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Epigenetic switches of tobacco transgenes associate with transient redistribution of histone marks in callus culture

Kateřina Křížová,[1] Ann Depicker[2,3] and Aleš Kovařík[1,*]

[1]Institute of Biophysics, Academy of Sciences; Královopolská, Brno, Czech Republic; [2]Department of Plant Systems Biology; VIB; Gent, Belgium; [3]Department of Plant Biotechnology and Bioinformatics; Ghent University; Gent, Belgium

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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-*npt*II) 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 eukaryotic marks. Compilation of available data on epigenetic modification of inactivated P35S in different systems is provided.

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,[1] or PTGS, which merely affects transcript stability and/or the translation rate and where cytosines of the transcribed region are methylated.[2,3] Despite these differences, both TGS and PTGS are mechanistically related and likely evolved from a common ancestral system.[4]

Histone amino acid modifications represent a layer of epigenetic information that is widespread in eukaryotes, including plants.[5] Repressive histone marks that are closely linked with transcriptional silencing include hypomethylation of H3K4 and dimethylation of H3K9.[6] By contrast, H3 and H4 acetylation usually mark active promoter states. At the cytogenetic level, H3K4 methylation marks actively transcribing regions (euchromatin), whereas H3K9 dimethylation associate with repressed genomic territories collectively called heterochromatin.[7] Genetic screens in Arabidopsis revealed several chromatin factors involved in RdDM, among which DNA methyltransferases and histone deacetylases were the most prominent.[8] Cooperation of DNA methylation, H3K9 dimethylation and several other factors were shown to be needed for stable robust TGS of transgenes[9] and endogenes[10] in Arabidopsis. DNA methylation and histone H3K9 dimethylation are also closely interconnected at the whole genome level.[11]

Although tobacco has long been a classical object of genetic studies, its histone modification patterns have been rarely studied. The tobacco SET [*Su(var)*, *E(z)* and *Trithorax* conserved] domain of protein NtSET1 binds chromatin and methylates H3K9.[12] Overexpression of NtSET1 causes defects in chromosome condensation/segregation in transgenic tobacco.[13] Transgenic tobacco plants expressing the Drosophila Polycomb (Pc) chromodomain show developmental abnormalities in leaves and flowers.[14] Recently, additional epigenetic genes have been cloned from *Nicotiana tabacum* including homolog of dicer DCL2 involved in small RNA biogenesis[15] and a family of DNA methytransferases.[16] These studies thus suggest that tobacco may use a similar epigenetic mechanism as the model organisms to control its developmental programs although there might be

*Correspondence to: Aleš Kovařík; Email: kovarik@ibp.cz
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Table 1. Summary of the P35S epilallelic variants reported in different systems and their molecular characteristics

Species	Locus	Epiallele	T-DNA copy	Epilalele generation	[1]Meiotic revers.	Type of silenc.	[2]P35S siRNAs	[3]DNA methyl.	Histone marks	Ref.
Nicotiana tabacum	[4]35S:*npt*II (Locus 1)		2			PTGS	-	Negligible (b.s.)	H3K9Ac, H3K4me3	44
		Locus 1E	2	Cell culture	No	TGS	-	Strong (b.s.)	H3K9ac, H3K4me	43,48
		Locus 1*	2	siRNA signals	No	TGS	-	Strong (b.s.)	H3K9Ac, H3K4me	46
	35S:*RIN* (Locus 271)		> 5			TGS	+	Strong (s.b.)	H3K9me2	26
		Locus 271d		Rearrangement, small deletion	No	active	-	Weak (s.b.)	n.d.	45
	35S:*hpt* Locus H		> 5				-	Weak (b.s.)	n.d.	76
		Locus H*		siRNA signals	Partial	TGS	-	Strong (b.s.)	n.d.	24
	35S:*npt*II (Locus 2)		1			active	-	Negligible (b.s.)	H3K9ac, H3K4me	44
		Locus 2*	1	siRNA signals	Yes	TGS	-	Strong (b.s.)	H3K9ac, H3K4me	46
Nicotiana benthamiana	35S-*GFP*		1			active	-	Negligible (s.b.)	n.d.	77
		35S-GFP*	1	siRNA signals (VIGS)	Partial	TGS	-	Strong (s.b.)	n.d.	77
Petunia hybrida	35S-*CHS*-A		2			PTGS		Negligible (s.b.)	n.d.	78
		35S-CHS-A*	2	siRNA signals (Inverted repeat)	n.d.	TGS	n.d.	Strong (s.b.)	n.d.	78
Petunia hybrida	[4]35S-*CHS*-A (C001)		2			PTGS	-	Weak (b.s.)	n.d.	28
		C002	2	Spontaneous conversion	No	TGS	-	Strong (b.s.)	n.d.	28
Gentian triflora x G. scabra	PS		1		n.d.	active	n.d.	Negligible (b.s.)	H3Kac, H3K4me3	23
		RS	1	Cell culture	n.d.	TGS	n.d.	Strong (b.s.)	H3K4me2, H3K9me2	23
Oryza stativa	35S:GFP (M65)		1			active	-	Negligible (b.s.)	H3ac, H4ac	22
		M65-Pi	1	siRNA signals	No	TGS	-	Strong (b.s.)	H3K9me2, H3K9me2	22

PTGS to TGS conversion occurred in two systems in which T-DNA was organized as an inverted repeat. [1]Meiotic stability of epiallele over generations following removal of a trigger factor (callus culture, siRNA). [2]siRNAs produced by a transgene locus and not by a hairpin trigger. [3]b.s., DNA methylation determined along the ~300 bp of P35S by bisulfite sequencing, levels: < 10%, negligible; 10–20%, weak; > 20%, strong; s.b., methylation determined by methylation-sensitive restriction enzymes in combination with Southern blot hybridization.

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.[17] 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,[18-20] whereas deposition of heterochromatic histone marks was not reported.[20,21] Conversely, TGS is

accompanied by DNA hypermethylation, H3K9 dimethylation and overall histone deacetylation of the promoter region.[22-26] Application of epigenetic inhibitors resulted in increased expression of silenced loci in most,[9,27,28] but not all, cases of silenced loci.[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.[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.[30] Aberrant promoter hypermethylation seems to be a ubiquitous feature of both animal and plant[31] cell cultures. By contrast, some repeated sequences within the heterochromatin tend to lose heterochromatic marks in cell cultures.[32] Alteration of spatial organization of chromosome territories has been noted in cytogenetic studies.[33,34] Additionally, the silencing potential of hairpin constructs seems to be less efficient in calli than in the differentiated leaf.[35] Although cell culture-induced epialleles do not necessarily persist in regenerated plants,[33,36] there are several examples of their transmission to regenerated plants and even transgeneration inheritance.[30,37-40] Alterations of DNA methylation patterns seem to be the most stable modification, probably due to the inheritance of symmetrical CG motifs.[41,42] In previous reports, we characterized epiallelic variants of tobacco PTGS transgenic locus 1 that arose at high frequency among cell culture regenerants.[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.

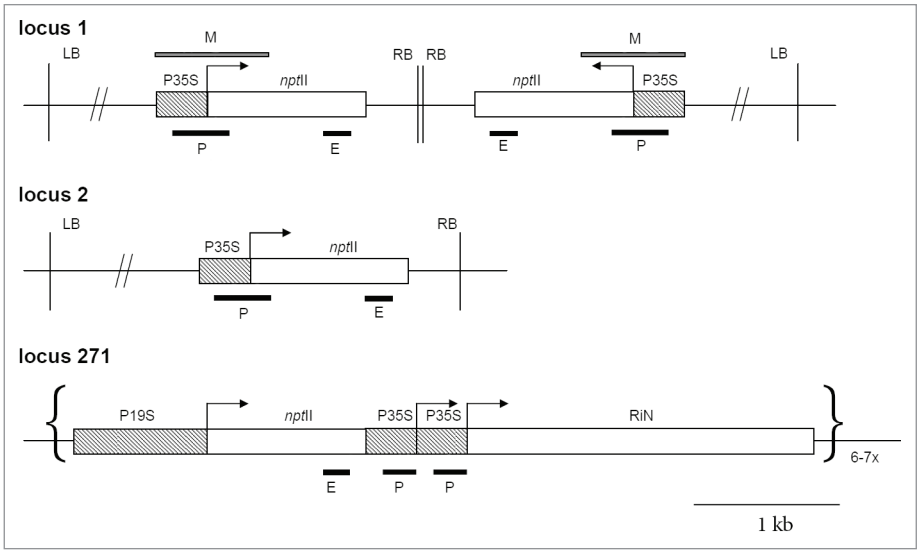

Figure 1. Schematic outline of T-DNA insertions in locus 1, locus 2 and locus 271. Locus 1 contains two copies of T-DNA that are arranged as an IR about the right border. Promoters are in hatched squares; P35S, CaMV 35S promoter; P19S, CAMV 19S promoter; *npt*II, *neomycin phosphotransferase* II gene; *RIN*, *nitrate reductase* gene in an antisense orientation. M bar indicates region analyzed by bisulfite genomic sequencing. P (promoter) and E (3' end) bars correspond to PCR fragments amplified after ChIP. RB, LB right and left border, respectively.

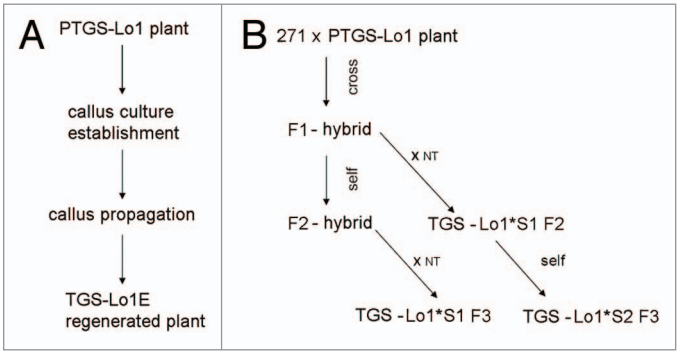

Figure 2. Schematic outline of P35S epialleles generation. (**A**) Epialleles generated spontaneously in callus culture. (**B**) Epialleles induced by siRNAs in 271 Locus 1 hybrids. S1, S2 represent plants of first or second generation after silencer locus segregation respectively. "x NT," cross to a non-transgenic tobacco.

Results

Organization of transgenic loci and experimental set up. Locus 1 (Lo1; **Fig. 1**) and locus 2 (Lo2) were described in detail previously.[19] T-DNA contain the *neomycin phosphotransferase* II reporter transgene driven by the 35S promoter (P35S:*npt*II) together with a non-silenced *hygromycine resistence* gene (*hpt*) close to the left border under the control of *nopaline synthase* promoter (Pnos) lying about 1 kb upstream of the P35S.[18] Expression of the *npt*II gene in Lo1 is silenced at the posttranscriptional level, DNA methylation occurs primarily at the 3' end region and the *npt*II-specific 21-to 25-nt siRNAs are able to induce silencing and methylation of unlinked homologous loci. The *npt*II gene is actively expressed and non-methylated in Lo2.[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 *npt*II transgene driven by a CaMV 19S promoter (**Fig. 1**). This 271 locus was shown to effectively silence in trans all

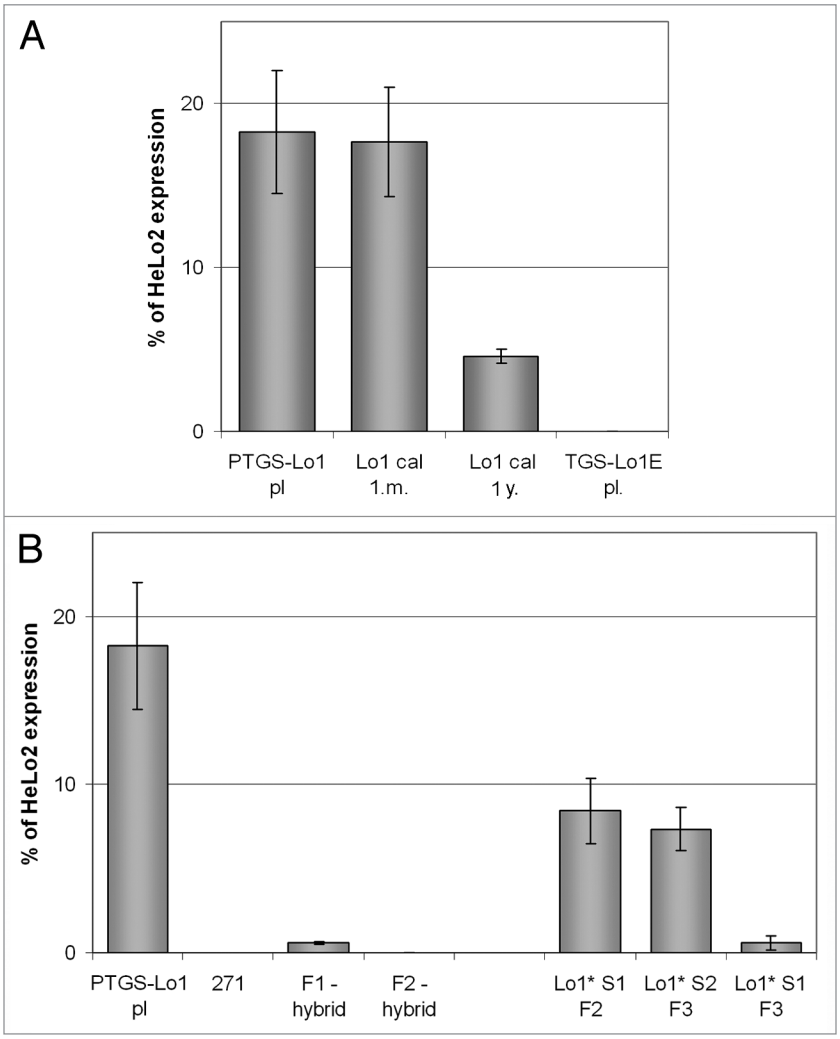

Figure 3. Expression analysis of the *npt*II reporter genes in parental plants and derived epiallelic variants. The *npt*II transcripts were analyzed by quantitative RT-PCR. (**A**) epialleles generated by callusogenesis. Results include data from two independent experiments (3 technical replicates); (**B**) transcription level of epialleles generated by siRNA signals. Values are expressed as percentages of the *npt*II levels in a non-silenced HeLo2 line.

transgenes driven by 19S and 35S promoters at the transcriptional level and silence the endogenous nitrite reductase gene at the posttranscriptional level.[24,26] These homologous interactions were mediated by small RNA molecules.[45]

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*S1 F3), containing only locus 1. Moreover, the first generation of segregants (Lo1*S1 F2) was selfed to obtain an additional generation without the silencer locus 271 (Lo1*S2 F3).[46]

Expression patterns of the *npt*II genes in epiallelic variants. We previously reported partial loss of in trans PTGS silencing by locus 1 on locus 2 target in hybrid line exposed to callus conditions.[47] To determine whether this is due to epigenetic changes at the silencer leading to TGS and the loss of the capacity to produce silencing RNA molecules, we now analyzed the *npt*II transgene expression level of locus 1 in a hemizygous Lo1 line, upon callus culture and upon regeneration. The parental leaf of PTGS-Lo1 plant and early callus had almost similar mRNA levels, corresponding to approximately 17% of the level of a non-silenced Lo2 plant line (**Fig. 3A**). By contrast, the 1-y old callus Lo1 had 4-fold lower *npt*II levels than the parental leaf, and virtually no transcription was found in a regenerated locus 1E plant. In conclusion, the epigenetic change imposed during callus culture lead to formation of epialleles with decreased expression of *npt*II genes.

The TGS epialleles of locus 1 were also generated by siRNA signals in 271 Lo1 hybrids. Expression patterns of two generations of hybrids (**Fig. 3B**, left panel) and segregating progenies (**Fig. 3B**, right panel) were shown in our prior results.[46] Briefly, the paramutated progeny segregating from the hybrids demonstrated reduced (Lo1*S1 F2, Lo1*S2 F3) or negligible (Lo1*S1 F3) transcription.

The silencer locus 271 failed to show substantial *npt*II expression, indicating TGS silencing of a P19S-driven *npt*II gene consistent with previous results.[26]

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 *npt*II 5' end (**Fig. 4**).

The promoter region of PTGS-Lo1 in leaf DNA was non-methylated whereas low level of methylation occurring at the *npt*II 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 *npt*II 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.[47] Compared with the callus, a regenerated TGS-Lo1E plant showed increased methylation of the promoter (~50%) and the *npt*II 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.[24] 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

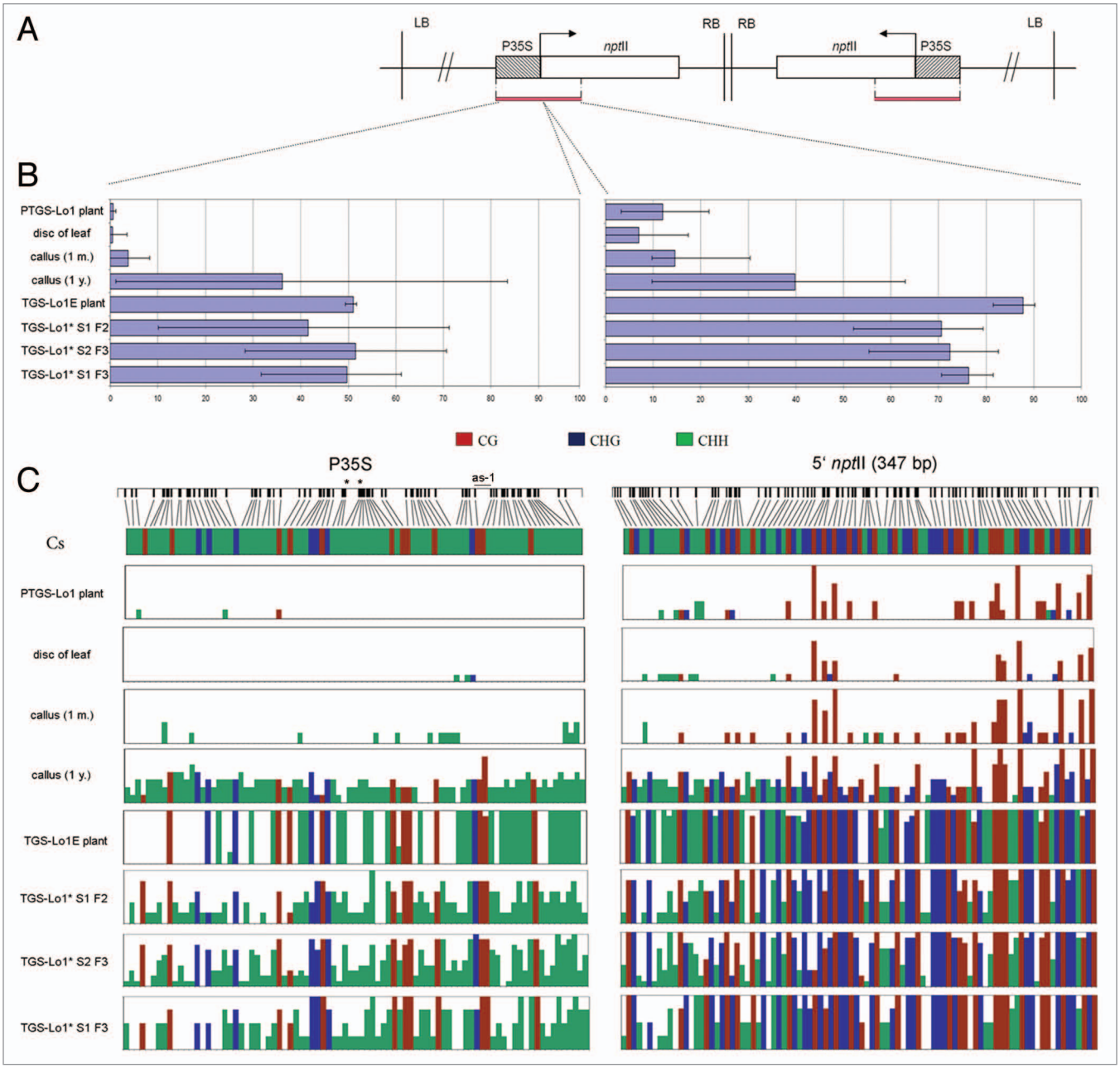

Figure 4. Detailed bisulfite methylation analysis of epiallelic variants. (**A**) Transgene subregions subjected to bisulfite sequencing. (**B**) Column graphs showing average methylation levels from 5–10 clones. Error bars represent clones with maximum and minimum level of DNA methylation. (**C**) Distribution 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.

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' *npt*II 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 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

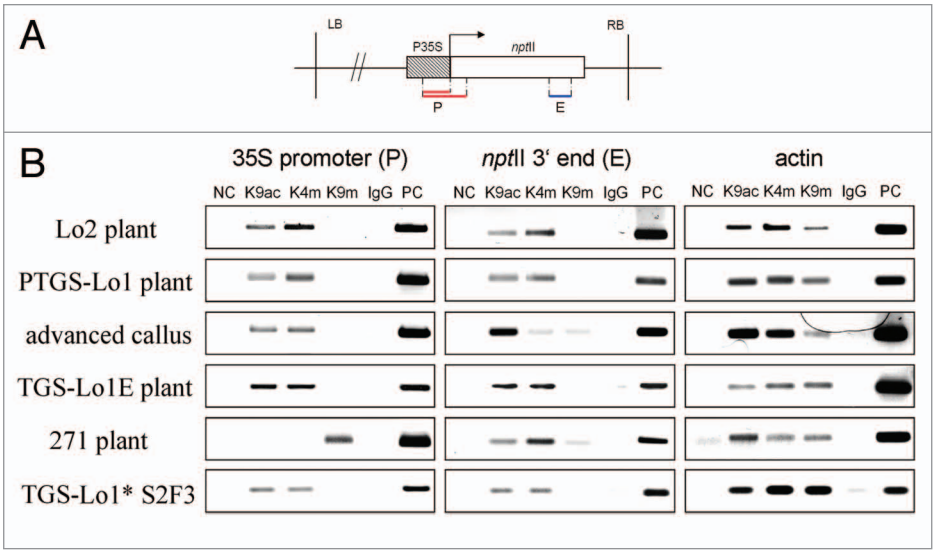

Figure 5. Histone modification patterns along the transgenes. (**A**) Schematic outline of subregions analyzed by ChIP: P (promoter) and E (3' end of *npt*II). The P subregion in locus 271 was shorter (upper line) than in loci 1 and 2 (bottom line). (**B**) Electrophoretic profiles of PCR products obtained by amplification of immunoprecipitated DNA. Results of a repeated experiment performed on an independent callus culture are shown in **Figure S3**.

reduction of methylation in some of its epialleles which may be explained by the absence of methylation-inducing siRNAs in TGS lines.[48] Nevertheless, considerable methylation of BamHI and NcoI sites was present in all locus 1 samples. In contrast, the sites in non-silenced locus 2 were unmethylated. Together the results of Southern blot and bisulfite analysis were in a good agreement.

Histone modifications in tobacco P35S-driven transgenes. We used ChIP analysis to monitor changes in histone modification during epiallelic conversion. Chromatin was immunoprecipitated with antibodies against euchromatic and heterochromatic histone marks. Two independently established calli were analyzed as biological replicates at the promoter (~349 bp, ~212 bp in locus 271) and at the 3' end of the *npt*II transcribed region (~174 bp) that appear to be critical for epiallele formation[49] (**Figs. 1 and 5A**). Both regions are separated by sequence length (~700 bp), allowing sufficient resolution of ChIP. Each immunoprecipitated DNA sample was amplified with P35S-, *npt*II- and actin-specific primers using conventional polymerase chain reaction (PCR; **Fig. 5**).

Histone modification changes during dedifferentiation: In a parental PTGS variant, only H3K9ac and H3K4me3 signals were visible in both the promoter and transcribed region (**Fig. 5B**). Similarly, both H3K9ac and H3K4me3 signals were visualized in a sample of TGS-Lo1 epialleles. The histone profiles in callus samples differed from those of parental and regenerated plants. The H3K4me3 was nearly lost from the *npt*II coding region, whereas faint H3K9me2 signals appeared (**Fig. 5B**). Semiquantitative evaluation of band intensity is shown in **Figure 6**. It is evident that H3K4me3 levels decreased in both analyzed calli compared with the parental leaf. Additionally, mostly repressive histone H3K27m1/K27m3 and H4K20m1 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 *npt*II coding region showed intermediate H3K9ac, H3K4me3 and H3K9me2 marks.

Locus 2: The non-silenced locus 2 carrying a similar T-DNA insertion as the silenced locus 1 showed stable euchromatic marks in both parental plant and derived callus (**Fig. 5; Fig. S3B**).

Controls: No amplified product was obtained after immunoprecipitation with normal mouse IgG. The endogenous family of 5S rRNA genes was immunoprecipited with anti H3K9me2 but not H3K4me3 (**Fig. S3C**) consistent with their heterochromatic nature[50] and heavy DNA methylation.[51] Actin genes showed intermediate signals with both euchromatic and heterochromatic signals, whereas in Arabidopsis, actin genes were strictly euchromatic.[52] The intermediate marks could be explained by the presence of pseudogenes and/or inactivated gene copies in allotetraploid tobacco nuclei.[53] 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 *npt*II gene in locus 1, dense DNA methylation appeared to be restricted mostly at the 3' transcribed region.[18,44,54] Consistent with this assumption, the 3' transcribed region was extensively methylated at both CG and non-CG motifs. By contrast, the 5' end of *npt*II 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.[55] Here we show that in the *npt*II transcribed region, only positively acting histone marks have been found, suggesting that the *npt*II 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

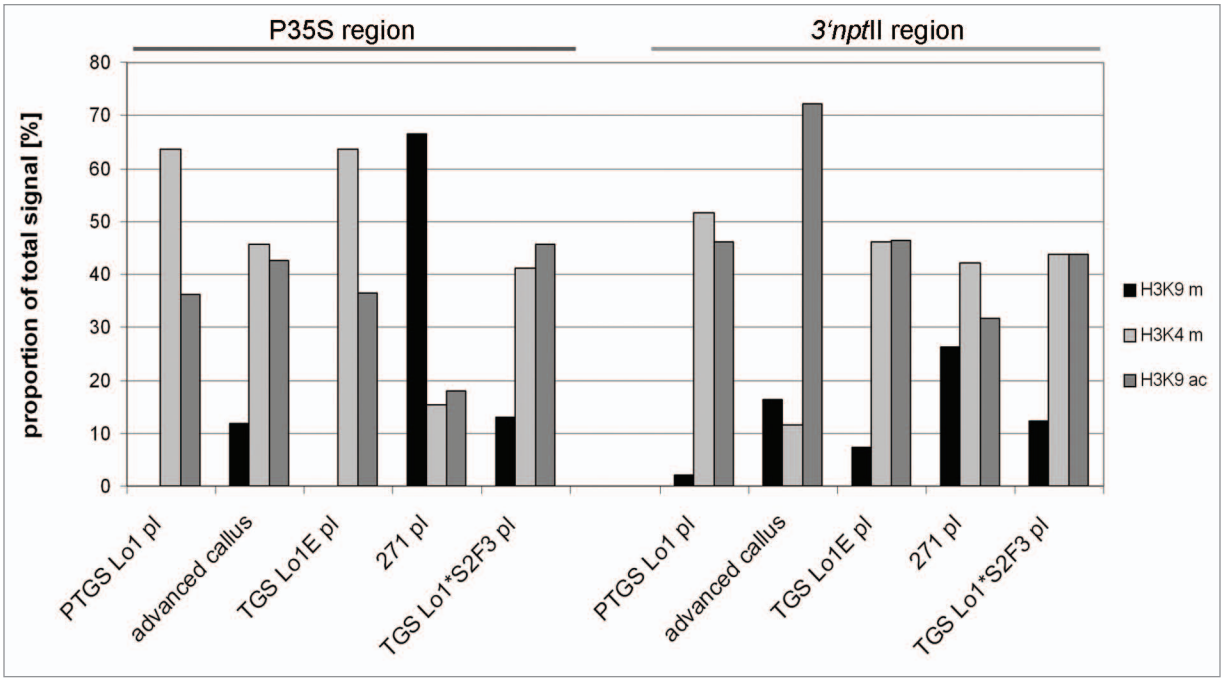

Figure 6. Semiquantitative evaluation of histone modification patterns along the transgenes. Fluorescent signals in gels (**Fig. 5**) were counted and the levels were expressed as percentages of total signal (a sum of H3K9me2, H3K4me3 and H3K9ac).

tobacco.[20] It is likely that siRNAs produced during the PTGS process methylate DNA but do not change the euchromatic histone environment.

Although methylation of non-CG motifs is only rarely found in coding regions,[56] a distinct class of endogenous genes seems to be regulated by this type of methylation.[57] In PTGS locus 1, we hypothesize that this type of methylation may have no or only a marginal effect on transcription at least under normal physiological conditions. First, nuclear run-on patterns of a non-silenced locus 2 and PTGS locus 1 were indistinguishable.[43] Second, treatment of cells with hypomethylation drugs showed only weak release of silencing.[58] Similarly, the inverted repeat of the PAI genes is transcribed despite heavy cytosine and H3K9 methylation.[52] The reason for why the 5' end is methylated primarily at CG, whereas the 3' end is methylated at both CG and non-CG, is not fully understood. The absence of siRNAs cannot account for these differences because experiments with transitive silencing showed that siRNAs are formed from both the central and 3' regions of the transgene.[59,60] In Arabidopsis, the IBM1 (increase in BONSAI methylation 1) factor was reported to actively remove non-CG methylation from coding regions.[61] One possibility is that the non-CG methylation is more actively removed from the 5' ends than from the 3' ends of the genes.

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 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,[26] whereas locus 1 is composed of two inverted complete T-DNA copies, each 5 kb in length.[44] 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,[45,46] while these are not detectable in TGS locus 1 epialleles.[46] Although RNA signals seem to rarely induce H3K9me2 in plants,[8] 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.[11] 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. In support, developmentally regulated telomerase genes were shown to possess active H3K4me3 histone mark in the presence of repressive H3K27me3 mark[64] suggesting complex chromatin variants in the genomes.

Consistent DNA methylation but variable siRNA and histone marks at epigenetically inactivated 35S promoters. Because many factors including T-DNA copy number,[65] insertion site,[66] local features,[67] and differential settings of epigenetic modifiers[68] may influence transgene activity, comparisons of chromatin patterns between different loci may be difficult. Epiallelic variants

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 DNA methyltransferases to the target.[69]

Transient histone modification changes induced by cell culture may trigger the epigenetic switch from PTGS to TGS. Previously, we showed that locus 1 bearing inverted repeated *npt*II transgenes reproducibly undergoes epigenetic switches from PTGS to TGS in callus culture regenerants.[43,47] Here, we studied this process in detail by analyzing histone and DNA modifications in the parental plant, callus culture and regenerated plants hereof. In the dedifferentiated callus, loss of 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[70] was not detectable in locus 1 epialleles, confirming its mostly euchromatic nature and indicating that polycomb repressive complexes are not involved in epigenetic switches of the transgene. Significantly, a non-silenced and non-methylated locus 2 did not undergo chromatin changes in callus suggesting that chromatin alterations were associated with PTGS state and/or inverted repeat character of locus 1. As both H3K4me3 and H4K9me2 are opposing chromatin marks labeling euchromatin and heterochromatin, respectively, are present, it suggests that contradictory (active and inactive) epigenetic marks are imposed on a transgene during cell culture cultivation. The intermediate histone marks in callus cells can be explained in two ways. On the one hand, a mosaic of cells carrying the transgene with either heterochromatic or euchromatic marks may exist, reflecting known epigenetic variability in the callus. In this context, we reported cell to cell DNA methylation heterogeneity of locus 1.[47] On the other hand, there may be variability at the single-nucleosome level. Under this scenario, a given histone octamer may harbor opposing modifying marks for asymmetrical distribution. Indeed, callus transgene chromatin completely lost H3K4me3 and retained high levels of H3K9ac (supporting the existence of nucleosomes with ambivalent marks).

One of the most prominent observations was a correlation between H3K4me3 demethylation at the 3' end and spreading of DNA methylation from this region into the promoter. It would be intriguing to determine whether these distally related changes are interrelated. Histone H3K4 methylation is regulated by a group of enzymes from the JmJ (Jumanji) family known to be involved in RNA-directed DNA methylation.[71,72] Significantly, some JmJ14 mutants released the transgene PTGS that was correlated with an increase in promoter methylation and retardation of transcription.[29] It is tempting to speculate that the activity of some JmJ proteins and perhaps other epigenetic factors, are modified in the callus, leading to a polarized (3'→5') methylation spreading over a distance that may be as long as 1 kb. Spreading of DNA methylation could be related to weakening of transcription initiation from P35S and reduced polymerase occupancy of the coding region. In support, epialleles generated by the targeting of P35S by RdDM showed similar de novo methylation of the 5' coding region to the callus-induced epialleles.

Materials and Methods

Plant material, hybridization and callus culture conditions. All transgenic tobacco (*Nicotiana tabacum*) SR1 plants were generated by *Agrobacterium*-mediated transformation.[18] 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.[73] The line hemizygous for the TGS locus 1E was obtained by plant regeneration from long-term HeLo1 callus cultures.[43] 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 Lo1 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.[46]

Calli were established from leaf explants by hormonal treatment and grown in 0.7% agar containing B5 salts supplemented with sucrose (30 gl^{-1}), α-naphthaleneacetic acid (2.0 mgl^{-1}) and 6-benzylaminopurine (0.2 mgl^{-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 mgl^{-1}) and 6-benzylaminopurine (2.0 mgl^{-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 lyophilized leaves or calli using the cetyltrimethylammonium bromide method as described previously.[74]

Bisulfite treatments were performed on purified genomic DNA using the EpiTect bisulfite kit (QIAGEN). Primers for amplification of the 35S promoter and 5' *npt*II region are as follows: forward primer: 5'-CAT TAC ATC 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 AAG AAA GTA G-3'. The forward primer matched the *npt*II 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

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.[75]

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 *npt*II level related to the actin transcripts was performed using the Fast Start SYBR Green Master (Roche) by the Rotorgene 6000 (QIAGEN). The *npt*II gene was amplified with the forward primer 5'-CGT TAC AAG AGA GAA ATC GCC-3' and the reverse primer 5'-TTC TAT CGC CTT CTT GAC GAG-3'; actin was amplified with the forward primer 5'-CTG GAT TTG CTG GTG ATG AT-3' and the reverse primer 5'-CYC TCT TGG ATT GAG CTT-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 *npt*II 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) into the 0.2- to 1-kb fragments. Immunoprecipitation was performed using commercially available antibodies anti-H3K4me3 (Abcam; catalog no. ab8580), anti-H3K9ac (Abcam; catalog no. ab10812) and anti-H3K9me2 (Millipore 07-212). Classical PCR was performed on ChIP samples. The P35S and the 5' coding sequence was amplified with the forward primer 5'-AAG GCT ATC GTT CAA GAT GCC-3' and the reverse primer 5'-GAT TGT CTG TTG TGC CCA GTC-3'. The positions are about +140 (forward) and –210 bp (reverse) relative to the transcription start site. Because of the locus 271 arrangement it was necessary to amplify its P35S region by another reverse primer 5'-TCT CCA AAT GAA ATG AAC TTC CTT AT-3'. The PCR program consisted of 3 min of initial denaturation at 94°C followed by 35 cycles of 20 sec at 94°C, 50 sec at 58°C and 50 sec at 72°C. The 3' *npt*II region and actin were amplified using the same primers as those used for quantitative RT-PCR analysis in the same PCR cycle (initial denaturation at 94°C for 2 min followed by 35 cycles of 20 sec at 94°C, 20 sec at 56°C and 30 sec at 72°C). 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 previously[46,47] with 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 *npt*II-coding sequence and the 35S promoter probes were prepared from the ~830-bp and ~980-bp inserts of the pGEM-*npt*II 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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