Plant genomic DNA methylation was analyzed by an improved SssI methyltransferase assay and by genomic sequencing with sodium bisulfite. Kanamycin, hygromycin, and cefotaxime (also called Clarofox) are commonly used as selective agents for the production of transgenic plants. These antibiotics caused DNA hypermethylation in tobacco plants grown in vitro, which was both time- and dose-dependent. An exposure of the plantlets to 500 mg/liter cefotaxime for 1 month caused the de novo methylation of 3 × 10^7 CpG sites/haploid genome of 3.5 × 10^9 base pairs. It occurred in high, moderate, and low repetitive DNA and was not reversible upon the removal of the antibiotics. Reversion was only observed in progeny grown in the absence of drugs. Analysis of the promoter regions of two single-copy genes, an auxin-binding protein gene and the class I chitinase gene, showed the hypermethylation to be heterogeneous but biased toward CpGs. The hypermethylation of the class I chitinase and the auxin-binding protein promoters was not a consequence of a drug-induced gene amplification.

In vertebrate cells treated with DNA synthesis inhibitors, there is a hypermethylation of genomic DNA and a silencing of specific genes (1). As an example, Nyce et al. (2) have shown that the treatment of Chinese hamster cells with 3-azido-3-deoxythymidine (AZT) for 24 h resulted in a 2-fold genome-wide hypermethylation. At the same time, they observed low frequency silencing of the thymidine kinase gene, which was linked to a progressive resistance to AZT. Thymidine kinase is essential for the conversion of AZT into AZT-5'-monophosphate, the active form of the inhibitor. The production of thymidine kinase-negative (TK−) epimutants was 40-fold more frequent in AZT-treated cells than in controls. The drug resistance was shown to be directly related to the methylation of the thymidine kinase gene. The TK− epimutants could be rapidly reverted to the wild type by exposing the cells to the demethylating agent 5-aza-cytidine. Concomitant with this reversion, the cells also regained the ability to convert AZT into its 5'-monophosphate. Other drugs such as 5-fluorodeoxyuridine, cytosine arabinoside, hydroxyurea, aphidicolin, and methotrexate (all inhibitors of DNA synthesis), or inhibitors of topoisomerase II and microtubule formation, had a hypermethylation effect on the DNA from various cells grown in tissue culture (3). However, serum deprivation or protein synthesis inhibitors like cycloheximide or RNA synthesis inhibitors such as actinomycin D had no effect on the hypermethylation of the DNA. The hypermethylation occurred predominantly in the newly synthesized DNA fraction and remained even after the removal of the drug. In the present paper we show that antibiotics such as hygromycin, kanamycin, and cefotaxime, which are commonly used as selective agents in the production of transgenic plants, also cause DNA hypermethylation in Nicotiana tabacum plantlets grown in vitro. The practical implications and the possible mechanisms of this DNA hypermethylation will be discussed.

**MATERIALS AND METHODS**

**Plant Cultivation and Treatment—Seeds from N. tabacum var. SR1 were surface-sterilized with 1.4% (w/v) NaOCl for 5 min, extensively washed in sterile water, and placed on a filter paper imbibed with Linsmaier and Skoog (LS) liquid medium, without hormones (4, 5). After 2 weeks of germination at 23 °C in the presence of light, 10–12 plantlets were placed onto a plastic grid in a 400-cm² plastic container (diameter 8 cm × height 9 cm; Phytotech Labor, Schwarzenburg, Switzerland) with a silicon sponge plug in the lid for air exchange. The plantlets were grown in 30 ml of liquid LS medium, without hormones, in a climatic cabinet (Termaks, Solheimsvik, Norway) at 23 °C, 50% relative humidity and a 16-h photo-period. The control plants were grown in parallel with the treated plants but without antibiotics in the culture medium. The media for the treated plants were supplemented with one of kanamycin monosulfate (Sigma), hygromycin B (Calbiochem), or the sodium salt of cefotaxime (also called Clarofox, Roussel) at the concentrations listed under “Results.” Cefotaxime solutions were prepared fresh before use. Kanamycin and hygromycin are usually used as selection agents for the production of transgenic plants, whereas cefotaxime is used as a bacteriostatic compound in Agrobacterium-mediated plant transformation. The medium was replaced every week. Wild type tobacco was used for the treatment with cefotaxime. Stable transgenic plant lines containing pTET1, a teiR-NPTII gene construct, or pAT2, a GUS-NPTII gene construct (6) conferring resistance respectively to kanamycin and hygromycin were used for treatment with the appropriate antibiotic.

**Isolation of Genomic DNA—**A modified version of the method of Sorensen (7) was used. The plant material was frozen in liquid nitrogen and ground into a fine powder with a pestle and mortar. One volume of powder was dissolved in 2 volumes of extraction buffer (20 mM Tris-HCl, pH 8.0, 0.5 M EDTA, 10 mM EGTA, 1% (w/v) Sarkosyl, 1% (w/v) polyvinylpyrrolidone K40, 500 μg/ml proteinase K) and incubated at 37 °C overnight. The sample was extracted six times with phenol and once with chloroform and then dialyzed against several changes of water (10 liters) at 4 °C for 2 days. The DNA was precipitated with isopropanol in the presence of 0.3 M sodium acetate, pH 5.0. The DNA was digested with 20 μg/ml pancreatic ribonuclease A in 1 mM EDTA and 0.1 mM EGTA at room temperature for 1 h. After a phenol and a chloroform extraction, the DNA was ethanol-precipitated and subsequently redissolved in water overnight. Concentrations were measured by optical density, and the quality of the DNA was determined by agarose gel electrophoresis.

**Quantification of Cytosine and 5-Methylcytosine at DNA Termini—**The method of Bestor et al. (8), combined with the two-dimensional thin layer chromatography system of Kuchino et al. (9), was used to determine the methylation state of MspI (CCGG) sites in total genomic DNA. Labeled cytosine (C) and 5-methylcytosine (mC) spots were identified from the position of non-radioactive markers localized under UV light. The image and relative intensity of the radioactive spots were obtained using a PhosphorImager (Molecular Dynamics). The percentage of mC

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1 To whom correspondence should be addressed. Tel.: 41-61-697-66-88; Fax: 41-61-721-40-91; E-mail: schmitt@fmi.ch.
2 The abbreviations used are: AZT, 3-azido-3-deoxythymidine; SAM, S-adenosylmethionine; ABP, auxin-binding protein.
was calculated using the formula: % M = \left(\frac{m(C(C + M))}{100}\right)\times 100.

**SssI Methylase Accepting Assay**—A modification of the methyl-accepting assay of Wu et al. (10) was used to determine the methylation status of DNA isolated from control and antibiotic-treated plants. 5–10 μg of genomic DNA were digested to completion overnight with EcoRI (100 U/μg of DNA) under the conditions recommended by the manufacturer. The DNA was then phenol- and chloroform-extracted, precipitated with ethanol, and resuspended in water. Its concentration was determined from its A_{260} nm value. The extent of the digestion was tested by size fractionation through a 1% agarose gel.

Digested DNA (300 ng, unless specified) was incubated, in triplets, in 25 μl of reaction mixtures (CpG methylase, New England Biolabs), 2 μM Sadenosyl-l-methyl-3H-methionine (‘HISAM, 75–85 Ci/mmol, Amersham, TRK581) and 2 μM non-radioactive S-adenosylmethionine (SAM, New England Biolabs) in the buffer supplied by the manufacturer. The reaction mixtures (25 μl) were incubated at 37°C for 2 h (unless specified). The reaction was stopped by heating at 65°C for 10 min. Each reaction product was spotted onto a separate DEAE-cellulose filter (2 × 2 cm, Schleicher & Schuell, NA 85) that had been preincubated in washing buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Nonidet P-40 (v/v)) for 1 h. After blotting away the excess moisture with Whatman 17 Ch paper, the pooled DEAE-cellulose filters were washed once in 200 ml of washing buffer for 15 min, under agitation, and then placed in a scintillation vial containing 9 ml of Ready Safe scintillation mixture (Beckman). The scintillant was quenched, in the dark, at 4°C for 1 h and then counted for 5 min. A DNA digested with HindIII and wild type tobacco leaf DNA digested with EcoRI were used as positive controls for the test. The background was determined using a reaction without DNA, and the test was considered acceptable when the background was less than 4% of the value obtained from the control tobacco DNA.

Calculations—The data obtained from the scintillation counter were used to calculate the number of picomoles of methyl groups incorporated/μg of DNA. Δpmol of CH₃ incorporated/μg of DNA was calculated using the formula: Δpmol of CH₃/μg of DNA = (pmol of CH₃/μg of control plant DNA) − (pmol of CH₃/μg of treated plant DNA). Variation between experiments was corrected by normalizing the relative data to the values obtained for the SR1 tobacco DNA used as an internal control. The genome size of tobacco is 3.5 × 10⁹ base pairs, and there is 3.9 pg of DNA/haploid genome (11).

**Fractionation of Genomic DNA**—Genomic DNA was fractionated into high, moderate, or low repeated DNA according to the method of Britten and Kohne (12). Genomic DNA was cleaved with EcoRI and sheared by injection through a 26-gauge needle. Upon phenol/chloroform extraction and ethanol precipitation, the DNA was resuspended in 0.4 M sodium phosphate, pH 6.8, and then immediately absorbed onto hydroxyapatite (Biofinity). The DNA was then phenol- and chloroform-extracted, precipitated with ethanol, and resuspended in water.

**Methylation of λ DNA by the SssI Methylase—10 μg of λ DNA, digested with HindIII, was methylated to completion with 10 units of SssI methylase in the presence of unlabeled SAM under the conditions recommended by the manufacturer. The extent of methylation was tested by HpaII digestion (the enzyme does not cut the sequence CCGG when the internal C is methylated).

**Genomic Sequencing**—Leaf genomic DNA was extracted as described in Doyle and Doyle (13) and purified on a CsCl density gradient. 5–10 μg of each DNA sample were subjected to chemical modification by the reaction with sodium bisulfite according to the procedure of Frommer et al. (14), as modified by Raizada et al. (15). The chemically modified DNA was amplified using a strand-specific PCR and inserted into a modified pBluescript vector (pUBS1, C. M. Lazarus, Bristol University) using unique HindIII and PstI sites that had been engineered into the primers. The plasmids were cloned in Escherichia coli strain MC1022 and positive colonies were identified by single colony PCR (method of Young and Blakesly: Ref. 16). Plasmid DNA was then extracted from the positive clones and purified using the QIAgen miniprep kit (QIAgen AG) and subjected to dideoxy-sequencing using either the Sequenase 2.0 kit (Amersham, plc) or an Applied Biosystems Inc. automated DNA sequencer.

**Southern Blot Analysis**—Genomic DNA was digested to completion with EcoRI, separated on a 1% agarose gel, and transferred to a Hybond N (Amersham) nylon membrane using standard procedure (17). The hybridization was carried out in phosphate buffer (18), and the blots were washed at high stringency (0.2 x SSC, 1% SDS, 65°C). The probe used for detection of the class I chitinase gene was the 540-base pair EcoRI fragment of the genomic clone LCH200 (19). The probe used for detection of the auxin-binding protein (ABP) gene was the first kilobase of nucleotide sequence upstream from the coding region of the 785 gene (EMBL accession no. X70902 (1993); S. Shimomura).

**RESULTS**

**Quantitative Determination of DNA Methylation by the SssI Methylase Accepting Assay**—SssI methylase (CpG methylase) is used to methylate position 5 of cytosines in CpG sites in DNA. When [³H]SAM is used as a substrate, the radioactivity incorporated into the DNA is proportional to the number of unmethylated CpG sites in the genome. The original method of Wu et al. (10) was modified in order to decrease the nonspecific background. After the SssI methylase reaction, the labeled DNA was bound to DEAE-cellulose filters and washed in low salt conditions as described under “Materials and Methods.” The maximal recorded value, in the absence of DNA, was 0.15% of the counts/min obtained for the λ DNA and 4% of the value for SR1 tobacco DNA (data not shown). The DNA concentration dependence of the incorporation of tritiated methyl groups into λ and tobacco DNA is shown in Fig. 1 (top panel).

The maximal incorporation of tritiated methyl groups (pmol/μg of DNA) was obtained between 0.1 and 0.5 μg of a control DNA and at 1 μg of tobacco DNA. Beyond these values, the incorporation was no longer proportional to the DNA concentration;
PhosphorImager. The image and the quantification of the spots were obtained using a PhosphorImager (Fig. 2A, two-dimensional thin layer chromatography analysis of the methylation of the internal cytosine at CCGG (MspI) sites from total genomic DNA from N. tabacum treated or not treated with cefotaxime. C stands for cytosine and mC for 5-methylcytosine. B, the table shows the relative intensity of the radioactive spots on the TLC plate. The image and the quantification of the spots were obtained using a PhosphorImager.

Therefore, 0.3 μg of tobacco DNA were used per reaction. The time dependence for the incorporation of labeled methyl groups into tobacco DNA (Fig. 1, middle panel) indicated that the reaction was complete after 2 h of incubation. The lower panel demonstrates that the incorporation of the tritiated methyl groups into λ DNA was inversely proportional to the initial state of methylation at CpG sites. When the picomoles of methyl groups incorporated/μg of DNA were plotted against the initial methylation state of the DNA substrate, the curve was approximately linear (r = 0.999), which supports the hypothesis that the number of labeled methyl groups incorporated into the DNA substrate is a true reflection of its initial methylation state. To confirm the reliability of the SsSI accepting assay, the relative percentage of mC in DNA was quantified using the method of Bestor et al. (8). DNA from plantlets treated with cefotaxime (500 mg/liter) and control plantlets (cefotaxime, 0 mg/liter) was analyzed at the MspI (CCGG) sites, and Fig. 2A shows a PhosphorImager picture of the labeled nucleotides separated by TLC. It is clear that, in the cefotaxime-treated plantlets, the proportion of mC is larger than in the control plantlets, and quantification of this difference using a PhosphorImager (Fig. 2B) confirmed the extent of the cefotaxime-induced hypermethylation. The same results were obtained when the same DNA was analyzed by the SsSI methylase accepting assay, as shown in the left panel of Fig. 3. The SsSI accepting assay can therefore be used to analyze the methylation status of tobacco DNA.

Cefotaxime, Kanamycin, and Hygromycin Cause DNA Hypermethylation—Plantlets exposed in culture to antibiotics such as cefotaxime, kanamycin, and hygromycin showed a dose-dependent increase in the mC content of their DNA (Fig. 3). The plantlets were treated for 1 month with cefotaxime at either 250 or 500 mg/liter, kanamycin at either 100 or 400 mg/liter, or hygromycin at 20 mg/liter. All three drugs gave varying degrees of DNA hypermethylation. The strongest hypermethylation of DNA was observed with cefotaxime at 500 mg/liter, where an increase of 3 x 10^7 methylated CpG haploid genome was observed. As seen in Fig. 4, DNA hypermethylation was time-dependent. An exposure of the plantlets to 20 mg/liter hygromycin for 15 days resulted in DNA hypermethylation of 2.7 ± 0.7 Δpmol of CH$_3$/haploid genome, and after 30 days of treatment the hypermethylation was 10.6 ± 1.3 Δpmol of CH$_3$/haploid genome.

**State of DNA Methylation after the Removal of Antibiotics**—To test whether the DNA methylation was reversible upon the removal of the antibiotics, tobacco plantlets were first cultivated for 5 weeks on media containing cefotaxime at 500 mg/liter and subsequently cultivated for 2 weeks without antibiotics in the media (Fig. 5). The organs produced during the period of antibiotic treatment were scored and tested separately from the ones produced after the period of antibiotic treatment. The results (Fig. 5) showed that the level of hypermethylation induced by the antibiotics during 5 weeks of treatment (15.6 ± 2.4 Δpmol of CH$_3$/μg of DNA) remained unchanged during the 2 weeks after removal of the drug. However, the organs produced after the removal of the antibiotic showed a reduced DNA hypermethylation (2.9 ± 0.2 Δpmol of CH$_3$/μg of DNA), indicating that for the newly synthesized organs there is, in absence of the drug, significantly less DNA hypermethylation. The same trend was also observed in plantlets cultivated in the presence of the antibiotics cefotaxime (500 mg/liter) and hygromycin (40 mg/liter) for 1 month, then transferred into soil and grown until seeds were produced. The seeds were germinated without exposure to drugs. The incorporation of tritiated methyl groups in the mather plant DNA after the culture period was 0.15 ± 0.03 pmol of CH$_3$/μg of DNA whereas the progeny gave a value of 2.9 ± 0.4 pmol of CH$_3$/μg of DNA.
a value comparable with that of the wild type plantlets. 

**Methylation of Different DNA Fractions**—DNA hypermethylation of cefotaxime-treated tobacco plantlets was also tested in DNA fractionated by their Cot values. Table I shows the results of the SssI accepting assay on highly, moderately or lowly repeated DNA fractions. The results indicate that hypermethylation affected all three DNA fractions. However, highly repeated DNA fractions showed more methylation than the average and low repeated DNA fractions showed less.

**Analysis of DNA Methylation of Single-copy Genes**—Sodium bisulfite reacts readily with unmethylated, but not methylated, cytosines causing the deamination of cytosine to uracil under acidic conditions. When the chemically modified DNA is cloned and sequenced any methylated cytosine will be observed in the “C” track, whereas an unmethylated cytosine will be read as a “T.” The methylation pattern of a 120-base pair region of the tobacco auxin-binding protein gene T85 (EMBL accession no. X70902 (1993)) was determined for DNA extracted from plantlets grown on liquid LS media, without hormones, as controls or in the presence of 500 mg/liter cefotaxime. Four clones from each DNA sample were analyzed by DNA sequencing. Fig. 6 shows that the DNA from untreated plantlets (grown in the absence of cefotaxime) was unmethylated in the promoter region, whereas, for plantlets grown in the presence of cefotaxime, substantial Cpg methylation was observed. Three out of four clones showed a strong bias toward de novo methylation of CpG sites with 100%, 40%, and 60% of all CpG sites being methylated. Aside from CpG sites, CpgpG and CptpG sites were also methylated. The clones 500a appeared to be completely methylated, even at non-symmetrical sites. These results were not due to an artifact of the bisulfite reaction, since other DNA samples treated at the same time and with the same batch of sodium bisulfite showed no DNA methylation (see Fig. 6, 0a, lane T). The complete conversion of the control DNA templates also demonstrated that there was no sequence-specific protection of the DNA that might prevent its complete reaction with the bisulfite. A 191-base pair region of the promoter for the class I chitinase gene was also analyzed for sequence-specific methylation by genomic sequencing of the bisulfite reaction products. Fifteen clones from each of soil-grown tobacco plants, plants grown in culture in the absence of cefotaxime (cef-0), and plants grown in culture in the presence of 500 mg/liter cefotaxime (cef-500) were sequenced (Table II). As with the ABP gene, T85, none of the clones from soil-grown plants showed any methylation in this region. Two of the clones from cef-0 plants showed a small amount of de novo methylation, and eight of the cef-500 clones showed the same (Table II). This de novo methylation did not appear to be directed to specific sites but instead showed considerable heterogeneity from clone to clone; however, an analysis of the context of this methylation (once site availability and the number of clones had been taken into account) showed that in both the ABP and class I chitinase genes the preferred target sites were CpG > Cpnpg > CpX and the approximate preferences for each site were, respectively, 1:0.5:0.25 (Table III). This indicates that while CpG is the preferred target for the methylation other sites are also affected. The fact that we were able to detect drug-induced methylation changes in two short regions of two independent sequences further serves to confirm the scale of this phenomenon.

**Analysis of Gene Amplification**—To test the possibility that a drug-induced gene amplification could be the cause of DNA hypermethylation, the copy number of the class I chitinase gene and the ABP gene, T85, were compared between plants cultivated in the presence or absence of cefotaxime. Southern blot analysis using the respective probes showed no increase in the copy number of either the class I chitinase or the ABP genes between the controls and the treated plants. We conclude that the DNA hypermethylation observed here was not due to gene amplification.

**DISCUSSION**

Somaclonal variations in plant DNA methylation patterns have been reported to arise frequently during tissue culture. Methylation changes are associated with differentiation processes such as embryogenesis (21) and plant regeneration, leading to epigenetic variation of certain traits as well as gene silencing (for reviews, see Refs. 22 and 23). For example, Ingebretsen et al. (24) have described a case of posttranscriptional silencing of reporter transgenes in primary transformants of tobacco. This silencing was correlated with DNA methylation, and this de novo methylation was not restricted to cytosines located in the CG or CNG context. The level of methylation is also known to be affected by exogenous chemicals such as AZT (which induces hypermethylation) and 5-azacytidine (which induces hypomethylation). The results presented in this paper show that the antibiotic hygromycin, kanamycin, and cefotaxime all induce a genome-wide hypermethylation in cultured tobacco plants. As all of these antibiotics are routinely used in the production of transgenic plants, this observation may be of critical importance in the study of certain types of gene silencing that plague this area of research (24, 25). In addition to any regulatory consequences that methylation may have on gene expression, it will also increase the risk of point mutations created by the spontaneous deamination of methyl cytosine into thymine, although this need not necessarily give rise to a specific phenotype.

The mechanism of antibiotic-induced methylation of DNA remains unclear; however, at least in the case of hygromycin and kanamycin (which both affect protein synthesis), it is possible that the drugs could act by slowing down the cell cycle to prolong the S phase. This would provide a wider window for the DNA methyltransferase to act on the DNA, but this cannot be
the entire explanation, as cefotaxime (a penicillin derivative) does not have any known cellular target. Another possibility is that there could be an overall increase in methyltransferase activity. Mammalian DNA methyltransferase can be induced into efficient de novo methylation directly by proteolytic cleavage. Induction could also occur as a consequence of an increase in methyltransferase activity.
in specific activators stimulating the enzyme or a decrease in the number of negative regulatory factors bound to non-methylated CpG sequences. The simplest model, however, would be that the DNA substrate becomes more susceptible to methylation by changes in its structure such as the appearance of heteroduplexes, single-stranded loops, or mismatches, all of which are known to stimulate methyltransferase activity (for reviews, see Refs. 26 and 27). The occurrence of single-stranded loops could account for the non-symmetrical methylation by creating hairpin structures bringing together C and G residues to form symmetrical CpG or CpNpG sites.

The observation that drug-induced hypermethylation only occurs during the application of the antibiotics is significant as it leads to the construction of a hypothesis which is currently being tested. Changes in methylation are usually accompanied by cell division, so the most likely targets for the antibiotics are the meristematic cells, which are constantly dividing and differentiating to produce new plant material. If the antibiotics interfere in the pathway toward differentiation in the daughter cells of the meristems, then they may become hypermethylated, whereas the meristem itself would remain unaffected and would not change its methylation status. Thus, once the antibiotics have been removed, any pre-existing cells will continue to be hypermethylated, but new growth will rapidly revert to

### Table II

Cefotaxime-induced cytosine methylation of the tobacco class I chitinase promoter

The circled numbers refer the number of each cloned bisulfite product from each of the DNA samples analyzed. The numbers in bold indicate the position of the sequence relative to the start of transcription. "Sequence" indicates the published sequence for this 192-bp region of the class I chitinase promoter from *N. tabacum.* "Leaf" shows the observed methylation pattern in clones derived from uncultured leaf material. "Cef-0" shows the observed methylation pattern in clones derived from plants cultured in the absence of the antibiotic cefotaxime. "Cef-500" shows the observed methylation pattern in clones derived from plants cultured in the presence of 500 mg/liter cefotaxime. Dots mark the positions of unmethylated cytosines, and "m" marks the positions of methylated cytosines.

| -587 | GTGCCTAGC AAAATATTA AAGATTATG AAAATCTTA GAGCCTCAT | Sequence |
|------|--------------------------------------------------|----------|
| Leaf | ...                                               |          |
| Cef-0 | ...                                               |          |
| Cef-500 | ...                                               |          |

| -587 | TTAATATATG TAACCTGCA GCCTATGTA CTGATATG TTCTGATA | Sequence |
|------|--------------------------------------------------|----------|
| Leaf | ...                                               |          |
| Cef-0 | ...                                               |          |
| Cef-500 | ...                                               |          |

| -587 | AGTAAGACAG GACCCTAGA TTGCCTGTT TGTTATGCA AGATGGAT | Sequence |
|------|--------------------------------------------------|----------|
| Leaf | ...                                               |          |
| Cef-0 | ...                                               |          |
| Cef-500 | ...                                               |          |

| -587 | TGTCGCTAGAG GTCGCGCCT CAACACTAA AATCTTCTTG | Sequence |
|------|--------------------------------------------|----------|
| Leaf | ...                                         |          |
| Cef-0 | ...                                         |          |
| Cef-500 | ...                                         |          |
**TABLE III**

**Sequence context of the cefotaxime-induced de novo methylation**

The preference of each potential site of methylation (CpX, CpG, and CNG) is calculated as a proportion of the total frequency of each site. The data were then normalized to give the preference of each site relative to CpG to ease comparisons between the two genes. ABP refers to the T85 auxin-binding protein gene, and chitinase refers to the class I chitinase gene from tobacco. The relative preference of each site is very close between the two genes (G/H, H/H, and I/H), but the extent of methylation in the ABP gene is approximately 6 times higher than that observed in chitinase gene (values G, H, and I).

|                      | ABP      | Chitinase |
|----------------------|----------|-----------|
| Cpxs methylated/no. of clones (A) | 5.5      | 0.27      |
| Cpgs methylated/no. of clones (B) | 3.75     | 0.33      |
| CNGs methylated/no. of clones (C) | 0.5      | 0.4       |
| No. of Cpxs/no. of Cs (D)         | 0.79     | 0.66      |
| No. of Cpgs/no. Cs (E)           | 0.18     | 0.09      |
| No. of CNGs/no. Cs (F)           | 0.04     | 0.25      |
| Cpx preference (A/D = G)         | 7.0      | 0.41      |
| Cpg preference (B/E = H)         | 20.8     | 3.52      |
| CNG preference (C/F = I)         | 14.0     | 1.6       |
| Relative Cpx preference (G/H)    | 0.34     | 0.12      |
| Relative Cpg preference (H/I)    | 1.00     | 1.00      |
| Relative CNG preference (I/H)    | 0.67     | 0.45      |

the wild type pattern of methylation. This hypothesis is supported by the experiment illustrated in Fig. 5. The property of meristem integrity has long been known and is fact often used as a means of rescuing plants from systemic viral (or other pathogen) infections. Several authors have suggested that DNA methylation could be involved in defense reactions, both in plants and animals, and, moreover, de novo methylation has been shown to be associated with retrovirus (28) and viroid (29) inactivation. As antibiotics are produced by fungi, which are pathogens of plants, a high dose of antibiotics could mimic a pathogen attack and induce the plant into a defensive posture. If this were the mechanism, then the proposed lack of meristem methylation would provide a way for the plant to “stand down” after surviving a fungal attack. However, if a methylation change did occur in the meristems, then it would be expected to be maintained irrespective of the presence or absence of antibiotics. Meyer et al. (20) have observed, in transgenic petunia, the selective methylation of foreign DNA integrated into an otherwise hypomethylated region of the genome. This methylation was probably a defense response of the plant to invading DNA, possibly by de novo methylation of the single-stranded T-DNA loop during integration, but the application of high concentrations of antibiotics at this stage may serve to exacerbate the situation, resulting in even more methylation and thus gene silencing.

Antibiotic-induced DNA hypermethylation should be considered as a drawback of classical selection procedures for plant tissue culture, and attempts should be made to reduce the use of such compounds in order to reduce their undesirable effects on plant development and gene expression. We suggest that perhaps the most critical stage for the long term phenotype of the plant may be early in its development, and the use of large concentrations of cefotaxime in particular immediately after co-cultivation of leaf pieces with Agrobacteria may be inadvisable.

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