Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Plant Biology

Enjoy the silence: Canonical and non-canonical RNA silencing activity during plant sexual reproduction

Jinping Cheng and German Martinez

Plants produce small RNAs that accomplish a surprisingly versatile number of functions. The heterogeneity of functions of plant small RNAs is evident at the tissue-specific level. In particular, in the last years, the study of their activity in reproductive tissues has unmasked an unexpected diversity in their biogenesis and roles. Here, we review recent findings about the biogenesis pathways and roles of small RNAs during plant sexual reproduction.

Addresses
Department of Plant Biology, Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Center for Plant Biology, Uppsala, Sweden

Corresponding author: Martinez, German (german.martinez.arias@slu.se)
(Martinez G.)

Current Opinion in Plant Biology 2024, **82:**102654

This review comes from a themed issue on **Epigenetics and gene regulation 2024**

Edited by: **Mark Zander** and **Javier Gallego-Bartolomé**

For a complete overview see the Issue and the Editorial

Available online xxx

https://doi.org/10.1016/j.pbi.2024.102654

Keywords
miRNA, siRNA, phasiRNA, Secondary siRNA, Mobile siRNAs, Heterochromatic siRNAs, RNA silencing, Transposable elements.

Introduction

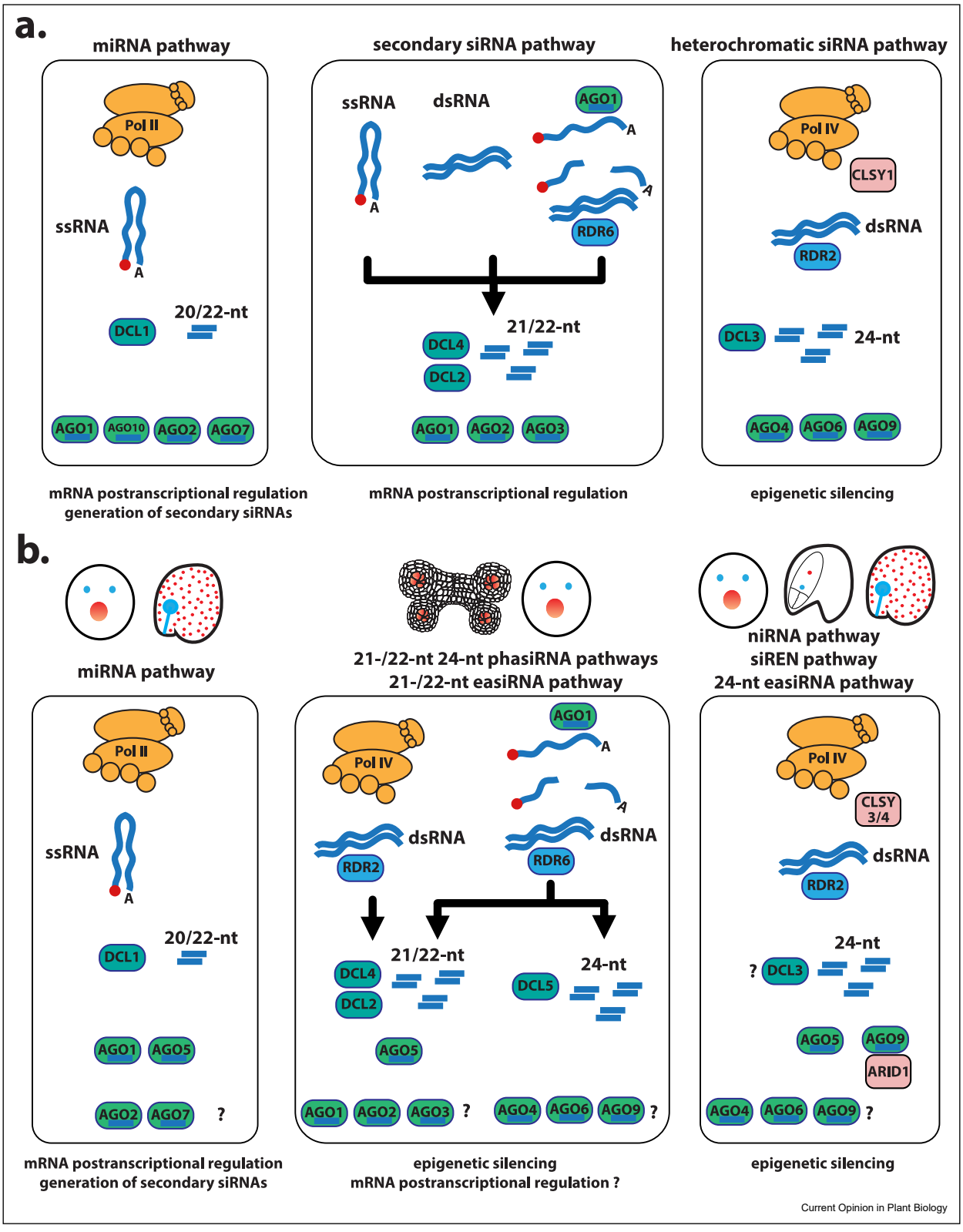
Plants produce a diversity of small RNAs (sRNAs) that are key for the posttranscriptional control of mRNAs and the epigenetic regulation of specific loci. The activity of sRNAs is essential for multiple aspects of plants' life cycle, including developmental regulation, response to stress, and maintenance of genome integrity [1]. sRNAs are categorized into microRNAs (miRNAs) and small interfering RNAs (siRNAs). Both sRNA categories share a size range from 20- to 25-nt and a biogenesis pathway that relies on DICER-LIKE (DCL) RNase-III enzymes and ARGONAUTE (AGO) proteins to effect their roles. miRNAs are characterized by their origin from single-stranded RNAs (ssRNAs) with partial self-complementarity and a biogenesis pathway that involves DCL1, which excises a single duplex from the ssRNA, and AGO1, AGO2, AGO7 and/or AGO10, which preferentially load one of the sRNAs from the excised duplex termed the mature miRNA. AGO-loaded miRNAs mediate the posttranscriptional regulation of complementary mRNAs. siRNAs, on the other hand, are a diverse and heterogeneous category that derive from dsRNAs of different origins. siRNAs can be further divided into secondary siRNAs and heterochromatic siRNAs (hc-siRNAs). Secondary siRNAs are characterized by a biogenesis route that is initiated by the miRNAs or siRNAs targeting a longer RNA that is then converted into dsRNA by RDR6. This dsRNA is further processed into a complex population of siRNA duplexes by DCL4, DCL2, and/or DCL5, which are loaded into AGO1, AGO2, and/or AGO3 to mediate the posttranscriptional regulation of complementary mRNAs. hc-siRNAs have a well-defined biogenesis pathway that, uses dsRNAs derived from the combined activities of a specialized DNA-dependent RNA polymerase, RNA polymerase IV (Pol IV) and its tightly associated RNA-dependent RNA polymerase, RDR2. These dsRNAs are then processed into hc-siRNAs by DCL3, which are loaded into AGO4/6/9 to introduce DNA methylation at complementary sites transcribed by Pol V [2,3]. The boundaries of these canonical pathways and the role of the different sRNA types have been increasingly blurred, partially due to the increased study of the role of these pathways in different tissues and developmental stages (summarized in Figure1) [4].

In particular, sRNAs play a pivotal role during reproduction, where they regulate sexual development and its associated molecular events. Recent fascinating examples of both canonical and non-canonical RNA silencing have revealed its crucial role for achieving reproductive success. In the following section, we review recent discoveries in the role and pathways mediating reproductive RNA silencing.

Subfunctionalization of miRNAs during sexual reproduction

miRNAs mediate the posttranscriptional regulation (termed posttranscriptional gene silencing, PTGS) through the endonucleolytic cleavage or translational repression of their target mRNAs [1]. In animals,

Figure 1



Canonical and non-canonical sRNA biogenesis pathways. a) Summary of the three canonical sRNA biogenesis pathways in somatic tissues. First (left panel), the miRNA pathway generates miRNAs ranging in size from 20- to 22-nt mediated by the processing of single-stranded RNAs (ssRNA) with self-complementarity by DICER-LIKE1 (DCL1). The miRNAs generated are mainly loaded into ARGONAUTE1 (AGO1), 10, 2 and 7. The secondary siRNA pathway (middle panel) generates 21-/22-nt siRNAs that can be initiated from different types of RNAs that include ssRNAs, dsRNAs generated by

miRNAs play integral roles during sexual reproduction, such as controlling gamete maturation and fertilization [5–8]. In plants, several components of the miRNA biogenesis pathway and specific miRNAs are implicated in the specification of the male and female germlines [9–11], but until recently, their activity in plant gametes was not well understood. This knowledge has been boosted due to the combined use of improved gametophyte-purification techniques and sRNA/RNA high-throughput sequencing. In Arabidopsis, miRNAs show a characteristic accumulation pattern during pollen gametogenesis [12] and embryogenesis [13]. In both tissues, miRNAs target diverse transcription factors and development-associated genes, similar to their role in somatic tissues, that are key for the proper development of reproductive structures [12,13].

miRNA signature in reproductive structures may be a consequence of the differential activity in their biogenesis pathways. Indeed, the study of the accumulation and role of AGO and DCL proteins in reproductive tissues has helped to identify a previously overlooked dynamism (summarized in Figure2). Analyses of AGO accumulation patterns in the gametophytes and embryo showed nearly all of these proteins accumulate in specific structures or cells (except AGO10 in the female gametophyte, and AGO3 and 10 in the male gametophyte) [14]. Importantly, AGO proteins belonging to the miRNA clade (composed of AGO1, 5, and 10), have a different hierarchy in reproductive structures which show accumulation and activity of AGO5 together with AGO1. Indeed, AGO1 and 5 accumulate in the gametic cells of both gametophytes, showing accumulation in the egg and central cells in the ovule, and the sperm cells in the pollen grain [14,15].

Additionally, the role of AGOs belonging to the miRNA clade has been inferred from recent direct analyses of the sRNA populations loaded into them [12,16]. Immunoprecipitation of AGO1 and 5 followed by sRNA sequencing from different stages of male gametogenesis has shown that in these tissues both AGOs retain their miRNA-loading ability and 5' nucleotide signature [12]. Interestingly, both AGOs are enriched in different members of the miR845 family, and target TEs of the ATGP and ATCOPIA superfamilies, similar to their role in somatic tissues [17,18]. Targeting of TEs by miRNAs in the male gametophyte correlates with a decrease of 24-nt and an increase of 21- and 22-nt TE-derived siRNAs [12] and might be a protective mechanism against TE activity. Indeed, this protective role is evident in maize mutants for the two AGO5 homologs (MALE-ASSOCIATED ARGONAUTE-1 and -2, MAGO1 and 2) [19]. In this species, MAGO1 and 2 protect against stress-associated TE reactivation by loading TE-derived phased-secondary siRNAs (phasiRNAs), an activity that is essential for the viability of maize pollen [19].

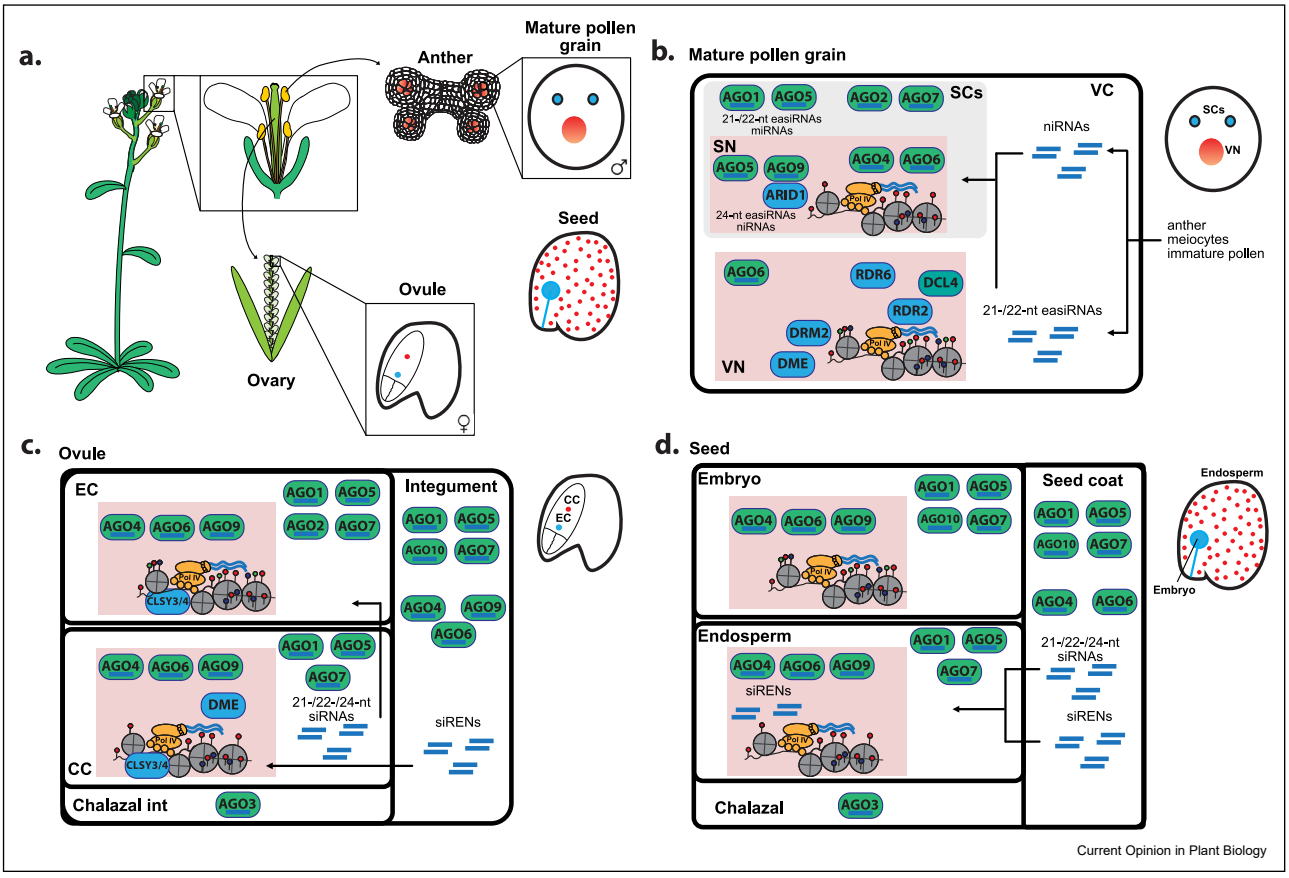
In animals, gamete-accumulating sRNAs can be transmitted during the fertilization process and exert their regulation transgenerationally [20,21]. In plants, this exciting role has not been identified until recently. In Arabidopsis, male-derived miR159 has been reported to act transgenerationally targeting the transcription factors MYB33/65 in the endosperm and promoting nuclear division [22]. This non-cell autonomous activity suggests a potential transgenerational role for, at least, pollen-derived miRNAs. Future studies are needed to determine if other sRNA classes share this ability. In summary, the canonical and non-canonical activities of the miRNA pathway are a fundamental aspect of both the regulation of the correct development of the reproductive tissues (pre- and postfertilization) and the maintenance of genome stability through the targeting of TEs.

Secondary siRNAs play a prime role during reproduction

Together with miRNAs, secondary siRNAs can also mediate PTGS of their target mRNAs. Similar to miRNAs, different types of secondary siRNAs show

RDR6 (as the ones resulting from amplification of certain miRNA-cleaved of mRNAs). These dsRNAs are processed by DCL4 and 2 into siRNAs, and are subsequently loaded into AGO1, 2 or 3. The canonical heterochromatic siRNA (hc-siRNA) pathway (right panel) generates DCL3-dependent 24-nt siRNAs from RNA polymerase IV (Pol IV) transcripts converted into dsRNA by RDR2 and subsequently loaded into AGO4/6/9. The chromatin remodeler CLASSY1 (CLSY1) interacts with Pol IV to produce the majority of hc-siRNAs. **b) Summary of the main canonical and non-canonical sRNA biogenesis pathways in reproductive tissues.** The miRNA pathway (left panel) has a similar composition as in somatic tissues with the difference of the increased activity of AGO5 in the loading of miRNAs (reported in the pollen grain). miRNA activity is also well-understood during embryogenesis. The involvement of other AGOs such as AGO2 and 7 is still not well-characterized. AGO10 is absent in all reproductive structures analyzed to date. The secondary siRNA pathway shows multiple variations in reproductive structures. In early pre-meiotic tissues of monocots, Pol II transcripts are processed by RDR6 into dsRNA and generate 21-/22-nt phasiRNAs by the action of DCL4 and 2. Additionally, in mature pollen grains in monocots, a non-canonical pathway where Pol II transcripts are processed by DCL5 to generate 24-nt phasiRNAs takes place. The involvement of the canonical RdDM-mediating AGOs (AGO4, 6, and 9 is not understood). Similarly, in Arabidopsis pollen, a non-canonical pathway uses Pol IV and RDR2 dsRNAs to generate 21-/22-nt epigenetically active siRNAs (easiRNAs) that are subsequently loaded into (at least) AGO5. The involvement of other AGOs in the loading of easiRNAs is not well understood. Finally (right panel) the hc-siRNA pathway in reproductive structures shows the involvement of different CLASY family members (CLSY3 and 4, which produce the majority of siRNAs in the endosperm, termed siRENs, in the ovule and endosperm, and CLSY3, which produces non-cell autonomous nurse cell-derived siRNAs, niRNAs, in the tapetal cells that accumulate in the mature pollen grain). The dsRNAs produced by Pol IV activity are processed by DCL3 into 24-nt hc-siRNAs and at least loaded in AGO5 and AGO9. In the mature pollen grain AGO9 interacts with the transcription factor ARID1 to mediate epigenetic silencing in connection with H3K9me2. The main reproductive structures where these pathways have been identified are shown on top of each panel. The role of each pathway is indicated at the bottom of each panel.

Figure 2



Tissue-specific localization of the factors involved in the canonical and non-canonical sRNA biogenesis pathways in reproductive structures. a) Cartoon depiction of the reproductive tissue location in Arabidopsis. Nuclei colored blue show actual gametes, while nuclei colored in red show companion cells. **b) Tissue-specific location of sRNA biogenesis pathways in the mature pollen grain.** Mature pollen grains contain two different cell types, sperm cells (SCs) and the vegetative cell (VC), each with its nucleus (sperm nucleus, SN, and vegetative nucleus, VN). In the mature pollen grain, AGO1, 5, 2, and 7 accumulate in the cytoplasm of the SCs. AGO4, 6, and 9 accumulate in the SN, but there is evidence of AGO5 activity in the SN mediating DNA methylation. Mobile (between the VC and the SCs) 21-and 22-nt easiRNAs are known to be generated in the VC or VN by a Pol IV-dependent pathway that involves RDR2. RDR6 is known to accumulate in the VC and could be involved in the generation of easiRNAs or other secondary siRNAs. Additionally, niRNAs derived from anthers and easiRNAs derived from meiocytes or immature pollen stages could be accumulated in the mature pollen and mediate their action in the SCs. This is known for niRNAs, which drive RdDM in the SCs. **c) Tissue-specific location of sRNA biogenesis pathways in the ovule.** Ovules contain different nuclei and tissues. Mainly, the egg cell (EC) and the central cell (CC) are involved in the generation of the embryo and endosperm after fertilization with the male-derived sperm cells. A mix of inner and outer integuments surround the ovule cells, including the chalazal integuments. All the canonical RdDM AGOs (AGO4, 6, and 9) have been detected in the EC and CC nuclei. Evidence of the activity of the RdDM pathway from sRNA sequencing and DNA methylation indicates that this pathway should be active in either the EC or the CC and driven by its interaction with CLSY3 and 4. miRNA and secondary siRNA associated AGOs such as AGO1, 5, and 7 accumulate in the cytoplasm of both the EC and CC. AGO2 and 8 accumulate only in the EC. The integuments show the accumulation of AGO1, 5, 7, and 10, while AGO3 accumulates only in the chalazal integuments. No activity of miRNAs or secondary siRNAs has been reported in the ovule, although there is evidence for the presence of mobile 21-/22-nt siRNAs, pointing to the activity of some of those pathways. **d) Tissue-specific location of sRNA biogenesis pathways in the seed.** After fertilization, the ovule generates the seed that contains two main tissues, the endosperm and the embryo. In the seed, the RdDM pathway should be active in either the endosperm and/or the embry. All the canonical RdDM AGOs (AGO4, 6, and 9) have been detected in the endosperm and the embryo. miRNA and secondary siRNA-associated AGOs such as AGO1, 5, and 7 accumulate in the cytoplasm of both the endosperm and the embryo at different times postfertilization. AGO10 accumulates only in the embryo postfertilization. The seed coat shows accumulation of AGO1, 4, 5,6, 7, and 10, while AGO3 accumulates only in the chalazal. miRNA activity is particularly important after fertilization and drives embryogenesis through all seed development. There is evidence of movement of 21-/22-nt secondary siRNAs at early seed developmental stages before the isolation of the seed coat between the seed coat and the endosperm. 24-nt siRENs accumulate in the seed coat and could potentially be transferred to the endosperm from this compartment at early seed development times. Additionally, male-transmitted miRNAs could mediate the regulation of transcripts in the seed, most likely in the endosperm.

tissue-specific accumulation in reproductive structures. Trans-acting siRNAs (tasiRNAs) are a class of secondary siRNAs that derive from the targeting of TAS transcripts by different miRNAs, including miR173, miR828 and miR390, which generate phased siRNAs [23]. TasiRNAs are well-characterized in somatic tissues where they control the expression of key developmental regulators such as auxin response factor genes (ARFs) [24]. In addition to their important functions in somatic tissues the activity of the canonical tasiRNA pathway is partially needed in the cells surrounding the megaspore mother cell (the pre-meiotic cell that will give rise to the female gametophyte) for the specification of the female germline in Arabidopsis [25–27]. While the function of tasiRNAs in the PTGS regulation of their targets [28–30] or the mediation of DNA methylation through a non-canonical pathway [31] is well described in somatic tissues, their direct targets in reproductive tissues remain uncharacterized.

In addition to tasiRNAs, another type of secondary siRNAs, reproductive phasiRNAs, are crucial for the development of the male gamete. Similar to ta-siRNAs, phasiRNAs of 21- and 24-nt derive from miRNA-cleaved transcripts (termed *PHAS* loci) that are processed in a phased manner by DCL [32]. 21-nt phasiRNAs are initiated by different miRNA families (including miR2118/412, miR11308, and miR14051) which in premeiotic anthers of the majority of monocots and some dicots target several transcripts (termed *21-PHAS*) [32–36] that differ from their targets in somatic tissues [32,37]. 21-nt phasiRNAs generated in the somatic layers of anthers mediate PTGS of a diverse group of transcripts (including genes and TEs) in the male germ cells of, at least, rice and maize [19,35]. On the other hand, 24-nt phasiRNAs are triggered by miRNAs (including miR2275, miR11308, miR2118/412, and miR14051), which drive their production from a set of transcripts termed *24-PHAS* mediated by the monocot-exclusive DCL5 [38,39]. In contrast to 21-nt phasiRNAs, 24-nt phasiRNAs accumulate at later anther developmental stages [34] and mediate canonical DNA methylation [40]. The elimination of phasiRNAs reduces plant fertility, indicating their importance for the development of the male gametophyte [32–35]. Interestingly, in some plant species, certain miRNAs can drive the generation of both 21- and 24-nt phasiRNAs from the same *PHAS* loci [36,38]. This indicates that other factors regulating the accessibility of the targeted transcript to different DCL proteins might exist and highlights the potential dynamism of this secondary siRNA class [41].

In addition to tasiRNA and phasiRNAs, secondary siRNAs derived from TEs show a characteristic accumulation pattern in reproductive structures [42]. Initially observed in Arabidopsis mutants displaying TE transcriptional reactivation, TE-derived siRNAs of 21-/22-nt (termed epigenetically activated siRNAs, easiRNAs) silence TEs when RdDM activity is compromised [18,43]. In line with this role, easiRNAs accumulate naturally in the mature pollen grain of *Arabidopsis thaliana* [43], which has low levels of DNA methylation and repressive histone marks [44,45]. In somatic tissues, easiRNAs can be generated by a diversity of pathways that include the processing of RDR6-generated dsRNAs by DCL4 (including their natural genomic disposition with inverted repeats) [46], non-canonical miRNA-mediated recognition of Pol II or Pol IV transcripts [12,17,18,47], and ribosome stalling due to the unfavorable codon usage of TE transcripts [48,49]. In Arabidopsis pollen, easiRNA accumulation is partially dependent on a non-canonical pathway that involves Pol IV [47,50], and DCL4 and 2 [17] (which also mediate the production of mobile secondary siRNAs [51]). easiRNAs are generated during pollen development [50], although certain populations could be transferred from meiocytes [17] or the tapetal cells, similar to nurse cell-derived 24-nt sRNAs [52]. In somatic tissues, easiRNAs use multiple overlapping mechanisms to silence TEs, including the PTGS of their transcripts via endonucleolytic cleavage [18,43,51] or translational repression [53], and transcriptional gene silencing (TGS) through H3K9me2 deposition [53,54] driven by non-canonical RDR6-RdDM. In the pollen grain, easiRNAs can, at least, mediate the PTGS of their targets [17,51], and also participate in non-canonical RdDM [50], although this later role needs further confirmation. In addition, easiRNAs might have functions that extend beyond the regulation of TE transcriptional and posttranscriptional activity. Indeed, their accumulation in the male gametophyte and their associated AGO proteins correlate with an increased intensity of the 'triploid block' hybridization barrier (which prevents interploidy crosses between tetraploid males and diploid females) in Arabidopsis [12,17,47,50]. Overall, these examples show the importance of secondary siRNAs for the correct development of reproductive tissues and genome stability in the gametes.

sRNA-mediated translational repression during sexual reproduction

sRNA-mediated translational repression is less understood than cleavage-based PTGS, in part due to the lack of technical approaches for studying this type of regulation in plants [55–57]. At the mechanism level, sRNA-mediated translational repression is independent of mRNA deadenylation or RNA decay [58] and is mediated by, at least, AGO1, AGO3, and AGO10, which interact with cytoskeleton-, processing bodies (PBs)- and/or endoplasmic reticulum-associated components [59–61]. Both miRNAs and siRNAs can mediate this type of regulation [59,60,62,63]. Interestingly sRNA-mediated translational repression is modulated with cell/tissue specificity being reported to be high in the mature pollen grain compared to other tissues [64], and reduced in

dark-grown plants [65]. The few reported events of sRNA-mediated translational repression in plants offer a treasure trove of fascinating PTGS examples. Several miRNAs induce the translational repression of their targets [66] and some of their translational repression events are developmentally modulated [67–69]. One of the most fascinating examples of this regulation was observed transgenerationally during reproduction. Wang et al. reported that a TE-derived siRNA (siRNA854), which naturally targets the PB component UBP1b to induce its cleavage [70], is sequestered by a paternally expressed gene (PEG2) transcript in the pollen grain. Interestingly, the effect of this sequestration is only evident transgenerationally in the seed, where appropriate amounts of siRNA854 are needed for proper seed development [63]. The mature pollen grain and the seed are known to exhibit strong translational repression [71,72]. Indeed, artificial miRNAs mediate strong translational repression of reporters in the mature pollen grain [64]. Future genome-wide analysis of the participation of miRNAs and siRNAs in the regulation of translational repression in reproductive tissues might reveal the overall relevance of this type of regulation.

Heterochromatic siRNAs diversity in reproductive tissues

Hc-siRNAs have a well-characterized genomic origin, biogenesis pathway, and role in mediating RdDM or TGS. Recent studies have shown that hc-siRNAs are produced with specificity for different cells and tissues. This specificity is regulated by the CLASSY (CLSY) proteins [73,74], a family of chromatin remodeling factors that interact with Pol IV [75,76]. This machinery is responsible for generating organ-specific populations of 24-nt siRNAs and is the main factor behind the production of ovule siRNAs in the endosperm (siren-RNAs) [77,78] and nurse cell-derived siRNAs in tapetal cells (niRNAs) [52]. Both types of 24-nt siRNAs can methylate targets with less perfect complementarity in trans [52,78], a feature that is not gametophyte-exclusive and was initially observed in somatic tissues [79].

Besides Pol IV-derived transcripts the RdDM pathway can accept non-canonical substrates. For example, in somatic tissues, Pol II-produced pre-miRNAs can generate 24-nt sRNAs that mediate the methylation in cis of their producing loci [80]. Furthermore, Pol II-transcribed loci containing inverted repeats, as the ones found in a member of the TE family Mutator, can enter the canonical RdDM and mediate the DNA methylation of loci with sequence complementarity [81]. In reproductive tissues, the use of non-canonical substrates in the generation of hc-siRNAs is exemplified by the use of Pol II-transcripts for the generation of 24-nt phasiRNAs [34,40], as explained above. It is unknown if other classes of reproductive 24-nt siRNAs can derive from similar non-canonical transcripts.

The analyses of the subcellular localization of the AGO proteins associated with hc-siRNAs have revealed a strong versatility and potentially important roles of this pathway in the gametophytes and embryo. AGO4, 6, and 9 accumulate in the egg and central cells in the female gametophyte, and in the sperm cells (with AGO6 accumulating also in the vegetative nucleus) in the male gametophyte [14]. Interestingly, AGOs that do not play a major role in regulating RdDM activity in somatic tissues are important in reproductive structures. For example, both AGO5 and AGO9 participate in the regulation of DNA methylation in the SAM and the male gametophyte [16,82]. This indicates that AGO5 has a dual role in the loading of 21-/22-nt miRNAs and TE-derived siRNAs, but also 24-nt TE-derived siRNAs [16]. Genetic analyses have shown that AGO5 and AGO9 play a partial role in the correct determination of the precursor cell (termed megaspore mother cell, MMC) that will give rise to the mature ovule [10,25]. The activity of these AGOs in the cells surrounding premeiotic ovules, most likely through canonical RdDM (at least for AGO9), is redundant with that of tasiRNAs [10,25].

Other forms of non-canonical RdDM such as RDR6-RdDM might be active in reproductive structures. Indeed, RDR6-RdDM shows an inflorescence-specific activity determined by the expression pattern of AGO6 [3,31,83–85]. AGO6 is also present in the sperm cells, the vegetative nucleus of pollen, and the central cell in the ovule [14]. This subcellular localization points to a potential role of non-canonical RdDM in the gametophytes or postfertilization tissues, but the influence of this form of RdDM remains unexplored. Interestingly, other tissue-specific factors that do not belong to the canonical RdDM pathway in the plant body can modulate its tissue-specific activity. In the male gametophyte, AGO9-mediated heterochromatic silencing in the sperm cells is promoted by its interaction with the pollen-exclusive transcription factor ARID1 [86]. Beyond their role in regulating RdDM in the gametophytes where they are produced, hc-siRNAs have also been connected to the transgenerational inheritance of epialleles in maize [87], although, in Arabidopsis, this class of sRNAs seems to have a limited transgenerational influence [50]. In sum, these findings suggest that hc-siRNA populations and activity in reproductive tissues are heavily influenced by tissue/cell-specific factors that might modulate RdDM activity according to their needs.

Concluding remarks

The role of RNA silencing during plant sexual reproduction involves a diverse array of canonical and non-canonical mechanisms. These mechanisms are determined both by the tissue-specific expression of its factors (with the examples of AGO5, AGO9, and CLSY

proteins as some of the most versatile components) and interactors (such as ARID1). Further, other components such as sRNA consumption during non-cell autonomous RNA silencing [88], the role of sRNA post-transcriptional modifications [89], or the incorporation of non-canonical sRNAs derived from functional non-coding RNAs (such as rRNAs and tRNAs) [90–93], are underexplored aspects that might play relevant roles during sexual reproduction. Yet, certain developmental stages such as pre-meiotic and meiotic tissues are understudied due to their relative inaccessibility which makes them incompatible with high-throughput sequencing. Future research on this topic will likely bear more fascinating examples of RNA silencing diversity expanding our understanding of the tissue-specific activities of this mechanism in eukaryotic organisms.

Funding

We thank Formas (2021-01161), the Swedish Research Council (VR 2021–05023), and the Knut and Alice Wallenberg Foundation (KAW 2019.0062) for supporting research in the Martinez group.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors want to thank Thamara Juárez-González and Filipe Borges for their input during the preparation of this manuscript.

Data availability

No data was used for the research described in the article.

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Borges F, Martienssen RA: **The expanding world of small RNAs in plants**. *Nat Rev Mol Cell Biol* 2015, **16**:727–741.

2. Matzke MA, Mosher RA: **RNA-directed DNA methylation: an epigenetic pathway of increasing complexity**. *Nat Rev Genet* 2014, **15**:394–408.

3. Cuerda-Gil D, Slotkin RK: **Non-canonical RNA-directed DNA methylation**. *Nat Plants* 2016, **2**, 16163.

4. Vaucheret H, Voinnet O: **The plant siRNA landscape**. *Plant Cell* 2024, **36**:246–275.

5. Presslauer C, Bizuayehu TT, Kopp M, Fernandes JMO, Babiak I: **Dynamics of miRNA transcriptome during gonadal development of zebrafish**. *Sci Rep* 2017, **7**.

6. Yuan SQ, Schuster A, Tang C, Yu T, Ortogero N, Bao JQ, Zheng HL, Yan W: **Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development**. *Development* 2016, **143**:635–647.

7. McJunkin K, Ambros V: **The embryonic mir-35 family of microRNAs promotes multiple aspects of fecundity in Caenorhabditis elegans**. *G3-Genes Genomes Genetics* 2014, **4**:1747–1754.

8. Eun SH, Stoiber PM, Wright HJ, McMurdie KE, Choi CH, Gan Q, Lim C, Chen X: **MicroRNAs downregulate Bag of marbles to ensure proper terminal differentiation in the male germline**. *Development* 2013, **140**:23–30.

9. Huang J, Zhao L, Malik S, Gentile BR, Xiong V, Arazi T, Owen HA, Friml J, Zhao DZ: **Specification of female germline by microRNA orchestrated auxin signaling in**. *Nat Commun* 2022, **13**.

10. Tucker MR, Okada T, Hu YK, Scholefield A, Taylor JM, Koltunow AMG: **Somatic small RNA pathways promote the mitotic events of megagametogenesis during female reproductive development in**. *Development* 2012, **139**:1399–1404.

11. Oliver C, Santos JL, Pradillo M: **On the role of some ARGONAUTE proteins in meiosis and DNA repair in**. *Front Plant Sci* 2014, **5**.

12. Oliver C, Annacondia ML, Wang Z, Jullien PE, Slotkin RK, Kohler C, Martinez G: **The miRNome function transitions from regulating developmental genes to transposable elements during pollen maturation**. *Plant Cell* 2022, **34**:784–801.

13. Plotnikova A, Kellner MJ, Schon MA, Mosiolek M, Nodine MD: **MicroRNA dynamics and functions during Arabidopsis embryogenesis**. *Plant Cell* 2019, **31**:2929–2946.

14. * Jullien PE, Schroder JA, Bonnet DMV, Pumplin N, Voinnet O: **Asymmetric expression of Argonautes in reproductive tissues**. *Plant Physiol* 2022, **188**:38–43.
This publication explored the technically challenging cloning and analysis of the subcellular localization of all AGO proteins in the reproductive tissues of *Arabidopsis thaliana*. This milestone work allowed the field to have a better understanding of the tissue-specific roles of RNA silencing in plants.

15. Borges F, Pereira PA, Slotkin RK, Martienssen RA, Becker JD: **MicroRNA activity in the Arabidopsis male germline**. *J Exp Bot* 2011, **62**:1611–1620.

16. ** Bradamante G, Nguyen VH, Incarbone M, Meir Z, Bente H, Dona M, Lettner N, Mittelsten Scheid O, Gutzat R: **Two ARGONAUTE proteins loaded with transposon-derived small RNAs are associated with the reproductive cell lineage in Arabidopsis**. *Plant Cell* 2023, **36**:863–880.
This outstanding work provides a meticulous description of the tissue-specific role of two AGO proteins (AGO5 and AGO9) that have both non-canonical and canonical functions. The authors describe how both AGO proteins which have tissue-specific expression in the shoot apical meristem and the male gametophyte mediate the RdDM pathway in those tissues, silencing transposable elements.

17. Borges F, Parent JS, van Ex F, Wolff P, Martinez G, Kohler C, Martienssen RA: **Transposon-derived small RNAs triggered by miR845 mediate genome dosage response in Arabidopsis**. *Nat Genet* 2018, **50**:186–192.

18. Creasey KM, Zhai J, Borges F, Van Ex F, Regulski M, Meyers BC, Martienssen RA: **miRNAs trigger widespread epigenetically activated siRNAs from transposons in Arabidopsis**. *Nature* 2014, **508**:411–415.

19. Lee YS, Maple R, Dürr J, Dawson A, Tamim S, del Genio C, Papareddy R, Luo AD, Lamb JC, Amantia S, *et al.*: **A transposon surveillance mechanism that safeguards plant male fertility during stress**. *Nat Plants* 2021, **7**.

20. Cecere G: **Small RNAs in epigenetic inheritance: from mechanisms to trait transmission**. *FEBS Lett* 2021, **595**:2953–2977.

21. Rechavi O, Lev I: **Principles of transgenerational small RNA inheritance in**. *Curr Biol* 2017, **27**:R720–R730.

22. Zhao YS, Wang SY, Wu WY, Li L, Jiang T, Zheng BL: **Clearance of maternal barriers by paternal miR159 to initiate endosperm nuclear division in**. *Nat Commun* 2018, **9**.

23. Yoshikawa M: **Biogenesis of trans-acting siRNAs, endogenous secondary siRNAs in plants**. *Gene Genet Syst* 2013, **88**: 77–84.

24. Felippes FF, Weigel D: **Triggering the formation of tasiRNAs in Arabidopsis thaliana: the role of microRNA miR173**. *EMBO Rep* 2009, **10**:264–270.

25. Olmedo-Monfil V, Durán-Figueroa N, Arteaga-Vázquez M, Demesa-Arévalo E, Autran D, Grimanelli D, Slotkin RK, Martienssen RA, Vielle-Calzada JP: **Control of female gamete formation by a small RNA pathway in**. *Nature* 2010, **464**. 628-U200.

26. Su ZX, Wang NN, Hou ZM, Li BY, Li DN, Liu YH, Cai HY, Qin Y, Chen XM: **Regulation of female germline specification via small RNA mobility in Arabidopsis**. *Plant Cell* 2020, **32**: 2842–2854.

27. Su ZX, Zhao LH, Zhao YY, Li SF, Won S, Cai HY, Wang LL, Li ZF, Chen PJ, Qin Y, *et al.*: **The THO complex non-cell-autonomously represses female germline specification through the TAS3-ARF3 module**. *Curr Biol* 2017, **27**:1597-+.

28. Howell MD, Fahlgren N, Chapman EJ, Cumbie JS, Sullivan CM, Givan SA, Kasschau KD, Carrington JC: **Genome-wide analysis of the RNA-DEPENDENT RNA POLYMERASE6/DICER-LIKE4 pathway in Arabidopsis reveals dependency on miRNA- and tasiRNA-directed targeting**. *Plant Cell* 2007, **19**:926–942.

29. Allen E, Xie Z, Gustafson AM, Carrington JC: **microRNA-directed phasing during trans-acting siRNA biogenesis in plants**. *Cell* 2005, **121**:207–221.

30. Rajagopalan R, Vaucheret H, Trejo J, Bartel DP: **A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana**. *Genes Dev* 2006, **20**:3407–3425.

31. Wu L, Mao L, Qi Y: **Roles of dicer-like and argonaute proteins in TAS-derived small interfering RNA-triggered DNA methylation**. *Plant Physiol* 2012, **160**:990–999.

32. Fei Q, Xia R, Meyers BC: **Phased, secondary, small interfering RNAs in posttranscriptional regulatory networks**. *Plant Cell* 2013, **25**:2400–2415.

33. Johnson C, Kasprzewska A, Tennessen K, Fernandes J, Nan GL, Walbot V, Sundaresan V, Vance V, Bowman LH: **Clusters and superclusters of phased small RNAs in the developing inflorescence of rice**. *Genome Res* 2009, **19**:1429–1440.

34. Zhai J, Zhang H, Arikit S, Huang K, Nan GL, Walbot V, Meyers BC: **Spatiotemporally dynamic, cell-type-dependent premeiotic and meiotic phasiRNAs in maize anthers**. *Proc Natl Acad Sci U S A* 2015, **112**:3146–3151.

35. Jiang P, Lian B, Liu C, Fu Z, Shen Y, Cheng Z, Qi Y: **21-nt phasiRNAs direct target mRNA cleavage in rice male germ cells**. *Nat Commun* 2020, **11**:5191.

36. Pokhrel S, Huang K, Belanger S, Zhan J, Caplan JL, Kramer EM, Meyers BC: **Pre-meiotic 21-nucleotide reproductive phasiRNAs emerged in seed plants and diversified in flowering plants**. *Nat Commun* 2021, **12**:4941.

37. Zhai JX, Jeong DH, De Paoli E, Park S, Rosen BD, Li YP, González AJ, Yan Z, Kitto SL, Grusak MA, *et al.*: **MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs**. *Gene Dev* 2011, **25**:2540–2553.

38. Pokhrel S, Huang K, Meyers BC: **Conserved and non-conserved triggers of 24-nucleotide reproductive phasiRNAs in eudicots**. *Plant J* 2021, **107**:1332–1345.

39. Xia R, Chen C, Pokhrel S, Ma W, Huang K, Patel P, Wang F, Xu J, Liu Z, Li J, *et al.*: **24-nt reproductive phasiRNAs are broadly present in angiosperms**. *Nat Commun* 2019, **10**:627.

40. Zhang M, Ma XX, Wang CY, Li Q, Meyers BC, Springer NM, Walbot V: **CHH DNA methylation increases at 24-PHAS loci depend on 24-nt phased small interfering RNAs in maize meiotic anthers**. *New Phytol* 2021, **229**:2984–2997.

41. Zheng J, Chen C, Li G, Chen P, Liu Y, Xia R: **Biogenesis of reproductive PhasiRNAs: exceptions to the rules**. *Plant Biotechnol J* 2023, **21**:241–243.

42. Martinez G, Slotkin RK: **Developmental relaxation of transposable element silencing in plants: functional or byproduct?** *Curr Opin Plant Biol* 2012, **15**:496–502.

43. Slotkin RK, Vaughn M, Borges F, Tanurdzic M, Becker JD, Feijo JA, Martienssen RA: **Epigenetic reprogramming and small RNA silencing of transposable elements in pollen**. *Cell* 2009, **136**:461–472.

44. He SB, Vickers M, Zhang JY, Feng XQ: **Natural depletion of histone H1 in sex cells causes DNA demethylation, heterochromatin decondensation and transposon activation**. *Elife* 2019, **8**.

45. Calarco JP, Borges F, Donoghue MTA, Van Ex F, Jullien PE, Lopes T, Gardner R, Berger F, Feijó JA, Becker JD, *et al.*: **Reprogramming of DNA methylation in pollen guides epigenetic inheritance via small RNA**. *Cell* 2012, **151**:194–205.

46. Slotkin RK, Freeling M, Lisch D: **Mu killer causes the heritable inactivation of the Mutator family of transposable elements in Zea mays**. *Genetics* 2003, **165**:781–797.

47. Martinez G, Wolff P, Wang Z, Moreno-Romero J, Santos-Gonzalez J, Conze LL, DeFraia C, Slotkin RK, Kohler C: **Paternal easiRNAs regulate parental genome dosage in Arabidopsis**. *Nat Genet* 2018, **50**:193–198.

48. * Kim EY, Wang L, Lei Z, Li H, Fan W, Cho J: **Ribosome stalling and SGS3 phase separation prime the epigenetic silencing of transposons**. *Nat Plants* 2021, **7**:303–309.
This work reveals a translation-dependent PTGS mechanism regulating TEs. mRNA transcripts from the TE *EVADE* are cleaved caused by ribosome stalling, leading to siRNA production derived from RNA fragments.

49. Oberlin S, Rajeswaran R, Trasser M, Barragán-Borrero V, Schon MA, Plotnikova A, Loncsek L, Nodine MD, Marí-Ordóñez A, Voinnet O: **Innate, translation-dependent silencing of an invasive transposon in**. *EMBO Rep* 2022, **23**.

50. Pachamuthu K, Simon M, Borges F: **Targeted suppression of siRNA biogenesis in Arabidopsis pollen promotes triploid seed viability**. *Nat Commun* 2024, **15**.

51. Martínez G, Panda K, Köhler C, Slotkin RK: **Silencing in sperm cells is directed by RNA movement from the surrounding nurse cell**. *Nat Plants* 2016, **2**.

52. ** Long J, Walker J, She W, Aldridge B, Gao H, Deans S, Vickers M, Feng X: **Nurse cell–derived small RNAs define paternal epigenetic inheritance in Arabidopsis**. *Science* 2021, **373**.
This outstanding work described for the first time the production of nurse cell-derived siRNAs (niRNAs), CLSY3-dependent mobile 24-nt siRNAs produced in the tapetum of the male gametophyte that mediate the silencing of TEs and genes. Similar to siRENs, niRNAs are involved in gene regulation through complementary homologous genic loci with less than two mismatches.

53. Lee SC, Ernst E, Berube B, Borges F, Parent JS, Ledon P, Schorn A, Martienssen RA: **Arabidopsis retrotransposon virus-like particles and their regulation by epigenetically activated small RNA**. *Genome Res* 2020, **30**:576–588.

54. Parent JS, Cahn J, Herridge RP, Grimanelli D, Martienssen RA: **Small RNAs guide histone methylation in Arabidopsis embryos**. *Gene Dev* 2021, **35**:841–846.

55. Schon MA, Kellner MJ, Plotnikova A, Hofmann F, Nodine MD: **NanoPARE: parallel analysis of RNA 5' ends from low-input RNA**. *Genome Res* 2018, **28**:1931–1942.

56. Addo-Quaye C, Eshoo TW, Bartel DP, Axtell MJ: **Endogenous siRNA and miRNA targets identified by sequencing of the Arabidopsis degradome**. *Curr Biol* 2008, **18**:758–762.

57. German MA, Pillay M, Jeong DH, Hetawal A, Luo S, Janardhanan P, Kannan V, Rymarquis LA, Nobuta K, German R, *et al.*: **Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends**. *Nat Biotechnol* 2008, **26**: 941–946.

58. Iwakawa H, Tomari Y: **Molecular insights into microRNA-mediated translational repression in plants**. *Mol Cell* 2013, **52**: 591–601.

59. Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O: **Widespread translational inhibition by plant miRNAs and siRNAs**. *Science* 2008, **320**:1185–1190.

60. Li SB, Liu L, Zhuang XH, Yu Y, Liu XG, Cui X, Ji LJ, Pan ZQ, Cao XF, Mo BX, *et al.*: **MicroRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in**. *Cell* 2013, **153**:562–574.

61. Yang L, Wu G, Poethig RS: **Mutations in the GW-repeat protein SUO reveal a developmental function for microRNA-mediated translational repression in Arabidopsis**. *Proc Natl Acad Sci U S A* 2012, **109**:315–320.

62. Lanet E, Delannoy E, Sormani R, Floris M, Brodersen P, Crété P, Voinnet O, Robaglia C: **Biochemical evidence for translational repression by Arabidopsis MicroRNAs**. *Plant Cell* 2009, **21**: 1762–1768.

63. Wang GF, Jiang H, de León GD, Martinez G, Köhler C: **Sequestration of a transposon-derived siRNA by a target mimic imprinted gene induces postzygotic reproductive isolation in Arabidopsis**. *Dev Cell* 2018, **46**:696-+.

64. Grant-Downton R, Kourmpetli S, Hafidh S, Khatab H, Le Trionnaire G, Dickinson H, Twell D: **Artificial microRNAs reveal cell-specific differences in small RNA activity in pollen**. *Curr Biol* 2013, **23**:R599–R601.

65. Jang GJ, Yang JY, Hsieh HL, Wu SH: **Processing bodies control the selective translation for optimal development of Arabidopsis young seedlings**. *Proc Natl Acad Sci U S A* 2019, **116**:6451–6456.

66. Schalk C, Cognat V, Graindorge S, Vincent T, Voinnet O, Molinier J: **Small RNA-mediated repair of UV-induced DNA lesions by the DNA DAMAGE-BINDING PROTEIN 2 and ARGONAUTE 1**. In *Proceedings of the national Academy of Sciences of the United States of America*, **114**; 2017. E2965–E2974.

67. Beauclair L, Yu A, Bouche N: **microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis**. *Plant J* 2010, **62**: 454–462.

68. von Born P, Bernardo-Faura M, Rubio-Somoza I: **An artificial miRNA system reveals that relative contribution of translational inhibition to miRNA-mediated regulation depends on environmental and developmental factors in**. *PLoS One* 2018, **13**.

69. Brosnan CA, Sarazin A, Lim P, Bologna NG, Hirsch-Hoffmann M, Voinnet O: **Genome-scale, single-cell-type resolution of microRNA activities within a whole plant organ**. *EMBO J* 2019, **38**.

70. McCue AD, Nuthikattu S, Reeder SH, Slotkin RK: **Gene expression and stress response mediated by the epigenetic regulation of a transposable element small RNA**. *PLoS Genet* 2012, **8**.

71. Bai B, Peviani A, van der Horst S, Gamm M, Snel B, Bentsink L, Hanson J: **Extensive translational regulation during seed germination revealed by polysomal profiling**. *New Phytol* 2017, **214**:233–244.

72. Honys D, Twell D: **Transcriptome analysis of haploid male gametophyte development**. *Genome Biol* 2004, **5**.

73. ** Zhou M, Coruh C, Xu GH, Martins LM, Bourbousse C, Lambolez A, Law JA: **The CLASSY family controls tissue-specific DNA methylation patterns in Arabidopsis**. *Nat Commun* 2022, **13**.

This work elucidates the distinct DNA methylation profiles observed in different tissues, which are linked to the tissue-specific expression patterns of CLASSY (CLSY) family proteins. Additionally, the study also reveals the locus-specific methylation patterns in ovules, mediated by the ovule-enriched CLSY3 and CLSY4, demonstrating a mechanism of tissue- and locus-specific DNA methylation regulation in plants.

74. Zhou M, Palanca AMS, Law JA: **Locus-specific control of the de novo DNA methylation pathway in Arabidopsis by the CLASSY family**. *Nat Genet* 2018, **50**:865–873.

75. Law JA, Vashisht AA, Wohlschlegel JA, Jacobsen SE: **SHH1, a homeodomain protein required for DNA methylation, as well as RDR2, RDM4, and chromatin remodeling factors, associate with RNA polymerase IV**. *PLoS Genet* 2011, **7**.

76. Ream TS, Haag JR, Wierzbicki AT, Nicora CD, Norbeck AD, Zhu JK, Hagen G, Guilfoyle TJ, Pasa-Tolic L, Pikaard CS: **Subunit compositions of the RNA-silencing enzymes Pol IV and Pol V reveal their origins as specialized forms of RNA polymerase II**. *Mol Cell* 2009, **33**:192–203.

77. ** Grover JW, Burgess D, Kendall T, Baten A, Pokhrel S, King GJ, Meyers BC, Freeling M, Mosher RA: **Abundant expression of maternal siRNAs is a conserved feature of seed development**. In *Proceedings of the national Academy of Sciences of the United States of America*, **117**; 2020:15305–15315.

78. ** Burgess D, Chow HT, Grover JW, Freeling M, Mosher RA: **Ovule siRNAs methylate protein-coding genes in trans**. *Plant Cell* 2022, **34**:3647–3664.

These two works described for the first time the production of siRNAs in the endosperm (siRENs) of multiple Brassicaceae species. These siRNAs are mainly involved in gene regulation through complementary homologous genic loci with less than two mismatches.

79. Fei Y, Nyikó T, Molnar A: **Non-perfectly matching small RNAs can induce stable and heritable epigenetic modifications and can be used as molecular markers to trace the origin and fate of silencing RNAs**. *Nucleic Acids Res* 2021, **49**:1900–1913.

80. Wu L, Zhou H, Zhang Q, Zhang J, Ni F, Liu C, Qi Y: **DNA methylation mediated by a microRNA pathway**. *Mol Cell* 2010, **38**:465–475.

81. Slotkin RK, Freeling M, Lisch D: **Heritable transposon silencing initiated by a naturally occurring transposon inverted duplication**. *Nat Genet* 2005, **37**:641–644.

82. Gutzat R, Rembart K, Nussbaumer T, Hofmann F, Pisupati R, Bradamante G, Daubel N, Gaidora A, Lettner N, Donà M, *et al.*: **Shoot stem cells display dynamic transcription and DNA methylation patterns**. *EMBO J* 2020, **39**.

83. Duan CG, Zhang H, Tang K, Zhu X, Qian W, Hou YJ, Wang B, Lang Z, Zhao Y, Wang X, *et al.*: **Specific but interdependent functions for Arabidopsis AGO4 and AGO6 in RNA-directed DNA methylation**. *EMBO J* 2015, **34**:581–592.

84. McCue AD, Panda K, Nuthikattu S, Choudury SG, Thomas EN, Slotkin RK: **ARGONAUTE 6 bridges transposable element mRNA-derived siRNAs to the establishment of DNA methylation**. *EMBO J* 2015, **34**:20–35.

85. Sigman MJ, Panda K, Kirchner R, McLain LL, Payne H, Peasari JR, Husbands AY, Slotkin RK, McCue AD: **An siRNA-guided ARGONAUTE protein directs RNA polymerase V to initiate DNA methylation**. *Nat Plants* 2021, **7**: 1461–1474.

86. * Wu WY, Li L, Zhao Y, Zhao YS, Jiang T, McCormick S, Zheng BL: **Heterochromatic silencing is reinforced by ARID1-mediated small RNA movement in Arabidopsis pollen**. *New Phytol* 2021, **229**:3269–3280.

This work provided another example of the cell-specific versatility of the RNA silencing machinery. Here, the authors describe a sperm cell-specific transcription factor (ARID1) that interacts with AGO9 and is key to mediating its role in the silencing of heterochromatin in that cell type. These results suggest that other factors, outside of the known members of the RdDM pathway, can influence tissue-specific RdDM activity.

87. Cao S, Wang LF, Han TW, Ye WX, Liu Y, Sun Y, Moose SP, Song QX, Chen ZJ: **Small RNAs mediate transgenerational inheritance of genome-wide-acting epialleles in maize**. *Genome Biol* 2022, **23**.

88. Devers EA, Brosnan CA, Sarazin A, Albertini D, Amsler AC, Brioudes F, Jullien PE, Lim P, Schott G, Voinnet O: **Movement and differential consumption of short interfering RNA duplexes underlie mobile RNA interference**. *Nat Plants* 2020, **6**: 789–799.

89. Herridge RP, Dolata J, Migliori V, de Santis Alves C, Borges F, Schorn AJ, Van Ex F, Parent JS, Lin A, Bajczyk M, *et al.*: **Pseudouridine guides germline small RNA transport and epigenetic inheritance**. *bioRxiv* 2023.

90. Martinez G: **tRNA-derived small RNAs: new players in genome protection against retrotransposons**. *RNA Biol* 2018, **15**: 170–175.

91. Chery M, Drouard L: **Plant tRNA functions beyond their major role in translation**. *J Exp Bot* 2022, **74**: 2352–2363.

92. Panstruga R, Spanu P: **Transfer RNA and ribosomal RNA fragments – emerging players in plant–microbe interactions**. *New Phytol* 2024, **241**:567–577.

93. Lalande S, Merret R, Salinas-Giegé T, Drouard L: **Arabidopsis tRNA-derived fragments as potential modulators of translation**. *RNA Biol* 2020, **17**:1137–1148.