

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

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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.

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## 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 heterochromatin 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<sup>\*</sup>], 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 a 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 *NRPDI*, 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\*,30\*,31,32,33]. The sites with highest methylation match regions that are demethylated in the endosperm [30\*], 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\*,30\*,31\*\*].

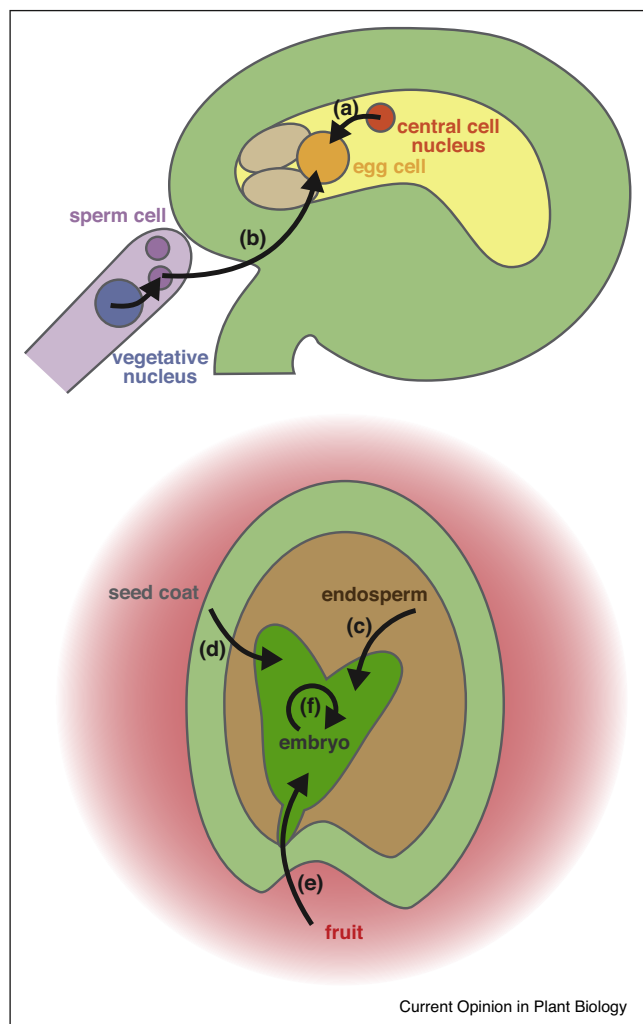
Indeed, demethylation at a number of promoters might facilitate upregulation of the germination program [30\*]. Conversely, mutants that maintain the embryogenic program following germination also maintain high CHH methylation [31\*\*], indicating that germination can occur despite elevated methylation, 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.

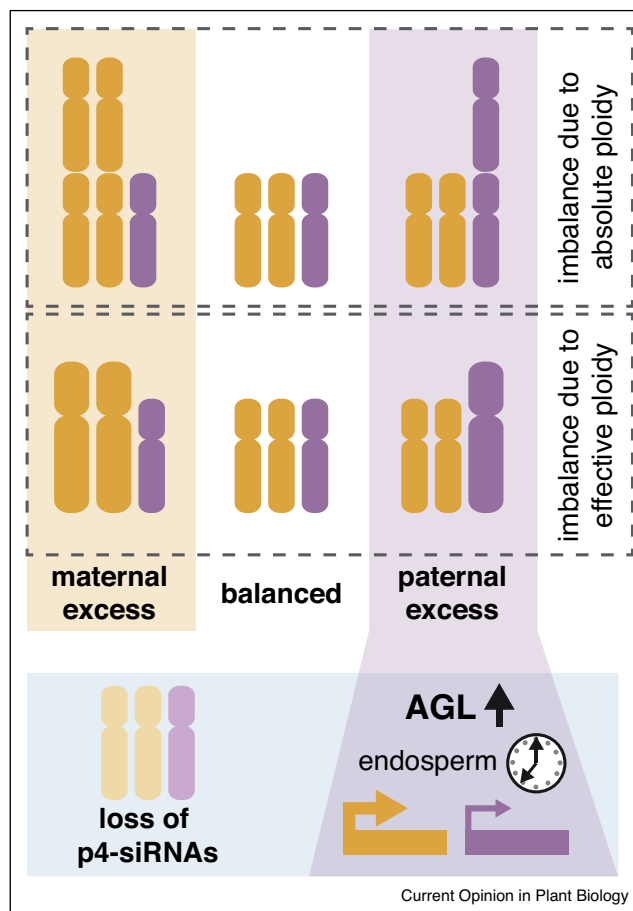
Figure 1



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).

If maternally derived p4-siRNAs increase maternal effective ploidy, what influences paternal effective ploidy? Loss of *NRPDI* 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 *NRPDI* 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 indicate that loss of paternal 21/22-nt siRNAs has little impact on the maternal expression bias observed in paternal excess

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.

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* intra-specific 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 crucial 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 *B. 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.

## Declaration of interests

Nothing declared.

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