Drug-induced hypomethylation of a posttranscriptionally silenced transgene locus of tobacco leads to partial release of silencing

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Abstract The effect of DNA methylation upon posttranscriptional gene silencing (PTGS) has been investigated in transgenic tobacco lines showing PTGS and methylation of the neomycin phosphotransferase II (*nptII*) reporter genes. Application of the hypomethylation drugs dihydroxypropyladenine or 5-azacytidine resulted in approximately 30% reduced methylation of cytosines located in a non-symmetrical context in the 3′ untranslated region of the *nptII* transgenes. The hypomethylation was accompanied by up to 12-fold increase in NPTII protein levels, suggesting that methylation of non-symmetrical motifs may account for an increased degree of PTGS. Models for the possible role of DNA methylation in PTGS are discussed.

Key words: DNA methylation; Gene silencing; Hypomethylation drug; T-DNA; Tobacco

1. Introduction

In plants, the posttransriptional gene silencing (PTGS) process is characterized by several features, including normal transcriptional activity of the promoter, RNA instability, the capacity to degrade homologous RNA from unlinked loci, and meiotic resetting (for review, see [1]). At the DNA level, silenced transgenes often possess a high level of cytosine methylation, particularly in the 3′ half of the transcribed region [2–4]. Methylation modification in the promoter region has been convincingly shown to be incompatible with transcription in vitro [5] and in vivo [6]. In vivo induced DNA hypomethylation by drug treatment or in particular genetic backgrounds leads almost in every case to activation of gene expression in both mammals [7] and plants [8–11], suggesting a causal relationship between transcription inhibition and promoter methylation. However, despite the good correlation between coding sequence methylation and reduction of steady-state mRNA levels, especially in transgenes with no homologous endogenes [1–3], the precise role of methylation in PTGS has not been established yet. In particular, it is not known whether DNA methylation is required to trigger and maintain silencing or whether this epigenetic modification is merely an outcome of silenced state established by other mechanisms. The evidence to distinguish between these two possibilities is often conflicting. The early appearance of methylation preceding the onset of silencing in virus-induced PTGS suggests rather a more active involvement of DNA methylation, at least in some cases of PTGS [12]. On the other hand, cosuppression (silencing of homologous endogenous gene by a transgene) occurs in some cases without extensive methylation of homologous endogenous sequences arguing against the primary role of DNA methylation in silencing triggering [13–15]. In *Neurospora*, the PTGS-related gene silencing in the vegetative phase is called 'quelling' and was shown to be methylation independent [16]. In the same organism, premature transcription termination caused by methylation of transcribed sequences resulted in reduced steady-state RNA levels of genes affected by repeat-induced point mutation [17]. Whether both methylation-dependent and -independent silencing systems occur in plants is not determined yet.

Previously, we have elaborated a system to inhibit DNA methyltransferase through the accumulation of the S-adenosylhomocysteine (SAH) metabolite, a natural inhibitor of transmethylation reactions. Application of (S)-9-(2,3-dihydroxypropyl)adenine (DHPA), a competitive inhibitor of SAH-hydrolase in tobacco cell cultures led to the dramatic accumulation of intracellular SAH [18] together with hypomethylation of repetitive sequences in tobacco and this with considerable specificity for CNG [19] and non-symmetrical motifs [20]. Also transgenic tobacco plants that expressed antisense SAH-hydrolase showed an increased accumulation of SAH and a hypomethylated genome [21]. The commonly used 5-azacytidine inhibits methylation because it is incorporated into DNA instead of cytosine and then covalently binds and inactivates enzyme molecules [22]. Therefore, 5-azacytidine incorporation has an effect on both symmetrical and non-symmetrical cytosines. Combination of two hypomethylation drugs with a different mode of action, 5-azacytidine and DHPA, offers a suitable tool to study methylation-dependent expression of genes under the limiting DNA methyltransferase activities.

We have previously characterized several tobacco transgenic lines carrying the neomycin phosphotransferase II (*nptII*) reporter gene [3,23,24]. Up to 1000-fold differences in expression of *nptII* were found between individual genotypes [23]. Nuclear run-on assays confirmed that silencing occurred at the posttranscriptional level [3,24] and that only transcribed sequences seemed to possess silencing capacities in *trans* [25].

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Abbreviations: DHPA, dihydroxypropyladenine; ELISA, enzyme-linked immunosorbent assay; HElo1, hemizygous for locus 1; HElo2, hemizygous for locus 2; HElo1/HElo2, hybrid of HElo1 and HElo2; NPTII, neomycin phosphotransferase II; PTGS, posttranscriptional gene silencing

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DNA methylation of both symmetrical and non-symmetrical motifs was specifically found in the 3′ end of the silenced *nptII* gene [3,4,23] and the degree of methylation correlated with changes in RNA synthesis levels during plant development [23]. Here, we assess the role of DNA methylation in PTGS through the experimentally induced hypomethylation in cell cultures of silenced transgenic tobacco lines.

2. Materials and methods

2.1. Plant material and culture conditions

Callus cell cultures were established from leaf explants by hormonal treatment according to standard procedures. Calluses were grown in 0.7% agar containing B5 salts supplemented with sucrose (30 g/l), α-naphthaleneacetic acid (2.0 mg/l), and 6-benzylaminopurine (0.2 mg/l). The drug treatments were performed on fully undifferentiated cells following continuous passage in culture for more than 90 days. The hypomethylation schemes were as follows: the cells were grown in the presence of 25 μM and 100 μM DHPA for 4–5 weeks, or in the presence of 200 μM 5-azacytidine for 6–8 weeks. DHPA was synthesized by organic synthesis according to the method described in [26].

2.2. DNA isolation and Southern blot hybridization

Total genomic DNA was extracted from approximately 10 g of wet callus tissue according to the modified procedure of Saghai-Maroof et al. [27]. Calluses were extensively lyophilized prior to homogenization in liquid nitrogen. After isopropanol precipitation, DNA was dissolved and subjected to RNase A (40 μg/ml, 15 min, 37°C) and proteinase K (20 μg/ml, 2 h, 50°C) treatments, extracted using phenol-chloroform-isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1), and precipitated with ethanol. The quantity and quality of DNA preparations were checked by absorbance at 260/280 nm.

Purified DNA was digested with an excess of enzyme (5 U/μg DNA) added at two 3-h intervals to the reaction mixture. Approximately 10–15 μg of digested DNA was loaded on gel, electrophoresed in a 0.8% agarose gel and alkali-blotted onto Hybond XL membrane (Amersham, Aylesbury, UK). The DNA on filters was hybridized with a ^{32}P-labelled DNA probe (DekaLabel kit, MBI, Fermentas, Vilnius, Lithuania) corresponding to the entire NPTII-coding sequence (a 0.8-kb *Bgl*II/*Bam*HI fragment). Southern hybridization was carried out in 0.25 M Na-phosphate buffer, pH 7.0, supplemented with 7% sodium dodecyl sulfate (SDS) at 65°C for 16 h followed by washing with 2×SSC (1×SSC, 150 mM NaCl, 15 mM Na_3-citrate, pH 7.0), 0.1% SDS (twice 5 min), 0.2×SSC, 0.1% SDS (twice 15 min). Completion of digests was checked by hybridization with a chloroplast probe. The hybridization bands were visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, CA, USA) overnight and autoradiography (1–2 weeks exposure). Quantification of hybridization signals was performed by ImageQuant software (Molecular Dynamics).

2.3. Protein extraction and NPTII ELISA

To extract soluble proteins, the lyophilized callus tissue (20–40 mg of dry weight) was ground in an Eppendorf tube with 200–400 μl extraction buffer (0.25 M Tris–HCl, pH 8.0, 1 mM phenylmethylsulfonylfluoride), then centrifuged at 20 000×*g* to remove cell debris. The protein concentration of the extracts was determined according to the Bio-Rad (Hercules, CA, USA) protein assay [28]. The NPTII ELISA was done according to the manufacturer's instructions (5 Prime→3 Prime, Boulder, CO, USA). Reading of the microtiter plate was done at 405 nm, using a kinetic program (5-min intervals for 2 h).

3. Results

3.1. Plant material

The three transgenic tobacco lines used to examine the relationship between methylation and PTGS are descendants of the primary transformant pGVCHS(287) [25]. The transgenic T-DNA construct contained the *nptII* reporter gene under control of the 35S cauliflower mosaic virus promoter. The characteristics of individual lines were as follows: in the line hemizygous for locus 1 (HElo1) two T-DNAs were integrated as an inverted repeat (designated locus 1) [25]; the *nptII* gene was highly methylated in the transcribed region and its expression was close to the background values [4]. In the line hemizygous for locus 2 (HElo2), the T-DNA was integrated as a single copy insertion (locus 2), no methylation was found within the restriction sites investigated and the *nptII* gene was highly expressed [4]. The HElo1/HElo2 hybrid line carrying both loci was obtained by crossing homozygous locus 1 (HOlo1) and homozygous locus 2 (HOlo2) lines and displayed strong locus 1-dependent silencing of the *nptII* gene expression from locus 2 [24]. The structure of the silencing locus 1 present in HElo1 and HElo1/HElo2 lines is depicted in Fig. 1 [25]. To inhibit DNA methyltransferase activity and consequently DNA methylation, callus cultures and the two hypomethylation drugs, DHPA and 5-azacytidine, were used. The methylation pattern and gene expression of the *nptII* transgenes were not grossly influenced by callus induction (data not shown).

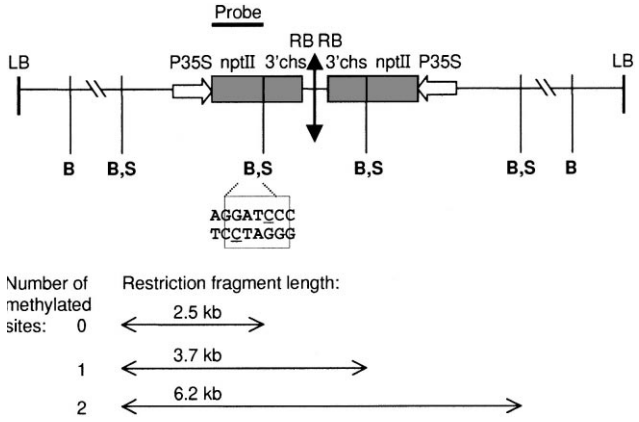

Fig. 1. Physical map of the *Bam*HI (B) and *Sma*I (S) sites in the silencing locus 1. Evidence for an inverted repeat character of the T-DNA insertion is given elsewhere [5]. The sequence context of the *Bam*HI site between the *nptII*-coding region and 3′chs untranslated region is shown to demonstrate the non-symmetrical position of methylation-sensitive cytosines within the site (underlined). The size of the diagnostic fragments for digestion at +876 (*Bam*HI) and +881 (*Sma*I) of the *nptII* gene are illustrated below. LB, T-DNA left border; RB, T-DNA right border; P35S, 35S promoter of cauliflower mosaic virus; 3′chs, 3′ chalcone synthase untranslated region from *Antirrhinum majus*.

3.2. Drug-induced hypomethylation of cytosines within the coding region of the nptII transgene

Callus cultures derived from the silenced (HElo1, HElo1/HElo2) and non-silenced (HElo2) transgenic lines were treated with 25 μM and 100 μM DHPA or with 200 μM 5-azacytidine (see Section 2). The extent of DNA hypomethylation was investigated within a 11-bp sequence located in the 3′ untranslated region of the transcribed region. At position +876/886 (relative to the transcription starting site) and 33 bp downstream of the *nptII*-coding sequence and 219 bp upstream of the polyadenylation site, this sequence contained the recognition sequences for *Bam*HI and *Sma*I methylation-sensitive restriction enzymes. DNA from the highly expressing cell line HElo2, which does not show methylation of *Bam*HI or *Sma*I sites, served as a control.

From the *nptII* sequence (Fig. 1), it follows that two meth-

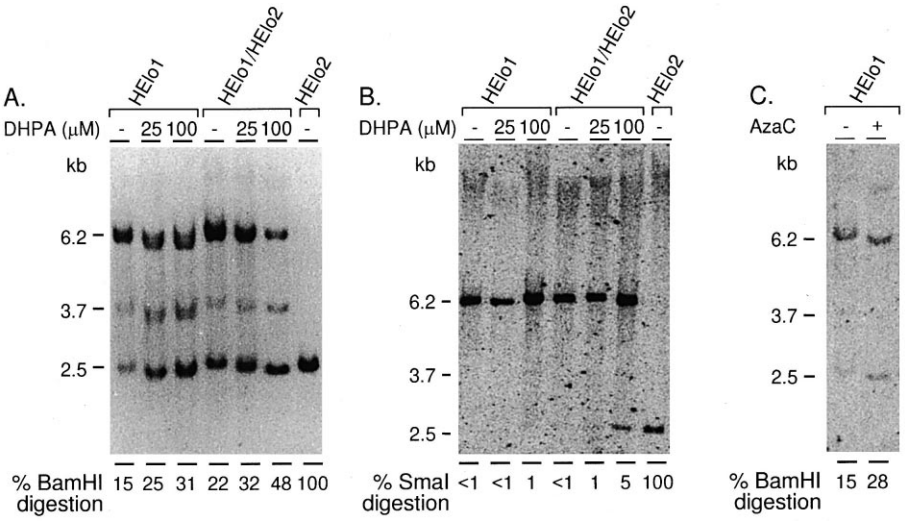

Fig. 2. Evidence for hypomethylation of *nptII*-coding sequences induced by two different drugs. Gel blots of DNA from calluses treated with DHPA (A and B) and 5-azacytidine (C). The methylation was analyzed by digestion of DNAs with *Bam*HI (A, C) and *Sma*I (B). The blots were hybridized with the *nptII*-coding region-specific DNA probe. The extent of the digestions at +876 (*Bam*HI) and +881 (*Sma*I) sites was quantitated for each lane and is expressed as the percentage of the intensity of the 2.5-kb band relative to the sum of the 6.2-kb and 3.7-kb bands by PhosphorImager analysis.

ylation-sensitive cytosines, one on each DNA strand within the *Bam*HI site, are located in non-symmetrical contexts. The hybridizing *Bam*HI fragments obtained with an *nptII*-coding sequence probe in digested DNA from the non-silenced HElo2 line yielded a 2.5-kb hybridization fragment corresponding with the predicted size for the non-methylated, completely restricted DNA. However, upon methylation hybridizing bands originating from DNA molecules with two, one, and no methylated *Bam*HI sites (bands of 6.2, 3.7, and 2.5 kb, respectively; Fig. 1) can be visualized in lanes loaded with DNA isolated from HElo1 and HElo1/HElo2 cells. In control digests of HElo1 and HElo1/HElo2 DNAs isolated from calluses that were not treated with hypomethylation drugs, the 6.2-kb hybridization fragment always appeared as the strongest band in electrophoretic profiles, suggesting abundant methylation of both internal *Bam*HI sites of the inverted repeat (Fig. 2A). Reproducibly and significantly, digestions of DNA samples from calluses treated with DHPA (Fig. 2A and B) or 5-azacytidine (Fig. 2C) were more complete, resulting in an increase of the intensity of the 2.5-kb fragment. To quantify the extent of hypomethylation, we measured the band intensities in individual lanes by a PhosphorImager. The digestibility of DNA (expressed as the amount of the 2.5-kb band relative to the total signal) seemed to be proportional to the concentration of DHPA in culture media. At the highest concentration of DHPA (100 μM) used, the methylation of the *Bam*HI sites was reduced by 16% and 26% for HElo1 and HElo1/HElo2 lines, respectively (Fig. 2A).

The *Sma*I enzyme that recognizes the CCCGGG sequence is sensitive to cytosine methylation at the symmetrical CG motif [29]. The 2.5-kb hybridization fragment corresponds to fully digested and non-methylated T-DNAs, whereas the 6.2-kb fragment indicates methylation of the *Sma*I site at the 3′ end of the two *nptII* genes. The presence of the 6.2-kb band and the absence of a 2.5-kb band in silenced genotypes (Fig. 2B) showed that the *Sma*I sites at +881 were completely methylated in virtually all the DNA molecules. Thus, the *Sma*I sites were noticeably more methylated than the *Bam*HI sites probably because of the symmetrical distribution of the methylation-sensitive cytosines in a CG motif. Similarly to the *Bam*HI digests, no additional hybridizing DNA fragments were visualized on the autoradiograph, indicating the absence of methylation at the *Bam*HI and *Sma*I sites in the 5′ upstream sequences (Fig. 2B). In the samples treated with demethylating drugs, the enhanced *Sma*I cleavage was observed only in DNA isolated from HElo1/HElo2 cells grown with

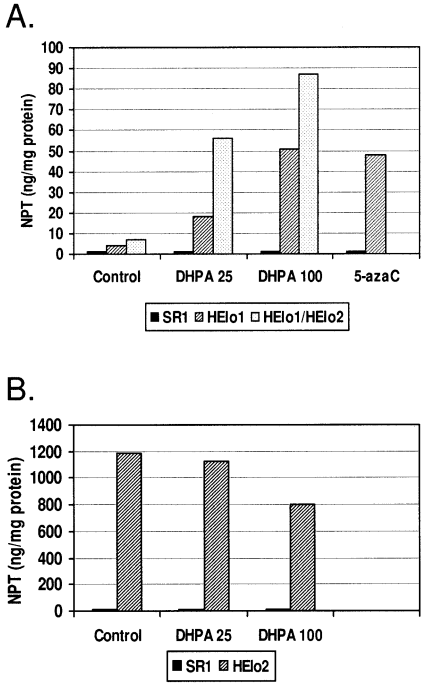

Fig. 3. NPTII protein accumulation levels in hypomethylated and control samples. Protein extracts from drug-treated and non-treated calluses were assayed for the NPTII protein content by ELISA. For each experiment with cell cultures, two parallel samples were analyzed. Each protein extract was assayed by three independent ELISAs and the results were expressed as an average.

100 μM DHPA, suggesting that the observed hypomethylation was derived from locus 2 on which methylation had been imposed by the presence of locus 1 in a hybrid HElo1/HElo2 line [4].

3.3. Expression of the nptII reporter gene in hypomethylated calluses

To assess the effects of methylation associated with PTGS genes, we analyzed the NPTII protein content in methylated, hypomethylated, and non-methylated calluses. Protein extracts prepared from aliquots of the same callus tissue used previously for DNA extraction were assayed for the NPTII protein concentration using ELISA (Fig. 3). In untreated samples, the NPTII accumulation in the silenced genotypes HElo1 and HElo1/HElo2 was at least 200-fold lower than that in the non-silenced HElo2 genotype. These values were similar to those in leaf tissue [25]. The drug treatments resulted in elevated accumulation levels of NPTII protein in the silenced genotypes of HElo1 and HElo1/HElo2. When different concentrations of DHPA were tested, the NPTII content correlated with the concentration of the applied drug and also with the extent of hypomethylation of the *Bam*HI site. The NPTII accumulation levels in the non-silenced HElo2 genotype remained essentially the same or were even slightly reduced after the DHPA treatment. Together, both drugs that induced hypomethylation by a different biochemical mechanism of DNA methyltransferase inhibition, caused higher accumulation of the protein product of a reporter gene. Therefore, we conclude that hypomethylation of transcribed sequences can partially release the established silencing by interfering either with the creation of the silencing-inducing signal or with the maintenance of an induced autocatalytic cycle.

4. Discussion

The results presented here indicate that methylation of transcribed sequences plays a role in PTGS. When methylation of the reporter *nptII* gene was partially inhibited either with DHPA or 5-azacytidine, we observed a concentration-dependent (6–12-fold) increase in the expression of silenced loci.

The methylation changes were monitored on a 11-bp sequence located at the 3′ end of the *nptII* reporter gene enabling methylation analysis of both symmetrical (CG) and non-symmetrical motifs lying next to each other. Application of DHPA led to a rather specific hypomethylation of non-symmetrically methylated cytosines without major hypomethylation of symmetrical CG motifs confirming our previous experiments with this drug [19]. However, the extent of hypomethylation of silent T-DNA loci was slightly lower than in other parts of genome. For example, at 25 μM DHPA some non-symmetrical sites within the 5S rRNA genes were completely demethylated [20], whereas that within the *Bam*HI site of the T-DNA was hypomethylated by approximately 10%. In the HElo1 genotype, the symmetrical CG motif located within the neighboring *Sma*I site was not hypomethylated, even at the highest concentration of the inhibitor. In highly methylated regions (for example, the 3′ end of the *nptII* genes in locus 1 [4]), the inhibition of methyltransferase activity resulted probably in an ordered hypomethylation of different sequence motifs: hypomethylation of non-symmetrical motifs before hypomethylation of symmetrical motifs. Interestingly, such hierarchy of hypomethylation also seems to hold true for genetically induced hypomethylation in *Arabidopsis* in which *ddm1*-induced reduction of cytosine methylation levels resulted in activation of an endogenous gene family [10]. Possibly de novo methylation (which needs to be established after each replication cycle) of non-symmetrical sites is sensitive to various stimuli including methyltransferase cofactor levels whereas the maintenance of the methylation of symmetrical sites could be more stable. In reminiscence, the methylation of a non-symmetrical motif (also in a 3′ located *Bam*HI site) correlated with observed developmental changes in *nptII* gene expression in tobacco transgenic lines [23]. Whereas the 3′ located *Sma*I sites in locus 1 remained hypermethylated, the *Sma*I site was hypomethylated to some extent in a silenced locus 2 in drug-treated hybrid HElo1/HElo2 calluses. Also inspection of the less methylated 5′ transcribed region of the *nptII* gene in the HElo1 line revealed some hypomethylated CG motifs (data not shown). The differential methylation (and hypomethylation) of the same restriction sites located within locus 1 and locus 2 possibly reflects different methylation mechanisms of silencing and in *trans*-silenced loci. The silenced locus 2 had a considerably lower degree of methylation in the *nptII*-coding sequence compared to the analogous region in locus 1 [4] and may be methylated by RNA-directed methylation [30]. On the other hand, the extensive methylation in locus 1 may be directed by the palindromic arrangement of the T-DNA insertion in which both RNA [30] and DNA [15] methylation signals may be active.

Reduction of silencing can be correlated with the extent of hypomethylation of cytosine residues located at the 3′ end of the gene. Control experiments in transgenic, non-silenced lines showed a similar or slightly reduced *nptII* expression after the drug treatments ruling out the possibility that global hypomethylation altered the expression of a putative 35S promoter-activating transcription factor. The possible hypomethylation of promoter sequences did probably not contribute to elevated expression because the methylation of the 5′ upstream sequences of posttranscriptionally silenced *nptII* genes was low or negligible [23]. It is necessary to stress that in our system the drug-induced hypomethylation did not increase the expression of the silenced reporter gene to the level seen in non-silenced lines. In fact, the 10-fold increased NPTII protein levels observed in silenced lines after hypomethylation induction correspond to approximately 10% of the NPTII protein levels found in non-silenced lines. This observation may be explained by the fact that only partial hypomethylation of the gene was achieved by drug treatments (most CG sites were not hypomethylated). Another possibility is that additional epigenetic mechanisms besides methylation affect and regulate the maintenance of the sequence-specific PTGS. However, hypomethylation of CNG motifs without concomitant hypomethylation of CG sites has been observed in activated transgenes of *Arabidopsis* mutated in the *sgs1-1* locus [31], suggesting that partial hypomethylation may be enough to trigger complete or partial expression. The effect of hypomethylating drugs on gene expression was more pronounced in a hybrid genotype than in that with the silencer locus alone, what may be related to less extensive methylation of silenced locus 2 and/or to weakening of the silencing signal induced by locus 1.

Most intriguing is what might be the mechanism linking RNA degradation and DNA methylation. Transient transfection experiments in tobacco protoplasts using in vitro meth-

ylated constructs showed inhibition of transcription elongation by methylation of the coding sequences [32]. In *Neurospora*, Rountree and Selker [17] elegantly showed inhibition of RNA elongation along DNA that contained nearly every cytosine methylated. The elongation block could be relieved by 5-azacytidine or by a mutation affecting the metabolism of methylation cofactors. The above described plant system shares apparent similarities with that in *Neurospora*: (i) silencing is induced posttranscriptionally, (ii) partial relief of silencing can be induced with hypomethylation drugs, and (iii) 3′ ends of transcribed sequences have an extremely high density of methylation located on both symmetrical and non-symmetrical cytosines. Adapting Selker's model, we propose that RNA elongation may slow down at the 3′ end either because of difficulties of the RNA polymerase machinery to get through methylated cytosines or more probably through a condensed chromatin that is possibly assembled on methylated DNA [33]. A decreased rate of elongation may result in 'jamming' of RNA molecules that are already present on the DNA at a high density because of frequent initiation of transcription by the strong 35S promoter. Decreased spacing between 'jammed' RNA molecules along the DNA would favor formation of intermolecular (rather than normal intramolecular) bonds between individual RNA molecules. Complexes of several RNA molecules might be recognized by cellular defence systems as 'aberrant' and consequently degraded [34]. Another and equally probable possibility is that transcription through densely methylated DNA regions interferes with the folding and processing of the pre-RNA. By doing so, incorrectly assembled or inverted repeat readthrough RNAs can accumulate generating the molecular signals for PTGS establishment. A third possibility is that methylation induces conformational chromatin changes and that the resulting heterochromatin might recruit an RNA degradation machinery to the target locus. Short RNA molecules formed as an outcome of degradation process or other aberrant RNAs may subsequently serve as templates for an RNA-dependent RNA polymerase [35] and initiate degradation of any homologous molecule in *trans* by a process that could already be methylation independent. Further studies will be needed to characterize the chromatin structures at the inverted repeat center and establish the role of DNA methylation in maintaining an inactive state.

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