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The expanding world of small RNAs in plants

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Abstract

Plant genomes produce a variety of small RNAs that function in distinct, yet overlapping, genetic and epigenetic silencing pathways. However, the abundance and diversity of small RNA classes varies in different plant species, suggesting co-evolution between environmental adaptations and gene silencing mechanisms. Small RNA biogenesis in plants is well understood, but we are just beginning to uncover their intricate regulation and activity. Here, we discuss the biogenesis of plant small RNAs, such as microRNAs, secondary small-interfering RNAs and heterochromatic small-interfering RNAs, and their diverse cellular and developmental functions, including reproductive transitions, genomic imprinting and paramutation. We also discuss the diversification of small RNA-directed silencing pathways through the expansion of RNA-dependent RNA polymerases, Dicer and **Argonaute** proteins.

Introduction

Small RNAs are involved in plant development, reproduction and genome reprogramming, and the large variety of small RNA pathways in plants is likely to contribute to their phenotypic plasticity. It is generally accepted that these pathways evolved as a cellular defense mechanism against RNA viruses and transposable elements, and later adapted to regulate the expression of endogenous genes. This is consistent with the fact that most small RNA classes have a recognized role in defense responses as well as in epigenetic regulation, but their relative importance and overlap varies between plant species[1]. Most plant small RNAs are produced as 21 to 24-nucleotide RNA molecules as a result of the activity of **DICER-LIKE (DCL)** proteins[2,3], which relies on the formation of double-stranded RNA (dsRNA) intermediates from either hairpin precursors, derived from overlapping sense and antisense transcripts or from the synthesis of dsRNA from ssRNA by RNA-DEPENDENT RNA POLYMERASEs (RDRs). Processed small RNA duplexes are loaded onto ARGONAUTE (AGO) proteins to target coding or non-coding RNAs (ncRNAs) by sequence complementarity. Depending on the nature of the target transcript and AGO protein involved, this process might lead to target cleavage and degradation, translational repression or recruitment of additional co-factors.

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Competing interests statement

The authors declare no competing financial interests.

In this review we discuss recent findings and the current understanding of the origin and biogenesis of small RNAs in plants, and the molecular pathways contributing to their diversification and function. The duplication of genes encoding DCL and RDR proteins resulted in the diversification of small RNAs[4,5], whereas the diversification of AGO proteins resulted in the development of distinct gene silencing processes based on differential AGO affinities to small RNA duplexes[6] (Box 1). Endogenous small RNAs in plants can be divided into several major classes: microRNAs (miRNAs), hairpin-derived small-interfering RNAs (hp-siRNAs), natural antisense siRNAs (natsiRNAs), secondary siRNAs and heterochromatic siRNAs (hetsiRNAs). All small RNAs in plants are modified at the 3′ end by 2′-O-methylation, including miRNA which lack this modification in animals. 2′-O-methylation is essential to confer stability and protection from 3′ uridylation and degradation. In plants, miRNAs are involved in post-transcriptional gene silencing (PTGS) by transcript cleavage or translational repression, and might trigger secondary siRNA production from Pol II-derived cleaved transcripts. While many small RNAs are involved in PTGS, the majority of siRNAs in plants are associated with RNA-directed DNA methylation (RdDM) and transcriptional gene silencing (TGS). Once established, TGS is maintained by 24-nucleotide (nt) hetsiRNAs, which regulate important epigenetic mechanisms such as imprinting and **paramutation**. Many small RNA biogenesis pathways have been genetically characterized in *Arabidopsis thaliana*, as these mutations are viable. In plants with larger genomes, hetsiRNAs play essential roles during reproductive transitions such as meiosis, gametogenesis and embryogenesis, likely associated with the more repetitive nature of the genome.

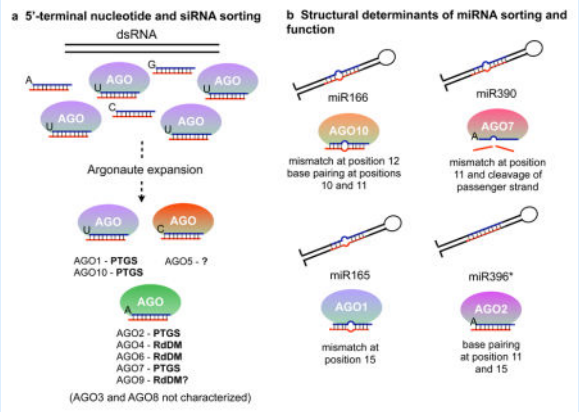
Box 1

Sorting small RNA onto Argonaute proteins

The ARGONAUTE (AGO) protein family in plants has diversified extensively giving rise to plant-specific AGO proteins[155]. The identity of the 5′ terminal nucleotide of plant small RNAs has a strong effect on the loading of small RNAs to specific AGO proteins, which determines their activity. This bias might be intrinsic to all AGOs, as in organisms with only one AGO (such as *Schizosaccharomyces pombe*) the protein has a strong bias towards small RNAs with 5′ uracil (see the figure, part **a**). In *Arabidopsis thaliana*, AGO1 and AGO10 also bind small RNAs with a 5′ uracil, whereas AGO2, AGO4, AGO6, AGO7 and AGO9 prefer adenines and AGO5 has a bias for cytosines[111,156,157] (see the figure, part **a**). This 5′ nucleotide specificity is determined by a structural loop lining the sRNA-binding pocket in the MID domain of AGO proteins[158]. An additional layer of complexity is particularly obvious for microRNA (miRNA) loading, which is affected also by the imperfect complementarity and bulges that characterize miRNA duplex structures. For example, AGO10 is a critical regulator of shoot apical meristem (SAM) specification because of its preferential interaction with miR166, which is mediated by one internal base mismatch flanked by two paired bases within the mature duplex[159–161] (see the figure, part **b**). This prevents the loading of miR166 onto AGO1 and silencing of the class III HD-ZIP transcription factors in the SAM[159,160]. Another example is miR390, which is preferentially loaded onto AGO7 (see the figure, part **b**), leading to the production of trans-acting small interfering RNAs (tasiRNAs) from *TAS3*

transcripts[67,162]. AGO7 association with miR390 involves preference for a 5′-A, but also a mismatch at position 11 of the miRNA duplex[163]. Importantly during the AGO7–miR390 interaction, AGO7-mediated cleavage of the complementary strand is required to establish a functional silencing complex[163] (see the figure, part **b**).

The miR393b duplex provides another interesting example of miRNA sorting in plants, as its guide strand is loaded onto AGO1, whereas the other strand, miR393b*, is loaded onto AGO2 and plays an essential role in mediating antibacterial defense[164]. Mechanistic insight into this type of small RNA sorting was recently reported, showing that the 15th nucleotide of a miR165 duplex directs miRNA loading onto both AGO1 and AGO2 through their PIWI domain (see the figure, part **b**). In contrast, AGO2 preferential binding to miR396* requires base pairing at position 11 and 15 of the duplex, whereas AGO1 tolerates mismatches at these central positions[165]. The importance of this mechanism was nicely illustrated using the miR165–miR165* duplex, where removing the 15th nucleotide mismatch in artificial miR165 stem-loops led to loading onto AGO2 instead of AGO1, down-regulation of miR165 target genes and partial suppression of the adaxialized phenotype, which is characteristic of *ago1* mutant alleles[165].



The biogenesis of small RNAs in plants

Processing of dsRNA into small RNAs requires the activity of Dicer enzymes. The minimal functioning Dicer is found in budding yeasts (though not in *Saccharomyces cerevisiae*), and is composed of a ribonuclease III domain and a dsRNA-binding domain (dsRBD), but lacks the DExD box helicase and PIWI/ARGONAUTE/ZWILLE (PAZ) domains found in higher eukaryotes[4,7]. The PAZ domain binds the 2-nucleotide 3′ overhang of dsRNAs, and is connected to the catalytic domain through an α-helical structure that acts as a ruler to determine small RNA size. The four DCL proteins in *A. thaliana* have been well characterized[2,3], indicating that the duplication of DCL genes has occurred early in plant evolution.

The synthesis of dsRNAs by RDRs contributed significantly to the expansion, diversification and evolution of small RNA functions. *RDR* genes are found in RNA viruses, plants, fungi, protists, and some lower animals, but are absent in flies, mice, and humans. In contrast to

DCLs, the RDR family seems to be more complex and many *RDR* genes remain uncharacterized[5]. Generally there are three main pathways responsible for the biogenesis of the vast majority of small RNAs in plants: one for the biogenesis of miRNAs, one for the biogenesis of 21 and 22 nucleotide secondary siRNAs and another for the biogenesis of 24 nucleotide hetsiRNAs (Fig. 1).

The origin and biogenesis of microRNAs

Plant miRNAs, typically 20 to 22 nucleotides in length, are endogenous genes transcribed by RNA Polymerase II (Pol II) into long primary microRNAs (pri-miRNAs), which are single-stranded and polyadenylated RNA molecules that fold into hairpin-like structures (Fig. 1a). The pri-miRNAs are cleaved by DCL1 into a smaller stem-loop structure called precursor microRNAs (pre-miRNAs), which are subsequently processed again by DCL1 to produce the mature miRNA duplexes consisting of the active miRNA strand and its complementary strand miRNA. MiRNA biogenesis pathways are particularly well described in *A. thaliana* where several DCL1 partners have been characterized as required for pri-miRNA processing (reviewed in [8]).

MicroRNAs mediate PTGS through mRNA cleavage or translation repression, and play essential roles in plant development. Null *dcl1* alleles in *A. thaliana* are embryonic lethal[9], whereas different hypomorphic alleles give rise to variable defects in integument, ovule, and floral development, and also have maternal effects[10]. *DCL1* knockdown in rice also results in defects in plant growth and in shoot, root, and leaf development, and ultimately leads to developmental arrest[11]. *fuzzy tassel (fzt)* mutants in maize have a broad range of vegetative and reproductive defects, and are hypomorphic *dcl1* alleles, as in these plants the abundance of some miRNAs is more dramatically reduced than others[12]. The vast majority of *MIRNA* genes are species- or family-specific, suggesting rapid evolution and a high turnover rate[13,14].

A subtype of miRNAs in *A. thaliana* and rice are the relatively rare, longer miRNAs, which are 23 to 25-nucleotide in length, are processed by DCL3 and function in transcriptional gene silencing (TGS)[15–17]. Furthermore, in *A. thaliana*, DCL4 was also found to process a class of newly evolved miRNAs[14,18,19]. These observations have led to the idea that pre-miRNA recognition by different DCL proteins might reflect the evolution of *MIRNA* genes: Some of these newer, DCL4-procesed pre-miRNAs are long and exhibit complementarity with their targets that extends beyond that of the mature miRNA, suggesting that *MIRNA* genes arose from inverted duplications of their target genes[14,20,21] and that DCL2, DCL3 and DCL4 processed these longer hairpins (or proto-MIRNA) into small RNAs of different sizes (hp-siRNA) (Fig. 1a). During evolution, the near-perfect sequence complementarity in proto-MIRNA hairpins has decreased and consequently refined into smaller transcripts that are now processed by DCL1 as a single miRNA duplex[22]. Another, similar origin for miRNAs has been proposed from miniature inverted-repeat transposable elements (MITEs) that, when transcribed, create hairpin RNAs resembling proto-MIRNAs[23,24].

The origin and biogenesis of small-interfering RNAs

Long dsRNAs that are the precursors of siRNAs can arise from the hybridization of sense and antisense transcripts, from the fold-back of an inverted-repeat sequence, from the hybridization of unrelated RNA molecules with sequence complementarity or, most commonly, following synthesis by RDRs (reviewed in [1]). Long dsRNA molecules can be synthesized by RDRs with or without initial priming[25,26], resulting in the amplification of a primary, small RNA-mediated silencing-triggering signal. The three major clades of eukaryotic RDRs are RDRα, RDRβ, and RDRγ; the RDRα clade is present in the fungal, plant and animal kingdoms, whereas RDRβ has been found in animals and fungi and RDRγ only in plants and fungi[5,27]. In *A. thaliana*, the RDRα clade is composed of RDR1, RDR2, and RDR6, while the RDRγ clade is composed of RDR3, RDR4, and RDR5. The RDRγ clade remains functionally uncharacterized in plants, but the presence and expression of at least one of its members in several other plant genomes and many fungi suggests functional significance[5,27]. Interestingly, efficient antiviral defense and viral siRNA biogenesis were detected in *rdr1, rdr2* and *rdr6* triple mutants in *A. thaliana*[28], indicating that RDR3, RDR4 and RDR5 might represent alternative pathways for antiviral defense. In addition, in the fission yeast *Schizosaccharomyces pombe*, RDRγ is involved in transcriptional gene silencing[29].

Endogenous siRNAs in plants are primarily processed by DCL2, DCL3 and DCL4, and have been categorized into secondary siRNAs (Fig. 1b) and hetsiRNAs (Fig. 1c). Secondary siRNAs include different subclasses such as trans-acting small interfering RNAs (tasiRNAs), phased small interfering RNAs (phasiRNAs), epigenetically-activated small interfering RNAs (easiRNAs) and natsiRNAs. The most abundant small RNAs are the 24-nucleotide hetsiRNAs, which mediate transcriptional silencing of transposons and pericentromeric repeats through RdDM (reviewed in [30,31]). The biogenesis of hetsiRNAs requires transcription by Pol IV followed by dsRNA synthesis by RDR2 and processing by DCL3 (Fig. 1c). By contrast, 21 and 22-nucleotide secondary siRNAs such as tasi-, phasi- and easiRNA are produced by DCL4 and DCL2, respectively, following Pol II transcription and dsRNA synthesis by RDR6 (Fig. 1b). DCL2 is often regarded as a backup for DCL4, as the former is recruited to dsRNA following the deletion of latter[32]. RDR1 activity is mostly associated with the amplification of exogenous, virus-derived small RNAs, being part of the main plant antiviral RNAi system together with DCL2 and DCL4[28,33]. Additional processing of siRNA requires SUPPRESSOR OF GENE SILENCING 3 (SGS3), which functions together with RDR6[34], and dsRNA binding protein 4 (DRB4), which interacts with DCL4 in the production of endogenous and exogenous 21-nucleotide siRNAs[35].

Small RNA modifications

Small RNA modifications can regulate their abundance and function, thus contributing to regulation of gene silencing. In plants, these modifications have been observed primarily at the 3′ end and are essential to confer stability and prevent small RNA degradation. Further mechanistic insight into these pathways in *A. thaliana* has suggested that protective RNA modifications are bypassed in certain tissues, cell types or growth conditions, thereby promoting small RNA diversity.

2′-O-methylation and uridylation

After processing, eukaryotic small RNA duplexes such as siRNAs, miRNAs and **piwi-interacting** RNA (piRNAs) can be modified by 2′-O-methylation, 3′ uridylation or adenylation, and adenosine deamination[36]. 2′-O-methylation of the terminal 3′ nucleotide is important for miRNA and siRNA stability, because unmethylated small RNAs are signaled for degradation by 3′uridylation[37,38]. Plant small RNAs are 2′-O-methylated at the 3′ terminal by HUA HENHANCER 1 (HEN1)[37–39] to prevent uridylation by the nucleotidyl transferase HEN1 SUPPRESSOR 1 (HESO1) [40,41]; uridylation is a signal for degradation via SMALL RNA DEGRADING NUCLEASE 1 (SDN1)[42] (Fig. 2). Loss of 2′-O-methylation activity in *A. thaliana* results in severe developmental defects, likely because essential miRNAs are depleted[39,43]. Small RNA 3′ uridylation was also observed in the algae *Chlamydomonas reinhardtii* and requires the nucleotidyl transferase MUT68[44], whereas in humans and *Caenorhabditis elegans*, many enzymes have been shown to uridylate miRNAs in a sequence-specific manner[45]. In human cells, poly-uridylation of pre-miRNAs is performed by terminal uridylyltransferase 4 (TUT4). The recruitment of TUT4 to pre-miRNAs by the RNA binding protein Lin-28 destabilizes the pre-miRNAs and reduces the levels of mature miRNAs[46,47]. In contrast to Lin-28-dependent poly-uridylation, Lin-28-independent mono-uridylation by TUTs is required for the processing of certain pre-miRNAs in human cells[48]. In *C. elegans*, the uridylation of some siRNAs restricts them to binding only the AGO protein CSR-1 thus reducing their abundance, which is required for proper chromosome segregation[49] and the recognition of self from non-self mRNA in the germline[50,51].

Although it is clear that these modifications play an essential role in regulating small RNA biogenesis and function, it remains poorly understood how these factors are recruited to miRNA processing complexes in plants. In *A. thaliana*, polyuridylation and degradation of the guide strand of miRNA duplexes requires loading onto AGO1, which has been shown to interact directly with HESO1[38]. This suggests that the primary role of 2′-O-methylation is to protect miRNAs from the AGO1-associated HESO1 activity that also uridylates 3′ ends of cleaved target transcripts and leads to their degradation[52] (Fig. 2). Recent studies have shown that HESO1 acts on most miRNAs, whereas the monouridylation of certain miRNAs requires another nucleotidyl transferase, UTP: RNA URIDYLYLTRANSFERASE (URT1)[53,54]. HESO1 seems to be more processive than URT1, perhaps because of their different substrate preference, which depends on the 3′ terminal nucleotide of the small RNAs: URT1 prefers 3′ adenine whereas HESO1 has strong preference for 3′ uridine, thus explaining why it polyuridylates its substrates. These enzymes also act synergistically to uridylate some miRNAs, as HESO1 acts on some monouridylated small RNAs derived from URT1 activity[54] (Fig. 2). Uridylation of miRNAs by URT1 and HESO1 is generally associated with reduced efficiency of target gene cleavage. An exception to this is the monouridylation of miR170 and miR171a by URT1; the resulting 22-nucleotide miRNA variants lead to the production of secondary siRNAs from their target transcripts and to efficient gene silencing[38,54] (see below). This indicates that tailed miRNAs loaded onto AGO complexes could be non-canonically functional.

Other types of miRNA tailings were found in mutants lacking both HESO1 and URT1 activities, including non-uridine nucleotides[53], suggesting that other small RNA modification pathways might exist in plants. There are eight nucleotidyl transferases that remain uncharacterized in *A. thaliana*, and these could have a role in miRNA modification or in processes utilizing secondary or heterochromatic siRNAs.

Novel small RNA modifications in plants

Recent efforts to discover novel small RNA modifications have identified base modifications by comparing mismatches between genomic sequences and sequencing reads of small RNAs, as some modifications result in preferential nucleotide misincorporation by reverse transcriptases during cDNA synthesis[55]. Such analyses revealed frequent A-to-G, G-to-A and U-to-C substitutions in *A. thaliana* and rice small RNA data[56,57]. U-to-C substitutions were also detected in small RNAs from rice anthers[58], and are likely the result of reverse transcriptase misincorporation at modified U, as editing enzymes responsible for U-to-C conversion have not been identified in plants. Such U-to-C mismatches in small RNA reads could be caused by pseudouridine, which is abundant in structured RNAs and is required for the stabilization and function of tRNAs and rRNAs[59]. Pseudouridylation in eukaryotic small RNAs has not been directly observed, but was recently reported in mRNAs in yeast and humans[60,61]. Putative functions for pseudouridine might be the stabilization and transport of small RNA duplexes, as pseudouridylation is essential for tRNA biogenesis and nuclear export in yeast[62,63].

Secondary siRNA biogenesis and control

PTGS in plants can be amplified when miRNA-mediated cleavage or aberrant processing of particular transcripts leads to the formation of dsRNA by RDR proteins, which is subsequently processed by DCLs into secondary siRNAs. This powerful silencing machinery is conserved within the plant kingdom, but notably, it has widely differing targets in different plant species such as *A. thaliana*, rice, maize and soybean, including mRNAs, ncRNAs and repeat-derived RNAs. Depending on the precursor mRNA, secondary siRNAs have been classified into different subclasses, such as tasiRNAs and phasiRNAs. While tasiRNA have been demonstrated to act in *trans*, phasiRNAs are secondary siRNAs of unknown function but identified as phased, and could therefore include coding and non-coding transcripts[64].

The biogenesis of tasiRNAs and phasiRNAs

The production of secondary, RDR-dependent small RNAs such as tasi- and phasiRNAs, requires transcript targeting by miRNAs[65] (Fig. 3). Targeting by two 21-nucleotide miRNAs at independent target sites along the transcript (“two-hits”), or targeting by a single 22-nucleotide miRNA (“one-hit”) can trigger the production of secondary RNA from transcripts[34,66,67] (Fig. 3a). In addition, the structure of particular miRNA duplexes may also influence siRNA biogenesis regardless of miRNA length[68]. Cleaved transcripts serve as templates for dsRNA synthesis by RDR6 and the production of 21- and 22-nucleotide siRNAs by DCL4 and DCL2 respectively, which can function in *trans* to target other transcripts. Secondary siRNAs are often “phased” so that their first nucleotide occurs every

21 or 22 nucleotides from the miRNA cleavage site[65,66,69]. This is because DCL2 and DCL4 digest the dsRNA processively (Fig. 3), and might even interact directly with RDR6. However, phasing can be difficult to detect when there are multiple miRNA cleavage sites, or multiple related template RNAs.

Secondary siRNAs are relatively rare in somatic cells of wild-type *A. thaliana*, even though many mRNA targets generate secondary siRNAs in this species[69]. In contrast, other plant genomes such as rice and maize contain thousands of tasi- and phasiRNA-generating loci that encode large families of ncRNAs[65]. TasiRNA biogenesis and functions have been well studied in *A. thaliana*, which has only four families of *TAS* genes. *TAS1a/b/c* and *TAS2* loci are targeted by miR173, while miR390 targets the *TAS3a/b/c*, and miR828 triggers *TAS4*-derived tasiRNAs[18,34,66]. *TAS3* is the most well conserved *TAS* locus, as it is also present in moss, rice, maize, and gymnosperms[65]. *TAS3*-derived siRNAs are designated tasi-ARFs, and their production requires a two-hit system with miR390 that is exclusively loaded to a specialized Argonaute protein (AGO7), to regulate auxin-related developmental responses (see Box 1 and Box 2).

Box 2

Intercellular movement and transgenerational inheritance of small RNAs

Different small RNA classes have been associated with non-cell autonomous signaling involving short (cell-to-cell) and long (between organs) distance movement of small RNAs[166,167]. Intercellular movement has broad implications for cell-to-cell communication and transgenerational inheritance of epigenetic signals[78,150]. Spatiotemporal coordination of cell fate decisions and tissue patterning in multicellular organisms also depend on intercellular communication by small RNAs. For example, adaxial-abaxial (upper-lower) patterning of lateral organs in plants requires two mobile small RNAs with opposing functions: miR165 and miR166 (produced in the lower side of the leaf) restrict the accumulation of the transcription factors HD-ZIP III to the upper ventral domain while tasi-ARFs (produced in the upper side) are able to diffuse and create a gradient to restrict their targets AUXIN RESPONSE FACTOR 3 and 4 (ARF3 and ARF4) to the lower ventral domain (reviewed in [168]). Non-cell-autonomous small RNAs are also required for radial patterning of root tissues, which is regulated in part by the transcription factors SHORT ROOT (SHR) and SCARECROW (SCR) that act together to induce the expression of miR165 and miR166[169]. Upon induction in the endodermal cell layer, miR165 and miR166 move into the central vascular cylinder and target the HD-ZIP III transcripts, creating a radial gradient that is important for the differentiation of the central vascular tissues[169].

Considering the primary evolutionary role of small RNAs as a genome defense mechanism against viruses and transposons, several studies have attempted to define the molecular requirements for biogenesis and systemic transmission of silencing signals in several organisms. In plants, siRNAs from reporter transgenes as well as endogenous sequences are able to spread systemically and induce gene silencing in recipient cells, but several questions remain regarding the biogenesis of small RNAs in response to environmental cues and the transgenerational inheritance of newly acquired epigenetic

states (reviewed in [170]). In the *Arabidopsis thaliana* male and female gametophytes, small RNA movement has been proposed to follow epigenome reprogramming, which results in transcriptional re-activation of transposable elements in germline companion cells[78,150]. These small RNA-directed mechanisms could provide surveillance of and protection against transposon activity during meiosis and epigenomic reprogramming in the germline[78,150], or in the seed after fertilization[171]. Additional sources of transposon-derived siRNAs during reprogramming in the gametophytes were also associated with genomic imprinting through targeted DNA demethylation and RdDM[172–174]. From these studies, it is clear that the high degree of reprogramming observed during plant gametogenesis provides numerous possibilities for establishing novel and beneficial epigenetic states by mobile small RNAs.

In maize anthers, phasiRNAs are produced from ncRNA precursors designated as *PHAS* ncRNA (Fig. 3b). There are thousands of *PHAS* loci in grass genomes, which are transcribed by Pol II, capped and polyadenylated, resembling protein-coding and trans-acting siRNA precursors (*TAS*) in this respect. In both rice and maize, internal cleavage directed by miR2118 triggers the production of 21-nucleotide phasiRNAs, whereas miR2275-directed cleavage triggers the production of 24-nucleotide phasiRNAs[70–72]. It is hypothesized that a complex containing homologs of RDR6 and SGS3 recognizes the 3′ end of cleaved *PHAS* transcripts and synthesizes dsRNA from the polyA tail to the cleavage site[73]. The dsRNAs are subsequently processed by DCL4 and DCL5 to generate 21- and 24-nucleotide phasiRNAs, respectively[74]. Both DCL4 and DCL5 in grasses have phased activity, generating populations of regularly spaced siRNAs from each *PHAS* precursor[65]. Although the expression of non-coding *PHAS* loci is anther-specific in grasses, the miR2118-482 superfamily is conserved in dicots and triggers phasiRNA production from coding NB-LRR genes in legumes and solanaceous species[64,75,76], and in some gymnosperms as well[64]. NB-LRR disease resistance genes encode innate immunity receptors, and these phasiRNAs in legumes and Solanaceae appear to be beneficial for plant-microbial interactions and plant immunity. It is likely that this elaborate defense mechanism was lost in grasses, to be replaced by a variety of non-coding *PHAS* loci producing different types of anther-specific phasiRNAs[65].

easiRNAs are produced from active retrotransposons

miRNAs are also able to trigger secondary siRNA biogenesis from transcriptionally re-activated transposable elements using a similar genetic pathway to tasiRNAs[77]. Re-activation of some transposons and easiRNA biogenesis occurs in wild-type *A. thaliana* pollen during epigenetic reprogramming[78], in cell cultures[79] or under stress conditions[80]. In DNA methylation mutants such as *DECREASE IN DNA METHYLATION 1* (*DDM1*) and *DNA METHYLTRANSFERASE 1 (MET1)*[78,81], as many as 2500 transposons are activated, and subsequently targeted by more than 50 miRNAs[77]. These miRNAs have well known functions in plant development, and are highly conserved, although targeting does not always result in easiRNA production[77]. It is possible therefore that miRNAs evolved originally to target transposons, and only subsequently adopted other functions, such as gene regulation and triggering the processing of tasi- and phasiRNA from non-coding precursors.

This hypothesis is consistent with a transposon origin of miRNA precursors[82] (see above), as transposable elements-derived proto-miRNA could of course target related transposons.

RNA interference and splicing

Secondary siRNA biogenesis pathways may also interact with additional cellular pathways that silence transposable elements and transgenes, such as RNA splicing. Recent work in yeast and flies have shown that stalled spliceosomes at weak splice sites and suboptimal introns could function as a signal to trigger RNAi, thus representing a way to discriminate between transposons or precursors of small RNAs and protein-coding transcripts[83,84]. In fact, previous studies in *A. thaliana* have shown that intron splicing is a potent suppressor of RDR6-dependent PTGS[85], and notably this requires SERRATE (SE) and the nuclear cap-binding complex ABA HYPERSENSITIVE 1 (ABH1), which are components of the miRNA biogenesis pathway[86–88]. However, SE function during intron splicing seems to be miRNA-independent, while it is unclear whether ABH1 and miRNAs are directly involved in splicing or else in the expression of specific splicing factors. It is possible that splicing suppresses PTGS by removing potential miRNA target sites located within introns, but this hypothesis awaits further investigation. Interestingly, splicing factors have also been associated with TGS, such as the SR45 protein that is required to establish RdDM and 24-nucleotide siRNA processing at *FLOWERING WAGENINGEN (FWA)* transgenes[89]. However, Pol IV-dependent transcripts involved in RdDM activity appear to be unspliced[90], supporting the idea that SR45 participates in RdDM indirectly, perhaps by regulating the splicing of RdDM components, as has been proposed in *S. pombe*[91–94].

RNA interference and RNA decay

RNA decay and PTGS are functionally linked, as RDR6-mediated PTGS of transgenes and endogenous genes is enhanced in mutants of non-sense mediated decay (NMD), decapping (which triggers RNA decay) and **exosome** factors in *A. thaliana*[95–101]. The interplay between RNAi and RNA decay was also observed in the fission yeast and fruit flies, where heterochromatic silencing of transgenes and transposons is promoted in the absence of the exosome[102,103].

In *A. thaliana*, loss of the NMD factor 5′ to 3′ exoribonuclease 4 (XRN4, also known as EIN5) enhances PTGS of thousands of endogenous genes upon viral infection[104]. This is mediated by the production of secondary siRNAs, which were designated virus-activated siRNAs (vasiRNAs) and, interestingly, their biogenesis relies on RDR1, DCL4 and AGO2, which is identical to the genetic pathway responsible for the production and activity of viral siRNAs[28,33,105]. Given that vasiRNAs are only observed upon infection with viruses lacking RNAi suppressors, vasiRNAs could represent an additional layer of antiviral defense[104], but this interesting idea needs further experimental evidence.

The decapping complex comprising DCP1, DCP2 and VARICOSE (VCS), which co-localize in **processing bodies (P-bodies)**, is also associated with PTGS[101]. The link between P-bodies and PTGS remains unclear, as RDR6-mediated PTGS occurs in distinct cytoplasmic **siRNA-bodies**[106]. However, a possible interaction between P-bodies and

siRNA-bodies was recently proposed[101], providing a direct subcellular connection between RNA decay and secondary siRNA biogenesis.

The switch to transcriptional silencing

Barbara McClintock coined the term "Changes of Phase" to describe how transposons switched between active and inactive forms[107], and recently there has been a lot of interest in finding distinctive features of transposon transcripts that trigger an epigenetically heritable state of TGS through RdDM. As discussed above, many retrotransposons in *A. thaliana* give rise to abundant 21 to 22-nucleotide easiRNAs when transcriptionally active, and recent evidence indicates that this could represent the entry point for TGS[108] through establishment of RDR6- and AGO6-mediated DNA methylation[109] (Fig. 4a). A PTGS-to-TGS transition would occur when Pol II transcription is replaced by the plant-specific RNA polymerases Pol IV and V, switching from 21 or 22-nucleotide to 24-nucleotide siRNA production and epigenetic silencing by RdDM[108,110] (Fig. 4b). Instead of using RDR6 and DCL2 or DCL4, Pol IV transcripts are processed by RDR2 and DCL3 into 24-nucleotide siRNAs, which are loaded onto AGO4, AGO6 or AGO9 to reinforce DNA methylation[31,111,112] (Fig. 4b). Of course, transcriptional silencing is only achieved when RdDM is able to spread into the promoter of the retrotransposon, which is found in the upstream long terminal repeat (LTR). This switch occurs immediately in *rdr6* and *dcl4* mutants indicating that 21nt siRNA might actually inhibit the production of 24nt siRNA[77,110]. An example is provided by the retrotransposon *Evadé (EVD)*[110] that generates increasing levels of RDR6-dependent dsRNA during multiple transposition events, which could eventually saturate the 21 and 22-nucleotide siRNA biogenesis enzymes DCL2 and DCL4, instead leading to DCL3 generating 24-nucleotide siRNAs from transcribed regions of the retrotransposon (Fig. 4a). In contrast to *EVD*, other retrotransposons are able to switch from producing 21-nucleotide to 24-nucleotide siRNAs following transient somatic re-activation by heat-stress, and independently of transposition[80]. The mechanisms responsible for these switches in siRNA biogenesis will require further investigation.

Nuclear functions of small RNAs

RdDM is an important RNAi-mediated epigenetic pathway in plants. It is involved in transcriptional silencing of transposons and repetitive sequences[31,112], and relies on a specialized transcriptional machinery that requires the plant-specific RNA polymerases Pol IV and Pol V[113]. Pol IV transcripts are rapidly processed into dsRNAs by RDR2, which are subsequently processed into 24-nucleotide siRNAs by DCL3 and exported to the cytoplasm. Once in the cytoplasm, they are incorporated into AGO4- (and other AGO-) containing complexes, and imported back to the nucleus to target nascent transcripts transcribed by Pol V at the same loci (Fig. 4b). While AGO4 is the most abundant AGO protein involved in RdDM, the related proteins AGO6 and AGO9 are also loaded with 24-nucleotide siRNAs and appear to be only partially redundant with AGO4, having specific functions (see below) [109,111,114]. In a plausible model, AGO4 is thought to recruit (in part) the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) to establish *de novo* DNA methylation at cytosines in all sequence contexts (CG, CHG and CHH, where H is A, C or T) (reviewed in [112 and 31]) (Fig. 4b).

Paramutation

Once established, DNA methylation can be passed on to other alleles in repulsion by a mechanism known as paramutation. First discovered in maize in the 1950s and 60s, paramutation has been more recently found in many other organisms[115,116]. During paramutation in maize, trans-homolog interactions between otherwise identical alleles can lead to heritable epigenetic changes mediated by small RNA[116,117]. Similarly, the *PAI2* gene in *A. thaliana* is epigenetically silenced in *trans* by siRNAs derived from an inverted duplication of *PAI* genes at another genomic locus, although silencing is not maintained in its absence[118,119]. In maize, mutations in one of several components of the RdDM pathway are defective in paramutation, including RDR2, Pol IV and V and chromatin remodelers related to the *A. thaliana* DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) and CLASSY 1 (CLSY1), which are also required for TGS[120]. Recent work also clarified that small RNA production is essential but not sufficient for paramutation, as DNA methylation also contributes to the strength of paramutation[121]. In *A. thaliana*, CHG methylation recruits the histone methyltransferase KRYPTONITE (KYP), which is responsible for the dimethylation of histone 3 Lys 9 (H3K9me2)[122]. This mark recruits the CHG chromomethylase CMT3, and both are required to initiate and maintain *PAI2* silencing[118,119]. Although a role in maize has yet to emerge, H3K9me2 in *A. thaliana* is recognized by the SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1), which recruits Pol IV and initiates siRNA biogenesis for the maintenance of gene silencing[123] (Fig. 4b). Thus higher levels of DNA methylation and H3K9me2 might result in stronger siRNA-mediated paramutation via Pol IV recruitment to paramutagenic alleles. As for paramutable alleles, it is still unclear how DNA methylation is established *de novo* in the presence of siRNAs, as in *A. thaliana*, Pol V recruitment also seems to require pre-methylated DNA[124]. One possibility is that the establishment of retrotransposon silencing in *A. thaliana* requires another RdDM pathway that involves 21 to 22-nucleotide siRNAs — the precursors of which are transcribed by Pol II from transposon LTRs — that are targeted to the chromatin by AGO6[109,110] (Fig. 4a). A similar model was proposed to explain stepwise TGS of the hypomethylated *FWA* **epiallele** by virus-induced gene silencing (VIGS), which also involves 21 and 22-nucleotide wsiRNAs, Pol V and DRM2[125]. This progressive silencing of transposable elements and epialleles by RdDM is reminiscent of paramutation.

Heterosis, polyploidy and hybrid incompatibility

Hybridization between different species (interbreeding) and the resulting **interspecific allopolyploids** are particularly common in plants[126]. Crops such as wheat, cotton, and canola are interspecific allopolyploids, whereas maize and sorghum are maintained as **intraspecific hybrids**. In both cases, maintaining heterozygosity is a key factor contributing to enhanced growth phenotypes, also known as hybrid vigor or heterosis[127]. The role of small RNAs during hybridization has been extensively studied in many plant species (reviewed in [128 and 129]), including stable cultivated Arabidopsis spp. allopolyploids generated by crossing tetraploid *A. thaliana* with *A. arenosa*, which resembles the natural allotetraploid *A. suecica*. Small RNAs, particularly miRNAs and tasiRNAs, were recognized as important genetic regulators of Arabidopsis allopolyploids[130]. Also in **introgression lines** derived from cultivated and wild relatives of tomato, miR395 was associated with beneficial

transgressive phenotypes associated with salt tolerance[131]. Paramutation is likely involved in this process as well, but previous studies in maize, rice, tomato and *A. thaliana* have reported contradictory evidence in this respect. In maize, loss of hetsiRNAs in *mop1* mutants (ortholog to *RDR2* in *A. thaliana*) does not influence hybrid vigor in reciprocal intraspecific crosses[132]. In fact, down-regulation of 24-nucleotide siRNAs in F1 hybrids seems to be a common observation at loci where both parents show differential accumulation of small RNAs[129]. These loci include genes and promoter regions that are often occupied by transposable elements, thus the loss of 24-nucleotide siRNAs could result in lower levels of DNA methylation and up-regulation of genes responsible for heterosis phenotypes. Intraspecific hybridization between ecotypes of *A. thaliana* had strikingly contrasting results, as increased levels of DNA methylation were observed in reciprocal crosses while 24-nucleotide siRNAs levels were unchanged[133]. In tomato, small RNAs were more abundant in introgression lines than in each parent and are associated with suppression of target genes and DNA hypermethylation[131], reminiscent of paramutation. Although these reports suggest a role for small RNAs during hybridization of genetically and epigenetically distinct genomes, their importance for growth and vigor remains largely unknown.

Epigenetic variation, polyploidy and small RNA diversity can ultimately lead to strong hybridization barriers in **wide crosses**, where transposon activity and genomic imprinting seem to respond in a parent- and dosage-dependent manner[134]. A simple illustration of small RNA-derived hybrid incompatibility in *A. thaliana*, is the truncated duplication of the essential gene *FOLT1,* which segregated within natural strains[135]. This truncated copy is able to produce siRNAs and trigger heritable silencing of active full-length copies elsewhere in the genome in *trans*, resulting in allelic incompatibilities in hybrid lines[135]. Similar observations in maize[136,137] suggest that segregation of epialleles and duplicate alleles may also be relevant in hybrid crops.

DNA damage repair

RNAi-mediated DNA damage repair occurs in plants[138], which goes in line with previous studies in the fungus *Neurospora crassa*[139] and *S. pombe*[140], in which rDNA-derived small RNAs and centromeric small RNAs, respectively, are induced upon DNA damage. Furthermore, both Dicer and AGO mutants in *S. pombe* are synthetic lethal with the key homologous repair protein Rad51 and have DNA damage response phenotypes[141]. Both AGO2 and AGO9 have a detectable effect on DNA repair efficiency in *A. thaliana*[138,142], where 21- and 24-nucleotide double-strand-break-induced siRNAs (diRNAs) are induced in the vicinity of double strand breaks (DSBs) and are produced by DCL2, DCL3 and DCL4[138]. The additional requirement of RDRs and Pol IV for diRNA biogenesis also suggests that *de novo* transcription and dsRNA amplification mechanisms are involved in DSB repair, although small amounts of diRNAs provide sufficient repair capacity in the absence of RDRs[138]. Notably, a role for small RNAs and AGO in DSB repair and specifically in homologues recombination was also found in human cells, *Drosophila melanogaster*, and *S. pombe*[141,143–145], suggesting an important and conserved role for small RNAs in DSB repair, possibly recruiting other protein complexes to DSB sites[138,146].

Small RNA in meiosis and gametogenesis

Some AGO proteins are preferentially expressed in reproductive tissues and enriched in germline cells[147–150], with specialized functions in chromosome segregation and cell fate specification. Small RNA sorting into different AGO proteins also contributes to functional diversity (Box 1), as unlike miRNAs, secondary siRNA duplexes have perfect complementarity between guide and passenger strands, so that sorting into different AGOs must rely exclusively on their 5′ terminal nucleotides. In rice and maize, phasiRNAs derived from thousands of non-coding precursors accumulate in meiotic and pre-meitoic cells. In *A. thaliana*, loss of heterochromatin in the vegetative nucleus is accompanied by accumulation of retrotransposon and other transposon easiRNAs in sperm cells[78]. In each case, intercellular transport is implicated in germline accumulation of small RNAs[78] (Box 2). The potential functions of these small RNAs in meiosis and gametogenesis remain enigmatic but are now being explored.

A recent study showed that 21-nucleotide phasiRNAs in maize anthers are expressed in meiocytes, and decline during gametophytic development, whereas 24-nucleotide phasiRNAs accumulate throughout meiosis and remain abundant in mature pollen[72] (Fig. 5a). A subset of 21-nucleotide phasiRNAs is loaded onto the *MEIOSIS ARRESTED AT LEPTOTENE 1* (*MEL1*) in rice[151], while its closest ortholog in maize is *AGO5c*, which seems to be expressed in a coordinated fashion with 21-nucleotide phasiRNAs in anthers[72]. By contrast, a binding partner of 24-nucleotide phasiRNAs has not yet been characterized, but transcriptional profiling suggests that *AGO18b* is the most promising candidate in maize, as it seems to be a recently evolved AGO, only found in monocot species[72]. Similarly in *A. thaliana* sperm cells, AGO1 (which binds most miRNAs and some secondary siRNAs) is largely replaced by its close homolog AGO5[149]. Null *ago5* mutants are fertile in *A. thaliana*[152], but mutations in *MEL1* lead to early meiotic arrest and male sterility[153]. MEL1 localizes to the cytoplasm of pre-meiotic cells[153], and like AGO5 in *A. thaliana*, shows selective binding of 5′ cytosine of small RNAs[151]. *mel1* mutants have abnormal tapetum and aberrant pollen mother cells (PMC) that arrest in early meiosis[153], suggesting that the subset of 21-nucleotide phasiRNAs bound to MEL1[151] are crucial for male fertility. The function of phasiRNAs in monocot plant species remains a mystery as they have no obvious targets in the genomes[72], but their peculiar accumulation dynamics observed in maize anthers is reminiscent of mammalian pachytene piRNAs, and might illustrate a possible convergent evolution of small RNAs in male gametogenesis[154].

Although phasiRNA and easiRNA biogenesis have only been reported in anthers and pollen, *AGO5* and *MEL1* are also expressed in ovules where other small RNA pathways have critical roles in reproduction. In maize, AGO104 (ortholog of AGO9 in *A. thaliana*) accumulates specifically in ovule somatic cells surrounding female meiocytes, and is involved in non-CG DNA methylation in heterochromatin[148]. In *ago104* mutants chromosome segregation is blocked during meiosis I and diploid female gametes arise at high frequencies[148] (Fig. 5b), whereas the formation of triads and microspores with multiple nuclei was also observed during male meiosis[148]. In *A. thaliana*, AGO9 binds 24-nucleotide siRNAs[111] and silences transposable elements in the egg cell[150]. AGO104 and AGO9 are active in somatic cells and regulate cell fate specification in a non-cell autonomous manner.

AGO104 represses somatic cell fate in germ cells[148]; conversely, AGO9 prevents sub-epidermal cells from adopting a megaspore-like identity[150] (Fig. 5b). These findings demonstrate a crucial role for small RNAs and epigenetic regulation during sexual reproduction in higher plants, and highlight an important link to apomictic development. Thus understanding these mechanisms might provide an excellent opportunity to use **apomixis** as a fast and efficient way to fix hybrid genotypes in crop species.

Conclusions and future perspectives

The diversification and specialization of gene silencing networks in plants is likely reflecting an important role for small RNAs in adaptation to a sessile life style. However, it remains unclear the contribution of most small RNA classes upon biotic and abiotic stress, as well as the transgenerational inheritance and stability of acquired small RNA-based responses. Most of our current understating of small RNA activity in plants comes from their prominent functions in plant development, starting from an essential role during the first embryonic divisions up until the regulation of meiosis and gametogenesis. Despite the extensive functional diversity in different plant species, the several pathways for small RNA biogenesis and function are evolutionary related, relying on tissue-specific expression patterns and sophisticated mechanisms to sort small RNA duplexes onto specific AGO proteins. We have been able to depict the complex molecular mechanisms involved in small RNA biogenesis and function in plants, but a complete understanding of the specificities and interplay between the different gene silencing machineries operating in plant cells will remain difficult until we are able to profile small RNAs in isolated cell-types and single cells. These future challenges are well underway, and will provide important new insight into small RNA-based gene regulation in a variety of cellular, developmental and transgenerational contexts.

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Glossary

Wide crosses
Crosses of related species or genera that naturally do not sexually reproduce with each other.

Intraspecific hybrids
Genetically divergent plants from the same species

Introgression lines
Population containing genetic material derived from similar species or wild relatives. They are generally produced through successive backcrossing and selection of single introgressed genomic segments from one of the parental lines.

Dicer-like (DCL)

Plant orthologs of other eukaryotic ribonucleose III Dicer enzymes; required for small RNA processing.

Argonaute (AGO)
The main effector proteins of gene silencing, which bind small RNA duplexes and promote small RNA-mediated target recognition and cleavage.

RNA-induced silencing complex
A protein complex that includes Argonaute proteins and small RNAs. The small RNAs hybridize to complementary target RNAs, which then undergo cleavage or translational repression, or recruit other factors, such as chromatin modifiers.

Paramutation
An inter-chromosomal sensing mechanism that initiates heritable epigenetic changes in *trans*. Small RNAs are often involved in this process by mediating RNA-directed DNA methylation (RdDM).

Transgressive phenotypes
Phenotypes in a hybrid progeny that are either superior or inferior to both parents. Transgressive phenotypes might facilitate hybrid specialization and are particularly important in crops when hybrid yields are higher than those of each parent.

piwi-interacting RNAs (piRNAs)
Large class of small RNAs produced in animal cells, which form functional silencing complexes by loading onto piwi proteins. piRNA complexes are mainly involved in the post-transcriptional gene silencing of retrotransposons in the germline.

Exosome
Multi-protein complex involved in 3′ to 5′ degradation of RNA molecules such as mRNAs or rRNAs.

Processing bodies (P-bodies)
Cytoplasmic foci that have essential roles in most mRNA decay mechanisms, including decapping and non-sense mediated decay, as well as in storing processed mRNAs to postpone their translation.

siRNA-bodies
Cytoplasmic foci in plant cells where RDR-DEPENDENT RNA POLYMERASE 6 (RDR6) and SUPPRESSOR OF GENE SILENCING 3 (RDR3) synthesize double-stranded RNA from single-stranded RNA.

Epiallele
A genetic locus where transcriptional activity is regulated by epigenetic silencing marks such as DNA methylation and histone modifications.

Interspecific allopolyploids
Polyploid organisms with two or more sets of genetically distinct chromosomes, which results from the cross between different species.

Apomixis

Natural ability of certain plants species to reproduce asexually through seed, producing offspring genetically identical to the parental plant.

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Biographies

Filipe Borges is a postdoctoral fellow in Rob Martienssen's laboratory at the Cold Spring Harbor Laboratory, New York, USA. He is studying reprogramming and transgenerational silencing of transposable elements in pollen by single-cell genomics and epigenomics, with a focus on the evolution of epigenetic regulation in eukaryotic systems. His training and graduate research at the Gulbenkian Institute in Lisbon, Portugal, focused on understanding the molecular mechanisms regulating germline specification in *Arabidopsis thaliana*, and developing new tools for purification of plant cells by fluorescence-activated cell sorting.

Robert A. Martienssen leads the plant biology group at Cold Spring Harbor Laboratory, where he focuses on epigenetic mechanisms that shape and regulate the genome and their impact on development and inheritance. His work on transposons, or 'jumping genes', in plants and in fission yeast revealed a link between heterochromatin and RNA interference. His laboratory currently focuses on mechanistic aspects of germline reprogramming and epigenetic inheritance, including DNA methylation, histone replacement and modification, and RNA interference.

Online summary

- Functional diversification and expansion of silencing pathways in plants relies on duplication of Dicer and Argonaute proteins.
- The main small RNA classes in plants are microRNAs (miRNAs), 21 to 22-nucleotide secondary small-interfering RNAs (siRNAs) and 24-nucleotide heterochromatic siRNAs (hetsiRNAs).
- All small RNAs in plants are modified at their 3′ end by 2′-O-methylation, including miRNAs, which lack this modification in animals. This modification confers stability and protection from degradation.
- Plant miRNAs are mainly involved in post-transcriptional gene silencing (PTGS) by transcript cleavage or translational repression, and also trigger secondary siRNA production from Pol II transcripts.
- Secondary siRNAs are produced as 21 and 22-nucleotide small RNAs involved in cleavage or translational repression or target transcripts in *cis* and *trans*. They are also able to initiate transcriptional gene silencing (TGS) by establishing DNA methylation at particular loci.
- The majority of siRNAs in plants are 24-nucleotide heterochromatic siRNAs and are involved in silencing repeats and transposable elements by RNA-directed DNA methylation (RdDM).
- Small RNAs in plants are involved in reproductive transitions such as meiosis and gametogenesis, and regulate important epigenetic mechanisms such as genomic imprinting and paramutation.

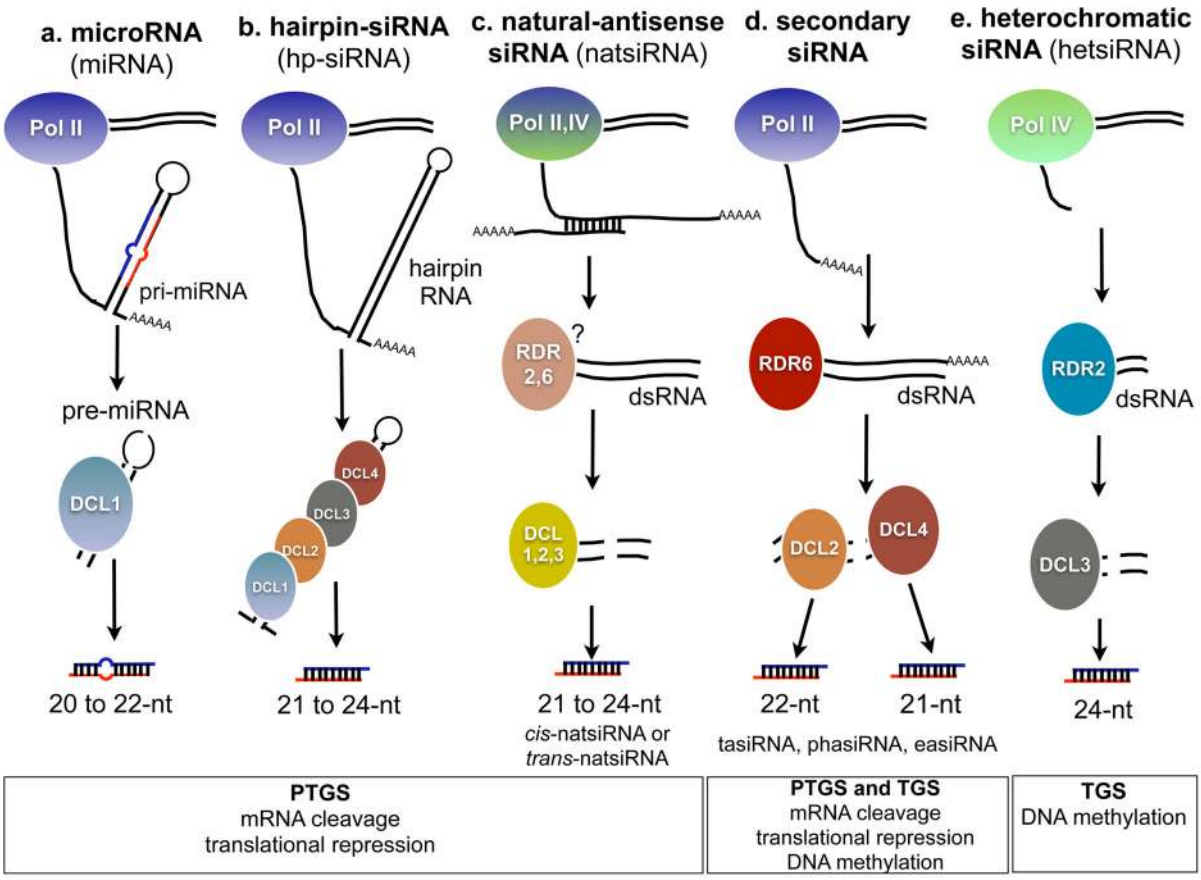

Figure 1. Main pathways for biogenesis of endogenous small RNAs in plants

a. Genes encoding microRNAs (miRNAs; left) are transcribed by RNA Polymerase II (Pol II) and fold into hairpin-like structures called primary (pri)-miRNAs, which are processed by DICER-LIKE 1 (DCL1) into a shorter stem-loop structure called precursor (pre)-miRNAs. Pre-miRNAs are processed again by DCL1 into the mature miRNA duplex. During miRNA processing, DCL1 is assisted by several proteins (reviewed in [8]). MiRNAs are involved in post-transcriptional gene silencing (PTGS) by mediating mRNA cleavage or translational repression. Longer Pol II-derived hairpins, termed hairpin-derived small-interfering RNAs (hp-siRNAs; middle), might originate from inverted repeats, and are originally processed by all DCLs. These hairpins might evolve into miRNAs, and are often designated as proto-MIRs. Natural-antisense small-interfering RNAs (natsiRNA; right) are produced from dsRNAs originating from overlapping transcription (*cis*-natsiRNA) or highly complementary transcripts originated from different loci (*trans*-natsiRNA)[175–177]. The biogenesis and function of natsiRNAs is still largely unclear. **b.** The precursors of secondary siRNAs are transcribed by Pol II, and may originate from non-coding loci, protein-coding genes and transposable elements. These transcripts are converted into double-stranded RNA (dsRNA) by RNA-DEPENDENT RNA POLYMERASE 6 (RDR6), and processed by DCL2 and DCL4 to produce siRNAs of 22- or 21-nucleotide (nt) in length, respectively. Secondary siRNAs are mostly involved in PTGS, but can also initiate RNA-directed DNA methylation (RdDM) at specific loci. They are subdivided into trans-acting siRNAs

(tasiRNA)[34,66,162,178], phased siRNA (phasiRNA)[65] or epigenetically-activated siRNA (easiRNAs) [77,179]. **c.** Heterochromatic siRNAs are derived from transposable elements and repeats located at pericentromeric chromatin. Their biogenesis requires Pol IV transcription and the synthesis of dsRNA by RDR2, which is subsequently processed into 24-nucleotide long siRNAs by DCL3. These small RNAs are involved in maintaining RdDM-mediated transcriptional gene silencing (TGS) (reviewed in [31]).

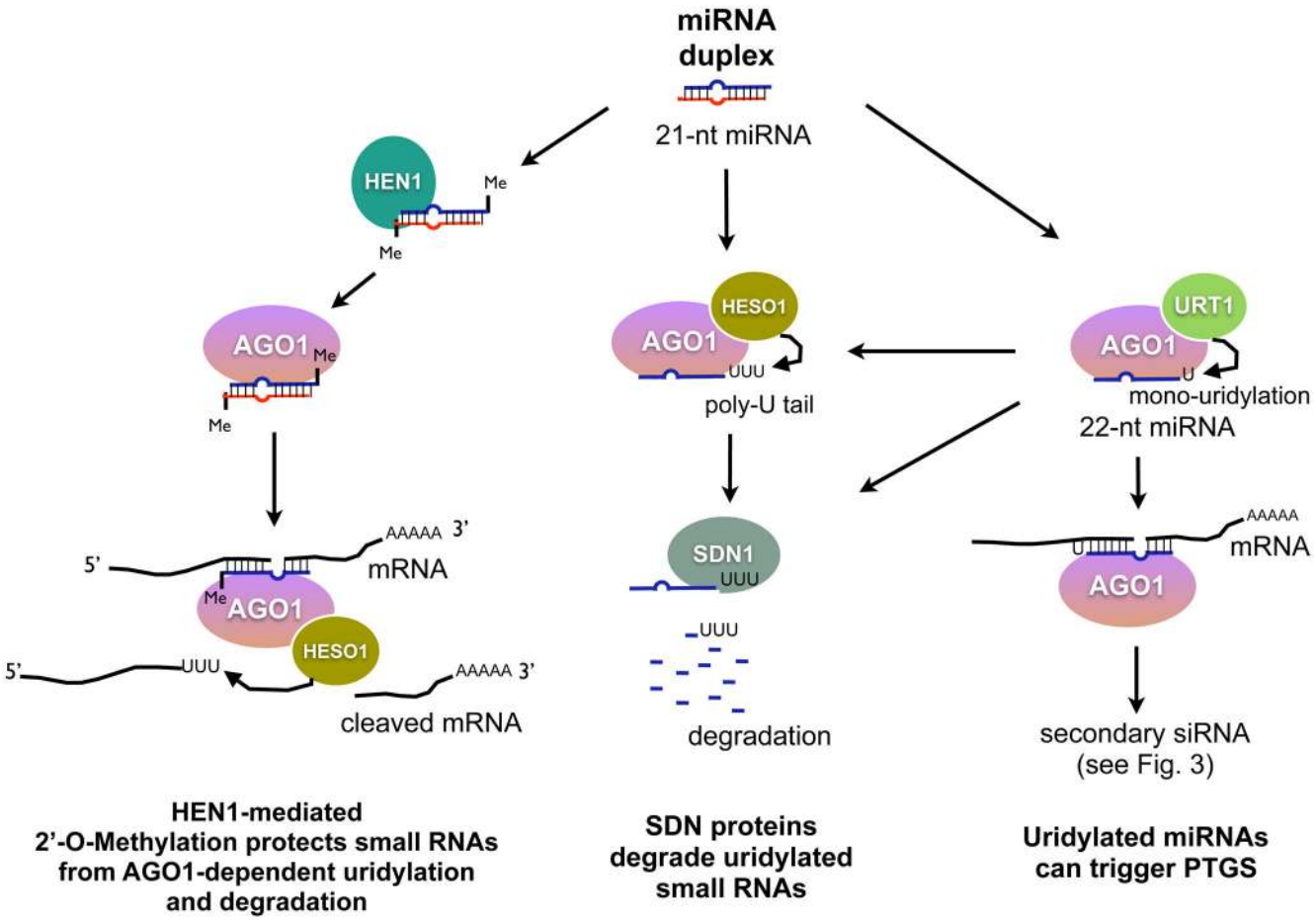

Figure 2. 2′-O-methylation, uridylation and degradation of miRNAs in *A. thaliana*

MicroRNA (miRNA) duplexes are 2′-O-methylated at both 3′ ends by HUA ENHANCER 1 (HEN1), which protects them from uridylation and degradation (left). HEN SUPRESSOR 1 (HESO1) and UTP:RNA URYDILTRANSFERASE 1 (URT1) are nucleotidyl transferases that uridylate unprotected 3′ ends of small RNAs, triggering their degradation by the 3′-5′ exonucleases SMALL RNA DEGRADING NUCLEASE (SDN; middle). ARGONAUTE 1 (AGO1) recruits HESO1 during mRNA target recognition and cleavage in order to polyuridylate and degrade the 3′ of cleaved target transcripts[52]. Thus, the 3′ methylation of miRNAs loaded onto AGO1 serves to protect them from HESO1 activity. Recent studies have shown that URT1 also interacts with AGO1 to establish mono-uridylation of particular miRNAs[53,54] (left), and this process may produce 22-nucleotide miRNA variants that are able to form functional **RNA-induced silencing complex**es and trigger post-transcriptional gene silencing (PTGS)[54] (see Fig. 3). HESO1 and URT1 have been shown to act both independently and synergistically, perhaps reflecting their different affinities for 3′ terminal nucleotides *in vitro*. HESO1 has preference for tailing 3′-uracil, whereas URT1 prefers 3′-adenine[54]. Although these features explain how these enzymes act synergistically at non-3′-uracil miRNA targets (URT1 forms substrates for HESO1), it does not fully account for their substrate preferences found *in vivo*[53,54].

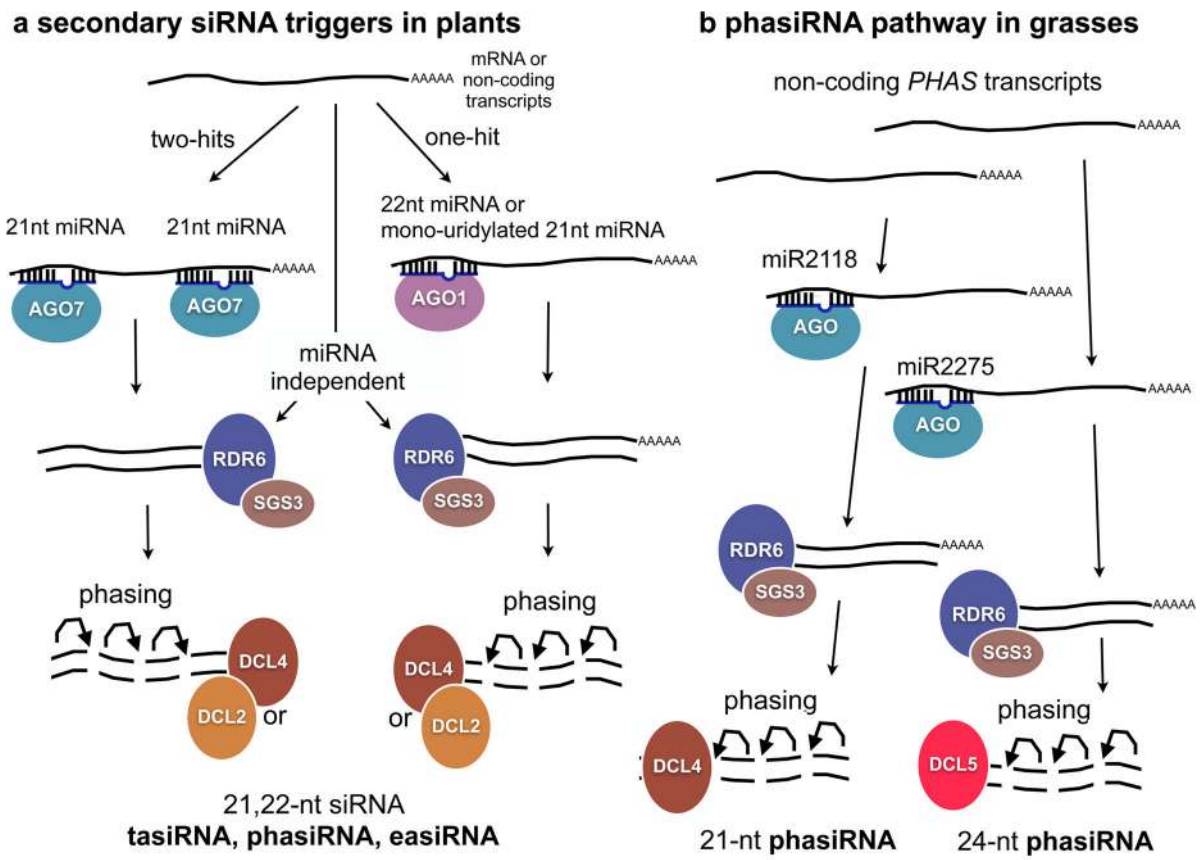

Figure 3. Triggers of secondary siRNA biogenesis

a. Plant microRNAs (miRNAs) target transcripts for cleavage or translational repression, and also trigger the production of secondary small-interfering RNAs (siRNAs) from mRNAs, non-coding RNAs and transposable elements. The most accepted mechanism for the biogenesis of trans-acting siRNA (tasiRNA), phased siRNA (phasiRNA) and epigenetically activated siRNA (easiRNA) relies on two distinct pathways. One consists a two-hit system utilizing two 21-nucleotide (nt) miRNAs per transcript, and requires the activity of an RNA-inducing silencing complex comprising ARGONAUTE 7 (AGO7). The second pathway consists of a one-hit system that usually involves a 22-nt miRNA loaded on AGO1, or 22-nt miRNA variants that are produced from monouridylation of 21-nt miRNAs (see Fig. 2). Both pathways are routed towards RNA-DEPENDENT RNA POLYMERASE 6 (RDR6)-mediated dsRNA synthesis aided by SUPPRESSOR OF GENE SILENCING 3 (SGS3), and processing of 21 and 22-nucleotide siRNAs by DICER-LIKE 4 (DCL4) and DCL2, respectively. RNA Polymerase II (Pol II)-derived transcripts might also produce miRNA-independent secondary siRNA via interactions with other RNA processing machineries such as the spliceosome[85], or during RNA decay[100,101], but these pathways are not fully understood. **b.** An additional phasiRNA biogenesis pathway was found in monocot plants such as maize and rice, which involves the transcription of non-coding *PHAS* transcripts form intergenic loci. Two miRNAs (miR2118 and miR2275) were found involved in cleavage of *PHAS* transcripts by an unknown AGO protein. These cleavage products are

converted into dsRNA by RDR6 and SGS3, and processed into 21- and 24-nucleotide phasiRNAs by DCL4 and DCL5, respectively (reviewed in [65]).

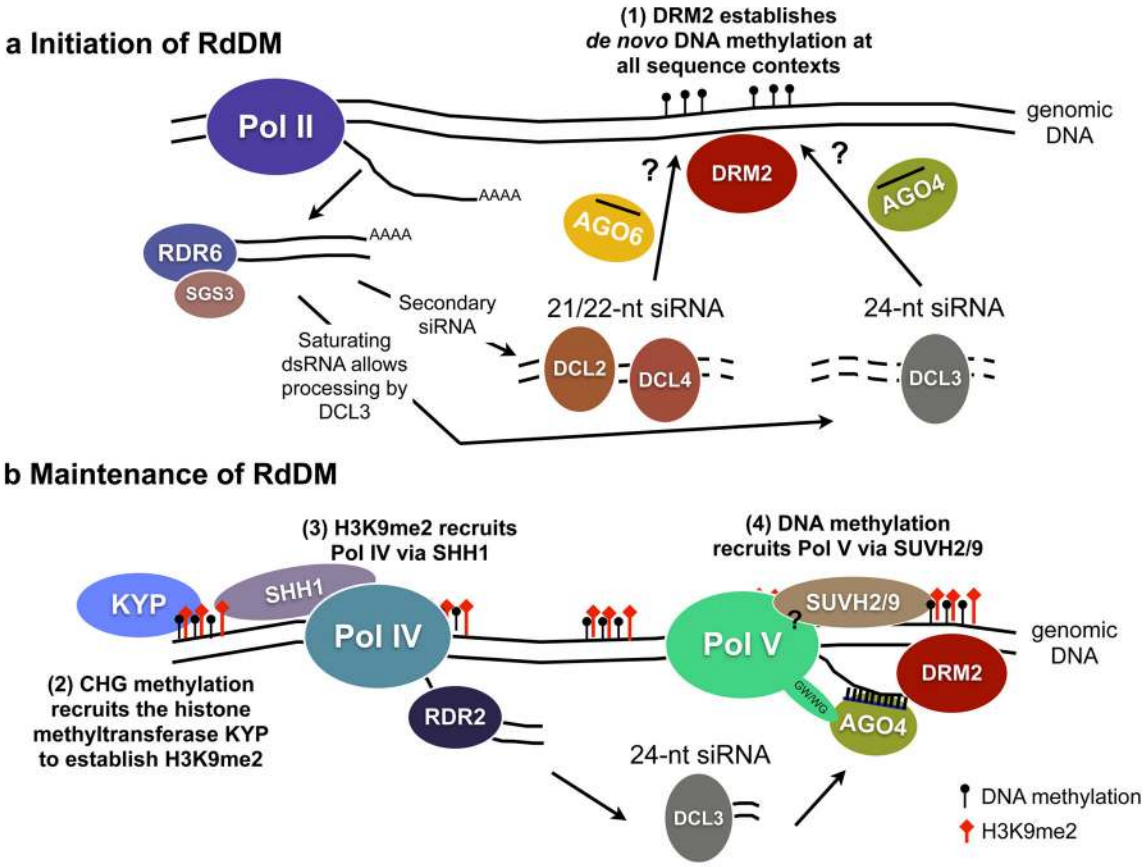

Figure 4. The transition from silencing by PTGS to silencing by TGS in transgenes, epialleles and active transposons

a. Post-transcriptional gene silencing (PTGS) by miRNAs is likely the major pathway triggering biogenesis of secondary 21 and 22-nucleotide (nt) siRNAs, in a process involving RNA-DEPENDENT RNA POLYMERASE 6 (RDR6), SUPPRESSOR OF GENE SILENCING 3 (SGS3), DICER-LIKE 4 (DCL4) and DCL2 (see Fig. 3). 21- and 22-nt siRNAs are required for the establishment of RNA-directed DNA methylation (RdDM) at particular transposable elements and epialleles, which at least at some loci requires the activity ARGONAUTE 6 (AGO6)[109]. This pathway is able to target nascent Pol II transcripts and recruit the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) to establish DNA methylation in all sequence contexts (1), but this interplay is not fully understood. An alternative pathway was proposed for transgenes and active retrotransposons, perhaps depending on their variable copy number and transcription levels. The accumulation of long dsRNA molecules might saturate both the DCL2 and DCL4 processing pathways, resulting in functional compensation by DCL3, which instead produces 24-nt siRNAs for the establishment of RdDM via AGO4[110]. **b.** CHG (H denotes A, C or T) methylation **previously established by DRM2,** is recognized by the histone methyltransferase KRYPTONITE (KYP), which reinforces the repressed chromatin state of methylated DNA by establishing the dimethylation of histone 3 Lys 9 (H3K9me2) [122] (2). A complete PTGS-to-TGS switch occurs when SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) binds H3K9me2 and recruits RNA Polymerase

IV (Pol IV) to initiate the biogenesis of 24-nt siRNAs through RDR2 and DCL3[123] (3). RdDM consolidation is achieved by the recruitment of Pol V to unmethylated DNA by SU(VAR)3–9 HOMOLOG 2 (SUVH2) and SUVH9[124] (4). This is followed by the recruitment of AGO4, mediated by sequence complementarity between the 24-nt siRNAs and the Pol V-nascent transcripts, and by the conserved GW/WG motif (also known as Ago hook) present in the carboxy-terminal region of the Pol V subunit NRPE1. Then AGO4 is able to recruit DRM2 to establish additional DNA methylation *de novo* (reviewed in [112 and 31]).

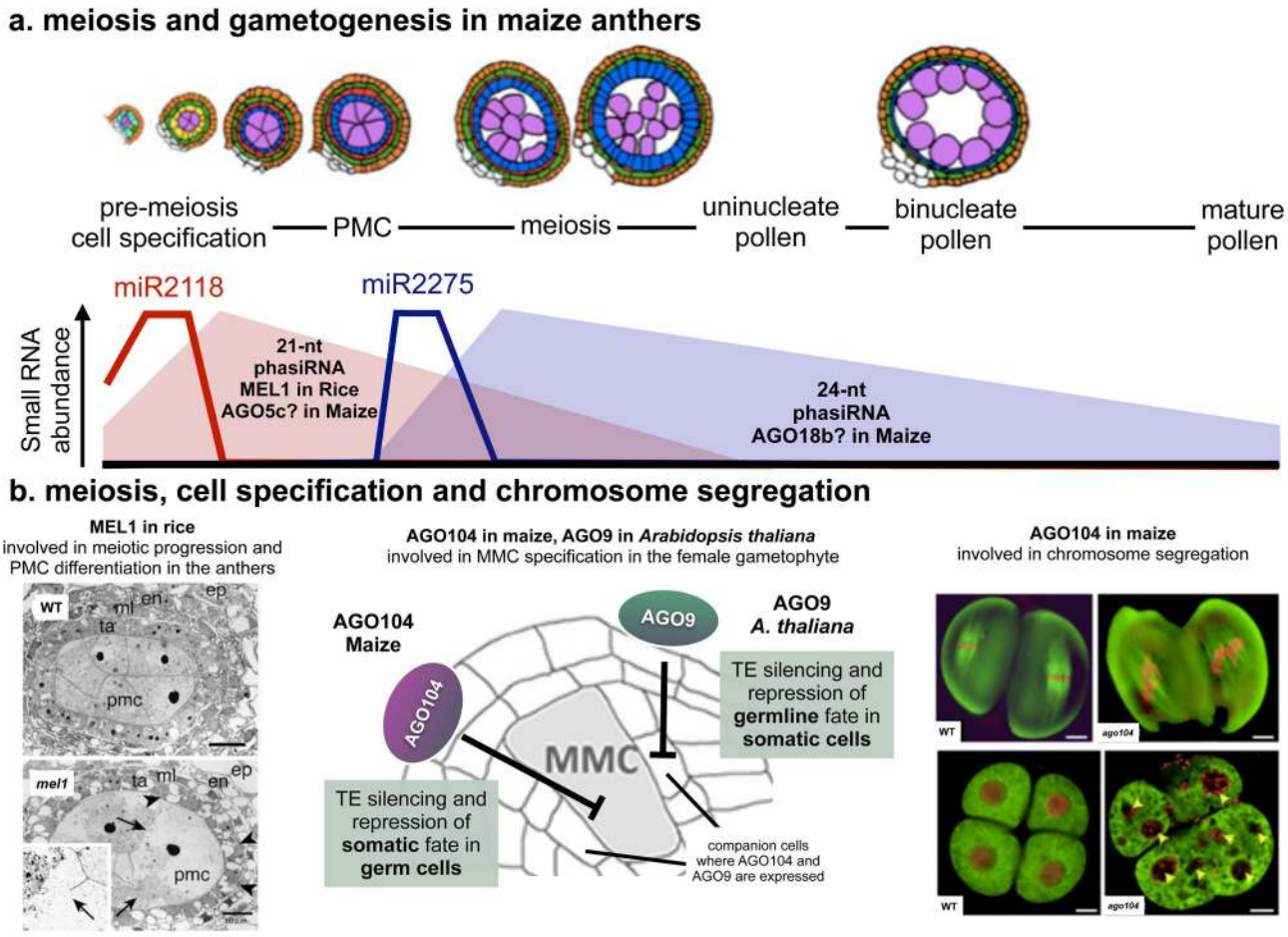

Figure 5. Small RNA functions in meiosis and cell fate specification

a. In grass anthers, two distinct small RNA classes are produced from non-coding PHAS transcripts: 21-nucleotide (nt) phasiRNAs are produced upon cleavage of *PHAS* transcripts by miR2118, whereas miR2275 triggers 24-nucleotide phasiRNA biogenesis from a different subset of *PHAS* loci (reviewed in [65]). The spatiotemporal dynamics of phasiRNA biogenesis was recently described throughout anther development in maize[72], showing a distinct and mostly non-overlapping accumulation patterns for both phasiRNA classes, which nicely coincides with the expression of their respective miRNA triggers. 21-nucleotide phasiRNAs are essentially pre-meiotic, whereas 24-nucleotide phasiRNAs peak during meiosis and decrease during pollen development. The function of these male-specific small RNAs remains unknown, but their different size and accumulation patterns suggest distinct biological activities. A subset of 21-nt phasiRNAs in rice is loaded onto the MEIOSIS ARRESTED AT LEPTONENE1 (MEL1) protein[151], which is the ortholog of AGO5 in *Arabidopsis thaliana*. The *mel1* mutants arrest during early meiotic stages, and produce dysfunctional pollen mother cells (PMCs) that appear frequently in developing anthers. **b.** ARGONAUTE (AGO) functions in meiosis, cell specification and chromosome segregation. (Left) In the female gametophyte, AGO104 in maize and AGO9 in *A. thaliana* were associated with non-cell-autonomous regulation of meiosis and germline specification, but the molecular pathways responsible for that are still unclear[148,150]. Despite both being

expressed in companion cells, AGO104 and AGO9 are involved in epigenetic silencing of transposable elements in the megaspore mother cells (MMC), perhaps through RdDM activity and mobile small RNA[148,150]. (Right) Importantly, *ago104* mutants also produce viable unreduced diploid gametes, indicating that AGO104 has a role meiotic chromosome segregation and establishing a direct link between small RNA regulation and apomixis[148]. Top image in panel **a** adapted from [72]. Right Image in panel **b** reproduced from [148] (arrowheads indicate micronuclei in abnormal tetrads).