

The *trans*-silencing capacity of invertedly repeated transgenes depends on their epigenetic state in tobacco

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ABSTRACT

We studied the *in trans*-silencing capacities of a transgene locus that carried the neomycin phosphotransferase II reporter gene linked to the 35S promoter in an inverted repeat (IR). This transgene locus was originally posttranscriptionally silenced but switched to a transcriptionally silenced epiallele after *in vitro* tissue culture. Here, we show that both epialleles were strongly methylated in the coding region and IR center. However, by genomic sequencing, we found that the 1.0 kb region around the transcription start site was heavily methylated in symmetrical and non-symmetrical contexts in transcriptionally silenced but not in posttranscriptionally silenced epiallele. Also, the posttranscriptionally silenced epiallele could *trans*-silence and *trans*-methylate homologous transgene loci irrespective of their genomic organization. We demonstrate that this *in trans*-silencing was accompanied by the production of small RNA molecules. On the other hand, the transcriptionally silenced variant could neither *trans*-silence nor *trans*-methylate homologous sequences, even after being in the same genetic background for generations and meiotic cycles. Interestingly, 5-aza-2-deoxy-cytidine-induced hypomethylation could partially restore signaling from the transcriptionally silenced epiallele. These results are consistent with the hypothesis that non-transcribed highly methylated IRs are poor silencers of homologous loci at non-allelic positions even across two generations and that transcription of the inverted

sequences is essential for their *trans*-silencing potential.

INTRODUCTION

Gene or transgene activity can be silenced at the transcriptional (TGS) or posttranscriptional (PTGS) level. Promoters of transcriptionally silenced genes are inactive and, thus, transcription is blocked, whereas those of posttranscriptionally silenced genes are active, but the transcripts are unstable and promptly degraded before the protein products can be made. The ability to communicate silencing information to homologous loci seems to be a characteristic feature of many transgene loci and is described as cosuppression. When homologous promoters are involved, the silencing is referred to as *trans*-TGS (1) and when the coding region is involved, as *trans*-PTGS (2). Several observations suggest that both TGS and PTGS may be mechanistically related (3,4). In plants, both types of interactions are frequently associated with DNA methylation of the homologous regions.

Highly expressed and convergently transcribed transgenes, as can be found on inverted T-DNA repeats, have particularly powerful silencing capacities of themselves and of homologous unlinked loci in both mammals and plants (5–8). The palindromic arrangement is important for self (*cis*)- and *trans*-silencing and methylation, which has been demonstrated in *Arabidopsis thaliana* by targeted deletion of one of the transgenes in the inverted repeat (IR) (9,10). The potent capacity of IR loci to methylate and silence homologous sequences *in trans* can be explained by two model mechanisms: (i) physical interaction of IR and homologous sequences via DNA–DNA pairing and (ii) involvement of aberrant RNA molecules that result from transcription through the IR center.

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In *Petunia* (*Petunia hybrida*), all the rare instances of PTGS caused by a promoterless chalcone synthase transgene were associated with both IR arrangement of the T-DNA and DNA methylation (7). A complex enhancerless transgene locus could be transcribed only in the presence of the homologous locus that carried a functional enhancer (11), suggesting that unlinked transgenes might be paired in the plant nucleus. Pairing interactions have also been proposed to be involved in paramutation, i.e. heritable *trans*-interacting effects of certain alleles in maize (12). In parallel, elegant experiments with forced transcription of promoter or cDNA sequences arranged in an IR structure that produce double-stranded hairpin RNA, imply that also RNA can mediate *in trans*-silencing (3). The *trans*-TGS effect induced by hairpin RNA products was accompanied by *de novo* methylation of cytosines in symmetrical and non-symmetrical context—a pattern known to be a hallmark of RNA-directed DNA methylation described in viroids (13). The powerful *in trans*-silencing capacity of IRs is not restricted to transgenes and has been observed in endogenous genes. For example, the strain Wassilewskija of *Arabidopsis* contains four copies of a gene coding for the tryptophan biosynthesis, of which two are arranged as an IR and two as unlinked copies. All four genes are densely methylated and the IR locus triggers *de novo* methylation and silencing of the unlinked homologous loci (14). Despite these observations, the presence of transgenes in IR arrangements was not sufficient in other transgenic systems to trigger the *in trans*-silencing process (15,16). For instance, *Arabidopsis* transgenes driven by the strong 35S promoter (P_{35S}) and arranged as IRs maintained high levels of expression. In this model system, PTGS was associated with dosage rather than with the arrangement of the transgenes (16). However, it should be noted that in this case the inverted transgenes were located far from the center of the palindrome and that two other genes were located in-between the assayed P_{35S} -driven transgenes.

Palindromic sequence arrangements are frequently found in multicopy loci that also contain both tandem repeats and truncated T-DNA insertions and even vector sequences that make the contribution of each structure difficult to assess. Previously, we have described a tobacco (*Nicotiana tabacum*) transgenic locus 1 that contains two copies of a T-DNA organized as a near perfect IR with no or little non-homologous sequence in the center. The residing P_{35S} :neomycin phosphotransferase II (*nptII*) reporter transgenes are organized in a tail-to-tail configuration within 1.8 kb from the IR center (Figure 1). Run-on analysis (17), experiments with inhibition of the chimeric potato (*Solanum tuberosum*) virus X:*nptII* replication (18) and dense methylation in the *nptII*-coding region (19) indicate that the *nptII* genes at the IR locus 1 are posttranscriptionally silenced. During *in vitro* propagation in callus culture, the mode of transgene silencing was changed from posttranscriptional into transcriptional. This event was accompanied by several changes in the distribution of the DNA methylation along the transgenes, hypermethylation of the 35S promoter being the most prominent (17). The novel epigenetic state was stably transmitted from the calli to regenerated plants and several plants carrying a TGS epimutant allele of locus 1 were recovered. Importantly, the PTGS or TGS epigenetic states are also stably transmitted to the next generation and, thus, are meiotically stable.

Here, we have characterized the TGS and PTGS epialleles of locus 1 in full detail and analyzed their silencing capacities. The expression of the *nptII* gene was studied at both the RNA and protein levels in hybrids by combining either the TGS or the PTGS allele of the 'IR silencer locus' with two normally expressed loci of the same transgene (locus 2 and locus B) and the levels of small RNA (smRNA) molecules were determined in silenced and non-silenced lines. Evidence is provided that the *in trans*-silencing potential is restricted to the PTGS version of the IR locus, while its TGS epiallele, despite heavy methylation, is not capable of *in trans*-silencing at either transcriptional or posttranscriptional level.

MATERIALS AND METHODS

Plant material

All transgenic tobacco (*Nicotiana tabacum*) SR1 plants were generated by *Agrobacterium tumefaciens*-mediated transformation (20). The plants hemizygous for the PTGS locus 1 (HeLo1; Figure 1) were obtained by crossing a plant homozygous for locus 1 with an untransformed SR1 tobacco (18). The line hemizygous for the TGS locus 1E was obtained by plant regeneration from long-term HeLo1 cell cultures as described (17). The line hemizygous for locus 2 (HeLo2; Figure 1) was obtained by crossing a plant homozygous for locus 2 with an untransformed SR1 tobacco (19). The plants hemizygous for locus B (HeB; Figure 1) were selected by screening the R2 generation (self-fertilization of the primary transformant GVCHS(320)-1 followed by the self-fertilization of the R1 plants) on antibiotic-containing medium (21).

The hybrids hemizygous for locus 1 and locus 2 (Lo1/Lo2), hemizygous for locus 1E and locus 2 (Lo1E/Lo2), hemizygous for locus 1 and locus B (Lo1/LoB) and hemizygous for locus 1E and locus B (Lo1E/Lo2) were obtained by crossing the respective parental plants. Crosses were performed by emasculating flowers manually and applying the pollen to the stigma. In all crosses, the Lo1 or Lo1E plants served as mother donor. Progeny plants were screened for the presence of the two transgenic loci by DNA gel-blot hybridization.

For hypomethylation experiments, seeds collected from the HeLo1E × HeLo2 cross were treated by 50 μM (water solution) 5-aza-2-deoxy-cytidine (Sigma-Aldrich, St Louis, MO, USA) for 7 days. The seedlings were transferred to soil and leaves were harvested for DNA and RNA analysis after 2 months. Plants were genotyped by blot hybridization.

DNA probes

In the hybridization experiments, the *nptII*-coding sequence and the 35S promoter probes were prepared from the ~830 and 980 bp inserts of the pGEM*nptII* and pGSJ290 plasmids, respectively (21). The cloned subtelomeric tobacco HRS60 repeat (22) was used as a probe for monitoring global methylation.

DNA isolation and DNA gel-blot hybridization

Total genomic DNA was isolated from lyophilized leaves by a cetyltrimethylammonium bromide method as described previously (23). DNA methylation was analyzed with methylation-sensitive restriction endonucleases. Approximately 10 μg of

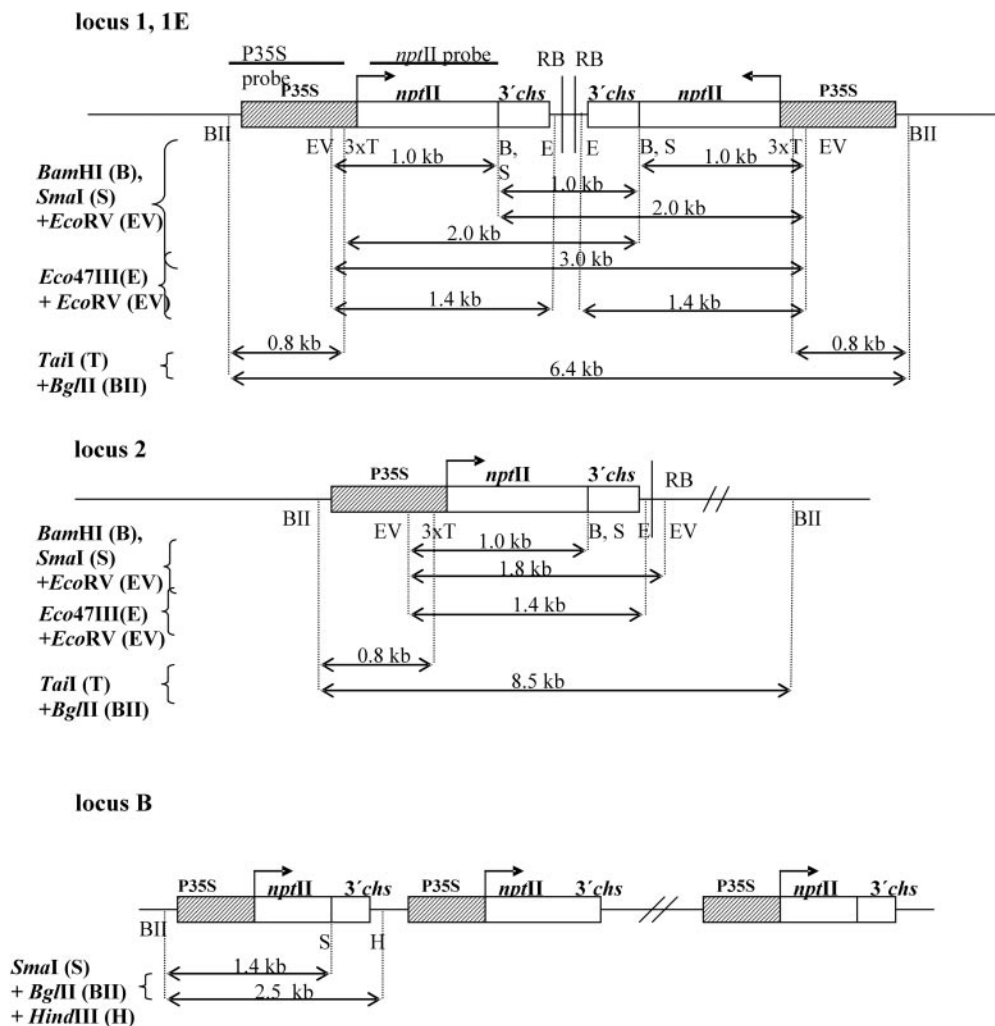


Figure 1. Schematic representation of the genomic organization of transgenic loci and physical maps of the restriction sites. The transgenic locus 1 has been previously reported as locus X (29). The methylation analysis of the promoter involved the three Tail sites; the diagnostic sites for the analysis of the *nptII*-transcribed region were *SmaI* and *BamHI*, and *Eco47III* for the non-transcribed sequences at the right border. Evidence for the IR character of the T-DNA insertions in locus 1 and locus 1E has been given elsewhere (18). *EcoRV*, *BglII*, and *HindIII* enzymes were used to dissect particular subregions of T-DNA. *P*_{35S}, promoter of the cauliflower mosaic virus; *nptII*, neomycin phosphotransferase II gene; RB, T-DNA right border; 3' chs, transcription termination sequence from the 3'-untranslated region of the chalcone synthase gene from snapdragon (*Antirrhinum majus*).

genomic DNA was digested with an enzyme excess (5 U per 1 µg DNA). After digestion, the DNA was separated by electrophoresis on a 1% (w/v) agarose gel. The gels were alkali-blotted onto a Hybond-XL membrane (GE-Healthcare, Little Chalfont, UK) and hybridized against ³²P-labeled DNA probes (DekaLabel kit; MBI, Fermentas, Vilnius, Lithuania) for at least 16 h at 65°C. After washing under high-stringency conditions [twice for 5 min in 2× standard saline citrate (SSC) + 0.1% (w/v) sodium dodecyl sulfate (SDS) and twice for 20 min in 0.2× SSC + 0.1% (w/v) SDS at 65°C; 1× SSC = 150 mM NaCl, 15 mM Na₃-citrate, pH 7.0], the hybridization bands were visualized with a PhosphorImager STORM and the data were processed with the ImageQuant software (GE-Healthcare).

RNA isolation and RNA gel-blot hybridization

Total RNA was isolated from young leaves with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the

manufacturer's instructions. The RNA quality was checked by electrophoresis on a 1% (w/v) agarose gel. After electrophoresis on a 1.2% (w/v) formaldehyde-agarose gel, the gel was washed for 10 min in sterile water to remove the formaldehyde. The RNA was denatured in 0.05 M NaOH and blotted onto a Hybond-XL membrane (GE-Healthcare) in 20× SSC. Radioactively labeled *nptII* DNA probes were hybridized in ULTRAhyb buffer (Ambion, Austin, TX, USA) for 24 h at 35°C. After washing under low-stringency conditions (twice for 15 min in 2× SSC + 0.1% (w/v) SDS at 50°C), the hybridization bands were visualized with a PhosphorImager STORM. To evaluate the *nptII* expression levels, the intensities of the *nptII* hybridization signals were normalized to the ethidium bromide-stained ribosomal RNA bands.

Bisulfite sequencing of the *P*_{35S}-5'-*nptII* region

Bisulfite sequencing was performed according to the protocol described by Grunau *et al.* (24). First, genomic DNA was

digested with an excess of the methylation-insensitive restriction enzyme *MseI* to disrupt the IR structure of locus 1. Of the digested DNA, 500 ng supplemented with 10 µg of carrier tRNA (Sigma-Aldrich) was denatured in 0.3 M NaOH at 42°C for 20 min. A fresh bisulfite solution was prepared by dissolving 5.41 g sodium bisulfite (Sigma-Aldrich) in 8 ml of distilled water without vigorous shaking. After adding 500 µl of 20 mM hydroquinone (Sigma-Aldrich), the solution was adjusted to pH 5 with 10 M NaOH, and the final volume was adjusted to 10 ml with distilled water. After complete dissolution of the bisulfite, the solution was passed through a 0.22 µm filter, and 1200 µl was added directly to the denatured DNA. The reaction was overlaid with 200 µl of mineral oil (Sigma-Aldrich) and incubated in the dark at 55°C for 4 h. The DNA was desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI) according to the manufacturer's protocol. The DNA was eluted in 110 µl of 10 mM Tris-HCl (pH 7.5), of which 100 µl was desulfonated with 11 µl of 3 M NaOH at 37°C for 20 min. The desulfonated DNA was neutralized with 47 µl of 10 M ammonium acetate, supplemented with 10 µg tRNA and precipitated with 500 µl of 95% ethanol at -20°C overnight. The DNA was resolved in 100 µl 10 mM Tris-HCl (pH 7.5) and stored at -20°C. The bisulfite-treated DNA was amplified by semi-nested PCR in a 50-µl reaction volume containing 1× PCR Rxn Buffer (MgCl₂; Invitrogen, Carlsbad, CA), 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 µM forward primer, 1 µM reverse primer, 5 U *Taq* DNA polymerase (Invitrogen) and 2.5 µl of template (bisulfite-treated DNA or first PCR). For amplification of the *P*_{35S}-5'-*nptII* region, the same forward primer 5'-CATTACATCACCCATAATAAATAC-TTTCTC-3', the first reverse primer 5'-GAATAGAGAGAAAGATATATTTTTTAAGAT-3' and the second reverse primer 5'-GTAATAGAGATTGGAGTTTTTAAGAAAGTAG-3' were designed using mismatches at positions complementary to potentially methylated cytosines. In parallel, the same primer sequences were converted into degenerate primers: forward primer 5'-CATTRCATCARC-CATRAATAACTTTCTC-3', the first reverse primer 5'-GAAYAYAGAGAAAGATATATTTYTYAAGAT-3' and the second reverse primer 5'-GTAATAGAGATTGGAGY-TYTAAAGAAAGTAG-3'. The PCR program consisted of 2 min of initial denaturation at 94°C followed by 5 cycles of 1 min at 94°C, 2 min at 50°C, 3 min at 72°C, subsequently followed by 25 cycles of 30 s at 94°C, 2 min at 50°C, 90 s at 72°C. The program was ended with an extension step for 10 min at 72°C. The PCR products were purified with the High Pure Purification Kit (Roche Diagnostics, Brussels, Belgium) according to the manufacturer's instructions. The amplicons were eluted in 60 µl elution buffer. After subcloning in pGEM-T (Promega) or pCR2.1 (Invitrogen), at least nine clones from each cloned amplicon were selected for DNA isolation and sequencing.

smRNA isolation and RNA gel analysis

Fractions of smRNA molecules were isolated as previously described (25) with minor modifications. Briefly, ~10 g of fresh young tobacco leaves was ground in liquid nitrogen and resuspended in RLT buffer (Qiagen). After double extraction with phenol/chloroform/isoamylalcohol (25:24:1, v/v), the sample was precipitated with 3 volumes of ethanol, the pellet

resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and heated to 65°C. The solution was incubated for 30 min on ice with polyethylene glycol (molecular weight 8000) at a final concentration of 5% and 0.5 M NaCl. During this procedure, nucleic acids of high molecular weight were precipitated and the fraction of smRNA molecules remained in the solution. After centrifugation, the supernatant was precipitated with ethanol and the pellet resuspended in formamide (Sigma-Aldrich). The quality and quantity of RNAs were checked by polyacrylamide gel electrophoresis followed by ethidium bromide staining. Only isolates with comparable electrophoretic profiles were used for further analysis.

The smRNAs were separated by electrophoresis (15% polyacrylamide, 7 M urea in 0.5× TBE; 1× TBE = 90 mM Tris-borate, 2 mM EDTA, pH 8.0), blotted onto a nylon Hybond-XL membrane (GE-Healthcare) with a semi-dry blot instrument (*trans*-Blot SD Semi-Dry Transfer Cell; Bio-Rad, Hercules, CA) in 0.5× TBE, and fixed by ultraviolet crosslinking. To estimate the size of the RNA bands, 20mer oligonucleotides were used as a marker. The single-stranded RNA probe was transcribed in sense orientation from a linearized pGen-3Zf(+)*npt* plasmid with ³²P-UTP using the RNAMaxx™ High Yield Transcription Kit (Stratagene, La Jolla, CA). Hybridization was performed as described (25,26), in ULTRAhyb buffer (Ambion) at 35°C for 48 h. The membrane was washed twice in 2× SSC + 0.1% (w/v) SDS for 30 min at 35°C and in 20 mM Tris-HCl, 5 mM EDTA (pH 8.0), 60 mM NaCl, 10 µg/ml RNaseA (for 1 h at 37°C) to remove unspecific background, and exposed to the Phosphor-Imager screen.

Protein extraction and NPTII ELISA

To extract soluble proteins, the lyophilized leaves (20–40 mg of dry weight) were ground in an Eppendorf tube with 200–400 µl extraction buffer (0.25 M Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonylfluoride) and centrifuged at 20 000 *g* to remove cell debris. The protein concentration of the extracts was determined according to the protein assay (Bio-Rad). The NPTII ELISA was done according to the manufacturer's instructions (5 Prime→3 Prime, Boulder, CO). The microtiter plates were read at 405 nm on the ELISA reader (GE-Healthcare), using a kinetic program (5 min intervals for 2 h).

RESULTS

Description of the transgenic loci used

The respective transgenic loci are schematically represented in Figure 1. The transgenic locus 1 in the line hemizygous for locus 1 (HeLo1) contains two copies of the GVchs287 T-DNA arranged as an IR with the residing *nptII* genes silenced at the posttranscriptional level (17). The isogenic line hemizygous for locus 1E (HeLo1E) contains an epimutated variant of locus 1, designated locus 1E, silenced at the transcriptional level. The transgenic tobacco lines hemizygous for locus 2 (HeLo2) and locus B (HeB) have been shown to express the *nptII* reporter gene to high levels (19,21). The line hemizygous for transgenic locus 2 (HeLo2) contains the GVchs287 T-DNA cassette integrated as a single copy and HeB carries the

transgenic locus B that consists of three GVchs287 T-DNA copies, two arranged as direct repeats and the third located in the same orientation at a distance of ~ 7 kb (21).

Previous results obtained by DNA gel-blot hybridization indicated differential methylation of the locus 1 TGS and PTGS epialleles (17). However, DNA gel-blot hybridization allows only the detection of methylated cytosines located in the target sites for the restriction enzymes. To obtain more detailed information about the distribution and density of methylated cytosine residues, we carried out a bisulfite genomic sequencing. The primers were designed to allow amplification of ~ 500 and 400 bp upstream and downstream of the transcription start site, respectively (Figure 2A). The DNAs from the HeLo1 and HeLo1E lines were treated with bisulfite. PCR amplifications were performed for the *nptII*-coding strand of the T-DNA. The PCR products were cloned into the pGemT or pCR2.1 vectors and several randomly selected clones were sequenced (Figure 2A), with each line representing the DNA methylation pattern from different plant

cells. The degree of methylation of the cytosines in a different sequence context is given in Figure 2B. In the clones recovered from the TGS epiallele, both the promoter and the transcribed region were methylated in all sequence contexts, i.e. symmetrical CG and CNG and non-symmetrical CNN. In contrast, in the clones from the PTGS epiallele, the sequences upstream of the transcription start site were not importantly methylated at any motif, while the immediate downstream region was methylated primarily at CG motifs. Although the sequence identity does not allow left and right part of the IR to be analyzed by genomic sequencing separately, the relative homogeneity of sequenced clones and blot hybridization data suggest that both parts of the repeat are methylated to a similar extent.

The epiallelism at locus 1 appeared to be meiotically stable: the progeny plants contained low *nptII* transcript levels (measured by RNA blot). DNA methylation patterns were inherited from the parental posttranscriptionally silenced (HeLo1) and transcriptionally silenced (HeLo1E) plants (data not shown).

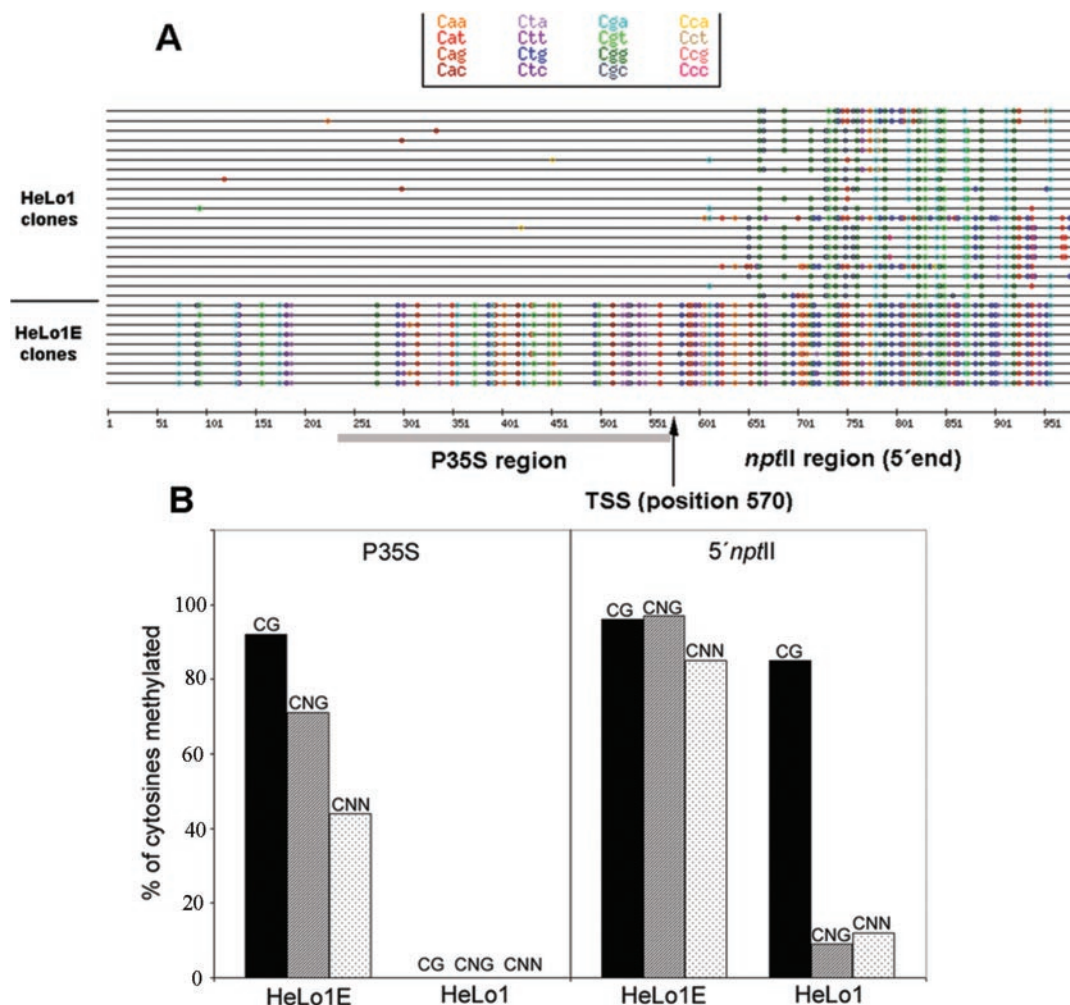


Figure 2. Distribution and density of cytosine methylation in the posttranscriptionally (HeLo1) and transcriptionally (HeLo1E) silenced epialleles of tobacco. (A) Genomic sequencing showing the methylation distribution along the 35S promoter and the 5'-*nptII*-coding region. The sequences obtained were processed through the MethTools software (24). The colored dots and horizontal grey bar indicate the positions of the methylated cytosines in trinucleotide contexts and the position of the core 35S promoter, respectively. TSS, transcription start site. (B) Percentages of methylated cytosines in symmetrical (CG, CNG) and non-symmetrical (CNN) contexts for the promoter ($-400/+1$) and the 5'-*nptII*-transcribed region ($+1/+400$). Data were assembled from 21 HeLo1 and 9 HeLo1E clones, each representing a pattern in individual cell.

Analysis of the *nptII* gene expression in hybrids

We analyzed the *nptII* RNA levels by RNA gel-blot hybridization (Figure 3A). The posttranscriptionally silenced HeLo1 line and its transcriptionally silenced HeLo1E derivative expressed negligible amounts of the *nptII* RNA (transcript), consistent with our previous study (17). Both non-silenced parental HeLo2 and HeB lines had a strong *nptII* hybridization signal. In the F1 plants that combined the PTGS locus 1 epiallele with either locus 2 (Lo1/Lo2 line) or locus B (Lo1/LoB), the steady-state *nptII* RNA levels were dramatically reduced (Figure 3A). No measurable signal was observed in Lo1/Lo2 plants, but some residual signal in all Lo1/LoB hybrids. However, there was a strong *nptII* RNA signal in the hybrid lines that combined the TGS locus 1E epiallele with both the non-silenced locus 2 and locus B (Lo1E/Lo2 and Lo1E/LoB lines). In these hybrids, the RNA signals were comparable with those present in parental non-silenced plants. To test the meiotic expression stability in Lo1E/Lo2 hybrids, a randomly selected Lo1E/Lo2 parental plant (mother donor) was crossed to the non-transgenic SR1 wild type. In the progeny that inherited

both loci in a hemizygous condition (H2 generation), the *nptII* RNA accumulation levels were not significantly different from those present in the F1 (Figure 3A and Table 1). Similarly, high expression was maintained in the third generation (H3) of Lo1E/Lo2 hybrids. A H3 generation was obtained from four independent crosses of H2 plants to non-transgenic SR1. No genetic instability of the IR was observed among more than 50 individuals that had inherited locus 1.

In selected plants, the expression of the *nptII* gene was also studied at the protein level. The NPTII protein content in leaf extracts was determined by NPTII ELISA (Figure 3B). The hybrids combining the PTGS variant of locus 1 had negligible amounts of NPTII protein, whose levels were close to the sensitivity limit of the method. The silencing factor (protein levels in hybrids compared to parental non-silenced lines) was more than two orders of magnitude, and no significant differences between silenced Lo1/Lo2 and Lo1/LoB lines were observed. In contrast, both hybrid lines that combined TGS variants of locus 1 with both locus 2 and locus B targets showed at least 100-fold higher expression than their

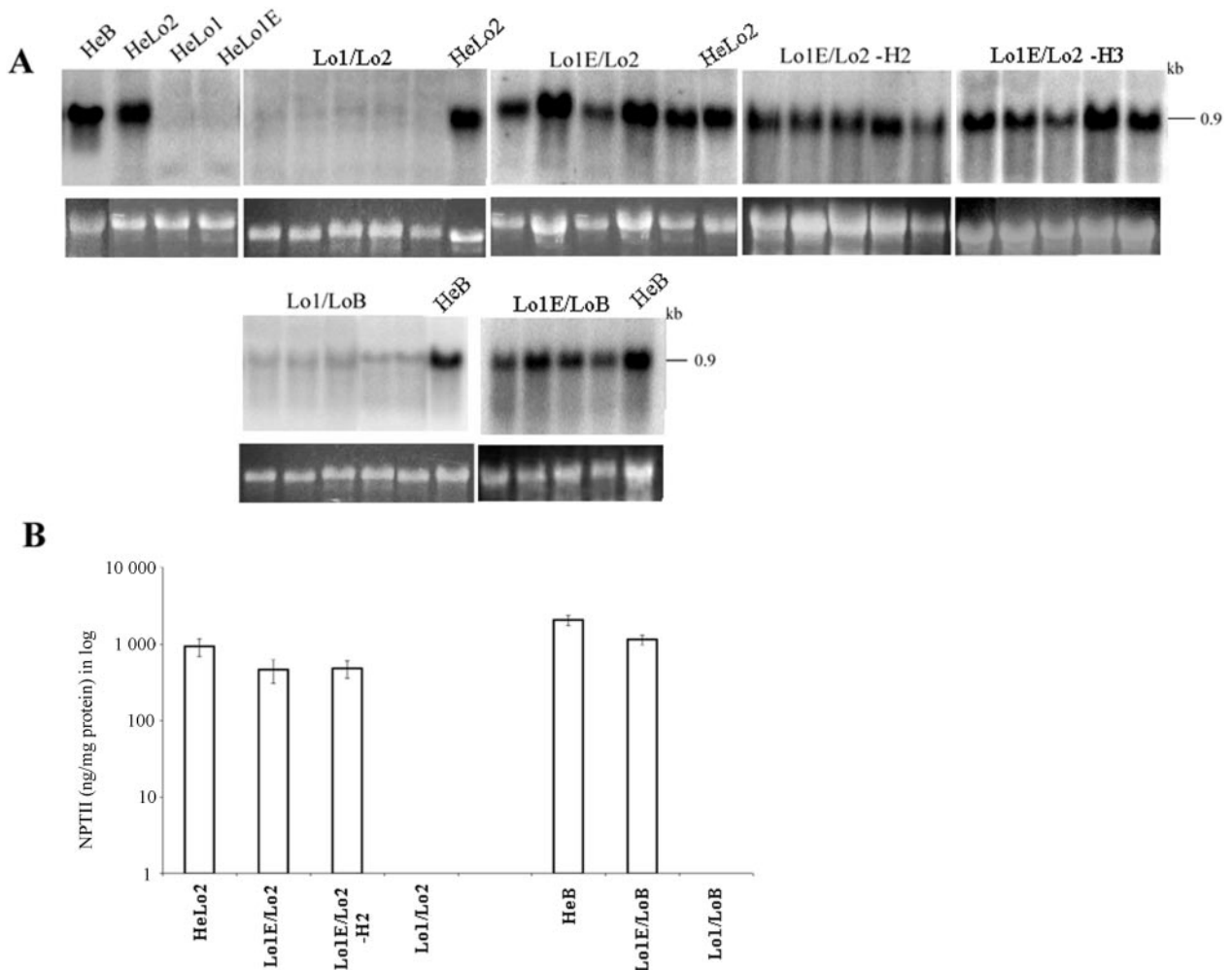


Figure 3. Expression analysis of the *nptII* reporter gene in the parental and hybrid lines. (A) Representative RNA gel-blot hybridizations of the *nptII* transcripts in silenced and non-silenced lines. Five micrograms of total RNA was loaded per lane and hybridized to the *nptII* DNA probe. RNA samples from paternal HeLo2 and HeB plants have been loaded to better compare hybridization bands intensities. The 25S rRNA ethidium bromide-stained bands are shown as loading controls. (B) NPTII protein accumulation level determined by ELISA. The silenced lines generally had NPTII protein levels below the detection limit of the method (5 ng/mg protein). The data represent averages from two to three ELISAs with two different protein extracts isolated from the same plant.

Table 1. Inheritance of expression and methylation patterns of target locus in hybrid lines

Line/genotype	Generation ^a	Numbers	Silencing	Frequency of <i>trans</i> -methylation (%)			
				Coding region BamHI	SmaI	IR center Eco47III	Promoter TaiI
Lo1E/Lo2	F1	21	No	0	9 ²	0	0
	H2	17	No	0	12 ²	0	0
	H3	8	No	0	7 ²	0	0
Lo1/Lo2	F1	12	Yes	100 ^b	100	100	0
	H2	12	Yes	ND ^c	100	ND	0

^aH2 and H3 plants are the progeny of the F1 parents. All lines were hemizygous for both silencer and target. The progeny was obtained by crossing of two F1 mother plants to non-transgenic SR1, followed by DNA gel-blot selection of individuals that inherited both loci in hemizygous conditions. In crosses involving the H2 generation of Lo1E/Lo2 hybrids, individuals with either partial or no *trans*-methylation of a SmaI site were used; there were no differences in the frequency of the SmaI site methylation among the progeny, indicating stochastic features of methylation at this site.

^bPartial methylation.

^cNot determined.

counterparts combining the PTGS epiallele. Compared with the levels found in parental HeLo2 and HeB plants, those in the Lo1E/Lo2 and Lo1E/LoB hybrids were 30–50% reduced.

Methylation analysis of *npI*II transgenes in hybrids carrying the PTGS epiallele of locus 1

In order to analyze methylation of parental T-DNA loci in hybrid plants separately, we carried out double or triple digests of genomic DNAs with methylation-insensitive and methylation-sensitive enzymes (Figures 1 and 4). In Lo1/Lo2 and Lo1/LoB hybrids, the parental loci were separated by EcoRV and by BglII and HindIII digestion, respectively. The methylation was studied with SmaI (mCCmCGGG), Eco47III (AGmCGCT) and BamHI (GGATmCC) with the methylcytosine-inhibiting cleavage indicated with mC. The blots were hybridized with the *npI*II probe. In silenced Lo1/Lo2 hybrid plants, SmaI (completely), Eco47III (partially) and BamHI (partially) failed to digest the 1.5 kb locus 2-specific EcoRV band, indicating *in trans*-methylation of these sites (Figure 4A–C). The difference in digestion efficiency of the SmaI versus the BamHI and Eco47III restriction enzymes could be explained by the variable number of methylation-sensitive cytosines within the target site: two methylation-sensitive cytosines (on each DNA strand) are present within the SmaI site for one within the BamHI and Eco47III sites.

The digestion of Lo1/LoB DNA with BglII and HindIII yielded the 6.4 and 2.8 kb bands, corresponding to locus 1 and locus B, respectively. The 2.8 kb BglII/HindIII band was not digested with SmaI, indicating *in trans*-methylation of this site in locus B (Figure 4D). These results are consistent with a strong *in trans*-methylation effect of PTGS locus 1 on homologous sequences.

Methylation analysis of *npI*II transgenes in hybrids carrying the TGS epiallele of locus 1

To analyze the methylation of parental loci in non-silenced Lo1E/Lo2 and Lo1E/LoB hybrids, we used the same strategy of double and triple digests as described above. The *npI*II probe hybridization profile of the EcoRV/SmaI-digested DNA from several Lo1E/Lo2 plants is shown in Figure 4A. In contrast to the situation in silenced Lo1/Lo2 hybrids, SmaI digested completely the 1.8 kb locus 2-specific band in most plants. In ~10% of the plants, we observed a 1.8 kb band in

addition to the 0.8 kb band after SmaI digestion indicating partial methylation of the site. The 1.8 kb EcoRV band was digested completely by Eco47III (Figure 4B) and BamHI (Figure 4C). Thus, *in trans*-methylation of the coding region sequences does not occur in Lo1E/Lo2 F1 hybrids to a significant extent.

In the second (H2) and third (H3) generation of Lo1E/Lo2 hybrid plants, no cytosine methylation was detected in the Eco47III and BamHI sites (Figure 4B; data not shown), the SmaI site was partially methylated in some plants (Figure 4A and Table 1).

The digestion of Lo1E/LoB DNA with BglII and HindIII yielded the 6.4 and 2.8 kb bands. The 2.8 kb locus B-specific band was digested with SmaI to a similar extent as that in the parental HeLoB plant (Figure 4D).

Because the TGS locus 1E was densely methylated in the 35S promoter (Figure 2), we examined the possibility of transmission of the methylation state from the 35S promoter of locus 1E to that of locus 2 in a hybrid. To discriminate between 35S promoters located in distinct transgenic loci, the DNA was predigested with BglII (Figures 1 and 5). The 8.5 kb BglII band contained 35S promoter molecules from locus 2 and the 6.4 kb band corresponded to locus 1 or 1E. The BglII fragments were subsequently digested with the restriction endonuclease TaiI, which is sensitive to methylation of cytosines located in a CG context (AmCGT). In the silenced Lo1/Lo2 line, both the 8.5 and 6.4 kb bands were digested with TaiI, indicating the lack of promoter methylation. In non-silenced Lo1E/Lo2 hybrid plants, the 6.4 kb BglII band remained undigested with TaiI, confirming the inheritance of locus 1E promoter methylation in the hybrid. However, the 8.4 kb band corresponding to locus 2 was completely digested into the 0.8 kb band, an indication that the homologous 35S promoter in locus 2 was not *in trans*-methylated in any of the hybrids. The locus 2 promoter was also fully digestible with other methylation-sensitive enzymes cutting the proximal (Sau96I and AluI) and distal (HpaII and NcoI) region (data not shown). No *in trans*-methylation of 35S promoter sequences occurred in plants of the H2 or of the H3 generation (data not shown).

Analysis of the smRNAs in hybrid plants

Because of growing evidence that small RNA (smRNA) molecules are involved in most, if not all, cases of

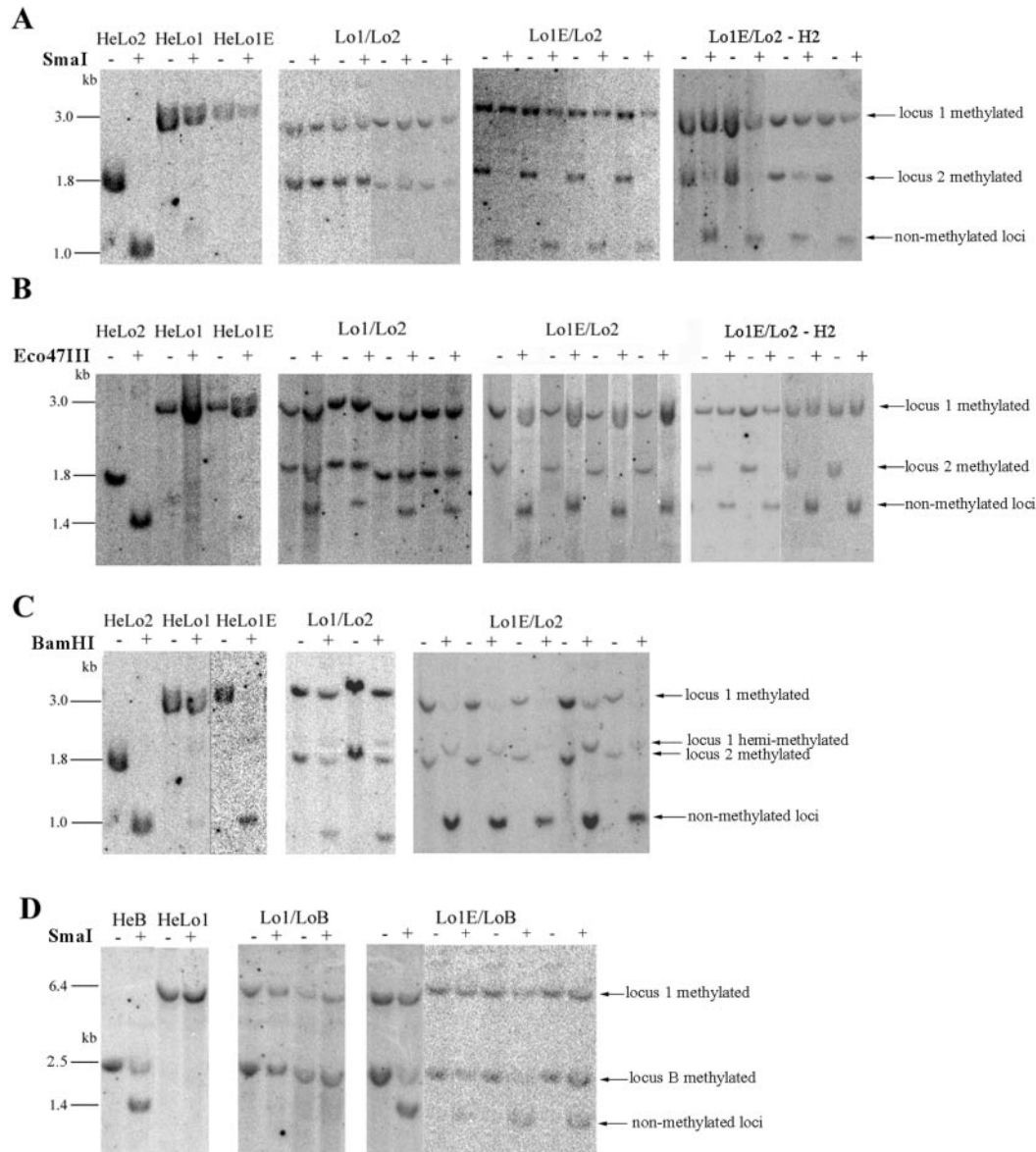


Figure 4. Methylation analysis of transgene loci in parental lines, F1 hybrids and subsequent generations. DNA gel-blot hybridization was carried out using the *npII* gene probe. Genomic DNAs were predigested with methylation-insensitive enzymes (–) to obtain locus-specific bands (indicated by arrows). Absence of digestion of locus-specific bands with methylation-sensitive enzymes (+) is indicative of transgene methylation. Expected lengths of restriction fragments are given in Figure 1. (A–C) Progeny of plants that inherited epiallelic variants of locus 1 and locus 2 (Lo1/Lo2 and Lo1E/Lo2). (D) Progeny of plants that inherited epiallelic variants of locus 1 and locus B (Lo1/LoB and Lo1E/LoB). (A) CG methylation in the *npII*-coding sequence. DNAs were digested with methylation-insensitive EcoRV followed by methylation-sensitive SmaI. (B) CG methylation in the non-transcribed downstream region. DNAs were digested with methylation-insensitive EcoRV followed by methylation-sensitive Eco47III. (C) Non-symmetrical methylation in the *npII*-coding sequence. DNAs were digested with methylation-insensitive EcoRV followed by methylation-sensitive BamHI. The sequence context C within the BamHI site is CCC and CCT on the top and bottom strand, respectively. (D) CG methylation in the *npII*-coding sequence. To separate the loci in Lo1E/LoB and Lo1/LoB hybrids, genomic DNA was digested with the HindIII/BglII enzymes and afterwards with the methylation-sensitive SmaI.

in trans-silencing (27), we analyzed the *npII* smRNA population in hybrid plants carrying either the PTGS or the TGS epiallele of locus 1. The smRNA fraction immobilized on the nylon membrane was hybridized against the radioactively labeled *npII* sense RNA probe. In both the Lo1/Lo2 and Lo1/LoB hybrids, a strong hybridization signal was obtained (Figure 6), but with some variation in the signal intensities among the Lo1/Lo2 but not Lo1/LoB plants (data not shown). Based on the 20 nt marker (M),

the size of the hybridizing fragments could be estimated to ~22–24 nt, a length in agreement with previous reports describing PTGS of the *npII* transgene (28) and other genes (29,30). A relatively weak signal was observed in the posttranscriptionally silenced HeLo1 line, homozygous for locus 1. In the non-silenced HeLo2, Lo1E/Lo2 and Lo1E/LoB lines, HeLo1E, and in non-transgenic SR1 tobacco, no hybridization signal was detectable, even after longer exposure of the blots.

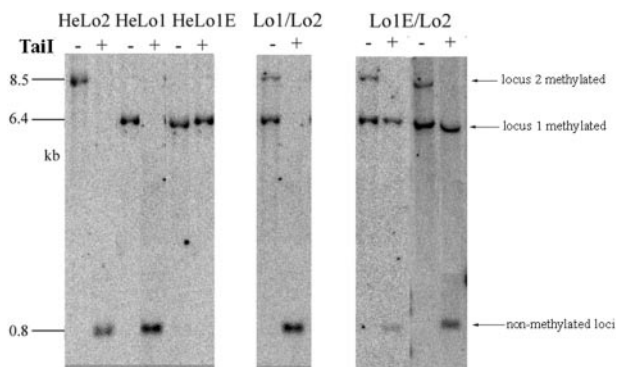


Figure 5. Absence of *trans*-methylation of promoter sequences in F1 hybrids carrying PTGS and TGS epialleles. Cytosine methylation was studied in the 35S promoter by DNA gel-blot hybridization of restricted DNAs. The sizes of the restriction fragments were calculated from the T-DNA sequence (Figure 1). The 0.8 kb fragment corresponds to the non-methylated BglIII/TaiI genomic fragment. The positions of the locus-specific bands after separation of the loci with methylation-insensitive BglIII enzyme are marked by arrows. Symbols above the blots indicate digestion with the methylation-insensitive enzyme only (–) or in combination with the methylation-sensitive Tail enzyme (+).

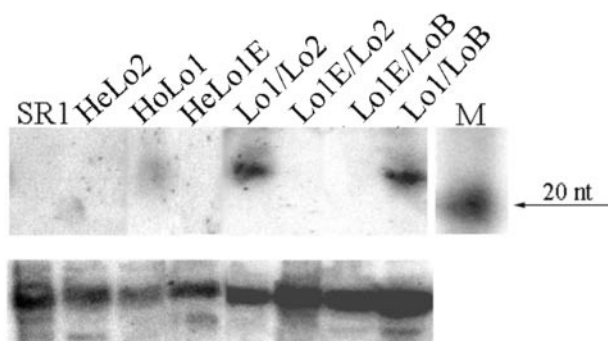


Figure 6. Detection of smRNAs. Low-molecular weight RNA fractions were blotted on a Hybond-XL membrane and hybridized with the hydrolyzed sense-specific *nptII* riboprobe. A 20 nt oligomer was used as a size marker. The loading control was a non-specific high molecular band. Approximately 50 μ g of RNA material was loaded into each lane. SR1, non-transgenic control; HeLo2, non-silenced line; HoLo1, PTGS single locus line; HeLo1E, TGS single locus line; Lo1/Lo2 and Lo1/LoB, PTGS F1 hybrids; Lo1E/Lo2 and Lo1E/LoB, non-silenced F1 hybrids.

Methylation and expression analysis of *nptII* transgenes in hypomethylated plants

Dense methylation of the 35S promoter of locus 1E might prevent transcription of its linked gene and consequently the generation of a *trans*-silencing RNA signal. To investigate the effect of locus 1E promoter hypomethylation on the *nptII* transgene expression in non-silenced Lo1E/Lo2 hybrids, we treated young Lo1E/Lo2 seedlings with 5-aza-2-deoxycytidine, a drug previously shown to induce overall genome hypomethylation in tobacco (31). The RNA blot in Figure 7A shows that the accumulation of the *nptII* RNA in 5-aza-2-deoxycytidine-treated plants is lower than that in non-treated controls, suggesting that resiliencing occurred after the drug treatment. There was some plant-to-plant variability in the degree of the *nptII* expression and in one plant the RNA level was negligible. The hypomethylation effect of the drug was demonstrated by DNA gel-blot hybridization of

an endogenous repetitive sequence (Figure 7B) and the 35S promoter (Figure 7C). The ladders of hybridization bands indicated hypomethylation of a subset of CCGG sites within the HRS60 repeat after the 5-aza-2-deoxycytidine treatment. The methylation of promoter sequences was analyzed by the BglIII/TaiI digestions as described above. The upper 6.4 kb BglIII/TaiI band comprised fully methylated molecules derived from locus 1, whereas the bottom 0.8 kb band is a sum of non-methylated promoter molecules derived from both locus 1 and locus 2. In all 5-aza-2-deoxycytidine samples, the signals in the 0.8 kb band were stronger than in the control. For quantification, we expressed the relative hypomethylation as a ratio between the 6.4 and 0.8 kb bands. Similarly as in the case of HRS60 hybridization, plants 2 and 5 had the highest degree of hypomethylation, suggesting a good correlation between global genome and promoter hypomethylation.

DISCUSSION

The TGS and PTGS epialleles differ in their methylation patterns

Methylation of the PTGS and TGS epialleles was studied by bisulfite genomic sequencing (Figure 2). In the PTGS variant, the methylated region that started \sim 50 bp downstream from the transcription start site was sharply separated from the unmethylated upstream sequences and the promoter region. Only a few clones showed rare methylation in the promoter at a frequency corresponding to 1 mC/per clone/500 bp. Such a frequency might reflect a general methylation noise and probably does not influence the promoter activity. The cytosines in the 5' transcribed region were highly methylated at the CG sites, while those in CNG and non-symmetrical context were less. Nevertheless, two HeLo1 clones had a high level of both symmetrical and non-symmetrical methylation, suggesting a certain epigenetic mosaicism among the cells. Interestingly, in these densely methylated molecules, methylation spread apparently further upstream close to the transcription start site.

In contrast to the PTGS locus 1, the TGS epiallele locus 1E was highly methylated in the promoter region: in the \sim 300 bp of the 35S core promoter, all clones displayed homogenous sequencing profiles and methylation appeared in all sequence contexts (92% CG, 71% CNG and 44% CNN). This pattern is similar to that of the transcriptionally silenced 35S promoter in another tobacco (32) and *Petunia* transgenic line (33), but clearly different from an inactive 35S promoter in *Arabidopsis* in which cytosines in non-symmetrical contexts were less methylated (34). The two TGACG motifs at –83 and –71 that are important for binding of the activation sequence factor (35) were completely methylated. The methylation density started to gradually decrease only at a distance of \sim 300 bp upstream from the transcription start site and the sites upstream of –500 were free of methylation. The sequences downstream from the transcription start site were more heavily methylated in the TGS than in the PTGS epialleles. Together, these results suggest that the tissue culture-induced epimutation of locus 1 (17) is caused by factors spreading the methylation and chromatin imprint from the IR center. The nature of the non-symmetrical methylation at the transcriptionally silenced promoter and the immediate downstream sequences remains enigmatic. This type of methylation is known to be

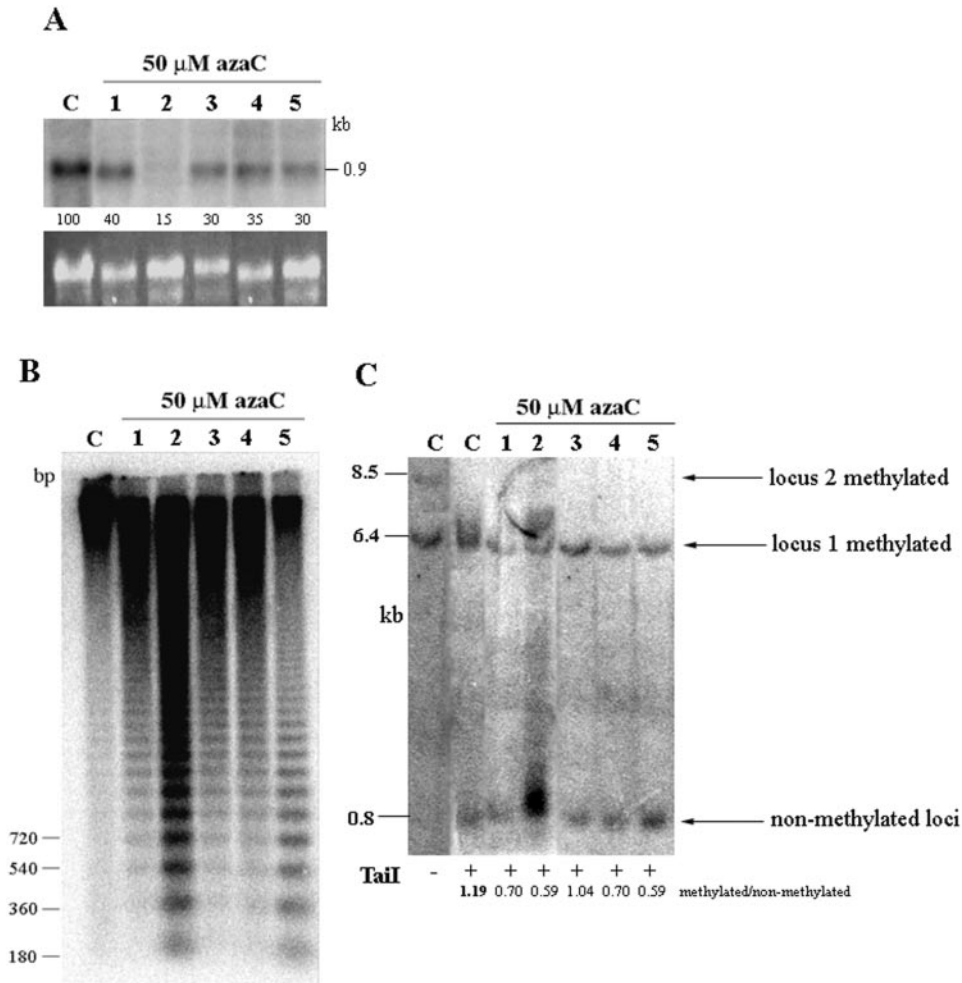


Figure 7. Expression and methylation analysis in hypomethylated Lo1E/Lo2 plants. (A) RNA gel-blot hybridization of the *nptII* RNA isolated from 5-aza-2-deoxy-cytidine (azaC)-treated (1–5) and non-treated (C) plants. The numbers below the lanes indicate relative signal intensities of the *nptII*-specific band. The signals were normalized by ethidium bromide fluorescence of the 25S rRNA gene (bottom panel). The signal in the control lane (C) was arbitrarily chosen as 100%. (B) DNA methylation in the endogenous HRS60 satellite analyzed by HpaII restriction enzyme. (C) DNA methylation in the 35S promoters analyzed by TaiI restriction enzyme (+) as described in Figure 5. DNAs were predigested with BglIII to separate both loci. The ratio between methylated/non-methylated fragments are indicated below the lanes.

mediated by RNA molecules. However, several lines of evidence argue against an RNA-directed mechanism of DNA methylation in the TGS epiallele. First, previous run-on assays (17) and more sensitive quantitative PCR (M. Fojtová, unpublished data) failed to show transcription of the linked *nptII* transgenes of the TGS locus 1E epiallele. Second, crossing experiments of the TGS HeLo1E to lines carrying homologous T-DNAs did not lead to *de novo* methylation of targets (see below), suggesting the absence of a diffusible signal emanating from a transcriptionally silenced 35S promoter. Perhaps the non-symmetrical methylation marks might rely on yet unidentified specific chromatin factors.

The TGS and PTGS epialleles of invertedly repeated transgenes differ in their *trans*-silencing and *trans*-methylation capacities

The hybrid lines combining PTGS or TGS epiallelic variants of the *P*_{35S}:*nptII* transgene organized as a tail-to-tail repeat had contrasting expression and DNA methylation features. First,

when the PTGS version was combined with any highly expressing homologous locus, the *nptII* transgene expression was more than 100-fold reduced, while the TGS variant did not or moderately *trans*-silenced. The expression outcome of various crosses was not influenced by repetitiveness and genomic organization of the target locus. The TGS *P*_{35S}:*nptII* epiallele did not induce transcriptional silencing of the 35S promoter in any line tested, despite the long homology region (5 kb) and the high level of methylation spreading up to 1.8 kb from the center of the IR. We have also tested the possibility that meiosis might facilitate transfer of silent information from the TGS *P*_{35S}:*nptII* epiallele to the target locus by homologous pairing. IRs have been proposed to be particularly sensitive to homology searches (36). However, Lo1E/Lo2 hybrids continued to express high levels of the *nptII* gene for at least three generations, while the hybrids combining the PTGS version of the transgene IR locus with locus 2 were stably silenced. Second, hybrids combining the PTGS but not the TGS version showed extensive *trans*-methylation of any homologous target locus. In silenced targets, both symmetrical and

non-symmetrical sites were *trans*-methylated to a similar extent, at least at the 3' end (Figure 4). This pattern of methylation has been proposed to be a hallmark of RNA-directed DNA methylation (37) and indeed smRNA molecules were found in silenced hybrids (Figure 6). Similarly, exposure of a wild-type *Arabidopsis SUPERMAN* allele to a heavily methylated and transcriptionally silenced epiallele did not result in *trans*-silencing (38), and transcriptionally silenced transgenes failed to cosuppress an endogenous gene by a PTGS mechanism in the same organism (39). Finally, Vaucheret *et al.* (40) found that only transcribed transgenic loci efficiently cosuppressed endogenous nitrate reductase in *N. plumbaginifolia*. Taken together, transgenes linked to transcriptionally silenced promoters, even when arranged as IRs, might be incapable of immediate *trans*-silencing interactions. Although we cannot exclude the possibility that a population level study might reveal pairing interaction at low frequency, the current data are more consistent with the idea that *trans*-silencing capacities of epialleles of IR loci are not dramatically influenced by repeated meiotic cycles, provided that their epigenetic state remains unaltered.

PTGS, but not TGS, epialleles of invertedly repeated transgenes produce smRNAs

Previously, we have shown by run-on assays that the parental locus 1 produces large amounts of *nptII* primary transcripts, while no transcripts were produced by its TGS locus 1E variant (17). Correspondingly, smRNA molecules derived from degraded *nptII* transcripts were detected in parental lines bearing the PTGS variant only. In hybrids, smRNAs were found among the silenced plants that inherited the PTGS but not TGS version of locus 1 (Figure 6). Taken together, the *trans*-methylation of locus 2 and locus B in hybrids with PTGS locus 1 probably occurs via smRNAs, although some uncertainty remains. For example, smRNA levels among the Lo1/Lo2 individuals varied somewhat despite strong and heritable PTGS (Table 1). Second, the parental silencer line containing locus 1 alone (both homozygous and hemizygous) produced relatively low amounts of smRNAs compared to those of hybrids [Figure 6 and (28)]. Perhaps, only a few *nptII* transcripts extend beyond a polyadenylation site, form double-stranded RNAs, and are eventually diced into smRNAs. The read-through model is supported by previous nuclear run-on studies indicating that RNA synthesis could proceed beyond the polyadenylation signal (41) and by *trans*-methylation of the Eco47III site downstream from it (Figure 4B).

Induced hypomethylation of the TGS epiallele partially restores its *in trans*-silencing capacity

Based on abundant data referring to a direct link between promoter methylation and its transcriptional activity (1,3,42,43), we tested the hypothesis that decreasing cytosine methylation in the 35S promoter region of the transgenic locus 1E would activate transcription of the downstream *nptII* transgene, potentially restoring its *in trans*-silencing capacity. Indeed, the plants in which the presence of the transcriptionally silenced locus 1E does not lead to *trans*-silencing of

locus 2, show reduced expression of the *nptII* reporter gene of locus 2 after treatment with a hypomethylation drug (Figure 7A). The reduction in expression seems to depend on dosage because plants with greater hypomethylation (2,5) had lower *nptII* RNA accumulation levels compared with plants with a lower degree of hypomethylation (1,3,4). Together with the observed hypermethylation of the promoter sequence of locus 1E, the most probable interpretation of this result is that the drug-induced hypomethylation of the promoter of locus 1E activates the transcription and re-establishes the RNA silencing signal, with reduced expression of the *nptII* gene of locus 2 as a consequence. In most reports, global hypomethylation led to elevated expression of genes or transgenes, whereas we observed the opposite, namely correlation of transcription restoration with production of silencing signals. We conclude that the impact of the global hypomethylation on expression patterns might depend on the experimental set up and the transgene copy number. Interestingly, relatively weak hypomethylation (20%) was apparently sufficient for resiliencing of the homologous transgene by a PTGS mechanism. Several reports in tobacco (32), *Petunia* (33) and *Arabidopsis* (34) show that partially methylated 35S promoters remained active. Saturation methylation of both symmetrical and non-symmetrical motifs is probably needed for complete shutdown of the transcription activity. It will be interesting to see whether changes in global methylation levels observed during development (44,45), stress (46–48) and tissue culture (49) would alter the balance between the TGS and PTGS states. Although TGS of locus 1E associated with its *trans*-silencing inability was fairly stable in plants over generations (Table 1), a progressive loss of *nptII* expression was observed in Lo1E/Lo2 calli (M. Fojtová, unpublished data), suggesting that cell culture can destabilize the expression patterns.

Silencing signals generated by the IR loci

Most current data indicate that immediate *trans*-silencing in F1 hybrids is almost always induced by DNA–RNA interactions, whereas DNA–DNA pairing might be involved in the cases where longer time and more generations are needed to manifest silencing. A moderate decrease of *nptII* expression in a few F1 hybrids bearing the TGS locus 1E variant (Figure 3A) could potentially be explained by infrequent or weak DNA–DNA interactions proposed to be involved in transvection and paramutation phenomena (50,51). However, we consider this possibility unlikely, because meiosis, known to stimulate homology searches, did not alter the expression and methylation patterns established in the second and third generation of Lo1E/Lo2 hybrids (Figure 3B). Therefore, we favor the hypothesis that occasionally incomplete repression of the strong 35S promoter within the locus 1E plants generates a weak RNA silencing signal, resulting in a moderate decrease in *nptII* expression in some Lo1E/Lo2 and Lo1E/LoB lines. Weak, often stochastic *trans*-methylation of the SmaI site at the 3' end of the coding region is consistent with this hypothesis. Although sensitive reverse transcription PCR failed to reveal transcripts close to the 3' end of the *nptII*-coding sequence in TGS locus 1E (data not shown), spurious transcripts at certain stages of plant development cannot be excluded. It is significant that the 35S promoter of the target

locus was not *trans*-methylated (and inactivated) by the highly methylated promoter of locus 1E even after being in contact for several generations, indicating that promoter sequences need to be transcribed to generate a *trans*-TGS signal (3). Also endogenous highly methylated R8.3 repetitive sequence failed to provoke silencing and *trans*-methylation of a transgene carrying a repeat unit in tobacco hybrids (52). Perhaps cryptic promoters (53) residing in chromosomal DNA or the T-DNA could account for many, if not all, examples for *trans*-TGS (32). Stable epialleles of transgenes have been reported to interact *in trans* in tetraploid, but not in diploid, plants (54) leading to silencing and progressive methylation of an active epiallele in the F2 generation. Meiosis-dependent epigenetic conversion has been proposed to be mediated by DNA pairing interactions (55). Hence, the capacity of the TGS locus 1E to *trans*-silence homologous sequences at an allelic position remains to be tested.

Several reports showed a strong correlation between *trans*-silencing capacity and an IR arrangement of the T-DNA (9,56), while no such correlation was observed in other studies (15,16). Our data suggest that the IR structure in itself and the heavy methylation are not sufficient to induce *trans*-silencing. However, transcription of the IR generates *trans*-silencing RNA signals. Dependence of *trans*-silencing capacities on the epigenetic properties of the trigger locus might explain the changes in transgene expression throughout several generations, especially in multi-locus systems. We speculate that our transgene model might be also applicable to regulatory networks of endogenous genes. For example, harmonized expression of multigenic families could be under the control of a single or a few 'master loci' bearing IR structures. So far, this hypothesis is best evidenced by the concerted regulation of phosphoribosylanthranilate isomerase (*PAI*) genes coding for tryptophan biosynthetic enzymes in *Arabidopsis* (57). Parallels are apparent between locus 1 and the PAI1-PAI4 IR. First, in both cases the *trans*-silencing capacities seem to depend on the activity of the upstream promoter. Second, the methylation of the PAI1-PAI4 IR is unaffected by the promoter activity, suggesting that methylation within the IR center does not depend on the status of the upstream promoter. However, despite these apparent similarities, there are also some differences. For example, first, the endogenous *PAI1* gene is highly expressed within the PAI1-PAI4 IR, while both PTGS and TGS epialleles of locus 1 are silenced. Second, small levels of smRNA molecules were detected in hybrids bearing the PTGS locus 1 (Figure 6), but not in *Arabidopsis* strains bearing the PAI1-PAI4 IR. Clearly, more studies are needed to clarify the issue. Another role for epiallelic variation could be envisaged during evolution of heritable resistance against viral infection (58). PTGS has been proposed to have primarily evolved as a defense system against replication of viral genomes (59). During the infectious phase, the actively replicating virus continues to express high amounts of RNA, because of the presence of strong promoters. However, upon occasional integration into the host chromosomes, endogenous viral sequences might acquire epigenetic marks leading to silencing. Indeed, tobacco and related species contain a large number of transcriptionally silenced and highly methylated repeats of viral origin (58,60). Hypothetically, replication of an exogenous virus could be blocked by interfering RNAs generated from an IR copy of its endogenous partner. The switch

between TGS and PTGS modes could have adaptive significance.

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