Introduction

Both transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) are associated with DNA methylation, often induced by small interfering RNAs (siRNAs) in a process called RNA-directed DNA methylation (RdDM). RdDM coincides with either TGS, where promoter methylation inactivates transcription, or PTGS, which merely affects transcript stability and/or the translation rate and where cytosines of the transcribed region are methylated. Despite these differences, both TGS and PTGS epialleles exhibit euchromatic (H3K4me3 and H3K9ac) histone modifications despite heavy DNA methylation in the promoter and transcribed region, respectively. However, in the TGS locus (271), abundant heterochromatic H3K9me2 marks and DNA methylation were present on P35S. Heterochromatic histone modifications are not automatically installed on transcriptionally silenced loci in tobacco, suggesting that repressive histone marks and cytosine methylation may be uncoupled. However, transient loss of euchromatic modifications may guide de novo DNA methylation leading to formation of stable repressed epialleles with recovered euchromatic marks. Compilation of available data on epigenetic modification of inactivated P35S in different systems is provided.

In plants, silencing is usually accompanied by DNA methylation and heterochromatic histone marks. We studied these epigenetic modifications in different epialleles of 35S promoter (P35S)-driven tobacco transgenes. In locus 1, the T-DNA was organized as an inverted repeat, and the residing neomycin phosphotransferase II reporter gene (P35S-nptII) was silenced at the posttranscriptional (PTGS) level. Transcriptionally silenced (TGS) epialleles were generated by trans-acting RNA signals in hybrids or in a callus culture. PTGS to TGS conversion in callus culture was accompanied by loss of the euchromatic H3K4me3 mark in the transcribed region of locus 1, but this change was not transmitted to the regenerated plants from these calli. In contrast, cytosine methylation that spread from the transcribed region into the promoter was maintained in regenerants. Also, the TGS epialleles generated by trans-acting siRNAs did not change their active histone modifications. Thus, both TGS and PTGS epialleles exhibit euchromatic (H3K4me3 and H3K9ac) histone modifications despite heavy DNA methylation in the promoter and transcribed region, respectively. However, in the TGS locus (271), abundant heterochromatic H3K9me2 marks and DNA methylation were present on P35S. Heterochromatic histone modifications are not automatically installed on transcriptionally silenced loci in tobacco, suggesting that repressive histone marks and cytosine methylation may be uncoupled. However, transient loss of euchromatic modifications may guide de novo DNA methylation leading to formation of stable repressed epialleles with recovered euchromatic marks. Compilation of available data on epigenetic modification of inactivated P35S in different systems is provided.
of P35S has been correlated with its increased DNA methylation, repressive histone marks and production of siRNAs (Table 1). All three characteristics seem to contribute to silencing, although each can operate in different phases, at various magnitudes and in diverse silencing systems. Posttranscriptional silencing of P35S-linked genes was associated with increased DNA methylation of transcribed regions, whereas deposition of heterochromatic histone marks was not reported. Conversely, TGS is differences in the setting and function of individual epigenetic tools.

The cauliflower mosaic virus 35S promoter (P35S) is the most widely used promoter for driving plant transgenes in both basic research and biotechnologies. Despite numerous studies showing epigenetic silencing of linked genes either at the transcriptional or posttranscriptional levels, the mechanisms of P35S inactivation are not fully understood. The epigenetic inactivation of P35S has been correlated with its increased DNA methylation, repressive histone marks and production of siRNAs (Table 1). All three characteristics seem to contribute to silencing, although each can operate in different phases, at various magnitudes and in diverse silencing systems. Posttranscriptional silencing of P35S-linked genes was associated with increased DNA methylation of transcribed regions, whereas deposition of heterochromatic histone marks was not reported. Conversely, TGS is
accompanied by DNA hypermethylation, H3K9 dimethylation and overall histone deacetylation of the promoter region.\textsuperscript{22-26} Application of epigenetic inhibitors resulted in increased expression of silenced loci in most,\textsuperscript{9,27,28} but not all, cases of silenced loci.\textsuperscript{23} Generally, TGS seems to be more sensitive to chromatin factor deficiencies than PTGS, although recent reports have suggested that certain histone modifications may function in PTGS as well.\textsuperscript{29} Despite numerous transgenic lines are available, histone modifications on epigenetically inactivated 35S promoters have not been studied yet in tobacco or related Petunia species (both Solanaceae).

Phenotypic variation known to occur in callus culture and regenerated plants (termed somaclonal variation) is likely to have a molecular background and involves an epigenetic modifications of chromatin.\textsuperscript{30} Aberrant promoter hypermethylation seems to be a ubiquitous feature of both animal and plant\textsuperscript{31} cell cultures. By contrast, some repeated sequences within the heterochromatin tend to lose heterochromatic marks in cell cultures.\textsuperscript{32} Alteration of spatial organization of chromosome territories has been noted in cytogenetic studies.\textsuperscript{33,34} Additionally, the silencing potential of hairpin constructs seems to be less efficient in calli than in the differentiated leaf.\textsuperscript{35} Although cell culture-induced epialleles do not necessarily persist in regenerated plants,\textsuperscript{33,36} there are several examples of their transmission to regenerated plants and even transgeneration inheritance.\textsuperscript{30,37-40} Alterations of DNA methylation patterns seem to be the most stable modification, probably due to the inheritance of symmetrical CG motifs.\textsuperscript{41,42} In previous reports, we characterized epiallelic variants of tobacco PTGS transgenic locus 1 that arose at high frequency among cell culture regenerants.\textsuperscript{43} The meiotically stable TGS variant (locus 1E) maintained inactive hypermethylated P35S over generations without detectable siRNA signals.

Epialleles represent an excellent system to study the correlation of chromatin modification with the expression state and inheritance of the silencing. Here, we studied chromatin histone marks imposed on tobacco transgene loci during the PTGS to TGS conversion induced by RNA signals or arising spontaneously during dedifferentiation of cells. Using chromatin immunoprecipitation (ChIP), we analyzed the distribution of histone marks along different regions of transgenes, addressing the relationships between expression activity, DNA methylation and histone modification.

**Results**

**Organization of transgenic loci and experimental set up.** Locus 1 (Lo1; Fig. 1) and locus 2 (Lo2) were described in detail previously.\textsuperscript{19} T-DNA contain the neomycin phosphotransferase II reporter transgene driven by the 35S promoter (P35S:nptII) together with a non-silenced hygromycine resistance gene (\textit{hpt}) close to the left border under the control of nopaline synthase promoter (Pnos) lying about 1 kb upstream of the P35S.\textsuperscript{18} Expression of the \textit{nptII} gene in Lo1 is silenced at the posttranscriptional level, DNA methylation occurs primarily at the 3’ end region and the \textit{nptII}-specific 21-to 25-nt siRNAs are able to induce silencing and methylation of unlinked homologous loci. The \textit{nptII} gene is actively expressed and non-methylated in Lo2.\textsuperscript{44} The homologous transgenic locus 271 consists of the complex insertion of six to seven copies of the tobacco nitrite reductase (NiR) sequence in an antisense orientation (RiN) driven by two 35S promoters and the \textit{nptII} transgene driven by a CaMV 19S promoter (Fig. 1). This 271 locus was shown to effectively silence in trans all
transgenes driven by 19S and 35S promoters at the transcriptional level and silence the endogenous nitrite reductase gene at the posttranscriptional level. These homologous interactions were mediated by small RNA molecules.

The outline of epiallele generation is schematically depicted in Figure 2 and involves (1) callus culture of PTGS-Lo1 line establishment, callus cultivation for 1 y and plant regeneration; and (2) two generations of hybrids (F1, F2) carrying the silencer locus 271 together with locus 1 were crossed with non-transgenic tobacco plants to obtain segregants (Lo1*S1 F2, Lo1*S2 F3) or negligible (Lo1*S1 F3) transcription.

The silencer locus 271 failed to show substantial nptII expression, indicating TGS silencing of a P19S-driven nptII gene consistent with previous results.

**DNA methylation analysis.** To study methylation changes accompanying formation of TGS epialleles, we performed bisulfite sequencing of locus 1. The sequenced region comprised the entire 35S promoter (344-bp) and the 347-bp subregion of the nptII 5' end (Fig. 4).

The promoter region of PTGS-Lo1 in leaf DNA was non-methylated whereas low level of methylation occurring at the nptII 5' region was limited to the CG context cytosines (Fig. 4C). Methylation of promoter sequences slightly increased in 1-mo-old microcalli, where in several individual clones a few non-symmetrical cytosines became methylated. DNA methylation gradually increased during callus propagation and in 1-y-old calli, approximately 30–40% of cytosines in P35S and approximately 40% of cytosines at the nptII 5' end were methylated (Fig. 4B). The de novo methylation in calli was accompanied by an increase of clone to clone variability in accordance with previous results. Compared with the callus, a regenerated TGS-Lo1E plant showed increased methylation of the promoter (~50%) and the nptII 5' end (80–90%), whereas the clone-to-clone variability decreased compared with the callus.

The in trans silencing activity of the silencing locus 271 is connected with methylation of target promoters. As shown previously, promoters of PTGS-Lo1 were non-methylated (Fig. 4). Bisulfite sequencing of locus 1* revealed the inheritance of cytosine methylation in CG and non-CG motifs in...
is sensitive to the cytosine methylation in nonsymmetrical CHH context. Two recognition Sau96I sites (GGNCC) are found within the P35 promoter (Fig. S2B). The PTGS plants showed a 0.91 kb Sau96I band corresponding to a non-methylated variant while in TGS variants and callus there was an additional 1.14 kb band representing methylated molecules. Methylation of nonsymmetrical sites in the 3’ region was analyzed using BamHI (GGATCC) and NcoI (CCATGG) restriction enzymes (Fig. S2C and D). While the PTGS locus 1 and TGS-Lo1* segregants displayed high level of CHH methylation, there was slight

both generations of segregants from Lo1 271 hybrids (Lo1*S1 F2 and Lo1*S1 F3) and their progenies (Lo1*S2 F3). Interestingly, significant de novo methylation also appeared in the 5’ nptII region that was not targeted by silencing siRNAs. Contrast to locus 1* segregants, locus 2* segregants from Lo2 271 hybrids have completely lost methylation and regained expression (Fig. S1).

To validate bisulfite results we inspected methylation of restriction sites using Southern blot hybridization. The methylation status of the P35S promoter was analyzed by Sau96I enzyme, which

Figure 4. Detailed bisulfite methylation analysis of epiallelic variants. (A) Transgene subregions subjected to bisulfite sequencing. (B) Column graphs showing average methylation levels from 5–10 clones. Error bars represent clones with maximum and minimum level of DNA methylation. (C) Distribution of mC along the sequenced fragment. Individual vertical columns represent the average methylation at particular position. Positions of the as-1 regulatory element and Sau96I restriction sites (asterisks) are indicated.
together with positive-acting H3K36m3 modifications were analyzed in parental plant and derived calli (Fig. S3A). No significant chromatin enrichment was obtained using these antibodies. The immunoprecipitation profiles of the leaf and callus were similar to results from an independently established callus (Fig. S3A).

Paramutated locus 1* epialleles: The Lo1*S2 F3 line showed enrichment after chromatin precipitation with euchromatic H3K9ac and H3K4me3 antibodies in both subregions of the transgene (Figs. 5 and 6).

Locus 271: The anti-H3K9me2 immunoprecipitated to P35S sequences of locus 271 produced a positive signal. Conversely, no immunoprecipitation signal was obtained from the anti-H3K9ac and anti-H3K4me3 samples. The nptII coding region showed intermediate H3K9ac, H3K4me3 and H3K9me2 marks.

Controls: No amplified product was obtained after immunoprecipitation with normal mouse IgG. The endogenous family of 5S rRNA genes was immunoprecipitated with anti-H3K9me2 but not H3K4me3 (Fig. S3C) consistent with their heterochromatic nature and heavy DNA methylation. Actin genes showed intermediate signals with both euchromatic and heterochromatic signals, whereas in Arabidopsis, actin genes were strictly euchromatic. The intermediate marks could be explained by the presence of pseudogenes and/or inactivated gene copies in allotetraploid tobacco nuclei. Homologous alleles inherited from both parents may be differentially imprinted in tobacco.

Discussion

Strict positive-acting histone marks at a PTGS locus despite coding region methylation. During PTGS of the nptII gene in locus 1, dense DNA methylation appeared to be restricted mostly at the 3’ transcribed region. Consistent with this assumption, the 3’ transcribed region was extensively methylated at both CG and non-CG motifs. By contrast, the 5’ end of nptII was not markedly methylated at non-CG motifs, whereas some CG sites were abundantly methylated (Fig. 4C). This pattern resembles typical gene body methylation observed in endogenous genes that is only exceptionally correlated with silencing. Here we show that in the nptII transcribed region, only positively acting histone marks have been found, suggesting that the nptII gene-specific siRNAs cannot alter the euchromatic status. Similarly, RdDM induced during a viroid RNA infection did not increase dimethylation of lysine H3K9 or decrease acetylation of H3 in...
heterochromatic H3K9me2 marks were found in locus 271, arguing that P35S is not refractory to heterochromatic histone modifications in tobacco. There may be several explanations. Locus 271 has a complex organization composed of several complete and incomplete T-DNA copies, whereas locus 1 is composed of two inverted complete T-DNA copies, each 5 kb in length. Perhaps heterochromatic histone modifications are more often connected with a multicopy character of the sequences rather than with the expression status (silencing). Another distinction between locus 1 and locus 271 is their capacity to produce siRNAs: locus 271 produces high levels of P35S-specific 24 nt siRNAs, while these are not detectable in TGS locus 1 epialleles. Although RNA signals seem to rarely induce H3K9me2 in plants, we cannot exclude the possibility that the deposition of H3K9me2 could be stimulated by siRNA signals in locus 271. In Arabidopsis, H3K9me2 and CHG DNA methylation are tightly interconnected, creating a self-reinforcing loop. It was surprising that the inactive P35S completely lacked dimethylated H3K9 despite methylation of the CHG motifs. It seems that the interplay between H3K9me2 and non-CG methylation may work at the genome-wide level, although there may be significant exceptions at the local level. For example, complex chromatin variants may influence transgene activity, comparisons of chromatin patterns between different loci may be difficult. Epiallelic variants...

Repressive histone marks occur in some but not all TGS epialleles. TGS is usually accompanied by heterochromatic histone marks and cytosine methylation (reviewed in refs. 6, 62 and 63). However, our findings indicate that epigenetic inactivation of the 35S promoter was not associated with repressive H3K9me2 histone marks in locus 1 epialleles. Instead, euchromatic H3K9 acetylation and H3K4 trimethylation were typically present on both active and inactive 35S promoters. However, abundant...
of transgene loci represent an excellent system to study the relationship between gene silencing and individual epigenetic marks. Table 1 shows P35S epigenetic variants of reported in different systems. Although dense CG and non-CG methylation always accompany P35S inactivation, other repressive marks, such as dimethylation of H3K9, demethylation of H3K4, H3K9 deacetylation and silencing siRNAs, are more variable in attributes and are even dispensable for TGS, at least in the maintenance phase. In many cases including TGS epialleles of locus 1, non-symmetrical methylation was not accompanied with detectable amounts of siRNAs. The question arises regarding how the inheritance of non-CG methylation can be explained if neither heterochromatic histones nor RNA signals are present. One possibility is that specific protein complexes binding to the enhancer recruit histone marks nor RNA signals are present. One possibility is that specific protein complexes binding to the enhancer recruit non-CG methylation.

The changes were highly localized to the 3′ transcribed region, whereas histone modification patterns at the promoter remained unaffected. The developmentally regulated H3K4 trimethylation was accompanied by partial gain of H3K9me2. The changes were highly localized to the 3′ transcribed region, whereas histone modification patterns at the promoter remained unaffected. The developmentally regulated H3K27 methylation was not detectable in locus 1 epialleles, confirming the presence of nucleosomes with ambivalent marks. Indeed, callus transgene chromatin completely lost H3K4me3 and retained high levels of H3K9ac (supporting the existence of nucleosomes with ambivalent marks).

Table 1

| Epigenetic Mark | P35S Epialleles | Locus 1 Epialleles | Locus 2 Epialleles |
|----------------|----------------|-------------------|-------------------|
| H3K4me3        | Reduced        | Absent            | Increased         |
| H3K9ac         | Increased      | Absent            | Increased         |
| H3K9me2        | Decreased      | Absent            | Absent            |

![Figure 1](https://example.com/figure1.png)

**Materials and Methods**

**Plant material, hybridization and callus culture conditions.** All transgenic tobacco (Nicotiana tabacum) SR1 plants were generated by Agrobacterium-mediated transformation. The plants hemizygous for the PTGS locus 1 (HeLo1; Fig. 1) were obtained by crossing a plant homozygous for locus 1 with an untransformed SR1 tobacco. The line hemizygous for the TGS locus 1E was obtained by plant regeneration from long-term HeLo1 callus cultures. Seeds of the tobacco transgenic plants homozygous for the transgenic locus 271 (Fig. 1) were obtained from INRA Versailles (a gift of Dr Hervé Vaucheret). All crosses were performed by emasculating flowers manually before they opened and applying pollen to the stamen. 271 Lol F1 hybrids were obtained by crosses of HoLo1 to 271; the experimental strategy to obtain hybrids and relevant segregants is depicted in Figure 2. Hybrid plants and segregants were genotyped by DNA gel blot hybridization.

Calli were established from leaf explants by hormonal treatment and grown in 0.7% agar containing B5 salts supplemented with sucrose (30 g l−1), α-naphthaleneacetic acid (2.0 mg l−1) and 6-benzylaminopurine (0.2 mg l−1). Calli were transferred onto fresh agar medium every 30 d. To obtain regenerated plants, calli were transferred onto shoot-inducing medium (for 1 mo) containing α-naphthaleneacetic acid (0.2 mg l−1) and 6-benzylaminopurine (2.0 mg l−1). After the rooting phase on growth medium without hormones, the plantlets were transferred into greenhouse conditions.

**DNA isolation and bisulfite genomic sequencing.** Total genomic DNA was isolated from lyophlized leaves or calli using the cetyltrimethylamonium bromide method as described previously.

Bisulfite treatments were performed on purified genomic DNA using the EpiTect bisulfite kit (QUAEG). Primers for amplification of the 35S promoter and 5′ nptII region are as follows: forward primer: 5’-CAT TAC ACC CAT AAT AAA TAC TTT CTC-3’; the first reverse primer: 5’-GAA TAG AGA GAA AGA TAT ATT TTT TAA GAT-3’; and the second reverse primer: 5’-GTA ATA GAG ATT GGA GTT TTT TAA AAA GTA G-3’. The forward primer matched the nptII coding sequence at about +410 (with respect to transcription start site). The reverse primers were located in a vector sequence at about −660 and −570, respectively. The PCR program consisted of 2 min of initial denaturation at 94°C, followed by 35 cycles of 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min for 30 cycles and a final extension at 72°C for 7 min.

**DNAsequencing.** The amplified DNA fragments were cloned into pCR2.1-Ampicillin vector (Invitrogen) for sequencing. Each sample was sequenced with the primers used for PCR amplification.
0.5 min at 94°C, 1.5 min at 45°C and 1 min at 72°C. The program was ended with an extension step for 10 min at 72°C. The PCR products were cloned into a TA vector (pDrive, QIAGEN) and between 5 and 10 clones from each sample were sequenced (Eurofins MWG Operon). The data were processed, and the methylation density was calculated using CyMATE software.17

RNA isolation and quantitative reverse transcription (RT)-PCR analysis. Total RNA was isolated from young leaves or calli using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer’s instructions and treated with DNaseI (TURBO DNA-free, Applied Biosystems/Life Technologies).

The cDNAs were prepared by reverse transcription of RNAs using Superscript II reverse transcriptase (Invitrogen/Life Technologies) and random nonamers (Sigma). Quantification of the nptII level related to the actin transcripts was performed using the Fast Start SYBR Green Master (Roche) by the Rotorgene 6000 (QIAGEN). The nptII gene was amplified with the forward primer 5'-CGT TAC AAG AGA GAA ATC GCC-3' and the reverse primer 5'-TTC TGG ATT GAG CTT-3'; actin was amplified with the forward primer 5'-CGT TAC AAG AGA GAA ATC GCC-3' and the reverse primer 5'-CYC TCT TGG ATT GAG CCTT-3' in the same PCR cycle (initial denaturation at 94°C for 10 min followed by 35 cycles of 20 sec at 94°C, 20 sec at 56°C and 30 sec at 72°C). The amount of nptII transcript was determined for two to three plants/calli of each line in several technical replicates.

Chromatin immunoprecipitation (ChIP). Immunoprecipitation of chromatin was performed using the EpiQuik™ Plant ChIP kit (Epigentek) according to the manufacturer’s instructions.

One gram of leaves or calli was cross-linked, and the isolated DNA was sonicated (6 × 10 sec; power setting 1) using the Branson Sonifier B-12 sonicator (Branson Sonic Power Company). 5'mlated DNA was sonicated (6 × 10 sec; power setting 1) using the Branson Sonifier B-12 sonicator (Branson Sonic Power Company) before the ChIP experiment was performed in two biological replicates.

Southern blot hybridization. The standard procedures involving DNA isolation, restriction enzyme treatments and Southern hybridization were described previously2,6,18 with the exception that the blots were washed under medium stringency conditions (2 × SSC, 55°C, 2 × 5 min and 0.6 × SSC, 55°C, 2 × 15 min). The nptII-coding sequence and the 35S promoter probes were prepared from the -830-bp and -980-bp inserts of the pGEM-nptII and pGSJ290 plasmids, respectively.19 The hybridization bands were visualized with a PhosphorImager Typhoon (GE Healthcare) and the data were processed with the ImageQuant software (GE Healthcare).

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/epigenetics/article/24613

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