Heavy de novo methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation

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ABSTRACT

Previous analysis of potato spindle tuber viroid (PSTVd) RNA-infected tobacco plants has suggested that an RNA–DNA interaction could trigger de novo methylation of PSTVd transgene sequences. Using the genomic sequencing technique, the methylation pattern associated with the RNA-directed DNA methylation process has been characterized. Three different PSTVd transgene constructs all showed a similar pattern of methylation. Most of the cytosines at symmetrical as well as non-symmetrical positions appeared to be methylated in both DNA strands of the viroid sequences. Heavy methylation was mostly restricted to the viroid cDNA sequences. Flanking DNA regions immediately adjacent to the viroid cDNA displayed a lower but significant level of cytosine methylation. The observation that the heavy methylation was essentially co-extensive with the length of the PSTVd cDNA sequences provided evidence that a direct RNA–DNA interaction can act as a strong and highly specific signal for de novo DNA methylation. These data also confirmed that de novo methylation was not limited to canonical CpG and CpNpG sites, but can also involve all the cytosine residues located in the genomic region where the RNA–DNA interaction takes place.

INTRODUCTION

The most common eukaryotic DNA modification is methylation of cytosine at position 5 (m5C). In plants and animals, DNA methylation has been identified as a powerful mechanism to regulate gene expression (reviewed in [1,2]) and is thought to play an essential role in a number of cellular processes, such as developmental control, genomic imprinting, control of parasitic elements and gene silencing (for reviews see 3–7). In animals, C residues at CpG dinucleotides are the preferred targets for DNA methylation, while methylation at both CpG and CpNpG sequences is common in plants (8). The symmetry of the CpG and CpNpG sites was proposed to be important for stable maintenance of methylation patterns throughout DNA replication cycles. After replication, a maintenance methyltransferase (MTase) could rapidly methylate C residues in the newly synthesized strand, if the parental strand contained an m5C in the complementary sequence (9–11). In this semi-conservative model, the methylation pattern at non-symmetrical sequences is not efficiently maintained and should be lost after several cell divisions. However, cytosine methylation of non-symmetrical sequences was recently reported in mammals (12,13), in fungi (14,15) and in plants (16–20) and could contribute to the regulation of gene expression (21). Therefore, non-symmetrical methylation patterns have to be maintained by a mechanism different to that proposed in the semi-conservative model or they have to be established de novo after each DNA replication cycle.

Little is known about the molecular mechanisms that target DNA sequences for de novo methylation. It is also not clear if the processes involved in de novo methylation of symmetrical sequences are different from those taking place in the de novo methylation of non-symmetrical sequences. Because of the difficulty in analyzing cells where de novo methylation is initiated, the frequent appearance of symmetrical methylation patterns may simply reflect that only these patterns are efficiently maintained.

A large part of the information about de novo methylation in higher eukaryotes comes from the characterization of transgenic plants. In plants, heavy de novo methylation and silencing of multiple transgene copies integrated at the same locus have been proposed to occur through a DNA–DNA pairing process (22–24). The best evidence for such a mechanism is based on the methylation induced premeiotically (MIP) phenomenon that was discovered in the fungus Ascomobolus immersus (25). In this filamentous fungus, tandemly duplicated sequences are very efficiently detected and methylated while variable methylation efficiencies are observed for duplicated sequences at ectopic positions. The observations that methylation is co-extensive with duplication and that either both duplicated sequences are affected, or neither of the two is, suggested that this premeiotic process required a direct interaction between the duplicated sequences (25,26). The methylation of an unmethylated sequence homologous to a methylated locus has been observed in different plant species (23,24,27). To account for this de novo methylation, involvement of a DNA pairing-dependent process termed ‘epigene conversion’ was proposed (24,28,29). In epigene conversion, strand interactions between methylated and unmethylated homologs produces hemimethylated intermediates which are the preferred substrates.

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of maintenance MTase (11). Other possible mechanisms for de novo methylation propose either that the MTase has affinity for certain types of structural features in DNA (28,30) or a specific detection of sequences such as transgenes because of their different isochore content (i.e. GC content) compared with the locus of integration (31). Recognition of specific CpG or CpNpG sites localized in a particular sequence context by the DNA MTase, in conjunction with cis- and/or trans-acting regulatory factors, was also considered as a mechanism to initiate de novo methylation (for a review see 32).

Recent findings in our laboratory suggested that an RNA–DNA interaction served as a signal that triggered de novo DNA methylation (33). Viroid cDNA copies integrated into the tobacco genome were methylated after autonomous viroid RNA–RNA replication had taken place in these plants. However, only fragmentary information was obtained on the RNA-directed methylation of the transgenes. Only a few C residues, all of which were located in CpG and CpNpG sequences, were analyzed for methylation by Southern blot analysis using methylation-sensitive endonucleases.

In this paper, our interest was to characterize the RNA-directed DNA methylation (RdDM) process and the pattern of transgene methylation. RdDM was examined by the bisulfite method, which allows the detection of all methylated C residues within a genomic DNA region of interest (34). Here, we report that most if not all the cytosines localized within the genome-integrated viroid sequences were methylated whatever their sequence context. This heavy methylation pattern was almost entirely restricted to the viroid sequences, indicating that the RNA-directed process was highly specific and led to de novo methylation of all the cytosines located in a putative RNA–DNA triplex region. A lower degree of cytosine methylation was observed in the DNA regions immediately flanking the viroid sequences and may partly result from the spreading of methylation from the heavily methylated viroid sequences into the adjacent regions.

**MATERIALS AND METHODS**

**Plant material**

Transgenic SRI-4(–), SRI-3(+), SRI-SB2(+) and SRI-SB2(+)/I tobacco plants containing genomically integrated PSTVd constructs were previously described by Wassenegger et al. (33).

**Plant DNA isolation and Southern blotting**

Plant nuclear DNA was isolated from 10–20 g of leaf material following the procedure of Bedbrook (35). Restriction analysis was performed with 15 µg of genomic DNA and 75–100 U of the appropriate endonuclease in the recommended buffer (Boehringer Mannheim). Digested DNA was electrophoresed in 0.8% agarose gels and transferred onto positively charged Qiabane membrane (Qiagen).

Southern hybridization was carried out according to the method of Amasino (36) using a PSTVd-specific DNA probe labeled with [α-32P]dCTP by a random-primed DNA labeling reaction (Boehringer Mannheim).

**Bisulfite conversion of DNA**

Several series of bisulfite treatments of genomic DNA were performed using two methods, essentially as described by Clark et al. (37) or Olek et al. (38). Prior to the treatment, DNA from SRI-4(–)-6, SRI-3(+)-9, SRI-SB2(+) and SRI-SB2(+)/I-4 plants was digested with SspI, phenol/chloroform extracted, ethanol precipitated and resuspended in water.

‘Classical’ treatment according to Clark et al. (37). Digested DNA (1 µg) was denatured in 0.3 M NaOH for 20 min at 37°C in a total volume of 70 µl. Denatured DNA was mixed with 400 µl of a freshly prepared 2 M sodium metabsulphite (Merck)/0.6 mM hydroquinone (Sigma), pH 5.0, solution (1.7 M/0.5 mM, final concentrations). The reaction mixture was incubated under mineral oil, in a thermal cycler (Appligene) for 18 h at 55°C with a 30 s denaturation step at 95°C every 2 h. DNA was then purified via a desalting column step (Promega Wizard DNA Clean-Up System) and the eluted DNA was incubated in 0.3 M NaOH for 15 min at 37°C. After neutralization with ammonium acetate to a final concentration of 3 M, the DNA was ethanol precipitated and resuspended in 50 µl of water (20 ng/µl).

Bisulfite treatment of agarose beads according to Olek et al. (38). Digested DNA (1 µg) was boiled for 10 min, quickly chilled on ice and subsequently incubated in 0.3 M NaOH for 15 min at 50°C. Denatured DNA was mixed with 2 vol of 2% LMP agarose dissolved in water to give a final volume of 180 µl. This mixture was directly pipetted into chilled mineral oil to form beads of 15 µl (100 ng of denatured DNA/bead). The beads were incubated in 200 µl of 2.5 M sodium metabsulphite (Merck)/1.25 mM hydroquinone (Sigma), pH 5.0, for 4 h at 50°C in the dark and the protocol of Olek et al. (38) was then strictly followed.

To demonstrate the efficiency of bisulfite treatment and the sensitivity of the transgene sequences to bisulfite conversion, the pPCV702SM transformation vectors that carry the PSTVd-4(–), PSTVd-3(+) and PSTVd-SB2(+) transgene constructs, which had been introduced into the SRI tobacco genome, were used as controls. Conversion controls were performed on 135 pg of SspI digested plasmid DNAs, mixed with 1 µg of SspI digested genomic DNA isolated from SRI-SB2(+)-4, SRI-4(–)-6 or SRI-3(+)-9 plants (i.e. ∼50 plasmid copies equivalent genome).

**PCR amplification, cloning and sequencing**

PCR was performed on 5 µl of converted DNA or on a single bead using a Crocodile II apparatus (Appligene). The amplifications were in a reaction volume of 100 µl containing 0.5 µl each primer, 0.2 mM each dNTP and 2.5 U of TaKaRa LA Taq in the recommended buffer. Samples were processed as follows: 1 cycle of 95°C for 3 min with annealing for 1 min at the Tmp (temperature corresponding to the lowest Tm of the primers used); 35 cycles of 72°C for 30 s, 95°C for 30 s, Tmp for 45 s; 1 cycle of 72°C for 5 min.

The following primers were used. Upper strand of p35S–PSTVd junction: PSTVd-4(–) construct, p350BiD (GTATAATTATGTGAGATAGTGG) and p180BiUS (TTTTCACCCTTCITCTTTC); PSTVd-3(+) construct, p710US (AAGY AAGTGGATTGATGTG) and p280BiR (ACRTTCARTTRRTTCCAC); PSTVd-SB2(+) construct, p710US (AAGYAAAGTGGATTGATGT) and p410BiR (CCTRRCRCCACTCCCAC). Lower strand of primer p355–PSTVd junction: PSTVd-4(–) construct, p580BiD (CTTCCTFAAAATRCATCATT) and p100BiLS (GAAYAYTGGAGYGAAYTG); PSTVd-3(+)SB2(+) construct, p700BiLS (ACRTTCACACACCTCTTCT) and p320LS (TTYAGTGTGTTTTYAYYGGGTTAG). Upper strand of PSTVd-pAnos junction: PSTVd-4(–) construct, p320LS (TTY-
AGTTGTTTYYAAYGGGTAG and pNOS1020BiUS (CTCTAATCCATGAACACTCTC); PSTVd-3(+)/SB2(+) constructs, p10BiUS (AYTYGTGGTTYYTGTGGT) and pNOS1020BiUS (CTCTAATCCATGAACACTCTC). Lower strand of PSTVd–pAnos junction: PSTVd-4(–) construct, p140BiR (CRTCRRCCACTCCCCAC) and p940LS (AGAAAATTTATGATATAGTAAAAGYAGYAGAAG) or pNOS1120BiLS (GGYAAATYYYGAAYTAGTAAAYAATAGA). PCR products were excised from the gel and subcloned into the PGEM-Teasy vector (Promega). Sequencing was performed using an automated sequencer (ALFExpress; Pharmacia Biotech) and the Cy5 AutoRead Sequencing Kit (Pharmacia Biotech).

RESULTS

Plant material

Transgenic tobacco lines SRI-4(–), SRI-3(+) and SRI-SB2(+) carrying the PSTVd-4(–), PSTVd-3(+) and PSTVd-SB2(+) transgene constructs, respectively (Fig. 1a), have been previously described (33). SRI-4(–) and SRI-3(+) plants contained viroid (PSTVd) replication initiation (VRI)-competent cDNA constructs while the SRI-SB2(+) plants were transformed with a dimeric VRI-incompetent PSTVd cDNA construct. For PSTVd infection, viroid-free SRI-SB2(+) tobacco cuttings were mechanically inoculated (39) and infected plants were named SRI-SB2(+/VRI). Replication of PSTVd RNA in SRI-4(–), SRI-3(+) and SRI-SB2(+/VRI)-4 plants was monitored by northern blot analysis (data not shown).

PSTVd replication is associated with methylation of viroid cDNA sequences

To determine whether PSTVd cDNA sequences were specifically methylated in PSTVd-infected plants, genomic DNA from different lines was analyzed using the methylation-sensitive HpaII endonuclease (restriction site CCGG, with digestion blocked by methylation at either C residue). The hybridization patterns observed with a PSTVd-specific probe on DraI/HpaII-restricted DNAs of SRI-3(+)-1 and -9 and SRI-4(–)-6 and -8 transformants revealed hybridizing fragments of ~1.7 and ~2 kb, respectively. No faint band of smaller size could be detected even after long film exposure (data not shown). This demonstrated full methylation of all HpaII sites within the PSTVd sequence (see maps in Fig. 1a). As for the SRI-SB2(+/VRI)-4 DNA, an additional PSTVd-hybridizing fragment could be detected.
of a larger size was observed for all the SRI-3(+) and SRI-4(−) DNAs, which clearly suggested that the PSTVd RNA-directed methylation was not strictly restricted to the PSTVd cDNA part of the transgene sequences. The −2 and −2.3 kb fragments detected with the SRI-3(+) and SRI-4(−) DNAs, respectively, were expected if there was only partial cleavage of the HpaII site located in the pAnos region (Fig. 1a).

DNA methylation status of the PSTVd-SB2(+) transgene in SRI-SB2(+)-4 and SRI-SB2(+)/I-4 plants

For bisulphite analysis, we achieved similar results using either of two methods, described by Clark et al. (37) and Olek et al. (38). DNAs of the plasmid vectors carrying the PSTVd-4(−), PSTVd-3(+) and PSTVd-SB2(+) transgene constructs were used as a control for monitoring complete conversion of unmethylated C residues into U residues (for details see Materials and Methods). Each bisulphite-treated plasmid DNA control was amplified with all the primer pairs subsequently used to analyze the corresponding genomic DNA and a total of 72 independent control clones (six clones for each amplification) were characterized. Sequencing analysis demonstrated that the only detectable m^5C residues were located at EcoRII sites (data not shown). This demonstrated that the bisulphite treatment was 100% efficient, because plasmid DNA is known to be methylated at EcoRII sites (Cm^5C/\(\text{Cy}^\text{G}\)) by the bacterial dcm methylase, and confirmed that the DNA regions we intended to analyze did not contain sequences that were intrinsically resistant to bisulphite conversion.

The DNA regions analyzed corresponded to the 5′ junction between the p35S promoter and PSTVd sequences and the 3′ junction between the PSTVd and pAnos sequences (Fig. 1a). The Southern blot analysis indicated that a high level of methylation could exist in the viroid cDNA and in the 5′ and/or 3′ adjacent sequences. Therefore, PCR primers were designed to allow amplification of methylated, unmethylated and partially methylated targets at the 5′ and 3′ junctions (see Materials and Methods).

Using bisulphite-treated SRI-SB2(+)-4 DNA, separate PCR amplifications were performed for the upper and lower strands at the 5′ and 3′ junctions. Sequence analysis of 32 clones showed that these regions were virtually free of cytosine methylation (data not shown). At the 5′ p35S–PSTVd junction, a total of 984 C residues, of which 529 are located within the PSTVd cDNA, were examined. Only three C residues, each at different positions in independent clones, were identified as potentially methylated. Similarly, the analysis of the PSTVd–pAnos junction showed only one potential m^5C out of 712 C positions analyzed (176 C positions within the PSTVd part). These results correspond to a frequency of cytosine methylation of ~0.2%, indicating that the PSTVd transgene sequences are hypomethylated in the SRI-SB2(+)-4 plant.

Sequence compilation of the 5′ and 3′ junctions that were amplified from the bisulphite-treated SRI-SB2(+)/I-4 DNA gave a completely different pattern of cytosine methylation (Figs 2 and 3). In both the upper and lower strand of the viroid-specific part of the 5′ p35S–PSTVd junction, 91.7 and 95.1% of all the C residues were found to be methylated (Fig. 2). Of the m^5C residues, 43.8% was found in a non-symmetrical sequence context, while 29.3% was detected at CpG and 26.9% at CpnPcG sites. This distribution closely reflected the relative representation of these sites in this region (42.7, 31.2 and 26.1%). Upper strand methylation ranged from 60 to 100% and lower strand from 72.5 to 100% for individual DNA molecules. Strikingly, 22 out of 28 DNA molecules displayed >90% methylation and included 11 molecules that were completely methylated. Moreover, the few non-methylated C residues identified seemed to have a random distribution along the viroid-specific sequence.

Cytosine methylation at symmetrical and non-symmetrical sites was also detected in 24 out of 28 individual DNA strands in the non-viroid-specific section of the p35S–PSTVd junction and it appeared to be restricted to the region immediately adjacent to the viroid sequence (positions −1 to −21). In this PSTVd-flanking region, the overall level of methylation rapidly decreased from 75 (−1 to −5) to 29.4% (−6 to −21) with increasing distance from the PSTVd sequence. In the proximal −1 to −21 p35S region, this corresponded to an average degree of C methylation of 38.9%, which was significantly lower than that detected for the viroid sequence (94.7%). In the region further upstream (−22 to −128), sparse methylation was detected in eight out of 28 independent clones. As expected from the Southern blot analysis (Fig. 1b, lane 1), the bisulphite analysis confirmed that the HpaII site located at the 5′ junction between the PSTVd and pAnos sequences was not fully methylated (Fig. 2).

For the SRI-SB2(+)/I-4 plant, the methylation pattern at the 3′ junction is shown in Figure 3 and was essentially similar to that found at the 5′ junction. However, the level of cytosine methylation in the PSTVd region was reduced compared with that at the 5′ junction and only 64% of symmetrical and 69.2% of non-symmetrical C residues were methylated. In individual clones, the methylation levels ranged from 10 to 100%, but the majority of clones (75%) showed >60% methylation. The overall level of methylation decreased to 32.8% in the pAnos region immediately adjacent to the PSTVd sequence (Fig. 3, positions +1 to +20) and, interestingly, seven out of 16 clones did not show any detectable methylation in this area. In the +21 to +117 region, m^5C residues were detected in six out of the 16 individual strands, mainly in symmetrical positions. For the viroid-free SRI-SB2(+)-4 plant, 16 individual clones were analyzed and no methylation was found in the corresponding 3′ junction. This indicated that a low but significant level of methylation was present within this 3′ region of the PSTVd-infected SRI-SB2(+)/I-4 plant. In particular, two out of the 16 clones displayed cytosine modification at the diagnostic HpaII,88 site, which was in good agreement with the low signal strength of the ~1.4 kb band identified in the Southern blot analysis (Fig. 1b, lane 1). Nevertheless, together with the situation observed in the promoter region, these results strongly suggest that in the SRI-SB2(+)/I-4 plant the PSTVd RNA-directed DNA methylation was almost entirely restricted to the PSTVd cDNA transgene sequences.

Methylation pattern of the viroid construct in the SRI-3(+)-9 plant

DNA fragments of 191 and 208 bp representing the upper and lower strands of the 5′ junction from the SRI-3(+)-9 plant were analyzed. These fragments differ from those of the SRI-SB2(+)/I-4 plants by the PSTVd sequence lying directly next to the p35S sequence (see Fig. 1a). Despite this difference, their methylation patterns were essentially the same as those detected for the 5′ junction fragments in the SRI-SB2(+)/I-4 plant (data not shown). Methylation was mainly limited to the viroid sequences, with an overall methylation frequency of 92% in the viroid region and of 39% in the −1 to −21 proximal p35S region.
The general pattern of methylation at the 3' junction was close to that observed in the SRI-SB2(+)/I-4 plant, but it was significantly increased (Fig. 4). In the viroid sequence, nearly all the C residues (99%) were methylated whatever their genomic context. The pAnos region immediately flanking the PSTVd sequence (positions +1 to +20) showed an overall methylation level of 71.9%, which was significantly higher than the 32.8% detected for the same area in the SRI-SB2(+)/I-4 plant. The +21 to +117 region was 16.7% methylated, which was ~7-fold higher than the value observed for the same SRI-SB2(+)I-4 pAnos

Figure 2. Distribution of cytosine methylation at the junction between p35S and PSTVd sequences in the SRI-SB2(+)/I-4 plant. For both strands, a region of the p35S ranging from –1 to –128, relative to the boundary between the p35S and the PSTVd cDNA sequences, was examined. The PSTVd-specific sequence, printed in bold, was analyzed from +1 to +60 for the upper strand and from +1 to +197 (only partially presented) for the lower strand. Cytosine methylation at symmetrical CpG and CpNpG sequences is indicated by filled squares, while methylation of non-symmetrical C residues is indicated by asterisks. Non-methylated C residues at symmetrical and non-symmetrical positions are represented by open squares and circles, respectively. The location of the HpaII site is shown. When the target sequence of the PCR primers (see Materials and Methods) is presented, the nucleotide residues (C or T) found at degenerate positions are indicated.
region, and 54% of the m^5C residues were found in CpG or CpNpG sites, which contain 48.6% of all the C residues. However, nine out of 24 strands did not show any methylation within this area whereas the majority of the DNA molecules displayed m^5C residues in the +1 to +20 pAnos region. In four out of 24 DNA strands, the HpaII +88 site appeared to be methylated and was resistant to HpaII digestion, as expected from the Southern blot analysis of SRI-3(+)-9 DNA (Fig. 1c, lane 2). Within the +40 to +117 region, half of the DNA strands displayed variable m^5C levels, mostly in the range of 7–23% (Fig. 4). DNA regions downstream of position +117 were also analyzed, but only sparse methylation could be detected (data not shown).

**Methylation pattern of the PSTVd transgene in the SRI-4(−)-6 plant**

The PSTVd-4(−) construct contained 4.4 PSTVd cDNA units that were arranged in an opposite orientation to the PSTVd-SB2(+) and PSTVd-3(+) constructs (Fig. 1a). The methylation level detected in the 5′ junction reached 98.2% for the PSTVd sequences (data not shown), a value comparable with those observed in SRI-SB2(+)/I-4 and -3(+) plants. However, cytosine methylation reached 74.3% in the proximal –1 to –21 p35S region while only ∼39% of the cytosines were methylated in SRI-3(+) plant (2/24). (ii) The +41 to +90 pAnos region for which only 10 out of the 22 clones displayed cytosine methylation (70% for clone 502 and 10–44% for the other clones). (iii) The +90 to +200 region which appeared virtually free of methylation (Fig. 5 and data not shown). Within the +1 to +90 pAnos region, 63.4% of the m^5C residues are localized at CpG and CpNpG sequences while the relative representation of these sites was only 47.4%. This obviously indicates that the overall distribution of m^5C residues was biased in favour of symmetrical sequences. The diagnostic HpaII site appeared to be methylated in a limited number of clones, which was suggested by the Southern blot analyses (Fig. 1c, lane 3).

To verify the bisulphite results, the methylation status of the SRI-4(−)-6 PSTVd sequence was examined using BamHI or AvaII endonuclease. BamHI is sensitive to cytosine methylation at non-symmetrical positions whereas AvaII is sensitive to methylation on both symmetrical and non-symmetrical cytosines (see Fig. 6a). An aliquot of 15 µg of genomic DNA was spiked with 5 ng of plasmid DNA (∼400 gene copy equivalents) prior to enzymatic digestion. A Southern blot of DraI/BamHI and DraI/AvaII restricted DNAs was first probed with a PSTVd-specific probe and autoradiographed for 72 h (Fig. 6b). After removal of the probe, the blot was rehybridized with a plasmid-specific probe and autoradiographed for 5 min (Fig. 6c). The hybridization patterns obtained with the plasmid probe perfectly correspond to those expected for the fully digested plasmid DNA (Fig. 6c), indicating the absence of restriction endonuclease inhibitors. With the PSTVd probe (Fig. 6b), cross-hybridization to the plasmid-specific restriction fragments was detectable on the 72 h exposed autoradiograph (compare Fig. 6b and c). The cross-hybridization resulted either from a contamination...
Figure 4. State of cytosine methylation at the PSTVd–pAnos junction of the SRI-3(+)–9 plant. The DNA region ranging from –65 to +117, relative to the border between PSTVd and pAnos sequences, is presented. The PSTVd-specific sequence is presented in bold. The location of the HpaII +88 site is indicated. Symbols are used as in Figure 2.

of the probe with traces of plasmid DNA or from the high CG content of the PSTVd sequence. For the Dral/BamHI digested DNA, detection of a major band of ~8.8 kb indicated that the BamHI sites within the viroid sequence were extensively methylated (Fig. 6b, lane 1). However, the presence of an ~6.8 kb faint band showed that, at least in some DNA molecules, the BamHI site located at the 3′ boundary of the PSTVd sequence was cut (Fig. 6a). The Dral/AvaII digestion revealed a unique PSTVd-specific fragment of ~3.2 kb (Fig. 6b, lane 2), demonstrating that the AvaII sites within the viroid sequence were also extensively methylated (see Fig. 6a). The absence of any larger hybridizing band further indicated that the AvaII site close to the 5′ junction (position –180) was not methylated. This result was in agreement with the bisulphite analysis (see above, Fig. 5). As observed for the Dral/BamHI restricted DNA, no hybridizing fragment corresponding to PSTVd monomers or multimers was detected. Thus, the Southern blot results strongly support the conclusion that a heavy methylation pattern was associated with the PSTVd transgene sequence.

DISCUSSION

DNA methylation is a well-characterized phenomenon which is involved in many diverse biological events, such as genomic imprinting and gene silencing. However, little is known about the molecular signals that regulate de novo methylation. Here, we demonstrate that de novo methylation of symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. We previously reported that during PSTVd RNA replication, PSTVd cDNA copies integrated into the tobacco genome are methylated (33). Viroids are plant pathogens consisting of circular, single-stranded, non-encapsidated RNA molecules, which do not encode any protein. The demonstration that viroids use an RNA–RNA pathway to autonomously replicate in the nucleus (reviewed in 40) led to the hypothesis that an RNA–DNA interaction could serve as a signal to direct de novo methylation (33).

In this study, the methylation pattern associated with viroid replication was characterized in transgenic plants using the genomic sequencing method (34). In the viroid-free SRI-SB2(+)-4 plant line, no significant methylation could be detected within the PSTVd transgene sequences. In contrast, the viroid-infected SRI-SB2(+)/I-4, SRI-3(+)–9 and SRI-4(–)/6 plants displayed methylation levels ranging from 90 to 100% within the PSTVd cDNA sequences. The only notable exception was observed at the 3′ junction in the SRI-SB2(+)/I-4 plant containing a lower level of m5C. This observation was not surprising because the PSTVd sequence comprised a 26 bp deletion (Fig. 1a), unlike the wild-type replicating viroid. Thus, formation of a RNA–DNA hybrid will be less efficient within this region than within the 5′ part where the PSTVd RNA and the viroid cDNA are entirely complementary. This would then lead to a lower level of C methylation at the SRI-SB2(+)/I-4-specific 3′ junction.

Although the PSTVd cDNAs of all the different constructs were not entirely sequenced, it is most likely that internal PSTVd
Figure 5. State of cytosine methylation at the PSTVd–pAnos junction of the SRI-4(–)-6 plant. An upper DNA strand region from –169 to +117 and a lower DNA strand from –59 to +117 are shown relative to the border between PSTVd and pAnos sequences. The locations of the \( \text{Hpa} \text{I} \) and the \( \text{Hpa} \text{II} \) sites are indicated. Symbols are used as in Figure 2.

Figure 6. Evidence of methylation at symmetrical and non-symmetrical positions in the PSTVd transgene sequence by restriction analysis. (a) Schematic map of the PSTVd-4(–) transgene. PSTVd cDNA units are represented by open boxes; the promoter and poly(A) terminator region are delimited by hatched boxes. Restriction sites for \( \text{Bam} \text{HI} \) (B) and \( \text{Ava} \text{II} \) (A) are indicated. Lengths of predicted restriction fragments are shown below (n.m. = not mapped). Restriction sites that are highly methylated are marked by asterisks. The sequence contexts of the \( \text{Bam} \text{HI} \) and \( \text{Ava} \text{II} \) sites in the PSTVd transgene are shown above the map; sites are boxed and cytosines that can inhibit enzymatic cleavage when methylated are in bold. (b) Southern blot of \( \text{Dra} \text{I}/\text{Bam} \text{HI} \) (lane 1) and \( \text{Dra} \text{I}/\text{Ava} \text{II} \) (lane 2) restricted genomic DNA from the SRI-4(–)-6 plant mixed with plasmid DNA. The blot was hybridized to \( ^{32} \text{P} \)-labeled PSTVd cDNA and autoradiographed for 72 h. (c) Reprobing of the blot with a \( ^{32} \text{P} \)-labeled plasmid probe after removal of the PSTVd-specific probe. The blot was autoradiographed for 5 min. The sizes of hybridized DNA fragments in kb are indicated.
sequences are also heavily methylated at symmetrical and non-symmetrical positions. As shown for the SRI-4(–)–6 DNA, this is supported by evidence that the AvrII, BamHII and HpaII restriction sites, which are distributed along the PSTVd-specific transgene sequence, are resistant to digestion (Figs 1 and 6). For most of the individual DNA strand molecules, heavy methylation was almost entirely restricted to the PSTVd sequences. The flanking DNA regions were either sparsely methylated or unmodified (see below). The demonstration of heavy methylation within the PSTVd-specific sequences shows the high specificity of the methylation machinery. It also provides a strong argument for de novo methylation being directed by unusual structures that could arise by pairing of RNA molecules with their genomic counterparts. As originally put forward (33), this process should be termed RNA-directed and not RNA-mediated DNA methylation (RdDM). This is to emphasize that only DNA sequences complementary to the directing RNA are specifically methylated.

In plants, the characterized DNA MTases are closely related to the putative mammalian maintenance enzyme (see 5,7). They show a high specificity for CpG and/or CpA/TpG sites and for hemimethylated DNA. These enzyme characteristics, together with the self-complementarity of CpG and CpNpG sites, explain the stable propagation of methylation patterns. This is in contrast to the RdDM pattern described here. Most, if not all, of the cytosines which are located in the putative RNA–DNA triplex region are methylated, irrespective of their sequence context. That this novel and strong pattern reflects the actual situation and does not result from experimental artifacts due to a lack of bisulphite conversion (41,42) was verified by the control experiments. The usage of the vectors containing the transgene constructs that had been introduced into the SRI-SB2(+), -3(-) and -4(–) plants mixed with the genomic DNAs demonstrated that none of the analysed C positions are intrinsically resistant to bisulphite conversion. In addition, the characterization of the SRI-SB2(+)-4 plant was an ideal control for the analysis of the PSTVd-infected SRI-SB2(+)/I–4 sister plant. The fact that the PSTVd transgene sequences specific for the SRI-SB2(+)-4 plant appeared nearly free of methylation strongly reinforces the validity of our results. Harrison et al. (42) recently reported that for different sequence motifs, C residues adjacent to methylated sites could give partial resistance to bisulphite conversion. The homogeneity of the methylation pattern observed within the viroid sequence argues against such bias in our case. Moreover, the results obtained by Southern blot analysis of the methylation status of different C residues located at symmetrical (CpG or CpNpG) and also non-symmetrical (CpA or CpC) sites are entirely consistent with the bisulphite data (see above; Figs 1 and 6).

The recognition of specific structures in DNA that are formed during the RdDM process may strongly stimulate the potential de novo activity of maintenance MTase(s), resulting in the loss of sequence specificity. Alternatively, putative MTases lacking the large N-terminal regulatory domain have recently been identified in Arabidopsis (METIII), mice, humans (Dnmt2) (7) and in the fungus Ascobolus immersus (Masc1) (43). These MTases might be involved in the establishment of de novo methylation patterns. In Ascobolus, Masc1 appeared to play an essential role in de novo methylation during the MIP process (43). In this fungus, DNA duplications are efficiently detected during the sexual reproduction cycle and are heavily methylated at cytosines (44). MIP certainly involves a DNA–DNA pairing step for the triggering of de novo methylation and was shown to affect all the cytosines in duplications >300–400 bp (15), a pattern reminiscent of that we observed for the RdDM process.

Viroids autonomously replicate in the nucleus via an RNA–RNA-dependent pathway, where they can accumulate to up to $3 \times 10^4$ copies, which are mostly localized in the nucleus (45). Mature circular viroid RNAs are plus-stranded molecules which are transcribed into an oligomeric minus-strand RNA. The minus-strand then acts as a template for the synthesis of oligomeric plus-strands which are processed to mature molecules by enzymatic cleavage and ligation steps (40). The detection of a similar pattern of heavy methylation in all the upper and lower PSTVd-specific strands could be an indication of de novo methylation events on both DNA strands after each replication cycle. However, the presence of both plus and minus PSTVd RNA in the nuclei of infected cells prevented the identification of the molecule(s) that directs the methylation. Furthermore, it is not known whether an RNA–DNA duplex or a triple helix structure is recognized by MTase(s). Theoretically, mature plus, oligomeric plus or minus PSTVd RNAs are all capable of binding complementary transgene DNA sequences. However, considering the large numbers of mature PSTVd molecules in the nucleus, we surmise that the primary determinant for methylation could involve interactions with partially denatured double-stranded, viroid molecules.

35S promoter-driven PSTVd replication initiation-competent cDNA constructs were introduced into SRI-3(+) and -4(–) plants. Therefore, viroid replication is expected to occur in every leaf cell. This could then lead to a strong de novo methylation of the PSTVd transgene sequences, a situation which corresponds well to our observations (see above). In the SRI-SB2(+)/I–4 plant, PSTVd infection was obtained by mechanical inoculation, but a pattern of methylation similar to those observed in the SRI-3(+)–9 and -4(–)–6 plants was detected throughout the cell population of infected leaves. In situ hybridization studies of PSTVd distribution in mechanically infected tomato leaves led Harders et al. (45) to conclude that the majority of cells (>80%) were uninfected and viroid free. However, our data indicated that viroid RNA-specific molecules were present in the majority of leaf cells. Several lines of evidence may explain the differences between our observations and those of Harders et al.

We cannot exclude that some classes of leaf cells may be resistant to our genomic DNA extraction procedure. However, it seems more likely that only a fraction of cells from infected leaves contain high levels of actively replicating PSTVd molecules. Systemic viroid infection requires cell-to-cell movement via plasmodesmata and long-distance movement through the phloem (46,47). We speculate that PSTVd RNA does reach most leaf cells, but is at a concentration too low to be detected by in situ hybridization. Importantly, the presence of low concentrations of PSTVd RNA molecules could still be sufficient to target specific de novo methylation of the genome-integrated viroid sequences. Alternatively, initiation of methylation in highly infected cells could produce a novel sequence-specific methylation signal, which could then systemically propagate. This signal could be comparable with the putative ribonucleoprotein complex that allows propagation of infectious viroid molecules. At least the nucleic acid component, required for the signal specificity, could differ from the mature viroid RNA and consist of partially degraded RNA molecules incapable of autonomous replication. In plants, such sequence-specific diffusible signals have been recently reported to be involved in the systemic spread of post-transcriptional gene silencing (48–50). A last, but less likely, possibility would implicate
high level maintenance of C methylation in the non-symmetrical context, a hypothesis which is difficult to reconcile with the general pattern of maintenance observed so far in plants (7).

Besides heavy methylation of the viroid sequences, most of the individual DNA strands displayed a significant but lower level of methylation within the 5′ and 3′ PSTVd-flanking regions. In all the three different constructs the extent of methylation appeared to be mainly restricted to the –1 to –21 promoter region and to the +1 to +40 pAnos region. It is conceivable that MTase(s) which is directed mainly restricted to the –1 to –21 promoter region and to the +1 to +40 pAnos region. It is conceivable that MTase(s) which is directed

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