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MINI-REVIEW

siRNA-mediated DNA methylation and H3K9 dimethylation in plants

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ABSTRACT

Heterochromatic siRNAs regulate transcriptional gene silencing by inducing DNA methylation and histone H3K9 dimethylation. Recent advances have revealed the distinct phases involved in siRNA mediated silencing pathway, although the precise functions of a number of factors remain undesignated, putative mechanisms for the connection between DNA and histone methylation have been investigated, and much effort has been invested to understand the biological functions of siRNA-mediated epigenetic modification. In this review, we summarize the mechanism of siRNA-mediated epigenetic modification, which involves the production of siRNA and the recruitments of DNA and histone methytransferases to the target sequences assisted by complementary pairing between 24-nt siRNAs and nascent scaffold RNAs, the roles of siRNA-mediated epigenetic modification in maintaining genome stability and regulating gene expression have been discussed, newly identified players of the siRNA mediated silencing pathway have also been introduced.

KEYWORDS epigenetic, DNA methylation, histone methylation, heterochromatic siRNA, argonaute

INTRODUCTION

The genomic DNA of eukaryotes is tightly wrapped on histone octamers and packaged into chromatin (Fig. 1). Nucleosome is the smallest structural unit of chromatin, which consists of an octamer of core histones comprised of two copies each of the four histone proteins H2A, H2B, H3, and H4 associated with 146 base pairs of double-stranded DNA. The covalent modification of chromatin, which determines the structure of chromatin and plays a crucial role in maintaining genomic stability and controlling gene expression include: DNA methylation at the cytosine nucleotide (Espada and Esteller, 2010; Inagaki and Kakutani, 2010) and post-translational histone modifications, such as methylation and acetylation (Fig. 1) (Lachner et al., 2003; Barth and Imhof, 2010). Significant progress has been made in recent years in understanding the functional roles of these chromatin modification marks in transcriptional regulation (Wang et al., 2008; Espada and Esteller, 2010; Inagaki and Kakutani, 2010); numerous noncoding (nc) transcripts, which control the structure and function of chromatin by chromatin modification, have been discovered (Buhler et al., 2007; Johnson et al., 2007; Zaratiegui et al., 2007). Noncoding (nc) transcripts are derived from heterochromatic loci, which are enriched for transposons and are thought to be transcriptionally inactive. Recent studies have found that siRNAs derived from transposon can mediate DNA methylation and histone H3 lysine 9 dimethylation (H3K9me2), and thus not only repress transposons, but also affects the expression of genes that are located next to them (Liu et al., 2004; Jia et al., 2009). In this review, we will focus on this siRNA mediated epigenetic modification and their functions in plants.

DNA METHYLATION

DNA methylation is a major epigenetic modification in the genomes of eukaryotes, which involves the addition of a methyl group to the 5 position of the cytosine pyrimidine ring (5meC) by DNA methyltransferase enzymes. Compared with animals, in which cytosine is methylated exclusively at CpG sequences (Bird, 1986), DNA methylation in plants occurs in all sequence contexts (CpG, CpHpG, and CpHpH, where H is adenine, cytosine, or thymine). In the *Arabidopsis* genome,

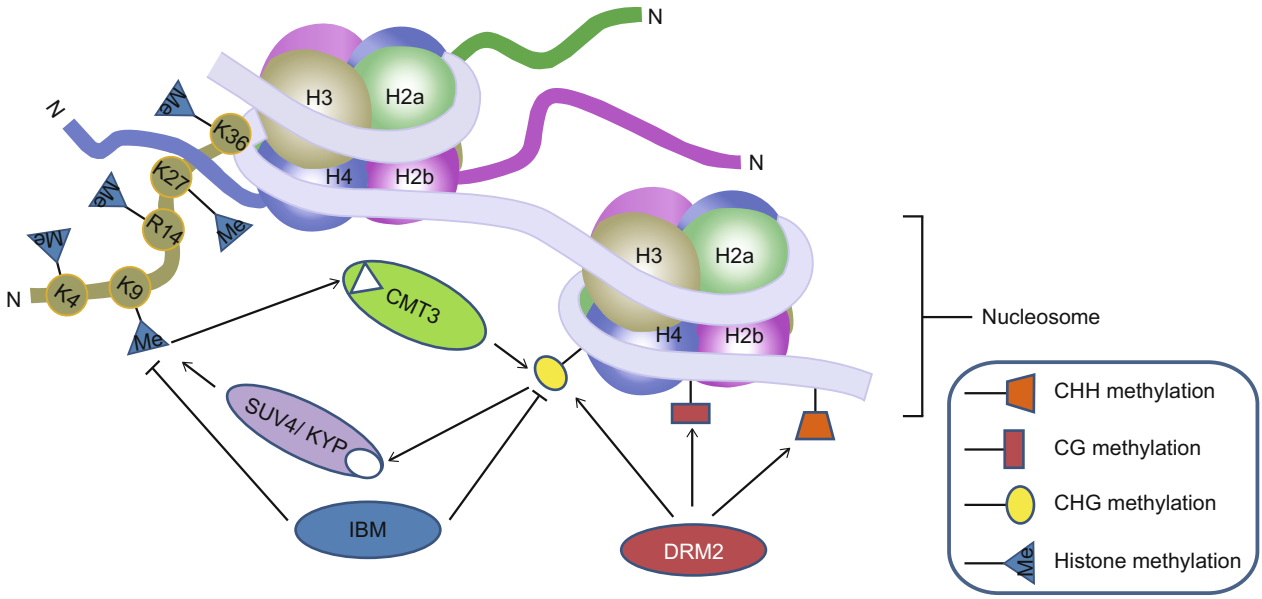

Figure 1. Schematic representation of the structure and modifications of chromatin. The basic unit of chromatin is nucleosome, which consists of an octamer comprised of two copies each of the four histone proteins associated with a segment of double-stranded DNA. Histone protein, which features a main globular domain and a long N-terminal tail, is highly post-translationally modified. Methylation of histone H3 occurs at several different lysine positions in the histone tail and H3K9me2 is performed by a family of SUVH enzymes. DRM2 initiates de novo DNA methylation at all CG, CHG, and CHH sites.The connection between DNA methylation and H3K9me2 could be due to both an active and a passive systems. In the active system, the SRA domains of SUV4/KYP are recruited to the methylated CHG target regions, and the SET domains of the enzyme methylates adjacent histones, while CMT3, which carries a chromodomain, can be recruited to the K9 dimethylated H3 tails and perform DNA methylation at adjacent CHG sites. IBM1, a histone demethylase, functions in the passive system by preventing the coupling of histone and DNA methylation.

about 24% of CG, 6.7% of CHG, and 1.7% of CHH are methylated (Cokus et al., 2008). DNA methylation mainly occurs in gene-coding regions (Tran et al., 2005; Zhang et al., 2006) and transposon-rich heterochromatic regions (Lippman et al., 2004; Zhang et al., 2006; Zilberman and Henikoff, 2007). In *Arabidopsis*, the maintenance of DNA methylation is primarily dependent on three methyltransferases: The DOMAIN REARRANGED METHYLTRANSFERASE 2 (DRM2), a DNMT3 homolog, catalyzes de novo DNA methylation (Zhang et al., 2006; Zilberman and Henikoff, 2007; Saze and Kakutani, 2011; Takuno and Gaut, 2012); METHLYTRANSFERASE1 (MET1), a DNMT1 homolog, is responsible for maintaining CG methylation after DNA replication (Saze et al., 2003; Zilberman and Henikoff, 2007); and CHROMOMETHYLASE3 (CMT3), a plant-specific DNA methyltransferase, functions in CHG (H is A, T, or C) (Fig. 1) methylation maintenance (Cao and Jacobsen, 2002a, 2002b). DNA methylation is a reversible process. A family of 5-methylcytosine DNA glycosylases catalyzes active DNA demethylation in plants (Barreto et al., 2007). Recent studies find that IDM1, a histone acetyltransferase, regulates DNA demethylation by binding methylated DNA thus creating a chromatin environment permissible for 5-methylcytosine DNA glycosylases to function (Qian et al., 2012).

HISTONE METHYLATION

The covalent modification of core histones is the other primary mode of chromatin modification. All four core histones contain a well-structured folded globular domain that is in close contact with the DNA and a less well-structured amino-terminal tail domain (Luger et al., 1997). The N-terminal tail of the core histone octamers protrudes out from the nucleosome particles and is susceptible to a variety of covalent modifications, including methylation, acetylation, and phosphorylation (Bernstein et al., 2007). It is important to point out that covalent modifications of histones, especially methylation is not limited in the N-terminal tails, they occur throughout all regions of the histones. Of the various modifications made to histones, methylation is one of the most complicated, both in terms of the nature of the signal and its biological consequences (Lachner and Jenuwein, 2002). Histone methylation can occur at different residues (lysine and arginine), distinct sites, and at different degrees, It is of note that not only the types and positions of residues, but also the numbers of methyl groups on the same residue affect molecular consequences of gene expression. In *Arabidopsis*, histone lysine methylation occurs mainly at Lys4 (K4), Lys9 (K9), Lys27 (K27), and Lys36 (K36) of histone H3

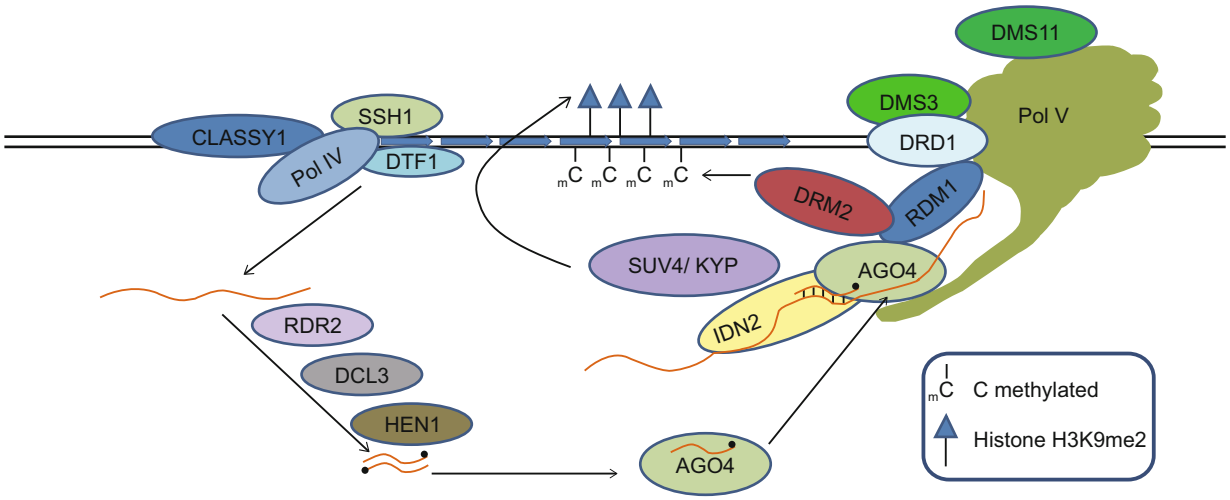

Figure 2. A scheme of siRNA production and siRNA-mediated Transcriptional Gene Silence (TGS). Single-stranded noncoding transcripts are produced by RNA polymerase IV from TE or repeats-containing loci aided by SSH1, DTF1, and chromatin remodeling proteins CLASSY1, 24-nt siRNAs are subsequently produced with RDR2, DCL3, and HEN1 proteins. Scaffold RNAs are produced predominantly by RNA polymerase IV facilitated by DMS11 and DDR complex, which is composed of DRD1, DMS3, and RDM1. De novo DNA methylation enzyme (DRM2) and histone methylation enzymes (SUV4 /KYP and other histone methyltransferases) are recruited to the target sequences assisted by complementary pairing between 24-nt siRNAs and nascent scaffold RNAs. IDN2 may play a role in stabilizing this siRNA-scaffold RNA interaction.

(Fig. 1) (Grossniklaus et al., 1998; Jackson et al., 2002; Ebbs et al., 2005; Ebbs and Bender, 2006; Cartagena et al., 2008; Saleh et al., 2008; Jacob et al., 2009). Histone methylation can be associated with either gene expression or repression. For example, methylation of histone H3 at K4 and K36 is associated with actively transcribed genes, whereas H3 methylation at K9 and K27 is enriched at constitutively condensed chromatin and developmentally inactive global genes (Berger, 2007). Mechanisms that control these various histone methylations are also different. For example, both H3K27 and H3K9 are associated with gene repression, but H3K27 is deposited at genes by the Polycomb group (PcG) proteins (Turck et al., 2007), while H3K9 is induced by heterochromatic siRNA, therefore, the histone methylation pattern we discuss in this paper will be focused on H3K9. Histone H3 lysine methylation is catalyzed by different histone methyltransferases with evolutionarily conserved SET domains (Liu et al., 2010); in *Arabidopsis*, three SET-domain methyltransferases-SU (VAR) 3–9 HOMOLOGUE4 (SUV4, also known as KYP) SUV5 and SUV6 are responsible for H3K9 dimethylation (Fig. 1) (Jackson et al., 2002). Like DNA methylation, histone methylations are not permanent marks either. A large number of enzymes have been discovered with the ability to demethylate methylated histone lysine residues via amine oxidation, hydroxylation, or deaimination (Li et al., 2010).

MECHANISMS OF siRNA DIRECTED DNA METHYLATION AND H3K9 DIMETHYLATION

The mechanism of siRNA directed DNA methylation and H3K9 dimethylation can be divided into the following phases: biogenesis of small interfering RNAs (siRNAs), production of scaffold RNAs, and recruit of silencing complex to target sequences (Fig. 2).

Biogenesis of siRNAs

Plants have several types of endogenous siRNAs, such as: trans-acting siRNAs, natural antisense siRNAs, and heterochromatic siRNAs, with different biogenesis pathways and functions. Heterochromatic siRNAs are the type of siRNAs responsible for mediating gene silencing by directing DNA and histone methylation machineries (Xie et al., 2004; Lu et al., 2005; Kasschau et al., 2007; Zhang et al., 2007; Mosher et al., 2008), therefore, we refer to heterochromatic siRNA as siRNA for the remainder of this paper. siRNAs are usually 24 nt in length and tend to be produced from repeats and transposable elements, although many are also found in intergenic regions. siRNA production, which requires a potential chromatin remodeling protein, CLASSY1 (Herr et al., 2005; Onodera et al., 2005), is initiated by a plant-specific DNA-dependent RNA polymerase: Pol IV, generating single stranded RNAs from a transposon or repeats-containing region (Herr et al., 2005; Onodera et al., 2005; Erhard et al., 2009; Sidorenko et al., 2009; Havecker et al., 2010). It has been recently discovered that the recruitment of Pol IV to target loci requires the assistance of certain proteins, such as SHH1 (Law et al., 2013) and DTF1 (Zhang et al., 2013). The Pol IV transcripts are converted by RNA-dependent RNA polymerase 2 (RDR2) into double-stranded RNAs, which are subsequently cleaved

by DICER-LIKE3 (DCL3) followed by the HEN1 methylation (Xie et al., 2004; Alleman et al., 2006). The resulting 24-nt siRNAs are then loaded onto AGO4 (argonaute 4) or its closely related argonaute proteins and are recruited back to homologous genomic loci (Chan et al., 2004; Ye et al., 2012). These argonaute-bound 24-nt siRNAs can then serve as sequence specific guides for methylation by pairing with complementary DNA or nascent scaffold RNA (Wierzbicki et al., 2009). It is important to point out that heterochromatic silencing may require two rounds of siRNA production. The secondary siRNAs are produced from methylated loci, which are associated predominately with short repetitive sequences in intergenic regions (Lee et al., 2012), for reinforcement and possibly spreading of methylation (Pontier et al., 2005; Matzke et al., 2009; Kanno et al., 2010). RDM1 was found to bind single-stranded methylated DNA and help the recruitment of the silencing complex to methylated DNA (Gao et al., 2010). AGO4 in the complex cuts Pol II and Pol V transcripts that are complementary to the initial trigger siRNAs. RDR2 copies the cleaved transcript fragments into double-strand RNAs, and DCL3 cleavage of double-strand RNAs leads to the production of secondary siRNAs at the methylated loci (Gao et al., 2010).

Production of scaffold RNAs

The DNA and histone H3K9 dimethylation requires that the AGO-siRNA complex and the chromatin methylation enzymes recruited to target loci. Production of long non-coding RNAs (lncRNAs), which act as scaffold RNAs, is a key step in this recruitment, which is independent of siRNA biogenesis shown in *Arabidopsis* mutants defective in NRPD1, RDR2, or DCL3 (Wierzbicki et al., 2008). Pol V plays predominant role in scaffold RNAs production, Pol II is also needed at certain loci (Wierzbicki et al., 2008; Zheng et al., 2009; Wierzbicki et al., 2012). The Pol V-dependent scaffold RNAs production requires a so-called DDR complex, which is composed of a chromatin-remodeling protein DRD1, a chromosome hinge domain protein DMS3 and a protein that binds single-stranded methylated DNA *in vitro* RDM1, to facilitate Pol V transcription (Kanno et al., 2005; Kanno et al., 2008; Law and Jacobsen, 2010). A GHKL-type ATPase, DMS11, is also needed for production of the Pol V transcript (Lorkovic et al., 2012).

Recruit of silencing machinery to target sequences

Mechanism about how the silencing machinery, including DNA methyltransferases and histone methyltransferases, is recruited to target sequences is not fully understood. The recruitment is assisted by complementary pairing between 24-nt siRNAs and nascent scaffold RNAs. Several models have been proposed to explain the recruitment mechanism (Wierzbicki et al., 2008). According to one of the models, after DCL3-generated 24-nt siRNAs being incorporated into AGO4, scaffold RNAs interact with the argonaute-bound 24-nt siRNAs complex by basepairing with siRNAs. IDN2, a recently identified component of the RdDM pathway with the ability to bind dsRNA with 5' overhangs (Zhang et al., 2012) may play a role in this siRNA-scaffold RNA interaction to stabilize interactions between an ARGONAUTE bound siRNA and a scaffold RNA synthesized at a target locus by the Pol V complex (Finke et al., 2012). The interaction of the siRNA with the scaffold RNAs might then direct the silencing machinery, including the de novo cytosine methyltransferase DRM2 and/or histone modifying activities, to the target loci. An alternative possibility is that scaffold RNAs may stabilize siRNA-DNA interactions by binding directly to AGO4. The third possibility is that scaffold RNAs may increase the interaction efficiency of AGO4 with target sequence by influencing structural features of heterochromatin (Wierzbicki et al., 2008). Recruitment of silencing machinery including DNA methyltransferases and histone methyltransferases to the target sequences results in the silencing of specific genomic loci.

A CONNECTION BETWEEN H3K9 AND DNA METHYLATION

A genome-wide analysis has showed a high level of co-localization of H3K9me2 and CHG methylation (Bernatavichute et al., 2008), suggesting a connection between these epigenetic marks. Possible explanations for the connection have been proposed as a result of the combined action of recruitment of CMT3-KYP to the target regions (Law and Jacobsen, 2010) and exclusion of H3K9me2 from genic regions by INCREASE IN BONSAI METHYLATION1 (IBM1) (Miura et al., 2009; Inagaki et al., 2010).

The connection between H3K9 and DNA methylation could be due to protein modules recognizing methylated cytosine and H3K9 residues respectively. KYP has a SRA domain, which is one of the three protein domains that recognize methylated cytosines, preferentially binding double-stranded oligonucleotides containing methylated cytosines in a non-CG context. This suggests that this histone modifier can read DNA methylation marks directly (Johnson et al., 2007). Furthermore, CMT3 carrying a chromodomain can recognize and bind K9 dimethylated H3 tails (Lindroth et al., 2004; Du et al., 2012). The interdependent recruitment of CMT3 and KYP couples the regulation of DNA methylation and histone H3K9 dimethylation by a positive feedback loop (Lindroth et al., 2004; Johnson et al., 2007).

The other putative mechanism includes removal of H3K9me2 by IBM1 (Saze et al., 2008). IBM1 encodes a histone demethylase that catalyzes the removal of H3K9 monomethylation and dimethylation in *Arabidopsis* (Inagaki et al., 2010). A loss-of-function of IBM1 causes ectopic H3K9 methylation at the BONSAI locus, leading to KYP- and CMT3-dependent non-CG DNA hypermethylation and gene silencing (Saze et al., 2008). Genome-wide profiling has shown that a large number of genes are hypermethylated at non-CG cytosines and histone H3K9 in IBM1 (Miura et al., 2009; Inagaki et al., 2010), suggesting that IBM1 protects protein-coding genes from repression via H3K9 and non-CG DNA methylation (Saze et al., 2008). Recent study suggested that IBM1 can also regu-

late gene expression by indirect silencing of gene expression through siRNA-dependent repression, in addition to direct silencing of gene expression through preventing the coupling of histone and DNA methylation (Fan et al., 2012).

EFFECTS OF siRNA-MEDIATED EPIGENETIC MODIFICATION

Effects of siRNA-mediated epigenetic modification have been well documented. It is important for genome stability over generations, as well as for regulation of gene expression. Maintaining genome integrity by silencing transposable elements is the major function of siRNA-mediated epigenetic modification. DCL3-generated 24-nt siRNAs are first incorporated into AGO4 and form a complex, which assist the recruit of DNA methyltransferases and histone H3K9 methyltransferases to the target sequence (Qi et al., 2006). Loss-of-function mutations in AGO4 can supress DNA methylation and histone H3K9 methylation, which results in reactivation of heterochromatic loci (Zilberman et al., 2003; Xie et al., 2004).

Although siRNA-mediated epigenetic modification typically participates in the suppression of transposable and repetitive sequences and in genome stability, it also plays an important role in the expression of a large number of endogenous genes. In *Arabidopsis*, variation in flowering time is due to an epiallele of FLOWERING LOCUS C (FLC) generated by siRNA mediated gene silencing. A transposon inserted into an intron of the FLC, from which siRNAs are generated. These siRNAs direct methylation of the transposon region, which spreads into the adjacent FLC gene to result in low levels of FLC expression and early flowering (Liu et al., 2004). Another example is the regulation of the FWA gene by a SINE transposon insertion in the promoter. These transposons give rise to siRNAs that trigger DNA methylation of the promoter, which lead to the repression of FWA gene (Soppe et al., 2000; Chan et al., 2006). When drm2 mutant plants are transformed with FWA, the introduced transgenes remain unmethylated, which results in FWA expression and late flowering (Chan et al., 2006). Regulation of SDC, a gene encodes an F-box protein and possesses seven direct repeats in its promoter, also displays the function of siRNA-mediated methylation. SDC repeats generate siRNA that trigger DNA methylation and transcriptional gene silencing (Henderson and Jacobsen, 2008). Loss of SDC silencing results in downward-curled leaves instead of flat leaves that are produced by wildtype *Arabidopsis*.

CONCLUSIONS AND PERSPECTIVE

It is clear that specific genomic loci are targeted for silencing through 24-nt heterochromatic siRNAs. siRNAs can mediate DNA methylation and/or H3K9 dimethylation of target sequences and thus affects the transcription of the target loci. siRNA-mediated epigenetic modification mechanism involves the biogenesis of small interfering RNAs (siRNAs), production of scaffold RNAs, and recruit of silencing complex to target sequences, these two siRNA-mediated epigenetic marks are conncected by the combined action of active and passive systems. Numerous evidences have proved the importance of siRNAs mediated DNA methylation and/or H3K9 dimethylation in regulating gene expression and genome stability. Despite all these accomplishments, several as yet unanswered questions remain in the siRNA-mediated silencing pathway. For example: How DNA methyltransferases and histone H3K9 methyltransferases are recruited by AGO4/siRNAs? How particular sequences are recognized or eliminated as substrates of DNA methyltransferases and histone methyltranfereases? what factors provide specificity to CMT3-KYP for recognition of methylated cytosines in specific regions? Are there more key players and targets involved in siRNA-mediated epigenetic modification? Future studies that address these questions will lead to a better understanding of siRNA-mediated silencing pathway.

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COMPLIANCE WITH ETHICS GUIDELINES

Chi Xu, Jing Tian and Beixin Mo declare that they have no conflict of interest.

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