Cell Culture-Induced Gradual and Frequent Epigenetic Reprogramming of Invertedly Repeated Tobacco Transgene Epialleles1[W]

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Using a two-component transgene system involving two epiallelic variants of the invertedly repeated transgenes in locus 1 (Lo1) and a homologous single-copy transgene locus 2 (Lo2), we have studied the stability of the methylation patterns and trans-silencing interactions in cell culture and regenerated tobacco (Nicotiana tabacum) plants. The posttranscriptionally silenced (PTGS) epiallele of the Lo1 trans-silences and trans-methylates the target Lo2 in a hybrid (Lo1/Lo2 line), while its transcriptionally silenced variant (Lo1E) does not. This pattern was stable over several generations in plants. However, in early Lo1E/Lo2 callus, decreased transgene expression and partial loss of Lo1E promoter methylation compared with leaf tissue in the parental plant were observed. Analysis of small RNA species and coding region methylation suggested that the transgenes were silenced by a PTGS mechanism. The Lo1/Lo2 line remained silenced, but the nonmethylated Lo1 promoter acquired partial methylation in later callus stages. These data indicate that a cell culture process has brought both epialleles to a similar epigenetic ground. Bisulfite sequencing of the 35S promoter within the Lo1 silencer revealed molecules with no, intermediate, and high levels of methylation, demonstrating, to our knowledge for the first time, cell-to-cell methylation diversity of callus. Regenerated plants showed high interindividual but low intraindividual epigenetic variability, indicating that the callus-induced epiallelic variants were transmitted to plants and became fixed. We propose that epigenetic changes associated with dedifferentiation might influence regulatory pathways mediated by trans-PTGS processes.

Plants regenerated from calli often display qualitative and quantitative phenotypic alterations, cytological abnormalities, sequence changes, and gene activation and silencing. These cell culture-induced changes, collectively called somaclonal variation, may be stable or unstable, irreversible or reversible, meiotically reset or transgenerationally transmitted (Karp, 1991; Phillips et al., 1994; Kaeppler et al., 2000). The variation in gene expression is believed to be mediated by epigenetic mechanisms involving methylation of DNA and chromatin modifications. Shifts from parental methylation states have been observed in many cell cultures and regenerated plants at both global and local levels (Kaeppler and Phillips, 1993; Smulders et al., 1995; Olhoff and Phillips, 1999; Jaligot et al., 2000; Kubis et al., 2003; Schellenbaum et al., 2008). Alleviation of silencing of ribosomal genes (Komarova et al., 2004; Koukalova et al., 2005), increased transposon activity (Hirochika, 1993; Grandbastien, 1998), and activation of cell cycle-controlling genes (Williams et al., 2003) were reported in studies linking changes in DNA methylation with changes in expression patterns. The important outcome of the cultivation of plant cells under suboptimal conditions is the formation of epialleles, epigenetically modified alleles of a gene (for review, see Finnegan, 2002; Arnholdt-Schmitt, 2004). For example, epigenetic changes (mainly in DNA methylation levels) were reported in response to water deficiency (Labra et al., 2002), osmotic stress (Kovarik et al., 1997), and heavy metal presence (Aina et al., 2004).

Stability of transgene expression is one of the key requirements for successful establishment of transgenic lines in agriculture and biotechnology. Routinely, stable epialleles are selected, ensuring high levels of the recombinant protein product. However, even metastable epialleles could be of potential interest, since attenuated expression may provide favorable phenotypic traits. The common features of unstable or metastable transgene loci are the repetitiveness of DNA sequences (presence of direct and inverted repeats), preexisting methylation, and/or distinct genetic environments (Kakutani, 2002; Richards, 2006). The influence of cell culture on gene expression and epigenetic modification has been studied in several

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results, often with contrasting outcomes: some loci acquired epigenetic silencing (Meng et al., 2006), while the other, originally silenced loci tend to increase expression (Mitsuhara et al., 2002) in the cell culture environment.

In plants, silencing may occur at transcriptional and posttranscriptional levels. Transcriptional gene silencing (TGS) is characterized by repression of transcription and is correlated with cytosine methylation of the promoter at symmetrical and nonsymmetrical motifs (Vaucheret and Fagard, 2001). The posttranscriptional silencing (PTGS) is defined as a mRNA degradation process triggered by double-stranded small RNA (smRNA) molecules (Vaucheret et al., 2001). In plants, it often associates with DNA methylation of coding sequences. Both PTGS and TGS may be influenced by environmental and developmental factors (De Neve et al., 1999). The factors underlying the direction of expression phenotype changes are largely unknown, but it is likely that T-DNA organization, preexisting epigenetic modifications, polyploidy, and type of silencing play a role. For example, reversion of PTGS into TGS phenotype has been reported in tobacco (Nicotiana tabacum; Fojtova et al., 2003) and petunia (Petunia hybrida; Kanazawa et al., 2007) transgenes containing inverted repeats.

To study the influences of cell culture stress on epiallelic stability, we designed a two-component transgene system containing a silencing trigger locus and a nonsilenced single-copy target locus serving as a sensitive reporter for the trans-silencing capacity of the silencing locus. In these hybrids, the trigger locus organized as an inverted repeat occurs in two meiotically stable epiallelic states, termed Lo1 and Lo1E (Van Houdt et al., 2000a; Fojtova et al., 2003). The neomycin phosphotransferase II (nptII) reporter gene within Lo1 is characterized by an active transcription, RNA degradation, and methylation of the coding region but not the promoter. The epiallelic variant Lo1E was previously obtained by regeneration of plants from Lo1 line callus (Fojtova et al., 2003). In these regenerants, the nptII reporter gene was found to be transcriptionally silenced and the linked 35S promoter contained heavy methylation of symmetrical and nonsymmetrical motifs. Both epiallelic variants Lo1 and Lo1E displayed high trans-generation stability of their methylation patterns. Only the Lo1 epiallele was able to trans-silence homologous loci by a PTGS mechanism, while the Lo1E epiallele did not trans-silence the homologous locus (Fojtova et al., 2006). This demonstrated that epigenetic modification of a silencer locus can dramatically influence the expression of unlinked genes. Here, we studied the stability of epiallelic variants of the silencer locus in callus culture. Evidence was obtained for diversification of epiallelic variants in tissue culture and erasure of parental methylation imprints in callus culture. Transition to the other extreme of epialleles was observed in approximately one-fourth of regenerated plants in both directions.

RESULTS

Description of the Transgenic Loci and Experimental Setup

Transgenic loci used for the construction of the tobacco hybrid lines are schematically depicted in Figures 1A and 2A. The transgenic line HeLo1 carries Lo1, which contains two copies of the GVchs287 T-DNA in an inverted repeat arrangement. The bacterial nptII reporter transgene driven by the 35S promoter is silenced at the posttranscriptional level in Lo1 (Van Houdt et al., 1997). The HeLo1E line containing epimutated transcriptionally silenced Lo1 (termed Lo1E) was obtained as a regenerated individual from the HeLo1 cell culture (Fojtova et al., 2003). Lo2 consists of a single-copy insertion of T-DNA and is characterized by high and stable nptII expression (Van Houdt et al., 2000a). Epialleles of the Lo1 were combined with the homologous transgenic Lo2 in hybrid plants. The PTGS epiallele of the Lo1 was shown to induce trans-silencing and trans-methylation of the Lo2 in Lo1/Lo2 hybrids, while its TGS counterpart (Lo1E) was unable to trans-silence the Lo2 (Fojtova et al., 2006).

The epiallelic stability of transgenes in hybrid lines during dedifferentiation and differentiation processes was investigated as shown in Figure 3; two independently established callus cultures from each hybrid line were analyzed. A total of 28 Lo1/Lo2 and 12 Lo1E/Lo2 regenerated plants were analyzed for reporter transgene expression and silencing locus promoter methylation.

Changes in Transgene Expression during Callus Culture and the Regeneration Process

The nptII transgene transcript levels in parental hybrid plants, calli, and regenerants were analyzed by RNA gel blot hybridization and real-time PCR analysis (Fig. 4). A strong hybridization signal was obtained with RNA extracted from the parental hybrid plant containing a TGS variant (Lo1E/Lo2) but not a PTGS variant (Lo1/Lo2) of Lo1. In the Lo1E/Lo2 callus, expression started to progressively deteriorate, and fully developed 3-month-old callus already had negligible transcription (Fig. 4A). The nptII expression in the Lo1/Lo2 line was not influenced by in vitro culture stress, and the nptII transcript levels were low in both plant and calli.

Plants were regenerated from the 12-month-old Lo1/Lo2 and Lo1E/Lo2 calli. There was more than 10-fold variation in nptII mRNA levels between the individuals. About three-fourths of Lo1E/Lo2 plants (Fig. 4B) showed high expression of the nptII gene. In these individuals, expression at both RNA and protein (Supplemental Fig. S1) levels was comparable to that of the parental plant. Most (about four-fifths) regenerated plants from Lo1/Lo2 calli retained a silenced phenotype, while six individuals (R2, R6, R14, R16, R23, and R24) showed partial relief of trans-silencing.
Analysis of the nptII-Derived smRNA Molecules

Next, we wished to investigate the nature of callus-induced expression changes. We have previously shown that communication between the PTGS Lo1 and Lo2 is mediated by nptII-derived smRNA molecules in Lo1/Lo2 hybrid plants, whereas these molecules are absent in the Lo1E/Lo2 (Fojtova et al., 2006). Here, we analyzed smRNAs in Lo1/Lo2 and Lo1E/Lo2 parental and selected regenerated plants. RNA samples enriched with a low molecular weight fraction were immobilized on the membrane and hybridized against the radioactively labeled nptII RNA sense probe (Fig. 5). The parental Lo1/Lo2 plant displayed the strong hybridization signal in the low molecular weight region; the length of the fragment was about 21 nucleotides. The signal was also detected in the 12-month-old Lo1/Lo2 callus and in the silenced regenerants (R4 and R9), while it was absent in the regenerants that expressed higher levels of the nptII mRNA (R2 and R6).

In the Lo1E/Lo2 hybrid line, the nptII hybridization signal was observed in the callus sample and in silenced regenerant (r3) but not in the nonsilenced parental plant and the nonsilenced regenerants (r2 and r6). These results indicate that the changes in expression patterns during dedifferentiation and plant regeneration can be correlated with changes in smRNA levels.

Figure 1. Analysis of the 35S promoter methylation in the Lo1/Lo2 and Lo1E/Lo2 parental hybrid plants, derived calli, and regenerated plants using methylation-sensitive restriction endonuclease. A, Genomic organization of transgenic loci and scheme of the digestion. BglII (BII) enzyme was used for the dissection of the particular regions of T-DNA in hybrid lines. Sau96I (S96I) restriction endonuclease was applied to analyze the methylation of cytosines in the nonsymmetrical sequence context. Arrows schematically delimit lengths of fragments that resulted after the digestion. P35S, Promoter from the Cauliflower mosaic virus; nptII, bacterial neomycin phosphotransferase II gene; 3' chs, transcription termination sequence from the 3' untranslated region of the chalcone synthase gene from snapdragon (Antirrhinum majus); RB, T-DNA right border. B, Southern-blot analysis of 35S promoter methylation. DNAs were predigested by BglII restriction enzyme to obtain locus-specific bands (+ lines). The samples in + lines were digested with methylation-sensitive Sau96I. The 6.4-kb BglII/Sau96I band indicates complete methylation of all four Sau96I sites within two 35S promoters of the Lo1. The 4.8-kb BglII/Sau96I band originated from methylation of one of the promoters on each site of the inverted repeat.

Analysis of the 35S Promoter Methylation by Southern-Blot Hybridization

We tested the hypothesis that the cell culture-induced alterations of RNA silencing signals might be explained by destabilization of the epiallelic states of the silencing trigger Lo1. It is known that strong methylation of a promoter can influence transcription...
of a linked gene. To study methylation changes accompanying dedifferentiation and plant regeneration, we carried out Southern-blot hybridization using a methylation-sensitive restriction endonuclease. A, Genomic organization of transgenic loci and scheme of the digestion. EcoRV (EV) methylation-insensitive enzyme was used for the dissection of the particular regions of T-DNA in hybrid lines. SmaI (S) enzyme is sensitive to methylated cytosines in the CG sequence context. Arrows schematically delimit lengths of restriction fragments. Abbreviations and symbols are as in Figure 1A. B, Southern-blot methylation analysis of the genic region. The experimental strategy was analogous to that described in Figure 1B. The 1.8-kb EcoRV/SmaI band represents a methylated Lo2 fragment.

Figure 2. Analysis of the 3′ nptII coding region methylation in the Lo1/Lo2 and Lo1E/Lo2 parental hybrid plants, derived calli, and regenerated plants using the methylation-sensitive restriction endonuclease. A, Genomic organization of transgenic loci and scheme of the digestion. EcoRV (EV) methylation-insensitive enzyme was used for the dissection of the particular regions of T-DNA in hybrid lines. SmaI (S) enzyme is sensitive to methylated cytosines in the CG sequence context. Arrows schematically delimit lengths of restriction fragments. Abbreviations and symbols are as in Figure 1A. B, Southern-blot methylation analysis of the genic region. The experimental strategy was analogous to that described in Figure 1B. The 1.8-kb EcoRV/SmaI band represents a methylated Lo2 fragment.

In Lo1/Lo2 plants regenerated from the 12-month-old callus, the methylated Sau96I fragments were found (Fig. 1B), suggesting that methylation patterns were transmitted from calli into plants. Interestingly, the most heavily methylated sites were found among the R2 and R6 plants. Both individuals showed elevated levels of the nptII transcripts (Fig. 4), supporting the hypothesis that inactivation of the Lo1 promoter is associated with loss of RNA silencing signal. The digestibility of the Lo2-specific 8.5-kb band remained unchanged (high), indicating the absence of methylation of the 35S promoter within Lo2. Similar to the Lo1/Lo2, the Lo1E/Lo2 regenerated plants showed interpopulation variation in methylation profiles. While in most individuals (represented by the r2, r5, and r6 regenerants) the 6.4-kb fragment was largely undigested (indicating heavy methylation of the Lo1 promoter), the DNAs from r3 and r4 regenerants showed increased sensitivity toward Sau96I.
Figure 3. Scheme of the experimental strategy. Lo1, plant line hemizygous for the transgenic locus 1, with expression of the nptII reporter transgene silenced posttranscriptionally; Lo1E, plant line hemizygous for the transgenic locus 1E, with expression of the nptII reporter transgene silenced transcriptionally; Lo2, plant line hemizygous for the transgenic locus 2, with active expression of the nptII reporter transgene silenced posttranscriptionally; Lo1E/Lo2, hybrid plant line combining locus 1 and locus 2; Lo1/Lo2, posttranscriptionally silenced hybrid plant line combining locus 1E and locus 2, with active expression of the locus 2 nptII transgene.

the r3 and r4 regenerants showed Lo2 silencing (Fig. 4), confirming an inverse correlation between Lo1 promoter methylation and trans-PTGS.

Methylation Patterns of the 35S Promoter Determined by Bisulfite Sequencing

Next, we wished to examine the homogeneity of methylation patterns at the silencing locus (Lo1) by bisulfite sequencing analysis in a parental Lo1/Lo2 hybrid, various stages of callus, and regenerated plants (for the tissues, see Supplemental Fig. S3). Since it was impossible to distinguish between clones derived from individual loci due to sequence homology, we separated both loci by gel electrophoresis after the BgIII digestion (there are two BgIII sites within Lo1 but only a single site within Lo2; Fig. 1A). The 35S promoter of Lo1 represented by the 6.4-kb band was isolated and subjected to bisulfite conversion, cloning, and sequencing. The distribution of methylcytosine residues along the individual clones is diagrammatically shown in Figure 6A, and the data are summarized in Figure 6B and Table I. The examined region contains 12, seven, and 66 cytosine residues at CG, CHG, and CHH sequence contexts, respectively. In accordance with Southern-blot hybridization (Fig. 1B), there was no or negligible methylation of the 35S promoters in the clones derived from the parental Lo1/Lo2 plant (top). Similarly, the green leaf discs used for callus induction did not acquire significant methylation. On the other hand, few clones derived from early callus showed increases of methylation, especially in nonsymmetrical contexts. The fully developed 12-month-old callus yielded clones with variable levels of methylation. There were clones with no (top lanes), intermediate (middle lanes), and high (bottom lanes) levels of methylation. The clones from regenerated plant R2 (nonsilenced by PTGS) showed relatively homogenous profiles with both CG and non-CG methylation. Clones derived from homologous Lo2 were nonmethylated (data not shown).

DNA Methylation in the Coding Region

The coding region methylation is considered a hallmark of PTGS (Ingelbrecht et al., 1994). We have studied methylation of the nptII coding region of both loci in the Lo1E/Lo2 hybrid using SmaI cutting between the nptII coding region and the 3′ untranslated region. The enzyme is sensitive to methylation of the outer and inner C within the CCCGGG recognition sequence. To separate transgenic loci, the DNAs were digested with EcoRV, providing 3.0- and 1.8-kb bands corresponding to Lo1 and Lo2 fragments, respectively (Fig. 2A). As shown in Figure 2B, the 1.8-kb band of Lo2 was efficiently digested in parental nonsilenced Lo1E/Lo2 plants, 12-month-old derived callus, and regenerated r2, r5, and r6 plants. Late-stage callus and both r3 and r4 regenerants that showed Lo2 silencing displayed an absence of SmaI digestion, indicating the presence of cytosine methylation in the Lo2 coding region in these individuals.

In Lo1/Lo2 hybrids, strong genic methylation of both transgenic loci observed in parental plants persisted during 24 months of the callus cultivation (Fig. 2B). In regenerated plants, R4 and R9 individuals with silenced Lo2 and low methylation of Lo1 promoter exhibited high levels of Lo2 trans-methylation at the SmaI restriction site. On the other hand, Lo2 methylation was decreased in weakly silenced R2 and R6 plants. The 3.0-kb Lo1-specific band remained methylated irrespective of the cultivation conditions. These results indicate that the methylation of the silencing Lo1 promoter was inversely correlated with trans-methylation of the SmaI site in the Lo2.

DISCUSSION

In this work, we used a two-component transgene model system to study the stability of RNA-mediated homologous transgene interactions in cell culture. We show that cell culture resulted in blurring of the parental epigenetic expression and methylation patterns at the silencing locus associated with increased epiallelic diversity. These changes influenced the production of smRNA molecules from a linked gene and...
silencing of a homologous unlinked locus. Despite a dominant PTGS phenotype in callus culture, a high proportion of nonsilenced individuals were found among regenerated plants. The regenerated plants have partially but not completely reestablished original expression patterns.

### Figure 4

Analysis of *nptII* expression in the parental plants, derived calli, and regenerants of the Lo1/Lo2 and Lo1E/Lo2 hybrid lines. A. Northern-blot analysis of the *nptII* reporter transgene expression. About 5 μg of total RNA was loaded per line and hybridized against the *nptII* DNA probe. The hybridization with the tobacco 18S rDNA probe is shown as a control for loading. B. Quantification of the *nptII* expression by real-time PCR. The *nptII* RNA levels were normalized to actin mRNA. The expression level of the nonsilenced Lo2 line was arbitrarily designated 100%. The transgenes whose expression exceeded 30% of that of Lo2 were considered “nonsilenced” (threshold indicated by the thick horizontal line).
Prevalent PTGS Phenotype in Hybrid Callus Cultures

The Lo1E/Lo2 line containing a TGS variant of the silencer had high NPTII levels in parental leaf. However, progressive silencing evolved in cell culture. The hybrid combining a Lo1 PTGS epiallele was silenced in both plants and calli. The presence of nptII gene-specific smRNAs and methylation of the coding region within the target indicated that silencing occurred at the posttranscriptional levels. This seems contradictory to previous reports demonstrating the loss of PTGS in calli and rapidly dividing cells (Mitsuhara et al., 2002; Correa et al., 2004). There might be several explanations. First, differences in the genomic organization of silencing loci need to be considered. It is known that trans-silencing elicited by hairpin constructs and viruses (Dalmay et al., 2000) does not require some components of RNA-processing machinery, including RNA-dependent RNA polymerase, that are needed for trans-PTGS. Perhaps there is a variation in the sensitivity of PTGS to environmental factors among the systems. Furthermore, unlike virus-induced PTGS (Correa et al., 2004), we did not detect any specific high molecular weight RNA species that would potentially indicate aberrant processing of the nptII-specific RNA signals (data not shown). We also found no evidence for the influence of the genomic location of target loci (Fojtova et al., 2006) recently reported for trans-TGS systems (Fischer et al., 2008). These data support the hypothesis that the trans-silencing RNA signals are produced only from the transcriptionally active Lo1, regardless of the developmental status and cultivation conditions. In conclusion, tobacco callus cultures probably contain all necessary components able to execute trans-PTGS triggered by invertedly repeated loci.

Cell Culture-Induced Methylation Mosaicism at the Silencing Locus Accounts for the Dominant PTGS Phenotype in Hybrids

Upon callus culturing in the Lo1E/Lo2 line, the methylated and inactive promoter epiallele (Lo1E) underwent hypomethylation. Previous experiments showed that the TGS 35S promoter is sensitive to hypomethylation, since the 5-azadeoxycytidine treatment resulted in elevated transcription of the linked nptII gene (Fojtova et al., 2006). It is likely that cell culture-induced hypomethylation activated the Lo1E promoter, as a result of which the production of trans-silencing RNA signal has been restored. In contrast to Lo1E, the active 35S promoter within Lo1 underwent progressive methylation in cell culture. After 12 months, the methylation profiles of promoters in the Lo1/Lo2 and Lo1E/Lo2 lines were almost indistinguishable, suggesting that cell culture had blurred differences in methylation imprints between the lines. The partial digestion of promoter fragments with methylation-sensitive enzymes indicated that intermediate methylation phenotypes have been established. This assumption has been further confirmed by bisulfite sequencing of the 35S promoter, which revealed astonishing clone-to-clone variation in methylation states of the callus clones but not those of parental leaf (Fig. 6). There were clones with no or marginal methylation, while some clones acquired full methylation of the 35S promoter.

Possible Mechanism of Callus-Induced Methylation Changes

The contrasting trends in methylation dynamics of the TGS and PTGS epialleles of Lo1 suggest that there might be two opposing forces acting on the promoter exposed to the cell culture environment. The reduced methylation in Lo1E may reflect a more general trend toward genome hypomethylation in plant cell cultures (Kaeppeler and Phillips, 1993; Kubis et al., 2003). On the other hand, the proximity of inverted repeats occupying distinct chromatin structure (Ebbes et al., 2005) and intensive transcription over the methylated coding region (van Blokland et al., 1997) could promote methylation spreading from the coding region into the promoter. Inverted repeat structure could potentially generate read-through transcripts, giving rise to
Figure 6. Bisulfite methylation analysis of the Lo1 in a Lo1/Lo2 plant, derived callus, and regenerated plants. Approximately 300 bp of the 35S promoter was sequenced. A. CyMATE output of the distribution of C methylation along the sequenced clones.
methylation-inducing smRNA molecules (Mette et al., 2000). Although we failed to identify promoter-specific smRNAs, the initial nonsymmetrical methylation imposed on a “naive” Lo1 promoter during callus induction (Fig. 6A; Table I) suggests that some type of RNA-directed process, acting in cis, might be involved. The symmetrical CG sites seem to be methylated later at more advanced stages of callus propagation. This mode of methylation dynamics is consistent with a gradual and perhaps stochastic process leading to denser methylation in advanced stages. In this context, methylation changes were more pronounced in plants regenerated from advanced stages of callus growth compared with those of earlier passages (Peredo et al., 2006).

Recently, a subset of endogenous promoters was reported to undergo hypermethylation in Arabidopsis (Arabidopsis thaliana) callus and suspension cultures (Berdasco et al., 2008). As in our system, the epigenetic changes were associated with transcriptional silencing of linked genes. However, endogenous promoters of Arabidopsis were methylated exclusively at CG dinucleotides, while the Lo1 35S promoters were methylated at both CG and non-CG motifs. Perhaps there might be differences in the mechanism of hypermethylation of endogenous genes and transgenes in callus cultures. It is necessary to stress that methylation changes affected 35S promoters within the PTGS Lo1 only, since a homologous promoter within the non-silenced Lo2 remained unmethylated (and active) throughout callus cultivation (Fig. 1B; Fojtova et al., 2003).

High Interindividual and Low Intraindividual Epigenetic Variability in Regenerated Plants

About three-fourths of regenerants from silent 12-month-old Lo1E/Lo2 callus showed expression levels of the nptII reporter gene comparable to that of the nonsilenced parental plant. Similarly, about one-fifth of regenerated plants from Lo1/Lo2 callus (established from a silenced Lo1/Lo2 plant) showed elevated reporter gene expression, although in this case its level was lower than that of the Lo1E/Lo2 regenerants. Thus, there was considerable interpopulation variability in expression patterns among regenerated plants (Fig. 4), likely reflecting epigenetic mosaicism of a parental callus. Indeed, bisulfite sequencing revealed a large clone-to-clone methylation variability in callus (Fig. 6), indicating that callus is a mixture of cells with different epigenetic phenotypes at the silencing trigger Lo1. The silenced phenotype was accompanied by coding region methylation and production of nptII-specific smRNAs, indicating a PTGS mechanism. On the other hand, these attributes were absent in nonsilenced regenerants, which had heavy methylation of the TGS 35S promoter at the silencing Lo1. These results support the hypothesis that the promoter activity of the Lo1 is a key player in the trans-silencing process in this system. Bisulfite analysis (Fig. 6) showed that all clones derived from a nonsilenced regenerant had nearly complete methylation of an as-1 regulatory element (containing two CG motifs at –67 and –79; Kanazawa et al., 2006) at the silencing trigger Lo1. Since a factor binding to as-1 was shown to be methylation sensitive, we can speculate that the methylation of the as-1 element accounts for promoter inactivity in regenerated plants. Interestingly, the intraindividual methylation variability among the sequenced clones was negligible, indicating that plants tend to maintain accurate setting of epigenetic states throughout mitosis. The uniform methylation profiles across the sequenced clones further support a single-cell rather than a multiple-cell origin of regenerants.

The increased frequency of methylated epialleles in regenerated plants possibly reflects the trend toward

![Figure 6](http://www.plantphysiol.org)
increased genome methylation in regenerated plants. For example, rDNA (Koukalova et al., 2005), some transposons (Grandbastien, 1998), and other repeats (Jaligot et al., 2000) appeared to be less methylated in callus compared with regenerated plants. Also, less differentiated cells contain a lower degree of methylation than fully differentiated mature leaf (Mathieu et al., 2003). It is not certain whether cells with highly methylated DNA are favored for regeneration and selected or whether hormonal factors (LoSchiavo et al., 1989) associated with the regeneration process increase overall genomic methylation.

It is becoming apparent that in eukaryotic genomes many genes occur in multiple families that are subjected to an orchestrated epigenetic regulation (Melquist et al., 1999) and that no gene exists in isolation from other genes (Matzke and Matzke, 1990). Our results show that epigenetic change in a single locus could potentially influence the expression state of many other homologous loci through RNA signals. We propose that “hidden” epigenetic variability unobserved by a dedifferentiation process might have its molecular background in promoters containing inverted and tandem repeats. It will be interesting to determine if phenotypes modified by cell culture (Meins and Thomas, 2003) originate from loci containing these DNA structures.

MATERIALS AND METHODS

Plant Material and Callus Culture Conditions

All transgenic tobacco (Nicotiana tabacum) SR1 plants were generated by Agrobacterium tumefaciens-mediated transformation (Engelbrecht et al., 1994). The plants hemizygous for the PTGS Lo1 (HeLo1; Figs. 1A and 2A) were obtained by crossing a plant homozygous for Lo1 with an untransformed SR1 tobacco plant (Van Houdt et al., 2000b). The line hemizygous for the TGS Lo1E was obtained by plant regeneration from long-term Lo1 callus cultures (Fojtova et al., 2003). The line hemizygous for Lo2 (HeLo2; Figs. 1A and 2A) was obtained by crossing a plant homozygous for Lo2 with an untransformed SR1 tobacco plant (Van Houdt et al., 2000a). The hybrids hemizygous for Lo1 and Lo2 (Lo1/Lo2) and hemizygous for Lo1E and Lo2 (Lo1E/Lo2) were obtained by crossing the respective parental plants. In all crosses, the Lo1 or Lo1E plant served as the mother donor. Progeny plants were screened for the presence of the two transgenic loci by DNA gel-blot hybridization.

Calli were established from leaf explants by hormonal treatment and grown in 0.8% agar containing B5 salts supplemented with Suc (30 g L⁻¹), α-naphthaleneacetic acid (2.0 mg L⁻¹), and 6-benzylaminopurine (0.2 mg L⁻¹). Callus was transferred onto fresh agar medium every 3 to 4 weeks.

DNA Isolation and Southern-Blot Hybridization

Total genomic DNA was isolated from lyophilized leaves or calli by the cetyltrimethylammonium bromide method as described previously (Kovarik et al., 2000). DNA methylation was analyzed with methylation-sensitive restriction endonucleases. Approximately 20 μg of genomic DNA was digested with an enzyme excess (5 units per 1 μg of 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) and hybridized against 32P-labeled DNA probes (DekaLabel kit; MBI Fermentas) for at least 16 h at 65°C. The nptII-coding sequence and the 35S promoter probes were prepared from the approximately 830- and 980-bp inserts of the pGEmntptII and pGJS290 plasmids, respectively (Van Houdt et al., 1997). After washing under high-stringency conditions (twice for 5 min in 2× standard saline citrate [SSC] + 0.1% [w/v] SDS and twice for 20 min in 0.2× SSC + 0.1% [w/v] SDS at 65°C), the hybridization bands were visualized with a STORM PhosphorImager (GE Healthcare) and the data were processed with ImageQuant software (GE Healthcare).

RNA Isolation, Northern-Blot Hybridization, and Real-Time PCR Analysis

Total RNA was isolated from young leaves with the RNaseasy Plant Mini Kit (Qiagen) and extensively treated by DEAE-cellulose-free; Applied Biosystems/Ambion) according to the manufacturer’s instructions. 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 (Applied Biosystems/Ambion) 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 STORM PhosphorImager. To evaluate the nptII expression levels, the intensities of the nptII hybridization signals were normalized to the 18S ribosomal RNA bands (Lim et al., 2000).

cDNAs were prepared by reverse transcription of RNAs using the SuperScript II Reverse Transcriptase (Invitrogen) and Random Nonamers (Sigma). Quantification of the nptII level related to the actin transcript was done using the Power SYBR Green PCR Master Mix (Applied Biosystems/Ambion) by the 7300 Real-Time PCR System (Applied Biosystems/Ambion). nptII was amplified with the forward primer 5′-CTCATCGCCCTTCTTGACGAG-3′ and the reverse primer 5′-TTTATCGCCCTTCTTGACGAG-3′; actin was amplified with the forward primer 5′-CTGATTTGCTGGTGATGAT-3′ and the reverse primer 5′-CCTCTGGATGATGATG-3′ in the same PCR cycle (initial denaturation at 94°C for 10 min followed by 35 cycles of 20 s at 94°C, 20 s at 56°C, and 30 s at 72°C). The SYBR Green I fluorescence was monitored consecutively after the extension step. The amount of nptII transcript was determined in three independent reactions for regenerated plants; for parental hybrid plants (Lo1/Lo2 and Lo1E/Lo2) and PTGS Lo1 plant, RNAs isolated from three individuals were analyzed in triplicate. nptII relative levels were expressed as percentage of the Lo2 nonsilenced transgenic plant expression.

smRNA Molecule Isolation and Northern-Blot Hybridization

Fragments of smRNA molecules were isolated as described previously (Hamilton and Baulcombe, 1999) with minor modifications (Fojtova et al., 2003).

The smRNAs were separated by electrophoresis (15% polyacrylamide, 7 M urea in 0.5× TBE; 1× TBE = 90 mM Tris-borate and 2 mM EDTA, pH 8.0), blotched onto a nylon Hybond XL membrane (GE Healthcare) with a semi-dry blotting instrument (trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad) in 0.5× TBE, 0.15× TBE, and 0.2× TBE. The smRNA molecules were isolated as described previously (Fojtova et al., 2003). Fractions of smRNA molecules were isolated as described previously (Hamilton and Baulcombe, 1999; Mette et al., 2000) in ULTRAhyb buffer (Applied Biosystems/Ambion) at 35°C for 48 h. The RNA was washed twice in 2× SSC + 0.1% (w/v) SDS for 30 min at 35°C, then in 20 mM Tris-HCl, 5 mM EDTA (pH 8.0), 60 mM NaCl, and 10 μg mL⁻¹ RNaseA (for 1 h at 37°C) to remove unspecific background, and exposed to the PhosphorImager screen.

Bisulfite Genomic Sequencing

Bisulfite treatments were carried out on purified genomic DNA using the EpiTect Bisulfite Kit (Qiagen). The primers for the amplification of the 35S promoter and 5′ nptII region are as follows: forward primer 5′-ATATCAATTCCCTATGACGAGAGATATATAT-3′, the first reverse primer 5′-GAATAGGAAGAAATATATATATATATGATGAT-3′, and the second reverse primer 5′-CGACCTTCGAGAAATATATATATATATGATGAT-3′.
5′-TATAAGAAGTTGACGTTTATAGAAGTA-3′. 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 54°C, and 1 min at 72°C. The PCR products were cloned into a TA vector (pDrive; Qiagen), and five to 18 clones from each sample were sequenced (Eurofins MWG Operon). The data were processed and methylation density was calculated using CyMATE software (Hetzl et al., 2007).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Analysis of the NPTII protein level in the Lo1E/Lo2 parental plant, callus, and regenerants.

Supplemental Figure S2. Southern-blot methylation analysis of the 35S promoter in 46-month-old Lo1/Lo2 and Lo1E/Lo2 calli.

Supplemental Figure S3. Description of tissues used for methylation analysis by bisulfite sequencing.

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