A pathogenic non-coding RNA induces changes in
dynamic DNA methylation of ribosomal RNA genes
in host plants

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ABSTRACT
Viroids are plant-pathogenic non-coding RNAs able
to interfere with as yet poorly known host-regula-
tory pathways and to cause alterations recognized
as diseases. The way in which these RNAs coerce
the host to express symptoms remains to be totally
deciphered. In recent years, diverse studies have
proposed a close interplay between viroid-induced
pathogenesis and RNA silencing, supporting the
belief that viroid-derived small RNAs mediate the
post-transcriptional cleavage of endogenous
mRNAs by acting as elicitors of symptoms expres-
sion. Although the evidence supporting the role of
viroid-derived small RNAs in pathogenesis is robust,
the possibility that this phenomenon can be a more
complex process, also involving viroid-induced
alterations in plant gene expression at transcrip-
tional levels, has been considered. Here we show
that plants infected with the ‘Hop stunt viroid’
accumulate high levels of sRNAs derived from ribo-
somal transcripts. This effect was correlated with an
increase in the transcription of ribosomal RNA
(rRNA) precursors during infection. We observed
that the transcriptional reactivation of rRNA genes
 correlates with a modification of DNA methylation in
their promoter region and revealed that some
rRNA genes are demethylated and transcriptionally
reactivated during infection. This study reports a
previously unknown mechanism associated
with viroid (or any other pathogenic RNA) infection
in plants providing new insights into aspects of
host alterations induced by the viroid infectious
cycle.

INTRODUCTION
Impelled by their need to optimize a humble (250–400 nt)
non-protein-coding genome to guarantee their infectious
cycle, viroids have evolved into versatile molecular entities
capable of interacting with the host-cell machinery at
diverse functional levels (1). In some cases, this crosstalk
can affect key host-regulatory pathways and cause pheno-
typic alterations recognized as plant diseases (2,3). Host
factors and/or mechanisms associated with basic aspects
of the life cycle of viroids, such as sub-cellular compart-
mentalization (4,5), replication (6–8) and movement
(9–12), have been thoroughly studied in the past years to
generate a relatively clear picture of the plant–viroid inter-
action (13). However, the way in which these tiny non-
coding RNAs (ncRNAs) subvert the plant cell machinery
by coercing the host to express symptoms remains a
conundrum (3).

As viroids lack mRNA activity, it was initially assumed
that viroid-induced pathogenesis must result from a direct
interaction between their genome and host factors
(proteins or nucleic acids). Based on this notion, early
pathogenesis models envisioned the plant-symptom
expression as a consequence of alterations in the endog-
uous RNA metabolism induced by base pair interactions
between viroid and host ncRNAs, like U1 (14) or 7S (15).
The identification of sequence/structural elements in the
viroid genome (pathogenicity determinants) (16,17)
supports the notion that these specific viroid domains
may interact with yet-to-be-identified host factors
interfering in their physiological role and consequently
incite disease (1,3). In recent years, and enhanced by the
advent of RNA silencing, diverse studies have provided
evidence for the existence of close interplay between
viroid-induced pathogenesis and this small RNA
(sRNA)-dependent regulatory mechanism. The idea that
viroid-derived sRNAs (vd-sRNAs) can mediate the

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post-transcriptional cleavage of endogenous mRNAs, acting as elicitors of symptom expression (18–20), was reinforced by the observation that the expression of host symptoms is associated with post-transcriptional RNA silencing in representative members of both Pospiviroidae (21) and AHSVd families. Despite strong evidence supporting the role of vRNA as inductors of host symptoms, the possibility that this phenomenon can be a more complex process, also involving viroid-induced alterations in plant gene expression at transcriptional levels, cannot be ruled out (3, 13).

Genome activity at the transcriptional level in plants is regulated by epigenetic modifications of DNA and histones. Cytosine DNA methylation in plants is a stable epigenetic mark, basic for the maintenance of genome stability, including regulation of coding and non-coding regions (23). Methylation takes place in three different sequence contexts, CG and CHG (symmetric) and CHH (asymmetric, where H refers to A, T or C) (24). De novo DNA methylation is regulated by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), with three different pathways being involved in its maintenance. DNA METHYLTRANSFERASE 1 (MET1) and DNA CHROMOMETHYLASE3 (CMT3), respectively, maintain CG and CHG methylation through DNA replication, whereas CHH methylation requires de novo continuous maintenance by DRM2 (24, 25). sRNAs are important in de novo establishment and reinforcement of stable DNA methylation because they can target homologous DNA sequences and induce cytosine DNA methylation in all three contexts. A specific RNA silencing pathway, termed RNA-directed DNA methylation (RdDM), mediates the biosynthesis of these epigenetically active sRNAs (26). In the Arabidopsis–RdDM pathway, single-strand transcripts originated by RNA polymerase IV (PolIV) are used by RNA-dependent RNA polymerase 2 (RDR2) as substrates to generate dsRNAs, which are subsequently processed by Dicer-like 3 (DCL3) into 23–24 nt siRNAs (25). These siRNAs guide Argonaute (AGO4/AGO6) proteins (likely through an siRNA-nascent PolV transcript base pairing mechanism) to target genomic regions (27). Next, DRM2 is recruited to direct both symmetric and asymmetric de novo methylation. Conversely, Arabidopsis encodes a family of DNA glycosylases/lyases that mediate the active demethylation of methylcytosines (28). Recently, an alternative RDR6 21/22-nt siRNA-dependent pathway has been described to mediate the initiation of de novo DNA methylation of transposable elements (29).

The first evidence linking viroid infection and transcriptional regulation in plants was provided by the demonstration that Potato spindle tuber viroid (PSTVd) replication directs de novo methylation of cognate DNA sequences in viroid-expressing transgenic tobacco plants (30). Furthermore, other studies have shown that diverse host genes exhibit a transcriptional alteration during PSTVd (31, 32), Citrus viroid III (33), Peach latent mosaic viroid (34) and Citrus exocortis viroid (35) infections. Although all this experimental evidence may support the idea that viroid infection incites alterations in plant gene expression at transcriptional levels (13), viroid-induced methylation of the host gene(s) or disruption of methylation pathways (which may eventually result in symptom expression) has not yet been reported in natural infections (3).

To address this issue, we first analyzed the alteration in the levels of endogenous sRNAs, associated with ‘Hop stunt viroid’ (HSVd) infection in cucumber (Cucumis sativus) plants. Our results indicate that HSVd-infected plants differentially accumulate high levels of the sRNAs derived from ribosomal transcripts (rb-sRNAs) associated with an increasing accumulation of ribosomal RNA precursors (pre-rRNAs) during infection. Finally, we observed that deregulation in rRNA transcriptional activity correlates with a dynamic modification of the DNA methylation level during the infection process to provide unprecedented insight into the potential endogenous regulatory pathways affected during the viroid life cycle in infected plants.

MATERIALS AND METHODS

Plant material

Cucumber (C. sativus cv Suyo) plants were agroinoculated with Agrobacterium tumefaciens strain C58C1 transformed with a binary pMOG800 vector carrying a head-to-tail infectious dimeric HSVd cDNA (Y09352) (36), as previously described (21) (Supplementary Materials). Mock-inoculated cucumber plants were used as a negative control. Plants were maintained in growing chambers and were analyzed at 10 (T1), 20 (T2) and 30 (T3) days post-infiltration (dpi) (Supplementary Figure S4A).

sRNA library information

The sRNA sequences used in this work were obtained from a library generated by starting from an sRNA population recovered from leaves and phloem exudates of healthy and HSVd-infected cucumber (C. sativus) plants and sequenced by 454 Life Science Technology (Lifesequencing, Branford, CT, USA; www.lifesequencing.com) [(37) - NCBI/SRA accession code SRP001408]. The vd-sRNAs, recovered from infected plants, were filtered out from this analysis.

RNA isolation

Total RNA was extracted from the leaves (~0.1 g) of different infected and healthy cucumber plants using the TRI reagent (SIGMA, St. Louis, MO, USA) according to the manufacturer’s instructions. The low-molecular-weight RNA (~200 nt) fraction was enriched from total RNA using MIRACLE (mRNA isolation Kit, STRATEGENE) according to the manufacturer’s instructions (Supplementary Materials).

Northern blot assays

Total RNA was analyzed by electrophoresis under denaturing conditions in 1% agarose gels with 0.25× Tris-Borate-EDTA (TBE) and 8 M urea (38). The RNA was blotted on nylon membranes (ROCHE Diagnostics GmbH, Mannheim, Germany) and was hybridized as previously described (11). Approximately 25 μg of low-molecular-weight RNA was loaded onto 20%
polyacrylamide gels with 0.25× TBE and 8 M urea. RNA was transferred to a nylon membrane (ROCHE Diagnostics GmbH, Mannheim, Germany). Hybridization was performed as previously described (39).

**Bisulfite conversion and sequencing**

Total genomic DNA was extracted from the leaves (~0.1 g) of different infected and healthy cucumber plants (Supplementary Figure S7A and B) (40). Bisulfite treatment was performed using the EpiTec Bisulfite kit (Qiagen) (Supplementary Material). Modified DNA was amplified by polymerase chain reaction (PCR) and was cloned (Supplementary Figure S7C). Between 13 and 24 clones were sequenced for each analyzed time in both the infected and mock-treated plants.

**RESULTS**

**rRNA-derived sRNAs are highly recovered from HSVd-infected plants**

To evaluate whether viroid infection induces alterations in the general profiles of host endogenous sRNAs, we compared the size distribution of the total reads recovered from HSVd-infected plants with the standard sRNA levels previously described for cucumber (41). As observed in Figure 1A (left), 21-nt sRNAs were the dominant size class in infected plants, whereas lengths of 22 and 24 nt were the most abundant in non-inoculated plants. The 23- and 25-nt-sized classes showed similar accumulation levels in both the control and HSVd-infected sRNA populations. This scenario was in general reproduced (except for the 22-nt reads) when the unique sequences recovered from both datasets were analyzed (Figure 1A, right), indicating that HSVd infection is associated with changes in the endogenous sRNA population levels, being the 21- and 24-nt species up- and downregulated in infected plants, respectively.

When plant endogenous sRNAs recovered from infected and healthy plants were analyzed by pairwise alignment against the cucumber transcript database, we observed that the sRNAs derived from 45S ribosomal RNA were the most differentially accumulated population of sRNAs in viroid-infected plants (Figure 1B). The accumulation of 45S rRNA-derived sRNAs (rb-sRNAs) was at least 4-fold higher in infected (27.4%) compared with control (6.8%) plants. This difference slightly increased (24.2 and 4.9%, respectively) when considering only sRNAs with expected DCL canonical sizes ranging from 21 to 24 nt (Figure 1C). In contrast, negligible differences were found when considering the levels of the unexpected size (<21 to >24 nt in length) reads (Figure 1D). Consistent with the global sRNA population (Figure 1A), the rb-sRNAs of 21 and 24 nt were, respectively, up- and down recovered from the HSVd-infected dataset in comparison with control cucumber reads (Figure 1E). To examine the distribution of the rb-sRNA set, all the potentially DCL-processed sequences (21–24 nt) recovered from the healthy and HSVd-infected cucumber plants were mapped on the 45S rRNA transcriptional unit (Figure 2A). Despite both sRNA populations displaying a relatively heterogeneous distribution pattern along the rRNA sequence (Figure 2B), the total number of sequences matching throughout the rRNA gene significantly increased in infected plants as compared with the reads recovered from mock-inoculated cucumbers (Figure 2C; parametric t-test: arithmetic means of 37.6 and 9.4, for infected and mock-inoculated plants, respectively, \( P < 2.2 \times 10^{-16} \)). Moreover, we noticed two considerable differences between rb-sRNA profiles in both analyzed samples. First, in the infected plants, the differentially recovered rb-sRNAs are predominantly mapping to two homologous regions of 300 nt located in the intergenic spacer (IGS) region and the 3′-end of the 25s rRNA (Figure 2B). The northern blot analyses, using a probe corresponding to this specific IGS region, revealed a remarkable accumulation of rb-sRNAs in infected plants (Figure 2D). Second, a major proportion of rb-sRNAs complementary to the rRNA transcripts from both polarities was recovered from the HSVd-infected plants (Figure 2E and F; Supplementary Figure S1), suggesting that the over-accumulation of rb-sRNAs observed in infected plants might originate from the rRNA-derived double-stranded RNAs processed in sRNAs.

Interestingly, the accumulation of sRNAs derived from 5S rRNA transcripts (5S sRNAs) was also increased in HSVd-infected (0.17%) compared with control (0.06%) plants (Supplementary Figure S2A). Consistent with observations in the 25S sRNA population, the 5S sRNAs of 21 and 24 nt were up- and downregulated in the HSVd-infected plants, respectively, and a major proportion of sRNAs complementary to 5S transcripts was recovered from the infected cucumber plants (Supplementary Figure S2B and C). When the 5S sRNAs were mapped onto their transcriptional unity, we observed that in the infected plants, the 5S sRNAs are heterogeneously distributed along the 5S rRNA sequence (Supplementary Figure S2D). However, the 5S sRNAs recovered from mock control disproportionately (72% of the analyzed reads) match to a region adjacent to the transcription start site. Importantly, the totality of these sRNAs was 24 nt long. All together, these results support the belief that in cucumber, HSVd infection is associated with a drastic alteration of rRNA-derived sRNA processing, mainly characterized by an increase in the accumulation of 21-nt-long sRNAs (potential products of the processing of ds-rRNAs) and a significant decrease of ribosomal-specific 24-nt sRNAs (assumed to be involved in maintenance of methylation status).

**rRNA precursors accumulate differentially during viroid infection**

In *Arabidopsis*, the 45S rRNA genes (Figure 2A) are tandemly arrayed by hundreds at the chromosomal loci known as nucleolus organizers (42). RNA polymerase I (PolI) transcribes primary transcripts (called pre-rRNAs) that are then extensively processed into 18S, 5.8S and 25S rRNAs by the sequential deletion of external and internal transcribed spacers (ETS and ITS, respectively) (43). Each rRNA gene is separated from its adjacent one by an IGS,
and its expression is regulated at several levels according to the physiological need for ribosomes, with one such level being the epigenetic on/off switch that controls the number of active rRNA genes (44). A deficiency in this regulatory mechanism, which controls rRNA transcriptional activity, has been recently associated with overproduction of rb-sRNAs in Arabidopsis mutants (45,46). To determine whether an alteration in the transcriptional activity of rRNA genes can be linked to the substantial accumulation of rb-sRNAs in HSVd-infected cucumber plants, we analyzed the accumulation of pre-rRNAs at specific points (T1: 10 dpi and T3: 30 dpi) during viroid infection. The reverse transcriptase-polymerase chain reaction (RT-PCR) assay was used to compare the pre-rRNA levels, analyzing specifically two regions in the 27S rRNA transcription unit (Figure 3A). Two different primers complementary to the 3’-end of 25S rRNA (25s-A) and ITS2 (ITS2-A) were used to generate the cDNA template. The pair 5.8s-Fw/5.8s-Rv was used to differentially amplify by PCR the unprocessed rRNAs. Significant differences in the pre-rRNA transcript accumulation levels were found in viroid-infected plants in comparison with mock-inoculated controls at infection time T3 when using both the 25s-A and the ITS2-A primers for RT (Figure 3B, B1 and B2, respectively). In the analysis performed at T1 (10 dpi), we observed only a selective accumulation of pre-rRNAs when the PCR was done from the ITS2-A-generated cDNA. This slight incongruence, however, can be attributed to poorer efficiency in the generation of sufficiently long cDNA templates when using the 25s-A primer, instead of ITS2-A, in the RT reaction. The differential accumulation of pre-rRNAs in infected plants at infection time T3 was corroborated by northern blot assays of 27S pre-RNA...
Viroid infection modifies dynamic rDNA methylation

Viroid infection modifies dynamic rDNA methylation

During diverse phases of the plants’ life cycle, rRNA genes are presumably in excess and are consequently silenced. At present, it is well accepted that the silencing of rRNA genes is a self-reinforcing regulatory phenomenon mediated by siRNA-directed cytosine methylation and heterochromatin formation (44,47). To investigate whether the transcriptional deregulation of rRNA genes (revealed as pre-rRNA accumulation) observed during viroid infection can be associated with reduced cytosine methylation, we analyzed by bisulfite sequencing, which identifies the position of methylated and unmethylated cytosines, a 131-bp region of genomic 45S rDNA (Figure 4A). Within this sequence, there were 9 symmetric (5 CG, 4 CHG) and 14 asymmetric (CHH) potential methylation sites (Figure 4B).

The DNA extracted from leaves of HSVd-infected and mock-inoculated cucumber plants at 10 dpi (T1) and 30 dpi (T3) was treated with bisulfite reagent to convert unmethylated cytosines into uracil, followed by PCR to amplify a specific rDNA sequence of 131 nt comprised between positions −115 and +15. PCR products were cloned, and the sequences of 13–24 clones were compiled for each time point. Methylation analysis revealed that at T1 point, HSVd infection resulted in a decrease in the relative number of methylated cytosine residues (83%) when compared with control plants. However, comparable relative methylation levels between infected and healthy plants were observed at 30 dpi (T3) (Figure 4C). When dynamic methylation was differentially analyzed for both symmetric and asymmetric pathways, we saw two different patterns. Symmetric methylation (CG/CHG) slightly decreased during infection and displayed lowered relative methylation levels between 10 and 12% at T1 and T3, respectively (Figure 4D and F; Supplementary Figure S3). These results indicate that HSVd infection is linked to CG/CHG demethylation, which may contribute to the transcriptional activation of normally silenced rDNA units, and can consequently induce the accumulation of pre-rRNA during pathogenesis. Conversely, in the asymmetric context (CHH), loss of relative methylation in infected plants at T1 was more significant than in the symmetric pathway, and a drastic increase in the relative methylation pattern of rDNA was observed at T3 of the HSVd-infected plants (Figure 4D and G; Supplementary Figure S4).

To provide a more accurate picture of the changes occurring in the CHH methylation during viroid infection, we next studied an intermediate point of the infective cycle, at 20 dpi (T2), by bisulfite sequencing (Supplementary Figure S3). As shown in Figure 4E, the level of asymmetric methylation progressively increased at the analyzed infection times, thus explaining the equilibrium in the total methylation level observed at point T3 when comparing the HSVd-infected and mock-inoculated plants (Figure 4C). The alteration in the methylation levels of infected plants at T3 infection time was validated by combined bisulfite and restriction analysis (Supplementary Figure S5). Taken together, these results indicate that in the analyzed rDNA regions, while CG and CHG sites maintain a constant hypomethylated status during infection, the CHH sites show a dual scenario, being hypomethylated at T1 and actively hypermethylated during HSVd infection development.
To obtain additional evidence supporting the influence of HSVd infection on host transcriptional activity, we also analyze by bisulfite sequencing a 149-bp region of 5S rDNA (Supplementary Figure S6A and B), another well-established target transcriptionally regulated by DNA methylation in plants (48). Consistent with observations for 45S rRNA, sequence analysis revealed that HSVd infection is also associated with alterations in the methylation status of the 5S rDNA. Methylation analysis revealed that at initial states of the infectious cycle (T1), the methylation levels observed in the analyzed region of the 5S rDNA were comparable for both analyzed samples (Supplementary Figure S6C). However, HSVd infection resulted in a significant reduction in the relative number of methylated cytosine residues in infected plants (53.6%) compared with mock-inoculated controls (62.4%) at the T3 analyzed infection time (Supplementary Figure S6D). The loss of relative methylation in infected plants at T3 was comparable for cytosine residues in both the symmetric and asymmetric sequence context (Supplementary Figure S6D, E and F). Collectively, our results provide unprecedented insights into alterations in the dynamics of the host DNA methylation status in viroid-induced pathogenesis.

**DISCUSSION**

Increasing evidence supports the notion that the study of viroid–host interactions can shed light on the regulatory pathways directed by ncRNAs in plants (2,13). Within this framework, deciphering the molecular basis of the viroid pathogenesis process emerges as a fundamental milestone to be fulfilled. Since the first viroid-induced disease was reported, diverse pathogenesis models have been proposed (3). The actual prevailing notion linking viroid pathogenesis and RNA silencing essentially considers the
interference of vd-sRNAs in endogenous RNA metabolism at a post-transcriptional level (19,20). However, the possibility that the expression of plant symptoms could eventually be a consequence of specific alterations in host regulatory mechanisms at a transcriptional level has also been envisioned (3,13,31–35). Interestingly, the data obtained in this work reveal that viroid-induced pathogenesis is also associated with transcriptional host alterations.

The initial observation that HSVd-infected cucumber plants show an unexpected hyper-accumulation of rb-sRNAs prompted us to speculate about the possibility

Figure 4. HSVd infection affects the methylation patterns in 45S rRNA genes. (A) Diagram of the rRNA gene intergenic region highlighting the promoter zone analyzed by bisulfite sequencing. The arrows represent the oligos used in the PCR assay and their relative position in the rRNA gene. (B) Graphic representation of the potential symmetric (CG-red bars and CHG-blue bars) and asymmetric (CHH-green bars) positions predicted to exist within the analyzed region. (C) Histogram documenting the relative (HSVd/mock) total DNA methylation levels at infection times T1 and T3. (D) Schematic representation of the differential analysis of both symmetric and asymmetric cytosine methylation at infection times T1 and T3 (paired t-test values T1: means symmetric methylation (mock) 0.73, (infected) 0.67, t = 1.604; means CHH methylation (mock) 0.22, (infected) 0.13, t = 2.180; T3: means symmetric methylation (mock) 0.72, (infected) 0.64, t = 2.481; means CHH methylation (mock) 0.11, (infected) 0.22, t = 3.047; *P < 0.05, **P < 0.01). (E) Evolution of the CHH methylation during HSVd infection in comparison with the level observed in the mock-inoculated plants. (F) Positions of methylcytosines in the analyzed regions displayed in the symmetric (CG and CHG) context. (G) Positions of methylcytosines in the analyzed regions displayed in the asymmetric context. The height of the bar represents the frequency at which cytosine was methylated at the analyzed infection times T1 (left) and T3 (right).
that, during infection, HSVd [as previously observed for a symptomatic variant of *Peach latent mosaic viroid* (49)] could interfere in a yet undetermined manner in the rRNA maturation process, and that the rb-sRNAs highly recovered from infected plants could be a product of the DCL-mediated degradation of aberrantly processed rRNAs. Nonetheless, the observation that mature forms of rRNAs, such as 25 S, accumulate at comparable levels in both healthy and infected plants (Figure 3B and C) was incongruent with this supposition. Consequently, we explored an alternative option that rb-sRNA accumulation in infected plants could be the result of the viroid-induced transcriptional deregulation of rRNAs, resembling that recently observed in *Arabidopsis* where rb-sRNAs overproduction has been linked to deficiencies in the on/off switch controlling the number of active rRNA genes (46). Increased accumulation of 27 S pre-rRNA in infected plants, in parallel to the maintenance of equivalent levels of processed rRNA forms (i.e. 25 S), strongly suggests that, during infection, HSVd may induce the upregulation of some of the normally inoperative rRNA transcriptional units.

Bisulfite sequencing clearly correlated HSVd infection with dynamic DNA methylation changes taking place in the analyzed regulatory region (–115/+15) of 45 S-rDNA. The symmetrical cytosine methylation progressively decreased during infection, whereas the asymmetric *de novo* cytosine methylation also decreased at the initial infection time but actively increased throughout infection development. Interestingly, it has been previously reported in *Arabidopsis* that changes in the expression of rRNA transcripts are related with the activation of silenced 45 S rRNA genes and correlate with reduced CG and CHG methylation in the position flanking the rRNA gene transcription initiation site (44,46). Accordingly, we favor the idea that 27 S pre-rRNA over-accumulation in HSVd-infected plants can result from activation of otherwise generally repressed rDNA genes by loss in maintenance of the methylation status in both symmetric and asymmetric motifs. Additional bisulfite sequencing approaches focused on analysis of the methylation status of 5 S rDNA unities (region –143/+5) revealed that HSVd infections also induce hypomethylation in both a symmetric and asymmetric context in this rDNA family, thus reinforcing the biological relevance of this HSVd-induced phenomenon. At this point, it is important to emphasize that our data were obtained from HSVd-agroinfected plants and not from transgenic plants constitutively expressing viroid RNAs. Consequently, we cannot exclude the possibility that the analyzed tissues could represent a mix of infected and non-infected cells, thus underestimating the effects of viroid infection on host DNA methylation. Our novel and unexpected result for the HSVd pathogenesis process is consistent with that previously reported for plant–bacteria interactions, where 5 S rRNA repeats were demethylated at a significant level in response to infection (50). Furthermore, dynamic changes in DNA methylation patterns have been recently described during antibacterial defense in rice (51), tobacco (52) and *Arabidopsis* (23,50). Finally, in a recent study using transgenic *Nicotiana benthamiana* plants expressing the replication-associated protein (Rep) of a geminivirus, it was proposed that this type of plant DNA viruses can induce a substantial reduction in the levels of host DNA methylation at CG sites in infected plants (53). Interestingly, the geminiviruses have, as HSVd do, a nuclear replication.

Speculations on the nature of the molecular mechanism regulating this active change in rDNA methylation during HSVd infection seem premature at this stage. Intriguingly, however, a similar scenario to that observed in infected cucumber plants has been described in *Arabidopsis* when a mutant for histone deacetylase 6 (HDA6), a key regulator of gene silencing that displays a complex interrelationship with DNA methylation, was analyzed (46). Earley *et al.* reported that *hda6* mutants lose the maintenance of symmetric methylation in 45 S rRNA promoter regions in parallel with a gain of *de novo* CHH methylation. Unexpectedly, and in concert with our results, increasing CHH methylation in *hda6* mutants, a typically repressive phenomenon, failed to suppress rRNA over-transcription. In addition, the siRNAs derived from the 45 S IGS region hyper-accumulated in the *hda6* mutant, resembling, at least in part, that found in viroid-infected cucumber plants (Figure 2B and C). Interestingly, it was also shown that the loss of HDA6 activity induced a decrease of the symmetrical methylation of 5 S rDNA and leads to the release of 5 S rDNA silencing in mutant *Arabidopsis* plants (54). On the other hand, it was also proposed that spurious transcription of ribosomal genes by PolIII can be associated with the elimination of the repressive modification regulating the rRNA expression in *Arabidopsis* (46). By bearing this in mind, it is important to consider that, during infection, HSVd reprograms PolIII activity to transcribe viroid RNA instead of the DNA template (55), which perhaps favors the spurious transcription of rRNAs in infected plants. Further studies are required to explore whether there is some interrelation between the observations herein and the rRNA genes mediated by HDA6 and PolIII in *Arabidopsis*. Moreover, we cannot rule out the possibility that viroid infection may induce changes in widespread dynamic DNA methylation, resembling what was observed in other pathogen–plant interactions (23,50–52). Broader analyses of DNA methylome on viroid-infected plants would help define the impact of viroid infection on DNA demethylation and host gene transcription.

In summary, the data shown here support the belief that during its pathogenesis process, HSVd induces changes in the DNA methylation of inactive rRNA genes, a previously non-described mechanism linked to viroid (or any other pathogenic RNA) infection in plants. Moreover, our findings provide new insights into aspects of the host alterations associated with the HSVd infectious cycle and constitute additional support for the emerging notion that viroid pathogenesis can be a consequence of a multilayered process involving diverse pathogen–host interactions at both the post-transcriptional and transcriptional levels.
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