RNA-directed DNA Methylation

Robert M. Erdmann\(^1\*,\) Colette L. Picard\(^2\*)

\(^1\) Center for Learning Innovation, University of Minnesota Rochester, Rochester, Minnesota, United States of America, \(^2\) Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, California, United States of America

* These authors contributed equally to this work.
\(^*\) rerdmann@r.umn.edu (RME); clpicard@ucla.edu (CLP)

Abstract

RNA-directed DNA methylation (RdDM) is a biological process in which non-coding RNA molecules direct the addition of DNA methylation to specific DNA sequences. The RdDM pathway is unique to plants, although other mechanisms of RNA-directed chromatin modification have also been described in fungi and animals. To date, the RdDM pathway is best characterized within angiosperms (flowering plants), and particularly within the model plant *Arabidopsis thaliana*. However, conserved RdDM pathway components and associated small RNAs (sRNAs) have also been found in other groups of plants, such as gymnosperms and ferns. The RdDM pathway closely resembles other sRNA pathways, particularly the highly conserved RNAi pathway found in fungi, plants, and animals. Both the RdDM and RNAi pathways produce sRNAs and involve conserved Argonaute, Dicer and RNA-dependent RNA polymerase proteins.

RdDM has been implicated in a number of regulatory processes in plants. The DNA methylation added by RdDM is generally associated with transcriptional repression of the genetic sequences targeted by the pathway. Since DNA methylation patterns in plants are heritable, these changes can often be stably transmitted to progeny. As a result, one prominent role of RdDM is the stable, transgenerational suppression of transposable element (TE) activity. RdDM has also been linked to pathogen defense, abiotic stress responses, and the regulation of several key developmental transitions. Although the RdDM pathway has a number of important functions, RdDM-defective mutants in *Arabidopsis thaliana* are viable and can reproduce, which has enabled detailed genetic studies of the pathway. However, RdDM mutants can have a range of defects in different plant species, including lethality, altered reproductive phenotypes, TE upregulation and genome instability, and increased pathogen sensitivity. Overall, RdDM is an important pathway in plants that regulates a number of processes by establishing and reinforcing specific DNA methylation patterns, which can lead to transgenerational epigenetic effects on gene expression and phenotype.

Biological functions of RdDM

RdDM is involved in a number of biological processes in the plant, including stress responses, cell-to-cell communication, and the maintenance of genome stability through TE silencing. An overview of some of the biological functions performed by RdDM is shown in Fig 1.
Transposable element silencing and genome stability

TEs are pieces of DNA that, when expressed, can move around the genome through a copy-and-paste or cut-and-paste mechanism. New TE insertions can disrupt gene expression or result in a mutant protein [1]. As a result, most organisms have mechanisms for preventing TE expression. This is particularly key in plant genomes, which are often TE-rich. Some plant species, including important crops like maize and wheat, have genomes consisting of upwards of 80% TEs [1,2]. RdDM plays a key role in silencing these mobile DNA elements in plants by adding DNA methylation over new TE insertions and constantly reinforcing DNA methylation over existing TEs, inhibiting
transposition and maintaining long-term genome stability [3]. Although the RdDM mechanism itself is unique to plants, using DNA methylation to silence TEs is a common strategy among eukaryotes [4].

RdDM primarily targets small TEs and TE fragments near genes, which are usually in open, accessible euchromatic regions of the genome that are permissive for gene expression [3,5]. In these regions, the ‘active’ chromatin state has a tendency to spread from expressed genes to nearby repressed regions, like TEs, which can cause these TEs to become activated and transpose [3]. Continuous activity by RdDM opposes the spread of active chromatin, maintaining a silent, repressive heterochromatic state over TEs in these otherwise euchromatic regions. In turn, RdDM activity recruits other pathways that help establish and propagate the silent, heterochromatic state (see ‘Interactions between RdDM and other chromatin modifying pathways’). Because of the self-reinforcing nature of these silencing pathways, excessive RdDM activity can also cause the silent, heterochromatic chromatin state over TEs to spread to nearby genes and repress them, with potentially harmful consequences for the organism [3,5]. Therefore, RdDM activity must be finely tuned to maintain a balance between repressing TEs and allowing expression of nearby genes [3].

In addition to maintaining stable silencing of TEs, RdDM can also initiate transcriptional silencing of foreign DNA, including novel TE insertions, virus-derived sequences, and transgenes (also see ‘Biotic stresses’ and ‘Transgene silencing’ below) [6–10]. When TEs integrate near genes, RdDM-mediated silencing of the TEs often affects gene expression [1,3]. However, this is not always deleterious, and can sometimes be overcome by other processes [11], or alter gene expression in ways beneficial to the plant. Over evolutionary time, beneficial TEs can become an important part of the mechanism by which a gene is regulated [1,3]. In one example, the gene ROS1 lies adjacent to a small helitron TE that is normally methylated by RdDM [12,13]. While DNA methylation is normally associated with transcriptional repression, this is not the case at the ROS1 locus. Instead, methylation of the helitron TE promotes ROS1 expression, so ROS1 expression is lost in mutants of the RdDM pathway that cannot methylate the TE [12,13]. Interestingly, ROS1 encodes a DNA glycosylase that functions to remove DNA methylation from the genome [14]. The link between ROS1 expression and RdDM activity at this TE ensures that DNA methylation and demethylation activities remain in balance, helping to maintain DNA methylation homeostasis genome-wide [12,13]. Thus, RdDM-mediated regulation of TEs can lead to beneficial regulatory outcomes.

Some TEs have evolved mechanisms to suppress or escape RdDM-based silencing in order to facilitate their own proliferation, leading to an evolutionary arms race between TEs and their host genomes. In one example, a TE-derived sequence was found to produce sRNAs that trigger post-transcriptional repression of a component of the RdDM pathway, inhibiting RdDM [15]. This sequence may have helped the original TE escape RdDM-based silencing and insert itself into the host genome.

Studying how RdDM targets and represses different types of TEs has led to many major insights into how the RdDM mechanism works. The retrotransposon EVADÉ (EVD) was one of the first TEs specifically shown to be repressed by RdDM-derived sRNAs [16]. Later work used EVD to trace the mechanism by which a novel TE insertion became silenced, revealing an important mechanistic link between post-transcriptional gene silencing and RdDM [9]. Studies of other retrotransposons, including ONSEN, which is regulated by both RdDM and heat stress [17,18], and Athila family TEs [10], among many others, have also provided valuable insights into RdDM-mediated TE silencing.
**Development and reproduction**

A number of epigenetic changes required for normal development and reproduction in flowering plants involve RdDM. In a well-studied example, RdDM is required for repression of the *FLOWERING WAGENINGEN (FWA)* gene, which allows for proper timing of flowering in Arabidopsis [19]. The *FWA* promoter contains tandem repeats that are usually methylated by RdDM, leading to transcriptional repression [20]. Loss of this methylation re-activates *FWA* expression, causing a late-flowering phenotype [19,20]. The loss of DNA methylation and associated late-flowering phenotype can be stably transmitted to progeny. Since the demethylated *fwa* allele leads to a stable, heritable change in the expression of *FWA* without any change to the DNA sequence, it is a classic example of an epiallele.

Mutations in the RdDM pathway can strongly affect gamete formation and seed viability, particularly in plant species with high TE content like maize and *Brassica rapa*, highlighting the importance of this pathway in plant reproduction [21–23]. During gamete formation, it has been hypothesized, and in some cases shown, that RdDM helps reinforce TE silencing in the germ cells [24,25]. In both pollen and ovules, a support cell undergoes epigenetic reprogramming, losing DNA methylation and other epigenetic marks at a number of loci, including TEs [24,26]. This causes TE re-activation and encourages the production of RdDM-derived sRNAs against these TEs in the support cells. The sRNAs are then thought to move from the support cell to the germ cell in order to reinforce TE silencing in the next generation. This phenomenon has been observed in pollen, but has yet to be shown definitively in the ovule [27,28]. This role for sRNAs in plants resembles the role of piRNAs in germline development in *Drosophila* and some other animals [29,30]. A similar phenomenon may also occur in roots to preserve TE silencing in important stem cell populations [31].

The RdDM pathway is also involved in regulating imprinted expression at some genes [32]. This unusual parent-of-origin-specific expression pattern occurs at several loci in the endosperm during seed development in flowering plants. A few factors involved in the RdDM pathway are themselves imprinted (favoring expression from the paternal allele) in diverse species, including *A. thaliana*, *A. lyrata*, *C. rubella*, and maize [33–36]. RdDM also plays a role in mediating the gene dosage effects seen in seeds derived from interploid crosses [37,38], though the mechanism for this remains largely unknown.

There is also evidence that RdDM plays a role in several other aspects of plant development, including seed dormancy [39], fruit ripening [40], and other pathways involved in flowering [41]. However, most of these data are correlative, and further study is necessary to understand the role of RdDM in these processes.

**Stress response**

**Abiotic stresses.** RdDM helps plants respond to a number of abiotic stresses, such as heat stress, drought, phosphate starvation, salt stress, and others [42]. Many TEs become upregulated under abiotic stress conditions [43,44], and thus one function of RdDM in stress response is to help counter this activation. In one example, the retrotransposon ONSEN is upregulated by heat stress, but normally remains suppressed by RdDM-associated sRNAs and can only transpose efficiently in heat-stressed plants that are also deficient in RdDM [17,18]. More generally, in plants exposed to heat stress, several components of the RdDM pathway become upregulated, and mutations in some components of the RdDM machinery reduce heat tolerance, suggesting RdDM plays an important role during heat stress [45,46]. In addition to regulating TEs under stress conditions, RdDM can also regulate genes in order to trigger appropriate stress responses. Under low humidity, leaves produce fewer stomata due to RdDM-mediated downregulation of two genes involved in stomatal development [47].
Similarly, RdDM becomes downregulated in response to salt stress, and this has been shown to trigger the expression of a transcription factor important in salt stress resistance [48].

**Biotic stresses.** RdDM was initially discovered as a response to infection by viroids [49], and along with RNAi plays an important role in defending the plant against viroids and viruses. The RdDM and RNAi machinery recognize viral RNAs and process them into sRNAs, which can then be used by both pathways to degrade viral RNA (RNAi) and silence viral DNA (RdDM) [50–52]. However, little is known about how the RdDM and RNAi machinery distinguish between viral RNAs and RNAs produced by the host plant. Mutants defective in RdDM and other methylation-deficient mutants are often hypersensitive to viral infection [53,54]. Virus-host interactions are another example of an evolutionary arms race, and many plant viruses encode suppressors of both RdDM and RNAi in an attempt to evade the host plant’s defenses [53,55–57].

RdDM is also involved in protecting the plant from other biotic stresses [50], including bacterial infections [58], fungal infections [59], and predation [60]. Loss of RdDM can have opposing effects on resistance for different pathogens. For example, some RdDM mutants have increased susceptibility to the bacterium *Agrobacterium tumefaciens* [61], but those same mutants have decreased susceptibility to the bacterium *Pseudomonas syringae* [58], highlighting the complexity of the different pathogen defense pathways and their interactions with RdDM [62].

**Transgene silencing.** In addition to naturally-occurring foreign nucleic acid stressors like TEs and viruses, artificially introduced DNA sequences, like transgenes, are also targeted for repression by RdDM [6,63]. Transgenes are widely used in genetics research to study gene function and regulation, and in plant breeding to introduce novel and desirable properties into a plant. Transgene silencing by RdDM and other mechanisms has therefore proved problematic for plant researchers. Efforts to understand how transgenes become silenced have ultimately helped reveal much of what we now know about the RdDM pathway (see ‘History and discovery of RdDM’). In one early example, researchers sequentially transformed plants with two different transgenes that shared some of their DNA sequence [64]. They found that transforming the second transgene into the plants led to the first transgene gaining DNA methylation and becoming inactivated [64]. This provided an early clue that there existed a trans-acting, sequence-based mechanism for transcriptional silencing of foreign DNA, later shown to be RdDM.

**Stress and RdDM-mediated epigenetic ‘memory’.** Due to the heritability of DNA methylation patterns in plants, and the self-reinforcing nature of RdDM and other DNA methylation pathways, any DNA methylation changes caused by environmental stressors have the potential to be maintained and transmitted to future generations. This can allow stress-induced DNA methylation changes to act as a ‘memory’ of the stressor and help prime the plant or its progeny to respond more efficiently to the stress if re-exposed [50,65]. For example, RdDM-derived sRNAs against TEs or viruses that have already integrated into the genome and been silenced serve as a ‘memory’ of those prior infections, protecting against future invasions by similar sequences. There is also evidence that DNA methylation changes due to other stressors, such as salt or heat stress, can persist in the progeny of stressed plants even in the absence of the original stressor [66]. In this study, the persistence of the stress-induced DNA methylation changes required several RdDM-related proteins, suggesting that RdDM was involved in maintaining the stress-altered DNA methylation patterns. In another example, resistance to insect attack was transmitted to progeny via DNA methylation changes, and this inheritance was also dependent on functional sRNA biogenesis pathways [50,60]. Thus, RdDM can potentially alter the plant epigenome in response to stress, and helps maintain these changes to modulate future stress responses in the affected plant and its descendants.
**Short and long-range signaling**

The sRNA molecules produced by RdDM and other pathways are able to move between cells via plasmodesmata, and can also move systemically through the plant via the vasculature [67–69]. They therefore have the potential to act as signaling molecules. This has been demonstrated in plants engineered to express green fluorescent protein (GFP) [70]. The GFP protein produced by these plants caused them to glow green under certain light conditions. When tissue from a second plant expressing a sRNA construct complementary to GFP was grafted onto the GFP-expressing plant, the GFP fluorescence was lost: after grafting, the sRNAs being produced in the second plant’s tissues were moving into the tissues of the first, GFP-expressing plant, and triggering silencing of GFP [70]. The same study showed that a subset of these mobile sRNAs were triggering the addition of DNA methylation to the GFP locus via RdDM. Therefore, sRNAs involved in RdDM can act as signaling molecules and trigger the addition of DNA methylation at complementary loci in cells far away from where the sRNAs were originally generated. Since then, studies have shown that sRNAs can move and direct RdDM both from shoot to root and root to shoot, though the silencing effect is more robust when sRNAs move from shoot to root [69–72].

Movement of sRNAs that drive RdDM activity plays an important role in plant development, including during reproduction [23,24,27] and root development [31]. In both cases, sRNA movement seems to function primarily as a way to reinforce DNA methylation and silencing of TEs in developmentally important cell types, like germ cells and stem cells. Silencing TEs and maintaining genome integrity in these cells is particularly important because they give rise to many other cells, all of which will inherit any defects or mutations in the original stem cell or germ cell. sRNA movement is also involved in plant-pathogen interactions: sRNAs can move from infected cells to distal uninfected tissues in order to prime a defense response, though to date this has only been shown for RNAi, not RdDM [73].

**Pathways and mechanisms**

This section focuses on the pathways and mechanisms by which RdDM leads to sequence-specific DNA methylation. The pathways presented here were characterized primarily in the model plant *Arabidopsis thaliana*, but are likely similar in other angiosperms. Conservation of RdDM in other plant species is discussed in more detail in 'Evolutionary conservation' below.

**RdDM and DNA methylation context**

RdDM is the only mechanism in plants that can add DNA methylation to cytosines regardless of sequence context [55]. DNA methylation in plants is typically divided into three categories based on the sequence context of the methylated cytosine: CG, CHG, and CHH, where H is any nucleotide except G (Fig 2). These reflect the different sequence contexts targeted by several DNA methylation pathways in plants. These context-specific pathways are primarily involved in maintaining existing DNA methylation patterns. The highly conserved methyltransferase MET1 (homolog of mammalian DNMT1) maintains DNA methylation in the CG context, while two conserved plant-specific methyltransferases, Chromomethylase 3 (CMT3) and CMT2, help maintain CHG and CHH methylation, respectively [74–77]. Unlike these pathways, RdDM leads to the addition of DNA methylation at all cytosines regardless of their sequence context. Like MET1, CMT2 and CMT3, RdDM is primarily involved in maintaining existing DNA methylation patterns [55]. However, RdDM is also the only pathway capable of adding DNA methylation *de novo* to previously unmethylated regions in plants.
Overview of the RdDM mechanism

The RdDM pathway can be split up into two main processes: the production of sRNAs, and the recruitment of DNA methylation machinery by those sRNAs to specific target loci in the DNA (Fig 3, top) \[55,78,79\]. These two activities together comprise RdDM, and ultimately lead to DNA methylation being added to cytosines at specific target loci.

Canonical RdDM

The canonical RdDM pathway is, as its name suggests, the most well-characterized RdDM pathway to date. Canonical RdDM is preferentially recruited to regions that are already DNA-methylated and heterochromatic, and acts to reinforce existing DNA methylation patterns at these loci, forming a positive feedback loop \[55,79\]. Canonical RdDM makes up the majority of RdDM activity in a cell \[79\]. The major steps of the canonical pathway are outlined below and in Fig 3 (top), while the various factors involved in the RdDM pathway are described in more detail in Table 1.
Fig 3. Schematic of the canonical RdDM pathway (top), and non-canonical RdDM and RNAi/PTGS (bottom). The canonical RdDM pathway can be broken into (1) sRNA production and (2) targeting DNA methylation to sites of sRNA production. The non-canonical RdDM pathway is closely related to RNAi and other PTGS pathways, and differs from canonical RdDM primarily in the source of sRNAs and sRNA processing. H3K9 = lysine 9 on histone H3; H3K4 = lysine 4 on histone H3; ssRNA = single-stranded RNA; dsRNA = double-stranded RNA, miRNA = microRNA.

https://doi.org/10.1371/journal.pgen.1009034.g003
sRNA production. The first part of the RdDM pathway revolves around the biogenesis of sRNAs. A plant-specific RNA polymerase complex, RNA Polymerase IV (Pol IV), is first recruited to silent heterochromatin via its interaction with CLASSY (CLSY) proteins and SAWAEEDEE homeodomain homolog 1 (SHH1) (also see ‘Interactions between RdDM and other chromatin modifying pathways’ below) [79–81]. Pol IV transcribes these regions to produce short single-stranded RNAs (ssRNAs) roughly 30 to 45 nucleotides in length, each of which is the precursor for a single sRNA [82–84]. These ssRNAs are converted into double-stranded RNAs (dsRNAs) co-transcriptionally by RNA-directed RNA polymerase 2 (RDR2), which physically associates with Pol IV [83]. The dsRNAs are then cleaved by the endoribonuclease Dicer-like 3 (DCL3) into 24 nucleotide (nt) sRNAs. Pol IV, RDR2, and DCL3 alone are sufficient for the production of 24 nt sRNAs in vitro [84], suggesting that while other factors involved in this part of the pathway may help increase efficiency or specificity, they are not required for Pol IV-mediated sRNA production.

While nearly all 24 nt sRNAs involved in RdDM are produced through the Pol IV-RDR2-DCL3 pathway, a small proportion are produced through other pathways. For example, some RNA Polymerase II (Pol II) transcripts that contain an inverted repeat sequence form double-stranded hairpin structures that can be directly cleaved by DCL3 to form 24 nt sRNAs (Fig 3, bottom) [79,85].

DNA methylation of target loci. In the second part of the pathway, the RdDM DNA methylation machinery is guided to DNA sequences complementary to the sRNAs generated in the first part of the pathway. One strand from each 24 nt double-stranded sRNA is loaded into Argonaute (AGO) proteins AGO4, AGO6, or AGO9 [55]. AGO3 may also be able to function in this pathway [86]. Argonautes are a large, highly conserved family of proteins that can bind sRNAs, forming a protein-sRNA duplex that enables them to recognize and bind other RNA sequences complementary to their sRNA partner [87]. Once formed, the AGO-sRNA duplex finds and binds complementary sequences along an RNA ‘scaffold’ produced by the plant-specific RNA Polymerase V (Pol V), with the help of interactions with Suppressor of Ty insertion 5-like (SPT5L), the Involved in de novo 2—IDN2 Paralog (IDN2-IDP) complex, and the Pol V subunit NRPE1 [88]. This leads to the recruitment of the DNA methyltransferase enzyme Domains Rearranged Methyltransferase 2 (DRM2), which methylates nearby DNA [55,79,89]. The mechanism by which the AGO-sRNA duplex recruits DRM2 is not yet well understood [90].

Non-canonical RdDM

Recent work has revealed a number of variations of the RdDM pathway, collectively referred to as non-canonical RdDM [79]. Unlike canonical RdDM, the non-canonical pathways are generally involved in establishing initial DNA methylation at new target loci, like novel TE insertions, rather than maintaining existing heterochromatin. Actively expressing elements like new TE insertions are normally strongly targeted by post-transcriptional gene silencing (PTGS/RNAi) pathways (Fig 3, bottom). Non-canonical RdDM occurs primarily as a byproduct of these PTGS pathways, leading to the initial establishment of a silent, heterochromatic state over the new TE or other target locus. Once that initial silent state is established, Pol IV can be recruited to the locus by CLSY and SHH1, and the canonical RdDM pathway takes over the long-term maintenance of silencing [79]. Therefore, the non-canonical RdDM pathways often act as a temporary bridge between initial post-transcriptional silencing of novel elements by RNAi, and long-term transgenerational transcriptional silencing via canonical RdDM [9,10,79]. Consistent with this role in initiation of novel silencing, non-canonical RdDM targets relatively few loci in comparison to canonical RdDM [79].
| Factor(s) | Factor type | Pathway | Role in RdDM | Known direct interactors | Description | References |
|-----------|-------------|---------|--------------|--------------------------|-------------|------------|
| NRPD1 and the Pol IV complex | RNA polymerase | Canonical RdDM | sRNA production | CLSY proteins, RDR2 | Pol IV is a plant-specific RNA polymerase complex and NRPD1, its largest subunit, is specific to the complex. Through its interaction with the CLSY proteins and SHH1, Pol IV is recruited to heterochromatic regions (specifically to H3K9me2- and H3K4me0-containing chromatin), and transcribes single-stranded RNAs precursors of the sRNAs used in the canonical RdDM pathway. | [80, 81, 93, 94] |
| NRPE1 and the Pol V complex | RNA polymerase | All RdDM | DNA methylation of target loci | Pol V is a plant-specific RNA polymerase complex and NRPE1, its largest subunit, is specific to the complex. Pol V transcribes non-coding RNAs that serve as scaffolds for several other RdDM components, most importantly the AGO-sRNA duplex, but also SPT5L, and the IDN2-IDP complex. Both NRPE1 and SPT5L contain an AGO hook motif that helps recruit AGO4 to Pol V transcripts. Mutating the AGO hook motifs on both proteins results in reduced DNA methylation at RdDM target loci, resembling nrpe1 null mutant phenotypes. Binding of the AGO-sRNA duplex to complementary sites along the Pol V transcript leads to recruitment of DRM2 and addition of DNA methylation to target loci. | [80, 81, 93–95] |
| RDR2 | RNA-dependent RNA polymerase | Canonical RdDM | sRNA production | Pol IV | Exists in a complex with Pol IV and converts the nascent Pol IV transcript to double-stranded RNA, which can then be processed by DCL3 to generate sRNAs for canonical RdDM. | [80, 83] |
| RDR6 | RNA-dependent RNA polymerase | PTGS, non-canonical RdDM | sRNA production | | Converts single-stranded RNAs to double-stranded RNAs for processing into 21–22 nt sRNAs by DCL2 and DCL4. Most of these sRNAs lead to PTGS, but some are loaded into AGO6 and participate in non-canonical RdDM. | [9, 80] |
| DCL1 | Endoribonuclease | PTGS, non-canonical RdDM | miRNA production, sRNA production | | An endoribonuclease that cleaves double-stranded RNA, primarily involved in the production of microRNAs that lead to PTGS via AGO1. Can also catalyze the production of 21 nt sRNAs from mRNAs containing inverted repeats, which can be used in either PTGS or non-canonical RdDM depending on the AGO protein they associate with. The four DCL proteins in A. thaliana (DCL1,2,3,4) compete for access to dsRNA substrates. | [81, 96–98] |
| DCL2 | Endoribonuclease | PTGS, Non-canonical RdDM | sRNA production | | An endoribonuclease that cleaves double-stranded RNA, resulting in 22 nt sRNAs that can be used in both PTGS and non-canonical RdDM. The four DCL proteins in A. thaliana (DCL1,2,3,4) compete for access to dsRNA substrates, and DCL2,4 can substitute for loss of DCL3 for most RdDM targets. | [80, 96, 97, 99] |

(Continued)
| Factor(s) | Factor type | Pathway | Role in RdDM | Known direct interactors | Description | References |
|-----------|-------------|---------|--------------|---------------------------|-------------|------------|
| **DCL3** | Endoribonuclease | Canonical RdDM | sRNA production | | An endoribonuclease that cleaves double-stranded RNA, resulting in 24 nt sRNAs used in canonical RdDM. Preferentially targets the short dsRNAs produced by Pol IV-RDR2, but can also slice other dsRNA substrates, including mRNAs containing inverted repeats or miRNA precursors. The four DCL proteins in *A. thaliana* (DCL1,2,3,4) compete for access to dsRNA substrates, and DCL2,4 can substitute for loss of DCL3 for most RdDM targets. When PTGS pathways via DCL2,4 become saturated, DCL3 can step in and process the DCL2,4 dsRNA substrates, triggering a switch from PTGS to RdDM-mediated TGS. | [9,80,96,97,99] |
| **DCL4** | Endoribonuclease | PTGS, Non-canonical RdDM | sRNA production | | An endoribonuclease that cleaves double-stranded RNA, resulting in 21 nt sRNAs that can be used for both PTGS and non-canonical RdDM. The four DCL proteins in *A. thaliana* (DCL1,2,3,4) compete for access to dsRNA substrates, and DCL2,4 can substitute for loss of DCL3 for most RdDM targets. | [96,97,99] |
| **AGO4** | Argonaute protein | Canonical RdDM | DNA methylation of target loci | NRPE1, SPT5L | The main Argonaute protein involved in canonical RdDM. AGO4 is partially redundant with AGO6, which can also function in this pathway, as well as with AGO9 in reproductive tissues. It binds the 24 nt sRNAs produced by the pathway to form an AGO4-sRNA duplex, which can recognize sequences complementary to the sRNA. Assisted by interactions with SPT5L, NRPE1, and the IDN2-IDP complex, the AGO4-sRNA duplex binds a single-stranded, noncoding RNA produced by Pol V, and helps recruit DRM2 to the DNA. | [80,93,100] |
| **AGO6** | Argonaute protein | All RdDM | DNA methylation of target loci | | An argonaute protein that can function in either canonical or non-canonical RdDM pathways. Partially redundant with AGO4 (the main canonical RdDM AGO). Can associate with either 24 nt or 21–22 nt sRNAs to trigger RdDM at complementary loci. By interacting with both 21–22 nt and 24 nt sRNAs, AGO6 helps in the transition from PTGS (normally mediated by 21–22 nt sRNAs) to stable silencing by RdDM (normally mediated by 24 nt sRNAs). Expressed particularly in the root and shoot meristems, which are the two main stem cell populations in plants. This may indicate that plants increase surveillance for novel TEs in order to ensure genome integrity in the key cells that will give rise to most of the other cells in the plant. | [10,80,93,100,101] |
| **AGO9** | Argonaute protein | Canonical RdDM | DNA methylation of target loci | | A highly specialized AGO expressed primarily in the germline, where it is required for proper female gamete formation. Interacts with 24 nt sRNAs to silence TEs in the germline, similar to the role of PIWI Argonaute proteins in animals. | [25,100,102] |
Table 1. (Continued)

| Factor(s) | Factor type | Pathway | Role in RdDM | Known direct interactors | Description | References |
|-----------|-------------|---------|--------------|--------------------------|-------------|------------|
| AGO1      | Argonaute protein | PTGS, non-canonical RdDM | sRNA production | Binds microRNAs or 21–22 nt sRNAs, which it uses to recognize complementary sequences on other RNAs. When an AGO1-sRNA duplex (often called the RISC) finds a complementary single-stranded mRNA, the RNA is cleaved by AGO1, destroying the mRNA and causing PTGS. The resulting RNA fragments can then be converted to dsRNAs by RDR6 and processed by DCL2,4 to form secondary 21–22 nt sRNAs. These are predominantly loaded back into AGO1, forming a self-reinforcing ‘RNAi loop’ (Fig 3). However, some of the 21–22 nt sRNAs are loaded into AGO6 instead, leading to RdDM. | [80,91,97,100] |
| DRM2      | DNA methyltransferase | All RdDM | DNA methylation of target loci | The main DNA methyltransferase involved in RdDM. Catalyzes the addition of a methyl group to cytosines in DNA. Recruited by the AGO4-sRNA duplex after it binds to a complementary sequence in a Pol V transcript, but the mechanism by which this happens is not well understood. | [80,103] |
| SHH1/DTF1 | DNA and chromatin binding protein | Canonical | sRNA production | CLSY1 | Required for Pol IV-derived sRNA production at a subset of RdDM loci. Via its SAWADEE domain, SHH1 binds histone H3 with specific modifications associated with heterochromatin and DNA methylation: methylation of the 9th lysine (H3K9me2) and unmethylated K4 (H3K4me0). By interacting with SHH1 via the CLSY proteins, Pol IV is recruited to heterochromatic/silent chromatin. To date, SHH1 has only been shown to directly interact with CLSY1. The ability of SHH1 to associate with Pol IV/NRPD1 is mostly abolished in clsy1,2 double mutants, so recruitment of Pol IV by SHH1 likely requires CLSY proteins. | [104,105,106,107] |
| CLSY1, CLSY2 | putative chromatin remodelers | Canonical | sRNA production | Pol IV, SHH1 | Required for SHH1 interaction with and recruitment of Pol IV to a subset of target loci. Mutually exclusive with loci regulated by CLSY3 and CLSY4. Together, the four CLSY proteins regulate nearly all Pol IV-derived sRNAs, and loss of all four results in a near total loss of 24-nucleotide sRNA production. Requires H3K9me2, likely through interaction with SHH1. sRNAs regulated by CLSY1,2 are enriched in the chromosome arms, while those regulated by CLSY3,4 are enriched in the pericentromere. | [107,108] |
| CLSY3, CLSY4 | putative chromatin remodelers | Canonical | sRNA production, Pol IV targeting | Pol IV | Involved in recruitment of Pol IV to a subset of target loci. Mutually exclusive with loci regulated by CLSY1 and CLSY2. Together, the four CLSY proteins regulate nearly all Pol IV-sRNAs, and loss of all four results in a near total loss of 24-nucleotide sRNA production. sRNAs regulated by CLSY3,4 are enriched in the pericentromere, while sRNAs regulated by CLSY1,2 are enriched in the chromosome arms. | [107,108] |
| HEN1      | RNA methylase | Both | sRNA production | none | Stabilizes sRNAs by adding methylation to the 3’-OH groups. | [109] |

(Continued)
| Factor(s) | Factor type | Pathway | Role in RdDM | Known direct interactors | Description | References |
|-----------|-------------|---------|--------------|--------------------------|-------------|------------|
| SUVH2, SUVH9 | methyl-DNA binding proteins | Both | DNA methylation of target loci | DDR complex, MORC1, MORC6 | A pair of closely related methyl-DNA binding proteins that interact with the DDR complex and are required for proper localization of the DDR complex and Pol V. By recruiting Pol V to regions with DNA methylation, which tend to be silent, heterochromatic regions, SU(VAR)3-9 homolog (SUHV) 2 and 9 help form a positive feedback loop that reinforces RdDM-mediated silencing. May also associate with MORCs. | [110] |
| DDR complex (RDM1, DMS3, DRD1) | putative chromatin remodeling complex | Both | DNA methylation of target loci | SUVH2, SUVH9 | The DDR complex, composed of DRD1, DMS3, and RDM1, is thought to facilitate access of Pol V to its target sites, possibly by unwinding DNA downstream of Pol V. Interacts with SUHV2,9, which bind methylated DNA, and this interaction may help recruit Pol V to regions of existing heterochromatin. RDM1 also binds single-stranded DNA, which may help unwind the DNA to facilitate recruitment of DRM2. | [88,110–113] |
| SPT5L/RDM3/ KTF1 | transcription factor | Both | DNA methylation of target loci | AGO4, Pol V transcripts | Interacts with AGO4 and helps recruit it to the RNA scaffold produced by Pol V. Like the Pol V subunit NRPE1, SPT5L contains an AGO hook motif in its C-terminal domain. The motifs on both NRPE1 and SPT5L redundantly help recruit AGO4 to loci being transcribed by Pol V. Mutating the AGO hook motifs on both proteins results in reduced DNA methylation at RdDM target loci, resembling nrpe1 null mutant phenotypes. Also required for co-transcriptional slicing of Pol V transcripts. | [95,114,115] |
| SWI/SNF complex | chromatin remodeling complex | Both | DNA methylation of target loci | IDN2 | The Switch/Sucrose non-fermentable (SWI/SNF) complex is a chromatin remodeling complex that is recruited to Pol V scaffolds by the IDN2-IDP complex, where it affects nucleosome positioning. SWI/SNF may promote RdDM by making the chromatin more accessible, which may facilitate access of DRM2 to DNA. | [116] |
| IDN2-IDP complex | dsRNA-binding protein | Both | DNA methylation of target loci | SWI/SNF complex | A complex composed of IDN2 and IDP1 (also called IDNL1) or IDP2 (IDNL2), IDN2, and possibly IDP1, can bind the dsRNA duplex formed when AGO-associated sRNAs hybridize with the Pol V scaffold. This complex is thought to help stabilize base pairing between the AGO-sRNA and Pol V scaffold RNA. IDN2-IDP may also facilitate recruitment of the SWI/SNF complex to Pol V scaffolds. Additionally, IDP1 can bind unmethylated DNA, which may help recruit DRM2 to regions lacking DNA methylation. | [116–118] |

(Continued)
The primary difference between the canonical and non-canonical RdDM pathways lies in the origin and biogenesis of the sRNAs involved. The canonical RdDM pathway involves 24 nt sRNAs, which are specific to that pathway and come predominantly from a single source (the Pol IV-RDR2 complex). In contrast, the non-canonical RdDM pathways involve 21–22 nt sRNAs from a variety of sources, allowing de novo DNA methylation to be initiated at many different types of loci. These 21–22 nt sRNAs are not specific to non-canonical RdDM, and also function in other PTGS pathways. In fact, only a small fraction of 21-22nt sRNAs are involved in RdDM, with the majority instead driving a positive feedback loop amplifying the PTGS response (Fig 3) [91]. The functional outcome of a specific 21–22 nt sRNA depends on the AGO protein it ultimately associates with: sRNAs that associate with AGO4, AGO6 or AGO9 result in RdDM and DNA methylation, while sRNAs that associate with other AGOs, like AGO1, primarily result in PTGS [55,79].
By using 21–22 nt sRNAs derived from a variety of sources, non-canonical RdDM can flexibly induce de novo DNA methylation and silencing at many different types of loci. One of the primary sources of 21–22 nt sRNAs is Pol II transcripts (Fig 3). Some of these transcripts, particularly those produced from TEs, viruses, or certain non-protein-coding transcripts, are targeted by PTGS pathways like miRNAs or RNAi, leading to cleavage of the transcript. The resulting fragments can be converted into dsRNA by RDR6 and then processed into 21–22 nt sRNAs by DCL2 or DCL4 [8]. Most of these 21–22 nt sRNAs are loaded into AGO1 and feed back into PTGS, amplifying PTGS efficiency [79]. However, some will instead associate with AGO6, leading to RdDM [10]. dsRNAs resulting from RDR6 activity can also sometimes be processed by DCL3 instead of DCL2/4 and trigger RdDM [9]. Additionally, some Pol II transcripts contain inverted repeat structures (Fig 3). These can be cleaved by DCL proteins independent of RDRs to produce either 21–22 nt or 24 nt sRNAs that can participate in RdDM [79]. Similarly, miRNA precursors, which also form hairpin structures and are normally cleaved by DCL1 to produce miRNAs, can instead be cleaved by other DCLs to form sRNAs for RdDM [79]. While most non-canonical RdDM occurs via AGO6 or AGO4, there is also a version of the pathway where sRNAs instead associate with AGO2, which together with the NERD complex (Needed for RDR2-independent DNA methylation) recruits DRM2 to target loci and triggers DNA methylation [92]. Since the non-canonical pathways are not yet as well characterized as the canonical RdDM pathway [79], there likely remain additional sources of sRNAs used for RdDM that have not yet been uncovered.

Factors involved in RdDM

A number of factors involved in RdDM are listed in Table 1, along with additional details about their function and corresponding references. Several factors primarily involved in PTGS (Fig 3) that sometimes participate in RdDM are also listed. Factors in bold are shown in Fig 3.

Interactions between RdDM and other chromatin modifying pathways

Different chromatin states, like active euchromatin or silent heterochromatin, are defined by a combination of specific histone modification and DNA methylation patterns. Repressive chromatin modifications, like DNA methylation, help promote DNA compaction and reduce DNA accessibility, while other modifications help open chromatin and increase accessibility. Methylation of the 9th lysine of histone H3 (H3K9), primarily in the form of H3K9 trimethylation (H3K9me3) in animals and H3K9 dimethylation (H3K9me2) in plants, is a highly conserved repressive modification [121,122]. Lack of H3K4 methylation (H3K4me0) is also associated with repression, along with several other histone modifications and variants. The combination of DNA methylation, H3K9me2, and H3K4me0 is strongly associated with heterochromatin in plants.

Since DNA methylation and repressive histone modifications together define heterochromatin, most DNA methylation pathways in plants recognize and interact with repressive histone marks and vice-versa, forming positive feedback loops that help maintain the repressive chromatin state [123]. The RdDM-associated protein SHH1 recognizes H3K4me0 and H3K9me2 at heterochromatic loci and recruits Pol IV to these loci to trigger additional DNA methylation at these regions [106]. Similarly, SUVH2 and SUVH9 help recruit Pol V to loci with DNA methylation [110]. Thus, both major parts of the canonical RdDM pathway are preferentially recruited to regions that are already in the silent, heterochromatic state marked by DNA methylation, H3K9me2, and H3K4me0. DNA methylation at these same heterochromatic loci is also recognized by the histone methyltransferases SUVH4/KYP, SUVH5, and
SUVH6, which bind to non-CG methylation and add H3K9me2 to nearby histones [123,124], closing the positive feedback loop. Similarly, CMT3 and CMT2, the two DNA methyltransferases involved in the maintenance of CHG and CHH methylation respectively (Fig 2) [75], both bind and add DNA methylation to H3K9me2-marked heterochromatin, forming their own feedback loop with SUVH4/5/6 [123,125]. These interactions help strongly reinforce silencing at TEs and other heterochromatic regions.

A similar feedback loop occurs in animals. HP1 plays a vital role in maintaining heterochromatin by propagating H3K9 methylation through a positive feedback loop with the H3K9 methyltransferase SUV39H [126]. H3K9 methylation recruits HP1, which recruits SUV39H to deposit more H3K9 methylation [126]. Though HP1 is conserved in plants, its function in this feedback loop is not conserved [127]. Instead, the positive feedback loops between H3K9me2 and the RdDM and CMT2/3 DNA methylation pathways fulfill a similar function in propagating H3K9me2. More recently, a plant-specific protein, Agenet Domain Containing Protein 1 (ADCP1), was also identified that may function analogously to HP1 in maintaining H3K9me2 levels in heterochromatin, facilitating heterochromatin formation [128].

Ultimately, the constant reinforcement of silencing chromatin modifications at heterochromatic loci creates a repressive chromatin state wherein the DNA and histones (nucleosomes) become tightly packed together. This helps silence gene expression by physically inhibiting access to the DNA, preventing RNA Polymerase II, transcription factors and other proteins from initiating transcription [129]. However, this same compaction also prevents factors involved in heterochromatin maintenance from accessing the DNA, which could lead to the silent, compact state being lost. This is particularly true in the dense constitutive heterochromatin surrounding the centromere. In these regions, the chromatin remodeler DDM1 plays a crucial role in DNA methylation maintenance by displacing nucleosomes temporarily to allow methyltransferases and other factors access the DNA [5,130,131]. However, since most RdDM targets are small TEs in open, accessible and gene-rich regions (see ‘TE silencing and genome stability’), few RdDM sites require DDM1 [5,99]. In fact, dense heterochromatin inhibits RdDM [5]. By contrast, CMT2 and CMT3 preferentially function in constitutive heterochromatin and depend strongly on DDM1 to maintain silencing over these regions [3,5,131]. Similarly, MET1, which maintains DNA methylation at CG sites after replication (Fig 2), requires DDM1 to access heterochromatin and maintain CG methylation in those regions [132]. Thus, DDM1 is a key regulator of DNA methylation in dense heterochromatin, but regulates sites mostly independently from RdDM [5,99].

Interactions between RdDM and the other three maintenance DNA methylation pathways (Fig 2) are limited and predominantly indirect. The DNA methyltransferase MET1 robustly maintains CG methylation genome-wide (Fig 2), including at RdDM target sites. In RdDM mutants, non-CG methylation at RdDM target sites is lost, but CG methylation is still maintained, suggesting that MET1 activity is independent of RdDM [99]. However, although met1 mutants lose CG methylation as expected, they also lose much of their non-CG methylation, including at RdDM target loci [99]. At these sites, silencing can still be initiated by RdDM in met1 mutants, but it is not maintained or transmitted to progeny, suggesting that MET1 is important for the maintenance, but not initiation, of silencing at a subset of RdDM target loci [120,133]. This effect is likely indirect: loss of MET1 leads to loss of H3K9me2 at some sites, which inhibits the recruitment of Pol IV and therefore prevents maintenance of DNA methylation via canonical RdDM, although the non-canonical pathways (which do not involve Pol IV) are not affected [99,120]. Loss of the histone deacetylase HDA6, which facilitates maintenance methylation by MET1 at some loci, has a similar effect, suggesting that multiple different
factors involved in maintaining heterochromatin likely facilitate RdDM-mediated DNA methylation maintenance [120].

Loss of RdDM leads to strong loss of non-CG methylation at TEs in gene-rich regions in the chromosome arms, but has little effect on DNA methylation levels in the constitutive heterochromatin around the centromere [3,5,99]. This suggests that CMT2 and CMT3, which function primarily to maintain CHG and CHH methylation in dense constitutive heterochromatin, do not depend on RdDM activity [3,5,99]. Similarly, in cmt2,cmt3 double mutants, many TEs in the chromosome arms remain methylated, presumably due to the persistent activity of RdDM, indicating that loss of CMT2/3 has little effect on RdDM activity [3,5]. This suggests that RdDM and CMT2/3 function mostly independently and at distinct loci: RdDM is the main pathway responsible for maintaining non-CG DNA methylation in euchromatic, gene rich regions, while CMT2 and CMT3 maintain non-CG DNA methylation in constitutive heterochromatin. In mutants defective in both RdDM and CMT2/CMT3, all non-CG methylation in the genome is eliminated [74], demonstrating that together RdDM and CMT2/CMT3 account for all non-CG methylation in the genome.

Balance between DNA methylation and demethylation

Most DNA methylation mechanisms in plants are self-reinforcing (see above), including RdDM: Pol IV and Pol V are both recruited to heterochromatic regions that already have DNA methylation, encouraging additional DNA methylation via canonical RdDM [55]. Positive feedback loops like these can cause DNA methylation activity to spread out from the intended methylated target sites into genes or other regulatory elements, which can negatively affect gene expression. To prevent this spreading, DNA methylation pathways are opposed by passive and active DNA demethylation. DNA methylation can be lost passively with each cell division, because newly-synthesized strands of DNA lack DNA methylation until it is re-added by one of the maintenance DNA methylation pathways [134]. DNA methylation can also be actively removed in plants by DNA glycosylases, which remove methylated cytosines via the base excision repair pathway. In Arabidopsis, there are four proteins responsible for removing DNA methylation: Repressor of silencing 1 (ROS1), Demeter (DME), Demeter-like 2 (DML2), and Demeter-like 3 (DML3) [135,136]. These DNA glycosylases help prevent the spread of DNA methylation from RdDM targets to active genes [14,137]. Loss of active DNA demethylation in ros1:dml2:dml3 triple mutants leads to a widespread increase in DNA methylation levels, whereas ectopic expression of ROS1 leads to progressive loss of DNA methylation at many loci [138], highlighting the importance of balancing DNA methylation and demethylation activity.

Interestingly, expression of the DNA demethylase ROS1 is directly tied to RdDM activity: DNA methylation over a TE targeted by RdDM in the ROS1 promoter is required for ROS1 expression [12,13], though other factors are also involved in regulating ROSI [139,140]. Since ROS1 expression is tied to DNA methylation at a specific TE, ROS1 expression is strongly reduced in plants with defective RdDM that lose the ability to methylate that TE [12]. This general mechanism helps maintain DNA methylation homeostasis by tuning DNA demethylation activity to DNA methylation activity, helping to ensure that DNA methylation patterns can be stably maintained over time.

Evolutionary conservation

Origins of RdDM pathway members

While all eukaryotes share three RNA polymerases (RNA Pol I, II and III), plants have two additional polymerases, Pol IV and Pol V. Both Pol IV and V share an evolutionary origin,
deriving from Pol II [94,141]. In other eukaryotic kingdoms that lack these two specialized RNA polymerases, Pol II transcribes the precursors of small RNAs used in silencing pathways—in fact, Pol II transcripts are also sometimes processed into sRNAs in plants (Fig 3). It has been hypothesized that the origin of both Pol IV and Pol V is rooted in “escape from adaptive conflict” [142]. The idea is that potential tensions between the “traditional” function of Pol II and the small RNA biogenesis function could be relieved by duplication of Pol II and subfunctionalization of the resulting multiple RNA polymerases.

Analyses of evolutionary lineage for Pol IV and Pol V are complicated to some extent by the fact that each enzyme is actually comprised of at least 12 subunits [141]. In Arabidopsis thaliana, some subunits are shared between Pol IV and Pol V, some are unique to each polymerase, and some are shared between Pol II, IV, and V [143]. Orthologs of certain Pol IV and V subunits have been found in all lineages of land plants, including ferns, liverworts, and mosses (Fig 4) [142,144]. These findings argue for a shared origin of Pol IV and V dating back to early land / vascular plants.

Much of the work done to elucidate the genes and proteins involved in the RdDM pathway has been performed in Arabidopsis thaliana, a model angiosperm. However, studies of Pol IV and V conducted in maize show some key differences with Arabidopsis. Maize Pol IV and V differ from each other in terms of only one subunit (the largest one). In Arabidopsis, Pol IV and V differ from each other in terms of three subunits [145]. However, maize utilizes a set of interchangeable catalytic subunits—two in the case of Pol IV and three in the case of Pol V—that provide additional specialization of polymerase functionality [145]. While differences exist, overall there is a broad overlap in RdDM functions and components between the different angiosperm species studied to date.

Outside of Pol IV and Pol V, a large proportion of key RdDM component proteins (for example, DCL3 and AGO4) have orthologs found within each class of land plants, which provides support for the hypothesis that some form of the RdDM pathway evolved early within the plant lineage (Fig 5) [142]. However, RdDM pathway functionality does appear to change to an appreciable extent between different plant species and lineages. For example, while gymnosperms have functional Pol IV and produce 24 nt small RNAs, the biogenesis of sRNAs within gymnosperms is much more heavily skewed towards 21 nt than 24 nt sRNAs [146]. This suggests that canonical RdDM may be rarer or less pronounced in gymnosperms than in angiosperms. Similarly, while orthologs of DRM2 are found in various angiosperms, there are no known DRM2 orthologs in other plant lineages [147]. One possibility is that angiosperms have the “most complete” version of the RdDM pathway, with all other plant lineages possessing robust and functional subsets of the pathway. However, since nearly all of the work on RdDM has been done in angiosperms, it is also possible that alternative versions of RdDM in other lineages have simply not yet been uncovered, particularly if these alternative versions include different proteins or proteins without clear homologs in angiosperms.

**Relationships with sRNA silencing pathways in other kingdoms**

All eukaryotic kingdoms host some form of small RNAs. One such class of sRNAs is the Piwi-interacting RNAs (piRNAs). Much like in RdDM, piRNAs primarily function to target and silence transposons, particularly in the germline [29,30]. However, piRNAs are only found in animals, are longer than the small RNAs functioning in RdDM (24–32 nucleotides), and mediate their functions through interactions with a different subclass of AGO proteins, the PIWI subfamily, which are absent from plants [29,30]. MicroRNAs (miRNAs) are another class of small RNA with silencing properties [148]. While miRNAs are in a similar size range as RdDM sRNAs (~21 nt), miRNAs associate with a distinct set of Argonaute proteins that
silence target RNAs by initiating their degradation or blocking their downstream translation into proteins, rather than recruiting DRM2 to add DNA methylation to nearby DNA. Both RdDM and the miRNA pathways involve related proteins from the Argonaute and Dicer families [148].

Perhaps the most analogous pathways to RdDM in another eukaryotic kingdom are the sRNA directed transcriptional gene silencing (TGS) and co-transcriptional gene silencing (CTGS) pathways in *Schizosaccharomyces pombe* [149]. In *S. pombe*, TGS directs methylation of H3K9, leading to heterochromatin formation, and is directed by sRNAs produced from the targeted regions [150]. Similar to canonical RdDM, this pathway is a positive feedback loop: sRNAs are generated preferentially from heterochromatin-rich areas of the genome, and these sRNAs direct the addition of K3K9 methylation to maintain/spread heterochromatin. Meanwhile, CTGS is directed by AGO1-bound sRNAs, similar to PTGS within plants, and results in

Fig 4. A schematic depicting the evolutionary conservation of selected Pol IV and V subunit orthologs within the plant kingdom. Subunits beginning with NRPD are Pol IV subunits, subunits beginning with NRPE are Pol V subunits, and subunits labeled as NRPD/E are found in both Pol IV and V. [141] A filled circle for a subunit indicates that an ortholog for that subunit has been identified within the associated lineage.

https://doi.org/10.1371/journal.pgen.1009034.g004
the inhibition of transcription by Pol II, as well as to Pol II release [151,152]. Unlike RdDM, TGS and CTGS in *S. pombe* do not rely on transcription from non-Pol II sources or lead to the addition of DNA methylation. However, the *S. pombe* pathways and RdDM share many of the same components, like RNA-directed RNA polymerases and sRNAs, and have similar functions in maintaining heterochromatin.

**History and discovery of RdDM**

Introducing transgenes into organisms has been a widely used tool in plant genetics research for decades. However, researchers often find that their introduced transgenes are not expressed as strongly as expected, or sometimes even at all, a phenomenon called transgene silencing [153]. The discovery of transgene silencing in the 1990s spurred a great deal of interest in understanding the mechanisms behind this silencing [154–156]. Researchers found that transgene silencing was ubiquitous, occurring in multiple species (including Arabidopsis,
Tobacco, and Petunia), and was associated with increased DNA methylation over and around the silenced transgene [157–159].

Around the same time in 1994, work in tobacco plants had revealed a new pathway involving RNAs that resulted in DNA methylation. Researchers found that when viroids were introduced into the plant and integrated into the plant genome, the viroid sequences, but not the host genome, gained DNA methylation [49]. The deposition of methylation over these foreign viroid sequences helped inhibit viroid replication, and was therefore thought to represent a plant pathogen defense mechanism. The evidence suggested that the viroid RNAs produced during viroid replication were being used by the plant as a template to help target DNA methylation to the viroid sequences. This mechanism was therefore named RNA-directed DNA methylation, or RdDM [49].

RdDM turned out to be the solution to the transgene mystery: like viroids and viruses, transgenes are foreign sequences, and as a result they are often recognized as foreign invaders and targeted for silencing by RdDM and PTGS. Since transgene silencing was a reliable marker of RdDM activity, researchers were able to design genetic screens to identify mutants that failed to trigger silencing at transgenes, reasoning that these genes were likely to be involved in the RdDM pathway. These experiments revealed many parts of the pathway, including RNA Pol IV and V, Dicer-like proteins, Argonautes, and others [6,160,161].

The involvement of sRNAs in RdDM was initially suspected due to the similarity between RdDM and RNAi, the latter of which had recently been shown to involve small RNAs [49,162]. To test whether sRNAs were involved in RdDM, RNA hairpin structures complementary to a specific gene promoter were introduced into Arabidopsis and Tobacco [163]. The hairpin RNAs were processed into sRNAs, which were able to trigger the addition of DNA methylation to the targeted promoter and silence the gene [163]. This demonstrated that sRNAs could direct DNA methylation to specific loci. Later efforts showed that the sRNAs involved in RdDM were approximately 24–26 nt long, while the sRNAs associated with RNAi were only about 21–22 nt in length [164,165]. Soon after, the identification of AGO4 and characterization of its role in RdDM led to predictions, later confirmed, that 24 nt sRNAs were associating with AGO4 and directing DNA methylation to complementary loci [165,166].

Early work on transgene silencing and RdDM also identified SDE4 as required for the production of most sRNAs involved in RdDM [167]. SDE4 would later be identified as the largest subunit of Pol IV, and renamed NRPD1. A number of studies published in quick succession from multiple research groups, utilizing both forward and reverse genetic approaches, went on to identify and characterize Pol IV and Pol V as highly specialized plant RNA polymerases involved in RdDM [168–171]. The Pol IV / Pol V naming convention was adopted shortly thereafter [88,141].

**Potential biotechnology applications of RdDM**

Since the mechanism underlying the sequence-specificity of RdDM is well known (Fig 3), RdDM can be ‘tricked’ into targeting and silencing endogenous genes in a highly specific manner, which has a number of potential biotechnological and bioengineering applications. Several different methods can be used to trigger RdDM-based DNA methylation and silencing of specific genes. One method, called virus-induced gene silencing (VIGS), involves inserting part of the promoter sequence of the desired target gene into a virus [172]. The virus will reproduce the chunk of promoter sequence as part of its own RNA, which is otherwise foreign to the plant. Because the viral RNA is foreign, it will be targeted for PTGS and processed into sRNAs, some of which will be complementary to the original target gene’s promoter (Fig 3). A subset of these sRNAs will recruit the RdDM machinery to the target gene to add DNA
methylation. In one study, researchers used this method with an engineered Cucumber Mosaic Virus to recruit RdDM to silence a gene that affected flower pigmentation in petunia, and another that affected fruit ripening in tomato [173]. In both cases, they showed that DNA methylation was added to the locus as expected. In petunia, both the gain of DNA methylation and changes in flower coloration were heritable, while only partial silencing and heritability were observed in tomato. VIGS has also been used to silence the FWA locus in Arabidopsis, which resulted in plants that flowered later than normal [172]. The same study also showed that the inhibitory effect of VIGS on FWA and flowering can become stronger over the course of successful generations [172].

Another method to target RdDM to a desired target gene involves introducing a hairpin RNA construct that is complementary to the target locus. Hairpin RNAs contain an inverted repeat, which causes the RNA molecule to form a double-stranded RNA (dsRNA) structure called an RNA hairpin. The dsRNA hairpin can be processed by DCL proteins into siRNAs which are complementary to the target locus, triggering RdDM at that locus (Fig 3). This method has been used in several studies [12,174,175].

Changes induced by RdDM can sometimes be maintained and inherited over multiple generations without outside intervention or manipulation, suggesting that RdDM can be a valuable tool for targeted epigenome editing. Recent work has even bypassed RdDM altogether by artificially tethering DRM2 (or other components of the RdDM pathway) directly to specific target loci, using either zinc finger nucleases or CRISPR [90,176]. In these experiments, tethering the RdDM machinery to a specific locus led to gain of DNA methylation at the target site that was often heritable for multiple generations, even once the artificial construct was removed through crossing. For all of these methods, however, more work on minimizing off-target effects and increasing DNA methylation efficiency is needed.

Genetically Modified Organisms (GMOs) have played a large role in recent agricultural research and practice, but have proven controversial, and face regulatory barriers to implementation in some jurisdictions. GMOs are defined by the inclusion of “foreign” genetic material into the genome. The treatment of plants with engineered RNAs or viruses intended to trigger RdDM does not change the underlying DNA sequence of the treated plant’s genome; only the epigenetic state of portions of the DNA sequence already present are altered. As a result, these plants are not considered GMOs. This has led to efforts to utilize RdDM and other RNA-mediated effects to induce agriculturally-beneficial traits, like altering pathogen or herbicide susceptibility, or speeding up plant breeding by quickly inducing favorable traits [177–179]. However, while this is an area of active interest, there are few broadly implemented applications as of now.

Supporting information
S1 Text. Version history of the text file.
(XML)
S2 Text. Peer reviews and response to reviews.
(XML)

References
1. Dubin MJ, Mittelsten Scheid O, Becker C. Transposons: a blessing curse. Curr. Opin. Plant Biol. 2018; 42:23–29. https://doi.org/10.1016/j.pbi.2018.01.003 PMID: 29453028
2. Wicker T, Gundlach H, Spannagl M, Uauy C, Borrill P, Ramírez-González RH, et al. Impact of transposable elements on genome structure and evolution in bread wheat. Genome Biol. 2018; 19:103. https://doi.org/10.1186/s13059-018-1479-0 PMID: 30115100
3. Sigman MJ, Slotkin RK. The First Rule of Plant Transposable Element Silencing: Location, Location, Location. Plant Cell. 2016; 28:304–13. https://doi.org/10.1105/tpc.15.00869 PMID: 26869697

4. Deniz Ö, Frost JM, Branco MR. Regulation of transposable elements by DNA modifications. Nat. Rev. Genet. 2019; 20:417–431. https://doi.org/10.1038/s41576-019-0106-6 PMID: 30867571

5. Zemach A, Kim MY, Hsieh PH, Coleman-Derr D, Eshed-Williams L, Thao K, et al. The Arabidopsis nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. Cell. 2013; 153:193–205. https://doi.org/10.1016/j.cell.2013.02.033 PMID: 23540698

6. Chan SW, Zilberman D, Xie Z, Johansen DK, Jacobsen SE. RNA silencing genes control de novo DNA methylation. Science. 2004; 303:1336. https://doi.org/10.1126/science.1095989 PMID: 14988555

7. Pérez-Hormaeche J, Potel F, Beaucal F, Le Masson I, Courtial B, Bouché N, et al. Invasion of the Arabidopsis genome by the tobacco retrotransposon Tnt1 is controlled by reversible transcriptional gene silencing. Plant Physiol. 2008; 147:1264–78. https://doi.org/10.1104/pp.108.117846 PMID: 18467467

8. McCue AD, Panda K, Nuthikattu S, Choudary 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. https://doi.org/10.15252/embj.201489499 PMID: 25388951

9. Williams BP, Pignatta D, Henikoff S, Gehring M. Methylation-sensitive expression of a DNA demethylase gene serves as an epigenetic rheostat. PLoS Genet. 2015; 11:e1005142. https://doi.org/10.1371/journal.pgen.1005142 PMID: 25826366

10. Harris CJ, Scheible W, Wongpalee SP, Liu W, Cornett EM, Vaughan RM, et al. A DNA methylation reader complex that enhances gene transcription. Science. 2018; 362:1182–1186. https://doi.org/10.1073/science.aar7854 PMID: 30523112

11. Lei M, Zhang H, Julian R, Tang K, Xie S, Zhu JK. Regulatory link between DNA methylation and active demethylation in Arabidopsis. Proc. Nat. Acad. Sci. U.S.A. 2015; 112:3553–7. https://doi.org/10.1073/pnas.1502279112 PMID: 25733903

12. Williams BP, Pignatta D, Henikoff S, Gehring M. Methylation-sensitive expression of a DNA demethylase gene serves as an epigenetic rheostat. PLoS Genet. 2015; 11:e1005142. https://doi.org/10.1371/journal.pgen.1005142 PMID: 25826366

13. Cho J. Transposon-Derived Non-coding RNAs and Their Function in Plants. Front Plant Sci. 2018; 9:600. https://doi.org/10.3389/fpls.2018.00600 PMID: 29774045

14. Mirozze M, Reinders J, Bucher E, Nishimura T, Schneberger K, Ossowski S, et al. Selective epigenetic control of retrotransposition in Arabidopsis. Nature. 2009; 461:427–30. https://doi.org/10.1038/nature08328 PMID: 19734882

15. Cavrak VV, Lettner N, Jamge S, Kosarewicz A, Bayer LM, Mittelsten Scheid O. How a retrotransposon exploits the plant's heat stress response for its activation. PLoS Genet. 2014; 10:e1004115. https://doi.org/10.1371/journal.pgen.1004115 PMID: 24497839

16. Soppe WJ, Jacobsen SE, Alonso-Blanco C, Jackson JP, Kakutani T, Koornneef M, et al. The late flowering phenotype of fwa mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. Mol. Cell. 2000; 6:791–802. https://doi.org/10.1016/s1097-2765(05)00090-0 PMID: 11090618

17. Wang G, Köhler C. Epigenetic processes in flowering plant reproduction. J. Exp. Bot. 2017; 68:797–807. https://doi.org/10.1093/jxb/erw486 PMID: 28062591
24. Martinez G, Köhler C. Role of small RNAs in epigenetic reprogramming during plant sexual reproduction. Curr. Opin. Plant Biol. 2017; 36:22–28. https://doi.org/10.1016/j.pbi.2016.12.006 PMID: 28088028

25. Olmedo-Monfíl V, Durán-Figueras N, Arteaga-Vázquez M, Demesa-Arévalo E, Autran D, Grimanelli D, et al. Control of female gamete formation by a small RNA pathway in Arabidopsis. Nature. 2010; 464:628–32. https://doi.org/10.1038/nature08828 PMID: 20208518

26. Slotkin RK, Vaughn M, Borges F, Tanurdić M, Becker JD, Feijó JA, et al. Epigenetic reprogramming and small RNA silencing of transposable elements in pollen. Cell. 2009; 136:461–72. https://doi.org/10.1016/j.cell.2008.12.038 PMID: 19203581

27. 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. https://doi.org/10.1038/nplants.2016.30 PMID: 27249563

28. Erdmann RM, Hoffmann A, Walter HK, Wagenknecht HA, Groß-Hardt R, Gehring M. Molecular movement in the Arabidopsis thaliana female gametophyte. Plant Reprod. 2017; 30:141–146. https://doi.org/10.1007/s00497-017-0304-3 PMID: 28695277

29. Siomi MC, Sato K, Pezic D, Aravin AA. PIWI-interacting small RNAs: the vanguard of genome defence. Nat. Rev. Mol. Cell Biol. 2011; 12:246–58. https://doi.org/10.1038/nrm3089 PMID: 21427766

30. Ernst C, Odom DT, Kutter C. The emergence of piRNAs against transposon invasion to preserve mammalian genome integrity. Nat Commun. 2017; 8:1411. https://doi.org/10.1038/s41467-017-01049-7 PMID: 29127961

31. Kawakatsu T, Stuart T, Valdes M, Breakfield N, Schmitz RJ, Nery JR, et al. Unique cell-type-specific patterns of DNA methylation in the root meristem. Nat Plants. 2016; 2:16058. https://doi.org/10.1038/nplants.2016.58 PMID: 27243651

32. Vu TM, Nakamura M, Calarco JP, Susaki D, Lim PQ, Kinoshita T, et al. RNA-directed DNA methylation regulates parental genomic imprinting at several loci in Arabidopsis. Development. 2013; 140:2953–60. https://doi.org/10.1242/dev.092981 PMID: 23760956

33. Waters AJ, Blinski P, Eichten SR, Vaughn MW, Ross-Ibarra J, Gehring M, et al. Comprehensive analysis of imprinted genes in maize reveals allelic variation for imprinting and limited conservation with other species. Proc. Natl. Acad. Sci. U.S.A. 2013; 110:19639–44. https://doi.org/10.1073/pnas.1309182110 PMID: 24218619

34. Pignatta D, Erdmann RM, Scheer E, Picard CL, Bell GW, Gehring M. Natural epigenetic polymorphisms lead to intraspecific variation in Arabidopsis gene imprinting. Elife. 2014; 3:e03198. https://doi.org/10.7554/eLife.03198 PMID: 24994762

35. Klosinska M, Picard CL, Gehring M. Conserved imprinting associated with unique epigenetic signatures in the Arabidopsis genus. Nat Plants. 2016; 2:16145. https://doi.org/10.1038/nplants.2016.145 PMID: 27643534

36. Hatorangan MR, Laenen B, Steige KA, Slotte T, Köhler C. Rapid Evolution of Genomic Imprinting in Two Species of the Brassicaceae. Plant Cell. 2016; 28:1815–27. https://doi.org/10.1105/tpc.16.00304 PMID: 27465027

37. Erdmann RM, Satyaki PRV, Klosinska M, Gehring M. A Small RNA Pathway Mediates Allelic Dosage in Endosperm. Cell Rep. 2017; 21:3364–3372. https://doi.org/10.1016/j.celrep.2017.11.078 PMID: 29262317

38. 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. https://doi.org/10.1105/tpc.19.00047 PMID: 31064867

39. Iwasaki M, Hyvärinen L, Piskurewicz U, Lopez-Molina L. Non-canonical RNA-directed DNA methylation participates in maternal and environmental control of seed dormancy. Elife. 2019; 8:e37434. https://doi.org/10.7554/eLife.37434 PMID: 30910007

40. Cheng J, Niu Q, Zhang B, Chen K, Yang R, Zhu JK, et al. Downregulation of RdDM during strawberry fruit ripening. Genome Biol. 2018; 19:212. https://doi.org/10.1186/s13059-018-1587-x PMID: 30514401

41. Guo X, Ma Z, Zhang Z, Cheng L, Zhang X, Li T. Small RNA-Sequencing Links Physiological Changes and RdDM Process to Vegetative-to-Floral Transition in Apple. Front Plant Sci. 2017; 8:873. https://doi.org/10.3389/fpls.2017.00873 PMID: 28611800

42. Fortes AM, Gallucci P. Plant Stress Responses and Phenotypic Plasticity in the Epigenomics Era: Perspectives on the Grapevine Scenario, a Model for Perennial Crop Plants. Front Plant Sci. 2017; 8:82. https://doi.org/10.3389/fpls.2017.00082 PMID: 28220131

43. Kumar A, Bennetzen JL. Plant retrotransposons. Annu. Rev. Genet. 1999; 33:479–532. https://doi.org/10.1146/annurev.genet.33.1.479 PMID: 10690416
44. Ito H, Kim JM, Matsunaga W, Saze H, Matsui A, Endo TA, et al. A Stress-Activated Transposon in Arabidopsis Induces Transgenerational Abscisic Acid Insensitivity. Sci Rep. 2016; 6:23181. https://doi.org/10.1038/srep23181 PMID: 26976262

45. Liu J, Feng L, Li J, He Z. Genetic and epigenetic control of plant heat responses. Front Plant Sci. 2015; 6:267. https://doi.org/10.3389/fpls.2015.00267 PMID: 25964789

46. Popova OV, Dinh HQ, Aufsatz W, Jonak C. The RdDM pathway is required for basal heat tolerance in Arabidopsis. Mol Plant. 2013; 6:399–410. https://doi.org/10.1093/mp/sst023 PMID: 23376771

47. Tricker PJ, Gibbings JG, Rodríguez López CM, Hadley P, Wilkinson MJ. Low relative humidity triggers RNA-directed de novo DNA methylation and suppression of genes controlling stomatal development. J. Exp. Bot. 2012; 63:799–813. https://doi.org/10.1093/jxb/ers076 PMID: 22424111

48. Xu R, Wang Y, Zheng H, Lu W, Wu C, Huang J, et al. Salt-induced transcription factor MYB74 is regulated by the RNA-directed DNA methylation pathway in Arabidopsis. J. Exp. Bot. 2015; 66:6597–6008. https://doi.org/10.1093/jxb/erv312 PMID: 26139822

49. Wasseneger M, Heimes S, Riedel L, Sänger HL. RNA-directed de novo methylation of genomic sequences in plants. Cell. 1994; 76:567–76. https://doi.org/10.1016/0092-8674 (94)90119-8 PMID: 8313476

50. Huang J, Yang M, Zhang X. The function of small RNAs in plant biotic stress response. J Integr Plant Biol. 2016; 58:312–27. https://doi.org/10.1111/jipb.12463 PMID: 27648943

51. Raja P, Jackel JN, Li S, Heard IM, Bisaro DM. Arabidopsis double-stranded RNA binding protein DRB3 participates in methylation-mediated defense against geminiviruses. J. Virol. 2014; 88:2611–22. https://doi.org/10.1128/JVI.02305-13 PMID: 24352449

52. Jackel JN, Storer JM, Coursey T, Bisaro DM. Arabidopsis RNA Polymerases IV and V Are Required To Establish H3K9 Methylation, but Not Cytosine Methylation, on Geminivirus Chromatin. J. Virol. 2016; 90:7529–7540. https://doi.org/10.1128/JVI.00656-16 PMID: 27279611

53. Diezma-Navas L, Pérez-González A, Artaza H, Alonso L, Caro E, Llave C, et al. Crosstalk between epigenetic silencing and infection by tobacco rattle virus in Arabidopsis. Mol. Plant Pathol. 2019; 20:1439–1452. https://doi.org/10.1111/mpp.12850 PMID: 31274236

54. Calil IP, Fontes EPB. Plant immunity against viruses: antiviral immune receptors in focus. Ann. Bot. 2017; 119:711–723. https://doi.org/10.1093/aob/mcw200 PMID: 27780814

55. Matzke MA, Mosher RA. RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. Nat. Rev. Genet. 2014; 15:394–408. https://doi.org/10.1038/nrg3683 PMID: 24805120

56. Wang MB, Masuta C, Smith NA, Shimura H. RNA silencing and plant viral diseases. Mol. Plant Microbe Interact. 2012; 25:1275–85. https://doi.org/10.1094/MPMI-04-12-0093-CR PMID: 22670757

57. Wang Y, Wu Y, Gong Q, Ismayil A, Yuan Y, Lian B, et al. Geminiviral V2 Protein Suppresses Transcriptional Gene Silencing through Interaction with AGO4. J. Virol. 2019; 93:e01675–18. https://doi.org/10.1128/JVI.00656-18 PMID: 30626668

58. Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, et al. Widespread dynamic DNA methylation in response to biotic stress. Proc. Natl. Acad. Sci. U.S.A. 2012; 109:E2183–91. https://doi.org/10.1073/pnas.1209329109 PMID: 22737382

59. López A, Ramírez V, García-Andrade J, Flors V, Vera P. The RNA silencing enzyme RNA polymerase v is required for plant immunity. PLoS Genet. 2011; 7:e1002434. https://doi.org/10.1371/journal.pgen.1002434 PMID: 22242006

60. Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, et al. Herbivory in the previous generation primes plants for enhanced insect resistance. Plant Physiol. 2012; 158:854–63. https://doi.org/10.1104/pp.111.187831 PMID: 22098732

61. Gohlike J, Scholz CJ, Kleitz S, Weber D, Fuchs J, Hedrich R, et al. DNA methylation mediated control of gene expression is critical for development of crown gall tumors. PLoS Genet. 2013; 9:e1003267. https://doi.org/10.1371/journal.pgen.1003267 PMID: 23408907

62. Espinosa NA, Saze H, Sajo Y. Epigenetic Control of Defense Signaling and Priming in Plants. Front Plant Sci. 2016; 7:1201. https://doi.org/10.3389/fpls.2016.01201 PMID: 27563304

63. Aufsatz W, Mette MF, van der Winden J, Matzke AJ, Matzke M. RNA-directed DNA methylation in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 2002; 99 Suppl 4:16499–506. https://doi.org/10.1073/pnas.162371499 PMID: 12169664

64. Matzke MA, Primig M, Trnovský J, Matzke AJ. Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J. 1989; 8:643–9. https://doi.org/10.1002/j.1460-2075.1989.tb03421.x PMID: 16453972

65. Gutzat R, Mittelsten Scheid O. Epigenetic responses to stress: triple defense? Curr. Opin. Plant Biol. 2012; 15:568–73. https://doi.org/10.1016/j.pbi.2012.08.007 PMID: 22960026
66. Boyko A, Blevins T, Yao Y, Golubov A, Bilichak A, Ilnytskyy Y, et al. Transgenerational adaptation of Arabidopsis to stress requires DNA methylation and the function of Dicer-like proteins. PLoS ONE. 2010; 5:e9514. https://doi.org/10.1371/journal.pone.0009514 PMID: 20200866

67. Mermigka G, Verret F, Kalantidis K. RNA silencing movement in plants. J Integr Plant Biol. 2016; 58:328–42. https://doi.org/10.1111/jipb.12423 PMID: 26297506

68. Lewsey MG, Hardcastle TJ, Melnyk CW, Molnar A, Valli A, Urich MA, et al. Mobile small RNAs regulate genome-wide DNA methylation. Proc. Natl. Acad. Sci. U.S.A. 2016; 113:E801–10. https://doi.org/10.1073/pnas.1510721113 PMID: 26787884

69. Tamiru M, Hardcastle TJ, Lewsey MG. Regulation of genome-wide DNA methylation by mobile small RNAs. Proc. Natl. Acad. Sci. U.S.A. 2016; 113:E801–10. https://doi.org/10.1073/pnas.1515072113 PMID: 26787884

70. Lewsey MG, Hardcastle TJ, Melnyk CW, Molnar A, Valli A, Urich MA, et al. Mobile small RNAs regulate genome-wide DNA methylation. Proc. Natl. Acad. Sci. U.S.A. 2016; 113:E801–10. https://doi.org/10.1073/pnas.1510721113 PMID: 26787884

71. Bai S, Kasai A, Yamada K, Li T, Harada T. A mobile signal transported over a long distance induces systemic transcriptional gene silencing in a grafted partner. J. Exp. Bot. 2011; 62:4561–70. https://doi.org/10.1093/jxb/err163 PMID: 21652532

72. Zhang W, Kollwig G, Stecyk E, Apelt F, Dirks R, Kragler F. Graft-transmissible movement of inverted-repeat-induced siRNA signals into flowers. Plant J. 2014; 80:106–21. https://doi.org/10.1111/tpj.12622 PMID: 25039964

73. Parent JS, Martínez de Alba AE, Vaucheret H. The origin and effect of small RNA signaling in plants. Front Plant Sci. 2012; 3:179. https://doi.org/10.3389/fpls.2012.00179 PMID: 22908024

74. Stroud H, Do T, Du J, Zhong X, Feng S, Johnson L, et al. Non-CG methylation patterns shape the epigenetic landscape in Arabidopsis. Nat. Struct. Mol. Biol. 2014; 21:64–72. https://doi.org/10.1038/nsmb.2735 PMID: 24336224

75. Law JA, Jacobsen SE. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat. Rev. Genet. 2010; 11:204–10. https://doi.org/10.1038/nrg2719 PMID: 20142834

76. Cuerda-Gil D, Slotkin RK. Non-canonical RNA-directed DNA methylation. Nat Plants. 2016; 2:16163. https://doi.org/10.1038/nplants.2016.163 PMID: 27808230

77. Matzke MA, Kanno T, Matzke AJ. RNA-Directed DNA Methylation: The Evolution of a Complex Epigenetic Pathway in Flowering Plants. Annu Rev Plant Biol. 2015; 66:243–67. https://doi.org/10.1146/annurev-arplant-043014-114633 PMID: 25494460

78. Matzke MA, Kanno T, Matzke AJ. RNA-Directed DNA Methylation: The Evolution of a Complex Epigenetic Pathway in Flowering Plants. Annu Rev Plant Biol. 2015; 66:243–67. https://doi.org/10.1146/annurev-arplant-043014-114633 PMID: 25494460

79. Wendte JM, Pikaard CS. The RNAs of RNA-directed DNA methylation. Biochim Biophys Acta Gene Regul Mech. 2017; 1860:140–148. https://doi.org/10.1016/j.bbagrm.2016.08.004 PMID: 27521981

80. Della Valle S, Pikaard CS. The RNAs of RNA-directed DNA methylation. Biochim Biophys Acta Gene Regul Mech. 2017; 1860:140–148. https://doi.org/10.1016/j.bbagrm.2016.08.004 PMID: 27521981

81. Zhai J, Bischof S, Wang H, Feng S, Lee TF, Teng C, et al. A One Precursor One siRNA Model for Pol IV-Dependent siRNA Biogenesis. Cell. 2015; 163:445–55. https://doi.org/10.1016/j.cell.2015.09.032 PMID: 26451488

82. Blevins T, Podicheti R, Mishra V, Marasco M, Wang J, Rusch D, et al. Identification of Pol IV and RDR2-dependent precursors of 24 nt siRNAs guiding de novo DNA methylation in Arabidopsis. Elife. 2015; 4:e09591. https://doi.org/10.7554/eLife.09591 PMID: 26430765

83. Singh J, Mishra V, Wang F, Huang HY, Pikaard CS. Reaction Mechanisms of Pol IV, RDR2, and DCL3 Drive RNA Channeling in the siRNA-Directed DNA Methylation Pathway. Mol. Cell. 2019; 75:576–589.e5. https://doi.org/10.1016/j.molcel.2019.07.008 PMID: 31398324

84. Panda K, Ji L, Neumann DA, Daron J, Schmitz RJ, Slotkin RK. Full-length autonomous transposable elements are preferentially targeted by expression-dependent forms of RNA-directed DNA methylation. Genome Biol. 2016; 17:170. https://doi.org/10.1186/s13059-016-1032-y PMID: 27506905

85. Zhang Z, Liu X, Guo X, Wang XJ, Zhang X. Arabidopsis AGO3 predominantly recruits 24-nt small RNAs to regulate epigenetic silencing. Nat Plants. 2016; 2:16049. https://doi.org/10.1038/nplants.2016.49 PMID: 27243648

86. Meister G. Argonaute proteins: functional insights and emerging roles. Nat. Rev. Genet. 2013; 14:447–59. https://doi.org/10.1038/nrg3462 PMID: 23732335
88. Wierzbicki AT, Haag JR, Pikaard CS. Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. Cell. 2008; 135:635–48. https://doi.org/10.1016/j.cell.2008.09.035 PMID: 19013275

89. Cao X, Jacobsen SE. Role of the arabidopsis DRM methyltransferases in de novo DNA methylation and gene silencing. Curr. Biol. 2002; 12:1138–44. https://doi.org/10.1016/s0960-9822(02)00925-9 PMID: 12121623

90. Gallego-Bartolomé J, Liu W, Kuo PH, Feng S, Ghoshal B, Gardiner J, et al. Co-targeting RNA Poly- merases IV and V Promotes Efficient De Novo DNA Methylation in Arabidopsis. Cell. 2019; 176:1068–1082.e19. https://doi.org/10.1016/j.cell.2019.01.029 PMID: 30739798

91. Voinnet O. Use, tolerance and avoidance of amplified RNA silencing by plants. Trends Plant Sci. 2008; 13:317–28. https://doi.org/10.1016/j.tplants.2008.05.004 PMID: 18565786

92. Pontier D, Picart C, Roudier F, Garcia D, Lahmy S, Azevedo J, et al. NERD, a plant-specific GW protein, defines an additional RNAi-dependent chromatin-based pathway in Arabidopsis. Mol. Cell. 2012; 48:121–32. https://doi.org/10.1016/j.molcel.2012.07.027 PMID: 22940247

93. Haag JR, Pikaard CS. Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing. Nat. Rev. Mol. Cell Biol. 2011; 12:483–92. https://doi.org/10.1038/nrm3152 PMID: 21779025

94. Zhou M, Law JA. RNA Pol IV and V in gene silencing: Rebel polymerases evolving away from Pol II’s rules. Curr. Opin. Plant Biol. 2015; 27:154–64. https://doi.org/10.1016/j.pbi.2015.07.005 PMID: 26344361

95. Lahmy S, Pontier D, Bies-Ethève N, Laudie M, Feng S, Jobet E, et al. Evidence for ARGONAUTE4-DNA interactions in RNA-directed DNA methylation in plants. Genes Dev. 2016; 30:2565–2570. https://doi.org/10.1101/gad.289553.116 PMID: 27986858

96. Henderson IR, Zhang X, Lu C, Johnson L, Meyers BC, Green PJ, et al. Dissecting Arabidopsis thaliana DICER function in small RNA processing, gene silencing and DNA methylation patterning. Nat. Genet. 2006; 38:721–5. https://doi.org/10.1038/ng1804 PMID: 16699516

97. Bologna NG, Voinnet O. The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis. Annu Rev Plant Biol. 2014; 65:473–503. https://doi.org/10.1146/annurev-arplant-050213-035728 PMID: 24579988

98. Wang J, Mei J, Ren G. Plant microRNAs: Biogenesis, Homeostasis, and Degradation. Front Plant Sci. 2019; 10:360. https://doi.org/10.3389/fpls.2019.00360 PMID: 30972093

99. Stroud H, Greenberg MV, Feng S, Bernatavichute YV, Jacobsen SE. Comprehensive analysis of silencing mutants reveals complex regulation of the Arabidopsis methylome. Cell. 2013; 152:352–64. https://doi.org/10.1016/j.cell.2012.10.054 PMID: 23313553

100. Fang X, Qi Y. RNAi in Plants: An Argonaute-Centered View. Plant Cell. 2016; 28:272–85. https://doi.org/10.1105/tpc.15.00920 PMID: 26869699

101. Eun C, Lorkovic ZJ, Naumann U, Long Q, Havecker ER, Simon SA, et al. AGO6 functions in RNA-mediated transcriptional gene silencing in shoot and root meristems in Arabidopsis thaliana. PLoS ONE. 2011; 6:e25730. https://doi.org/10.1371/journal.pone.0025730 PMID: 21998686

102. Durán-Figueroa N, Vielle-Calzada JP. ARGONAUTE9-dependent silencing of transposable elements in pericentromeric regions of Arabidopsis. Plant Signal Behav. 2010; 5:1476–9. https://doi.org/10.4161/psb.5.11.13548 PMID: 21057207

103. Cao X, Aufsatz W, Zhilberman D, Mette MF, Huang MS, Matzke M, et al. Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. Curr. Biol. 2003; 13:2212–7. https://doi.org/10.1016/j.cub.2003.11.052 PMID: 14680640

104. 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:e1002195. https://doi.org/10.1371/journal.pgen.1002195 PMID: 21811420

105. Zhang H, Ma ZY, Zeng L, Tanaka K, Zhang CJ, Ma J, et al. DTF1 is a core component of RNA-directed DNA methylation and may assist in the recruitment of Pol IV. Proc. Natl. Acad. Sci. U.S.A. 2013; 110:8290–5. https://doi.org/10.1073/pnas.1300585110 PMID: 23637343

106. Law JA, Du J, Hale CJ, Feng S, Krajevski K, Palanca AM, et al. Polymerase IV occupancy at RNA-directed DNA methylation sites requires SHH1. Nature. 2013; 498:385–9. https://doi.org/10.1038/nature12178 PMID: 23636332

107. 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. https://doi.org/10.1038/s41588-018-0115-y PMID: 29736015
108. Yang DL, Zhang G, Wang L, Li J, Xu D, Di C, et al. Four putative SWI2/SNF2 chromatin remodelers have dual roles in regulating DNA methylation in Arabidopsis. Cell Discov. 2018; 4:55. https://doi.org/10.1038/s41421-018-0056-8 PMID: 30345072

109. Ji L, Chen X. Regulation of small RNA stability: methylation and beyond. Cell Res. 2012; 22:624–36. https://doi.org/10.1038/cr.2012.36 PMID: 22410795

110. Liu ZW, Shao CR, Zhang CJ, Zhou JX, Zhang SW, Li L, et al. The SET domain proteins SUVH2 and SUVH9 are required for Pol V occupancy at RNA-directed DNA methylation loci. PLoS Genet. 2014; 10:e1003948. https://doi.org/10.1371/journal.pgen.1003948 PMID: 24465213

111. Wierzbicki AT, Ream TS, Haag JR, Pikaard CS. RNA polymerase V transcription guides ARGONAUTE4 to chromatin. Nat. Genet. 2009; 41:630–4. https://doi.org/10.1038/ng.365 PMID: 19377477

112. Zhong X, Hale CJ, Law JA, Johnson LM, Feng S, Tu A, et al. DDR complex facilitates global association of RNA polymerase V to promoters and evolutionarily young transposons. Nat. Struct. Mol. Biol. 2012; 19:870–5. https://doi.org/10.1038/nsmb.2354 PMID: 22864289

113. Pikaard CS, Haag JR, Pontes OM, Blevins T, Cocklin R. A transcription fork model for Pol IV and Pol V-dependent RNA-directed DNA methylation. Cold Spring Harb. Symp. Quant. Biol. 2012; 77:205–12. https://doi.org/10.1101/sqb.2013.77.014803 PMID: 23567894

114. He XJ, Hsu YF, Zhu S, Wierzbicki AT, Pontes O, Pikaard CS, et al. An effector of RNA-directed DNA methylation in Arabidopsis is an ARGONAUTE 4 and RNA-binding protein. Cell. 2009; 137:498–508. https://doi.org/10.1016/j.cell.2009.04.028 PMID: 19410546

115. Liu W, Duttkje SH, Hetzel J, Groth M, Feng S, Gallego-Bartolome J, et al. RNA-directed DNA methylation involves co-transcriptional small-RNA-guided slicing of polymerase V transcripts in Arabidopsis. Nat Plants. 2018; 4:181–188. https://doi.org/10.1038/s41477-017-0100-y PMID: 29379150

116. Zhu Y, Rowley MJ, Böhmderger G, Wierzbicki AT. A SWI/SNF chromatin-remodeling complex acts in noncoding RNA-mediated transcriptional silencing. Mol. Cell. 2013; 49:298–309. https://doi.org/10.1016/j.molcel.2012.11.011 PMID: 23246435

117. Ausin I, Mockler TC, Chory J, Jacobsen SE. IDN1 and IDN2 are required for de novo DNA methylation in Arabidopsis thaliana. Nat. Struct. Mol. Biol. 2009; 16:1325–7. https://doi.org/10.1038/nsmb.1690 PMID: 19915591

118. Xie M, Ren G, Zhang C, Yu B. The DNA- and RNA-binding protein FACTOR of DNA METHYLATION 1 requires XH domain-mediated complex formation for its function in RNA-directed DNA methylation. Plant J. 2012; 72:491–500. https://doi.org/10.1111/j.1365-313X.2012.05092.x PMID: 22757778

119. Jullien PE, Susaki D, Yelagandula R, Higashiyama T, Berger F. DNA methylation dynamics during sexual reproduction in Arabidopsis thaliana. Curr. Biol. 2012; 22:1825–30. https://doi.org/10.1016/j.cub.2012.07.061 PMID: 22940470

120. Blevins T, Pontvianne F, Cocklin R, Podicheti R, Chandrasekhar C, Yerneni S, et al. A two-step process for epigenetic inheritance in Arabidopsis. Mol. Cell. 2014; 54:30–42. https://doi.org/10.1016/j.molcel.2014.02.019 PMID: 24657166

121. Peters AH, Kubicek S, Mechtler K, O’Sullivan RJ, Derijck AA, Perez-Burgos L, et al. Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol. Cell. 2003; 12:1577–89. https://doi.org/10.1016/s1097-2765(03)00477-5 PMID: 14690609

122. Jackson JP, Johnson L, Jasencakova Z, Zhang X, PerezBurgos L, Singh PB, et al. Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in Arabidopsis thaliana. Chromosoma. 2004; 112:308–15. https://doi.org/10.1007/s10577-004-0075-7 PMID: 15014946

123. Du J, Johnson LM, Jacobsen SE, Patel DJ. DNA methylation pathways and their crosstalk with histone methylation. Nat. Rev. Mol. Cell Biol. 2015; 16:519–32. https://doi.org/10.1038/nrnm4043 PMID: 26296162

124. Li X, Harris CJ, Zhong Z, Chen W, Liu R, Jia B, et al. Mechanistic insights into plant SUVH family H3K9 methyltransferases and their binding to context-biased non-CG DNA methylation. Proc. Natl. Acad. Sci. U.S.A. 2018; 115:E8793–E8802. https://doi.org/10.1073/pnas.1809841115 PMID: 30150382

125. Du J, Zhong X, Bernatavichute YV, Stroud H, Feng S, Caro E, et al. Dual binding of chromomethylase domains to H3K9me2-containing nucleosomes directs DNA methylation in plants. Cell. 2012; 151:167–80. https://doi.org/10.1016/j.cell.2012.07.034 PMID: 23021223

126. Lachner M, O’Carroll D, Rea S, Mechtler K, Jenuwein T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature. 2001; 410:116–20. https://doi.org/10.1038/35065132 PMID: 11242053

127. Mylne JS, Barrett L, Tessadori F, Mesnage S, Johnson L, Bernatavichute YV, et al. LHP1, the Arabidopsis homologue of HETEROCROMATIN PROTEIN1, is required for epigenetic silencing of FLC. Proc. Natl. Acad. Sci. U.S.A. 2006; 103:5012–7. https://doi.org/10.1073/pnas.0507427103 PMID: 16549797
Zhao S, Cheng L, Gao Y, Zhang B, Zheng X, Wang L, et al. Plant HP1 protein ADCP1 links multivalent H3K9 methylation readout to heterochromatin formation. Cell Res. 2019; 29:54–66. https://doi.org/10.1038/s41422-018-0104-9 PMID: 30425322

Klemm SL, Shipony Z, Greenleaf WJ. Chromatin accessibility and the regulatory epigenome. Nat. Rev. Genet. 2019; 20:207–220. https://doi.org/10.1038/s41576-018-0089-8 PMID: 30675018

Vongs A, Kakutani T, Martienssen RA, Richards EJ. Arabidopsis thaliana DNA methylation mutants. Science. 1993; 260:1926–8. https://doi.org/10.1126/science.8316832 PMID: 8316832

Jeddeloh JA, Stokes TL, Richards EJ. Maintenance of genomic methylation requires a SWI2/SNF2-like protein. Nat. Genet. 1999; 22:94–7. https://doi.org/10.1038/8803 PMID: 10319870

Kankel MW, Ramsey DE, Stokes TL, Flowers SK, Haag JR, Jeddeloh JA, et al. Arabidopsis MET1 cytosine methyltransferase mutants. Genetics. 2003; 163:1109–22. PMID: 12663548

Jones L, Ratcliff F, Baulcombe DC. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Curr. Biol. 2001; 11:747–57. https://doi.org/10.1016/s0029-2215-z PMID: 11373834

Chan SW, Henderson IR, Jacobsen SE. Gardening the genome: DNA methylation in Arabidopsis thaliana. Nat. Rev. Genet. 2005; 6:351–60. https://doi.org/10.1038/nrg1601 PMID: 15861207

Li Y, Kumar S, Qian W. Active DNA demethylation: mechanism and role in plant development. Plant Cell Rep. 2018; 37:77–85. https://doi.org/10.1007/s00299-017-2215-z PMID: 29026973

Choi Y, Gehring M, Johnson L, Hannon M, Harada JJ, Goldberg RB, et al. DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in arabidopsis. Cell. 2002; 110:33–42. https://doi.org/10.1016/s0092-8674(02)00807-3 PMID: 12105995

Zhu J, Kapoor A, Sridhar VV, Agius F, Zhu JK. The DNA glycosylase/lyase ROST functions in pruning DNA methylation patterns in Arabidopsis. Curr. Biol. 2007; 17:54–9. https://doi.org/10.1016/j.cub.2006.10.059 PMID: 17309187

Williams BP, Gehring M. Stable transgenerational epigenetic inheritance requires a DNA methylation-sensing circuit. Nat Commun. 2017; 8:1242. https://doi.org/10.1038/s41467-017-02219-3 PMID: 29242266

Wang J, Blevins T, Podicheti R, Haag JR, Tan EH, Wang F, et al. Mutation of Arabidopsis SMC4 identifies condensin as a corepressor of pericentromeric transposons and conditionally expressed genes. Genes Dev. 2017; 31:1601–1614. https://doi.org/10.1101/gad.301499.117 PMID: 28882854

Córdoba-Cañero D, Cognat V, Ariza RR, Roldán Arjona T, Moliner J. Dual control of ROS1-mediated active DNA demethylation by DNA damage-binding protein 2 (DDB2). Plant J. 2017; 91:1710–11. https://doi.org/10.1111/tpj.13753 PMID: 29078035

Ream TS, Haag JR, Wierzbicki AT, Nicora CD, Norbeck AD, Zhu JK, et al. Subunit compositions of the RNA-silencing enzymes Pol IV and Pol V reveal their origins as specialized forms of RNA polymerase II. Mol. Cell. 2006; 33:192–203. https://doi.org/10.1016/j.molcel.2006.12.015 PMID: 19110459

Huang Y, Kendall T, Forsythe ES, Dorantes-Acosta A, Li S, Caballeró-Pérez J, et al. Ancient Origin and Recent Innovations of RNA Polymerase IV and V. Mol. Biol. Evol. 2015; 32:1788–99. https://doi.org/10.1093/molbev/msv060 PMID: 25767205

Tucker SL, Reece J, Ream TS, Pikaard CS. Evolutionary history of plant multisubunit RNA polymerases IV and V: subunit origins via genome-wide and segmental gene duplications, retrotransposition, and lineage-specific subfunctionalization. Cold Spring Harb. Symp. Quant. Biol. 2010; 75:285–97. https://doi.org/10.1103/sqb.2010.75.037 PMID: 21447813

Luo J, Hall BD. A multistep process gave rise to RNA polymerase IV of land plants. J. Mol. Evol. 2007; 64:101–12. https://doi.org/10.1007/s00239-006-0093-z PMID: 17160640

Haag JR, Brower-Toland B, Krieger EK, Sikorsenko L, Nicora CD, Norbeck AD, et al. Functional diversification of maize RNA polymerase IV and V subtypes via alternative catalytic subunits. Cell Rep. 2014; 9:378–390. https://doi.org/10.1016/j.celrep.2014.08.067 PMID: 25284785

Ma L, Hatlen A, Kelly LJ, Becker H, Wang W, Kovarik A, et al. Angiosperms Are Unique among Land Plant Lineages in the Occurrence of Key Genes in the RNA-Directed DNA Methylation (RdDM) Pathway. Genome Biol Evol. 2015; 7:2648–62. https://doi.org/10.1093/gbe/evv171 PMID: 26338185

Yaari R, Katz A, Domb K, Harris KD, Zemach A, Ohad N. RdDM-independent de novo and heterochromatin DNA methylation by plant CMT and DNMT3 orthologs. Nat Commun. 2019; 10:1613. https://doi.org/10.1038/s41467-019-09496-0 PMID: 30962443

Moran Y, Argon M, Praher D, Technau U. The evolutionary origin of plant and animal microRNAs. Nat Ecol Evol. 2017; 1:27. https://doi.org/10.1038/s41559-016-0027 PMID: 28529980

Castel SE, Martienssen RA. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. Nat. Rev. Genet. 2013; 14:100–12. https://doi.org/10.1038/nrg3555 PMID: 23329111
150. Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science. 2002; 297:1833–7. https://doi.org/10.1126/science.1074973 PMID: 12193640

151. Bühlér M, Verdel A, Moazed D. Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell. 2006; 125:873–86. https://doi.org/10.1016/j.cell.2006.04.025 PMID: 16751098

152. Zaratiegui M, Castel SE, Irvine DV, Kloc A, Ren J, Li F, et al. RNAi promotes heterochromatic silencing through replication-coupled release of RNA Pol II. Nature. 2011; 479:135–8. https://doi.org/10.1038/nature10501 PMID: 22002604

153. Fagard M, Vaucheret H. (TRANS)GENE SILENCING IN PLANTS: How Many Mechanisms? Annu. Rev. Plant Physiol. Plant Mol. Biol. 2000; 51:167–194. https://doi.org/10.1146/annurev.arplant.51.1.167 PMID: 15012190

154. Napoli C, Lemieux C, Jorgensen R. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell. 1990; 2:279–289. https://doi.org/10.1105/tpc.2.4.279 PMID: 12354959

155. van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell. 1990; 2:291–9. https://doi.org/10.1105/tpc.2.4.291 PMID: 2152117

156. Depicker A, Montagu MV. Post-transcriptional gene silencing in plants. Curr. Opin. Cell Biol. 1997; 9:373–82. https://doi.org/10.1016/s0955-0674(97)80010-5 PMID: 9159078

157. Assaad FF, Tucker KL, Signer ER. Epigenetic repeat-induced gene silencing (RIGS) in Arabidopsis. Plant Mol. Biol. 1993; 22:1067–85. https://doi.org/10.1007/BF00028978 PMID: 8400126

158. Ingelbrecht I, Van Houdt H, Van Montagu M, Depicker A. Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc. Natl. Acad. Sci. U.S.A. 1994; 91:10502–6. https://doi.org/10.1073/pnas.91.22.10502 PMID: 7937983

159. Meyer P, Heidmann I. Epigenetic variants of a transgenic petunia line show hypermethylation in trans-gene DNA: an indication for specific recognition of foreign DNA in transgenic plants. Mol. Gen. Genet. 1994; 243:390–9. https://doi.org/10.1007/BF00280469 PMID: 8202084

160. Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science. 1999; 286:950–2. https://doi.org/10.1126/science.286.5441.950 PMID: 10542148

161. Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJ. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J. 2000; 19:5194–201. https://doi.org/10.1093/emboj/19.19.5194 PMID: 11013221

162. Xie Z, Johansen LL, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, et al. Genetic and functional diversification of small RNA pathways in plants. PLoS Biol. 2004; 2:E104. https://doi.org/10.1371/journal.pbio.0020104 PMID: 15024409

163. Zilberman D, Cao X, Jacobsen SE. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science. 2003; 299:716–9. https://doi.org/10.1126/science.1079695 PMID: 1252258

164. Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC. An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell. 2000; 101:543–53. https://doi.org/10.1016/s0092-8674(00)80868-8 PMID: 10850496

166. Herr AJ, Jensen MB, Dalmay T, Baulcombe DC. RNA polymerase IV directs silencing of endogenous DNA. Science. 2005; 308:118–20. https://doi.org/10.1126/science.1106910 PMID: 15692015

167. Onodera Y, Haag JR, Ream T, Costa Nunes P, Pontes O, Pikaard CS. Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. Cell. 2005; 120:613–22. https://doi.org/10.1016/j.cell.2005.02.007 PMID: 15766525

168. Kanno T, Huettel B, Mette MF, Aufsatz W, Jaligot E, Daxinger L, et al. Atypical RNA polymerase sub-units required for RNA-directed DNA methylation. Nat. Genet. 2005; 37:761–5. https://doi.org/10.1038/ng1580 PMID: 15924141

169. Pontier D, Yahubyan G, Vega D, Bulski A, Saez-Vasquez J, Hakimi MA, et al. Reinforcement of silencing at transposons and highly repeated sequences requires the concerted action of two distinct RNA
polymerases IV in Arabidopsis. Genes Dev. 2005; 19:2030–40. https://doi.org/10.1101/gad.348405 PMID: 16140984

172. Bond DM, Baulcombe DC. Epigenetic transitions leading to heritable, RNA-mediated de novo silencing in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U.S.A. 2015; 112:917–22. https://doi.org/10.1073/pnas.1413053112 PMID: 25561534

173. Kanazawa A, Inaba JI, Shimura H, Otagaki S, Tsukahara S, Matsuzawa A, et al. Virus-mediated efficient induction of epigenetic modifications of endogenous genes with phenotypic changes in plants. Plant J. 2011; 65:156–168. https://doi.org/10.1111/j.1365-313X.2010.04401.x PMID: 21175898

174. Dalakouras A, Moser M, Zwiebel M, Krczal G, Hell R, Wassenegger M. A hairpin RNA construct residing in an intron efficiently triggered RNA-directed DNA methylation in tobacco. Plant J. 2009; 60:840–51. https://doi.org/10.1111/j.1365-313X.2009.04003.x PMID: 19702668

175. Pignatta D, Novitzky K, Satyaki PRV, Gehring M. A variably imprinted epiallele impacts seed development. PLoS Genet. 2018; 14:e1007469. https://doi.org/10.1371/journal.pgen.1007469 PMID: 30395602

176. Papikian A, Liu W, Gallego-Bartolomé J, Jacobsen SE. Site-specific manipulation of Arabidopsis loci using CRISPR-Cas9 SunTag systems. Nat Commun. 2019; 10:729. https://doi.org/10.1038/s41467-019-08736-7 PMID: 30760722

177. Dalakouras A, Wassenegger M, Dadami E, Ganopoulos I, Pappas M, Papadopoulou KK. GMO-free RNAi: exogenous application of RNA molecules in plants. Plant Physiol. 2019; 182:38–50. https://doi.org/10.1104/pp.19.00570 PMID: 31285292

178. Regalado A. The Next Great GMO Debate. MIT Technology Review. 2015. Available from https://www.technologyreview.com/s/540136/the-next-great-gmo-debate/.

179. Gohlke J, Mosher RA. Exploiting mobile RNA silencing for crop improvement. Am. J. Bot. 2015; 102:1399–400. https://doi.org/10.3732/ajb.1500173 PMID: 26391704