RNA (2000), *6*:55–65. Cambridge University Press. Printed in the USA.

A DNA target of 30 bp is sufficient for RNA-directed DNA methylation

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ABSTRACT

In higher plants, RNA–DNA interactions can trigger *de novo* methylation of genomic sequences via a process that is termed RNA-directed DNA methylation (RdDM). In potato spindle tuber viroid (PSTVd)-infected tobacco plants, this process can potentially lead to methylation of all C residues at symmetrical and nonsymmetrical sites within chromosomal inserts that consist of multimers of the 359-bp-long PSTVd cDNA. Using PSTVd cDNA subfragments, we found that genomic targets with as few as 30 nt of sequence complementarity to the viroid RNA are detected and methylated. Genomic sequencing analyses of genome-integrated 30- and 60-bp-long PSTVd subfragments demonstrated that *de novo* cytosine methylation is not limited to the canonical CpG, CpNpG sites. Sixty-base-pair-long PSTVd cDNA constructs appeared to be densely methylated in nearly all tobacco leaf cells. With the 30-bp-long PSTVd-specific construct, the proportion of cells displaying dense transgene methylation was significantly reduced, suggesting that a minimal target size of about 30 bp is necessary for RdDM. The methylation patterns observed for two different 60-bp constructs further suggested that the sequence identity of the target may influence the methylation mechanism. Finally, a link between viroid pathogenicity and PSTVd RNA-directed methylation of host sequences is proposed.

Keywords: co-suppression; epigenetic change; gene regulation; posttranscriptional gene silencing; transcriptional silencing; transgenic plants; viroid-host interaction

INTRODUCTION

The regulation of gene expression is a vital process for living cells. The hypothesis that RNA can regulate gene expression in eukaryotes through epigenetic modification was recently proposed, after the identification of RNA-directed DNA methylation (RdDM) (Wassenegger et al., 1994a; Pélissier et al., 1999). In these studies, heavy *de novo* methylation of viroid cDNA transgenes was demonstrated to occur only in tobacco plants displaying viroid accumulation. Because viroids consist of untranslated RNA molecules that autonomously replicate in the nucleus via the RNA–RNA pathway (for review see Sänger, 1987), it was proposed that methylation can be mediated by the recognition of unusual RNA–DNA pairing structures (Wassenegger et al., 1994a). The demonstration that the methylation was essentially coextensive with the length of the viroid cDNA sequences strongly supported this proposal (Pélissier et al., 1999).

In transgenic plants, RdDM was considered as an attractive mechanism to account for the frequent association of DNA methylation with posttranscriptional gene silencing (PTGS) (Ingelbrecht et al., 1994; Dougherty & Parks, 1995; Meyer, 1995; Baulcombe, 1996; Prins & Goldbach, 1996; Sijen et al., 1996; Depicker & Van Montagu, 1997; Stam et al., 1997; Wassenegger & Pélissier, 1998). More recent investigations showing that RdDM is not peculiar to the viroid system (Jones et al., 1998; Mette et al., 1999) have reinforced the speculation that RdDM represents a broadly important mechanism for gene regulation (Wassenegger & Pélissier, 1998).

Transgenes and cytoplasmic RNA viruses can both activate PTGS, resulting in a sequence-specific degradation of potentially all the RNA species with sufficient homology to the triggering RNA molecules (Stam et al., 1997; Ruiz et al., 1998). This activation has been proposed to proceed via excessive accumulation of a particular RNA (Lindbo et al., 1993) or production of aberrant RNA (abRNA) molecules (English et al., 1996). Although both hypotheses could be valid, a general re-

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quirement for accumulation of abRNA above a critical threshold is now favored (Depicker & Van Montagu, 1997; Vaucheret et al., 1998; Wassenegger & Pélissier, 1998; Waterhouse et al., 1998). Aberrant RNAs may correspond to truncated or incorrectly processed mRNAs, but the demonstration of the involvement of double-stranded RNAs (dsRNA) in initiating PTGS has gained in importance (Montgomery & Fire, 1998; Waterhouse et al., 1998; Wassenegger & Pélissier, 1999; for recent and attractive reviews see Fire, 1999; Kooter et al., 1999). Aberrant RNAs would represent preferred templates for a cellular RNA-directed RNA polymerase (RdRP) (Schiebel et al., 1998). As a consequence, <100-nt-long complementary RNAs (cRNAs) would be synthesized, and these cRNAs would ultimately target cytoplasmic mRNAs for degradation by dsRNA-specific RNases.

It is possible that a feedback effect of the cRNAs (or abRNAs) in the nucleus could lead to transgene methylation via RdDM (Dougherty & Parks, 1995; Sijen et al., 1996; Depicker & Van Montagu, 1997; Wassenegger & Pélissier, 1998). As a result, methylation may enhance the synthesis of abRNAs and enhance the stability of PTGS. In addition, cRNAs may be a component of the sequence-specific silencing signal. Such a signal is required for the spread of silencing from single cells, where PTGS was initiated, to the whole plant (Jorgensen et al., 1998; Voinnet et al., 1998; Palauqui & Balzergue, 1999; Wassenegger & Pélissier, 1999). In nonsilenced receiving cells, cRNA-directed DNA methylation may alter (trans)gene transcription, allowing permanent production of the silencing signal and maintenance of PTGS (Voinnet et al., 1998; Palauqui & Balzergue, 1999).

Further evidence for a role of RdDM in silencing came from a study of PTGS-mediated virus resistance in transgenic pea plants. In these plants, sequence-specific *de novo* methylation of the viral transgene and resistance occurred as a consequence of the presence of homologous RNA (Jones et al., 1998). Interestingly, Mette et al. (1999) recently reported an example of transcriptional gene silencing (TGS) for which an aberrant RNA transcribed from a locus containing promoter sequences was responsible for the methylation of an unlinked homologous promoter *in trans*. Although homology-dependent TGS was thought to be based on DNA–DNA interactions (for review see Matzke et al., 1996), the work of Mette et al. (1999) supports the hypothesis that TGS and PTGS can operate through a similar RNA-based mechanism (Park et al., 1996; Wassenegger & Pélissier, 1998).

To investigate the minimal target size for RdDM, we introduced PSTVd-specific cDNAs of 30, 60, 98, and 160 bp into the tobacco genome. After mechanical inoculation with PSTVd, the methylation state of the transgenes was characterized by Southern blot and bisulphite analyses. Our results show that a DNA target with as few as 30 nt of sequence complementarity to the PSTVd RNA is specifically methylated, demonstrating the remarkably high efficiency of the process. *De novo* methylation at symmetrical and nonsymmetrical sites was detected for all integrates. The methylation patterns also suggest that the identity of the target may influence the mechanism. These results strengthen the view that short cRNAs play a central role in both the degradation and the methylation steps associated with homology-dependent silencing phenomena.

RESULTS

Analysis of RdDM in transformed plants

Our rationale was to investigate the minimal target size necessary for RdDM by introducing PSTVd subfragments of various lengths into the tobacco SRI genome. The five transgene constructions that were used are shown in Figure 1A. To initiate PSTVd infection, a cutting of each individual transformant was mechanically inoculated and autonomous PSTVd RNA replication was monitored by Northern blot analysis. After the plants were proved to be PSTVd-infected (data not shown), genomic DNA was extracted from leaves that emerged from infected tissues. For control experiments, genomic DNA was isolated from viroid-free transformants.

Methylation of genome-integrated PSTVd cDNA subfragments

To obtain initial evidence for PSTVd RNA-directed *de novo* methylation of PSTVd cDNA subfragments, genomic DNAs from the PSTVd-30- (TR1) and PSTVd-60BH- (TR15) transformed plants were used for Southern blot analysis. Hybridization patterns typically obtained with a p35S-specific probe on *Dra*I-*Sma*I restricted DNAs are shown in Figure 1B. For the DNA from viroid-free plants, a single hybridizing band of ~5.3 kb was detected (Fig. 1B, lanes 2 and 4), indicating that the restriction site of the methylation-sensitive *Sma*I endonuclease located within the PSTVd sequence was not methylated (Fig. 1A). In contrast, an additional band of ~7.3 kb was detected with *Dra*I-*Sma*I restricted DNA from the PSTVd-infected TR1 and TR15 plants (Fig. 1B, lanes 1 and 3). The ~7.3-kb fragment was expected if the *Sma*I site within the PSTVd sequences was not cleaved (Fig. 1A). Although most of the TR1 plant DNA was digested at the *Sma*I site within the 30 bp PSTVd-specific transgene sequences (Fig. 1B, lane 1), this site appeared to be extensively methylated within the overlapping 60-bp-long PSTVd subfragment (see Fig. 1A) carried by the TR15 plant (Fig. 1B, lane 3). Characterization of the PSTVd-infected TR6 plant containing the PSTVd-60 construct (Fig. 1A) is presented in Figure 1C. Hybridization of *Ssp*I-*Nci*I-digested DNA with a p35S-specific probe (Fig. 1C, lane 1) revealed that the methylation-sensitive *Nci*I endonuclease failed

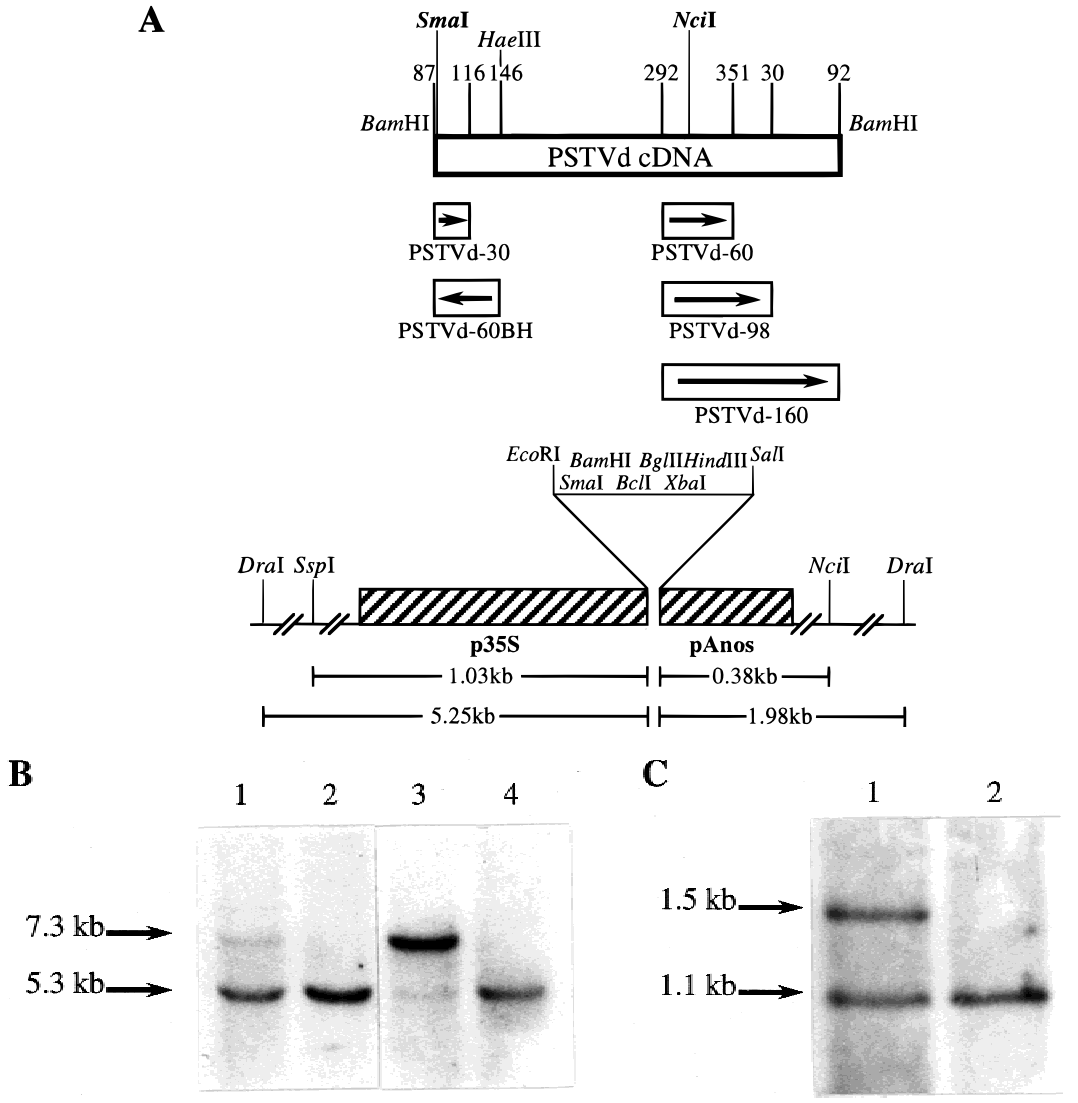

FIGURE 1. Evidence for methylation of the genome-integrated PSTVd-specific cDNA subfragments by Southern analysis. **A**: Schematic representation of the transgene constructions. The subfragments used to generate the PSTVd-30, PSTVd-60BH, PSTVd-60, PSTVd-98, and PSTVd-160 constructs are shown under a schema of a (+)-oriented *Bam*HI PSTVd KF 440-2 cDNA unit. The positions of the subfragments within the PSTVd cDNA unit are indicated by numbers (numbering according to Schnölzer et al., 1985). The *Sma*I and *Nci*I sites that were used to analyze the methylation status of the PSTVd-specific cDNA constructs are indicated in bold. A simplified map of the binary plant transformation vector pPCV702SM is shown at the bottom. The orientations of the PSTVd cDNA subfragments relate to the cauliflower mosaic virus 35S promoter (p35S), and are indicated by arrows. Cloning of the subfragments into the expression cassette is described in Materials and Methods. The termination region of the nopaline synthase gene is referred as pAnos. **B**: Southern blot of *Dra*I-*Sma*I-digested genomic DNA of transformants TR1 and TR15. TR1 and TR15 plants were transformed with the PSTVd-30 and PSTVd-60BH construct, respectively. Lane 1: PSTVd-infected TR1; lane 2: viroid-free TR1; lane 3: PSTVd-infected TR15; lane 4: viroid-free TR15. **C**: Southern blot of *Ssp*I-*Nci*I genomic DNA of the PSTVd-infected (lane 1) and viroid-free (lane 2) TR6 plants. The TR6 transgenic plant carries the PSTVd-60 construct. The size of hybridizing DNA fragments are indicated. Filters were probed with an α-^{32}P-dCTP-labeled p35S-specific DNA fragment.

to completely cleave the unique *Nci*I site within the 60-bp PSTVd-specific cDNA sequence (see Fig. 1A). Similar results were obtained using plants that harbored the PSTVd-98 and PSTVd-160 transgene constructs (Fig. 1A; data not shown). These transgenes both displayed a longer sequence homology with the PSTVd genome. Altogether, these results suggested that the PSTVd RNA can specifically target transgenes with as few as 30 bp homology to the viroid genome for methylation. To confirm this assumption, the methylation pattern of the transgenes was examined in detail using the bisulphite method. Bisulphite treatment allows the conversion of all cytosines to uracil, except those that are 5-methylated (Frommer et al., 1992). Following strand-specific PCR amplification and sequencing, all modified cytosines appear as thymine residues, whereas 5-methylated cytosines (m^5Cs) are still resolved as cytosines.

Methylation status of the PSTVd-60BH and PSTVd-60 transgenes

*Ssp*I-digested DNA from viroid-free and PSTVd-infected TR15 and TR6 plants were used for bisulphite treatment. At first, 31 independent clones, corresponding to upper or lower strands, were amplified from bisulphite-treated DNAs of noninfected TR15 (21 clones) and TR6 (10 clones) plants. The amplified products covered the PSTVd cDNA subfragments as well as part of the p35S and pAnos adjacent regions. Sequence analysis of these clones showed that, for both DNAs, this region was virtually free of cytosine methylation (data not shown). For the viroid-free TR15 plant, only three cytosines, each at different positions, appeared as potentially methylated on a total of 1,430 C residues examined. A similar frequency of cytosine methylation of ~0.2% was detected for the viroid-free TR6 plant (1m^{5}C/380 C positions analyzed), indicating that the 60-bp-long PSTVd-specific transgene sequences are hypomethylated in the viroid-free TR15 and TR6 plants.

This absence of methylation represented an ideal control for the analysis of the PSTVd-infected sister plants. Indeed, it substantiated the efficiency of the bisulphite treatment and demonstrated that none of the analyzed C positions is intrinsically resistant to bisulphite conversion in the genomic context of the TR15 and TR6 plants.

Sequence compilation of the strand-specific PCR amplifications obtained from the PSTVd-infected TR15 plants is presented in Figure 2, and these results are summarized in Table 1. Three additional DNA molecules with a methylation pattern identical to that of CL794 and one identical to CL795 are not included in Figure 2. In the upper and lower strands of the viroid-specific part, 85 and 61.9% of all C residues appeared to be methylated, that is, an overall methylation level of 76.6% (Table 1). Only 3 out of 29 individual DNA strands did not show any detectable methylation in this PSTVd sequence, but one (CL 722) of these molecules displayed m^{5}Cs in the region immediately downstream of the PSTVd-specific sequence region (Fig. 2). For the other DNA samples, upper-strand methylation ranged from 64 to 92% and lower-strand from 42.9 to 92.9%. m^{5}Cs were distributed at both symmetrical and nonsymmetrical sites over the entire PSTVd sequence. The *Sma*I site within this region was found to be methylated for most of the DNA strands (Fig. 2) and this DNA was resistant to *Sma*I digestion. These findings were consistent with the results obtained from Southern blot analysis (Fig. 1B, lane 3).

For the PSTVd upstream region, cytosine methylation was restricted to the 23 bp (upper strand) and 11 bp (lower strand) lying next to the PSTVd-specific part (Fig. 2; Table 1). Although the lower-strand-specific methylation pattern was analyzed up to position −159 (Fig. 2), no further methylation was detected. By contrast, cytosine methylation was found to extend up to 50 bp downstream of the viroid sequence (Fig. 2; Table 1). In this region, two individual strands showed no methylation, whereas all of the others displayed methylation levels ranging from 7.7 to 80%. This corresponded to an overall methylation within this 3′ region of 40%, which was significantly lower than that observed in the 60-bp-long viroid sequence (76.6%). DNA regions downstream of position +50 were analyzed, but no significant methylation could be detected (Fig. 2). Finally, no bias in favor of the symmetrical positions was found, neither in this proximal 3′ region, nor in the PSTVd-specific sequence.

Data of 20 independent clones, representing the methylation pattern of the PSTVd-60 transgene sequences in the PSTVd-infected TR6 plant, are summarized in Table 1. Cytosine methylation was essentially restricted to the 60-bp-long viroid region, with an overall methylation frequency of 35.3%. A similar degree of methylation was found in the proximal 3′ region (Table 1). Within the viroid sequence, m^{5}Cs were present in 18 out of the 20 individual strands, and 54.9% of them were located in symmetrical sites, which contain 43.1% of all the C residues. It should be noted that the observed methylation was not homogeneous along the

TABLE 1. Overview of the methylation of the different PSTVd transgenes.

	% m^{5}C per individual strand for PSTVd cDNA	Overall methylation level[a] for PSTVd cDNA (%)	Methylation in PSTVd-upstream region[b]	Methylation in PSTVd-downstream region[b]
PSTVd-3(+) (SRI-3(+)-9)[c]	90–100	>95	up to −21	up to +117
PSTVd-60 (TR6)	0–75	35.3	none	up to +14
PSTVd-60BH (TR15)	0–92.9	76.6	up to −23	up to +50
PSTVd-30 (TR1)	0–58.3	16	none	up to +22
PSTVd-30 (TR1)[d]	16.7–100	63.5	up to −21	up to +27

[a] Percentage of m^{5}C for the set of DNA strands analyzed.
[b] At least two different strands displaying methylation at the same position.
[c] These data are from Pélissier et al. (1999).
[d] Prior to bisulphite treatment, DNA was digested by *Ssp*I-*Sma*I.

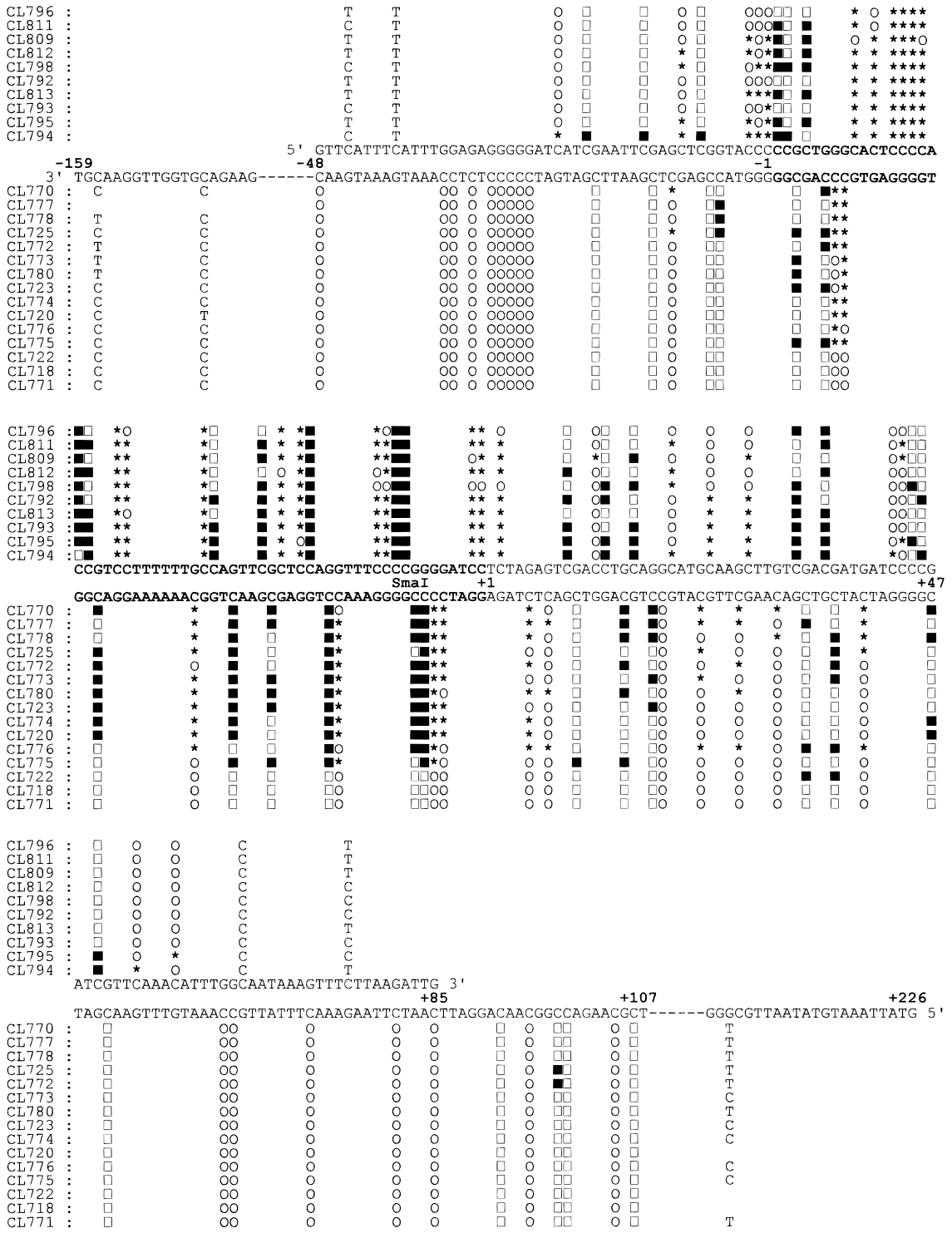

FIGURE 2. Methylation pattern of the PSTVd-60BH construct. For the upper strand, PSTVd-flanking regions ranging from −1 to −48 (p35S) and from +1 to +85 (pAnos) were examined. For the lower strand, the analyzed regions ranged from −1 to −159 (p35S) and from +1 to +226 (pAnos) (only partially presented). The PSTVd-specific sequence is printed in bold and the location of the *Sma*I site is shown. Cytosine methylation at symmetrical CpG and CpNpG sequences is indicated by filled squares, whereas methylation of nonsymmetrical C residues is indicated by asterisks. Nonmethylated C residues at symmetrical and nonsymmetrical positions are represented by open squares and circles, respectively. When the target sequence of the PCR primers (see Materials and Methods) is presented, C or T indicate the nucleotide residues that were found at degenerate positions.

viroid sequence. The 25-bp-long region that is located 5′ to the *Nci*I site (see Fig. 1A) displayed an overall methylation of 16.5%, and only 7 out of the 20 individual strands showed m^5Cs. In contrast to this poorly methylated region, the 3′ region comprising 35 bp displayed an overall methylation level of 42.1%, and 18 out of the 20 clones contained unconverted C residues. Finally, as expected from the Southern blot analysis of PSTVd-infected TR6 DNA (Fig. 1C, lane 1), the *Nci*I site appeared to be methylated and was resistant to *Nci*I digestion in 10 out of the 20 analyzed DNA strands.

Methylation pattern of the 30-bp-long PSTVd cDNA subfragment

Bisulphite analysis of genomic DNA isolated from the viroid-free TR1 plant revealed no transgene methylation (data not shown). However, as for the TR15 and TR6 plants, PSTVd infection of the TR1 plant was associated with specific methylation of the PSTVd cDNA and the immediately flanking sequences (Fig. 3; Table 1). In contrast to the 60-bp cDNA fragments, the 30-bp-long viroid cDNA displayed a significantly reduced overall methylation (Table 1). This is largely due to the fraction of DNA molecules (9/20) that did not contain any m^5C within this region (Fig. 3). Among those molecules that contained m^5C, the level of methylation within the PSTVd region mainly ranged from 8.3 to 37.5%. In particular, methylation at the *Sma*I site was found in a limited number of strands, confirming the results obtained by Southern blot analysis (Fig. 1B, lane 1). The 3′ flanking region, for which 10 out of the 20 characterized DNA molecules contained m^5Cs, presented an overall methylation frequency comparable to that of the viroid-specific part (Table 1). Moreover, three of the upper DNA strands exhibited m^5Cs exclusively within this 3′ flanking region (Fig. 3, CL 864, 867, and 868).

Interestingly, the *Sma*I site of the 30 bp fragment was found to be nonmethylated in all upper strands of the TR1 plant (Fig. 3). To investigate whether these C positions can be methylated in a subgroup of leaf cells, bisulphite analysis was intentionally biased. Prior to bisulphite treatment, the genomic DNA of the PSTVd-infected and viroid-free TR1 cuttings was digested with *Sma*I. No PCR products could be amplified from the viroid-free plant DNA, which confirmed that methylation, at least at the *Sma*I site, was absent from the PSTVd transgenic sequences in the noninfected leaves.

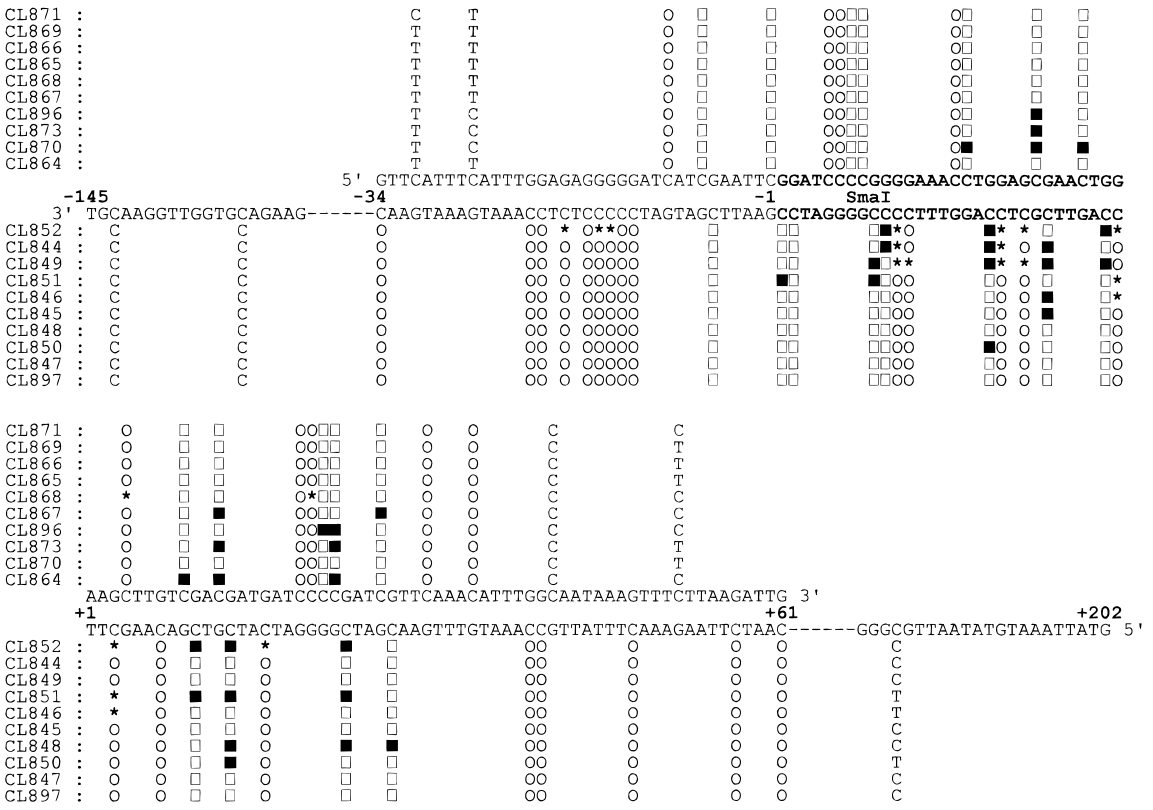

FIGURE 3. State of cytosine methylation of the PSTVd-30 construct. For the upper strand, PSTVd-adjacent regions ranging from −1 to −34 (p35S) and from +1 to +61 (pAnos) were examined. For the lower strand, the analyzed regions ranged from −1 to −145 (p35S) and from +1 to +202 (pAnos) (only partially presented). The PSTVd-specific sequence and the *Sma*I site are indicated in bold. Symbols are used as in Figure 2.

The data obtained from the DNA of the PSTVd-infected TR1 plant are reported in Table 1. An overall methylation level of 63.5 and 43.3% could be detected within the viroid sequence and the 3′ flanking region, respectively (Table 1). For both the upper- and lower-specific strands, the majority of the individual DNA molecules analyzed (17/20) showed m^5Cs distributed over the complete PSTVd sequence, with a methylation frequency ranging from 50 to 100%. In contrast to the methylation patterns previously observed (see, for example, the methylated upper-strand-specific molecules, Fig. 3), this suggests that widely divergent patterns of methylation were present in the PSTVd-infected TR1 plant cells. Thus, densely methylated transgene sequences appeared to be present in a subgroup of leaf cells.

DISCUSSION

In eukaryotes, DNA methylation is thought to play an essential role in various biological processes and is often correlated with gene inactivation. Despite its importance, information about the mechanisms that target particular regions of eukaryotic genomes for *de novo* methylation is scarce. We recently reported that transgenes corresponding to multimeric cDNA units of the 359-nt-long PSTVd RNA acquired a specific and dense methylation, probably as a consequence of pairing interactions between the PSTVd RNA and the genome-integrated cDNA copies (Wassenegger et al., 1994a; Pélissier et al., 1999). In the present study, we show that constructs comprising PSTVd-cDNA subfragments with a size varying from 160 to only 30 bp were all specifically methylated in PSTVd-infected plants. Southern blot analysis revealed that a methylation-sensitive diagnostic site within the viroid sequences was partially protected against digestion. Bisulphite treatment of DNA from the TR1, TR6, and TR15 plants substantiated these data and gave insight into the RdDM process.

In viroid-free plants, no significant methylation could be detected for any of the 30- and 60-bp constructs. In contrast, when these plants were infected with PSTVd, all constructs displayed cytosine methylation that was not confined to canonical CpG and CpNpG sites. The two 60-bp constructs containing different parts of the PSTVd cDNA showed different methylation patterns. Methylation levels within the PSTVd sequence mainly ranged from 43 to 93% for PSTVd-60BH and from 12 to 50% for the PSTVd-60 construct. Significant methylation was detected up to 50 bp downstream of the PSTVd region for the PSTVd-60BH transgene, whereas methylation within the PSTVd-60 construct was almost entirely restricted to the viroid sequence. In contrast to the PSTVd-60BH construct, m^5Cs were concentrated within the 3′ region of the viroid sequence of the PSTVd-60 transgene. Taken together, these observations strongly suggested that the sequence identity of the target influenced the methylation process.

The replication pathway of viroids implies that mature, circular plus-stranded, viroid RNAs and oligomeric minus- and plus-stranded molecules exist in the nucleus of infected cells (Sänger, 1987). Although any of these molecules could be potentially involved in RNA–DNA interactions, we proposed that mature PSTVd molecules actually trigger the methylation process (Pélissier et al., 1999). Mature molecules adopt a highly double-stranded rod-like secondary structure. Thermodynamic studies showed that viroids can undergo structural transitions from this rod-like structure to a metastable, branched structure with a marked loss of base pairing (Loss et al., 1991). This structure displays three particularly stable hairpins which, at higher temperatures, dissociate independently from each other in order of their individual thermal stability. Assuming that such metastable, partially denatured PSTVd RNA molecules interact with genomic DNA, the occurrence of viroid cDNA subfragment-specific methylation patterns can be explained. Three major factors may have an impact on the PSTVd RNA–DNA interaction and as a consequence of this on methylation: (1) RNA–DNA pairing requires a single-stranded RNA that is complementary to the target DNA. Thus, a genome-integrated cDNA could be more prone for interactions with the PSTVd RNA when the cDNA sequence corresponds to a premelting region of the viroid RNA. *Vice versa*, sequences corresponding to the stable hairpins may be less targeted by the PSTVd RNA. (2) Provided that RNA–DNA pairing is once established, the stability of this duplex could be dependent on the stability of the metastable structure of the PSTVd RNA. Transition of the PSTVd RNA proceeds stepwise from the rod-like structure to the completely open structure. However, this transition is strongly biased toward the rod-like RNA molecule. Thus, PSTVd RNA folding into the rod-like structure counterbalances the stability of the RNA–DNA duplex. (3) Finally, the stability of an RNA–DNA duplex could be influenced by the sequence context. Stability of the duplex will increase with the GC content of the hybridizing nucleic acids. It should be noted that the assumptions made above do not involve nucleic acid–protein interactions. However, it is conceivable that binding of proteins to the PSTVd RNA, as, for example, histones or RNA polymerase II, may also contribute to stabilization or destabilization of particular structures. Although there is only little information about viroid–protein interactions, the dependence of PSTVd replication (Schindler & Mühlbach, 1992) and processing (Tabler et al., 1992) on host enzymes is well documented. Sequence comparisons of the two 60-bp transgenes with the PSTVd genome (not shown) showed that partial hybridization of the PSTVd RNA with the genomic 3′ flanking region may extend the length of the interacting region in the PSTVd-60BH transgene. This could, at least par-

tially, explain the frequent occurrence of m^5Cs within this area (Fig. 2; Table 1).

Target sequences consisting of multimeric PSTVd cDNA units displayed a homogeneous and heavy pattern of cytosine methylation. Almost all the cytosines within the viroid sequences appeared to be methylated (Pélissier et al., 1999; see Table 1, first line). In contrast, transgenes comprising PSTVd-cDNA subfragments displayed lower levels of cytosine methylation (Table 1). The assumption that RdDM efficiency is dependent on the length of the target sequence is further supported by the comparison of the methylation patterns of the PSTVd-60BH and the PSTVd-30 constructs. Although the viroid sequences of the PSTVd-60BH construct completely overlapped the 30-bp-long PSTVd cDNA (see Fig. 1A), 30-bp-long PSTVd-specific DNA molecules that were methylated (i.e., subjected to RdDM) displayed a reduced level of methylation as compared to the 60-bp PSTVd subfragment. Application of the "biased bisulphite analysis" suggested that only a subgroup of leaf cells of the TR1 plant contained densely methylated PSTVd-specific 30-bp transgene (see Results; Table 1, line 5). Most importantly, shortening of the sequence complementarity from 60 to 30 nt caused a significant increase in the frequency of unmethylated DNA strands. This may reflect that the minimal target size for RdDM is close to 30 nt. Importantly, our data demonstrated a remarkably high efficiency of the RdDM process. It cannot be ruled out that a particular integration locus might have an effect on the efficiency of the RdDM process. This, however, does not challenge our data that demonstrate that small segments of genomic DNA can be targeted for methylation by RNA.

In situ hybridization studies suggested that the majority of leaf cells (>80%) are viroid free in mechanically inoculated plants (Harders et al., 1989). For the 60-bp constructs, dense methylation was found for the vast majority of the analyzed DNA strands, and no marked bias was detected in favor of symmetrical sites (Fig. 2). As previously discussed in detail (Pélissier et al., 1999), our results show that the viroid-specific RNAs seem to reach most of the leaf cells. Even if they are at a concentration too low to be detectable by in situ hybridization, these PSTVd RNA molecules could be capable to direct *de novo* methylation. Alternatively, initiation of methylation in highly infected cells might produce a novel sequence-specific methylation signal, which could then systemically propagate (Pélissier et al., 1999).

Despite numerous attempts over the past 20 years, the molecular mechanism of viroid-induced pathogenicity is still enigmatic and unresolved. As viroids do not encode any proteins, their pathogenicity must be based on specific interactions between the viroid RNA molecules and a host cell component(s). The nature of the host target(s) is not known. It could be a protein(s), RNA, DNA, or complexes of these molecules. The identification of the RdDM mechanism prompted us to propose a model to explain viroid pathogenicity (Sänger et al., 1996), and our present data support this hypothesis. A host gene(s), exhibiting as few as 30 nt of sequence complementarity with the PSTVd RNA, could be partially methylated upon viroid infection. Methylation-mediated silencing of the corresponding gene(s) would then initiate disease symptom expression. In this context, an intriguing feature of PSTVd infection in tomato has to be considered. Various PSTVd strains, which show remarkable difference in their pathogenicity, possess only subtle sequence alterations along the virulence-modulating (VM) region. Thermodynamic analyses of different PSTVd strains revealed that bending of the VM region was enhanced in severe strains when compared to intermediate strains. Bending might influence the affinity of the viroid to a host protein and modulate the pathogenic response of the infected plant (Schmitz & Riesner, 1998). Alternatively, we propose that bending could affect the capability of PSTVd RNA to recognize and/or to pair with complementary DNA. Conceivably, the stability of the VM region or its potential to bind a host factor(s) both might influence in vivo-specific transitions from the extended rod-like structure to "melted" structures. As a consequence, each PSTVd strain could be associated with a specific expression pattern of the putative target gene(s). According to the expression pattern(s), symptoms may vary from mild to severe PSTVd strains.

DNA sequences of ~30 bp are subject to cytosine methylation, and methylation is not restricted to symmetrical sequence contexts. This efficiency of the RdDM process contrasts with the potential sensitivity of DNA pairing-mediated *de novo* methylation mechanisms. The most convincing evidence for such a DNA-mediated mechanism comes from the MIP (methylation induced premeiotically) process acting in *Ascobolus immersus*. In this filamentous fungus, duplicated sequences are readily detected and methylated. The observations that methylation is exactly coextensive with the duplicated sequence and that either both repeats are affected, or neither of the two is, suggests that MIP involves a direct pairing between the duplications (Rossignol & Faugeron, 1994). In duplications longer than 400 bp, the methylation resulting from MIP affects all the cytosines (Goyon et al., 1994) whereas mainly CpG dinucleotides were methylated in 300–400-bp-long repeats (Goyon et al., 1996). In *Ascobolus* as well as in higher eukaryotic genomes, a minimal size of ~300 bp is probably required to enable direct interactions of homologous DNAs (Rossignol & Faugeron, 1994).

In plants, it appeared likely that most of the methylation patterns being associated with processes such as homology-dependent gene silencing (HdGS) or control of parasitic sequence elements [e.g., transposable elements, (TEs); proviral DNA] result from DNA–DNA

or RNA–DNA interactions (Kooter et al., 1999). Nevertheless, in most of the studies, the involvement of either of these interactions lacks definitive evidence. For example, a key role for a DNA–DNA pairing mechanism was often favored to account for the control of TEs (Bestor & Tycko, 1996; Matzke et al., 1996; Bender, 1998). It was assumed that physical interactions between ectopic TE sequences promote either *de novo* methylation of both interacting repeats or directional transfer of methylation from a methylated repeat to an unmethylated one (Matzke & Matzke, 1995; Bestor & Tycko, 1996). However, recent data indicated that repression of the I element in *Drosophila*, an organism lacking methylation, could be mediated by RNA molecules (Jensen et al., 1999). Particularities in the establishment of repression and its transmission to progeny could best be explained if the triggering RNA molecules induce changes in chromatin structure of the homologous nuclear sequences (Jensen et al., 1999). This study indicates that RNA-directed DNA methylation and/or RNA-directed chromatin structure modification could contribute to regulate the activity of some TE families in eukaryotes. In plants, this might include elements like SINEs (short interspersed nuclear elements) (Yoshioka et al., 1993; Deragon et al., 1994), whose length is too short to trigger direct DNA pairing (see also Kooter et al., 1999).

Finally, our results support the hypothesis for a dual function of short cRNAs in PTGS. Although we cannot exclude that the full-size PSTVd RNA is required to direct methylation, we propose that RNA molecules smaller than 359 nt would be sufficient to trigger RdDM. The fact that viroids accumulate in the nucleus argues for the importance of concentrating the methylation-directing RNA molecules within the nucleus. Assuming a feedback mechanism (Dougherty & Parks, 1995; Sijen et al., 1996; Wassenegger & Pélissier, 1998; Kooter et al., 1999), RdRP-synthesized cRNAs can be transported from the cytoplasm to the nucleus. Subsequently, cRNAs could directly induce methylation of genomic sequences. Alternatively, cRNA and hnRNA could form partially double-stranded molecules (Metzlaff et al., 1997) that might be more prone to direct genomic methylation (Pélissier et al., 1999; for review see Kooter et al., 1999).

In this context, a simplistic analysis using methylation-sensitive endonucleases might not be sufficient to demonstrate the absence of transgene methylation (Van Blokland et al., 1994; Goodwin et al., 1996). However, amplification of bisulphite-treated genomic DNA segments of more than 500 bp is often problematic. In addition, the bisulphite method requires large-scale controls to demonstrate complete conversion of all non-methylated Cs. Therefore, utilization of endonucleases remains a suitable method to assess the methylation status of large DNA regions. If appropriate restriction sites are located within the DNA segment of interest, restriction analysis can give a rough survey about the methylation status of a great number of different tissues or individuals. By contrast, genomic sequencing may provide basic data about the mechanisms of *de novo* methylation. In the case of our studies on RdDM, detection of all m^5Cs was essential to substantiate the high specificity of the methylation machinery for RNA–DNA hybrid structures (Pélissier et al., 1999; this article).

To date, evidence for the implication of RNA as a sequence-specific methylation signal is reported only for plants (Wassenegger et al., 1994a; Jones et al., 1998; Mette et al., 1999; Pélissier et al., 1999). However, RNA-directed epigenetic modifications are suggested to be involved in processes of gene silencing in fungi (Cogoni et al., 1996; Schuurs et al., 1997), in the control of TEs in fruit flies (Jensen et al., 1999), and in imprinting (Reik & Constancia, 1997) and X inactivation (Lee et al., 1999) in mammals. Future studies will show whether such a mechanism functions throughout eukaryotes as a mechanism of epigenetic gene regulation.

MATERIALS AND METHODS

Plasmid constructions

Constructs containing cDNA subfragments of the PSTVd variant KF440-2 genome (Schnölzer et al., 1985) were generated as follows: for the 30-bp construct (PSTVd-30), two complementary primers were annealed to create a *Eco*RI-*Hin*dIII fragment comprising nt 87–116 of the PSTVd RNA genome (for nomenclature see Schnölzer et al., 1985), which was cloned into the *Eco*RI-*Hin*dIII sites of the binary plant transformation vector pPCV702SM (Wassenegger et al., 1994b). The 60-bp *Bam*HI-*Hae*III fragment (nt 87–146) was obtained after *Bam*HI-*Hae*III digestion of a multimeric PSTVd-3(+) cDNA construct (Wassenegger et al., 1994a); after subcloning into *Bam*HI-*Sma*I-restricted pT3T7 vector (Boehringer Mannheim), the viroid segment was introduced into the *Eco*RI-*Hin*dIII sites of pPCV702SM (construct PSTVd-60BH). The other 60-bp (nt 292–351), 98-bp (nt 292–359 and 1–30), and 160-bp (nt 292–359 and 1–92) PSTVd-specific fragments were PCR amplified using the multimeric PSTVd-3(+) cDNA construct as template. PCR products were first subcloned into vector pTPCR (Wassenegger et al., 1994b), then introduced into the *Eco*RI-*Sal*I sites of pPCV702SM (constructs PSTVd-60, PSTVd-98, and PSTVd-160, respectively).

Plant tissue culture and transformation

Introduction of the T-DNA into the tobacco genome (*Nicotiana tabacum* cv. Petit Havana SRI) was performed as previously described (Wassenegger et al., 1994a).

Viroid infection, RNA, and DNA analyses

For viroid infection, one cutting was taken from each primary transformant. After they had been rooted, cuttings were mechanically inoculated and PSTVd replication was monitored

by Northern analysis (Wassenegger et al., 1994a). Plant nuclear DNA was isolated from leaf material after Bedbrook (1981). Southern hybridization was carried out according to Amasino (1986). Sequencing analyses were performed using an automated sequencer (ALFexpress; Pharmacia Biotech) and the Cy5 AutoRead Sequencing Kit (Pharmacia Biotech).

Bisulphite analysis

"Classical" bisulphite treatment was performed on 1 μg of *Ssp*I-digested DNAs essentially as described (Pélissier et al., 1999), but in the presence of urea (Paulin et al., 1998); the reaction mixture included sodium metabisulphite, urea, and hydroquinone to final concentrations of 1.74 M, 5.42 M, and 0.5 mM, respectively.

PCR was performed under standard conditions using 100 ng of treated DNA as template. Upper-strand-specific amplification was performed with the primers p710 BiUS: 5′-AAGYAAGTGGATTGATGTG-3′ and pNOS1020 BiUS: 5′-CTCTAATCATAAAAACCCATCTC-3′; to obtain products for cloning, a second round of PCR was performed with the nested primers p800 BiUS: 5′-GTTYATTTYATTTGGAG AGG-3′ and pNOS910 BiUS: 5′-CAATCTTAARAAACTTTA TTRCC-3′. For PSTVd-60BH-specific amplification only one round of PCR with the primers p800 BiUS-pNOS910 BiUS was performed. For the lower strand, PCR primers were as follows: p700BiLS: 5′-ACRTTCCAACCACRTCTTC-3′ and pNOS1050BiLS: 5′-GTATTAAATGTATAATTGYGGG-3′. PCR products were subcloned into pGEM-T Easy vector (Promega) before sequencing.

ACKNOWLEDGMENTS

We are grateful to N. Emans for critical reading of the manuscript (www.sciwriters.org). We also thank A.M. Bauer and L. Biock-Oswald for taking care of plants in the greenhouse.

Received September 13, 1999; returned for revision October 6, 1999; revised manuscript received October 21, 1999

REFERENCES

Amasino RM. 1986. Acceleration of nucleic acid hybridization rate by polyethylene glycol. *Anal Biochem 152*:304–307.

Baulcombe DC. 1996. RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol Biol 32*:79–88.

Bedbrook J. 1981. A plant nuclear DNA preparation procedure. *Plant Mol Biol News 2*:24.

Bender J. 1998. Cytosine methylation of repeated sequences in eukaryotes: The role of DNA pairing. *Trends Biochem Sci 23*:252–256.

Bestor HB, Tycko B. 1996. Creation of genomic methylation patterns. *Nat Genet 12*:363–366.

Cogoni C, Irelan JT, Schumacher M, Schmidhauser TJ, Selker EU, Macino G. 1996. Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA–DNA interactions or DNA methylation. *EMBO J 15*:3153–3163.

Depicker A, Van Montagu M. 1997. Post-transcriptional gene silencing in plants. *Curr Opin Cell Biol 9*:373–382.

Deragon J-M, Landry BS, Pélissier T, Tutois S, Tourmente S, Picard G. 1994. An analysis of retroposition in plants based on a family of SINEs from *Brassica napus*. *J Mol Evol 39*:378–386.

Dougherty WG, Parks TD. 1995. Transgenes and gene suppression—Telling us something new. *Curr Opin Cell Biol 7*:399–405.

English JJ, Mueller E, Baulcombe DC. 1996. Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell 8*:179–188.

Fire A. 1999. RNA-triggered gene silencing. *Trends Genet 15*:358–363.

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA 89*:1827–1831.

Goodwin J, Chapman K, Swaney S, Parks TD, Wernsman EA, Dougherty WG. 1996. Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. *Plant Cell 8*:95–105.

Goyon C, Barry C, Gregoire A, Faugeron G, Rossignol JL. 1996. Methylation of DNA repeats of decreasing sizes in *Ascobolus immersus*. *Mol Cell Biol 16*:3054–3065.

Goyon C, Nogueira TIV, Faugeron G. 1994. Perpetuation of cytosine methylation in *Ascobolus immersus* implies a novel type of maintenance methylase. *J Mol Biol 240*:42–51.

Harders J, Lukács N, Robert-Nicoud M, Jovin TM, Riesner D. 1989. Imaging of viroids in nuclei from tomato leaf tissue by *in situ* hybridization and confocal laser scanning microscopy. *EMBO J 8*:3941–3949.

Ingelbrecht I, Van Houdt H, Van Montagu M, Depicker A. 1994. Post-transcriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. *Proc Natl Acad Sci USA 91*:10502–10506.

Jensen S, Gassama MP, Heidmann T. 1999. Taming of transposable elements by homology-dependent gene silencing. *Nature Genet 21*:209–212.

Jones AL, Thomas CL, Maule AJ. 1998. De novo methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. *EMBO J 17*:6385–6393.

Jorgensen RA, Atkinson RG, Forster RLS, Lucas WJ. 1998. An RNA-based information superhighway in plants. *Science 279*:1486–1487.

Kooter JM, Matzke MA, Meyer P. 1999. Listening to the silent genes: Transgene silencing, gene regulation and pathogen control. *Trends Plant Sci 4*:340–347.

Lee JT, Davidow LS, Warshawsky D. 1999. *Tsix*, a gene antisense to *Xist* at the X-inactivation centre. *Nat Genet 21*:400–404.

Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG. 1993. Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. *Plant Cell 5*:1749–1759.

Loss P, Schmitz M, Steger G, Riesner D. 1991. Formation of a thermodynamically metastable structure containing hairpin II is critical for infectivity of potato spindle tuber viroid RNA. *EMBO J 10*:719–727.

Matzke MA, Matzke AJM. 1995. How and why do plants inactivate homologous (trans)genes. *Plant Physiol 107*:679–685.

Matzke MA, Matzke AJM, Eggleston WB. 1996. Paramutation and transgene silencing: A common response to invasive DNA? *Trends Plant Sci 1*:382–388.

Mette MF, van der Winden J, Matzke MA, Matzke AJM. 1999. Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. *EMBO J 18*:241–248.

Metzlaff M, O'Dell M, Cluster PD, Flavell RB. 1997. RNA-mediated RNA degradation and chalcone synthase A silencing in petunia. *Cell 88*:845–854.

Meyer P. 1995. Understanding and controlling transgene expression. *Trends Biotechnol 13*:332–337.

Montgomery MK, Fire A. 1998. Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. *Trends Genet 14*:255–258.

Palauqui J-C, Balzergue S. 1999. Activation of systemic acquired silencing by localised introduction of DNA. *Curr Biol 9*:59–66.

Park YD, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke AJM, Matzke MA. 1996. Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. *Plant J 9*:183–194.

Paulin R, Grigg GW, Davey MW, Piper AA. 1998. Urea improves

efficiency of bisulphite-mediated sequencing of 5′-methylcytosine in genomic DNA. *Nucleic Acids Res 26*:5009–5010.
Pélissier T, Thalmeir S, Kempe D, Sänger HL, Wassenegger M. 1999. Heavy de novo methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. *Nucleic Acids Res 27*:1625–1634.
Prins M, Goldbach R. 1996. RNA-mediated virus resistance in transgenic plants. *Arch Virol 141*:2259–2276.
Reik W, Constancia M. 1997. Making sense or antisense? *Nature 389*:669–671.
Rossignol J-L, Faugeron G. 1994. Gene inactivation triggered by recognition between DNA repeats. *Experientia 50*:307–317.
Ruiz T, Voinnet O, Baulcombe DC. 1998. Initiation and maintenance of virus-induced gene silencing. *Plant Cell 10*:937–946.
Sänger HL. 1987. Viroid replication. In: Diener TO, ed. *The viroids*. New York: Plenum Press. pp 117–166.
Sänger HL, Schiebel W, Riedel L, Pélissier T, Wassenegger M. 1996. The possible links between RNA-directed DNA methylation (RdDM), sense and antisense RNA, gene silencing, symptom-induction upon microbial infections and RNA-directed RNA polymerase (RDRP). In: Stacey G, Mullin B, Gresshoff PM, eds. *Biology of plant-microbe interactions*. St. Paul, Minnesota: International Society for Molecular Plant-Microbe Interactions. pp 533–540.
Schiebel W, Pélissier T, Riedel L, Thalmeir S, Schiebel R, Kempe D, Lottspeich F, Sänger HL, Wassenegger M. 1998. Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. *Plant Cell 10*:2087–2101.
Schindler IM, Mühlbach HP. 1992. Involvement of nuclear DNA-dependent RNA polymerases in potato spindle tuber viroid replication: A reevaluation. *Plant Sci 84*:221–229.
Schmitz A, Riesner D. 1998. Correlation between bending of the VM region and pathogenicity of different potato spindle tuber viroid strains. *RNA 4*:1295–1303.
Schnölzer M, Haas B, Ramm K, Hofmann H, Sänger HL. 1985. Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV). *EMBO J 4*:2181–2190.
Schuurs TA, Schaeffer EAM, Wessels JGH. 1997. Homology-dependent silencing of the *SC3* gene in *Schizophyllum commune*. *Genetics 147*:589–596.
Sijen T, Wellink J, Hiriart JB, van Kammen A. 1996. RNA-mediated virus resistance: Role of repeated transgenes and delineation of targeted regions. *Plant Cell 8*:2277–2294.
Stam M, Mol JNM, Kooter JM. 1997. The silence of genes in transgenic plants. *Ann Bot 79*:3–12.
Tabler M, Tzortzakaki S, Tsagris M. 1992. Processing of linear longer-than-unit-length potato spindle tuber viroid RNAs into infectious monomeric circular molecules by a G-specific endoribonuclease. *Virology 190*:746–753.
Van Blokland R, Van der Geest N, Mol JNM, Kooter JM. 1994. Transgene-mediated suppression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. *Plant J 6*:861–877.
Vaucheret H, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel J-B, Mourrain P, Palauqui J-C, Vernhettes S. 1998. Transgene-induced gene silencing in plants. *Plant J 16*:651–659.
Voinnet O, Vain P, Angell S, Baulcombe DC. 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell 95*:177–187.
Wassenegger M, Heimes S, Riedel L, Sänger HL. 1994a. RNA-directed de-novo methylation of genomic sequences in plants. *Cell 76*:567–576.
Wassenegger M, Heimes S, Sänger HL. 1994b. An infectious viroid RNA replicon evolved from an in vitro-generated noninfectious viroid deletion mutant via a complementary deletion in-vivo. *EMBO J 13*:6172–6177.
Wassenegger M, Pélissier T. 1998. A model for RNA-mediated gene silencing in higher plants. *Plant Mol Biol 37*:349–362.
Wassenegger M, Pélissier T. 1999. Signalling in gene silencing. *Trends Plant Sci 4*:207–209.
Waterhouse PM, Graham MW, Wang MB. 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc Natl Acad Sci USA 95*:13959–13964.
Yoshioka Y, Matsumoto S, Kojima S, Ohshima K, Okada N, Machida Y. 1993. Molecular characterization of a short interspersed repetitive element from tobacco that exhibits sequence homology to specific tRNAs. *Proc Natl Acad Sci USA 90*:6562–6566.