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REVIEW

RNA-directed DNA methylation in plants

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Abstract In plants, many small interfering RNAs (siRNAs) direct de novo methylation by DNA methyltransferase. DNA methylation typically occurs by RNA-directed DNA methylation (RdDM), which directs transcriptional gene silencing of transposons and endogenous transgenes. RdDM is driven by non-coding RNAs (ncRNAs) produced by DNA-dependent RNA polymerases IV and V (PolIV and PolV). The production of siRNAs is initiated by PolIV and ncRNAs produced by PolIV are precursors of 24-nucleotide siRNAs. In contrast, ncRNAs produced by PolV are involved in scaffolding RNAs. In this review, we summarize recent studies of RdDM. In particular, we focus on the mechanisms involved in chromatin remodeling by PolIV and PolV.

Keywords siRNAs · DNA methylation · RdDM · PolIV · PolV · RDM · DRM

Abbreviations

lncRNAs	Long non-coding RNAs
siRNAs	Small interfering RNAs
PTGS	Post-transcriptional gene silencing
TGS	Transcriptional gene silencing
RdDM	RNA-directed DNA methylation
AGO	ARGONAUTE
PolII	RNA polymerase II
RDR2	RNA-dependent RNA polymerase 2
dsRNAs	Double-stranded RNAs
DRM2	Domain rearranged methyltransferase 2
RDM	RNA-directed DNA methylation
RITS	RNA-induced transcriptional silencing complex

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Introduction

Overview of RdDM and its function

Zemach et al. (2013) reported that DNA methylation is a conserved epigenetic process in plants and animals. In plants, many 80-nucleotide, long non-coding RNAs (lncRNAs) and 24-nucleotide, small interfering RNAs (siRNAs) direct de novo DNA methylation, which is involved in post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS) stimulated by biotic or abiotic stresses. This process is described as RNA-directed DNA methylation (RdDM) (Onodera et al. 2005) and is a conserved phenomenon mediated by TGS in plants, animals, and fungi (Zhang et al. 2013; Castel and Martienssen 2013). Indeed, RdDM is an essential process for suppressing transposons, DNA damage caused by stress, and DNA nucleases. The process of DNA methylation is an important mechanism for the suppression of repetitive elements (transposons) and stabilization of the genome by silencing endogenous genes (Zhang and Zhu 2011). Small RNAs (sRNAs) 20–40 nucleotides in length that are associated with PIWI-interacting RNAs (piRNAs) catalyze heterochromatic histone modification or DNA methylation recruited by lncRNAs, leading to chromatin remodeling (Zhang et al. 2013).

In plants, the RdDM pathway causes the formation of siRNAs involved in TGS. Some small binding proteins with conserved biochemical activities, such as ARGONAUTE (AGO), play similar roles in various organisms (Vaucheret 2008). In animals, non-coding RNAs (ncRNAs) are generated only by DNA-dependent RNA polymerase II (PolII), but are generated by PolII, PolIV, and PolV in plants. RNA-dependent RNA polymerase 2 (RDR2) is a small protein that receives transcribed messenger RNAs (mRNAs) from PolIV to form stem–loop structures and double-stranded RNAs (dsRNAs) (Haag et al. 2012). Some minor protein factors, known as ATP-dependent chromatin-remodeling (CLSY1) proteins, help RDR2 form dsRNAs using nuclear nucleotides (Smith et al. 2007). After forming dsRNAs 70–80 nucleotides in length, Dicer-like 3 (DCL3) protein, which is a member of the ribonuclease III (RNase III) family of enzymes, cleaves stem–loop mRNAs to form 20–24 nucleotide siRNAs with a 2-nucleotide 3′ overhang. HUA ENHANCER (HEN1) protein, which is a methyltransferase enzyme, methylates siRNAs at the 3′ end to prevent cellular nuclease activities. The presence of the RNA-induced silencing complex (RISC) transfers the 3′ overhang duplex siRNAs to the target mRNA resulting in direct silencing of expression. The RISC binds to duplex siRNAs and unwinds them with its helicase activity (Yang et al. 2006). This process is associated with the PAZ domain, which is found in the AGO, P-element-induced wimpy testis (PIWI), and ZWILLE proteins (Baumberger and Baulcombe 2005). PAZ endonuclease activity separates siRNA duplexes, resulting in the formation of appropriate antisense single-stranded siRNAs that are complementary to target mRNAs. Finally, these siRNAs are loaded onto AGO4/6/9 which leads to RdDM. RdDM targets specific sites to recruit domain rearranged methyltransferase 2 (DRM2), which is an important plant ortholog of the mammalian de novo DNA methyltransferase DNMT3, to initiate de novo DNA methylation (Cao 2002). De novo methylation initiated by DRM2 is associated with DNA methyltransferase 1 (MET 1). In plants, MET1 assists chromomethylase 3 (CMT3), a plant-specific methyltransferase enzyme, to methylate specific CG and CHG sites to cause de novo methylation (Zhu 2008; Aufsatz et al. 2004; Martinez de Alba 2013; Zhu et al. 2013).

The RdDM pathway

PolII depends on PolIV and PolV and has a unique function in the RdDM pathway. PolIV generates single-stranded RNAs (ssRNAs), which are presumed to be precursors of siRNAs. Most 20–24 nucleotide siRNAs are derived from repetitive sequences or transposons. The biogenesis of these siRNAs depends on the plant-specific RdDM pathway in which methylated single-stranded siRNAs are associated with AGO4/6/9 proteins (Zhang and Zhu 2011; Martinez de Alba 2013; Zhu et al. 2013; Havecker et al. 2010). Altered functions among AGO4/6/9 proteins are related to the location of expression in the plant. According to Havecker et al. (2010) AGO4 is typically expressed in plants from buds to roots, but AGO6 is expressed in the apical meristems of shoots or roots and in regenerating tissues. Association of AGO4/6/9 with methylated single-stranded siRNAs triggers de novo methylation leading to transcriptional silencing of repetitive sequences and transposons (Henderson and Jacobsen 2007; Zaratiegui et al. 2007).

As shown in Fig. 1, PolIV associates with both SNF2-like chromatin-remodeling factor, called CLSY1 (Pontier et al. 2005; Kanno et al. 2005; Herr et al. 2005; Onodera et al. 2005), and a small protein known as SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1), which detects histone H3K9me2 (Law et al. 2011; He et al. 2009). DNA transcription factor 1 (DTF1) associates with SHH1 and contributes to the detection of the exact target by PolIV. This protein may assist DNA-directed RNA polymerase IV subunit 1 (NRPD1) which is the largest subunit of PolIV (Law et al. 2011). The *DTF1* gene is plant specific and encodes a DTF1 protein 167 amino acids in length, and contains a cryptic homeodomain in the N-terminal region and a C-terminal domain called SAWADEE that is conserved in plants (Mukherjee et al. 2009). The cryptic homeodomain is a conserved DNA-binding domain with a three-helix structure (Billeter 1996). In contrast, the SAWADEE domain adopts a specific chromo barrel structure involved in detecting and binding to modified histones (Zhang et al. 2013; Law et al. 2013). Mutations in genes encoding DTF1 and PolIV subunit NRPD1 have significant effects on siRNA levels. The *nrpd1* mutant exhibited a reduction in the level of siRNAs of less than 10 %, whereas the *dtf1* mutant exhibited a decrease of approximately 28 % in the level of siRNAs. Studies on mutations also showed that the mutation in *nrpe1* leads to a decrease in the level of siRNAs of ~50 % (Zhang et al. 2013). Based on these results, mutations in downstream genes involved in RdDM cause greater decreases in siRNA levels than mutations in upstream genes and lead to decreased DNA methylation across the entire genome. These studies also showed that association of DTF1 protein with PolIV is necessary for the initiation of RdDM. The specific function of the SAWADEE domain is the detection of histone H3 peptides methylated at lysines 2, 3, and 9 (Zhang et al. 2013). Thus, DTF1 recruits PolIV to methylate histone H3K9 (Law et al. 2013).

As shown in Fig. 1, methylated DNA is transcribed into ssRNAs by PolIV, which recruits RDR2 leading to the

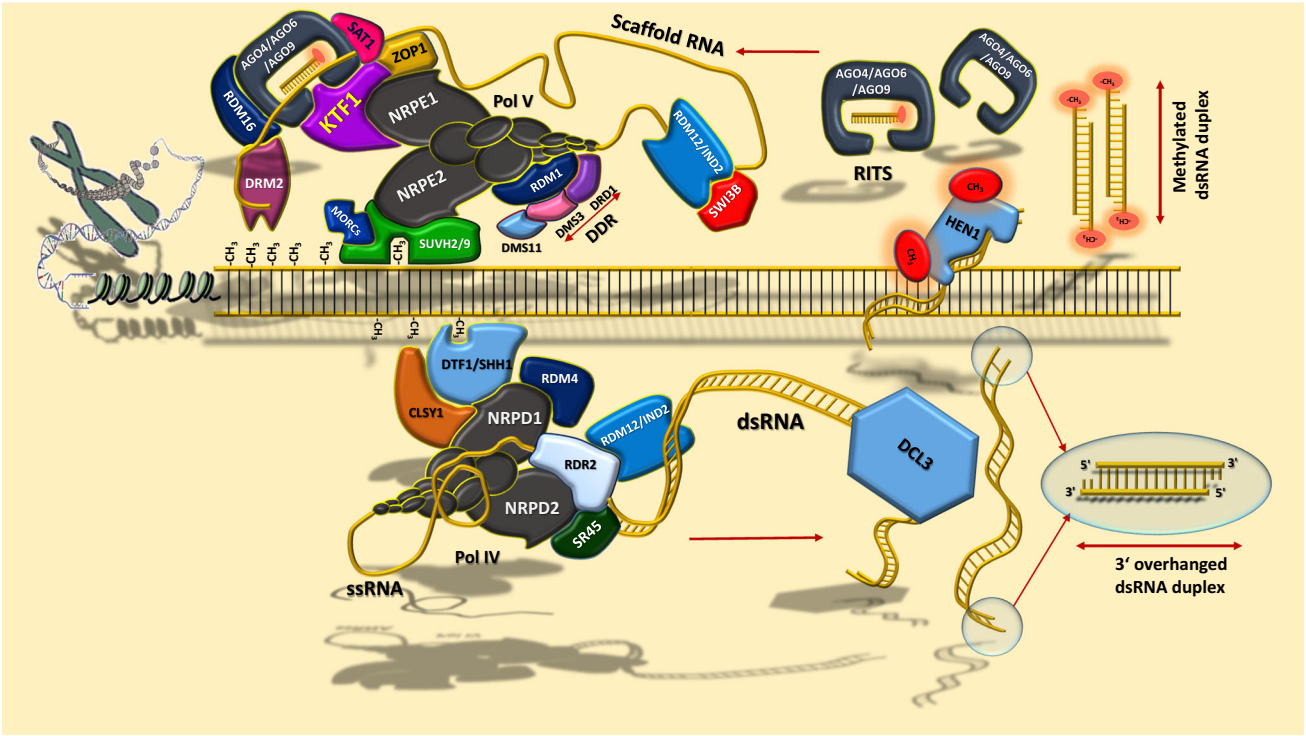

Fig. 1 RNA-directed DNA methylation in plants. The SHH1/DTF1 complex recruits PolIV to chromatin and CLSY1 causes it to initiate RdDM. PolIV transcribes methylated DNA, transposable elements, or repetitive sequences into ssRNA. RDR2 converts ssRNA into dsRNA by interacting with IDN2/RDM12. The SR45 splicing factor acts on PolIV-RDR2 upstream of RdDM to drive siRNA biogenesis. The dsRNA is cleaved by DCL3 into 24-nucleotide siRNA duplexes with 3′ overhangs, which are methylated by HEN1. SiRNA duplexes are converted to one strand and loaded into the RITS complex containing AGO. In contrast, PolV transcribes scaffold RNA from the DNA template. The DDR complex containing RDM1, DRD1, DMS3, and DMS11 associates with PolV to mediate scaffold RNA transcription to recruit DRM2, leading to DNA methylation at specific target loci. KTF1 interacts with the AGO–siRNA complex factor associated with RDM16 leading to the recruitment of STA1, POZ1 splicing factors. RDM12/IDN2 associates with SWI3B to stabilize the interactions between siRNAs and the scaffold RNA to regulate nucleosome positioning

formation of dsRNAs from ssRNAs using RNA-directed DNA methylation 4 (RDM4) protein. RDR2 recruits the INVOLVED IN DE NOVO 2 (IDN2) protein, which is associated with the RNA-directed DNA methylation 12 (RDM12) protein (Xie et al. 2004). Subsequently, DCL3 cleaves the dsRNAs into 24-nucleotide siRNA duplexes to recruit HEN1 to methylate 3′ overhang siRNA duplexes resulting in the avoidance of endonuclease enzyme activities (Xie et al. 2004). Then, methylated siRNA duplexes are loaded onto a RISC-like complex known as the RNA-induced transcriptional silencing (RITS) complex (Verdel et al. 2004). The RITS complex, which includes AGO4/6/9 protein, directs both CG and non-CG DNA methylation leading to the detection of the target loci on the RNA scaffold. This function is related to transcription of the RNA scaffold by PolV interacting with RITS resulting in the recruitment of the DDR complex (Law et al. 2010). The DDR complex is an association of proteins that act widely in silencing but are defective in distinct areas. PolV recruits chromatin-remodeling proteins including defective RNA-directed DNA methylation 1 (DRD1), defective meristem silencing 3 (DMS3), and RDM1 (Law et al. 2010; Kanno et al. 2004).

A chromatin-remodeling factor called SNF2 binds to RDM1 leading to the formation of DMS3, DMS11/GHKL ATPase complex (Lorkovic et al. 2012). In contrast, PolV recruits de novo methyltransferase proteins such as domain rearranged methyltransferase 2 (DRM2) during transcription of the RNA scaffold and interacts with AGO4/6/9–siRNA complex, resulting in linkage of RDM3 and KOW domain-containing transcription factor 1 (KTF1), which is a conserved PolII transcriptional elongation factor (He et al. 2009). The RDM12/IDN2 complex acts as a stabilizer to regulate interactions between AGO4/6/9–siRNAs and PolV (Ausin et al. 2009).

In addition, IDN2 stimulates the switch subunit 3B (SWI3B) protein, which is a subunit of the SWI/SNF DNA remodeling complex, to interact with PolIV, resulting in regulation of the position of DNA methylation inside the nucleus (Zhu et al. 2013). Recently, PolII has been also suggested to play a key role in the RdDM pathway. PolII recruits PolIV and PolV dependent on siRNAs in the exact

DNA target (Zheng et al. 2009). Many coordinators regulate the activity of effector proteins in RdDM. In yeast, for example, a transcription factor complex containing RNA PolII (IWR1)/RDM4/DMS4 regulates interactions between PolII, PolIV, and PolV (He et al. 2009; Kanno et al. 2010). In addition, an ATPase domain termed Morpheus' molecule 1 (MOM1), which is a protein homologous to the SWI2/SNF2 chromatin remolding complex, interacts with PolIV and PolV, resulting in regulation of TGS (Yokthongwattana et al. 2010). According to Zhang et al. (2013), the complex of the zinc finger (ZnF) and OCRE domain-containing Protein 1 (ZOP1) is another splicing factor that affects RdDM directly. Zhang et al. (2013) reported that ZOP1 is expressed downstream of RdDM and is necessary for the pathway (Fig. 1). Moreover, immunofluorescence assays showed that mutation of the *ZOP1* gene in the *zop1* mutant led to decreased PolIV levels and an eventual reduction in RdDM function (Zhang et al. 2013). ZOP1 has been reported to regulate the RdDM pathway directly in association with NPRE1 and DRM2 (Huang and Zhu 2014). In contrast, ZOP1 appears in condensed nucleolus foci including Cajal bodies, which function as the small ribonucleoprotein particle (snRNP) assembly center containing the U1, U2, U5, and U4/U6 snRNAs (Sanford et al. 2005; Morris 2008) and is required for AGO4/6/9 complex formation with 3′ overhang siRNAs (Li et al. 2008). Although, ZOP1 affects PolII and regulates its role in pre-mRNA splicing separate from RdDM (Zhang et al. 2013), recent studies showed that PolII also participates in the RdDM pathway through the generation of noncoding transcripts, leading to the recruitment of AGO4/6/9 (Zheng et al. 2009).

TGS and DNA methylation in the RdDM pathway require specific DNA methyltransferases such as MET1, CMT3, and DRM2 (Kankel et al. 2003). The balance between DNA methylation and DNA demethylation is a requirement for cell survival and is the reason that the action of DNA methyltransferase enzymes is balanced with the action of DNA demethylating glycosylase enzymes, such as Demeter (DME), Demeter-like 2 (DML2), and Demeter-like 3 (DML3) or repressor of silencing 1 (ROS1) and ROS3 (Zheng et al. 2008).

In histone modification, lysines 9 and 27 of histone H3 can be either methylated or acetylated through chromatin remodeling. Some components implicated as being involved in histone modifications are histone deacetylase 6 (HDA6), decreased in DNA methylation 1 (DDM1), ubiquitin protease 26 (UBP26, which deubiquitinates histone H2B and is required for DNA methylation, especially heterochromatic histone H3 methylation), DRD1, and histone methyltransferase SU-(Var)-2-9 homolog (SUVH2-9) (Ebbs and Bender 2006; Ebbs et al. 2005; Aufsatz et al. 2002; Sridhar et al. 2007).

Histone methyltransferase SUHV4 is necessary for the methylation of CHG sites through binding of the SRA methyl-cytosine-binding domain and CMT3, which binds to histone H3 methylated via the chromo domain tail of SUVH4 (Johnson et al. 2007). Thus, histone modification plays an important role in DNA methylation through both methylation and acetylation.

Studies of the RdDM pathway have shown that the RDM family proteins are important regulatory factors with various functions that regulate this pathway. For instance, RDM4 encodes a conserved transcription factor that affects both PolV by transcription of DNA intergenic sequences and PolII by transcription with a pleiotropic effect so, regulates PolII and PolV (He et al. 2009). Nevertheless, the RDM12 protein, which is identified as IDN2 and includes XS domain-containing SGS3 protein, regulates PolIV leading to transcription of 5′ overhang dsRNAs via a facilitator for RDR2, but it functions as a stabilizer of the interaction between the complex of AGO-3′ methylated overhang siRNAs and PolV results in transcription of scaffold RNAs (Ausin et al. 2009; Zhu et al. 2013). Additional studies revealed that RDM16 participates in pre-mRNA splicing and affects the RdDM pathway directly (Huang et al. 2013). A mutation in *RDM16* caused PolV transcripts to decrease but chromatin immunoprecipitation (ChIP) assays showed that the content of RDM16 in PolV target loci was rich (Huang et al. 2013). This observation emphasizes that PolV recruits RDM16 resulting in the regulation of PolV transcription (Huang et al. 2013) (Fig. 1).

Splicing factors play critical roles in the RdDM pathway. The *SR45* gene encodes the SR45 splicing factor, which contains a domain that is rich in serine and arginine and is essential for pre-mRNA splicing associated with PolIV and directs RDR2 leading to the production of dsRNAs (Huang and Zhu 2014). This factor is also suggested to be recruited by PolIV and RDR2 downstream of siRNA biogenesis (Huang and Zhu 2014). Ausin et al. (2012) stated that the mutation in this gene in the *sr45* mutant caused DNA methylation to decrease and caused a decreased flowering phenotype. The *sr45* mutation also caused a decrease in the AGO4 content and removed it from the RdDM pathway upstream of siRNA biogenesis (Li et al. 2006). Based on this result, SR45 should be expressed upstream of the RdDM pathway, leading to regulation of the pathway (Ausin et al. 2012).

As shown in Fig. 1, SRA1 (U5 snRNP) acts as an additional splicing factor involved in RdDM (Dou et al. 2013). Recent studies showed that SRA1 regulates the content of siRNAs by affecting PolV. Also, these studies indicated that SRA1 appears in Cajal bodies similar to POZ1 and associates with NRPE1 (Dou et al. 2013). Thus, SRA1 functions downstream of the RdDM pathway,

leading to effects on PolV transcription and the regulation of siRNA biogenesis.

Conclusion

Many questions remain to be answered regarding RdDM and gene silencing by TGS and epigenetics in cells. For instance, which additional specific proteins are involved in the interaction with DRM2? Which additional specific enzymes such as splicing factors are involved in this pathway? Furthermore, many components of the RdDM pathway remain to be identified using biochemical and biological approaches and genetic screening.

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