RNA-directed DNA Methylation and sexual reproduction: expanding beyond the seed

Hiu Tung Chow*, Tania Chakraborty*, and Rebecca A. Mosher

The School of Plant Sciences, The University of Arizona, Tucson, AZ 85721

* These authors contributed equally

Corresponding author

Rebecca A. Mosher

rmosher@email.arizona.edu

Box 210036

The University of Arizona

Tucson, AZ 85721-0036

Keywords

RNA-directed DNA Methylation, siRNA, Pol IV, Pol V, seed development, epigenetics, parental bias

Highlights

- We have an increasing understanding of DNA methylation dynamics during reproduction, but the exact role for RdDM in these dynamics is unclear
- New phenotypes indicate that RdDM is involved in seed development, perhaps by mediating parental dosage balance
- New model systems, including non-flowering plants, will allow a better understanding of the ancestral roles of RdDM

Abstract

Two trends are changing our understanding of RNA-directed DNA methylation. In model systems like Arabidopsis, tissue-specific analysis of DNA methylation is uncovering dynamic changes in methylation during sexual reproduction and unraveling the contribution of maternal and paternal epigenomes to the developing embryo. These studies indicate that RNA-directed DNA Methylation might be important for mediating balance between maternal and paternal contributions to the endosperm. At the same time, researchers are moving beyond Arabidopsis to illuminate the ancestral role of RdDM in non-flowering plants that lack an endosperm, suggesting that RdDM might play a broader role in sexual reproduction.

Introduction

In plants *de novo* DNA methylation is performed through RNA-directed DNA Methylation (RdDM) [1]. Unlike symmetric methylation, which is maintained through the recognition of hemi-methylated sites following DNA replication, CHH methylation (where H=A, T, or C) must be placed on unmethylated templates following each round of replication [2]. In constitutive heterochromatin CHH methylation is maintained by CHROMOMETHYLTRANSFERASE2, which recognizes heterochromatic marks such as H3K9me2 [3]. In euchromatin, or at the boundaries between hetero- and euchromatin, CHH methylation is instead placed by RdDM [4,5].

RdDM begins when RNA Polymerase (Pol) IV and RNA-dependent RNA Polymerase 2 (RDR2) generate short double-stranded transcripts that are then trimmed to 24 nt in length by DICER-LIKE3 (DCL3). The resulting siRNAs are termed p4-siRNAs due to their initiation by Pol IV. P4-siRNAs bind to ARGONAUTE4 (AGO4) or one of its close paralogs and seek out homologous loci transcribed by RNA Pol V. Finally, AGO4 recruits DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) to catalyze CHH methylation at these loci. In addition to this canonical RdDM pathway, several variations on RdDM operate in specific circumstances [6].

There have recently been great advances in the biochemistry of RdDM, including debate about whether the AGO4/p4-siRNA complex binds to DNA or nascent RNA [7,8], analysis of the catalytic function of Pol IV and Pol V [9,10], and analysis of the non-catalytic carboxy-terminal domains of these polymerases [11-13]. Unfortunately, space limitations preclude the discussion of this biochemical work. We will instead focus on the role of RdDM during seed development, and how an evolutionary perspective might help us understand its function.

Reproductive development is characterized by dynamic DNA methylation

Whole genome bisulfite sequencing of isolated cell types has recently provided a detailed look at changes in methylation levels during sexual reproduction [14-22]. This analysis has been easiest for the microspore and male gametophyte due to relatively easier isolation of homogenous cell populations, but information about the megaspore and female gametophyte are also increasing. However, DNA methylation offers only an indirect assessment of RdDM activity. Assessment of sexual lineage methylation in RdDM mutants is needed to better understand RdDM's precise role in these changes.

In the microspore mother cell (the diploid cell that will eventually undergo meiosis to create the haploid microspore) CHH methylation is reduced, suggesting reduced activity of RdDM [18]. Despite this overall reduction, RdDM appears at novel genic locations where it influences gene expression and impacts microsporogenesis [18]. A transient decrease in CHH methylation has also been observed in the megaspore mother cell [23], and genetic evidence indicates RdDM might also influence megasporogenesis [24]. Whether RdDM has a particular role in these tissues, or whether it has been co-opted to create cell-type specific expression patterns in a variety of specialized cells is an open question.

Following meiosis the haploid microspore develops into the 3-celled male gametophyte which contains two sperm cells encased in the vegetative cell. The vegetative nucleus is actively demethylated and loci with reduced CG methylation in the vegetative nucleus display increased CHH methylation in the sperm cells [14,15,17]. This pattern suggests p4-siRNAs might be produced from demethylated regions in the vegetative nucleus and move to the sperm cells to induce methylation. Transposon siRNAs produced in the vegetative nucleus can move into sperm cells and silence reporters expressed there [25], but it is not clear whether these siRNAs are canonical p4-siRNAs or other types of small RNAs [22,25,26]. CHH methylation is not

elevated overall in sperm cells [15,17,20,22], so either movement of p4-siRNAs is not widespread, mobile siRNAs cause predominantly post-transcriptional gene silencing, or something restricts the activity of mobile p4-siRNAs in the sperm cells [21]. Alternatively, CHH methylation in the sperm cells and demethylation of the vegetative nucleus might occur at the same locations due to parallel action of autonomous pathways.

Like the vegetative nucleus in the male gametophyte, the central cell of the female gametophyte is also actively demethylated [17], and could produce siRNAs that function in the egg cell. Although such movement has not been directly observed, injection of fluorescent reporters into central cells indicates that siRNA movement to the egg cell is possible [27]. Similarly, fluorescent reporters indicate that methylation in the egg cell requires *NRPE1* and *DRM2*, but not *NRPD1*, suggesting that p4-siRNA reception, but not production of p4-siRNAs, is required for egg cell methylation [23]. Alternatively, methylation in the egg cell might rely on non-canonical RDR6-RdDM, which utilizes Pol V but not Pol IV [6].

RdDM is highly active during embryogenesis

Following fertilization CHH methylation increases in both the endosperm and the young embryo [15,17,23,28], indicating that RdDM is occurring. CHH methylation continues to rise during embryogenesis, reaching levels in the mature embryo that are higher than almost any other tissue tested [29-33]. The sites with highest methylation match regions that are demethylated in the endosperm [31], suggesting either that demethylation in one tissue triggers p4-siRNA production and movement into an adjacent tissue, or that demethylation and methylation pathways are targeted to the same loci in endosperm and embryo, respectively. The level of CHH methylation immediately declines upon imbibition and germination, indicating that elevated methylation might be designed to achieve transcriptional quiescence for dormancy [29-31]. Indeed, demethylation at a number of promoters might facilitate upregulation of the germination

program [30]. Conversely, mutants that maintain the embryogenic program following germination also maintain high CHH methylation [31], indicating that germination can occur despite elevated methylation, and suggesting that hyperactive RdDM is an inherent part of the embryogenesis program. However, Arabidopsis mutants lacking RdDM have only subtle seed phenotypes, indicating that hyperactive RdDM is not required for embryogenesis in all species [34].

An important outstanding question is “which tissues generate the p4-siRNAs directing embryo RdDM?” Although most research has focused on the idea that p4-siRNAs might enter the embryo either with the sperm nucleus or from the endosperm, it is also possible that maternal sporophytic tissue could be a source of siRNAs. RdDM in the sporophyte is required for seed development in *Brassica rapa* [34], and many p4-siRNA are produced in seed coat [35]. DNA methylation patterns are also dynamic during fruit development [36-38], suggesting that these seed-adjacent tissues might influence the embryo epigenome. Alternatively, RdDM in the embryo might be entirely autonomous (Figure 1). Indeed, soybean embryogenic tissue culture shows hypermethylation of CHH sites despite a lack of interaction with maternal somatic tissue [39].

RdDM might influence parental balance in developing endosperm

Several studies suggest that RdDM’s largest impact on seed development occurs in the endosperm, particularly in cases of parental dosage imbalance [22,26,35,40-42]. Because the endosperm develops from the fertilization of the 2n central cell by a 1n sperm cell, endosperm is triploid with a 2:1 maternal:paternal ratio. Distortion of this ratio, by altering either the actual ploidy or the effective ploidy of the parents, results in changes to endosperm developmental timing (Figure 2). Paternal excess results in a prolonged phase of endosperm proliferation and produces larger seeds with a high abortion rate, due in part to overexpression of AGAMOUS-

LIKE transcription factors [35,40,43,44]. Loss of maternal RdDM also causes AGAMOUS-LIKE overexpression, suggesting that maternally-expressed p4-siRNAs might contribute to maternal effective ploidy [35,40]. In balanced (2:1 maternal:paternal) but RdDM-deficient endosperm there is slight bias toward maternal expression generally, suggesting that RdDM might repress maternal dosage [41]. However, recent work suggests endosperm has a "buffering" system that increases maternal expression and decreases paternal expression during paternal excess [42], raising the possibility that the maternal transcriptional shift in *nrpd1* endosperm is due to the perception of a slight paternal excess due to reduction in maternal effective ploidy.

If maternally-derived p4-siRNAs increase maternal effective ploidy, what influences paternal effective ploidy? Loss of *NRPD1* in pollen represses seed abortion in paternal excess crosses [22,26,41,42], suggesting that Pol IV might promote paternal effective ploidy. Surprisingly, there are few 24-nt p4-siRNAs in pollen; instead *NRPD1* is required for 21/22-nt siRNA production from transposons [22,26]. These siRNAs might be carried by the sperm cell to the central cell during fertilization to balance the maternal 24-nt p4-siRNAs [26]. However, allele-specific transcriptome data indicates that loss of paternal 21/22-nt siRNAs has little impact on the maternal expression bias observed in paternal excess endosperm [42], suggesting that these pollen siRNAs impact seed abortion without changing the ratio of effective parental ploidies.

RdDM mediates trans-chromosomal methylation

A fundamental aspect of sexual reproduction is the combining of different haploid genomes. Recently, we are learning that epigenetically-distinct alleles can influence each other after fertilization in a process called Trans-Chromosomal Methylation (TCM) [45]. Hybridization between different genotypes or between genetically identical but epigenetically distinct parents (epi-hybrids) results in gain or loss of methylation, especially at loci that epigenetically vary between parents [46-54]. P4-siRNAs are required to establish TCM in F1 *Arabidopsis*

intraspecific hybrids, suggesting that p4-siRNAs produced from one allele act *in trans* at the homologous allele [50,52]. Indeed, production of siRNAs at a non-allelic site is sufficient to trigger heritable methylation in tomato [54], indicating that chromosome pairing is not required. Loss of methylation following hybridization (Trans-Chromosomal demethylation, TCdM) suggests that there might be a critical p4-siRNA dosage needed to sustain methylation [50]. Interestingly, loci exhibiting TCdM have higher genetic variation, supporting the model that p4-siRNAs from one allele function *in trans*, but only at perfectly complementary sites [52]. Addition of a similar but distinct locus must be sufficient to dilute *cis*-acting p4-siRNAs and trigger loss of methylation.

In light of the discussion of RdDM during gametogenesis and early embryogenesis, it is interesting to note that there is no evidence for parental bias in TCM [47,48], suggesting that methylation is transferred at a point when both parental genomes contribute equally to p4-siRNA production. The identification of TCM in F1 leaves indicates it occurs during embryogenesis or early vegetative development [46-48,50-53], although there is also evidence that TCM at the H06 locus in tomato occurs during reproductive development [54].

RdDM pre-dates the evolution of flowering plants

The emphasis on seed and endosperm development in RdDM research might lead one to believe that RdDM is an angiosperm-specific pathway. In fact, most subunits of Pol IV and Pol V exist in all land plants, including bryophytes like *Marchantia polymorpha* and *Physcomitrella patens* [55-57] (discussed further below). Small RNA sequencing also indicates that 24-nt siRNAs are present throughout land plants, including gymnosperms [58-60], ferns [61], lycophytes [62], and bryophytes [56,63,64], although their accumulation is substantially lower than in angiosperms. In gymnosperms and bryophytes, 24-nt siRNAs are associated with CHH methylation at transposons and other repetitive DNA, suggesting that the molecular role of the

pathway is largely conserved [56,58,63,65]. In *P. patens*, genetic analysis of *nrpd1*, *nrpe1*, *rdr2*, and *dcl3* mutants demonstrated that 23/24-nt siRNAs are produced in a manner analogous to angiosperms and should be considered canonical p4-siRNAs [56]. In addition, *P. patens dcl3* mutants lose CHH methylation from p4-siRNA producing loci, while maintaining CG and CHG methylation at the same sites [63], further indicating that the basic mechanism of RdDM predates the radiation of land plants.

24-nt siRNAs are found primarily in reproductive tissues in gymnosperms, suggesting that RdDM has a role in seed development outside of double fertilization [58,59]. Reproduction is fundamentally different in bryophytes, which have a haploid-dominant life cycle: the zygote has only a short lifespan before meiosis, and the gamete grows vegetatively before differentiation of the reproductive structures [66]. Nevertheless, 24-nt siRNAs are more abundant in *M. polymorpha* reproductive structures [64], indicating that RdDM might have an ancient role in sexual reproduction. Consistent with this, CHH methylation increases during sexual reproduction in *M. polymorpha*, with high levels in the male and female reproductive structures [67]. CHH methylation also increases during maturation of the sporophyte, which mirrors the increase in CHH methylation during Arabidopsis embryogenesis [67]. RdDM might have roles outside of reproduction as well, as *P. patens nrpd1*, *rdr2*, *dcl3*, and *nrpe1* mutants have altered gametophyte growth and development [56,63].

Duplication of RdDM components leads to elaboration of RdDM-like pathways

Although the RdDM pathway appears to have an ancient role in *de novo* methylation of transposons, particularly during sexual reproduction, the earliest land plants did not have the same RdDM machinery as angiosperms. Bryophytes contain dedicated *NRPD1*, *NRPE1*, and *NRP(D/E)7*, but do not encode for *NRP(D/E)4* or *NRPE5*, which first arose in angiosperms and seed plants, respectively [55,57]. Non-seed plants also do not have a dedicated *RDR2*, but

rather have *RDR1/2*, which is equally related to *RDR1* and *RDR2* [55]. Whether the *RDR1* function of viral siRNA evolved later, or the *RDR1/2* in non-seed plants has dual functions is unknown. *DCL3* and the *AGO4* clade both existed in the earliest land plants [55].

There are a number of lineage-specific duplications of RdDM components, but rarely are these paralogs conserved outside of close relatives. One exception is duplication of *DCL3* to create *DCL3* and *DCL5* in monocots [68,69]. Both enzymes produce 24-nt siRNAs from dsRNA precursors; DCL3 dices Pol IV/RDR2 products while DCL5 dices longer dsRNA initiated by Pol II [69,70]. Duplication of *DCL3* appears to be a case of subfunctionalization, as the 24-nt phasiRNAs it generates are found in dicots, which lack *DCL5* [71]. Whether these 24-nt phasiRNAs target *de novo* methylation to homologous sites, and thereby perform a type of non-canonical RdDM, is unknown, however production of 24-nt phasiRNAs is required for complete male fertility in maize [72].

Other interesting duplications of RdDM machinery are *NRPE1* and *NRP(D/E)2* in the grass family [73]. These duplications are conserved in nearly all grass species and show signs of neofunctionalization, suggesting the formation of a sixth DNA-dependent RNA Polymerase [73]. It will be interesting to discover whether this putative Pol VI has a function within canonical RdDM or has evolved for a novel function like *DCL5*.

Conclusions

Like much plant molecular biology, RdDM research is moving into a new phase of exploring non-model species. Recently-described RdDM mutants in tomato [74,75], rice [76-78], and *Brassica rapa* [34] demonstrate that the RdDM pathway is essential for reproductive success. Understanding the function of RdDM in additional species – especially those with haploid-dominant lifecycles – will not only help us determine the developmental significance of RdDM,

but also uncover variations and related pathways.

Figures

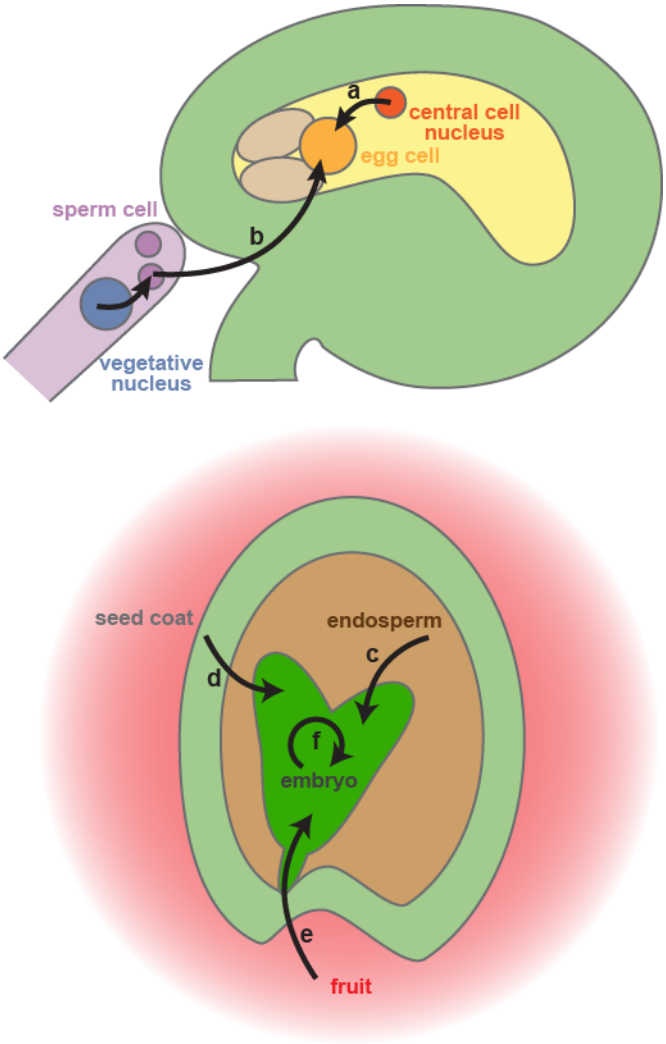

Figure 1. Routes for siRNA programming of RdDM during embryogenesis. siRNAs might enter the egg cell from the central cell before fertilization (a), or be delivered with the sperm cell during fertilization (b) to initiate RdDM during embryogenesis. Alternatively, programming of embryogenic RdDM might be ongoing through movement of siRNAs from the endosperm (c), seed coat (d), or other maternal tissues such as fruit (e). RdDM in the embryo might also be entirely autonomous and directed by siRNAs produced in the embryo (f).

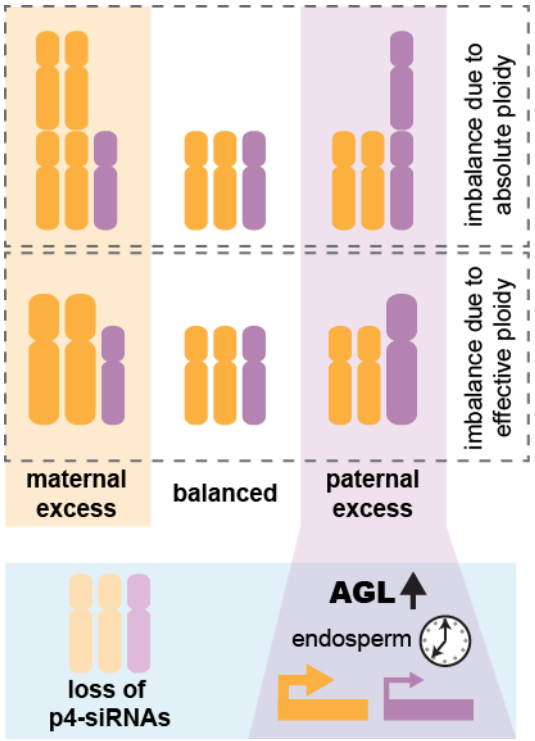

Figure 2. Endosperm development is determined by the ratio of maternal:paternal effective ploidy. Endosperm is unbalanced when the maternal:paternal ratio deviates from 2:1. Maternal excess (orange box) can arise due to excess chromosomes (absolute ploidy) or due to increase maternal effective ploidy. Similarly, paternal excess (purple box) can be due to changes in absolute or effective ploidy. The factors influencing effective ploidy are unknown, but crosses that lack p4-siRNAs mimic several features of paternal excess crosses, including increase expression of AGAMOUS-LIKE transcriptional factors, delayed endosperm cellularization, and maternal bias of many transcripts.

Acknowledgements

We apologize to our colleagues whose research could not be included due to space constraints. The Mosher lab is grateful that this work was supported by the National Science Foundation (IOS-1546825 and MCB-1929678).

Highlighted References

Bouyer:

•• Together with [29] and [30], this paper describes the elevated methylation in mature embryos and highlights the connection between early endosperm epigenomic changes and elevated embryo methylation.

Erdmann:

•• Together with [26] and [42], this paper demonstrates that tetraploid *nrpd1* fathers do not trigger paternal excess lethality like wild-type tetraploid fathers. The manuscript also reports a maternal bias in endosperm transcription following loss of RdDM.

Grover:

• This research describes seed development phenotypes associated with loss of RdDM in *Brassica rapa*, highlighting the importance of diverse experimental systems to understand RdDM.

Ingouff:

• The authors develop novel methylation-binding fluorescent reporters to study context-specific methylation patterns during early reproductive development.

Kawakatsu:

• Together with [31] and [30], this manuscript uncovers elevated methylation in mature embryos, which is dramatically lost following germination.

Martinez:

• Together with [41], and [42] this paper reports that seed lethality due to paternal genome excess can be overcome by loss of paternal NRPD1. The authors propose that Pol IV produces 21/22-nt siRNAs in pollen that counteract Pol IV dependent-siRNAs in endosperm.

Narsai:

• Together with [31] and [29], this paper demonstrates the extensive demethylation that occurs during germination.

Satyaki:

• Together with [41] and [26], this paper defines the machinery necessary for lethality in paternal excess crosses. It also demonstrates that the levels of mRNA and DNA methylation in endosperm do not substantially changed in lethal paternal excess crosses compared to viable paternal excess crosses, and that paternal excess endosperm has a maternal transcriptional bias.

Teng:

•• This paper describes the phenotype of a *dcl5* mutant in maize, highlighting the functional diversification that follows duplication of RdDM components and demonstrating the importance of studying small RNA pathways in multiple species.

References

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

2. Law JA, Jacobsen SE: **Establishing, maintaining and modifying DNA methylation patterns in plants and animals.** *Nat Rev Genet* 2010, **11**:204–220.

3. Zemach A, Kim MY, Hsieh P-H, Coleman-Derr D, Eshed-Williams L, Thao K, Harmer SL, Zilberman D: **The Arabidopsis nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin.** *Cell* 2013, **153**:193–205.

4. Li Q, Gent JI, Zynda G, Song J, Makarevitch I, Hirsch CD, Hirsch CN, Dawe RK, Madzima TF, McGinnis KM, et al.: **RNA-directed DNA methylation enforces boundaries between heterochromatin and euchromatin in the maize genome**. *Proc Natl Acad Sci USA* 2015, **112**:14728–14733.

5. Schoft VK, Chumak N, Mosiolek M, Slusarz L, Komnenovic V, Brownfield L, Twell D, Kakutani T, Tamaru H: **Induction of RNA-directed DNA methylation upon decondensation of constitutive heterochromatin**. *EMBO reports* 2009, **10**:1015–1021.

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

7. Lahmy S, Pontier D, Bies-Etheve N, Laudié M, Feng S, Jobet E, Hale CJ, Cooke R, Hakimi M-A, Angelov D, et al.: **Evidence for ARGONAUTE4-DNA interactions in RNA-directed DNA methylation in plants.** *Genes Dev* 2016, **30**:2565–2570.

8. Liu W, Duttke SH, Hetzel J, Groth M, Feng S, Gallego-Bartolome J, Zhong Z, Kuo HY, Wang Z, Zhai J, et al.: **RNA-directed DNA methylation involves co-transcriptional small-RNA-guided slicing of polymerase V transcripts in Arabidopsis.** *Nat Plants* 2018, **4**:1–188.

9. Marasco M, Li W, Lynch M, Pikaard CS: **Catalytic properties of RNA polymerases IV and V: accuracy, nucleotide incorporation and rNTP/dNTP discrimination.** *Nucleic Acids Res* 2017, **45**:11315–11326.

10. Singh J, Mishra V, Wang F, Huang H-Y, Pikaard CS: **Reaction mechanisms of Pol IV, RDR2 and DCL3 drive RNA channeling in the siRNA-directed DNA methylation pathway**. *bioRxiv* 2019, **45**:11315–40.

11. Trujillo JT, Beilstein MA, Mosher RA: **The Argonaute-binding platform of NRPE1 evolves through modulation of intrinsically disordered repeats.** *New Phytol* 2016, **212**:1094–1105.

12. Wendte JM, Haag JR, Singh J, McKinlay A, Pontes OM, Pikaard CS: **Functional Dissection of the Pol V Largest Subunit CTD in RNA-Directed DNA Methylation.** *Cell Rep* 2017, **19**:2796–2808.

13. Wendte JM, Haag JR, Pontes OM, Singh J, Metcalf S, Pikaard CS: **The Pol IV largest subunit CTD quantitatively affects siRNA levels guiding RNA-directed DNA methylation.** *Nucleic Acids Res* 2019, doi:10.1093/nar/gkz615.

14. Ibarra CA, Feng X, Schoft VK, Hsieh T-F, Uzawa R, Rodrigues JA, Zemach A, Chumak N, Machlicova A, Nishimura T, et al.: **Active DNA demethylation in plant companion cells reinforces transposon methylation in gametes**. *Science* 2012, **337**:1360–1364.

15. Calarco JP, Borges F, Donoghue MTA, Van Ex F, Jullien PE, Lopes T, Gardner R, Berger F, Feijo JA, Becker JD, et al.: **Reprogramming of DNA Methylation in Pollen Guides Epigenetic Inheritance via Small RNA.** *Cell* 2012, **151**:194–205.

16. Hsieh P-H, He S, Buttress T, Gao H, Couchman M, Fischer RL, Zilberman D, Feng X: **Arabidopsis male sexual lineage exhibits more robust maintenance of CG methylation than somatic tissues.** *Proc Natl Acad Sci USA* 2016, **113**:15132–15137.

17. Park K, Kim MY, Vickers M, Park J-S, Hyun Y, Okamoto T, Zilberman D, Fischer RL, Feng X, Choi Y, et al.: **DNA demethylation is initiated in the central cells of Arabidopsis and rice**. *Proc Natl Acad Sci USA* 2016, **113**:15138–15143.

18. Walker J, Gao H, Zhang J, Aldridge B, Vickers M, Higgins JD, Feng X: **Sexual-lineage-specific DNA methylation regulates meiosis in Arabidopsis.** *Nat Genet* 2018, **50**:130–137.

19. Sprunck S, Urban M, Strieder N, Lindemeier M, Bleckmann A, Evers M, Hackenberg T, Möhle C, Dresselhaus T, Engelmann JC: **Elucidating small RNA pathways in Arabidopsis thaliana egg cells**. *bioRxiv* 2019, doi:10.1101/525956.

20. Kim MY, Ono A, Scholten S, Kinoshita T, Zilberman D, Okamoto T, Fischer RL: **DNA demethylation by ROS1a in rice vegetative cells promotes methylation in sperm.** *Proc Natl Acad Sci USA* 2019, **116**:9652–9657.

21. Li C, Xu H, Fu F-F, Russell SD, Sundaresan V, Gent JI: **Reprogramming of 24nt siRNAs in rice gametes**. *bioRxiv* 2019, **43**:349–51.

22. Borges F, Parent J-S, Van Ex F, Wolff P, Martínez G, Köhler C, Martienssen RA: **Transposon-derived small RNAs triggered by miR845 mediate genome dosage response in Arabidopsis.** *Nat Genet* 2018, **109**:14746–192.

23. Ingouff M, Selles B, Michaud C, Vu TM, Berger F, Schorn AJ, Autran D, Van Durme M, Nowack MK, Martienssen RA, et al.: **Live-cell analysis of DNA methylation during sexual reproduction in Arabidopsis reveals context and sex-specific dynamics controlled by noncanonical RdDM**. *Genes Dev* 2017, **31**:72–83.

24. 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 Arabidopsis**. *Nature* 2010, **464**:628–U200.

25. 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**:16030.

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

27. Erdmann RM, Hoffmann A, Walter H-K, Wagenknecht H-A, Groß-Hardt R, Gehring M: **Molecular movement in the Arabidopsis thaliana female gametophyte.** *Plant Reprod* 2017, **30**:141–146.

28. Moreno-Romero J, Jiang H, Santos-González J, Köhler C: **Parental epigenetic asymmetry of PRC2-mediated histone modifications in the Arabidopsis endosperm.** *The EMBO Journal* 2016, **35**:1298–1311.

29. Kawakatsu T, Nery JR, Castanon R, Ecker JR: **Dynamic DNA methylation reconfiguration during seed development and germination.** *Genome Biol* 2017, **18**:171.

30. Narsai R, Gouil Q, Secco D, Srivastava A, Karpievitch YV, Liew LC, Lister R, Lewsey MG, Whelan J: **Extensive transcriptomic and epigenomic remodelling occurs during Arabidopsis thaliana germination.** *Genome Biol* 2017, **18**:172.

31. Bouyer D, Kramdi A, Kassam M, Heese M, Schnittger A, Roudier F, Colot V: **DNA methylation dynamics during early plant life.** *Genome Biol* 2017, **18**:179.

32. An Y-QC, Goettel W, Han Q, Bartels A, Liu Z, Xiao W: **Dynamic Changes of Genome-Wide DNA Methylation during Soybean Seed Development.** *Sci Rep* 2017, **7**:12263.

33. Lin J-Y, Le BH, Chen M, Henry KF, Hur J, Hsieh T-F, Chen P-Y, Pelletier JM, Pellegrini M, Fischer RL, et al.: **Similarity between soybean and Arabidopsis seed methylomes and loss of non-CG methylation does not affect seed development.** *Proc Natl Acad Sci USA* 2017, **114**:E9730–E9739.

34. Grover JW, Kendall T, Baten A, Burgess D, Freeling M, King GJ, Mosher RA: **Maternal components of RNA-directed DNA methylation are required for seed development in Brassica rapa.** *Plant J* 2018, **94**:575–582.

35. Kirkbride RC, Lu J, Zhang C, Mosher RA, Baulcombe DC, Chen J: **Maternal small RNAs mediate spatial-temporal regulation of gene expression, imprinting, and seed development in Arabidopsis.** *Proc Natl Acad Sci USA* 2019, **116**:2761–2766.

36. Gallusci P, Hodgman C, Teyssier E, Seymour GB: **DNA Methylation and Chromatin Regulation during Fleshy Fruit Development and Ripening.** *Frontiers in Plant Science* 2016, **7**:807.

37. Cheng J, Niu Q, Zhang B, Chen K, Yang R, Zhu J-K, Zhang Y, Lang Z: **Downregulation of RdDM during strawberry fruit ripening.** *Genome Biol* 2018, **19**:212.

38. Huang H, Liu R, Niu Q, Tang K, Zhang B, Zhang H, Chen K, Zhu J-K, Lang Z: **Global increase in DNA methylation during orange fruit development and ripening.** *Proc Natl Acad Sci USA* 2019, **116**:1430–1436.

39. Ji L, Mathioni SM, Johnson S, Tucker D, Bewick AJ, Do Kim K, Daron J, Slotkin RK, Jackson SA, Parrott WA, et al.: **Genome-Wide Reinforcement of DNA Methylation Occurs during Somatic Embryogenesis in Soybean.** *Plant Cell* 2019, **31**:2315–2331.

40. Lu J, Zhang C, Baulcombe DC, Chen J: **Maternal siRNAs as regulators of parental genome imbalance and gene expression in endosperm of Arabidopsis seeds**. *Proc Natl Acad Sci USA* 2012, **109**:5529–5534.

41. Erdmann RM, Satyaki PRV, Klosinska M, Gehring M: **A Small RNA Pathway Mediates Allelic Dosage in Endosperm.** *Cell Rep* 2017, **21**:3364–3372.

42. Satyaki PRV, Gehring M: **Paternally Acting Canonical RNA-Directed DNA Methylation Pathway Genes Sensitize Arabidopsis Endosperm to Paternal Genome Dosage.** *Plant Cell* 2019, **31**:1563–1578.

43. Walia H, Josefsson C, Dilkes B, Kirkbride R, Harada J, Comai L: **Dosage-Dependent Deregulation of an AGAMOUS-LIKE Gene Cluster Contributes to Interspecific Incompatibility**. *Current Biology* 2009, **19**:1128–1132.

44. Roth M, Florez-Rueda AM, Städler T: **Differences in Effective Ploidy Drive Genome-Wide Endosperm Expression Polarization and Seed Failure in Wild Tomato Hybrids.** *Genetics* 2019, **212**:141–152.

45. Greaves I, Groszmann M, Dennis ES, Peacock WJ: **Trans-chromosomal methylation**. *Epigenetics* 2012, **7**:800–805.

46. Groszmann M, Greaves IK, Albertyn ZI, Scofield GN, Peacock WJ, Dennis ES: **Changes in 24-nt siRNA levels in Arabidopsis hybrids suggest an epigenetic contribution to hybrid vigor.** *Proc Natl Acad Sci USA* 2011, **108**:2617–2622.

47. Greaves IK, Groszmann M, Ying H, Taylor JM, Peacock WJ, Dennis ES: **Trans chromosomal methylation in Arabidopsis hybrids.** *Proc Natl Acad Sci USA* 2012, **109**:3570–3575.

48. Shen H, He H, Li J, Chen W, Wang X, Guo L, Peng Z, He G, Zhong S, Qi Y, et al.: **Genome-wide analysis of DNA methylation and gene expression changes in two Arabidopsis ecotypes and their reciprocal hybrids.** *Plant Cell* 2012, **24**:875–892.

49. Shivaprasad PV, Dunn RM, Santos BACM, Bassett A, Baulcombe DC: **Extraordinary transgressive phenotypes of hybrid tomato are influenced by epigenetics and small silencing RNAs**. *Embo J* 2012, **31**:257–266.

50. Greaves IK, Eichten SR, Groszmann M, Wang A, Ying H, Peacock WJ, Dennis ES: **Twenty-four-nucleotide siRNAs produce heritable trans-chromosomal methylation in F1 Arabidopsis hybrids.** *Proc Natl Acad Sci USA* 2016,

113:E6895–E6902.

51. Rigal M, Becker C, Pélissier T, Pogorelcnik R, Devos J, Ikeda Y, Weigel DD, Mathieu O: **Epigenome confrontation triggers immediate reprogramming of DNA methylation and transposon silencing in Arabidopsis thaliana F1 epihybrids.** *Proc Natl Acad Sci USA* 2016, **113**:E2083–92.

52. Zhang Q, Wang D, Lang Z, He L, Yang L, Zeng L, Li Y, Zhao C, Huang H, Zhang H, et al.: **Methylation interactions in Arabidopsis hybrids require RNA-directed DNA methylation and are influenced by genetic variation.** *Proc Natl Acad Sci USA* 2016, **113**:E4248–56.

53. Junaid A, Kumar H, Rao AR, Patil AN, Singh NK, Gaikwad K: **Unravelling the epigenomic interactions between parental inbreds resulting in an altered hybrid methylome in pigeonpea.** *DNA Res.* 2018, **25**:361–373.

54. Gouil Q, Baulcombe DC: **Paramutation-like features of multiple natural epialleles in tomato.** *BMC Genomics* 2018, **19**:203.

55. Huang Y, Kendall T, Forsythe ES, Dorantes-Acosta A, Li S, Caballero-Perez J, Chen X, Arteaga-Vázquez M, Beilstein MA, Mosher RA: **Ancient Origin and Recent Innovations of RNA Polymerase IV and V.** *Mol Biol Evol* 2015, **32**:1788–1799.

56. Coruh C, Cho SH, Shahid S, Liu Q, Wierzbicki AT, Axtell MJ: **Comprehensive Annotation of Physcomitrella patens Small RNA Loci Reveals That the Heterochromatic Short Interfering RNA Pathway Is Largely Conserved in Land Plants.** *Plant Cell* 2015, **27**:2148–2162.

57. Wang Y, Ma H: **Step-wise and lineage-specific diversification of plant RNA polymerase genes and origin of the largest plant-specific subunits.** *New Phytol* 2015, **207**:1198–1212.

58. Nystedt B, Street NR, Wetterbom A, Zuccolo A, Lin Y-C, Scofield DG, Vezzi F, Delhomme N, Giacomello S, Alexeyenko A, et al.: **The Norway spruce genome sequence and conifer genome evolution.** *Nature* 2013, **497**:579–584.

59. Liu Y, El-Kassaby YA: **Landscape of fluid sets of hairpin-derived 21-/24-nt-long small RNAs at seed set uncovers special epigenetic features in Picea glauca**. *Genome Biol Evol* 2017, **9**:82–92.

60. Ujino-Ihara T, Ueno S, Uchiyama K, Futamura N: **Comprehensive analysis of small RNAs expressed in developing male strobili of Cryptomeria japonica.** *PLoS One* 2018, **13**:e0193665.

61. Berruezo F, de Souza FSJ, Picca PI, Nemirovsky SI, Martínez Tosar L, Rivero M, Mentaberry AN, Zelada AM: **Sequencing of small RNAs of the fern Pleopeltis minima (Polypodiaceae) offers insight into the evolution of the microrna repertoire in land plants.** *PLoS One* 2017, **12**:e0177573.

62. Banks JA, Nishiyama T, Hasebe M, Bowman JL, Gribskov M, dePamphilis C, Albert

VA, Aono N, Aoyama T, Ambrose BA, et al.: **The Selaginella genome identifies genetic changes associated with the evolution of vascular plants.** *Science* 2011, **332**:960–963.

63. Cho SH, Addo-Quaye C, Coruh C, Arif MA, Ma Z, Frank W, Axtell MJ: **Physcomitrella patens DCL3 is required for 22-24 nt siRNA accumulation, suppression of retrotransposon-derived transcripts, and normal development.** *PLoS Genetics* 2008, **4**:e1000314.

64. Tsuzuki M, Nishihama R, Ishizaki K, Kurihara Y, Matsui M, Bowman JL, Kohchi T, Hamada T, WATANABE Y: **Profiling and Characterization of Small RNAs in the Liverwort, Marchantia polymorpha, Belonging to the First Diverged Land Plants.** *Plant and Cell Physiology* 2016, **57**:359–372.

65. Ausin I, Feng S, Yu C, Liu W, Kuo HY, Jacobsen EL, Zhai J, Gallego-Bartolome J, Wang L, Egertsdotter U, et al.: **DNA methylome of the 20-gigabase Norway spruce genome.** *Proc Natl Acad Sci USA* 2016, **113**:E8106–E8113.

66. Bowman JL, Sakakibara K, Furumizu C, Dierschke T: **Evolution in the Cycles of Life.** *Annu Rev Genet* 2016, **50**:133–154.

67. Schmid MW, Giraldo-Fonseca A, Rövekamp M, Smetanin D, Bowman JL, Grossniklaus U: **Extensive epigenetic reprogramming during the life cycle of Marchantia polymorpha.** *Genome Biol* 2018, **19**:9.

68. Margis R, Fusaro AF, Smith NA, Curtin SJ, Watson JM, Finnegan EJ, Waterhouse PM: **The evolution and diversification of Dicers in plants.** *FEBS letters* 2006, **580**:2442–2450.

69. Kakrana A, Mathioni SM, Huang K, Hammond R, Vandivier L, Patel P, Arikit S, Shevchenko O, Harkess AE, Kingham B, et al.: **Plant 24-nt reproductive phasiRNAs from intramolecular duplex mRNAs in diverse monocots.** *Genome Res* 2018, **28**:1333–1344.

70. Song X, Li P, Zhai J, Zhou M, Ma L, Liu B, Jeong D-H, Nakano M, Cao S, Liu C, et al.: **Roles of DCL4 and DCL3b in rice phased small RNA biogenesis**. *Plant J* 2012, **69**:462–474.

71. 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.

72. Teng C, Zhang H, Hammond R, Huang K, bioRxiv BM, 2018: **Dicer-like 5 deficiency confers temperature-sensitive male sterility in maize**. *biorxiv.org* [no date], doi:10.1101/498410.

73. Trujillo JT, Seetharam AS, Hufford MB, Beilstein MA, Mosher RA: **Evidence for a Unique DNA-Dependent RNA Polymerase in Cereal Crops.** *Mol Biol Evol* 2018, **35**:2454–2462.

74. Gouil Q, Baulcombe DC: **DNA Methylation Signatures of the Plant Chromomethyltransferases**. *PLoS Genetics* 2016, **12**:e1006526.

75. Kravchik M, Damodharan S, Stav R, Arazi T: **Generation and characterization of a tomato DCL3-silencing mutant.** *Plant Sci.* 2014, **221-222**:81–89.

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

77. Moritoh S, Eun C-H, Ono A, Asao H, Okano Y, Yamaguchi K, Shimatani Z, Koizumi A, Terada R: **Targeted disruption of an orthologue of DOMAINS REARRANGED METHYLASE 2, OsDRM2, impairs the growth of rice plants by abnormal DNA methylation.** *Plant J* 2012, **71**:85–98.

78. Wei L, Gu L, Song X, Cui X, Lu Z, Zhou M, Wang L, Hu F, Zhai J, Meyers BC, et al.: **Dicer-like 3 produces transposable element-associated 24-nt siRNAs that control agricultural traits in rice.** *Proc Natl Acad Sci USA* 2014, **111**:3877–3882.