Cross-talk between posttranscriptionally silenced neomycin phosphotransferase II transgenes

Helena Van Houdt[a], Ales Kovařík[b], Marc Van Montagu[a], Anna Depicker[a,*]

[a] *Departments of Molecular Genetics and Plant Genetics, Flanders Interuniversity Institute for Biotechnology, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium*
[b] *Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic*

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Abstract Tobacco plants containing a transgene locus with two chimeric neomycin phosphotransferase II (*nptII*) genes in tail-to-tail orientation (locus 1) show posttranscriptional gene silencing. The silenced *nptII* transgenes of locus 1 can downregulate the expression of homologous *nptII* transgenes in hybrid plants. The 3′ region of the silenced *nptII* genes located in the center of the inverted repeat locus 1 is extensively methylated. Moreover, 3′ segments of in *trans*-inactivated transgenes also become methylated, suggesting cross-talk between homologous posttranscriptionally silenced genes. Our results are in accordance with the hypothesis that this cross-talk can be mediated by specially featured RNAs.

Key words: Cytosine methylation; Gene silencing; Inverted repeat; Posttranscriptional gene silencing; T-DNA; Tobacco

1. Introduction

Sequence duplications obtained by introducing multiple transgene copies or transgenes with homology to endogenes in plants frequently result in homology-dependent gene silencing (HDGS). HDGS can act at the transcription initiation level (transcriptional gene silencing or TGS) or at the post-transcriptional level (PTGS) (reviewed by [1–4]). TGS inhibits production of mRNA from genes driven by homologous promoters whereas PTGS drastically reduces the accumulation of homologous RNA species. Complex transgene loci have been implicated in both TGS and PTGS. In particular, the presence of inverted repeats has been strongly correlated with silencing [5–10]. Several reports on silencing in untransformed plants support the idea that inverted repeats are extremely potent in activating silencing [11–13]. Posttranscriptionally silenced transgenes can inactivate sequences in *trans* that are partially homologous in the transcribed regions and that are normally expressed in the absence of a silencing locus [1]. In some cases, PTGS is correlated with cytosine methylation in the transcribed region [14–21]. Whether there is a functional or even a causal relationship between DNA methylation and PTGS still remains unclear. However, virus resistance assays that induce transgene silencing accompanied by de novo methylation show that methylation precedes the onset of resistance [22]. Also, the partial release of PTGS obtained by drug-induced hypomethylation suggests a functional role of methylation in the silencing mechanism [23]. Further, little is known about the mechanisms that establish these de novo methylation patterns. In *Neurospora* and *Ascobolus*, duplicated sequences are methylated by the mechanisms of repeat-induced point mutation or methylation induced premeiotically, via DNA–DNA pairing [24,25]. In the case of TGS in plants, in *trans*-silencing and methylation of an unmethylated sequence homologous to a methylated locus was also ascribed to a DNA pairing-dependent process termed 'epigene conversion' [26,27]. Also RNA–DNA interactions could serve as a signal for de novo methylation [28,29]. Viroid cDNA copies integrated into the tobacco genome were methylated only after autonomous replication of viroid RNA–RNA had taken place in these plants [28,29]. Recently, TGS as a consequence of promoter methylation has been shown to depend on the production of an aberrant promoter transcript [30]. Therefore, RNA–DNA interactions can result in de novo methylation leading to HDGS [30]. It is tempting to speculate that post-transcriptionally silenced transgenes become methylated by a similar RNA-directed fashion.

Here, we analyzed the capacity of a neomycin phosphotransferase II (*nptII*) silencer locus, showing PTGS, to induce in *trans* silencing and methylation in hybrid plants that contain the silencer and a non-silencer locus. Our results support the idea of an efficient and continuous cross-talk between silenced genes within the cell.

2. Materials and methods

2.1. Plasmids

The T-DNA-derived plant transformation vectors pGVCHS(287) and pGVCHS(320) harbor two chimeric genes between the T-DNA borders (Fig. 1A: locus 1 and locus 2 containing the pGVCHS(287) T-DNA; locus B with the T-DNA of pGVCHS(320)), the first being the selection marker composed of the hygromycin phosphotransferase II coding sequence (*hpt*) under control of the nopaline synthase promoter (P*nos*) and 3′-untranslated region (3′*nos*) and the second chimeric gene is the *nptII*-coding sequence under control of the cauliflower mosaic virus 35S promoter (CaMV 35S) and a fragment of the 3′ region of the *Antirrhinum majus* chalcone synthase gene (3′*chs*) (287 bp and 320 bp in the T-DNA vectors pGVCHS(287) and pGVCHS(320), respectively).

2.2. Plant material

Transgenic tobacco plants containing the pGVCHS(287) or pGVCHS(320) T-DNA were obtained as described by Ingelbrecht et

*Corresponding author. Fax: (32)-9-2645349.
E-mail: anna.depicker@rug.ac.be

Abbreviations: HDGS, homology-dependent gene silencing; NPTII, neomycin phosphotransferase II; PTGS, posttranscriptional gene silencing; RB, right border; TGS, transcriptional gene silencing; UTR, untranslated region

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al. [31]. Homozygous transgenic plants (HOlo1, HOlo2, and HOloB) were obtained after several rounds of self-fertilization starting from the silenced primary transformants GVCHS(287)-1 (for HOlo1 and HOlo2 [32]) and GVCHS(320)-1 (for HOloB [19]). Hemizygous plants (HElo1, HElo2, and HEloB) were obtained by back-crossing the respective homozygous parental plants to an untransformed SR1 tobacco plant and the hybrids HElo1/HElo2 and HElo1/HEloB by intercrossing the respective homozygous parental plants. Crosses were performed by emasculating flowers manually and by applying the pollen to the stigma. Callus cell cultures were established from leaf explants by hormonal treatment according to standard procedures. Calluses were grown on Gamborg's B5 medium containing 0.7% agar and supplemented with sucrose (30 g/l), α-naphthalene acetic acid (2.0 mg/l), and benzylaminopurine (0.2 mg/l).

2.3. DNA isolation and Southern blot hybridization

DNA isolation from leaf tissue and hybridizations were mainly done as described previously [19]. Alternatively, total genomic DNA was extracted from approximately 10 g of wet callus tissue according to the modified procedure of Saghai-Maroof et al. [33]. Calluses were extensively lyophilized prior to homogenization in liquid nitrogen. After isopropanol precipitation, DNA was subjected to RNase A (40 μg/ml, 15 min, 37°C) and proteinase K (20 μg/ml, 2 h, 50°C) treatments. Some probes were labelled using the Gene Images random prime labelling kit (Amersham, Aylesbury, UK) and detected with the Gene Images CDP-Star module (Amersham).

3. Results

3.1. In cis methylation of the posttranscriptionally silenced nptII genes in locus 1

Transgenic tobacco plants that contained a transgene locus with two T-DNAs that harbors each a p35S-*nptII*-3′*chs* gene and that are organized in inverted orientation about the right

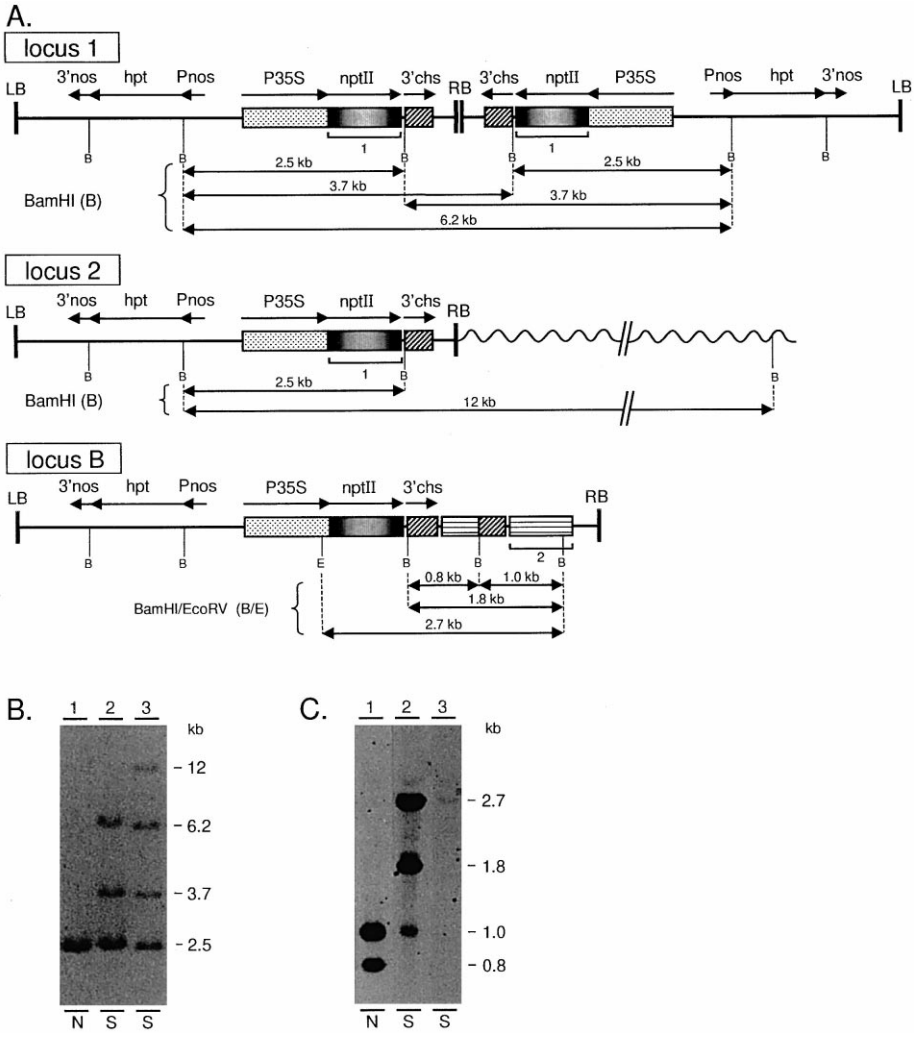

Fig. 1. *Bam*HI methylation in posttranscriptionally silenced neomycin phosphotransferase II (*nptII*) genes. A: Schematic outline of transgene locus 1 and locus 2 and of the T-DNA in locus B with indication of the obtained hybridizing DNA fragments in Southern blot analyses (B and C). The *nptII* genes are under control of the cauliflower mosaic 35S promoter (P35S). The chimeric *nptII* genes in locus 1 (lo1) and 2 (lo2) contain 287 bp of the 3′ signalling sequences of the chalcone synthase gene (3′*chs*) of *Antirrhinum majus* (T-DNA vector pGVCHS(287)), and those in locus B (loB) 320 bp of the same 3′ signalling sequences (T-DNA vector pGVCHS(320)). Locus 1 contains two invertedly repeated GVCHS(287) T-DNAs, whereas locus 2 harbors only a single copy of the same T-DNA. Locus B consists of three tandemly organized GVCHS(320) T-DNAs [19]. B: Southern blot analyses of *Bam*HI-digested genomic DNA. Lane 1, HElo2 (N); lane 2, HElo1 (S); lane 3, HElo1+HElo2 (S). Fragment 1 is used as a probe. C: Southern blot analyses of *Eco*RV/*Bam*HI-digested genomic DNA. Lane 1, HEloB (N); lane 2, HElo1+HEloB (S); lane 3, HElo1 (S). Fragment 2 is used as a probe. (S) silenced, (N) normally expressed. 3′*chs*, 3′ untranslated region of *A. majus* chalcone synthase gene; 3′*nos*, 3′-untranslated region of nopaline synthase; *nptII*, neomycin phosphotransferase II coding sequence; *hpt*, hygromycin phosphotransferase coding sequence; P35S, cauliflower mosaic virus 35S promoter; P*nos*, nopaline synthase promoter; LB, left border; RB, right border.

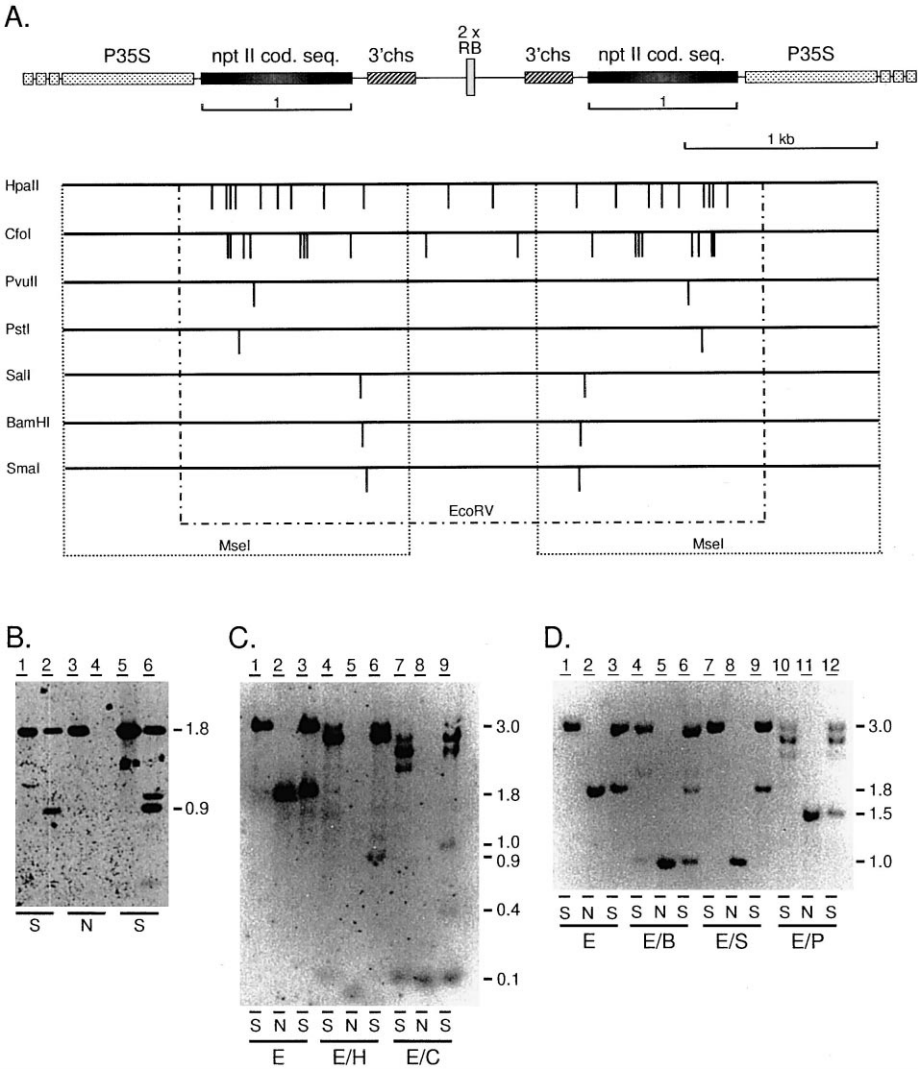

Fig. 2. Methylation study of the silencing locus 1 and in *trans*-silenced locus 2. A: Schematic representation of the central part of locus 1 and the analyzed restriction sites. *Eco*RV (E) and *Mse*I (M) are methylation-insensitive and were used to delimit certain DNA fragments for analysis. The *nptII* probe used for detection in Southern blot analyses presented in panels B, C, and D is indicated by the bar numbered 1. For abbreviations, see Fig. 1. B: Southern blot analysis of *Mse*I- and *Mse*I/*Hpa*II-digested genomic DNA. Lane 1, HElo1 *Mse*I-digested; lane 2, HElo1 *Mse*I/*Hpa*II-digested; lane 3, HElo2 *Mse*I-digested; lane 4, HElo2 *Mse*I/*Hpa*II-digested; lane 5, HElo1+HElo2 *Mse*I-digested; lane 6, HElo1+HElo2 *Mse*I/*Hpa*II-digested. C: Southern blot analysis of HElo1, HElo2, and HElo1+HElo2 genomic DNA digested with *Eco*RV (E) (lanes 1, 2, and 3, respectively), with *Eco*RV/*Hpa*II (E/H) (lanes 4, 5, and 6, respectively), and with *Eco*RV/*Cfo*I (E/C) (lanes 7, 8, and 9, respectively). Fragment 1 is used as a probe. D: Southern blot analyses of HElo1, HElo2, and HElo1+HElo2 genomic DNA digested with *Eco*RV (E) (lanes 1, 2, and 3, respectively), with *Eco*RV/*Bam*HI (E/B) (lanes 4, 5, and 6, respectively), *Eco*RV/*Sma*I (E/S) (lanes 7, 8, and 9, respectively), and *Eco*RV/*Pst*I (E/P) (lanes 10, 11, and 12, respectively). Fragment 1 is used as a probe. The length of some hybridizing DNA fragments is indicated in kb. (S) silenced, (N) normally expressed.

T-DNA border (RB) (locus 1; Fig. 1A) showed PTGS of the *nptII* transgenes (10 ng NPTII/mg total soluble protein). Transgenic tobacco plants with only a single copy of the same T-DNA (locus 2; Fig. 1A) expressed *nptII* normally (1000–1500 ng NPTII/mg total soluble protein; [10,32]). We determined the methylation status of several cytosines in the silenced *nptII* transgenes of locus 1 by Southern blot analyses using different methylation-sensitive restriction enzymes. Analysis of *Bam*HI-digested DNA of a HElo1 plant, using the *nptII*-coding sequence as a probe, showed three hybridizing DNA fragments (2.5 kb, 3.7 kb, and 6.2 kb), although only the 2.5-kb hybridizing DNA fragment would be expected after complete digestion of the *Bam*HI restriction sites (Fig. 1A,B, lane 2). The 3.7-kb and 6.2-kb hybridizing bands originated from incomplete digestion because of cytosine methylation. The cytosine is asymmetrically located in the *Bam*HI recognition sequence (G**GGATCC**T) in the 3′ untranslated region (UTR) and is methylated in one or both *nptII* genes of the inverted repeat locus 1. To test for simultaneous methylation of multiple cytosines located in the silenced *nptII* transgenes of locus 1, DNA of HElo1 was analyzed after double digestion with *Mse*I/*Hpa*II, *Eco*RV/*Hpa*II, and *Eco*RV/*Cfo*I. Both methylation-insensitive enzymes, *Eco*RV and *Mse*I, cut outside the *nptII* coding sequence (Fig. 2A). In all cases, hybridizing DNA fragments larger than those expected by complete digestion could be visualized when the *nptII* coding sequence was used as a probe (Fig. 2A–D). In a *Mse*I/*Hpa*II double digest, the hybridizing 1.8-kb band testi-

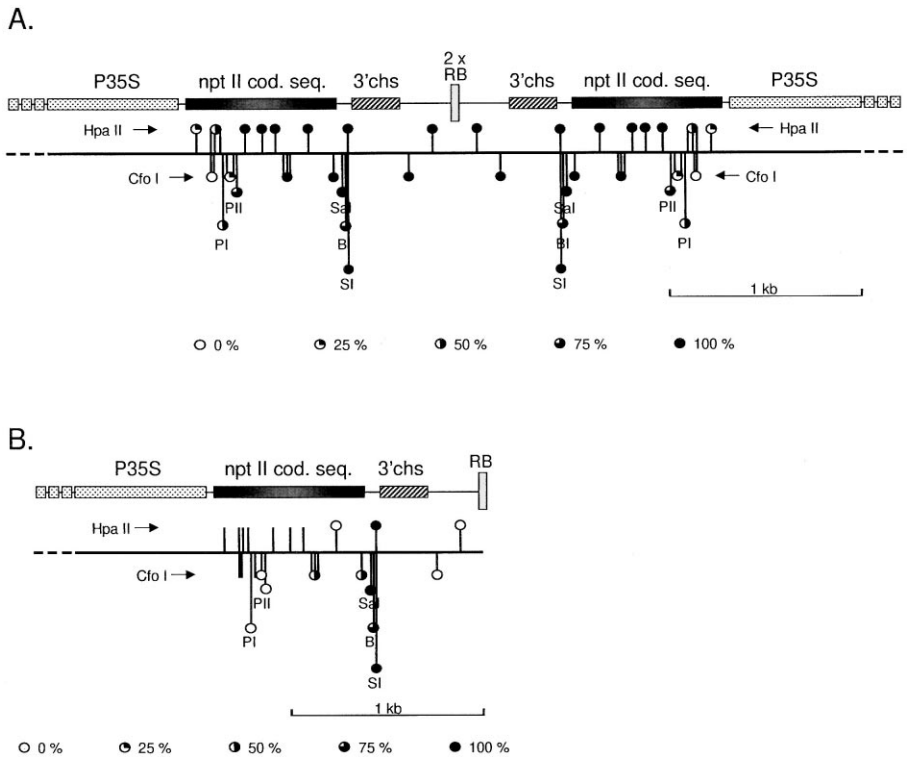

Fig. 3. Summary of the methylation status of the silencer locus 1 and of the in *trans*-silenced locus 2. A: Methylation status of the silencer locus 1 in HElo1 genomic DNA. B: Methylation status of the in *trans*-silenced locus 2 in HElo1+HElo2 genomic DNA. The analyzed sites are indicated with a vertical bar. The *Hpa*II sites above the bar and the *Cfo*I sites below the bar are indicated with an arrow. SI, *Sma*I; BI, *Bam*HI; SaI, *Sal*I; PII, *Pvu*II; PI, *Pst*I. The differently filled circles give an estimation of the percentage of methylation of that particular site or of a group of sites when they are very closely linked. Abbreviations as in Fig. 1.

fied to complete methylation of all nine *Hpa*II restriction sites between the indicated *Mse*I restriction sites (Fig. 2A,B, lane 2); the 0.9-kb band resulted from coordinate methylation of several *Hpa*II sites in the 3′ region, but digestion of at least one unmethylated *Hpa*II site in the 5′ region of the *nptII* coding sequence. The large size of the *Eco*RV/*Hpa*II-hybridizing fragments (2.7 kb and 3.0 kb) indicated that the *Hpa*II site downstream of the polyadenylation site and close to the RB was methylated in both T-DNA copies of the inverted repeat (Fig. 2A,C, lane 4). The large *Eco*RV/*Cfo*I-hybridizing bands also demonstrate extensive and coordinate methylation of at least 10–14 cytosines in the center of the inverted repeat (Fig. 2A,C, lane 7). These results showed that methylation was very extensive near the central RB junction of the inverted repeat and that methylation became weaker in the 5′ region of the coding sequence of both *nptII* genes. These observations were confirmed by the analysis of the 3′ UTR-located *Sma*I and *Sal*I restriction sites, which are completely methylated in HElo1 genomic DNA, and of the 5′ located *Pst*I restriction site, which only showed approximately 50% methylation (Fig. 2A,D, lanes 7 and 10; data not shown). As a control, the methylation study was also performed using genomic DNA isolated from normally expressing HElo2 tissue. No or only faint hybridizing DNA fragments of relatively low molecular mass were detected upon analysis of HElo2 genomic DNA using the three double digests (*Mse*I/*Hpa*II, *Eco*RV/*Hpa*II, and *Eco*RV/*Cfo*I) (Fig. 2B, lane 4; Fig. 2C, lanes 5 and 8), although single digestion with the methylation-insensitive enzymes *Mse*I and *Eco*RV allowed an easy detection of hybridizing fragments (Fig. 2B, lane 3; Fig. 2C, lane 2). Very small DNA fragments were probably produced in the double digests, demonstrating that the *Hpa*II sites in the *nptII* gene of locus 2 were free of methylation. Taken together, the results allowed us to conclude that primarily the 3′ half of the *nptII* coding sequence and the downstream sequences up to the center of the inverted repeat in locus 1 showed extensive cytosine methylation (Fig. 3A), whereas non-silenced *nptII* genes are not methylated.

3.2. *In trans methylation induced by the posttranscriptionally silenced locus 1*

We further investigated in *trans* methylation by locus 1 of two homologous transgene loci, locus 2 and locus B, in the hybrids HElo1/HElo2 and HElo1/HEloB. Locus 2 harbors one GVCHS(287) T-DNA identical to those present in locus 1 (Fig. 1A) and gives rise to high *nptII* mRNA and NPTII protein levels (ca. 1000 ng NTPII/ng total soluble protein). The highly *nptII*-expressing transgene locus B (ca. 2000 ng NPTII/mg total soluble protein) contains three tandemly organized GVCHS(320) T-DNAs of which the *nptII* chimeric gene contains a slightly longer 3′*chs* sequence and additional downstream sequences [19]. When locus 1 is combined with either of these high expressing loci, it is able to downregulate very efficiently their *nptII* expression by PTGS activated by locus 1 resulting in approximately 10 ng NPTII/mg total soluble protein in the hybrid tissue.

Southern blot analysis of *Bam*HI-digested genomic DNA of HElo1/HElo2 and of the non-silenced control HElo2 was performed. HElo2 showed a 2.5-kb hybridizing DNA fragment, whereas the hybrid HElo1/HElo2 plant had four hybridizing

DNA fragments (2.5 kb, 3.7 kb, 6.2 kb, and 12 kb) (Fig. 1A,B, lanes 1 and 3), of which the 12-kb band was unique for the hybrid. The 2.5-kb, 3.7-kb, and 6.2-kb fragments were also visualized by using genomic DNA of HElo1. Therefore, the 12-kb hybridizing band originated most probably from locus 2 that contained a methylated *Bam*HI restriction site in the 3′ UTR of the *nptII* transgene. Digestion of the genomic DNA of HElo1/HElo2 with the methylation-insensitive *Eco*RV enzyme resulted in two hybridizing fragments of 3.0 kb and 1.8 kb, originating from locus 1 and locus 2, respectively (Fig. 2A,C, lanes 1–3). Using *Eco*RV in combination with methylation-sensitive enzymes allowed us to determine the methylation status of locus 2 in the hybrid by tracing digestion of the 1.8-kb band. *Eco*RV/*Bam*HI digestion of HElo1/HElo2 genomic DNA confirmed that in the 3′ UTR the *Bam*HI site located in the *nptII* gene of locus 2 was partially methylated (Fig. 2D, lane 6). Because *Sma*I was unable to digest the 1.8-kb *Eco*RV fragment in the genomic DNA of HElo1/HElo2, while it was completely cut in that of HElo2, we could conclude that the 3′ UTR-located *Sma*I recognition sequence showed 100% methylation in locus 2 of the hybrid plant (Fig. 2A,D, lanes 9 and 8, respectively).

In contrast, the *Pst*I recognition sequence located 5′ in the *nptII* gene was completely cut in locus 2 using the genomic DNA of both HElo2 and HElo1/HElo2 (Fig. 2A,D, lanes 11 and 12, respectively). Digestion by *Hpa*II and *Cfo*I of the 1.8-kb band genomic DNA of HElo1/HElo2 was obvious because of its absence, but was incomplete because larger hybridizing bands were obtained than in identical analysis of HElo2 genomic DNA (Fig. 2C, lanes 6 and 9 and lanes 5 and 8, respectively). The size of these hybridizing bands indicated that the first *Hpa*II site downstream of the stop codon of *nptII* was methylated, which also holds for the two *Cfo*I sites upstream of the stop codon (Fig. 3B). Closer inspection of the intensity and size of the hybridizing bands of locus 1 in the double digests of the genomic DNA of HElo1/HElo2 revealed that the recognition sequences of methylation-sensitive enzymes were less efficiently cleaved in the hybrid than in HElo1 (compare lanes 6 and 2 in Fig. 2B, lanes 6 and 4, and lanes 9 and 7 in Fig. 2C, and lanes 12 and 10 in Fig. 2D). This result suggests that locus 1 became more extensively methylated in the presence of locus 2.

To assay in *trans* methylation of the highly homologous locus B that was silenced by locus 1, Southern blot analysis was performed of *Eco*RV/*Bam*HI double-digested DNA of HEloB and HElo1/HEloB, by using a probe which only hybridizes with locus B (fragment 2). Two hybridizing DNA fragments of 0.8 kb and 1.0 kb were detected for HEloB, whereas hybridizing DNA fragments of higher molecular mass were found for the hybrid HElo1/HEloB (Fig. 1A, locus B and Fig. 1C, lanes 1 and 2). The 1.0-kb hybridizing DNA fragment was only weakly visualized while the 1.8-kb and 2.7-kb fragments were very prominent, suggesting very extensive in *trans* methylation in the 3′ region of the *nptII* genes of locus B in HElo1/HEloB. No hybridizing DNA fragment was observed for *Bam*HI/*Eco*RV double-digested DNA of HElo1, because of the lack of homology between locus 1 and the probe used (Fig. 1C, lane 3). In conclusion, when locus 2 and locus B were combined with locus 1, the *nptII* transgenes in the in *trans*-silenced loci become methylated. Thus, locus 1 could not only reduce dramatically expression of ectopically located *nptII* transgenes in *trans*, but also impose its methylation status upon the in *trans*-silenced transgenes.

4. Discussion

In the parental tobacco transformant GVCHS(287)-1, carrying both the inverted repeat locus 1 and the single T-DNA copy locus 2, the posttranscriptionally silenced *nptII* transgenes show extensive methylation of the 3′ end-located *Bam*HI restriction site (data not shown). Upon segregation, locus 2 regains normal *nptII* expression and loses *Bam*HI methylation immediately, while silencer locus 1, which is responsible for the silencing phenotype, remains methylated. Combining locus 1 and locus 2 again by crossing results in efficient in *trans* silencing and remethylation of locus 2 in the hybrid. Furthermore, the highly homologous *nptII* genes of locus B are also methylated upon silencing by locus 1. Detailed methylation analyses were performed of the silencer locus 1 and of the non-silencer loci 2 and B in the absence or presence of locus 1. Locus 1 turned out to carry *nptII* genes that are extensively methylated in the 3′ half of the coding sequence, in the 3′*chs* signalling sequence, and downstream in the center of the palindromic sequence (Fig. 3A). The hypomethylated non-silencer loci 2 and B become methylated in the presence of locus 1; methylation is pronounced in the 3′ region of these in *trans*-silenced *nptII* genes. Moreover, the data indicate that in hybrid plants also locus 1 becomes more densely methylated. A first major question concerns the signal that triggers methylation of locus 1. It has been proposed that integrated DNA may acquire methylation patterns of neighboring genomic sequences [34]. However, in the studied case we consider this possibility improbable because the regions of the transgene locus juxtaposed to plant DNA are not methylated. It is more plausible that the palindromic sequence organization in the inverted repeat locus 1 may act as an intrinsic signal for de novo methylation as suggested by Stam et al. [35], because mainly the center of the palindrome is extensively methylated. Also Luff et al. [36] reported that an inverted repeat structure per se provides methylation signals. In the third possibility, the convergent transcription of the *nptII* transgenes, driven by the strong constitutive p35S promoter, would rather induce methylation. Such transcription-coupled methylation could be caused by pausing of the transcriptional machinery because of transcriptional interference resulting in a more extended transient formation of single-stranded DNA, which is known to be an excellent substrate for de novo methylation [37]. In a fourth possibility, methylation is triggered by RNA, as first shown by Wassenegger et al. [28]. The inverted repeat locus 1 might produce aberrant *nptII* transcripts, such as readthrough transcripts, because of improper 3′ processing. These aberrant self-complementary transcripts might trigger cytosine methylation. The different possible pathways are not mutually exclusive; it is even tempting to hypothesize that such an extensive methylation is very probably caused by a combination of factors. Another puzzling question is how homologous sequences silenced in *trans* become methylated. The observation of in *trans* methylation suggests nuclear cross-talk between posttranscriptionally silenced *nptII* transgenes. But, how this cross-talk is accomplished is unclear. The sequence specificity suggests that nucleic acids are involved. DNA–DNA as well as DNA–RNA interactions have been

shown to be implicated in triggering methylation of genomic DNA [24,28].

In our previous study, the dynamics of in *trans* methylation of posttranscriptionally silenced *nptII* transgenes suggested that an RNA signal rather than DNA–DNA pairing enables communication between ectopically located *nptII* transgenes [19]. In analogy, Mette et al. [30] described a transgene locus that silences and methylates homologous promoter sequences in *trans* via the production of aberrant transcripts. These findings strongly indicate that an RNA signal mediates the cross-talk between silencing and in *trans*-silenced transgenes in the nucleus. The nature of RNA signalling molecules, which direct genomic DNA methylation, is not known. The results of Mette et al. [30] indicate that only RNAs with special features can mediate the cross-talk. The most probable feature involved is the presence of hairpin structures based on the transcription of inverted repeats. Our results conform with the observations of Mette et al. [30] because locus 1 is a very good candidate for the production of transcripts with hairpin structures. Originally, Dougherty and Parks [38] and Wassenegger and Pélissier [39] proposed that small RNAs complementary to the degraded transcripts are not only involved in triggering degradation of sense transcripts, but also play a role in directing methylation of genomic DNA when they are in excess. On the other hand, Stam et al. [35] ascribe in *trans* methylation to DNA–DNA interactions. This proposal is based on the result that promoterless silencing loci, which probably do not produce RNA, but always contain multiple transgenes in inverted orientation [7], can direct in *trans* methylation of homologous single-copy transgene loci. However, their methylated transgenes are transcriptionally silenced and methylation is not erased after segregation of the methylation-inducing locus. Therefore, cross-talk between (trans)genes might occur at different levels and in different ways, depending on the nature of the signalling sequence and on the level at which transgene expression is hindered.

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