although the corresponding enzymes remain unknown (Lu *et al.*, 2009). Synthesized miRNA oligonucleotides with 3′ adenylation were degraded at a slower rate in plant extracts than were those without it (Lu *et al.*, 2009), indicating that adenylation contributes to miRNA stabilization.

4. AGO proteins in miRNA stability

In addition to its key role in miRNA-mediated activities, AGO1 shelters its associated miRNAs from degradation, based on the reduced abundance of many miRNAs in *ago1* null mutants (Vaucheret *et al.*, 2004). It is therefore counterintuitive that the weak allele *ago1-11* suppresses the 3′ truncation and 3′ uridylation of miRNAs in the *hen1* mutant (Zhai *et al.*, 2013). In addition, both truncated and tailed miRNA species associate with AGO1 *in vivo* (Zhao *et al.*, 2012a; Zhai *et al.*, 2013). This implies that, during miRNA degradation, SDN1 and HESO1/URT1 act on AGO1-bound miRNAs. Indeed, both HESO1 and URT1 are able to tail AGO1-bound miRNAs *in vitro*, and the tailed miRNAs remain associated with AGO1 (Ren *et al.*, 2014; Tu *et al.*, 2015; Wang *et al.*, 2015). The interactions between HESO1/URT1 and AGO1 are evidenced by reciprocal co-immunoprecipitation (Ren *et al.*, 2014; Wang *et al.*, 2015). Although SDN1–AGO1 interaction has not been reported, SDN1 acts on AGO1-bound miRNAs *in vitro* to generate truncated miRNAs of heterogeneous sizes that remain bound to AGO1 (Yu *et al.*, 2017). Given that 2′-*O*-methylation of miRNAs completely inhibits the activity of HESO1 and URT1, but not SDN1 (Ramachandran & Chen, 2008; Zhao *et al.*, 2012b; Tu *et al.*, 2015), one possibility is that SDN1 and HESO1/URT1 cooperate in degrading AGO1-bound miRNAs that are methylated: SDN1 removes the methyl group from these miRNAs, and HESO1/URT1 cause subsequent uridylation. This would lead to miRNA degradation by an unknown exonuclease that prefers U-tailed RNAs (Fig. 3). This hypothesis is supported by the following observations: 3′ truncated-only and 3′ truncated-and-tailed miRNA species are reduced in the *hen1 sdn1 sdn2* triple mutant compared with *hen1*, whereas 3′ tailed species are reduced in *hen1 heso1* with the concomitant increase in 3′ truncated-only forms (Yu *et al.*, 2017). Free miRNAs, on the other hand, can be degraded solely by SDN1 or sequentially by SDN1 and HESO1/URT1.

As the closest paralog of AGO1 amongst the 10 *Arabidopsis* AGO proteins, AGO10 is only expressed in certain cells and acts in stem cell maintenance in the shoot apical meristem (SAM) and in leaf polarity specification (Moussian *et al.*, 1998; Lynn *et al.*, 1999; Mallory *et al.*, 2004). Such functions were found to be achieved through repression of the activity of miR165/166 (Liu *et al.*, 2009; Zhu *et al.*, 2011). AGO10 has a higher binding affinity than AGO1 to miR165/6 and, rather than protecting this miRNA, AGO10 promotes its degradation (Zhu *et al.*, 2011; Zhou *et al.*, 2015). In *ago10* mutants, miR165/6 accumulation is sufficiently increased that it can be detected by *in situ* hybridization in *AGO10*-expressing cells, which is not the case in the wild-type (Liu *et al.*, 2009). *AGO10* overexpression results in the degradation of miR165/6 by SDN1 and SDN2 (Yu *et al.*, 2017). An *in vitro* assay further suggested that AGO10-bound miR165/6 is more susceptible than AGO1-bound miR165/6 to SDN1-mediated truncation (Yu *et al.*, 2017). Promotion of miR165/6 degradation probably contributes to AGO10-mediated maintenance of stem cells and the specification of leaf polarity.

5. Effect of target transcripts on miRNA stability

Although the enzymes for miRNA 3′ truncation or 3′ uridylation act on many miRNAs, specificity in miRNA degradation may be achieved through target RNAs or non-coding RNAs. In *Arabidopsis*, miR399 is regulated by a native transcript with a miR399 binding site from the *IPS1* (*INDUCED BY PHOSPHATE STARVATION 1*) locus (Franco-Zorrilla *et al.*, 2007). A 3-nt bulge at the cleavage site within the *IPS1* transcript abolishes miR399-mediated cleavage, thereby rendering the *IPS1* transcript a target mimic (TM) that sequesters miR399 from its other targets and reduces its activity (Franco-Zorrilla *et al.*, 2007). Genome-wide bioinformatic analyses indicate that many transcripts, from either non-coding genomic regions or annotated genes, can serve as potential endogenous TMs to regulate miRNA activity (Ivashuta *et al.*, 2011; H.J. Wu *et al.*, 2013). Intriguingly, in transgenic lines with artificial TMs, the levels of the corresponding miRNAs are reduced (Ivashuta *et al.*, 2011; H.J. Wu *et al.*, 2013). Similar results were observed in transgenic lines expressing short tandem target mimic (STTM) RNAs, which contain two tandem miRNA binding sites with mismatches at the cleavage positions (Tang *et al.*, 2012; Yan *et al.*, 2012). STTM-triggered miRNA degradation requires the activity of SDN1 and SDN2 *in vivo* (Yan *et al.*, 2012).

Target-induced miRNA turnover is conserved across flies and mammals. In animals, miRNAs recognize their targets through pairing at the seed region (miRNA nucleotides 2–7) (reviewed in Bartel, 2009). Extensive pairing between miRNAs and artificial target transcripts leads to 3′ trimming and tailing of miRNAs in *Drosophila* and humans (Ameres *et al.*, 2010; Cazalla *et al.*, 2010; Marcinowski *et al.*, 2012). Based on crystal structure analysis in *Thermus thermophilus* (Sheng *et al.*, 2014), the conformation of AGO is altered after a highly complementary target is in RISC such that the 3′ end of the guide is released from the binding pocket in AGO. Thus, it is deduced that the 3′ end of an AGO1-bound miRNA would be exposed to SDNs, HESO1 or URT1 on recognition of highly complementary targets in *Arabidopsis*.

6. Subcellular sites of miRNA turnover

The subcellular localization of SDNs, HESO1 and URT1 may offer clues for the subcellular sites of miRNA turnover. In addition, because AGO1-bound miRNAs can be truncated and uridylated, AGO1 localization is another important indicator for the sites of miRNA turnover. Although the localization patterns of SDNs are unknown, HESO1 and URT1 colocalize in cytoplasmic foci, where AGO1 is also localized (Wang *et al.*, 2015). In addition, both enzymes interact with AGO1, and uridylated miRNAs remain bound by AGO1 (Ren *et al.*, 2014; Wang *et al.*, 2015). Based on these findings, the cytoplasmic foci are potential sites of miRNA degradation. In addition to uridylating unmethylated miRNAs, HESO1 and URT1 also catalyze the uridylation of the 5′ cleavage fragments from miRNA target transcripts, leading to their degradation (Ren *et al.*, 2014). Given that a fraction of AGO1-mediated cleavage takes place on MBPs (S. Li *et al.*, 2016), the undefined cytoplasmic foci may contain MBPs.

The post-translational regulation of AGO1 protein may also provide a clue about the sites of miRNA turnover (Fig. 3). In pathogenic and viral contexts, AGO1 is ubiquitinylated by the polerovirus-encoded F-box protein P0 and subsequently degraded through autophagy, a process in which cytosolic proteins are delivered to lysosomes for degradation (Derrien *et al.*, 2012). AGO1 is also regulated by another F-box protein, F-box and WD-repeat domain-containing protein 2 (FBW2), which also leads to AGO1 degradation via autophagy (Earley *et al.*, 2010). The colocalization of AGO1 and AUTOPHAGY 8 (ATG8), an autophagosomal membrane protein (Derrien *et al.*, 2012), further indicates that AGO1 is associated with autophagosomes. As AGO1 degradation would indisputably impair the stability of its associated miRNAs, miRNA degradation may occur concomitantly with AGO1 autophagy.

IV. Concluding remarks

Although many players involved in miRNA biogenesis, degradation and activity have been discovered, much is unknown regarding the subcellular locations in which these processes take place. For example, it is unknown how D-bodies containing the dicing complex are formed, how AGO1, a presumably soluble protein, associates with ER and membrane-bound polysomes, and how membrane-bound polysomes affect miRNA-guided phasiRNA biogenesis. As AGO1 associates not only with miRNAs, but also with siRNAs from endogenous sequences, such as transposons and phasiRNA loci, as well as from exogenous sequences, such as viruses and transgenes, the subcellular partitioning of AGO1 between the cytosol and endomembranes and between the nucleus and the cytoplasm probably influences the activities of various types of small RNAs. The limited knowledge of the subcellular locations of miRNA biogenesis, degradation and activity precludes a full understanding of miRNAs, as well as the crosstalk between miRNAs and siRNAs.

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