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REVIEW

MicroRNA-mediated DNA methylation in plants

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Abstract **DNA methylation, a major event in epigenetics, plays an essential role in the control of gene expression. Increasing evidence suggests that long and short non-coding RNAs are involved extensively in plants to direct the establishment, spread, and removal of DNA cytosine methylation throughout their genomes. Yet, little has been known about the role of microRNAs (miRNAs) in DNA methylation although the role of small interfering RNAs (siRNAs) in DNA methylation has been well established. Several recent studies, however, provided the evidence for miRNA-directed DNA methylation in plants, and the working mechanisms still need to be fully explored. In this review, we highlight the key features of miRNA-directed DNA methylation in plants and provide insight into the complexities of such an event in plants. The interaction between miRNAs and the epigenetic machinery and the future potential research questions are briefly discussed.**

Introduction

In plants, epigenetic changes are frequently induced by DNA methylation, histone modifications, and chromatin remodeling (Wolffe and Matzke, 1999; Henderson and Jacobsen, 2007; Chinnusamy and Zhu, 2009; Ahmad et al., 2010). DNA methylation occurs most on a cytosine and is achieved through the addition of a methyl group onto the 5 position of the cytosine pyrimidine ring by methyltransferases. Cytosine DNA methylation is a typical epigenetic modification of DNA that can induce stable and heritable repression of transcription. DNA methylation plays an important role in the epigenetic regulation of genes although most methylated DNA sequences found in genomes are transposable elements and DNA repeats (Chan et al., 2005). Small RNAs (sRNAs) are essential triggers in directing DNA methylation on cytosine residues of different motifs including CpG, CpNpG, and CpHpH, emerging as potent regulators in the gene transcriptional networks in most eukaryotes (Zilberman et al., 2003; Onodera et al., 2005). Cellular sRNAs exist in a similar structure but with different names termed microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs), depending on their precursor structures, biogenesis pathways, and subsequent functions (Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009).

The most relevant and extensively studied small RNAs in relation to DNA methylation are siRNAs. Production of methylation-related siRNAs requires specific members of siRNA biogenesis and RNA transcription pathways such as RNA-dependent RNA polymerase 2 (RDR2) (Xie et al., 2004), Dicer-like 3 (DCL3), Argonaute 4 (AGO4), and also two DNA-dependent RNA polymerases, Pol IV and Pol V (Kanno et al., 2005; Matzke et al., 2009). While it is well established that siRNA pathways govern DNA methylation at transposing and repetitive DNA loci (Herr et al., 2005; Onodera et al., 2005; Cokus et al., 2008; Pontes et al., 2009; Zheng et al., 2009), a similar role of miRNA in DNA methylation was observed in an insignificant number of cases.

MiRNAs, the products of small non-coding genes, originated from hairpin-like single-stranded RNA precursors (pre-miRNAs and pri-miRNAs) transcribed mainly by Pol II (Lee et al., 2002; Bartel, 2004). In plants, mature miRNA is produced predominantly by the type III endoribonuclease Dicer-like 1 (DCL1) (Park et al., 2002; Liu et al., 2005; Sunkar et al., 2005a). Previously, miRNAs were shown to affect gene expression primarily at the post-transcriptional level through mRNA cleavage or translational repression (Llave et al., 2002; Tang et al., 2003; Chen, 2004; Brodersen et al., 2008; Lanet et al., 2009; Voinnet, 2009; Wu et al., 2009; Chellappan et al., 2010). Recent studies, however, have identified a novel population of miRNAs (23–27 nt), which are longer and different from the canonical miRNAs

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(20–22 nt) in *Arabidopsis*, rice and moss (*Physcomitrella patens*) (Zhu et al., 2008; Vazquez et al., 2008; Wu et al., 2009; Chellappan et al., 2010; Khraiwesh et al., 2010). These long miRNAs are generated by DCL3 and specifically associated with AGO4 protein. More importantly, the MIR-derived 23- to 27-nt species can guide cytosine DNA methylation not only at their own gene loci *in cis,* but also at their target gene loci *in trans*, occasionally resulting in transcriptional gene silencing. Here, we conduct a brief review on the recent advances in miRNA-directed DNA methylation in the plant kingdom over the past seven years.

miRNA-directed DNA methylation in different plant species

miRNA-related siRNA-directed DNA methylation in the dicot *Arabidopsis*

With a sequenced genome, *Arabidopsis thaliana* provides an excellent model for groundbreaking studies. The first evidence for DNA methylation mediated by miRNA in the plant kingdom was obtained from an experiment using *Arabidopsis* in 2004 (Bao et al., 2004). Bao et al. (2004) reported that *Arabidopsis* miR165/166 were shown to direct DNA methylation of several locations about 1759 bp and 1648 bp downstream (exons 13 and 14) of the miRNA binding sites (part of the 3′ end of exon 4 and the 5′ end of exon 5) on *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) coding regions, respectively. In *PHB* and *PHV* genes, the binding site of miR165/166 is split by an intron and the coding sequences were heavily methylated downstream of the miRNA binding site in the differentiated, but not the undifferentiated tissues/cells of the wild-type *Arabidopsis*. Furthermore, methylation is reduced in *phv-1d* and *phb-1d* gain-of-function mutants, which have an in-frame altered miRNA recognition motif that could not be targeted by miR165/166 for cleavage. This suggests that miR165/166 needs to bind to nascent spliced *PHV* and *PHB* transcripts to trigger both the mRNA cleavage and the DNA methylation for the target gene silencing. In this situation, target transcripts appear in a close association with the target gene loci to trigger the DNA methylation. However, a direct interaction between miR165/166 and the target DNA seems unlikely (Ronemus and Martienssen, 2005). A population of 24 nt miR165/166 was previously revealed in *Arabidopsis* (Vazquez et al., 2008) and it was suggested recently that the 24 nt miR165/166 was potentially the trigger to direct the methylation of *PHB* and *PHV* in *Arabidopsis* (Wu et al., 2010). Nevertheless, the 24 nt miR165/166 was a minor population in *Arabidopsis* (Vazquez et al., 2008) and the correlation of 24 nt miR165/166 with the methylation of *PHB* and *PHV* was not yet defined. The deep mechanism for miR165/166-triggered target gene methylation downstream of the miRNA binding site and its impact on target gene expression remains to be explored.

Subsequently, no further evidence for miRNA-directed DNA methylation has been reported in *Arabidopsis* until recently when Chellappan et al. (2010) discovered that two populations (23 to 27 nt) of sRNAs were produced from the same site of a novel class of miRNA genes (miR2883 and miR2328) and the longer sRNA could trigger the DNA methylation around the binding sites of their target genes (Chellappan et al., 2010). Small RNA biogenesis analyses showed that traditionally canonical miRNAs are 20 to 22 nt sRNA species that are DCL1-, but not RDR- and Pol IV-dependent. In contrast, the 23 to 27 nt sRNA species are similar to siRNAs that are produced by DCL3, RDR2 and Pol IV from a typical heterochromatic siRNA (hc-siRNA) biogenesis pathway. Supporting evidences for this scenario is the accumulation of the 21 nt sRNA species of miR2883 and miR2328 was unaffected in *rdr2-2*, *rdr6-15*, *nrpd1-3* mutants, but drastically reduced in the *dcl1-7*/ *fwf2*, *hen1-1* and *hyl1-2* mutants (Chellappan et al., 2010). In contrast, the 24 nt sRNA species of miR2883 and miR2328 were lost in expression not only in *dcl3-1*, but also in *rdr2-2* and *nrpd1-3*. This indicates that these 24 nt sRNA species are not miRNAs, but rather miRNA-related siRNAs generated by DCL3/RDR2/Pol IV through the hc-siRNA pathway (Chellappan et al., 2010). Moreover, these Pol IV/DCL3/RDR2-dependent hc-siRNAs are associated with AGO4 and direct the DNA methylation.

These new findings hypothesize that some MIR genes could regulate their target expression in dual modes, that is, via siRNA-mediated DNA methylation on their target gene loci and the traditional miRNA-mediated target transcript cleavage or translational attenuation. As a result from both modes of regulations, the expression of At4g16580, a target of miR2328, and At5g08490, a target of miR2831-5P (miR2831 generated from the 5′ end of the miRNA stem-loop region), were increased not only in *nrpd1-3* mutant, but also in *dcl1* mutant as compared with the corresponding wild-type *Arabidopsis* (Chellappan et al., 2010). Chellappan et al. (2010) demonstrated that a significant number of MIR genes in *Arabidopsis* have such dual functions of generating both 20 to 22 nt and 23 to 27 nt sRNAs from the same gene loci and control their target gene expressions via such dual modes of regulations. Similar populations of small RNAs have also been found in *Medicago* plants (Lelandais-Briere et al., 2009). These discoveries add new roles for miRNA genes in a complex regulation of their target genes in plants.

DNA methylation mediated by miRNA pathway found in the monocot rice

Different from *Arabidopsis*, rice (*Oryza sativa*) is a distinct model system for the study of monocots. Bioinformatics analysis and sRNA deep sequencing have identified many conserved and non-conserved 21 nt miRNAs in this species (Liu et al., 2005; Sunkar et al., 2005b; Sunkar et al., 2008; Zhu et al., 2008; Wu et al., 2009). Earlier than in *Arabidopsis*,

two populations of miRNA-related sRNAs were also reported in rice (Wu et al., 2009, 2010). In addition to the traditional 21 nt canonical miRNAs (cmiRNAs), a novel kind of 24 nt long miRNAs (lmiRNAs) has been seen to direct the DNA methylation in rice, despite the fact that underlying mechanisms may differ from those in *Arabidopsis* described above (Wu et al., 2010).

First, Wu et al. (2010) found three classes of AGO4-associated 24 nt miRNAs. Two classes of these 24 nt miRNAs arise from the 21 nt miRNA sites. One of the classes requires both DCL1 and DCL3 for its biogenesis, while the other requires only DCL3. Most interestingly, a subset of pri-miRNAs was found to give rise to both cmiRNAs and lmiRNAs through the coordinated actions of DCL1 and DCL3. For example, the pri-miR820.1 encodes two miRNA species, miR820.1 (21 nt) and miR820.2 (24 nt). miR820.1 and miR820.2 are incorporated into AGO1 and AGO4 clade proteins, respectively. Furthermore, bisulphite sequencing results showed that unlike *Arabidopsis*, AGO4-associated 24 nt lmiRNA can direct DNA methylation not only at their target sites, but also at the locus from which it is produced. For methylation at miRNA locus, the methylated sites were centered on the miRNA or miRNA* regions, and few methylated sites were detected at the sequences outside of the stem-loop region. For methylation at their target loci, the methylation was confined within a ~80 nt region around the target sites. By using *dcl3a-17* and *rdr2-2* mutants, they demonstrated that the biogenesis of lmiRNA is RDR2-independent, and the methylation directed by the lmiRNAs was not mediated by the known RDR2-dependent siRNA pathway but by the miRNA pathway.

DNA methylation mediated by miRNA pathway in moss

The moss, *Physcomitrella patens,* is another useful model system in plant molecular biology. A potentially related process of miRNA-directed DNA methylation was also discovered in *P. patens* by Khraiwesh et al. in 2010. However, the underlying mechanisms of miRNA action may diverge from those in the *Arabidopsis* and rice. In moss, PpDCL1a, an ortholog of AtDCL1, is the major *P. patens* DCL protein for miRNA biogenesis, while PpDCL1b is required for miRNA-guided RNA cleavage (Khraiwesh et al., 2010). Although a null Δ*PpDCL1b* mutant accumulated normal levels of miRNAs, the corresponding miRNA targets were not cleaved. Even so, levels of the miRNA targets were drastically reduced in Δ*PpDCL1b* mutant in contrast to the increase of miRNA targets in Δ*PpDCL1a* mutant. A plausible explanation for such observations is that a transcriptional regulation was involved in this scenario. Indeed, methylation-specific PCR together with evidence from nuclear run-on assay showed that target DNA methylation happened in Δ*PpDCL1b* mutant but not in the WT control. It was speculated that the miRNA:target-RNA duplexes were hypothesized to lead to the DNA methylation and the subsequent downregulation of the corresponding miRNA target genes in Δ*PpDCL1b* mutant. This was reminiscent of the case of miR165/166-target interaction in *Arabidopsis*. The binding site of miR166 on the target *PpC3HDZIP1* and *PpHB10* was similarly split by an intron in moss. Heavy methylation of *PpC3HDZIP1* and *PpHB10* genes in Δ*PpDCL1b* mutant, but not in the WT, suggests that the initiation of methylation upon defective target cleavage involves the miRNA:target-RNA duplexes through interactions of miR166 with its target RNAs (Khraiwesh et al., 2010).

Moreover, the methylation was observed to be miRNA-dosage dependent and the ratio of a miRNA to its target RNA was crucial for the initiation of DNA methylation at the target locus because the methylation and subsequent silencing of miRNA target loci were not limited to the knockout Δ*PpDCL1b* mutant but also occurs in the transgenic WT that ectopically expresses high levels of an amiRNA (Khraiwesh et al., 2010). A support for this scenario came from the fact that *P. patens* WT expressing amiR-GNT1 caused methylation of PpGNT1. Intriguingly, this pathway of miRNA-dosage dependent DNA methylation occurs also in the wild-type under plant hormone abscisic acid (ABA) treatment in moss. This finding raises the possibility that miRNAs also act in the epigenetic control of stress-responsive genes (Khraiwesh et al., 2010).

The possible relation of miRNA and methylation in moss proposed by Khraiwesh et al. (2010) is that ratios of the miRNA to the target RNA triggered DNA methylation at the target locus. In cases where miRNA concentrations exceed a threshold, the miRNA may interact directly with its target forming stable miRNA:mRNA duplex, and then the duplex might be recruited to a DNA methylation silencing complex that directs DNA methylation. Alternatively, in systems where the excess miRNA might be loaded to a DNA methylation silencing complex such as RNA-induced transcriptional silencing (RITS), triggering duplex formation which initiates and spreads DNA methylation.

Evolution and specialization of miRNA-mediated DNA methylation

miRNA-induced DNA methylation and its potentially related novel population of sRNAs have been discovered in at least four representative species throughout the plant kingdom. Many MIR genes were shown to have the dual functions of giving rise to both 20 to 22 nt and 23 to 27nt sRNA species in *Arabidopsis*, *Medicago*, rice, and moss (Zhu et al., 2008; Lelandais-Briere et al., 2009; Chellappan et al., 2010; Khraiwesh et al., 2010; Wu et al., 2010). Previously, the majority of mature miRNAs was found to be 20–23 nt in length in moss (Axtell et al., 2007; Fattash et al., 2007). Subsequent examination of sRNA deep sequencing data sets from moss also revealed the existence of 23 to 27 nt miRNAs in this species, although their function in DNA methylation

was not experimentally determined (Chellappan et al., 2010). Nevertheless, the dual functions of miRNAs in moss seem clearly defined by two distinct forms of DCL1 proteins, PpDCL1a and PpDCL1b (Khraiwesh et al., 2010).

Arabidopsis, *Medicago*, rice, and moss are evolutionarily distant from one another. Moss is among the earliest non-flowering land plants to evolve from single cell organisms; *Arabidopsis* and *Medicago* are dicotyledonous flowering plants while rice belongs to flowering monocotyledons. The existence of the dual-function MIR genes in these plants suggests that dual-function MIRs are broadly present in the plant kingdom, and the fundamental mechanism of DNA methylation may be conserved. The fact that such miRNA genes exist in moss alludes to their possible evolutionary origin in this ancient land plant. Moreover, dual-function MIRs have similar conserved functions in regulating target gene expression at both transcriptional and post-transcriptional levels by using the dual modes of action—23 to 27 nt long miRNA-mediated DNA methylation and 20 to 22 nt canonical miRNA-mediated mRNA degradation and/or translational inhibition.

Intriguingly, a similar link between miRNA and transcriptional gene silencing has also been demonstrated in mammals although the proposed mechanisms differed from the models presented in plants (Gonzalez et al., 2008; Kim et al., 2008). For example, in mammals, miR17-5p (miR17 from the 5′ end of the miRNA stem-loop region), miR20, and miR320 could target and remodel the promoter regions of specific genes for transcriptional silencing of the genes (Gonzalez et al., 2008; Kim et al., 2008). Nascent RNAs transcribed from the promoter regions seem essential for the miRNAs to target them for the gene transcriptional silencing (Gonzalez et al., 2008). Interestingly, miRNAs are also the negative regulators in DNA methylation through targeting the methyltransferases in mammals. The extraordinary example is that miR153 targets the methyltranferase DNMT1 for downregulation, which induced the DNA demethylation of DNMT1 targeted genes and so affected neural cell differentiation in SK-N-BE cells (Das et al., 2010). Transcriptional gene silencing mediated by miRNAs, either through DNA (de)methylation on coding regions in plants or through DNA (de)methylation and/or chromatin changes on promotor regions in mammals, suggests that the functions of miRNAs in gene regulation may be fundamentally conserved in almost all cellular organisms.

Another notable finding, revealed from *Arabidopsis*, rice and moss sRNA deep sequencing data sets, is that 43% of rice miRNA loci can produce 23 to 26 nt RNA population and almost half (49.1%) of them have more reads of the 23 to 26 nt population than those of the 21 nt population. In contrast, 42% of *Arabidopsis* and 36% of moss miRNA loci expressed the 23 to 26 nt population RNA, and compared to rice, fewer such loci produced more 23 to 26 nt population than the 21 nt population (Chellappan et al., 2010). This opposite distribution of the two sRNA populations suggests that 23 to 26 nt sRNAs may play more important roles in rice than in *Arabidopsis*. Furthermore, different plant species are likely to have different sRNA biogenesis pathways with distinct modes of action in transcriptional and post-transcriptional gene regulations through different populations of small RNAs. Supporting evidence for this assumption comes from the fact that the biogenesis of 24 nt lmiRNAs in rice does not require RDR2 and the 24 nt miRNAs can guide DNA methylation at both their generation sites *in cis* and their target sites *in trans* in rice. In contrast, MIR-derived 23 to 27 nt sRNAs in *Arabidopsis* required RDR2 for its biogenesis and guide DNA methylation mostly at their target loci *in trans*, and little at their generating sites *in cis*.

Taken together, although it is a widespread phenomenon that many dual-functional MIR genes were found in various plant species, each of which may have its own distinct pathway for generating MIR-derived small RNAs and have its own mode in directing DNA methylation in gene regulation (Fig. 1). Analyses of the sRNA size distribution and proportion from MIR loci, and their impact on the methylation status of the MIR and the target loci from more extensive plant species will shed light on the big question of the evolution and role of miRNAs in DNA methylation.

The differences between siRNA-directed DNA methylation (siRdDM) and miRNA-directed DNA methylation (miRdDM)

In plants, the most abundant class of sRNAs is heterochromatic siRNAs (hc-siRNAs) that are produced from perfectly double-stranded RNA (dsRNA) originating from either transposon loci or DNA repeats. Hc-siRNAs are predominantly 24 nt in length and their biogenesis depends on the activities of a plant-specific DNA-dependent RNA polymerase Pol IV and an RNA-dependent RNA polymerase RDR2 (Matzke et al., 2009). They direct *de novo* cytosine DNA methylation in all sequence contexts (CG, CHG, and CHH where H is A, C, or T) at the loci from which they are produced, a process termed RNA-directed DNA methylation (RdDM) (Chan et al., 2005; Matzke et al., 2009). In the RdDM pathway, transcripts from transposons and other repetitive DNA sequences are produced presumably by Pol IV. These transcripts serve as templates for RDR2 to generate double stranded RNAs (dsRNAs) which, in turn, are processed by DCL3 into the 24 nt siRNAs for the spreading of DNA methylations along the heterochromatic regions (Xie et al., 2004). This clade of siRNAs are primarily incorporated into the herterochromatin-related AGO4 clade protein to guide DNA methylation in *Arabidopsis* (Zilberman et al., 2003; Li et al., 2006; Qi et al., 2006). The related DNA methylation is mainly catalyzed by the *de novo* DNA methyltransferase DRM2 (Cao and Jacobsen, 2002; Matzke et al., 2009), and the resulting methylation can be at loci

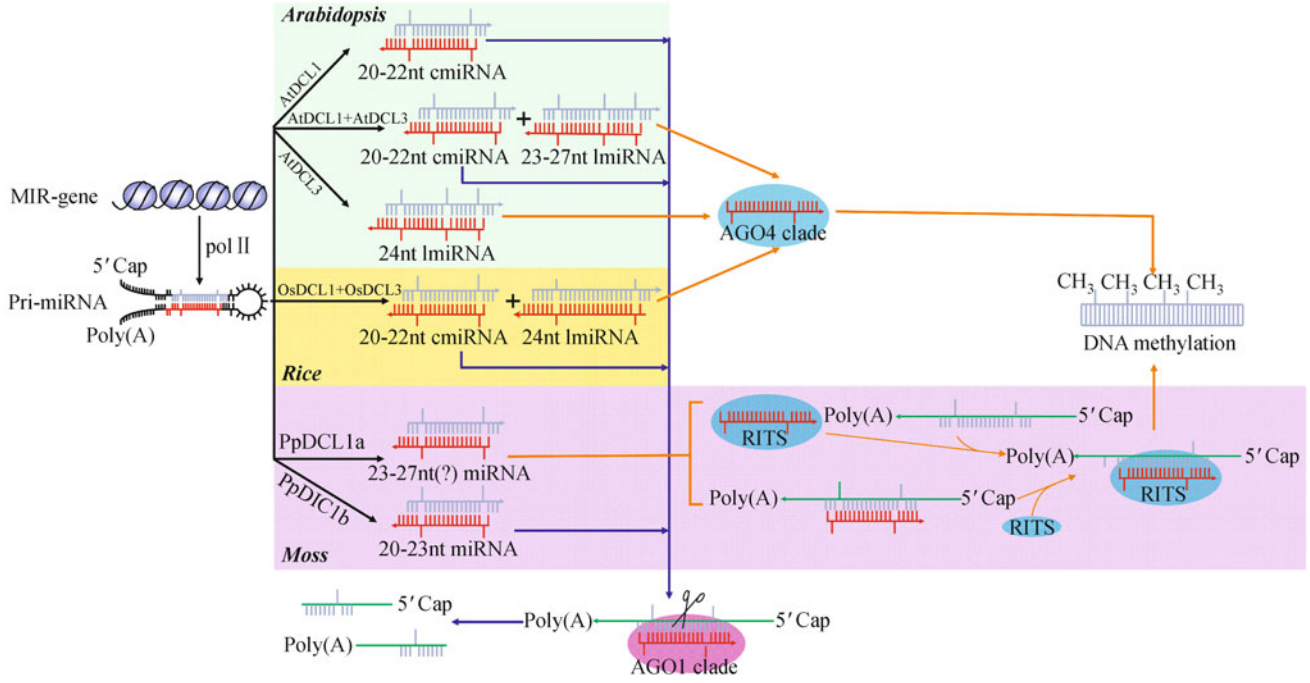

Figure 1 MicroRNA-related DNA methylation pathways in plants. Different sizes of miRNA-related sRNAs were discovered in different plant species and they are different in their biogenesis and maturation through different sets of enzymes and are channeled to different clades of AGO proteins for post-transcriptional gene silencing (PTGS) or transcriptional silencing (TGS). Specifically, in *Arabidopsis* and rice, 20–22 nt miRNAs are generated by DCL1 and specifically loaded into AGO1 clade proteins to form effector complexes to direct the cleavage of their target mRNAs posttranscriptionally. 24 nt and 23–27 nt miRNAs are processed by DCL3 with or without the coordination of DCL1 and bound to AGO4 clade proteins to guide methylation of the adjacent DNA transcriptionally. In moss, two types of miRNAs are produced via two different types of PpDCL1 proteins, PpDCL1a and PpDCL1b. PpDCL1b is responsible for the miRNAs that can form a cleavage-competent RNA-induced silencing complex (RISC), while PpDCL1a is for miRNAs to form non-cleaving RISC or RNA-induced transcriptional silencing complex (RITS). To trigger the target DNA methylation in moss, RITS is believed to form either before the long miRNA binding to the target RNA or after the long miRNA formed a duplex structure with the target RNA. The cleaving RISC directs PTGS while the non-cleaving RISC mediates the TGS in moss. *Arabidopsis*, rice, and moss pathways for miRNA-directed gene regulation are highlighted with the background of blue, yellow, and purple, respectively. Blue arrowed lines indicate PTGS pathways, and orange arrowed lines indicate TGS pathways in different plants. MIR, miRNA; Pol II, RNA polymerase II; Pri-miRNA, primary miRNA; AtDCL, *Arabidopsis* Dicer-like protein; OsDCL, rice Dicer-like protein; PpDCL, moss Dicer-like protein; AGO, Argonaute; Poly(A), poly (A) tail; RITS, RNA-induced transcriptional silencing complex; CH3, methyl group.

ranging from several hundred to several thousand nucleotides.

Similar to hc-siRNAs, MIR-derived siRNAs or lmiRNAs are also DCL3- and AGO4-dependent and direct cytosine DNA methylation (Wu et al., 2010). Nevertheless, key distinctions can be made between these two types of sRNAs in terms of their biogenesis and the targets with which they interact. Different from siRNAs that are normally produced from a long dsRNA in a heterogeneous manner, the production of MIR-derived siRNAs or lmiRNAs is site-specific and their structures are almost identical to the miRNAs. In other words, MIR-derived siRNAs or lmiRNAs are structurally the 3′ end lengthened miRNAs that are generated from the same sites as the mature miRNAs. There has been no detection of heterogeneous MIR-derived siRNAs or heterogeneous lmiRNAs from MIR loci.

The length of MIR-derived siRNAs is 23 to 27 nt and lmiRNAs 24 nt. The precursors of both MIR-derived siRNAs and lmiRNAs are presumably catalyzed by Pol II. The synthesis of lmiRNAs in rice does not require RDR2 activity. However, MIR-derived siRNAs in *Arabidopsis* do (Chellappan et al., 2010). Unlike hc-siRNAs-guided DNA methylation at loci ranging from several hundred to several thousand nucleotides, lmiRNAs-direct DNA methylation in rice is limited to approximately an 80 nt region around the lmiRNA and target binding sites (Wu et al., 2010) with the exception of miR165/166-directed downstream methylation on the *PHB* and *PHV* genes (Bao et al., 2004). While lmiRNA may act not only on its own loci *in cis,* but also on their target loci *in trans* to signal DNA methylation, MIR-derived siRNAs mainly guide DNA methylation at their target site *in trans* and seldom trigger DNA methylation at their generating sites *in cis* (Chellappan et al., 2010; Wu et al., 2010). Strikingly, miRNA-directed DNA methylation in moss can spread significantly outwards from the initiation region that produces the small RNAs – for example, from a

coding region into upstream promoter sequences, mirroring the spreading of hc-siRNAs-guided DNA methylation (Khraiwesh et al., 2010). Taken together, these studies suggest distinct pathways involving siRNA and miRNA-mediated DNA methylation.

Conclusion and outstanding questions

Plants possess a complex network of diverse small RNA pathways. These sRNA pathways are different in the sRNA biogenesis from distinct loci with different mechanisms and the subsequent sRNA recruitment to different effector complexes (Xie et al., 2004; Chapman and Carrington, 2007; Xie and Qi, 2008). DNA methylation and miRNA-directed post-transcriptional regulation of gene expression are two important aspects of plant epigenetics. The discovery of miRNA-mediated DNA methylation in moss, rice, and *Arabidopsis* presents an exciting connection between the two aspects and extends the range of miRNA function in overall gene regulation. The transcriptional control mediated by miRNA genes points to the existence of intriguing crosstalks between small RNA pathways. A similar link between miRNA and DNA methylation in the basal plant moss (Khraiwesh et al., 2010) suggests that miRNA-directed DNA methylation is likely an ancient pathway for gene regulation being conserved in the plant lineage and evolution. Nevertheless, we are now only beginning to understand the mechanism that MIR-derived sRNA species contribute to epigenetic regulation in plants. Many more unknowns and open questions await further explorations to determine the more specific roles of miRNAs and their biogenesis correlations in DNA methylation.

For example, why do *Arabidopsis* and rice present opposite distributions of the two distinct populations of miRNA-related sRNAs? What are the potential RDRs responsible for the production of 24 nt miRNAs in rice if the 24 nt miRNAs are actually siRNAs similar to those from the miRNA gene loci in *Arabidopsis*? In the case of miR165/166 directed PHB and PHV methylation, why are the methylated sites far downstream of the miR165/166 binding sites and distinct from other miRNAs directed target methylations in *Arabidopsis*? What are the specific DCLs and AGOs for miR165/166 to trigger such distinct patterns of the target DNA methylations in *Arabidopsis*? Are the *PHB* and *PHV* gene (de)methylations miR165/166-dependent in wild-type *Arabidopsis* that has normal DCL1 and AGO1 activities? What is the equivalent or distinct mechanism for miRNAs-directed DNA methylations in moss? What are factors that determine the spreading or not spreading of the DNA methylations triggered by the miRNA-related sRNAs in different plants species? Do animals and plants have conserved molecular pathways for sRNA-triggered DNA methylations? Does miRNA-directed DNA methylation have significant roles in plant physiology and development control? Answers to these questions will certainly shed light on the beginning-to-unfold mysteries of miRNA-directed DNA methylations.

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