Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants

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ABSTRACT

Tobacco plants were transformed with a chimeric transgene comprising sequences encoding β -glucuronidase (GUS) and the satellite RNA (satRNA) of cereal yellow dwarf luteovirus. When transgenic plants were infected with potato leafroll luteovirus (PLRV), which replicated the transgene-derived satRNA to a high level, the satellite sequence of the GUS:Sat transgene became densely methylated. Within the satellite region, all 86 cytosines in the upper strand and 73 of the 75 cytosines in the lower strand were either partially or fully methylated. In contrast, very low levels of DNA methylation were detected in the satellite sequence of the transgene in uninfected plants and in the flanking non-satellite sequences in both infected and uninfected plants. Substantial amounts of truncated GUS:Sat RNA accumulated in the satRNA-replicating plants, and most of the molecules terminated at nucleotides within the first 60 bp of the satellite sequence. Whereas this RNA truncation was associated with high levels of satRNA replication, it appeared to be independent of the levels of DNA methylation in the satellite sequence, suggesting that it is not caused by methylation. All the sequenced GUS:Sat DNA molecules were hypermethylated in plants with replicating satRNA despite the phloem restriction of the helper PLRV. Also, small, sense and antisense ~ 22 nt RNAs, derived from the satRNA, were associated with the replicating satellite. These results suggest that the sequence-specific DNA methylation spread into cells in which no satRNA replication occurred and that this was mediated by the spread of unamplified satRNA and/or its associated 22 nt RNA molecules.

Keywords: double-stranded RNA; gene silencing; RNA-directed DNA methylation; luteovirus; RNA interference; transcription elongation

INTRODUCTION

RNA-directed DNA methylation (RdDM) was first demonstrated by Wassenegger et al. (1994), who showed that nuclear replication of potato spindle tuber viroid (PSTVd) induced heavy *de novo* methylation of a PSTVd sequence integrated into the tobacco genome. More recently, Jones et al. (1998, 1999) showed that cytoplasmically replicating RNA of plant poty- and potex-viruses can also induce methylation of homologous nuclear viral transgene sequences. An important characteristic of RdDM is its sequence specificity: only the DNA sequences that are homologous to the replicating RNAs become hypermethylated (Jones et al., 1999; Pélissier et al., 1999). This suggests an involvement of

direct RNA/DNA interaction, possibly RNA/DNA pairing, in the process of RdDM (Jones et al., 1999; Pélissier et al., 1999).

The biological role of RdDM remains unclear. Based on their finding that DNA sequences as short as 30 bp could be targeted for methylation by PSTVd, Pélissier and Wassenegger (2000) proposed that RdDM induced by plant viroids may account for their pathogenicity: replicating viroid RNA may cause methylation of homologous sequences and consequently down-regulation of certain endogenous genes, leading to disease symptoms. It was also proposed that the *de novo* DNA methylation induced by replicating viral RNA is responsible for the maintenance of virus-induced nuclear gene silencing (Jones et al., 1999). In this case, DNA methylation is thought to be required for the production of silence-inducing aberrant transgene RNA after the virus is removed from the plant cells (Jones et al., 1999). *De novo* DNA methylation of coding se-

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quences is frequently found to be associated with post-transcriptional gene silencing (PTGS) in plants (Stam et al., 1997). Although DNA/DNA interactions may account for some cases of this *de novo* methylation (Luff et al., 1999), the coding sequence-specific nature of the methylation suggests that, in many cases of PTGS, the methylation is also RNA directed.

Although the methylation of the promoter region of a gene can lead to transcriptional inactivation (Stam et al., 1997), the effect on gene expression of methylation in the coding region remains unclear. DNA methylation can inhibit elongation of transcription in *Neurospora crassa* (Rountree & Selker, 1997) and in another fungus, *Ascobolus immersus*, methylation of the coding region has been associated with the production of truncated RNAs (Barry et al., 1993). The sizes of these transcripts indicated that the truncations had occurred in the region of methylated DNA, also suggesting that methylation can block transcript elongation. However, there is no clear evidence that DNA methylation leads to premature termination of transcription in plants, and the role of DNA methylation in PTGS remains elusive. Furthermore, whereas RdDM is associated with some cases of virus-induced gene silencing (Jones et al., 1999), it has yet to be demonstrated that viroid-induced methylation can cause gene silencing.

In this study, we examined whether replication of the viroid-like satellite RNA (satRNA) of cereal yellow dwarf luteovirus (CYDV) could affect the epigenetic status of nuclear genes so as to induce PTGS in plants. PTGS can be systemically transmissible in plants, possibly involving mobile signal RNA molecules (Jorgensen et al., 1998; Wassenegger & Pélissier, 1999) and such RNAs may resemble viroids (Voinnet et al., 1998). The CYDV satRNA (Miller et al., 1991) is viroid-like, as it is small (322 nt), circular, has extensive self-complementarity, and uses the rolling-circle replication strategy (reviewed in Symons, 1997). In this replication, the plus strand RNA is copied to produce minus strand RNA and vice versa; both strands undergo self-cleavage to produce unit-length RNAs (and multimers by inefficient cleavage), and the unit-length RNAs are circularized. However, unlike viroid RNA, which replicates autonomously using host-encoded RNA polymerase and is nonen-capsidated, the satRNA replication depends entirely on the presence of a helper virus, which provides the RNA-dependent RNA polymerase required for replication and coat protein for encapsidation. Because the helper virus only replicates in the cytoplasm of phloem and phloem-associated cells, the replication of the satellite RNA presumably also has these restrictions. This is in contrast to the nuclear-localized replication of many viroids, including PSTVd, in a wide range of cells.

We found that the satRNA of CYDV, when replicating in the presence of a helper virus, efficiently induced dense *de novo* methylation of its nuclear transgene sequence. Bisulphite-assisted genomic sequencing

(Clark et al., 1994) revealed that the satRNA-induced methylation was mostly confined to the satellite region of the GUS-satellite fusion transgene, resembling the RdDM induced by PSTVd and by poty-/potexviruses. We also detected a substantial amount of truncated transgene RNAs in the satRNA-replicating plant, and the sites of RNA truncation coincided with the 5' region of the satellite sequence of the chimeric transgene. Our results support the notion that double-stranded RNA is an effective trigger of both RdDM and PTGS in plants.

RESULTS

Transgene-derived CYDV satRNA could be replicated by PLRV

Two constructs (Fig. 1A) were introduced into tobacco through *Agrobacterium*-mediated transformation. One

FIGURE 1. A: Schematic diagrams of constructs used in tobacco transformation. 35S: cauliflower mosaic virus 35S promoter. Ocs3': 3' region of *Agrobacterium* octopine synthase gene. Sat: the full-length sequence of monomeric form of the CYDV satRNA. Sat3': the 3' half of the satellite sequence from nt 148 to the 3' end. Sat5': the 5' half of the satellite sequence from the 5' end to nt 147. GUS: β -glucuronidase gene sequence. E and H: *EcoRV* and *HindIII* restriction sites used in Southern blot hybridization. **B:** The sequences around the GUS:Sat and the Sat:Ocs3' fusion sites in the p35GUS:Sat transgene. The bold letters indicate sequences from the polyliner and Ocs3' in the fusion transgene that correspond to the regions required for formation of the ribozyme cleavage domain of the plus-strand CYDV satRNA. The numbers above the sequences correspond to the nucleotides of the monomeric form of CYDV satellite RNA.

(p35Sat) contains the full-length sequence of the monomeric form of CYDV satRNA, fused at the 5' end with the 3' half of the satellite sequence and at the 3' end with the 5' half of the satellite sequence. Following transcription, this construct should generate two native self-cleaving ribozyme domains of the plus-strand CYDV satRNA (Miller et al., 1991), allowing the production of functional monomeric satRNA. The second construct (p35GUS:Sat) contains a transcriptional fusion between a β -glucuronidase (GUS) sequence and the full-length monomer satellite sequence. In p35GUS:Sat, the sequence postulated to be optimal for the formation of ribozyme structures, which is required for efficient plus-strand cleavage of the native CYDV satRNA, does not occur in the regions flanking the satellite sequence (Fig. 1B). This fusion gene was therefore not expected to produce self-cleaving transcript and hence monomeric satRNA.

Tobacco plants transformed with p35Sat produced modest levels of the expected 322-nt monomeric form of CYDV satRNA (Fig. 2, lanes 3 and 4). The larger bands in these samples presumably represent various partial-cleavage products of the primary transcript. When the T1 progeny of transgenic p35Sat plants was infected with satRNA-free potato leafroll virus (PLRV), which belongs to the same luteovirus family subgroup as CYDV (Wang et al., 1998; Mayo & D'Arcy, 1999), large amounts of satRNA in mono- and polymeric forms were detected (Fig. 2, lanes 6 and 7). This indicates that the transgene-derived satRNA was being replicated in a rolling circle manner by PLRV.

Surprisingly, we found that the p35GUS:Sat plants also gave high levels of satRNA replication when infected with PLRV (Fig. 2, lane 5). They must have produced RNA molecules, despite lacking intact self-cleavage domains, that could be amplified by PLRV into functional satRNA, perhaps arising by nonspecific degradation of the GUS:Sat RNA.

CYDV satRNA replication correlated with dense DNA methylation in the satellite sequence of the GUS:Sat fusion transgene

Four independent p35GUS:Sat transgenic lines (GS1, GS2, GS3, and GS4) were used to study the effect of satellite replication on transgene methylation. Southern analysis (e.g., Fig. 4A) and selectable marker inheritance showed that the T1 siblings within each line inherited identical transgene complements (two copies for lines GS1 [data not shown] and GS3, two to three copies for line GS2, and three to four copies for line GS4) as single loci. The methylation of the transgenes was first examined in uninoculated or PLRV-infected, antibiotic-resistant T1 siblings of these lines using bisulphite sequencing. Bisulphite treatment converts unmethylated C residues in genomic DNA to U residues,

FIGURE 2. Evidence of expression of the monomeric form of CYDV satRNA in primary p35Sat transformants, and replication of the transgene-derived satRNA in primary p35Sat and p35GUS:Sat transformants by PLRV. Total RNA (30 μ g each) was separated in 1.3% formaldehyde agarose gel, blotted to Hybond-N membrane, and hybridized with in vitro-transcribed, 32 P-labeled, antisense CYDV satRNA. Sat RNA, in vitro sense transcript of the Sat3'-Sat-Sat5' fusion sequence in pGEM.Sat, mixed with 30 μ g total RNA from nontransgenic plant. The arrow indicates the monomeric form of CYDV satRNA.

but does not affect methylated C residues; thus cloning and sequencing the PCR amplification products from bisulphite-treated DNA detects the methylation status of each cytosine (Clark et al., 1994). The transgene DNA from uninfected p35GUS:Sat plants (Fig. 3A) had low levels of DNA methylation, of which all but one of the methylated cytosines were in the symmetrical CG or CNG context. In contrast, the satellite sequences of the transgenes in the PLRV-infected, satRNA-replicating, p35GUS:Sat plants were densely methylated (Fig. 3B). All of the 86 cytosines in the upper strand and 73 of the 75 cytosines in the lower strand within the satellite sequences were either fully or partially methylated. These included cytosines in both symmetric and nonsymmetric contexts, although those in the symmetrical contexts were methylated at higher frequencies (85.2% on average) than in the nonsymmetrical contexts (56.6%

on average). The methylation was mostly restricted to the satellite sequences, with only a few cytosines in the immediate flanking sequences being methylated.

The satellite sequence in the p35SGUS:Sat transgene was analyzed by bisulphite sequencing in genomic DNA from PLRV-infected and uninfected plants of all four independent transgenic lines. The degree of methylation in individual PCR clones from uninfected plants ranged from 0 to 15% (6.0% on average). In contrast, every PCR clone from PLRV-infected plants had between 36 and 93% of its cytosines (symmetric and nonsymmetric sites) methylated. This suggests that the hypermethylation occurred in most if not all cells in the PLRV-infected, satRNA-replicating, plants.

To confirm these methylation results from a larger population of molecules, two approaches were taken. One approach used bisulphite conversion and PCR amplification, but replaced the cloning and sequencing with hybridization of the PCR product to a probe containing the original satellite sequence. The other used Southern analysis of the transgene DNA treated with the restriction enzyme McrBC, which digests only methylated DNA (Sutherland et al., 1992). With the first approach, the PCR products from all the uninfected p35SGUS:Sat plants and the control plasmid showed very low levels of hybridization with the original satellite sequence (Fig. 4B, lanes 2, 3, 4, and 8), indicating that most of the C residues in the satellite sequence had been converted to U residues by bisulphite treatment. In contrast, the PCR products from the infected plants gave strong hybridization signals (Fig. 4B, lanes 5–7), indicating that most of the cytosines in the satellite sequence of the transgenes were methylated and resistant to bisulphite conversion.

The second approach also confirmed the correlation between dense methylation of the satellite sequence in the p35SGUS:Sat transgenes and satellite replication. Genomic DNA from both PLRV-infected and uninfected p35GUS:Sat plants was treated with the restriction enzyme *EcoRV*, to release a 1.4-kb fragment that included the full-length satellite sequence (Fig. 1A), and with the enzyme McrBC. The DNA was then hybridized with the satellite sequence. As shown in Figure 4C, the McrBC-treated DNA from uninfected plants gave significant levels of hybridization signals (the decreased size of the hybridization band in comparison to untreated DNA might be due to partial digestion by McrBC), whereas that from the satRNA-replicating plants gave little or no hybridization signals. This demonstrates that the latter had much higher levels of methylation in the satellite sequence than the former.

A small satellite RNA species was detected in satRNA-replicating plants

A small, approximately 25-nt RNA has been found to be correlated with PTGS in plants (Hamilton & Baul-

combe, 1999). To examine whether such RNA species exist in satRNA-replicating plants, RNA was isolated from the same virus-infected and uninfected p35GUS:Sat plants shown in Figure 4B, separated in polyacrylamide gel, and hybridized with sense (Fig. 5A) or antisense (Fig. 5B) satellite probes. Small satellite-derived RNAs of both sense and antisense orientation of ~22 nt were detected in all three plants that contained replicating satRNA, but not in the three uninfected plants. Probing the filter for GUS sequences gave no detectable signal for RNAs in this size range (data not shown).

Accumulation of truncated RNA in p35GUS:Sat plants was associated with satRNA replication

To examine the effect of satRNA replication on the expression of the p35GUS:Sat transgene, total RNA was isolated from PLRV-infected or uninfected p35GUS:Sat plants and analyzed by northern blot hybridization. Infected and uninfected tobacco plants expressing a GUS gene without the CYDV satellite sequence were included as controls. In the p35GUS:Sat plants, the levels of full-length GUS:Sat transcript were, on average, significantly lower in PLRV-infected than in uninfected plants (Fig. 6, lanes 1–8 versus lanes 13–20). Curiously, a reduction of transcription upon PLRV infection was also apparent in the control GUS plants, perhaps as part of a general gene down-regulation due to virus infection. However, the reduction of GUS transcript level (Table 1) was greater in the p35GUS:Sat plants (1.9-fold) than in the GUS plants (0.8-fold). Despite the presence of the satellite sequence in its 3' region, the p35GUS:Sat fusion gene still gave moderate levels of GUS activity. The GUS expression levels determined by fluorometric assay (Jefferson et al., 1987) in infected and uninfected p35GUS:Sat and GUS plants correlated with the abundance of full-length GUS-containing transcript (Table 1).

In the p35GUS:Sat plants in which the satRNA was replicating, we observed a substantial amount of less-than-full-length GUS:Sat transcript (Fig. 6, lanes 1–7). Surprisingly, the majority of this shorter transcript appeared to be of a similar size, forming a relatively unique hybridizing band (band 2). This band could not be seen for the p35GUS:Sat plants (Fig. 6, lanes 13–20) not infected with PLRV or plant GS2-3 (Fig. 6, lane 8), which was infected with PLRV but showed no detectable satRNA replication (it is possible that satRNA replication occurred in this plant but at an undetectable level at the time of assay). RNA band 2 was also absent from the control GUS plants, which instead contained a number of truncated signals with smaller sizes. A further northern blot analysis showed that these smaller bands were common to all the p35GUS:Sat and control GUS plants with or without PLRV infection. The size of band 2 (approximately 1.9 kb) appears to

A

FIGURE 3. Methylation status of CYDV satellite sequence and its flanking GUS-polylinker and Ocs3' sequences in transgenic p35GUS:Sat plants without (**A**, plants GS1-8 or GS1-10) or with (**B**, plants GS1-1 or GS2-2) replicating CYDV satRNA (infected with PLRV for 10 weeks). The empty circles indicate unmethylated cytosine residues, the filled circles the methylated Cs at symmetric (CG or CNG) sites, and the star symbols the methylated Cs at nonsymmetric sites. The number of circles or star symbols in each column indicates the number of sequenced DNA molecules, and the circles or star symbols in each line indicate the methylation status of an individual molecule. The satellite sequence is underlined. (*Figure continues on facing page.*)

be close to that expected for a full-length GUS transcript without the additional sequences from the satRNA and the transcribed region of the Ocs terminator. This suggests that truncation had occurred around the GUS:Sat fusion site.

The 3' ends of the truncated RNAs coincided with the 5' region of the satellite sequence

To examine if RNA truncation in satRNA-replicating p35GUS:Sat plants indeed occurred around the GUS:

B

FIGURE 3. Continued.

Sat fusion area, RNA from the infected plants GS1-2, GS1-5, and GS2-1 and the uninfected plant GS2-4 (Fig. 6, lanes 2, 5, 6, and 18) and from several other infected or uninfected plants of the transgenic lines GS1, GS2, GS3, and GS4 was analyzed by RNA-oligonucleotide ligation and subsequent RT-PCR (Fig. 7A,B). The GUS-specific primer used in the RT-PCR corresponds to the nucleotides around 330 bp upstream of the GUS/satellite junction in the p35GUS:

sat transgene (Fig. 7C). Therefore, the RT-PCR products should be approximately 350 bp (including the two primers) in size if the truncation of RNA occurs around the fusion site between the GUS and the satellite sequences, and approximately 800 bp for the full-length transcript. As predicted, two major RT-PCR products were detected from the satRNA-replicating plants, one around 340 bp and the other around 700–900 bp (Fig. 7A). The smearing of the full-length RNA band

FIGURE 4. **A:** Transgene copy number analysis of T1 progeny of p35GUS:Sat lines GS2, GS3, and GS4 infected (for 17 weeks) with PLRV (Sat+) or uninfected (Sat-). Genomic DNA (20 μ g each) was digested with *Hind*III, separated in 1% agarose gel, blotted onto Hybond-N+ membrane, and hybridized with 32 P-labeled Ocs3' sequence. The asterisks indicate the plants that were analyzed in **B**, in which the same DNA used in **A** was treated with bisulphite, the satellite and 100-bp flanking sequences were amplified by PCR, and the PCR products separated in 1.5% agarose gel (upper panel), blotted onto Hybond-N+ membrane, and hybridized with the satellite sequence (lower panel). **C:** Methylation analysis using *Msp*I digestion. Genomic DNA isolated from plants uninfected (Sat-) or infected (Sat+) with PLRV for 17 weeks (GS3-4, GS2-7), 10 weeks (GS1-1, GS2-2) or 15 months (GS1-2) was digested with *Eco*RV in the presence (+) or absence (-) of the *Msp*I enzyme (New England Biolabs), separated in 1% agarose gel, blotted onto Hybond-N+ membrane, and hybridized with the satellite sequence. NC: non-transgenic control. Marker: DNA size marker. Plasmid: plasmid DNA of p35GUS:Sat.

may reflect the different lengths of the poly(A) tails in the RNA population. This result is in good agreement with that of the northern blot analysis (Fig. 6). The

FIGURE 5. Detection of small satellite RNAs. Total RNA (20 μ g each), isolated using TRIzol from T1 p35GUS:Sat plants (same as those in Fig. 4B) uninfected (Sat-) or infected (Sat+) with PLRV for 17 weeks, was separated in 15% denaturing polyacrylamide gel, blotted onto Hybond-N filter, and hybridized with in vitro-transcribed, 32 P-labeled, sense (**A**) or antisense (**B**) satellite RNA. Marker: size markers comprised of DNA oligonucleotides.

uninfected p35GUS:Sat plant GS2-4 showed little signal around 340 bp (Fig. 7A,B), although when the primary RT-PCR products were reamplified by PCR using the same primers, bands with different sizes were observed in the PCR products for the uninfected as well as the infected plants (Fig. 7B).

To identify the exact 3' ends of the truncated RNA molecules, the DNA (290–400 bp) around the 340 bp band as shown in Figure 7A and B was purified from the gel, cloned into pGEM-T Easy vector and sequenced. As shown in Figure 7C, the majority (37 out of 40) of the truncated RNA molecules from the satRNA-replicating plant had 3' ends that correspond to nucleotides within the first 65 bp of the satellite sequence. Only two of the RT-PCR clones had been truncated outside the satellite sequence. None of the truncations was at the ribozyme cleavage site, indicating that they were not due to ribozyme-mediated self-cleavage of the full-length transcript. An attempt to identify the 3' ends of the low-level truncated RNAs from uninfected plants as shown in Figure 7B was unsuccessful because cloning of the RT-PCR products failed to yield GUS sequence-containing clones.

RNA truncation was independent of the levels of DNA methylation in the satellite sequence

To examine whether the RNA truncation was due to methylation of the satellite sequence, we analyzed the

FIGURE 6. Northern blot analysis of T1 p35GUS:Sat (GS) or control GUS (G) plants uninfected or infected with PLRV (for 10 weeks). Total RNA (25 μ g each) was separated in 1.3% formaldehyde-agarose gel, blotted to Hybond-N membrane, and hybridized with in vitro-transcribed, 32 P-labeled, antisense GUS RNA (A) or CYDV satRNA (C). B: RNA loading control for A. The two arrows show the full-length GUS:sat RNA (1) and the truncated RNA (2) bands.

RNA isolated from p35GUS:Sat plants infected with PLRV for over 13 months (Fig. 8A–C, lanes 1–4) or for 5 weeks (Fig. 8A–C, lanes 5–10). SatRNA replication was evident in the plants 13 months after infection (Fig. 8C, lanes 1–4), but the levels of satRNA were generally low, probably because the tissue was relatively old and unfavorable for helper virus (PLRV) replication. Plants GS3-2 and GS4-1, infected for 5 weeks, showed very high levels of satRNA replication (Fig. 8C, lanes 6 and 7). The other plants infected at that time had low or undetectable levels of satRNA, but the satRNA replication reached high levels similar to that seen in GS3-2 and GS4-1 when analyzed 17 weeks after PLRV infection (data not shown). As shown in Figure 8A and C, truncated RNA was clearly detected in the two plants that had high levels of satRNA replication (lanes 6 and 7). None of the other infected plants showed the truncated RNA signal, despite the presence of replicating satRNA in most of these plants. Interestingly, the level of full-length GUS:Sat RNA in the two plants with truncated RNA (i.e., GS3-2 and GS4-1) was not significantly different from the other plants in Figure 8A that had little or no replicating satRNA and showed no truncated RNA.

High levels of satRNA replication could lead to high levels of DNA methylation in the satellite sequence, which could account for the presence of truncated RNA production. We investigated this by examining the

methylation status of the DNA from plant GS2-2 and from plants GS3-2 and GS4-1 through hybridization of PCR products amplified from bisulphite-treated DNA with the satellite sequence (Fig. 8D). Plant GS2-2 showed a high level of DNA methylation (lane 1) despite the low level of satRNA replication. Sequencing of the PCR products indicated that the levels of methylation in the satellite sequence was comparable to those shown in Figure 3B (50% to 76% of the cytosines in each molecule were methylated). In contrast, plants GS3-2 and GS4-1, which had very high levels of satRNA replication, showed very low levels of DNA methylation (Fig. 8D, lanes 2 and 3). Taken together, these results indicate that widespread, dense DNA methylation induced by satRNA did not occur immediately upon satRNA replication, and suggest that this methylation of the satellite sequence is not the prime cause of the RNA truncation.

DISCUSSION

RNA-directed DNA methylation has been demonstrated with potato spindle tuber viroid (Wassenegger et al., 1994) and plant poty- and potexviruses (Jones et al., 1998, 1999). Here we show that the satellite RNA of cereal yellow dwarf luteovirus, when replicating in plant cells, is also capable of directing high levels of *de novo* DNA methylation. The methylation is highly sequence specific, being restricted mostly to the satellite sequence of the GUS:Sat fusion transgene. Our results thus provide a new example of RdDM and further evidence of the general existence of RdDM in plants. In addition, we present evidence that ~22-nt sense and antisense RNAs are associated with the RdDM-directing satRNA and that DNA methylation in the coding sequence does not cause RNA truncation. This provides insights into the induction and spread of RdDM, and into its possible biological role in plants.

Induction of RdDM

All three types of RNA species (virus, viroid, and now satellite), with the proven ability to direct *de novo* DNA methylation, replicate or are replicated in the plant. Perhaps replication is needed to produce sufficient levels of RNA for efficient RdDM. Alternatively, the double-stranded nature of the RNA, which is expected to exist during the RNA replication process, is the effective inducer of RdDM, as suggested by Kooter et al. (1999). This latter concept is supported by Mette et al. (1999) who showed that transcription of an inverted repeat locus encoding a nopaline synthase promoter (NOSpro), to presumably produce a self-complementary transcript, appeared to direct methylation of other copies of the NOSpro sequence in the genome. It is also con-

TABLE 1. Comparison of full-length transgene RNA levels and GUS activities in PLRV infected (PLRV+) and uninfected (PLRV-) plants

Virus infection	Plants	Relative GUS activity ^a	Relative abundance of full-length RNA ^b	Average values	
				GUS activity	RNA abundance
PLRV+	GS1-1	0.87	1.4	0.50 (0.11) ^c	3.2 (0.4)
	GS1-2	1.10	2.3		
	GS1-3	0.32	4.1		
	GS1-4	0.16	3.9		
	GS1-5	0.27	2.1		
	GS2-1	0.25	2.6		
	GS2-2	0.46	3.8	5.91 (0.41)	23 (3)
	GS2-3	0.54	5.2		
	G1-1	5.75	17		
	G1-2	4.94	19		
	G2-1	5.73	22	1.18 (0.19)	9.3 (0.9)
	G2-2	7.23	35		
PLRV-	GS1-6	1.72	6.4		
	GS1-7	1.13	6.8	8.88 (0.64)	41 (4)
	GS1-8	0.93	11.0		
	GS1-9	1.03	11.9		
	GS1-10	2.32	14.1		
	GS2-4	0.95	8.7		
	GS2-5	0.65	9.2		
	GS2-6	0.72	6.3		
	GS1-3	9.67	43		
	GS1-4	9.15	46		
	GS2-3	9.99	46		
	GS2-4	6.73	28		

^aReading of kinetic fluorometric GUS assay of 1 μ g protein extract.^bRelative signal intensity of the full-length hybridization bands in Figure 6 calculated using the ImageQuant computer program.^cThe figures in brackets are standard errors.

sistent with the results of Dalmay et al. (2000) showing that a release of transgene methylation in *Arabidopsis* plants deficient in PTGS and RNA-dependent RNA polymerase (RdRP) correlated with the proposed inability to produce dsRNA to mediate PTGS.

RdDM signal and spread

PLRV, the helper virus for CYDV satRNA in tobacco, replicates only in the cytoplasm of phloem and phloem-associated cells (Shepardson et al., 1980; Taliansky & Barker, 1999) so the amplification of CYDV satRNA is expected to be similarly restricted. However, our results from a range of analyses suggest that the satellite sequence of the p35GUS:Sat transgene was methylated in most (if not all) cells of the satRNA-replicating plants. This suggests that the satRNAs, unaided by the helper virus, or secondary RNA signals produced from the satRNA were capable of moving from cell to cell and entering nuclei to interact with homologous DNA sequences. The existence of such a diffusible signal for RdDM has been suggested by previous results with PSTVd and viruses. PSTVd-directed DNA methylation

was found to occur in the majority of leaf cells although a large portion of them was expected to be viroid-free (Pélissier et al., 1999) and there is evidence that potyvirus-directed *de novo* DNA methylation can occur in cells of shoot meristems in which the potyvirus has not replicated (Jones et al., 1998).

What is the signal molecule? The presence of ~25-nt RNAs has been found to be perfectly correlated with posttranscriptional gene silencing in plants and has been proposed to be a possible component of the systemic signal for this type of silencing (Hamilton & Baulcombe, 1999). In our study, small, ~22-nt, sense and antisense satellite-derived RNAs could be readily detected in plants showing PLRV-replicated satRNA and heavily methylated satellite transgene sequences, but not in uninfected plants. This makes these small RNAs likely candidates as signal molecules for spreading and directing RdDM. They may also play a similar role in directing sequence-specific RNA degradation as 21–23 nt RNAs have been shown to mediate RNAi (or dsRNA-induced mRNA degradation) in *Drosophila* (Zamore et al., 2000), suggesting that the small RNAs are directing both PTGS and RdDM in plants.

FIGURE 7. Analysis of 3' ends of truncated p35GUS:Sat RNA from T1 plants uninfected (Sat⁻) or infected (Sat⁺) with PLRV for 10 weeks (GS1 and GS2 plants, same as those in Fig. 6) or 5 weeks (GS3-2 and GS4-1, same as those in Fig. 8). Portions of the initial RT-PCR (**A**) or secondary PCR (**B**) reactions were separated in 1.5% agarose gel, blotted onto Hybond-N⁺ filter, and hybridized with ³²P-labeled GUS sequence. NC: nontransgenic control. M: DNA size markers. **C**: Sequencing analysis of RNA-oligonucleotide ligation/RT-PCR products. The arrows indicate the 3' termini of truncated RNA sequences, and each arrow represents one RT-PCR clone that was sequenced. The bold letters indicate the polylinker region between the GUS and the satellite sequences.

Truncation of transgene transcripts associated with satRNA replication

Replication of CYDV satRNA was associated with the accumulation of truncated GUS:Sat RNA, and the truncation occurred within the 5' region of the satellite sequence. Previous studies with *Neurospora* (Rountree & Selker, 1997) and *Ascombolus* (Barry et al., 1993) suggest that DNA methylation may block transcription elongation, which could lead to the production of truncated RNA. However, our results show that the presence of the truncated GUS:Sat RNA was independent of the

degree of DNA methylation in the satellite sequence. This makes it unlikely that methylation was responsible for the production of the truncated RNA observed in the satRNA-replicating plants. The results favor an involvement of direct RNA/RNA or RNA/DNA interactions in the production of truncated RNA. The truncation of GUS:Sat RNAs could be due to specific removal of the satellite region from mature or primary GUS:Sat transcripts through RNAi-like degradation. In *Drosophila*, RNAi-mediated cleavage of mRNA occurs only within the region of identity to the inducer dsRNA (Zamore et al., 2000). However, in the plants where truncated

FIGURE 8. RNA truncation versus satRNA replication and DNA methylation. Total RNA (30 μ g each) isolated from T1 p35GUS:Sat plants uninfected or infected with PLRV for 13 months or 5 weeks was separated in 1.2% formaldehyde agarose gel, blotted onto Hybond-N membrane, and hybridized with in vitro-transcribed, 32 P-labeled antisense GUS (A) or satellite (C) RNA. B: loading control. D: Genomic DNA from plants GS2-2, GS3-2, and GS4-1 (as indicated by arrowheaded lines) was treated with bisulphite, the satellite sequence and 100 bp flanking sequences were amplified by PCR, the products separated in 1.5% agarose gel (upper panel), blotted onto Hybond-N+ membrane, and hybridized with the satellite sequence (lower panel).

RNA occurred, substantial amounts of full-length GUS:Sat RNA persisted, suggesting that, if RNAi-mediated degradation was operational, it was relatively inefficient.

Alternatively, the truncated GUS:Sat RNA could have been produced by transcription that was prematurely terminated by the formation of RNA/DNA triplexes. Triplex-forming oligonucleotides have been shown to inhibit transcription elongation in animal cells (Faria et al., 2000). The satellite-derived ~ 22 nt RNAs in the PLRV-infected p35GUS:sat plants may similarly form triplexes with the homologous DNA sequences in the transgene

and inhibit transcription elongation. The coexistence of both full-length GUS:Sat RNA and truncated RNA, that we observed, might have been due to different levels of the satRNA and the resultant transcription-terminating ~ 22 -nt RNAs in different cell types; the levels of these RNAs could be relatively high in the phloem and phloem-associated cells where the virus replicates, leading to significant levels of transcription termination and truncated RNA, whereas the RNAs that spread into the uninfected cells could be at insufficient concentrations to cause premature termination. Pooling the transcripts from all the cell types, as we did by extracting from whole-tissue samples, would then be expected to give a population of both truncated and full-length transcripts.

The biological role of RdDM

The independence of RNA truncation on DNA methylation observed in this study is consistent with our previous finding that GUS gene silencing by a dsRNA-encoding inverted-repeat transgene is associated with increased DNA methylation in the GUS sequence but is not reversed by demethylation treatment (Wang & Waterhouse, 2000). In both cases, the hypermethylation appears to be a footprint of dsRNA-mediated PTGS, but does not contribute to the reduction in RNA levels. This suggests that, although PTGS and RdDM are likely to be induced by the same dsRNA, they may have distinct biological roles. It has been suggested that PTGS is a natural mechanism developed by plants to fight against viral infection (Covey et al., 1997; Kooter et al., 1999; Waterhouse et al., 1999). DNA methylation, on the other hand, has been suggested to play a role in plant defense against selfish DNA (i.e., transposon elements) by silencing the transposons through methylation-induced chromatin condensation (Ashraf & Ip, 1998; Kooter et al., 1999). It has been recently found that, in *Caenorhabditis elegans*, mutations of several genes involved in RNAi can cause reactivation of transposons (Tabara et al., 1999). This indicates that dsRNA is involved in both RNA degradation and transposon silencing. Our result suggests that this may also be the case in plants; dsRNA confers viral resistance by triggering PTGS against viral RNA, while it can silence the transposons by inducing RdDM in the target sequences.

MATERIALS AND METHODS

Plasmid construction

The self-cleavable CYDV satellite sequence in p35Sat was constructed as follows: the 5' half, the 3' half, and the full-length satellite sequences were amplified by PCR from a cDNA clone (Miller et al., 1991) using the following three combinations of primers, respectively: 5'-CGCGGATCCGTT AACAGAGCGCGTACTGTCTG-3' (primer 1) and 5'-GCCG

AGCTCAAGTCTCCTCACTTCAAAG-3' (primer 2), 5'-GCG CTGCAGCTTTACGTGTTTGTTCAGC-3' (primer 3) and 5'-GCGGGATCCGATATCCACGAAATAGGAAGTCG (primer 4), and primer 1 and primer 4. The three PCR fragments were assembled together in the order of the 3' half, the full-length, and the 5' half sequences through ligations between the *EcoRV* and *HpaI* sites introduced in primers 1 and 4 to form pGEM.Sat. This fusion sequence mimics the native multi-meric form of the plus-strand CYDV satRNA and re-creates the ribozyme domain for plus-strand cleavage. The fusion satellite sequence in pGEM.Sat was excised with *HindIII*/*EcoRI*, treated with Klenow polymerase, and cloned into the *SmaI* site of pART7 (Gleave, 1992), forming p35Sat. To make the GUS:Sat fusion gene, the CYDV satellite sequence was amplified by PCR using the primers 1 and 4 shown above, and cloned along with a full-length GUS sequence into pART7. For *Agrobacterium*-mediated plant transformation, the constructs were inserted into the *NotI* site of pART27 (Gleave, 1992).

Plant transformation

Tobacco (*Nicotiana tabacum* Wisconsin 38) was transformed essentially as described by Ellis et al. (1987).

Northern blot hybridization

Total RNA was isolated from tobacco tissue by the phenol extraction method essentially as described by de Vries et al. (1988) and quantified by UV spectrophotometry. RNA (20–30 μ g) was separated in formaldehyde-agarose gel (Sambrook et al., 1989) and blotted to Hybond-N membrane (Amersham Life Science) using 10 $\times$ SSC. The blot was hybridized with 32 P-labeled RNA probes obtained by in vitro transcription using radioactive UTP from the respective sequences cloned into pGEM plasmids (Promega). Hybridization was performed at 55 °C using buffer B as described in Promega's "Protocols and Application Guide."

Detection of small sense and antisense satellite RNA

Total RNA was isolated from approximately 50 mg of tobacco leaf tissue using TRIzol (Gibco BRL) following the manufacturer's instructions (the overall small RNA detection protocol was kindly provided by Dr. Jan Kooter). Approximately 20 μ g of total RNA were separated in a 15% denaturing polyacrylamide gel, and then electroblotted and UV crosslinked onto Hybond-N membrane. To detect the small satRNA, the membrane was hybridized with sense or antisense satellite RNA probe transcribed from linearized pGEM.Sat plasmid. Hybridization was carried out at 45 °C using the same solution for the northern hybridization described above.

Virus infection

Young plants grown from T1 seed of p35Sat and p35GUS:Sat tobacco were inoculated in a contained room with PLRV by placing approximately 10–20 viruliferous aphids onto each

plant. After 2–5 days, the aphids were killed by application of systemic or contact insecticide, and the infected plants were then transferred to the glasshouse.

Southern blot hybridization

Total genomic DNA was isolated from tobacco leaves using a CTAB (cetyl triethylammonium bromide) method (Draper & Scott, 1988). Approximately 10–20 μ g DNA were digested with appropriate restriction enzymes, separated in agarose gel, blotted onto Hybond-N+ filter (Amersham Pharmacia Biotech) using 0.4 N NaOH solution, and hybridized in formamide-containing buffer with appropriate sequences labeled with 32 P.

Bisulphite conversion and sequencing

The methylation status of the cytosine residues was determined using the bisulphite conversion method described in Clark et al. (1994). Four micrograms DNA each from expanded leaves of satRNA-replicating or nonreplicating plants was treated with bisulphite and purified with the Wizard DNA Clean Up system (Promega). The DNA was resuspended in 100 μ L water, and 5–10 μ L was used in a 100- μ L PCR reaction. A hot-start PCR was performed using the Ampli Taq Gold™ DNA polymerase (Perkin Elmer) with the following reaction profile: 12 min at 94 °C followed by 10 cycles of 1 min at 94 °C, 2:30 min at 50 °C, 1:30 min at 72 °C, and 30 cycles with 1 min at 94 °C, 1:30 min at 55 °C, 1:30 min at 72 °C, with a final extension of 10 min at 72 °C. A 500-nt region of both the upper and the lower strands of DNA, which covers all of the satellite sequence along with 100 nt flanking GUS-polylinker and Ocs3' sequences, was amplified using primers specially designed to preferentially hybridize with the bisulphite-converted molecules. The primers used were: 5'-TAATAATTTTTTGGTGTATTATTGTTGGT-3' (Gus-upper-forward), 5'-TTAAAAACAAATATCATACATCATAAACA-3' (Ocs3'-upper-reverse), 5'-ATAAAAAACCAACAAAAA CAAACAATA-3' (Gus-lower-forward), and 5'-AGTTATATATG TTTAGGTTTTTAT-3' (Ocs3'-lower-reverse). PCR products were purified using the Wizard PCR preps DNA Purification system (Promega) and cloned into pGem-T Easy vector (Promega). Plasmid DNA was prepared from *Escherichia coli* using Wizard Plus Minipreps DNA Purification System (Promega), and sequenced with an automatic DNA sequencer using the M13 reverse primer.

RNA-oligonucleotide ligation-based RT-PCR (3' RACE) and DNA sequencing

Ligation of the oligonucleotide 5'-CCACGTGAGCTCTGATT AAG-3' onto the 3' termini of RNA was performed overnight at 16 °C following the procedure described in Litière et al. (1999). Reverse transcription was performed at 42 °C for 1 h using SupScript reverse transcriptase (GIBCO BRL) and a primer that was complementary to the above oligonucleotide. The subsequent PCR reaction was carried out using a GUS-specific primer, 5'-GACCCTGCAGTGAAGAGTATCAGTGT GC-3', and the primer used for reverse transcription, with the following reaction cycles: 3 min at 94 °C, 1 cycle; 45 s at

94°C, 45 s at 60°C, 1 min at 72°C, 10 cycles; 45 s at 94°C; 45 s at 56°C, 60 s at 72°C, 30 cycles. A portion of the RT-PCR product was separated in 1.5% agarose gel, blotted onto Hybond-N+ filter, and hybridized with ³²P-labeled GUS probe. The RT-PCR product was reamplified by PCR with the same primers, separated in 1.5% agarose gel, and the DNA of approximately 290–400 bp was purified from the gel, cloned into pGEM-T Easy vector (Promega) and sequenced by the Big Dye Terminator method (PE Applied Biosystems) using the GUS-specific primer, 5'-TGGATATGTATCACCGCGTC-3'.

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