Alterations in *nptII* and *gus* Expression following Micropropagation of Transgenic M.7 Apple Rootstock Lines

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ABSTRACT. Seven *nptII* and *gus* transgenic lines of the apple (*Malus ×domestica* Borkh.) rootstock Malling 7 (M.7) were examined by glucuronidase (GUS) histochemical testing and a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). These lines had different amounts of neomycin phosphotransferase II (NPTII). The amounts of NPTII among lines was positively correlated with the ability of the transgenic lines to regenerate in the presence of kanamycin, paromomycin, or geneticin. Regenerants derived from transgenic lines also varied greatly in GUS expression. The apical portion of regenerant stem tissues had stronger GUS staining than the basal portion of stem. All regenerated tissue of T1, a transgenic line originally classified as a uniform GUS staining line, showed non-GUS staining, while the regenerated tissues of chimeric transgenic lines showed nonstaining, chimeric staining, or uniform GUS staining, indicating the potential to select uniform GUS staining lines from chimeras. Polymerase chain reaction (PCR) indicated the *gus* gene was present in GUS negative (nonstaining) lines. Negative PCR results with primers derived from *vir G* of *Agrobacterium tumefaciens*, and failure to isolate *A. tumefaciens* from M.7 transgenics indicated that PCR and GUS staining results were not due to *A. tumefaciens*. A modified PCR methylation assay (MPMA) indicated that methylation of cytosines of the CCGG site in the *gus* gene, and in the border between the CaMV35S promoter and the *gus* gene, was positively correlated with complete *gus* gene silencing (nonstaining lines). However, the MPMA indicated that methylation was not always associated with variable GUS expression, suggesting that chimeric staining could be due to a mixture of transformed and nontransformed cells in some transgenic lines.

In the apple scions 'Greensleeves' (James et al., 1989), 'Delicious' (Sriskandarajah et al., 1994), and 'Royal Gala' (Yao et al., 1995) and the rootstocks M.26 (Maheswaran et al., 1992) and M.7 (Momol et al., 1996; Norelli et al., 1994), transgenic plants have been obtained through *Agrobacterium*-mediated gene transformation. Transformation of apple requires a selectable marker to select transformed cells and a screenable marker to confirm gene transfer. The *nptII* gene and the *gus* gene have been regarded as marker genes with stable gene expression (Martin et al., 1990; McDonnell et al., 1987).

However, variation in marker gene expression has been reported in transgenic lines and has been observed in apple transgenics (Yao et al., 1995). In GUS histochemical assays to assess transformation, false positive results due to *Agrobacterium* contamination, false negative results from *gus* gene silencing, and variation of GUS staining due to tissue specificity and incomplete gene insertion could decrease the efficiency of screening for transformants.

The tissue sampled when doing GUS histochemical assays is important, as variation of *gus* gene expression has been reported to depend on the tissue or cell type, and tissue culture conditions in transgenic rice (*Oryza sativa* L.) and tomatillo (*Physalis ixocarpa* Brot.) (Assad-García et al., 1992; Battraw and Hall, 1990). Yao et al. (1995) reported that GUS staining was more intense in the vascular tissues of transgenic 'Royal Gala' apple plants than in any other stem tissue. In addition, the promoter region will affect gene expression (Mitsuhara et al., 1996). The possibility of *gus* gene silencing has been reported in petunia (*Petunia hybrida* L.) (Linn

et al., 1990), potato (*Solanum tuberosum* L.) (Ottaviani et al., 1993), maize (*Zea mays* L.) (Kaeppler and Phillips, 1993), and other crops. Several reports suggested that gene silencing is positively correlated with *gus* gene methylation (Ottaviani et al., 1993; Pua, 1994).

Expression of the *nptII* gene has been reported to vary in cotton (*Gossypium hirsutum* L.) depending on the organs sampled per line and among transgenic lines (Özcan et al., 1993; Rogan et al., 1992). These variations in expression of the *nptII* gene and the *gus* gene could be problematic for the antibiotic selection of transformed cell lines and for confirmation of transformation in screening for uniform GUS-staining lines, respectively. We attempted to design a reliable assay to detect the variation of marker gene expression in selection and screening procedures. The objectives of this study were to determine 1) variation in the expression of the *nptII* gene among transgenic lines, 2) whether the amount of NPTII is correlated with shoot regeneration rate, 3) variation in GUS staining among different stem portions, and 4) whether lack of GUS expression is correlated with DNA sequence methylation within the promoter region of the transgene.

Materials and Methods

PLANT MATERIALS. Plant materials were obtained from stocks maintained at the Dept. of Plant Pathology, Cornell Univ., New York State Agricultural Experiment Station, Geneva. Nontransgenic M.7 and seven transgenic apple rootstock lines of M.7 that had been subcultured on proliferation media for >2 years were studied. The plants were transgenic for *nptII*, *gus* gene, and a gene encoding a lytic peptide, attacin E or SB-37, and were derived using *Agrobacterium*-mediated transformation (Nordeen et al., 1992; Norelli et al., 1994) (Table 1). Previously, these transgenic lines were classified as uniform or chimeric on the basis of uniformity and nonuniformity of staining in GUS histochemical assays, respectively.

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Table 1. Transgenic apple rootstock lines obtained after transformation of M.7 with *A. tumefaciens*. All transgenic lines contained *nptII* and *gus*.

Transgenic line ^a	T1	T34	T55	T56	T57	T58	T66
<i>A. tumefaciens</i> ^a strain	LBA4404	EHA105	EHA105	EHA105	EHA105	EHA105	EHA105
Useful molecule ^a	Attacin E	SB-37	SB-37	SB-37	SB-37	SB-37	SB-37
Vector plasmid ^a	pLDB15	pBPRB37	pBCCB37	pLDB10	pBPRB37	pBCCB37	pBCCB37
Selecting antibiotics ^a	Pa10	Km100	Km100	Km100	Km100	Km100	Km100
Chimera analysis ^a	Uniform	Uniform	Chimera	Chimera	Chimera	Chimera	Chimera
E	+	+	c	c	c	—	c
VC	+	+	c	+	c	—	c
P	+	+	+	+	+	s	+

^aT1 (Norelli et al., 1994).

^a*Agrobacterium* strains that were used to transfer vector plasmid into apple plant.

^aBactericidal proteins encoded by transferred genes; attacin E (Norelli et al., 1994) and SB-37 (Nordeen et al., 1992) controlled under CaMV 35S with duplicated upstream signals.

^aVectors are based on pBI121, where *nptII* and *gus* are controlled under NOS and CaMV 35S promoter, respectively. pLDB15 and pLDB10 (Norelli et al., 1994); pBPRB37 and pBCCB37 [pLDB10 containing the coding region for the signal of the PR1 protein of tobacco and the signal peptide in the Cecropin B protein of *Hyalophora cecropia*, respectively, fused to the NH₂-terminal and of SB-37 (L. Destéfano-Beltrán, personal communication)].

^aPa10 = 10 µg·mL⁻¹ of paromomycin, Km100 = 100 µg·mL⁻¹ of kanamycin.

^aAs described in the GUS histochemical assay section; E = epidermis and cortex, VC = vascular cylinder, P = pith.

^a(+)Uniform GUS staining, c = chimeric GUS staining, and (—)non-GUS staining. This evaluation was conducted before subculturing.

SHOOT REGENERATION. Leaf discs from each transgenic plant were recultured on a regeneration medium with three antibiotic concentrations. Regeneration medium consisted of Murashige and Skoog's (1962) inorganic salts, Linsmaier and Skoog's (1965) vitamins, sucrose (30 g·L⁻¹), 1*H*-indole-3-acetic acid (IAA) (10 mg·L⁻¹), thidiazuron (TDZ) (1 mg·L⁻¹), and cefotaxime (250 µg·mL⁻¹), solidified with agar (7 g·L⁻¹). The pH of the medium was adjusted to 5.7 with 1 N KOH. Twelve antibiotic treatments—kanamycin (10, 50, 75, and 100 µg·mL⁻¹), paromomycin (50, 100, 200, and 250 µg·mL⁻¹), and geneticin (1, 2.5, 10, and 50 µg·mL⁻¹)—were used as selection antibiotics. The shoot regeneration rate of each transgenic line was recorded as the percentage of leaf pieces forming shoots at this selection culture stage. Since regeneration rates varied among experiments, regeneration was expressed as a percentage of the amount of regeneration in control plants (in the absence of any antibiotics). After 4 to 5 weeks on regeneration medium, the shoots were transferred to tubes containing regeneration medium modified with casein hydrolysate (0.4 µM), paromomycin (100 µg·mL⁻¹), 6-benzylaminopurine (BAP) (1 µg·mL⁻¹), and naphthaleneacetic acid (NAA) (1 µg·mL⁻¹) instead of TDZ and IAA. After 3 to 4 weeks, the shoots were anchored in a tangential orientation in proliferation medium (Norelli et al., 1988) containing cefotaxime (250 µg·mL⁻¹).

ELISA TEST. Leaf tissue from the transgenic lines and nontransgenic M.7 was harvested from shoots maintained on proliferation medium for 3 to 5 weeks. Protein was extracted by grinding 10 to 20 mg of young leaf tissue in an extraction buffer that consisted of 10 mM sodium sulfite, 2% (w/v) polyvinylpyrrolidone (mw 24,000 to 40,000), 3 mM sodium azide, 10.2% (w/v) bovine serum albumin, and 2% (v/v) Tween-20. NPTII antibody, biotinylated NPTII antibody, and streptavidin conjugated with alkaline phosphatase were obtained from 5Prime-3Prime Inc (Boulder, Colo.) and sandwich ELISA was conducted following the manufacturer's recommended procedure. The experiment was performed twice and, in each experiment, four leaf samplings were made of each line. The absorbance was read using a microplate reader (MR700; Dynatech Laboratories, Chantilly, Va.). The LSD test was conducted to determine whether there were differences in NPTII absorbance among the transgenic lines.

GUS HISTOCHEMICAL ASSAY. Regenerants derived from seven transgenic M.7 apple lines, T1, T34, T55, T56, T57, T58, and T66

were assayed for GUS staining. After 3 to 5 weeks on proliferation medium, GUS histochemical assays were conducted using 5-bromo-4-chloro-3 indolyl glucuronide (X-Gluc; Research Organics, Cleveland) as described by Jefferson (1987). One shoot was harvested randomly from each tube containing regenerated shoots; 429 shoots were assayed. The shoots were cross-sectioned into segments 0.2 to 0.4 mm thick with a sharp blade. The stem cross-sections were of two groups: apical and basal. The apical sample was within 2 mm of the apical meristem. The basal sample was from within 2 mm of the sample stem junction with the main stem.

Stem cross-sections were placed in 96-well plates containing X-Gluc solution consisting of 100 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1% (w/v) X-Gluc. The plates were incubated overnight, then examined with a dissecting microscope to determine GUS activity in three tissue layers: E (epidermis and cortex), VC (vascular cylinder), and P (pith). The staining of the cross-sections was evaluated as uniform (+), with complete GUS staining over one layer, chimeric (c), with incomplete GUS staining, and nonstaining (—), with no GUS staining. Lines with all three layers stained uniformly (+) were classified as uniform lines, while lines where no layers were stained were classified as nonstaining lines. Lines where only one or two layers were stained or partially stained were classified as chimeric lines.

ISOLATION AND DETECTION OF AGROBACTERIUM. To confirm that transgenic lines were not contaminated with residual *Agrobacterium*, 10 mg of tissue culture samples from T1, T57, T58, and T66, which had been stored at -80 °C for 1 year, were ground in 120 µL of 5 mM phosphate buffer, pH 6.5. The ground plant materials were plated on potato dextrose agar (PDA) medium (Schaad et al., 1993) containing 50 mg·L⁻¹ of kanamycin. After a 48-h incubation at 28 °C, the plates were observed for bacterial growth. To confirm that bacterial growth was *A. tumefaciens*, the plate surface was rinsed with 2 mL of sterile distilled water, and wash water was subjected to PCR amplification using the *virG* primers (forward: GAGTACTCTCTTCCGCGACG, reverse: TTGGAA-TATCAGACTTTGCGG). To determine the sensitivity of the isolation and detection procedure, nontransgenic M.7 plant tissue was ground in 5 mM phosphate buffer containing *A. tumefaciens* EHA105 (pLDB15) populations that ranged from 9 to 9 × 10⁶ cfu/g at power of 10 intervals and grindates were plated as described above.

Fig. 1. Restriction map of the CaMV35S promoter 2647-bp fragment containing the CaMV35S-GUS gene. The restriction sites of enzymes used for the methylation assay by modified polymerase chain reaction are described. (A) The 210-bp fragment is amplified by 35S F (forward) and GUS R108 (reverse) primers. (B) The 1165-bp fragment is amplified by 35S F (forward) and GUS R1063 (reverse) primers.

MODIFIED PCR METHYLATION ASSAY. Genomic DNA was extracted as described by Norelli et al. (1994) from leaf tissues of transgenic lines (T1), and chimeric lines (T55, T56, T58, and T66) and their regenerants, maintained on proliferation medium for 3 to 5 weeks. DNA was digested with restriction enzymes that were methylation-sensitive isochizomeric pairs of endonuclease (*MspI/HpaII*), *HhaI*, and *MboI* according to the strategy of Weber et al. (1990) (Fig. 1). A programmable thermal controller (PTC-100; MJ Research, Watertown, Mass.) was used for PCR. Each PCR reaction was carried out in 50 μ L containing 5 μ L of 200 μ M dNTP, 1.5 mM magnesium chloride, 5 mM each primer, 100 ng restricted genomic DNA, and 1.25 units of Taq DNA polymerase. DNA was amplified for 40 cycles of 1.5 min at 94 $^{\circ}$ C, 2 min at 55 $^{\circ}$ C and 2 min at 72 $^{\circ}$ C. Two sets of primers were used for amplification. Both used the same forward primer that originated in the 35S promoter (5'-CCCACTATCCTTCGCAAGACCC-3'). The first reverse primer (5'-CAGCAGCAGTTTCATCAATCA-3') started at base pair 1063 of the GUS coding region and produced a 1165-bp fragment. The second reverse primer (5'-TTCCCACCA-ACGCTGATC-3') started at base pair 108 of the GUS region and yielded a 210-bp fragment, which amplified the border region between the 35S promoter and the *gus* gene (Fig. 1). The amplified DNA was stained with ethidium bromide after electrophoresis on a 1% agarose gel in Tris-borate buffer (45 mM Tris-borate and 1 mM EDTA), detected by UV light, and photographed. To verify that the PCR bands obtained were not due to *A. tumefaciens* contamination, unrestricted DNA was amplified with primers for *vir G* as described above.

Results and Discussion

VARIATION OF NPTII LEVEL IN APPLE TRANSGENIC LINES. Transgenic M.7 lines differed significantly in the amount of NPTII they produced (Fig. 2). T55 and T66 had the highest absorbance values, T56 and T57 had intermediate absorbance, and T1 and T58 had the lowest absorbance. Nontransgenic M.7 had a very low absorbance level that was attributed to background. These results were consis-

tent with studies in cotton, where individual transgenic lines had varying levels of NPTII (Rogan et al., 1992). Variation of *nptII* expression level might be due to the amount of gene transcript, which is influenced by the gene copy number; position effects, where adjacent plant DNA affects the expression of randomly inserted and introduced genes (Hobbs et al., 1990); post transcriptional control (Ingelbrecht et al., 1994); and the quality and integrity of the transgene sequence.

THE CORRELATION BETWEEN NPTII LEVEL AND REGENERATION RATE. Since shoot regeneration is a critical step in transformation, we determined whether shoot regeneration rate in the presence of antibiotics was correlated with NPTII expression level. Correlations were tested within a given concentration of each antibiotic. In general, with all antibiotics tested there was a significant positive correlation

between the level of NPTII and the rate of shoot regeneration (Fig. 3). The transgenic lines with highest NPTII levels (T55 and T66) had the highest shoot regeneration rate (Fig. 3). T56 and T57 had intermediate NPTII levels and shoot regeneration rates. T1 and T58 had the lowest NPTII level. T1 had the least shoot regeneration, while T58 was intermediate. Correlation coefficients between NPTII level and regeneration rate on kanamycin media ranged from 0.74 to 0.93.

At 75 μ g·mL⁻¹ kanamycin, the coefficient was the highest (Fig. 3a). On paromomycin medium, the correlation coefficient ranged from 0.39 to 0.77 (Fig. 3b). On geneticin medium, the correlation coefficient ranged from 0.50 to 0.72 (Fig. 3c). The highest correlation was observed on media containing kanamycin compared to the correlations observed with the other antibiotics.

These results indicate that, in apple transgenic lines, greater levels of *nptII* expression probably result in more shoot regeneration in the presence of a selection antibiotic. The increased shoot regeneration on selection media is most likely due to increased

Fig. 2. NPTII activity (absorbance) of *nptII* transgenic M.7 apple lines. Absorbance was measured using a microplate reader (MR 700; Dynatech Laboratories) after a NPTII double-sandwich ELISA using alkaline phosphatase. "1st Exp." and "2nd Exp." indicate results from the first and second experiment, respectively. In each experiment four different leaf samplings from different jars of each line were made. Presented values of each line were the mean value of samples of each line in each experiment. Treatments within an experiment followed by the same letter did not differ significantly [LSD ($p = 0.05$)].

detoxification of the antibiotics by phosphorylation catalyzed by NPTII (Yoshikura, 1989). It is unclear if transgenic lines with high *nptII* expression are more desirable for obtaining nonchimeric lines. McHughen and Jordan (1989) speculated that extremely high *nptII* expression level could protect nontransformed cells, resulting in selection of chimeras.

TISSUE LAYER SPECIFICITY IN *gus* GENE EXPRESSION. Two of the lines, T1 and T34, were uniform GUS staining, and five were chimeric GUS staining (T55, T56, T57, T58, and T66). Although shoot tissues of T1 and T34 initially showed uniform GUS stain-

Fig. 3. Correlation between shoot regeneration and NPTII level (absorbance) in *nptII* transgenic apple lines. Shoot regeneration rate was measured as the percentage of leaf segments forming shoots. The rate is expressed as a percentage of the amount of regeneration that occurred in the M.7 control without antibiotics in the medium. Each graph shows the correlation between shoot regeneration and NPTII level (absorbance) at the concentration of each antibiotic that had the highest correlation coefficient value ($p = 0.05$).

ing, T1 ceased producing GUS (Fig. 4a) and T34 lost uniform GUS staining during subsequent culturing. Variation in *gus* gene expression was examined using GUS histochemical assays. In transgenic M.7 apple lines, the intensity of GUS staining differed among the cell layers of the stem (Fig. 4b). The vascular tissue stained more intensely than other parts of the stem. This agrees with results in transgenic tobacco (*Nicotiana tabacum* var. Samsun) (Jefferson et al., 1987), transgenic tomatillo (Assad-García et al., 1992), and transgenic apple (Srisankarajah et al., 1994; Yao et al., 1995). The stronger staining in vascular tissues may be due to the greater cell number per unit area (Jefferson et al., 1987).

GUS expression can also occur from contamination of tissue culture material with *A. tumefaciens* during the transformation process (Martin et al., 1990; Cassells, 1991; Matzk et al., 1996). The transgenic plants used in this study were subcultured on proliferation medium for >2 years with no evidence of *Agrobacterium* growth. However, Cassells (1991) argued that sampling tissue is necessary to detect residual *Agrobacterium*. Graham and McNicol (1990) crushed plant material and plated it onto nutrient rich medium to screen for residual *Agrobacterium* and Hamill et al. (1990) suggested using PCR amplification to detect *Agrobacterium* contamination. To determine if the M.7 transgenic lines contained residual *Agrobacterium* from transformation, plating of plant material on rich media was combined with PCR amplification (Schaad et al., 1993). No *Agrobacterium* was observed on plates containing grindates of transgenic lines or nontransgenic M.7, and no *vir* gene fragment was detected from PCR amplification of washings of these plates. However, *Agrobacterium* was detected when nontransformed M.7 was ground in buffer containing 9 cfu/g of *A. tumefaciens* EHA105 (pLDB15). The amount of tissue sampled per regenerant (10 mg) was determined by a limited availability of material. Matzk et al. (1996) found that tissue culture plants have high moisture contents that provide an ideal environment for the survival and growth of plant-associated bacteria. For this reason the sample was believed to adequately reflect the *Agrobacterium* content of the entire regenerant.

When DNA was isolated from fresh or frozen tissue culture material and subjected to PCR amplification using *gus* gene primers, no *gus* fragment was detected from the nontransgenic M.7 line, while *gus* fragments were detected in transgenic lines. These results indicate that the transgenic shoots were not contaminated with *Agrobacterium*.

Frozen samples were stored at -80°C without a cryoprotectant, and the issue of *Agrobacterium* surviving the freeze-thaw cycle might be questioned. van Asma (1995) found that *Agrobacterium* survived better at -80°C than 0 or -20°C for long-term storage. This work was done using a cryoprotectant. *Agrobacterium tumefaciens* was isolated from grapevine cane tissues collected in the Finger Lakes region of New York during February 1982 and 1983. In this region, during February, the temperature is usually $\approx 0^{\circ}\text{C}$ (Burr and Katz, 1984). T.J. Burr (personal communication) routinely stores *A. tumefaciens*-infected grape tissues at -20°C and recovers *A. tumefaciens* from thawed tissues with no loss of viability. We see no reason why there should be more freeze-thaw damage in tissue stored at -80 than at -20°C , since freezing and thawing occurs at ≈ 0 to -5°C , and not between -20 and -80°C .

Variable metabolic activity with corresponding differences in rates of transcription and translation between cell types also may affect GUS staining (Benfey and Chua, 1989; Jefferson et al., 1987). Because vascular layers have young cells and active cell division, the metabolic activity would be high, resulting in greater amounts of β -glucuronidase. For example, chimeric line T57 had

Fig. 4. Variation of GUS staining in stem cross-sections of transgenic M.7 apple rootstock lines. (A) Loss of detectable GUS staining after tissue culture and regeneration of regenerants of transgenic M.7 rootstocks. The two blue stems on the left are from a uniform GUS staining line, the two stems in the middle are from nonstaining regenerants of T1 and T66, and the stem on the right is from nontransgenic M.7. (B) Uniform, chimeric, and nonstaining classification as described in the materials and methods. The cross-sectioned stems on the left are uniform staining, that in the middle is chimeric, and those on the right are nonstaining. (C) Variation in GUS blue staining between the cross-sectioned stem of the apical and basal portion. The cross-sectioned stems on the left were sampled from the apical portion and those on the right were sampled from the basal portion. Blue staining is stronger in the apical portion of the stem than in the basal portion.

no GUS staining of xylem tissue, while phloem tissue was stained. This is consistent with observations in tomatillo (Assad-García et al., 1992), where phloem stained strongly but xylem tissue stained weakly or not at all. A possible explanation is that phloem has a higher density of active cells than xylem. The variation in GUS staining could also be explained by the existence of chimeras comprised of a mixture of transformed and nontransformed cells (Dong and McHughen, 1993; Lowe et al., 1995; Peña et al., 1995).

VARIATION OF *gus* GENE EXPRESSION IN APICAL AND BASAL STEM SAMPLES. GUS histochemical assays were conducted to determine the effect of tissue location on the variation of *gus* expression. GUS staining was stronger in the apical portion of the shoot sampled from regenerants of transgenic lines (Fig. 4c). Within transgenic regenerants, of the apical portions 572 were uniform staining, 639 were chimeric, and 76 were nonstaining, while of the basal portions 417 were uniform, 678 were chimeric, and 192 were nonstaining. Chi-square tests were conducted to compare GUS staining between apical and basal portions. The ratio of uniform, chimeric, and nonstaining sections differed significantly between the apical and basal portions. The apical portion had stronger GUS staining than the basal portion ($P < 0.005$).

There are several possible explanations for GUS staining differences due to age effects in staining. The metabolic activity of cells in the apical portion is higher than in the basal portion (Benfey and Chua, 1989). Jefferson et al. (1987) reported that the amount of β -glucuronidase could be higher in the apical portion because the cell population was higher. The cell size of the basal portion is larger than the cell size of the apical portion. Jefferson et al. (1987) and Benfey et al. (1989) found that differences in cell size could change the degree of staining observed; small cells have more intense blue staining, while large cells have less intense staining. Finally, the CaMV35S promoter may express the *gus* gene differently in young versus old tissue or between cell types (Jefferson et al., 1987). If the CaMV35S promoter is preferentially active in cells during the S phase of the cell cycle, as argued by Nagata et al. (1987), then the pattern of GUS staining may depend on cell division activity in these tissues.

A previous report (Jefferson et al., 1987) and this study showed that GUS histochemical assays were affected by the age of the tissue sampled in apple. Therefore, young apical tissues should be used for GUS assays to confirm transformation and in screening for transgenic lines in apple.

CORRELATION BETWEEN THE INHIBITION OF THE *gus* GENE AND METHYLATION OF THE CCGG SITE IN *gus* GENE AND THE BORDER BETWEEN THE 35S PROMOTER AND THE *gus* GENE. No GUS staining was observed in tissue from any T1 regenerants (Fig. 4a) or from some regenerants derived from transgenic lines T56 and T66. PCR results indicated that the nonstaining tissue had the *gus* gene present. In T1 transgenic tissue the presence of the *gus* gene was confirmed using Southern blotting (Norelli et al., 1994). A modified PCR methylation assay (MPMA) was used to determine if the lack of staining could be due to inhibition of *gus* gene expression caused by methylation of the *gus* gene or methylation between the border of the 35S promoter and the *gus* gene (Linn et al., 1990; Meyer and Heidmann, 1994; Meyer et al., 1992). The MPMA results are summarized in Table 2.

*Mbo*I was used as a restriction enzyme that is not sensitive to methylation of cytosine. Digestion of DNA treated with this enzyme eliminated detectable PCR template activity except for a 210-bp fragment amplified in second replicate of T55 C (Table 2). This weak band could have resulted from incomplete restriction procedure or DNA quality. When DNA was not digested with *Mbo*I, the 1165- and 210-bp fragments were amplified in GUS

uniform and nonstaining lines. These results indicate that DNA was digested when a methylation-insensitive enzyme was used. No bands were observed in nontransgenic M.7, indicating that there was no contamination.

The restriction enzymes *MspI*, *HpaII*, and *HhaI* are methylation sensitive, so, if methylation is present at these sites, the DNA available for PCR amplification is not cut and a band is present. With *HhaI*, the 1165-bp fragment was amplified in all transgenic lines tested, indicating that methylation of cytosine of GCGC sites is not critical for *gus* gene expression in these lines. Because the *HhaI* restriction site is not present on the 210-bp sequence, all lines should be positive for this band (Fig. 1).

MspI is inhibited by methylation of the external cytosine at the CCGG site, while *HpaII* is sensitive to methylation of both cytosines of the CCGG site. With *MspI*, the 1165- and 210-bp fragments were amplified in all GUS nonstaining lines and were not amplified in the uniform and chimeric lines tested, except for one of three replicates of solid line T55 Km10 for the 1165-bp fragment, two of three replicates of solid line T55 Km10 for the 210-bp fragment, and all replicates of solid line T55 C for the 210-bp fragment. These amplified bands could be due to partial digestion.

With *HpaII*, the 1165- and 210-bp fragments were amplified in all GUS nonstaining transgenic lines, while, in uniform and chimeric lines, no bands or weak bands were observed. T55 Km75 had amplified bands (1165 and 210 bp), in the first experiment, but not in the second experiment. The several positive bands and weak

bands observed with the 210-bp sequence could be due to partial digestion, since there is only one restriction site in this sequence. Also, GUS uniform or chimeric lines could have methylation in a small fraction of cells. Southern blot analysis has indicated some methylation of GUS uniform transgenic lines of potato, tobacco, and maize (Kaeppeler and Phillips, 1993; Ottaviani et al., 1993; Weber et al., 1990). However, the degree of methylation was higher in GUS nonstaining transgenic lines (Kaeppeler and Phillips, 1993; Ottaviani et al., 1993; Weber et al., 1990).

These results indicate that the GUS nonstaining lines tested had methylation on the external or internal cytosine of the CCGG site, while uniform GUS staining lines and chimeric lines had no or less methylation. Cytosine methylation is known to occur principally at the sequence CG and CXG in higher plant cells and may be correlated with gene inactivation (Gruenbaum et al., 1981; Ingelbrecht et al., 1994; Meyer et al., 1993). In the present work with apple, methylation of the external or internal cytosine of CCGG appears to be important in *gus* gene inactivation. Also, these results indicate that the loss of GUS activity in the transgenic line T1 and the absence of GUS activity in regenerants of T1 are correlated with methylation of the *gus* gene. PCR of DNA isolated from GUS-staining tissue amplified *gus* fragments but did not amplify *vir* fragments, indicating that detection of *gus* by PCR was not due to contaminating *A. tumefaciens*.

EVIDENCE THAT CHIMERIC GUS EXPRESSION IS DUE TO A MIXTURE OF TRANSFORMED AND NONTRANSFORMED CELLS. Since partial digestion has a dominant effect on the presence of a PCR band, if lines

Table 2. Results of modified polymerase chain reaction (PCR)-methylation assay with isochizomeric endonuclease restriction enzymes.

Transgenic lines	Type ²	Enzyme							
		<i>MboI</i>		<i>HhaI</i>		<i>MspI</i>		<i>HpaII</i>	
		1165 ^y	210 ^x	1165	210	1165	210	1165	210
T1 Km50 ^v	—	— ^u	—	+	+	+	+	+	+
T1 Pa50	—	—	—	+	+	+	+	+	+
T1 P ^t	—	—	—	+	+	+	+	+	+
T56 Pa200	—	—	—	+	+	+	+	+	+
T66 Pa200	—	—	—	+	+	+	+	+	+
T55 Km75	+	—	—	+	+	—	—	+	+
First		—	—	+	+	—	—	+	+
Second		—	—	w	w	—	—	—	—
T55 Km 10	+	—	—	+	+	—	—	—	—
First		—	—	+	+	—	—	—	—
Second		—	—	+	+	+	+	+	+
Third		—	—	+	+	—	w	—	w
T55 C ^r	+	—	—	+	+	—	w	w	w
First		—	—	+	+	—	w	w	w
Second		—	w	+	+	—	+	—	+
T55 Km50	c	—	—	+	+	—	—	—	w
First		—	—	+	+	—	—	—	w
Second		—	—	+	w	—	—	—	—
T55 P	c	—	—	+	w	—	—	—	—
T58 C	c	—	—	+	+	—	—	—	w

²GUS histochemical assay after proliferation or regeneration subculturing: (—)GUS negative (nonstaining) in all layers, (+)GUS positive (uniform staining) in all layers, c = GUS chimeric or negative staining in at least one layer.

^yThe size of the amplified fragment is 1165 bp. Primers were 35S F forward and GUS R1063 reverse primers.

^xThe size of the amplified fragment is 210 bp. Primers were 35S F forward and GUS R 108 reverse primers.

^u(+)Band present, (—)band absent, w = weak band, +/-w = predicted restriction fragments.

^vAntibiotic treatment: T1 Km50, where T1 indicates transgenic lines of R₀ regenerants, Km indicates kanamycin selection, 50 indicates the antibiotic concentration.

^tT1 P indicates transgenic line of R₀ regenerants.

^rThe experiment was replicated at least two times. If results varied, the results from each replicated experiment are shown.

^cC = control (no selection): T55 C, where T55 indicates transgenic line of R₀ regenerants.

appearing chimeric in the GUS histochemical assay were due to methylation of the *gus* gene of only some cells, results of MPMA would be the same as the nonstaining lines (Table 2). However, in the MPMA the chimeric lines were similar to the uniform lines, indicating that the chimeric appearance of the tissue in the GUS assay was not due to methylation. These MPMA results suggest that T55 and T58 could be genetic chimeras. McHughen and Jordan (1989) have suggested that uniform staining transgenic tissue might be obtained from chimeric lines by antibiotic selection procedures. On medium containing antibiotic we were able to select regenerants that appeared uniform staining for GUS expression from all of the transgenic lines (T55, T56, T57, T58, and T66) except T1. In this study we observed that the highest recovery of uniform GUS-staining regenerants (73 of 93 regenerants), as determined by GUS histochemical assays, was obtained with one of the highest NPTII expressors, T55, and the lowest recovery of uniform GUS-staining regenerants (2 of 80 regenerants) was obtained with the lowest NPTII expressor, T58, suggesting that high levels of NPTII expression favor the selection of uniform staining lines. However, the rate of recovery of uniform GUS-staining regenerants was variable among the transgenic lines and ranged from 3% to 78% of all regenerants. In addition, no trends could be detected between the recovery of uniform GUS staining regenerants and the type or amount of antibiotic used, with different transgenic lines responding differently to each antibiotic.

This study demonstrates that the micropropagated shoots from apple transgenic lines varied in *nptII* and *gus* expression. The selected trait, antibiotic resistance, showed a certain range of variation in NPTII levels by ELISA. Also, the nonselected trait, GUS staining, was absent in some lines and showed varied strength and tissue specificity in other lines. In addition, *gus* gene methylation appeared to be correlated with one nonstaining transgenic line but not in lines that appeared chimeric in GUS histochemical assays. Initial studies indicated that high GUS activity regenerants could be selected from cultured cells of chimeric lines under antibiotic selection. In screening transformants, sampling procedures must be optimized when using GUS histochemical assays. Younger apical tissue, rather than older basal tissue, should be chosen for GUS histochemical assays to avoid an effect of cell age or density on *gus* gene expression. Due to the variability of the assay, a reasonable number of replicates should be used. Furthermore, when using the expression of the GUS reporter gene as an indicator of transformation, lack of gene expression could be due to nontransformed tissue, a tissue age effect, gene insertion quality, or gene silencing resulting from transcription or post-transcription inhibition. Although this study examined the expression of the *nptII* and *gus* genes, the results suggest that silencing of agriculturally useful genes must also be considered when screening transgenic plants.

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