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An update on RNA-mediated DNA methylation (RdM) mechanism in plants: Biology, evolution and biotechnological applications-a review

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ABSTRACT

DNA methylation is considered to be the most important epigenetic mark in eukaryotes. Plant epigenetic mechanisms are remarkably complex when compared to mammals. The RdDM mechanism serves multiple functions including genome defense, imprinting, paramutation and gene expression control in response to growth, developmental or stress signals. The RdDM involves the production of 24-nt-long siRNAs that can be produced from heterochromatin repetitive sequences, inverted repeats or overlapping transcription. The hallmark of the RdDM is that cytosine methylation occurs in all sequence contexts. The sources and targets of RdDM reside in high-density heterochromatic regions of transposons and repetitive sequence and with less density in euchromatic regions. The core genes of RdDM epigenetic pathway are conserved between monocots and dicots and the mutation in any of these genes exhibits similar phenotypes. However, recent data show that methylation patterns and levels may vary in different genomes. Thus, RdDM plays much wider and more pronounced roles in complex genomes. The current review is aimed at reviewing the RdM mediated mechanisms and its role in plant biology. Further the evolutionary and biotechnology applications are described. As the technology for whole-genome microarrays and high throughput sequencing and profiling of DNA methylation are being used to analyze several plant genomes, comparative epigenomic approaches are identifying newer RdM players and targets. These developments will enable basic gene expression studies and enable biotechnological applications to improve plants for several traits.

Keywords: *DNA methylation, genome defense, paramutation, RdDM, plant biology.*

1.0. Introduction

Methylation and demethylation machinery interact to fine-tune the epigenetic states in response to environmental or developmental cues, or to upregulate or downregulate particular genes [58]. This dynamic regulation of methylation and demethylation machinery preserves the flexibility of the plant epigenome. Mendelian heredity might not be enough for plants to flourish and adapt in a dynamic and changing environment [136].

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The epigenome is fine-tuned by epigenetic responses to changes in growth circumstances and environmental stimuli. Plants are able to live for many generations in tough or nutrient-poor environments because of the way their epigenome is adjusted [1]. However, compared to mammals, plant epigenetic systems seem to be very complex. The most significant epigenetic marker is thought to be DNA methylation [4]. Genome defense, imprinting, paramutation, and gene expression regulation in response to growth, developmental, or stress signals are just a few of the roles played by the RNA dependent D methylation (RdDM) [215]. Given that the RdDM change is reversible and that the putative targets are located in proximity to genes in the euchromatin, likely, RdDM contributes far more to the regulation of gene expression in response to environmental or developmental stimuli than previously believed [11;200]. Monocots and dicots share the basic genes of the RdDM epigenetic pathway, and mutations in either of these genes result in symptoms that are comparable. However, new research indicates that different genomes may have distinct methylation levels and patterns. For instance, the rice genome's promoters are more methylated than those in Arabidopsis [144].

The architecture of the genome influences epigenetic regulation, and larger and more complex genomes may call for more extensive functions for epigenetic silencing. The majority of the DNA sequences found in the genomes of plants and mammals are converted into non-coding RNAs (ncRNAs) [144].

Our knowledge of molecular, cellular, developmental, and evolutionary biology has been completely transformed by the recently identified functions of ncRNAs in gene regulation mechanisms [205]. Plants have a wide variety of ncRNA-based gene silencing mechanisms. In response to a disease state or unfavorable environmental cues, the ncRNAs and their silencing machinery may operate in a cell or tissue-specific manner, at a particular developmental stage [188; 194].

The current review is centered around the RNA mediated DNA silencing in plants given the unique features of this mechanism in comparison other mechanisms. We use PubMed, Google scholar, ResearchGate and Ph. D thesis to source the literature. The initial section provides a basic over view of RNA silencing and RNA epigenetics mechanism along with historical and evolutionary perspectives. The components and mechanism of RdM are described in detail to describe the differences and unique pathways. The third section involves description of the cellular functions of RdM in plants. The last section is targeted at the plausible application of the mechanism in plant biotechnology and engineering.

2.0. RNA-mediated and Epigenetic mediated RNA silencing

A basic cellular mechanism that regulates gene expression and protects cells from viruses and genomic parasites is RNA-mediated silencing, also referred to as RNA interference (RNAi) or RNA silencing [39]. Double-stranded RNA (dsRNA), which is cleaved into short RNAs that recognize and target sequences for downstream regulation by complementarity, is the first step in silencing. Although RNA targets are often considered as post-transcriptional gene silencing (PTGS), small RNAs can also regulate DNA and chromatin sequence-specific modifications [48]. In eukaryotes, RNA-mediated gene silencing began as a defense mechanism against viruses and transposable elements [179]. They have developed to identify chromosomal alterations and control the expression of a sizable portion of endogenous genes through microRNAs [119]. Apart from plants, various fungi [29], flies [123], nematodes [30], and mammals [159] use RNA silencing, indicating its significance and preservation. In short, the silencing mechanism involves the following steps: The nuclease Dicer cleaves dsRNA, separating the short strands and incorporating the antisense strand into the RNA-induced silencing complex (RISC), where it is duplexed with a matching mRNA. The duplex in this complex is bound and cleaved by the Argonaute protein (Ago) [39]. Instead, RNA-dependent RNA polymerases (RdRPs) can use the mRNA as a template to extend the duplex in some organisms, such as nematodes and plants [186]. This new dsRNA increases the strength and duration of silence by serving as a substrate for the production of new short interfering RNAs. Histone or DNA methylation events that suppress transcription are mediated by nuclear RNAi pathways [82]. RNA-directed DNA methylation was first connected to the RNAi by studies in *Arabidopsis thaliana* that showed a correlation between the generation of small interfering RNAs (siRNAs) and PTGS and the corresponding DNA methylation of target loci [221]., Summary of RNA silencing pathways in plants are represented in Table-1.

Small non-coding RNA molecules can cause heritable or persistent alterations in gene expression without changing the underlying DNA sequence [98]. This mechanism is known as epigenetically mediated RNA silencing. Briefly, the mechanisms involve breaking of dsRNA to roughly 21–24 nucleotide fragments.

The cell's own genes are endogenously regulated by miRNAs, or microRNAs. piRNAs (PIWI-interacting RNAs) and siRNAs (small interfering RNAs) enable defense against viruses and transposable elements. This is made possible by PTGS one of the mechanisms of epigenetically mediated RNA silencing. This method involves the RISC complex binding to messenger RNA (mRNA) either fully or partially, which causes the mRNA to be physically blocked from translating or directly cleaved (degraded). This stops the mRNA from becoming a protein. Next is the TGS, where the short RNAs enter the nucleus and instruct enzymes to change the chromatin structure of the promoter of the target gene [39]. To regulate chromatin structure and adjust stress-related pathways, plants use four main epigenetic processes: DNA methylation, histone modification, silencing through small RNA molecules, and the remodeling of chromatin [209]. The creation of RNA scaffolds is a common process by which lncRNAs and short RNAs alter chromatin structure and inhibit transcription [152]. The basic motifs and concepts are preserved throughout eukaryotes, from fission in yeast to mammals, although the machinery that uses RNA scaffolds has undergone significant divergence during evolution. Cellular development and genome stability depend on this mechanism, which serves as a strong genomic defense system [199]. It aids in shielding cells against parasitic DNA (transposons) and viral infections [222]. Without the original RNA trigger, these loops have the potential to become self-reinforcing, passing on the "silent" epigenetic state to subsequent generations [196].

It has also been demonstrated that RNA-mediated gene silencing mechanisms are essential for controlling the expression of endogenous genes and preserving chromatin structure, which ensures genome stability [51]. It plays a part in transposable element control and X chromosomal inactivation, among other processes [88; 178]. A plausible question arises how could the cell ascertain whether the indigenous and imported sequences were the same? Only if an indigenous gene had a sequence that was the same as the inserted sequence would it will be silenced? A direct complimentary connection between the "co-supressed genes" is one potential method [184]. This might occur through direct base pairing between the DNAs; the separated DNAs could form heteroduplexes with strands from the two genomic loci after looping out of intervening segments and melting the DNA duplex. Naturally, sequence information is also carried by RNA, but in the quelling demonstration, it seems that sequences that were not translated into detectable RNA were highly successful in silencing the endogenous gene [85]. Recruitment via nascent RNA is the first step in the mechanism of RNA epigenetics. Next, small-RNA-guided AGO complexes or site-specific RNA-binding proteins associate with nascent coding RNA or non-coding RNA scaffolds to recruit effector complexes that methylate histones or DNA. The function of RNA as a part of self-reinforcing positive feedback loops is carried out in the second stage. These loops play important roles in the epigenetic inheritance of DNA and histone methylation patterns and are exclusive to small-RNA systems with an amplification component [4]. The location of the small-RNA amplification machinery on developing transcripts and their activation by the histone or DNA methylation processes brought on by the short RNAs themselves constitute the crucial event [27]. Figure 1. illustrates the mechanism of RNA-mediated chromatin modification. A vital surveillance mechanism that controls gene expression and protects the genome from alien genetic material is epigenetic RNA silencing [134].

Transposons, which are mobile genetic elements, have the ability to "hop" across the genome and cause mutations [57]. These sequences are identified by small RNAs, which then silence/ "freeze" them at the locus [16]. RNA interference (RNAi) is a mechanism used by cells to target and eliminate the genetic material of invasive viruses [133]. Small RNAs guide DNA methyltransferases to particular genomic loci in plants, stabilizing the chromatin and transferring genetic resistance to subsequent generations [201]. RNA molecules act as structural scaffolds, actively recruiting chromatin-modifying enzymes to keep DNA tightly packed and inaccessible to unusual transcription. When these mechanisms fail, the genome becomes unstable. Loss of RNA silencing pathways can lead to massive transposon mobilization, heightened mutation rates [148], and developmental abnormalities, playing a central role in disease states like cancer and immunodeficiency syndromes [121]. Major RNA silencing technologies in plants is summarized in Table-2.

3.0. Components and proteins of RdM

Plant-specific Pol IV and Pol V polymerases- Higher plants have two RNA polymerases, RNA Pol IV and Pol V, which primarily operate in gene silencing [228]. Unlike Pol II, the catalytic subunits are not essential for viability. The largest and second largest catalytic subunits of these two polymerases have been identified and characterized as NRPD1 and NRPD2 for RNA Pol IV [28]. Forward and reverse genetics screens have established that RdDM is associated with RNA Pol IV and Pol V [193]. Cytosine methylation and siRNA accumulation have been investigated in Pol IV and Pol V mutants. siRNA accumulation is eliminated in Pol IV mutants but not in Pol V mutants, demonstrating that Pol IV operates upstream of Pol V and at separate stages of the RdDM [71]. Pol IV is also involved in the creation of natural antisense siRNAs (nat-siRNA) from the dsRNA generated by overlapping 3' ends of gene pairs [129]. RDM2 has a similar sequence to the fourth subunit of RNA Pol II (RPB4) [71], however it is not an orthologue of RPB4 and has evolved to have a different function in Pol IV and Pol V complexes. Domains Rearranged Methyltransferase 2 (DRM2) is known as NRPD4/NRPE4 for the Pol IV and Pol V complexes, respectively [74]. NRPD4 is necessary for high-level siRNA accumulation, DNA methylation, and transcriptional silencing of transposons and repetitive regions [202]. According to the current understanding of the RdDM process, Pol IV first transcribes methylated DNA to form the first transcripts, which travel into the nucleolus and are converted into dsRNA by RDR2 [168]. The siRNAs are then generated by endoribonuclease DICER-LIKE 3(DCL3) dicing dsRNA and methylating it with HUA ENHANCER 1 (HEN1) [195]. The siRNAs are loaded onto Argonaute RISC Component 4 (AGO4) which can bind to NRPE1 and exit the nucleolus together to find and base pair with the Pol V transcripts at the target loci [226].

AGO proteins- Argonautes are key participants in RNA-based gene silencing mechanisms [165]. They are found in bacteria, archea and eukaryotes, and varies within species [95]. The proteins are divided into three classes according to their evolutionary relationships and capacity to bind short RNAs. Group 1, known as AGO proteins binding to miRNA and siRNAs. Group 2, known as PIWI interacts with RNAs (piRNAs), while the group 3 members are exclusively found in worms and bind to secondary siRNAs [107].

Arabidopsis and rice genomes have 10 and 18 group 1 argonaute-like genes, respectively. Argonaute proteins function similarly to RNAse H endonucleases, but instead of DNA, they target RNA molecules [110]. The proteins have four different functional domains viz., N-terminal, PAZ, MID, and PIWI domains. PAZ, MID, and PIWI domains play essential roles in small RNA pathways [167]. The PAZ domain detects the 3' end of short RNAs [32], while the MID domain binds to the 5' phosphate [96]. The PIWI domain has an endonuclease activity similar to that of RNase H enzymes [72]. AGO1 was initially discovered in Arabidopsis mutants with pleiotropic developmental abnormalities and later found to be involved in PTGS [15]. AGO4 and AGO6 were found to be involved in transcriptional gene silencing [183]. AGO4 was discovered during a forward genetic screen for mutants that are faulty in transcriptional gene silencing at the SUPERMAN (*SUP*) gene locus [53]. In *Arabidopsis*, AGO4 was found to interact with NRPE1.7. The NRPE1 C-terminal domain forms a complex with AGO4 and siRNA [92]. AGO4 may recruit other RdDM components to induce methylation or employ its enzymatic activity to create secondary siRNAs that reinforce silence. AGO6 was discovered in a forward genetic screen for second site TGS suppressors in the ros1-1 mutant [62].

DNA cytosine methyl transferases-In the *Arabidopsis* genome, up to 50% of the cytosines are methylated [31]. DNA methylation is a key epigenetic change. In addition to the core DNA sequence, chromatin structure plays an important role in determining gene transcription [2]. The methylation of cytosine nucleotides contributes to the formation of heterochromatin [6]. Thus, DNA methylation causes gene repression, while demethylation causes gene expression. For example, significant loss of methylation causes large reactivation of transposons [189]. Furthermore, the position of the methylation marks relative to the gene determines how DNA methylation controls gene expression. Methylation in promoter sequences tends to suppress gene expression [46], whereas loss of gene body methylation may not result in considerably greater gene expression [20]. These mechanisms assist fine-tune expression in response to developmental or environmental cues. Cytosine methyltransferases catalyze the methylation of cytosine nucleotides across the genome. The methylation of cytosine nucleotides regulates the extent of heterochromatin and thus the level of gene expression [91]. The methylation mark on DNA can attract methyl-binding proteins (MBP), which can then be used to recruit additional chromatin-modifying and remodeling complexes [154]. Cytosines are found in three sequence contexts: 5' CpG 3', 5' CpHpG 3', and 5' CpHpH 3' respectively. Cytosine in CpG and CpHpG is known as symmetric cytosine [13], while CpHpH is known as asymmetric cytosine [117]. Maintenance methyltransferases can establish methylation on the unmethylated strand of CpG and CpHpG using information from the old methylated strand [50]. This methylation can occur even in the absence of the original methylation signal. First, methylation targets transposable elements and repeat-rich sequences like as centromeric repeats and ribosomal DNA sequences (rDNA) [118]. Second, methylation at CpG loci is extremely abundant, followed by CpHpG and CpHpH loci, respectively [89]. A typical cytosine methyltransferase has four key domains: a binding domain for SAM, a binding site for the DNA target, a catalytic domain that catalyzes the methyl transfer event, and a genome targeting domain [175]. Three primary cytosine methyltransferase classes have been identified in plants.

The Methyltransferase 1 (MET1) [115], chromomethyltransferase (CMT) [60] and the domain-rearranged methyltransferase (DRM) respectively [97]. MET1 is the primary methylation maintenance enzyme at CpG dinucleotides. Met1 mutants have significantly reduced CpG methylation, resulting in morphological abnormalities such as delayed blooming and reduced size [25]. A chromodomain motif located in the C-terminal domain distinguishes the universal and plant-specific CMT chromomethylase. Forward genetic screens have revealed that CMTases are involved in non-CpG methylation. DRM has all of the domains seen in mammalian DNMT3, albeit in a different order. The drm1 and drm2 double mutant shows no morphological abnormalities and very minor alterations in methylation patterns [131]. DRM establish DNA methylation on non-methylated target sequences.

Histone Proteins-To modulate chromatin structure, histone proteins can be methylated, acetylated, deacetylated, phosphorylated, or ubiquitinated [112]. Histone methylation can affect gene expression or repression. Lysines in histones can be monomethylated, demethylated, or trimethylated, which adds another degree of intricacy [73]. Histone modifications related to gene expression include the di- or trimethylation of histone H3 lysine 4 (H3K4me2/3) [192] and the di- or trimethylation of histone H3 lysine 36 (H3K36me2/3) [137]. However, trimethylation of lysine 27 of histone H3 (H3K27me3) is a histone alteration associated with gene repression [230]. The majority of coding sequences are found in euchromatin regions, while repetitive and transposable elements reside in heterochromatin regions. Usually, heterochromatin is subjected to tight and somewhat permanent silencing, whereas euchromatin is subjected to both silencing and activation mechanisms [132]. Chromatin is made up of nucleosome units, which are made up of a DNA helix wrapped around histone octamers (two copies of each H2A, H2B, H3, and H4) [126]. At the molecular level, cytosine methylation and histone changes interact. Silent genes were found to be DNA methylated and deacetylated at histones H3 and H4 [77]. Cytosine methylation can be utilized to trigger histone changes. The *Arabidopsis* AtHDA6 deacetylates the histones of RdDM-silenced promoters. Forward genetics screenings have identified AtHDA6 as a critical maintainer of the silent state, as well as an essential component of RdDM that causes sequence-specific covalent modifications of histones [195]. Chromatin remodeling factors use ATP to shift, destabilize, or slide nucleosomes. Chromatin remodelers regulate gene transcription, DNA replication, repair, and recombination [120]. There are three types of ATP-dependent chromatin remodeling complexes: switching defective/sucrose nonfermenting (SWI/SNF) ATPases [75], initiation switch (ISWI) ATPases [12], and chromodomain and the helicase-like domain (CHD) helicases [5]. The *Arabidopsis* genome contains 41 SWI/SNF2-like chromatin remodeling proteins [103]. The DRD1 subfamily of SNF2-like chromatin remodeling proteins is present only in plants and shares similarities with the Rad54, ATRX, and JBP2 class of SNF2-like family of ATP-dependent chromatin remodeling and motor proteins [14]. DRD1 primarily functions in RNA de novo DNA methylation of cytosines throughout all sequence contexts in the target promoter. Interestingly, the drd1 mutant plants maintained more CG methylation in the target promoter than wild type plants, implicating that DRD1 was required for complete deletion of CG methylation from the target promoter [67].

DRD1 has dual function, in the presence of an RNA trigger it aids in methylation, and in the absence of an RNA trigger, it aids in methylation erasure [220]. The ddm1 mutant, like met1, exhibits demethylation of FWA and a late blooming phenotype [3]. Several loci, including SUP gene, the PAI1-PAI4 inverted repeat (IR), and the NOStro (nopaline synthase) promoter have been demonstrated to sustain DNA methylation even in the absence of DDM1 [223]. The loci of these sequences may be accessible to the methylation machinery without the need for DDM.

Sources and targets of RdDM-SiRNAs measuring 24 nucleotides in length can be produced from a variety of dsRNA sources. These sources include RNA Pol II transcription of inverted repetitions [61] or overlapping transcription of a single transcript [63], replication of ssRNA viruses [130], aberrant RNAs [218], and Pol V-generated ssRNAs [226] from methylation DNA regions. Furthermore, all DNA sequences that complement the isolated siRNAs could be targeted for methylation. In both wild-type and mutant contexts, endogenous targets of the RdDM pathway were identified using genome-wide methylation and transcript profiling [117]. RdDM has been found to primarily target intergenic areas, [103], transposons, repetitions [160], and plant genes in euchromatin [204]. Reactivation of the Copia-type LTRCO family's soloLTR resulted in decreased cytosine methylation and enhanced expression of surrounding sequence targets implicating the role of RdM [141]. The chromatin state influences the reactivation of targets, with highly upregulated targets located in euchromatin whereas targets with modest reactivation are found in heterochromatin [185]. The transposons soloLTR, LTR1, and LTR3 contain similar DNA sequences, but their reactivation differs depending on their chromatin position [41].

Demethylation Machinery-The action of DNA methyltransferases and DNA demethylases determines the genome's final DNA methylation pattern, whether in a specific tissue or during development [50]. Specific de novo DNA methylation that results in a desired methylation pattern may not necessitate the use of DNA demethylases. The plant genome can undergo both passive and active methylation. DNA is hemi-methylated following replication, and the newly manufactured DNA strand requires the action of maintenance DNA methyltransferases to preserve its methylation pattern [116]. Passive demethylation happens when the maintenance DNA methyltransferases fail to methylate the newly produced strand [164]. Active DNA demethylation is the enzymatic removal of the methyl group from 5-methyl cytosine (5-meC). Several DNA glycosylases with demethylase activity have been found in Arabidopsis, including members of the DEMETER family [214]. These proteins include the repressor of silence (ROS1), DEMETER (DME), DEMETER like 2 (DML2), and DEMETER like 3. The most extensively investigated and proven method is base excision repair, which is triggered by 5-meC DNA glycosylases [157]. In plants, DNA demethylases prevent RdDM of transgenes and endogenous genes, regulate imprinting and transposons, and contribute to 5S rDNA chromatin decondensation [219]. Repressor of silencing 1 (ROS1), a DNA glycosylase/lyase, prevents promoter methylation via the RdDM pathway, hence maintaining transgenic and homologous indigenous gene expression [176]. Several maternally imprinted genes rely on demethylases to express themselves. The DEMETER (DME) is a DNA demethylase that regulates the expression of the maternally imprinted MEDEA (MEA) polycomb group gene in endosperm tissue [143].

Plants under stress exhibit lower methylation patterns, regardless of DNA replication, indicating the involvement of active DNA demethylation [17]. DNA demethylases operate in chromatin decondensation to allow gene expression at specific loci. In plants, it has been shown that the levels and activities of the demethylases are tightly regulated and coupled to the levels and activities of methyltransferases. RdDM and chromatin remodeling proteins silence the 5S rDNA repeats seen in centromeric heterochromatin [231]. ROS1 promotes active DNA demethylation and decondenses the 5S rDNA chromatin, allowing gene expression during periods of high protein synthesis demand [100].

4.0. Biology and mechanisms of RdDM

The RdDM pathway is divided into two major steps: First the synthesis of sRNAs and second the recruitment of DNA methylation machinery to particular target loci in the DNA [140]. These two actions combine to form RdDM, which eventually results in DNA methylation of cytosines at specific target loci. Canonical RdDM is the most well studied RdDM accounting for the majority of RdDM activity in a cell. RdDM is attracted preferentially to regions that are already DNA methylated and heterochromatic, where it reinforces existing DNA methylation patterns, resulting in a positive feedback loop [142]. The first stage of the RdDM pathway focuses on the synthesis of sRNAs. A plant-specific RNA polymerase complex, RNA Polymerase IV (Pol IV), is initially recruited to silent heterochromatin via its association with CLASSY (CLSY) proteins and SAWADEE homeodomain homolog 1 (SHH1) [229; 93]. Pol IV transcribes these regions to form short single-stranded RNAs (ssRNAs) of around 30 to 45 nucleotides in length, each of which is the precursor for a single sRNA. RDR2, which physically connects with Pol IV, converts these ssRNAs into doublestranded RNAs (dsRNAs) during co-transcription [129]. The endoribonuclease Dicer-like 3 (DCL3) cleaves the dsRNAs into 24 nucleotide (nt) sRNAs. Almost all 24 nt sRNAs involved in RdDM are created by the Pol IV-RDR2-DCL3 pathway, with a minor proportion produced via alternative pathways [22]. For example, some RNA Polymerase II (Pol II) transcripts with an inverted repeat sequence create double-stranded hairpin structures that can be directly cleaved by DCL3 to produce 24 nt sRNA [155]. In the second part of the pathway, the RdDM DNA methylation machinery is directed to DNA sequences that complement the sRNAs produced in the first part. One strand of each 24 nt double-stranded sRNA is loaded into Argonaute (AGO) proteins AGO4, AGO6, or AGO9 [108]. AGO3 may also be able to function along this pathway. Once generated, the AGO-sRNA duplex seeks and binds complementary sequences along an RNA'scaffold' created by the plant-specific RNA Polymerase V (Pol V) and interacts with SPT5L, IDN2-IDP complex, and the Pol V component NRPE1[68; 182]. This interaction results in the recruitment of the DNA methyltransferase enzyme DRM2 which methylates adjacent DNA.

Unlike canonical RdDM, non-canonical pathways are primarily engaged in generating initial DNA methylation at new target loci, such as novel TE insertions, rather than sustaining heterochromatin [109]. Actively expressing TE insertions, are typically heavily targeted by post-transcriptional gene silencing (PTGS/RNAi). Non-canonical RdDM is predominantly a consequence of the PTGS pathway, resulting in the first creation of a silent heterochromatic state over the new TE or other target locus [180].

Once established, CLSY and SHH1 then recruit Pol IV to the locus, and the canonical RdDM pathway enables long-term silencing maintenance [54]. Some of these transcripts, particularly those derived from TEs, viruses, or certain non-protein-coding transcripts, are targeted by PTGS mechanisms such as miRNAs or RNA interference, resulting in transcript cleavage. RNA-dependent RNA polymerase 6 (RDR6) can convert the resultant fragments into dsRNA, which is then processed into 21-22 nt sRNAs by DCL2 or DCL4 [208]. Most of these 21-22 nt sRNAs are loaded into AGO1 and fed back into PTGS, increasing its efficiency [42]. However, few will associate with AGO6, resulting in RdDM. RDR6-induced dsRNAs can sometimes be processed by DCL3 rather than DCL2/4 [41]. In addition, some Pol II transcripts contain inverted repeat sequences that can form double-stranded hairpin structures. These can be cleaved by DCL proteins in the absence of RDRs to yield either 21-22 nt or 24 nt sRNAs capable of participating in RdDM [173]. The main distinction between the canonical and non-canonical RdDM pathways is the origin and biogenesis of the sRNAs involved. The canonical RdDM route uses 24 nt sRNAs that are unique to that pathway and primarily originate from a single source (the Pol IV-RDR2 complex) [66]. In contrast, non-canonical RdDM pathways use 21-22 nt sRNAs from a variety of sources, allowing de novo DNA methylation to occur at a wide range of loci [23]. These 21-22 nt sRNAs are not limited to non-canonical RdDM; they also function in additional PTGS pathways. The functional outcome of a certain 21-22 nt sRNA depends on the AGO protein it eventually associates with: sRNAs that associate with AGO4, AGO6, or AGO9 result in RdDM and DNA methylation [145], while sRNAs that associate with other AGOs, such as AGO1, largely result in PTGS [122]. Figure-2 illustrates the mechanism of RdM and its components.

Interaction of RdM components with the genome

Because DNA methylation and restrictive histone modifications characterize heterochromatin, most DNA methylation processes in plants identify and interact with repressive histone marks and vice versa, producing positive feedback loops that aid in the maintenance of the repressive chromatin state [235]. The RdDM-associated protein SHH1 detects H3K4me0 and H3K9me2 at heterochromatic loci and recruits Pol IV to these areas, causing further DNA methylation [115]. Similarly, SUVH2 and SUVH9 aid in the recruitment of Pol V to sites containing DNA methylation [80]. Similarly, CMT3 and CMT2 bind and contribute to DNA methylation to H3K9me2-marked heterochromatin, generating their own feedback loop with SUVH4/5/6 [213]. These interactions contribute to strengthen silencing at TEs and other heterochromatic areas. Constant reinforcement of suppressing chromatin changes at heterochromatic loci results in a repressive chromatin state in which DNA and histones (nucleosomes) are densely packed together. This helps to silence gene expression by physically blocking access to the DNA, preventing RNA Polymerase II, transcription factors, and other proteins from commencing transcription [21]. DDM1, a chromatin remodeler, plays an important function in DNA methylation maintenance by temporarily displacing nucleosomes to provide methyltransferases [210] and other factors access to the DNA. However, because the majority of RdDM targets are small TEs located in open, accessible, and gene-rich areas, few RdDM sites require DDM. Dense heterochromatin suppresses RdDM [31], whereas CMT2 and CMT3 mainly act in constitutive heterochromatin and rely heavily on DDM1 to maintain silencing in these locations [79].

Similarly, MET1 needs DDM1 to reach heterochromatin and sustain CG methylation in those locations. MET1 robustly maintains CG methylation across the genome, including at RdDM target sites [86]. Loss of MET1 causes loss of H3K9me2 at some locations, which inhibits Pol IV recruitment and so precludes maintenance of DNA methylation via canonical RdDM, although non-canonical routes remain unaffected [115]. Loss of the histone deacetylase HDA6, which enhances MET1-mediated DNA methylation maintenance at some loci, has a comparable impact [217], implying that numerous mechanisms involved in heterochromatin maintenance likely contribute to RdDM-mediated DNA methylation maintenance. CMT2 and CMT3, which are principally responsible for maintaining CHG and CHH methylation in dense constitutive heterochromatin, are not dependent on RdDM activity. In the cmt2, cmt3 double mutants, numerous TEs on the chromosome arms remain methylated [172], most likely due to RdDM's persistent activity, indicating that CMT2/3 loss does not affect RdDM activity. This shows that RdDM and CMT2/3 operate primarily independently and at separate loci.

Plant DNA methylation mechanisms are mostly self-reinforcing. RdDM Pol IV and Pol V are both recruited to heterochromatic areas with existing DNA methylation, promoting further DNA methylation by canonical RdDM [56]. Positive feedback loops like these can cause DNA methylation activity to expand beyond the intended methylated target sites and into genes or other regulatory elements, negatively affecting gene expression. Passive and active DNA demethylation processes work in opposition to DNA methylation pathways [177]. DNA glycosylases can also actively remove methylated DNA in plants. DNA glycosylases aid in the prevention of DNA methylation from RdDM targets to activated genes [59]. Loss of active DNA demethylation in ros1; dml2; dml3 triple mutants results in a widespread increase in DNA methylation levels [18], whereas ectopic ROS1 expression causes progressive loss of DNA methylation at many loci [197], emphasizing the importance of balancing DNA methylation and demethylation activity. Because ROS1 expression is linked to DNA methylation at a specific TE, it is significantly reduced in plants with deficient RdDM that lose the ability to methylate that, TE [44]. This general process contributes to DNA methylation homeostasis by matching DNA demethylation activity to DNA methylation activity, ensuring that DNA methylation patterns can be maintained stably over time.

5.0. Biological roles of RdM in Plants

As a critical mediator between the genome and environmental factors, the epigenome is intrinsic to plant survival and successful adaptation. RNA-directed DNA methylation (RdDM) contributes to a wide range of biological processes in plants, including the regulation of stress responses, intercellular communication, and the maintenance of genome integrity by suppressing transposable elements (TEs) [156]. In the genome of *Arabidopsis*, studies have revealed that DNA methylation exerts a dosage-dependent influence on gene expression regulation and transposable element (TE) silencing. Moreover, CG and non-CG methylation act redundantly to regulate diverse biological processes, including flowering, trichome development, vascular development, meristem formation, and root cell differentiation [104]. Knockdown of all DNA methyltransferase gene resulted in severe developmental defects such as extreme growth retardation and no floral transitions in Arabidopsis, highlighting the role of RdDM.

RdDM is a conserved epigenetic pathway that mediates siRNA-dependent (TGS) in plants. Experimental analysis in grape (*Vitis vinifera* L.) identified RdDM-related genes belonging to gene families, with conserved gene structures and motif compositions [200]. Expression profiling of these genes exhibited tissue-specific and stress-responsive expression patterns and were differentially regulated by phytohormones and during seed development. Notably, the genes *VvIDN2a*, *VvDRD1a*, *VvRDR1a*, and *VvRDR6* were strongly associated with seed development and ovule abortion, highlighting their potential roles in grape growth, development, and molecular breeding. In woodland strawberry (*Fragaria vesca*), the *FveFDM1* gene was found to play a crucial role in plant growth and development [224]. Loss of *FveFDM1* function caused smaller leaves, flowers, and fruits due to reduced cell division and DNA methylation. Altered expression of genes involved in hormone biosynthesis and the cell cycle was also observed. Although the hormones gibberellin and auxin levels were reduced, external application of these hormones did not restore normal organ size. These results indicate that *FveFDM1* gene regulates organ development mainly through RdDM-mediated methylation [224]. Further investigation in species of Strawberry highlighted that the *FaQR3* gene enables production of furanone a key compound responsible for strawberry aroma. Lower DNA methylation increased *FaQR3* expression and furanone production during fruit ripening, proving that the RdDM pathway is necessary for activity [102].

Epigenetic mutations occurring in nature can significantly affect plant growth and development by modifying gene expression without altering the DNA sequence. For instance, DNA methylation-mediated silencing of the *Lcyc* gene in *Linaria vulgaris* changes floral symmetry, while promoter methylation of *LeSPL-CNR* delays fruit ripening in tomato [113]. Similar to these natural epialleles the *ect-pMADS3* is stably inherited through maintenance of DNA methylation across generations, promoting stable, heritable epialleles, providing a mechanism for creating natural phenotypic variation and contributing to plant evolution [170]. Plants can adapt to environmental stress by developing stress memory, which enable them to respond faster to future stress episodes. Studies implicate in *Arabidopsis thaliana*, siRNAs promote stress memory, regulating gene expression by degrading target mRNAs and promoting RdDM [169]. However, heat stress reduces siRNA production and DNA methylation, leading to the activation of stress-related genes and the *ONSEN* transposable element. Loss of siRNA production leads to increased *ONSEN* activity and are inherited [139]. In *Arabidopsis thaliana* rising temperatures induce epigenetic mechanisms enabling plants to cope with heat stress and transmit stress memory to their offspring. Further, heat stress activates the gene *HSFA2*, which upregulates the histone demethylase gene *REF6*. These two factors reinforce each other's expression through a heritable positive feedback loop [111]. The *REF6–HSFA2* pathway also induces *SGIP1*, which promotes the degradation of *SGS3*, thereby suppressing tasiRNA production [135]. Reduced tasiRNA levels activate *HTT5*, resulting in early flowering but decreased immune responses. This coordinated network of histone modification, transcriptional regulation, and small RNA pathways enables plants to establish transgenerational thermomemory and improve adaptation to heat stress [111]. Table-3 summarizes landscape of RNA mediated stress remodeling in plants.

Advanced studies in *Arabidopsis thaliana* on tissue-specific DNA methylation analyses have revealed dynamic epigenetic changes during sexual reproduction and highlighted the roles of maternal and paternal epigenomes in embryo and endosperm development [74]. These findings suggest that RdDM enables regulate the balance between parental genomic contributions. In addition to the above studies, studies in non-flowering plants indicate that RdDM has an evolutionarily conserved role in sexual reproduction, extending beyond its function in endosperm development [36]. In case of Petunia plants, the activation of pMADS3 through RdDM create stable, active epialleles that may contribute to plant evolution and reveal a regulatory DNA element involved in controlling flower specific gene expression [203]. During endosperm development RDDM play a significant role by regulating the expression of maternal and paternal genes. The endosperm contains high levels of small interfering RNAs (siRNAs), and disruption of RdDM components such as *Pol IV* results in the misexpression of thousands of genes, including imprinted genes [36]. Research findings implicate that RdDM establishes epigenetic marks before fertilization, particularly in the female gametophyte, which influence gene expression after fertilization. Loss of maternal *Pol IV* reduces maternal siRNA accumulation and alters the expression of several parent-specific genes, that maintains genomic imprinting [49]. Furthermore, *Pol IV*-dependent siRNAs contribute to balancing maternal and paternal genome dosage during seed development. Defects in this pathway disrupt DNA methylation, alter imprinted gene expression, and can lead to abnormal endosperm development and reduced seed viability [36].

Another role of RdM mechanism in plant is in senescence. In *Arabidopsis thaliana*, leaf senescence is associated with reduced expression of chromatin-silencing genes, resulting in the activation of transposable elements and relaxation of heterochromatin structure promoting nutrient remobilization from vegetative tissues to reproductive organ [217]. Trejo-Arellano, et.al, 2019, study indicate DNA methylation remained unchanged, with differences mainly confined suggesting that chromatin remodeling, have significant role during leaf senescence. When the plant organs age, DNA methylation gradually decreases, leading to reduced TE silencing [65]. However, this change does not occur in the stem cells of the shoot apical meristem, allowing newly formed organs to maintain stable epigenetic patterns with the transcription factors such as *TCX5* and *TCX6* [40]. Thus, plants maintain different DNA methylation patterns in different tissues depending on their age and function. RdDM also have effect on flowering pattern in plants, as demonstrated in the model plant *Arabidopsis thaliana*. RdDM methylation of tandem repeats in the *FLOWERING WAGENINGEN (FWA)* promoter suppresses the expression of repeats, ensuring normal flowering time. Knock-out studies implicate loss of promoter methylation reactivates *FWA*, resulting in delayed flowering [99].

In plant species with high load of transposable elements (TE), such as Maize and *Brassica rapa* RdDM has roles in reproduction that ensures proper gamete development and seed viability [52]. During gametogenesis, support cells surrounding the germ cells undergo epigenetic reprogramming, resulting in the temporary activation of TEs and the production of TE-derived small RNAs (sRNAs). These sRNAs are thought to move into the germ cells, where they reinforce TE silencing and safeguard genome stability for the next generation in pollens.

Similar RdDM-mediated regulation has also been proposed in root stem cell maintenance. [99]. *Arabidopsis thaliana* studies carried out to understand the involvement of siRNAs and RNA-directed DNA methylation (RdDM) in female germline development have valuable yielded insights [43]. It is a challenging investigation because the female reproductive cells are enclosed within maternal somatic tissues. However, Genetic pattern studies have shown that RdDM components such as RDR2, DCL3, AGO9, and DRM are required to ensure the formation of a single megaspore mother cell (MMC) [127]. Loss of these genes leads to the appearance of multiple MMC-like cells within an ovule. Since AGO9 is expressed in somatic cells surrounding the MMC [147], RdDM is thought to act in these neighboring cells to suppress female germline identity. This regulation occurs through repression of SPL/NZZ via TGS [9]. Furthermore, trans-acting siRNAs (tasiRNAs), which mediate post-transcriptional gene silencing, are also necessary to restrict MMC initiation, indicating that several small RNA pathways work together during early female reproductive development [198].

The disruption of the RdDM pathway during viral infection alters the plant transcriptome and changes disease symptoms. Endogenous sRNA-directed DNA methylation contributes to symptom development independently of plant defence, highlighting an important epigenetic role in plant–virus interactions [187]. Beyond antiviral defence, RdDM also participates in plant responses to bacterial, fungal, and insect pathogens. Interestingly, the impact of RdDM on disease resistance varies among pathogens, as disruption of the pathway can either enhance or reduce susceptibility depending on the type of pathogen, highlighting its complex role in regulating plant immune responses [7]. The figure 3 illustrates the biological roles RdM in plants.

6.0. Evolutionary Perspective of RNA-Directed DNA Methylation

Sequence-specific transcriptional gene suppression via short RNAs is made possible by RNA-directed DNA methylation (RdDM), which is considered a significant evolutionary advance in plants. This mechanism first appeared as a genomic immune system to protect against transposable elements (TEs) from an evolutionary standpoint. RdDM reduces insertional mutations and chromosomal instability by methylating and silencing mobile genomic elements. This innate defense mechanism was appropriated over the course of evolution to control endogenous gene expression, promote genome evolution, and enable adaptive reactions to environmental stress. Pol IV and Pol V, two plant-specific RNA polymerases, most likely originated after the route evolved from primitive transcription mechanisms dependent on RNA polymerase II. These specialized polymerases have developed through gene duplication and functional divergence of RNA polymerase II subunits, allowing plants to develop an efficient mechanism for using DNA methylation to target repetitive DNA and transposable elements [83]. Different selection pressures have acted on NRPF1 and NPRE1 after duplication. According to the findings on a study by Freeling et al. (2015), the branch that leads to the NRPF1 clade is longer Positive selection on particular substitutions may have propelled the evolution toward a novel function, or relaxed selection allowing the accumulation of substitutions may have led to a longer branch length. Similar duplication event is reported by Zhang et al. (2015), in the expansion of the AGO family proteins.

The results implicate a functional diversification of AGO proteins presumably due to expanding small RNA-directed regulatory pathways. The recruitment and specialization of auxiliary proteins such RDR2, DCL3, AGO4, DRM2, and SHH1 also contributed to the evolution of the RdDM pathway. Together, these proteins create 24-nucleotide small interfering RNAs (siRNAs), drive ARGONAUTE proteins to homologous genomic loci, and catalyses de novo cytosine methylation. According to research conducted by Bélanger et al. in 2023, the RDR3 proteins appeared before RDR1/2/6. Since RDR6 is present in all terrestrial plants and filamentous green algae, it is possible that the evolution of phased siRNAs and RDR6 proteins are related. The American sweet flag (*Acorus americanus*), the earliest divergent living monocot species, is the source of the 24-nt reproductive phased siRNA-associated DCL5 protein. The study identified multiple duplication events of AGO genes that were lost, retained, or further duplicated in subgroups, indicating that the evolution of AGOs is complex in monocots.

The complexity of RdDM components varies among evolutionary lineages, but they are conserved among terrestrial plants, according to comparative genomic investigations. However, the canonical RdDM pathways in flowering plants are quite specialized. Stress tolerance (such as salt stress), developmental transitions, and transgenerational epigenetic inheritance are all impacted by the canonical RdDM machinery of angiosperms, with ancestral mechanisms found in basal plants, gymnosperms, or green algae; bryophytes have simpler methylation systems [190]. While the basic machinery is typically retained, RdDM varies across gymnosperms and angiosperms (flowering plants). There are notable differences in DNA methylation patterns, genomic structure, and transposable element composition. While angiosperms usually exhibit dynamic DNA methylation associated with development and environmental responses [78], gymnosperms have incredibly large genomes packed with repetitive DNA and transposable elements, resulting in broad methylation landscapes [191]. Further comparative epigenomic research is required to comprehend the evolutionary divergence of seed plants because gymnosperm. Methylomes are still relatively less understood, especially at single-base resolution. Variations in genomic size, developmental complexity, and species diversity among seed plants have all been influenced by variations in RdDM activity. Although gymnosperms still have a large number of RdDM-associated genes, their distinct DNA methylation landscapes and transposable element regulation suggest lineage-specific divergence after the split between gymnosperms and angiosperms [124]. Ausin et al. (2016), study report a correlation between repeat element and methylation percentage in different contexts including three gymnosperms; *G. biloba, P. taeda, G. montanum* and four angiosperms; *A. thaliana, O. sativa, Z. mays, A. trichopoda*. The study revealed a strong positive correlation in CG and CHG contexts (r = 0.914 and r = 0.759) respectively while CHH methylation displayed a correlation coefficient (r = 0.00003) consistent with similar findings that global and gene body mCG and mCHG positively correlate with genome size. Because of this well-coordinated mechanism, which also maintains genomic integrity, plants have an effective epigenetic defence against transposable elements [16].

From an evolutionary standpoint, the primary selective benefit of RdDM is the suppression of transposable elements. RNA-directed methylation silences transposons because they can disrupt gene function and jeopardize genomic integrity, protecting the genome against harmful insertions.

The emergence of land plants, or embryophytes, led to the development of highly complex silencing mechanisms. To safeguard meiotic genomes, terrestrial plants developed RNA-directed DNA methylation (RdDM) and extended particular chromatin remodeling pathways, such as those controlled by CHROMOMETHYLASES [19]. In order to trap transposons in heterochromatin, plants were able to create and preserve extremely persistent repressive marks (such as H3K9me2 and DNA methylation at CG, CHG, and CHH sites). Over the course of evolution, this mechanism aided in maintaining genome integrity, organizing chromosomes, controlling repetitive DNA, and diversifying plant genomes. Short interfering RNA is used RdDM to mediate de novo DNA methylation, an evolutionarily conserved chemical modification of cytosine bases. Plants use DNA methylation to regulate development, repress transposable elements (TEs), and control gene [190]. Certain epigenetic marks are specific to plants, even though they share many characteristics with fungi. The same epigenetic mark may have distinct activities in various organisms, or separate epigenetic marks may perform identical functions. Additionally, the enzyme systems that produce or remove epigenetic marks are frequently conserved, however there are instances in which they differ significantly across plants and other creatures [213].

Research on a variety of plants suggests that RdDM regulates not just genome defence but also developmental genes, reproductive processes, genomic imprinting, and responses to environmental stresses. Transposable elements make up a large percentage of plant genomes, and their proliferation has been crucial to the diversification and growth of genomes, including RNA repertoire [55]. According to a 2026 study by Liu et al. regulatory innovation and polyploid adaptation in cereal crops are driven by TE-induced expansion of enhancer RNA repertoires. RdM involvement in these additional activities suggests that the system has undergone continuous evolutionary development, allowing plants to combine epigenetic control with environmental adaptability [125]. As a result, RdDM developed as a genome-defense mechanism that maintains chromosomal integrity while silencing these mobile genetic elements. In CG, CHG, and CHH sequence contexts, cytosine methylation is established by the collaboration of specialized proteins and the canonical RdDM pathway. They stress that with the history of seed plants; this pathway has grown more complex and represents a singular evolutionary novelty of plants. Further, the RdDM pathway's evolutionary changes have influenced genome architecture, enhanced genomic stability, and made it easier for seed plants to respond to shifting environmental conditions. Comparative genomic analyses show that while the complexity of RdDM components varies among evolutionary lineages, they are conserved across terrestrial plants. While flowering plants have highly specialized canonical RdDM pathways, bryophytes have simpler methylation systems [151]. Many RdDM-associated genes are still present in gymnosperms, but they exhibit different DNA methylation landscapes and transposable element control, indicating lineage-specific divergence following the split between gymnosperms and angiosperms.

7.0. Biotechnological applications of RdM

RdDM is a key epigenetic mechanism in plant biotechnology. It turns off certain genes without changing the underlying DNA sequence by using siRNAs to direct targeted DNA methylation. It is well regarded for maintaining genomic stability and controlling characteristics.

Stable "epialleles" (heritable features without DNA sequence alterations) can be bred and selected to get beyond stringent GMO restrictions which is one of RdM's benefits in crop biotechnology. Plants are able to adapt to changing environmental conditions because epigenetic alterations are reversible, in contrast to irreversible genetic mutations. Traditional breeding and domestication processes can be accelerated by activating latent transposable elements or altering RdDM machinery, which can produce abrupt, heritable phenotypic alterations. RdDM has been utilized in a number of studies to stabilize genetically modified crops (GMC) by inhibiting "leaky" transgenes, or foreign genes that have been added, to avoid unforeseen phenotypic or environmental impacts. Foreign genes that have been inserted into GMC and express proteins or RNA at low levels when they should be switched off can interfere with research, depleting cellular resources, or have harmful side effects [207]. According to a study by Li et al. (2024), tethering MORC proteins to the unmethylated *FWA* gene promoter in the few mutants via protein fusion to an artificial zinc finger protein 108 (ZF) resulted in effective methylation of the promoter through recruitment of the RdDM machinery at endogenous RdDM sites. Furthermore, the effective de novo methylation and silencing of FWA transgenes are compromised by mutations in the MORC proteins.

Environmental elements like heat or drought can activate RdDM pathways. It helps plants regulate autoimmune defences without compromising development by acting as a "dial" for stress reactions. Target genes can be precisely and reversibly silenced or activated by employing CRISPR-Cas systems coupled to epigenetic modifiers to actively induce or remove methylation at specific loci rather than changing the primary DNA sequence [76]. The study by Chinreddy et al. (2026), shows successful modification of genes governing fruit attributes and stress responses to improve tomatoes. "Epigenetic memory" is based on the RdDM pathway. RdDM can strengthen particular chromatin states that enable plants to withstand future stress when they are subjected to abiotic conditions like drought, cold, or high salinity (Araújo, et al., 2025). Climate-resilient crop varieties can be produced by the stable transmission of these stress-induced epigenetic changes to future generations [149]. An application of this method is demonstrated in adaptability of rice plants to drought conditions is mediated by transgenerational epimutations caused by multi-generational drought imposition [227].

RdDM can be used to control the expression of plant immune receptors or to silence the genes of invasive viruses presenting a viable method for creating crops with strong disease resistance without the need for foreign transgenes. Sequences that target particular viral genes are carried by the altered harmless viral vectors. When a plant becomes infected, the RdDM pathway is activated, which silences the invasive virus [158]. The Papa ring spot virus (PRSV) serves as an example of the application of this technique [24]. In a different work, Kanazawa et al. (2011), used Cucumber Mosaic Virus to recruit RdDM to silence a gene that affects tomato fruit ripening and petunia flower pigmentation. Additionally, the study demonstrated that VIGS's inhibitory impact can intensify with successive generations. Biotechnologists can enhance commercially significant features of crops by modifying the RdDM pathway. Controlling flowering time, enhancing fruit ripeness and flavour, and boosting nutritional content are a few examples. In plants like Arabidopsis, the epigenetic silencing of certain floral repressor genes (like *FWA* or *FLC*) is successfully demonstrated [87]. This entails changing DNA methyltransferases and small interfering RNAs (siRNAs) to control when the plant goes into flowering [171]. Study of Xue et al. (2025), is an example in Chrysanthemum demonstrating CmFDa-mediated epigenetic regulation of flowering According to Cheng et al. (2018), RdDM functions as a developmental switch that either activates or represses ripening-related genes that control texture, fragrance, and pigment production. Hence through genetic engineering of genes in the pathways leading to these traits crops with altered traits can be produced. Small interfering RNAs (siRNAs) guide de novo methylation to specific cytosine sites repressing or activating key transcription factors (e.g., MADS-box genes, *NAP*, *SPL*, *WRKY*, and *NAC*) and ethylene biosynthesis genes such as (*MdACS1*, *MdACS3a*, and *MdACO1*) [101]. A hairpin RNA construct corresponding to the target locus is introduced as part of RdDM to a desired target gene [197]. In premier rice cultivars, hairpin RNA expression has provided broad-spectrum antiviral resistance [146]. Advances in this field of plant biotechnology include the use of CRISPR to artificially tie DRM2 (or other RdDM pathway components) directly to particular target loci. For horticultural plants, RNA silencing technologies have been applied to boost resistance against diseases and pests, modify plant structure and flowering time, enhance the commercial qualities of fruits and flowers, increase nutritional content, eliminate toxic substances and allergens, and create valuable industrial products [64]. Even when the artificial construct is eliminated, the technique permits numerous generation and locus-specific signals. However, additional work on minimizing off-target effects and increasing DNA methylation efficiency is needed before the wide spread applications of the technology.

8.0. Discussion

RNA silencing is an evolutionarily conserved mechanism in eukaryotes. The last two decades have seen multiple overlapping but functionally distinct RNA silencing pathways being unraveled in plants such as the posttranscriptional microRNA and small interfering RNA pathways and the transcriptional RNA-directed DNA methylation pathway [90]. Several of these findings have in turn been exploited for developing artificial RNA silencing technologies such as hairpin RNA, artificial microRNA, intrinsic direct repeat 3' UTR inverted repeat, artificial trans-acting siRNA, and virus-induced gene silencing technologies [38]. While several of these findings have aided research work in basics of gene regulation several of these RNA silencing technologies, such as the hairpin RNA technology, have already been widely used for genetic improvement of crop plants in agriculture [166;64]. RNA silencing is induced by double-stranded RNA (dsRNA) or hairpin structured RNA (hpRNA), involving common factors including Dicer or Dicer-like (DCL) and Argonaute (AGO) family proteins. The RNA silencing pathway has greatly diversified in plants to cope with different functional requirements. According to the source of dsRNA or hpRNA precursor and the functional target of sRNAs, RNA silencing in plants can be classified into 4 overlapping but functionally distinct pathways: microRNA (miRNA) pathway [153], trans-acting small interfering RNA (tasiRNA) pathway [114], RNA-directed DNA methylation pathway [125], and exogenic RNA silencing pathway [47]. Associated with the diversification of RNA silencing pathways, plants have evolved multiple RNA silencing factors.

The components of the mechanism have different players in plants and numbers. For instance, the model plant Arabidopsis encodes four DCLs, six RNA-dependent RNA polymerases (RDRs), and ten AGOs, plus several other factors [150]. These changes and modifications enable specific functions in plants.

Diverse classes of RNA, ranging from small to long non-coding RNAs, have emerged as key regulators of gene expression, genome stability and defense against foreign genetic elements [30]. Small RNAs modify chromatin structure and silence transcription by guiding Argonaute containing complexes to complementary nascent RNA scaffolds and then mediating the recruitment of histone and DNA methyltransferases. In addition, recent advances suggest that chromatin-associated long non-coding RNA scaffolds also recruit chromatin-modifying complexes independently of small RNAs. These co-transcriptional silencing mechanisms form powerful RNA surveillance systems that detect and silence inappropriate transcription events [69], and provide a memory of these events via self-reinforcing epigenetic loops [206]. RNA-directed DNA methylation to the RNAi pathway has been described in *Caenorhabditis elegans*, in fission yeast *Schizosaccharomyces pombe*, protozoa *Tetrahymena thermophila*, as well as in animal germline and somatic cells. Implicating conservation of general role for RNAi and related mechanisms in heterochromatin formation or DNA methylation. Several advantages of RdM RNA silencing technologies are useful tool in gene function analysis and crop improvement for several reasons. i) They are now well-established technologies and easy to use. ii) Complete knock-out of essential genes is lethal to plants and therefore such mutants cannot be recovered by CRISPR/Cas9-like mutagenesis technologies. However, mutants of such genes could be recovered for gene function analysis by incomplete gene knockdown [161]. iii) RdM allow for tissue-specific silencing of a gene [70]. iv) enables simultaneously silence multiple genes using transgenes iv) The RdM mechanism by permanently "remembering" dangerous or inappropriate transcription events, enable robustly protect genome integrity over time [138]. Also, the epigenetic memory units adapt to both internal biological states and external environmental changes, helping cells maintain selective gene repression [26].

Every technology has its limitations. 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 Therefore, RdDM activity must be finely tuned to maintain a balance between repressing TEs and allowing expression of nearby genes. There is also evidence that RdDM plays a role in several other aspects of plant development, including seed dormancy fruit ripening and other pathways involved in flowering. However, most of these data are correlative, and further study is necessary to understand the role of RdDM in these processes. Plausible solutions include combinations of RNA silencing technologies based on the different RNA silencing pathways could enhance the efficiency of silencing. Combining RNA surveillance and RNA-directed DNA Methylation (RdDM) creates a powerful cellular mechanism for epigenetic gene regulation, genome defense, and molecular diagnostics [156]. Cells use this intersection to identify aberrant transcripts and stably silence their source at the DNA level. Researchers can design modular RNA interference (RNAi) systems to combine post-transcriptional silencing with chromatin modifications, allowing for multiplexed, targeted gene regulation without altering the underlying DNA sequence.

Several important questions about the biogenesis and function of non-coding RNAs remain unanswered. The mechanisms that distinguish between different types of transcription and that trigger the generation of different classes of small RNAs remain to be fully understood, although the available evidence indicates a major role for RNA processing events that act co-transcriptionally to determine whether a nascent transcript becomes a functional mRNA or is marked for processing by RNAi and other surveillance mechanisms. Finally, the mechanisms by which lncRNAs participate in the recruitment of Polycomb proteins and other chromatin modifying activities, particularly the molecular basis of specificity, remain poorly defined. We can look forward to answers to these questions and, if the recent past is a guide, to more exciting and unexpected discoveries about the roles of RNA in gene regulation.

Table 1: RNA silencing pathways in plants

Pathway	Salient features	Functions	Reference
miRNA Pathway	20-24-nt sRNAs, sequence complementarity with their target mRNA	Plants regulatory genes such as transcription factor genes. plant development.	Xu and, Chen 2021
Trans-acting siRNA Pathway	21-nt sRNAs derived from non-coding transcript, back-up mechanism to post-transcriptionally silence TEs,	stress responsive	Jacobs T2015
RNA-directed DNA Methylation (RdDM) Pathway	24-nt siRNAs, the principal function of RdDM is to silence TEs and repetitive DNA to maintain genome stability.	Development, stress-biotic and abiotic response.	Erdmann 2020
RNA Silencing Induced by Exogenic Nucleic Acids	invading nucleic acid molecules	Exogenic RNA silencing overlaps with the endogenous siRNA and RdDM pathways	Kryovrysanaki 2020
Sense Transgene-induced RNA Silencing	associated with DNA methylation at promoters of transgenes	Crop improvement, virus resistance	Chen 2018
Anti-viral RNA Silencing	viral 21, 22, and 24- nt siRNAs. Mechanisms such as binding to siRNA duplexes to prevent RISC formation and inducing degradation of AGO1 and stable secondary structures and/or specific subcellular localization are unique features	genetic modification (Host-Induced Gene Silencing), environmentally friendly viral resistance.	Gupta and Mukherjee2019

Table 2: Major RNA silencing technologies in plants

Methodology	Salient features	Applications	Example/reference
hairpin RNA (hpRNA) transgene	contains an inverted repeat sequence	Gene function and trait improvement	Construct Targeting Soybean *FAD2-1* Mroczka, et al., 2010
Artificial miRNAs (amiRNAs)	specific, multiplexed gene knockdown while minimizing the off-target effects	Pest and pathogen resistance, abiotic stress tolerance.	Nicotiana benthamiana phytoendesaturase gene. Golubeva 2025
Artificial tasiRNAs	silence specific target genes.	Metabolic engineering and gene function study	TAS1c locus to impair *Plum pox virus.* Zhao, 2015
3' UTR inverted-repeat (IR) transgene	stem-loop (hairpin) structure triggers RNA interference (RNAi) or post-transcriptional gene silencing to downregulate target genes	High throughput gene silencing and crop improvement	Transgenic Maize Liu 2009
Virus-Induced Gene Silencing (VIGS)	"Knock down" or silence specific target genes without requiring time-consuming stable genetic transformation	Functional genomics, crop improvement	Chili Pepper Arce-Rodríguez 2020.

Table 3: Landscape of RNA mediated stress remodeling in plants

Stress	Pathway	Function
Carbon stress	RdM and CMT2/3 mediated	Transgenerational memory, accelerated growth
Ozone stress	Referential CHG demethylation	Activation of defense and repair genes
Heavy metal stress	RdM	Demethylation and upregulation of detox transporters
Light stress		ROS and chloroplast signaling
Nutrient stress	Te hypermethylation and siRNA guided CHH changes	Fe/zn levels
Drought stress	RdM	Heritable memory
Temperature stress	RdM	Heritable memory
Salt stress	RdM	Cultivar specific methylation

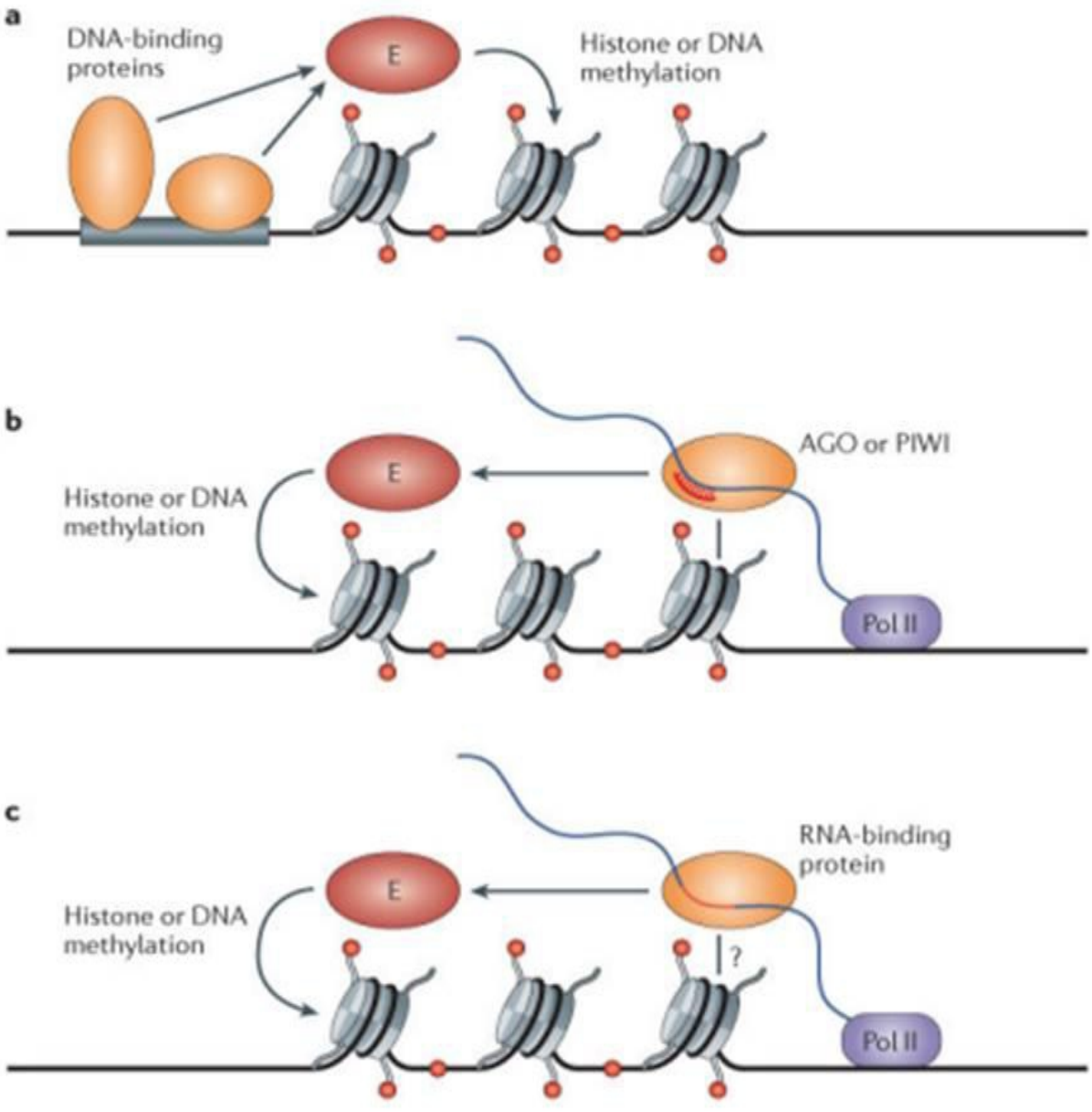

Figure 1: Various paths of Methylation in epigenetic gene expression

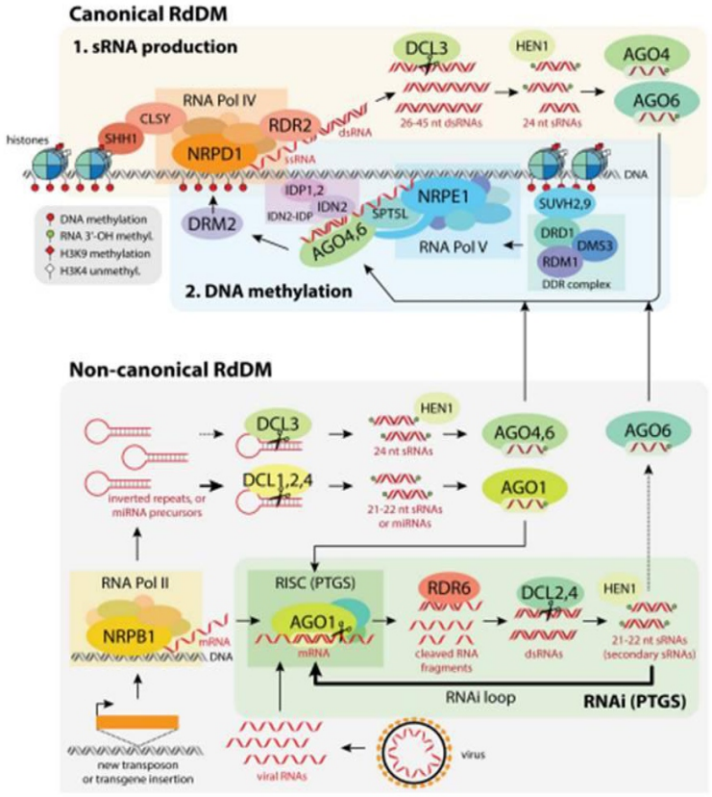

Figure 2: Illustration of the RdM mediated Mechanisms of methylation

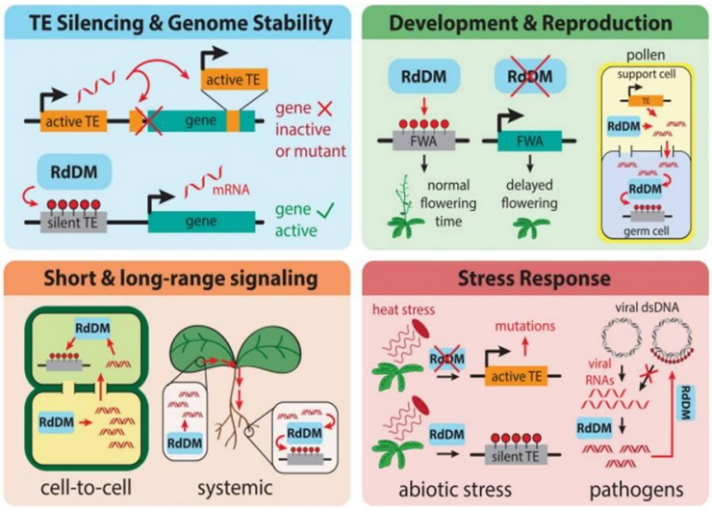

Figure 3: Illustration of various roles of RdM methylation in plants

Conclusion

RNA surveillance functions to sieve out short-lived, long-lived, and nonfunctional RNAs as per cellular requirements and could be a central mode of gene regulation culminating in the control of developmental schemes in plants. RNA surveillance pathways ensure strong control of their target RNAs. Understanding how cells police transcripts is fundamental for advancing both human medicine and agriculture. It bridges messenger RNA surveillance pathways (like nonsense-mediated mRNA decay or NMD) with epigenetic regulation (like RdDM), which are essential for preventing cancer, tackling crop diseases, and discovering new therapeutic targets.

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