Review

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Viroid-induced DNA methylation in plants

Abstract: In eukaryotes, DNA methylation refers to the addition of a methyl group to the fifth atom in the six-atom ring of cytosine residues. At least in plants, DNA regions that become *de novo* methylated can be defined by homologous RNA molecules in a process termed RNA-directed DNA methylation (RdDM). RdDM was first discovered in viroid-infected plants. Viroids are pathogenic circular, non-coding, single-stranded RNA molecules. Members of the Pospiviroidae family replicate in the nucleus through double-stranded RNA intermediates, attracting the host RNA silencing machinery. The recruitment of this machinery results in the production of viroid-derived small RNAs (vd-sRNAs) that mediate RNA degradation and DNA methylation of cognate sequences. Here, we provide an overview of the cumulative data on the field of viroid-induced RdDM and discuss three possible scenarios concerning the mechanistic details of its establishment.

Keywords: bisuflite sequencing; potato spindle tuber viroid; RNA-directed DNA methylation; small RNAs.

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Introduction: viroids

Viroids are non-encapsidated, single-stranded (ss), 250–400 nucleotide (nt)-long circular RNA molecules that do not encode proteins (1, 2). They are classified into two families, the Pospiviroidae and the Avsunviroidae, whose members replicate in the nucleus and the chloroplast, respectively (3). Pospiviroidae have a wide host range, and Pospiviroidae species can comprise more than 200 variants (4). Pospiviroidae's type species, potato spindle tuber viroid (PSTVd), has a rod-like secondary structure ~360-nt-long genome that can be functionally and structurally divided into five domains: (i) terminal left, (ii) pathogenicity (P), (iii) central (C) containing the conserved central region, (iv) variable (v) and (5) terminal right (TR) (5). When PSTVd enters the plant cell, a specific signal seems to direct it into the nucleus. Presumably, this process involves the interaction between the host's bromodomain-containing viroid RNA-binding protein 1 (VIRP1) and the viroid's TR domain (6). In the nucleus, PSTVd replicates via an asymmetric rolling circle mechanism (7). The circular monomeric ssRNA(+) is transcribed by DNA-dependent RNA polymerase II (POLII) into linear oligomeric single-stranded RNA (ssRNA) of negative orientation (ssRNA(-)). Probably anchored in the nucleoplasm, ssRNA(-) serves as a template for POLII for the production of linear oligomeric ssRNA of positive orientation (ssRNA(+)). The ssRNA(+), likely transferred into the nucleolus, is finally processed by a type III RNase and a DNA LIGASE into unit-length circular RNAs (8) (Figure 1). The mature viroid then exits the nucleus into the cytoplasm and moves to neighboring cells through the plasmodesmata (9) and to distant parts of the plant through the phloem (10).

Viroids and RNA silencing

The interaction between viroid and its host activates the plant RNA silencing machinery (11, 12). At least in *Nicotiana benthamiana*, all four dicer-like endonucleases (DCLs) seem to process PSTVd into small interfering RNAs (siRNAs) that, in turn, affect the viroid titer (13). Interestingly, in *N. benthamiana*, PSTVd needs to be processed by DCLs to enable its efficient systemic spreading (13). Yet, the nature of viroid RNA molecules serving as templates for DCLs is still elusive. Potentially, both the mature viroid RNA resembling microRNA (miRNA) precursors and double-stranded RNA (dsRNA) replication intermediates could be processed by DCLs. In any case, DCL1 and DCL4 are predominantly generating 21-nt siRNAs that are generally loaded onto ARGONAUTE1 (AGO1) and are involved in post-transcriptional gene silencing

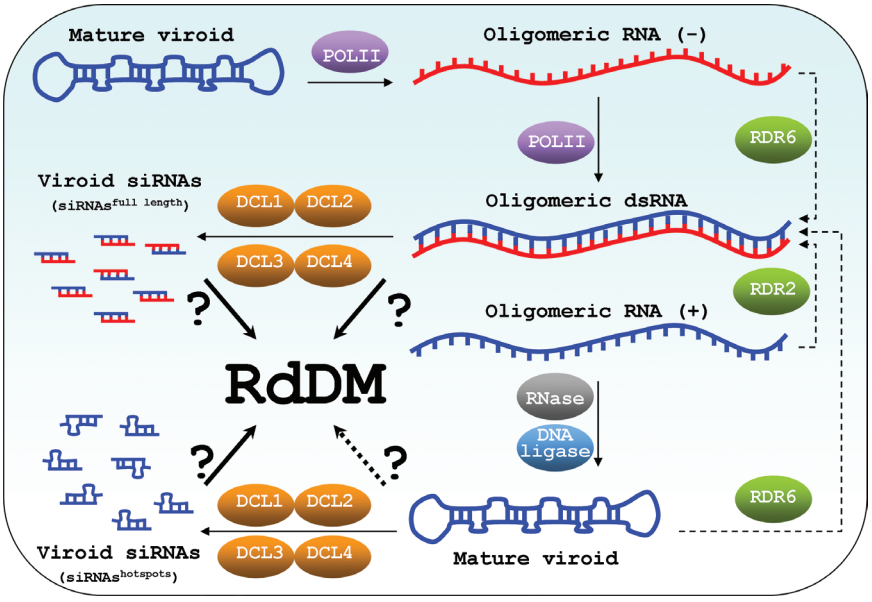

Figure 1 Life cycle of Pospiviroidae. Upon inoculation, mature Pospiviroidae RNA molecules enter the plant cell nucleus. During the asymmetric replication circle, POLII transcribes circular viroid RNA into oligomeric RNA of (-)-orientation. In the nucleoplasm, this RNA is transcribed into oligomeric (+) RNA, again by POLII. Subsequently to its transport into the nucleolus, a type III-like RNase and a DNA ligase are recruited to assemble the mature viroid molecule. Oligomeric (+) and (-) replication intermediates may form dsRNA molecules. Alternatively, dsRNA molecules may be produced by RDR6-mediated transcription of mature viroid RNA and/or of oligomeric (-) in the nucleoplasm. In addition, it cannot be excluded that, in the nucleolus, dsRNA is synthesized by RDR2 transcription of oligomeric (+) RNA. All four DCLs produce vd-sRNAs. However, it is not clear whether only dsRNAs or, due to the high capacity of viroid RNA to form intermolecular secondary structures, also single-stranded viroid RNA is processed. If dsRNA is processed, fully complementary 21–24-nt siRNAs are produced that ideally cover the full length of the viroid (siRNAs$^{full\ length}$). Both polarities of vd-sRNAs could be loaded onto AGOs. If ssRNA viroid is processed, siRNAs will most probably be produced only from hotspot regions displaying secondary structures that are recognized by DCLs (siRNAs^{hotspots}). They are not perfect duplexes and may be only of (+) polarity. Whether dsRNAs, siRNAs$^{full\ length}$ or siRNAs^{hotspots} guide the RdDM machinery to cognate DNA is not clear.

(PTGS) (14). Twenty-two-nucleotide siRNAs, essentially produced by DCL2, are associated with transitive silencing (15, 16). Finally, DCL3 generates 24-nt siRNAs that are mainly loaded onto AGO4 and are involved in RdDM (17–19). In tomato leaves, 21-, 22- and 24-nt vd-sRNAs are accumulating to almost equal levels at an early stage of PSTVd infection. However, in symptomatic leaves, a shift from 21- to 24-nt sRNAs is detectable (20). Similar to endogenous siRNAs and miRNAs, vd-sRNAs are phosphorylated at their 5′-end and methylated at their 3′-end by HEN1 (Hua enhancer 1) (21). Notably, both polarities of vd-sRNAs have been detected (22, 23). Deep-sequencing data of sRNAs from viroid-infected plants revealed that vd-sRNAs(+) map to hotspot regions along the viroid genome, while vd-sRNAs(-) are distributed more homogeneously over the viroid genome (24, 25). In any case, vd-sRNAs appear to be biologically active since they were shown to trigger PTGS (26). However, the viroid itself is considered to be resistant to vd-sRNA-mediated degradation, presumably due to its secondary structure (24). Nevertheless, in tomato plants, PSTVd was targeted for PTGS by hairpin-derived siRNAs (27).

Hallmarks of viroid-induced RdDM

In addition to activation of PTGS, Pospiviroidae have the potential to trigger the RdDM of homologous sequences. In fact, RdDM itself was discovered in viroid-infected tobacco plants that contained multimeric genome-integrated PSTVd cDNA copies (28). However, the overall biological significance of viroid-induced DNA methylation during natural infection is not clear. Several host genes exhibit transcriptional alteration during viroid infection (29). Viroid symptoms could be related to global epigenetic changes. However, such changes have not been reported, so far (30). Thus, we will focus on viroid-induced DNA methylation of transgenes and will provide a brief overview of major hallmarks in this field.

RdDM discovery

Transgenic tobacco plants that carried an expression cassette containing three head-to-tail-linked,

positive-oriented, full-length PSTVd cDNA copies [SR1-PSTVd 3(+)] were generated (Figure 2) (28). In these plants, PSTVd-3(+) primary transcripts were processed to mature, circular, infectious PSTVd molecules. Southern blot analysis of genomic DNA extracted from these plants revealed that the PSTVd-specific cDNA was severely resistant to methylation-sensitive restriction endonucleases. Yet, whether PSTVd-3(+) cDNA methylation was due to DNA-DNA interactions or due to viroid replication was not clear. To this end, SR1-PSTVd-SB2(+) plants were generated. These plants contained two head-to-tail-linked, positive-oriented, PSTVd cDNA copies. Importantly, both copies carried a 26-bp deletion (Figure 2). Thus, PSTVd-SB2(+) primary transcripts were not processed to infectious mature viroid molecules. Southern blot analysis revealed that, in viroid-free plants, the PSTVd-SB2(+) locus was not methylated, indicating that the presence of tandem PSTVd cDNA repeats was not sufficient to trigger *de novo* DNA methylation. However, when SR1-PSTVd-SB2(+) plants were PSTVd infected, the PSTVd-SB2(+) locus became heavily methylated. Since viroid replication exclusively proceeds through RNA intermediates, it was evident that an RNA molecule triggered the methylation of homologous DNA sequences. The phenomenon was termed RNA-directed DNA methylation (28).

CHH methylation as a hallmark of *de novo* RdDM

Further studies on viroid systems employing bisulfite sequencing provided important insights into the RdDM mechanism. In PSTVd-infected SR1-PSTVd-SB2(+) plants, methylation analysis of two DNA strands revealed that both were equally methylated (31). This finding suggested that the RNA trigger interacted with both DNA strands, indicating its double-stranded nature. The assumption that dsRNA indeed activates RdDM was demonstrated by Mette et al. (32, 33). Importantly, in PSTVd-infected plants, the PSTVd-SB2(+) locus exhibited cytosine methylation (C methylation) at any sequence context; CG, CHG and CHH

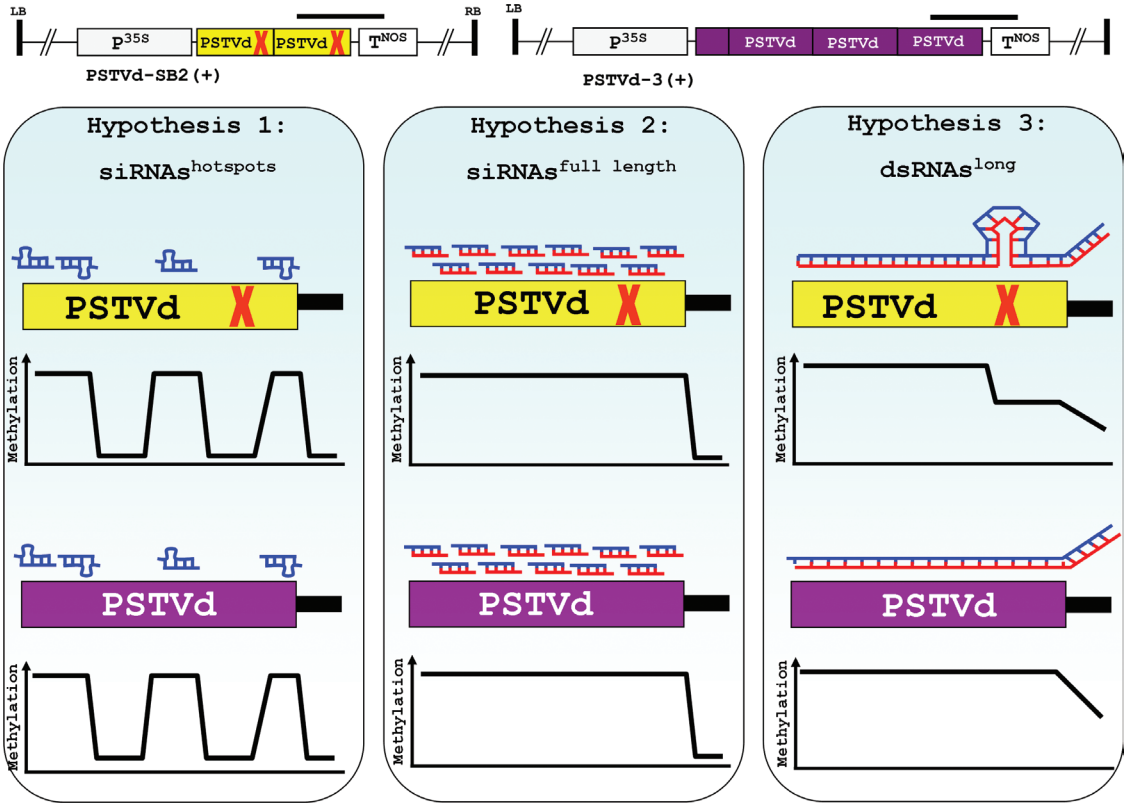

Figure 2 Models of viroid-induced RdDM. As exemplified by the PSTVd-SB2(+) and PSTVd-3(+) transgenes, three different hypotheses are described. Both transgenes are under the control of the *Cauliflower mosaic virus* (CamV) 35S promoter and of the nopaline synthase (NOS) terminator. The PSTVd-SB2(+) transgene contains a dimeric PSTVd cDNA with a 26-bp deletion (red X). Its primary transcript is not infectious. PSTVd-3(+) carries a trimeric PSTVd, and its transcripts are further processed into mature viroid RNA. The expected methylation patterns of the transgene regions (indicated by a black bar) that are established upon viroid infection are discussed (see main text).

(where H=A, C or T) (31). This was in contrast to mammalian systems where C methylation is predominantly found at CG sites (34). In plants, RdDM is essentially mediated by DRM1/2 (domains rearranged methyltransferase 1/2), with the assistance of MET1 (methyltransferase 1) and the plant-specific CMT3 (chromomethylase 3) (35, 36). In the absence of RdDM guide molecules, methylation at CG sites and, to a lower extent, at CHG sites is maintained by MET1 and CMT3, respectively. However, methylation in CHH contexts is lost (37, 38). Thus, asymmetric CHH methylation can be considered as a hallmark of ongoing RdDM (31, 39).

Maintenance of CG methylation

CG methylation may be maintained by MET1. However, such maintenance mechanisms were observed for transgenic promoter sequences, but not for transgenic coding/transcribed regions (40). In order to check this assumption, we analyzed the methylation status of PSTVd-SB2(+)-transcribed region in viroid-infected plants and in their viroid-free progeny (41). Upon viroid infection, the PSTVd-SB2(+) locus became, as expected, densely methylated at all Cs. In the viroid-free progeny plants, the PSTVd-SB2(+) locus retained, for at least two generations, CG, but not CHG or CHH methylation (41). Notably, this maintenance of methylation was not associated with histone H3 dimethylation and/or H3 acetylation (41). These data were in agreement with observations made in other systems (42), and they clearly showed that transgenic coding/transcribed regions can also retain CG methylation in the absence of RdDM guide molecules.

Minimal target size for RdDM

To investigate the minimal target size for RdDM, transgenic tobacco plants carrying non-infectious PSTVd cDNA subfragments of 30, 60, 98 and 160 bp in size (PSTVd-30, PSTVd-60, PSTVd-98 and PSTVd-160, respectively) were generated and PSTVd-infected by mechanical inoculation. In the infected plants, the methylation status of each transgene was analyzed by bisulfite sequencing. PSTVd-60, PSTVd-98 and PSTVd-160 exhibited dense RdDM patterns, with the density of C methylation gradually increasing with the length of the cDNA subfragments. PSTVd-30 was still methylated but at a basal level, reflecting that approximately 30 bp is the minimal target size for RdDM initiation (39, 43).

RdDM interactional limitations

It appeared that there is a threshold of thermodynamic and/or kinetic interaction between RdDM guide molecules and their targets. Yet, this threshold seems to be influenced not only by the extent of base pair interactions but also by the established nucleic acid structures. When a 134-bp PSTVd cDNA subfragment (PSTVd-134) was inserted into a chimeric satellite of *Tobacco mosaic virus* (STMV) cDNA construct (STMV/PSTVd-134), it was poorly targeted by RdDM upon viroid infection (44). In contrast, the same PSTVd-134 sequence became heavily methylated when not flanked by satellite cDNA sequences. Thus, in the case of STMV/PSTVd-134, the limiting factor for RdDM was not the extent of base pair interactions between RdDM guide molecules and the target region, but the inhibitory effect of the flanking sequences. Replicating and non-replicating satellite sequences are known to exhibit complex secondary structures that may render them significantly resistant to RNA silencing (12). Thus, one may hypothesize that complex secondary structures of the target DNA (or its nascent transcript) exhibited little thermodynamic and/or kinetic interactions with RdDM guide molecules (44).

Trans-RdDM of related viroids

In the above examples, PSTVd transgenes were used as RdDM targets triggered by PSTVd infection. The extent to which infection by another member of Pospiviroidae would induce RdDM on PSTVd transgenes was not analyzed. To investigate the potential of a PSTVd-related viroid to mediate the RdDM of a PSTVd cDNA, tomato apical stunt viroid (TASVd) was used as an RdDM trigger. PSTVd and TASVd share 81% sequence homology and exhibit only two regions of continuous 24- and 29-nt sequence homology, respectively. Thus, only one (covering the 24-nt region) and six overlapping (covering the 29-nt region) TASVd 24-nt siRNA species could potentially target PSTVd-SB2(+) transgene with perfect complementarity. In TASVd-infected SR1-PSTVd-SB2(+) plants, RdDM was monitored on the PSTVd-SB2(+) transgene (unpublished observations). C methylation was weaker than methylation induced by PSTVd infection (almost 90%), but still significant (40–50%). Importantly, regions outside the continuous 24- and 29-nt homology regions were methylated. In homologous and non-homologous regions, methylation was established at almost equal levels. These data showed that, if 24-nt siRNAs are the RdDM guide molecules, they do not need perfect

complementarity with the DNA target to induce methylation. Alternatively, and most probably, they imply that longer RNAs may be the actual RdDM guide molecules (see below).

Nature of the viroid-induced RdDM guide molecules

In the following, we will briefly discuss three different views that may explain how RdDM can be triggered. The discussion will be based on data derived from examples of viroid-induced RdDM as described above for PSTVd-SB2(+) and PSTVd-3(+) transgenes (Figure 2).

In the first hypothesis (*siRNAs*^{hotspots}), the mature viroid is processed by DCLs only at hotspot regions (24) (Figure 2, hypothesis 1). In this case, the produced siRNAs^{hotspots} have to necessarily be only of (+) polarity. Importantly, none of the produced 21–24-nt siRNAs^{hotspots} will be perfect dsRNA, since such regions do not exist in the mature viroid. Most probably, such bulge-containing, miRNA-resembling siRNAs could be loaded onto AGOs, but the extent to which they can trigger RdDM is unknown. In general, miRNAs do not trigger RdDM, although, in rare cases, some 24-nt miRNAs have been shown to trigger *cis* and *trans* RdDM (45). Thus, it cannot be generally excluded that such bulge-containing siRNAs^{hotspots} could also trigger RdDM. However, such a scenario would imply that only those PSTVd cDNA regions corresponding to siRNAs^{hotspots} become methylated. Methylation of the full-length PSTVd cDNA would not be expected (Figure 2). Yet, the experimental data are in contrast to this assumption. The PSTVd-SB2(+) and, in particular, the PSTVd-3(+) transgene exhibited dense methylation along the entire sequences. Methylation was clearly not restricted to hotspot regions (31, 41). In addition, Cs on both DNA strands were densely methylated (31), an observation that could not be easily explained by the accumulation of only (+)-polarity siRNAs^{hotspots}. In summary, the first scenario of siRNA^{hotspots}-guided RdDM lacks experimental support.

According to the second hypothesis (*siRNAs*$^{full\text{-}length}$), vd-sRNAs are not derived from processing of mature viroids, but primarily from dsRNA replication intermediates (Figure 1). Thus, not only (+)- but also (-)-polarity siRNAs^{PSTVd} should be detectable (13, 20). In this case, the accumulating vd-sRNAs would ideally fully cover the complete PSTVd sequence (Figure 2, hypothesis 2). Although not considered here, it should be noted that DCL-mediated processing of dsRNA does not result in the production of siRNAs fully matching the dsRNA region (46). In our discussion, it is not taken into account either that siRNAs involved in RdDM need to have a 5′ adenine to be preferentially and efficiently loaded onto AGO4 (47). Finally, only the guide strands of siRNA duplexes are retained on AGOs. Thus, only one strand has the potential to hybridize with the target DNA (or nascent transcripts of the target DNA). Despite these obvious system-inherent limitations, it is, nevertheless, assumed here that (1) DCLs produce 21–24-nt siRNAs$^{full\text{-}length}$ covering the entire viroid sequence, (2) all siRNAs$^{full\text{-}length}$ are loaded onto AGO4 and trigger RdDM and (3) both strands of the siRNA$^{full\text{-}length}$ duplexes are equally loaded onto AGOs. Under these presuppositions, the produced siRNAs$^{full\text{-}length}$ may recognize both strands along the entire viroid cDNA and induce RdDM of all Cs.

However, despite these admissions, this scenario is still in contrast to experimental observations. In viroid-infected SR1-PSTVd-SB2(+) and SR1-PSTVd-3(+) plants, methylation was not strictly constricted to viroid sequences, but showed a significant spread into sequences up- and downstream of the viroid cDNA (up to 30 and 50 bp, respectively) (Figure 2) (31, 41, 44). No siRNAs corresponding to these regions can be generated from any PSTVd RNA. Thus, vd-sRNA can hardly trigger RdDM of the flanking regions. By using an intronic hairpin system, it was shown that *de novo* methylation is highly specific. With a single base precision the dsRNA inducer seemed to function as a ‘ruler’ defining the region that became methylated (48). Thus, the observed spreading of methylation into regions flanking the viroid cDNA are probably not due to the inaccuracy of *de novo* methyltransferases. In summary, the *siRNA*$^{full\text{-}length}$ working hypothesis also fails to account for certain aspects of the experimental data.

According to the third hypothesis (*dsRNA*^{long}), long dsRNA molecules guide the *de novo* DNA methylation machinery to its target (Figure 2, hypothesis 3). One may assume that the interaction of long dsRNA molecules with DNA recruits the RdDM machinery to such aberrant RNA/DNA structures. In the case of viroid-induced RdDM, long dsRNA may primarily derive from the POLII-produced viroid replication intermediates (Figure 1). A second source of dsRNA could be based on RDR6-mediated transcription of oligomeric viroid RNA and/or of aberrant primary transgene transcripts (Figure 1) (22, 49, 50). In particular, the transcription of aberrant transcripts containing sequences up- and downstream of the viroid cDNA could explain the spread of methylation into these regions. Yet, RDR2 transcription of oligomeric RNAs(+) (Figure 1) cannot be excluded since they

both co-localize in the nucleolus (51, 52). We propose that RdDM guide RNA is double and not single stranded. *De novo* C methylation is induced at both DNA strands indicating that they both have to interact with guide molecules (39). In the case of the PSTVd-SB2(+) and PSTVd-3(+) transgenes, long viroid dsRNAs may interact with homologous cDNA sequences (Figure 2, hypothesis 3). However, since the dsRNA derives most probably from replication intermediates, it could be oligomeric (Figure 1). Thus, in physical terms, interaction of the oligomeric dsRNA with the dimeric PSTVd-SB2(+) or trimeric PSTVd-3(+) transgenes will necessarily exhibit some flanking 'tails' up- and downstream of the putative RNA/DNA hybrids. It is not very likely that the first one or first two mismatches within the regions directly flanking the fully complementary hybrid will initiate an immediate disassociation of the RNA from the DNA. Instead, these tails could tolerate some mismatches. However, increasing numbers of mismatches will lead to gradual weakening of the interaction between the guide RNA and the target DNA (Figure 2, hypothesis 3). As a consequence, after about 50 bp (depending on the number of mismatches) up- and downstream from the perfect hybrid, the RdDM machinery is no longer efficiently recruited (31, 39). An additional interesting point is that, in PSTVd-SB2(+) transgene, the homogeneous and dense methylation pattern is disrupted at sequences flanking the 26-bp deletion region (Figure 2C), indicating that the deletion affected a putative RNA/DNA hybridization. In the case of a siRNA/DNA hybridization, disruption should be only observed at a maximum of 23 bp up- and downstream of the deletion. Assuming that a homogenous population of 24-nt siRNAs covers the PSTVd-SB2(+) transgene, siRNAs matching the sequences up- and downstream of the deletion should reinitiate RdDM. However, decreased methylation was found about 50 bp up- and downstream of the deletion (31, 41). Thus, the interaction between the trigger and the target appeared to be impaired along sequence stretches longer than 24 bp. This may indicate that RdDM-triggered RNA molecules are longer than 24 bp. Additional evidence for this suggestion came from the observation that a gradual increase in the density of methylation positively correlated with the length of DNA target (43). If a 24-nt siRNA has the potential to recruit the RdDM machinery, one would expect that a 30-bp and a 60-bp target (see the subsection 'Minimal Target Size for RdDM') will be equally methylated. This was in contrast to the finding that the 30- and 60-bp target regions displayed a total C methylation level of 16% and 77%, respectively (43). Thus, it is reasonable to assume that a longer-than-24-nt RNA interacts with the target.

Revisiting the current RdDM model

The mechanism of RdDM has been studied not only using viroids but also using numerous other viral and transgenic plant systems. The generally accepted view is that the RdDM pathway is initiated by the activity of POLIV (53). It is suggested that POLIV generates transcripts serving as templates for RDR2. The putative chromatin remodeler and/or helicase CLASSY 1 (CLSY1) facilitates this process (54). The generated dsRNA is then processed by DCL3 into 24-nt siRNAs that are methylated at their 3′ end by HEN1 and loaded onto AGO4 (51, 55). Independently of siRNA biogenesis, POLV is assumed to transcribe the target DNA, in a process requiring DRD1 (defective in RNA-directed DNA methylation 1), DMS3 (defective in meristem formation 3) and RDM1 (required for DNA methylation 1) (all three collectively termed DDR complex) (56). Presumably, AGO4 loaded with 24-nt siRNAs recognizes POLV transcripts (57–59). There is experimental evidence that a complex between AGO4, the carboxy-terminal domain of POLV, KTF1 (KOW-domain-containing transcription factor 1) and IDN2 (involved in de novo 2) is formed (60). Importantly, it is suggested that the RDM1 protein of the DDR complex binds AGO4 and DRM2, guiding this complex to POLV-transcribed regions finally initiating RdDM (34, 56, 61).

A major concept of the current models is the assumption that 24-nt siRNAs recognize homologous DNA (or nascent transcripts) and thus guide RdDM (34, 56). However, at least for viroid-induced RdDM, the 24-nt siRNA-guided RdDM hypothesis is inconsistent with experimental data. Moreover, a growing body of evidence in non-viroid systems also suggests the need for a critical revisiting of previous assumptions (62). For example, RdDM of certain loci was not abolished in *Arabidopsis thaliana dcl3* knockout mutants, where 24-nt siRNAs were depleted (19, 63, 64). Due to DCL redundancy, 21- and 22-nt siRNAs are also produced, but they were shown to ineffectively trigger RdDM (56). Thus, not only the absence of 24-nt siRNAs still allows RdDM to take place, but, reversely, the presence of 24-nt siRNAs does not ensure RdDM establishment (47). Overall, we propose that sRNAs are not the actual RdDM guide molecules, but that they are involved in an intermediate step producing longer dsRNA molecules that would guide the RdDM machinery to DNA targets (62). In this view, sense RdDM (S-RdDM) and inverted repeat RdDM (IR-RdDM) should be separately discussed. In S-RdDM, the role of 21–24-nt sRNAs would be indispensable for RdDM. Sense transcripts, e.g., POLII-, POLIV- and/or POLV-produced transcripts, are *per se* not capable to trigger RdDM. However, AGO-bound 21–24-nt siRNAs (65) may associate with such transcripts to trigger the generation of dsRNA through the activity of RDRs and/or

POLs. The resulting long dsRNA would then guide the RdDM machinery. In contrast, in IR-RdDM, dsRNAs may be directly produced by transcription of hairpin RNAs or by viroid/virus replication. Provided that dsRNA is efficiently targeted into or retained in the nucleus, it could directly trigger RdDM without the need for sRNAs (48). In any case, further experiments are needed to ascertain which RNA molecules guide the RdDM machinery.

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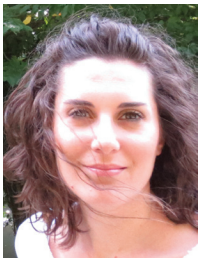

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