The Role of Aminoglycoside Antibiotics in the Regeneration and Selection of Neomycin Phosphotransferase-transgenic Apple Tissue

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Abstract. Regeneration from apple (*Malus × domestica* Borkh.) M.26 leaf tissue was completely inhibited by ($\mu\text{g}\cdot\text{ml}^{-1}$) 1 geneticin, 5 kanamycin, 10 to 25 paromomycin, and 100 neomycin. nptII-transgenic M.26 had an increased tolerance to all four of the antibiotics tested, with inhibition of regeneration occurring at ($\mu\text{g}\cdot\text{ml}^{-1}$) 2.5 geneticin, 100 kanamycin, 375 paromomycin, and 375 neomycin. Paromomycin (100 to 250 $\mu\text{g}\cdot\text{ml}^{-1}$) and neomycin (250 $\mu\text{g}\cdot\text{ml}^{-1}$) significantly increased the amount of regeneration from nptII-transgenic M.26 apple leaf tissue. p35SGUS-INT, a plasmid with a chimeric b-glucuronidase gene containing a plant intron, was useful for studying the early events of apple transformation by eliminating GUS expression from *Agrobacterium tumefaciens*. It was used to determine that the optimal aminoglycoside concentrations for the selection of nptII-transgenic M.26 cells were ($\mu\text{g}\cdot\text{ml}^{-1}$) 2.5 to 16 kanamycin, 63 to 100 neomycin, and 25 to 63 paromomycin. Geneticin was unsuitable as a selective agent.

The neomycin phosphotransferase (EC 2.7.1.69) gene (*nptII*) of the transposable element Tn5 has been used widely as a selectable marker in plant transformation vectors (Fraley et al., 1986). Neomycin phosphotransferase (NPT; EC 2.7.1.95) inactivates kanamycin A by phosphorylating the 3'OH of its 6-deoxy-6-aminoglucose-1-alpha sugar residue (Yoshikura, 1989). Due to its specificity, NPT is active against a limited group of aminoglycoside antibiotics including kanamycin, geneticin (G418), neomycin, and paromomycin (Yoshikura, 1989). Kanamycin is the antibiotic most frequently used for the selection of nptII-transgenic plants.

James et al. (1989) produced nptII-transgenic apple plants of the cultivar Greensleeves using a disarmed binary vector plasmid in *Agrobacterium tumefaciens* followed by kanamycin selection. Regeneration of transgenic plants was greatly reduced in leaf discs exposed to selection with 60 μg kanamycin/ml for more than 5 days (James et al., 1989). Yepes (1990), in our laboratory, extended transformation studies to several other apple cultivars and similarly found selection of nptII-transgenic plants with kanamycin to be inefficient and difficult. One of the purposes of this study was to evaluate the effects of various aminoglycoside antibiotics that are susceptible to NPT phosphorylation on the regeneration of nptII-transgenic and nontransgenic apple tissues.

Previous attempts to study the early events of transformation in apple using a storable B-glucuronidase (GUS; EC 3.2.1.31) marker gene (Jefferson et al., 1987) were of limited success because GUS activity in transformed cells and in still present *A. tumefaciens* could not be distinguished (Martin et al., 1990). In this

study, we used the GUS marker to determine optimal antibiotic concentrations for the selection of nptII-transformed cells by using a chimeric β -glucuronidase gene containing a plant intron (Vancanneyt et al., 1990) that eliminates GUS activity in *A. tumefaciens*.

Materials and Methods

Chemicals. Benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), geneticin (antibiotic G418), gibberellic acid (A_3 90% of total gibberellins) (GA), indolebutyric acid (IBA), kanamycin sulfate; 4-methylumbelliferone (MU), 1-naphthaleneacetic acid (NAA), and neomycin sulfate were obtained from Sigma Chemical Co., St. Louis; 4-methylumbelliferyl-B-D-glucuronide (MUG) was obtained from Clontech Labs, Palo Alto, Calif.; 3,5-dimethoxy-4-hydroxyacetophenone (acetosyringone) was obtained from Aldrich Chemical Co., Milwaukee, Wis.; carbenicillin disodium (Geopen, TM) was obtained from Roerig, Pfizer, New York; cefotaxime sodium (Claforan, TM) was obtained from Hoechst-Roussel, Somerville, N.J.; and paromomycin sulfate was obtained from Parke-Davis.

Plant material. The apple rootstock 'Malling 26' (M.26) and its transgenic derivative T1 (Norelli et al., unpublished) were used in this study. T1 is transformed with *nptII*, a β -glucuronidase gene, and a gene encoding the lytic peptide attacin E. T1 was obtained from M.26 leaf tissue inoculated with LBA4404 (pLDB15) (Destéfano-Beltrán et al., 1990) and regenerated on medium that contained 250 μg cefotaxime/ml and 10 μg paromomycin/ml. Southern analysis indicated that T1 most probably contains a single T-DNA insert with at least partial duplication of *nptII* (Norelli et al., unpublished).

Plant tissue culture. Methods and media used for shoot tip proliferation and rooted-plant culture were as previously described (Norelli et al., 1988), except that proliferation medium contained ($\mu\text{g}\cdot\text{ml}^{-1}$) 1.0 BA, 0.3 IBA, and 0.2 GA, and rooting medium contained 0.2 IBA. The modified N_6 medium (Chu et al., 1975) described by Welander (1988) containing 5 μg BA/ml, and 0.1 μg

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Abbreviations: GUS, b-glucuronidase; NPT, neomycin phosphotransferase.

NAA/ml was used for plant regeneration from leaf pieces. All regeneration media, except cocultivation medium, contained 250 µg cefotaxime/ml. Leaves used for regeneration were harvested from rooted plant tissue cultures and were fully unfolded, yet still in an active stage of leaf expansion. Leaves were sliced transversely into segments 3 to 5 mm wide with a scalpel, and segments were placed abaxial surface up on regeneration medium. Leaf pieces on regeneration medium were first placed in darkness at room temperature (21 to 30C) for 2 weeks, transferred to dim light (3.5 to 6 µmol·m⁻²·s⁻¹ 16 h day) at 22C for 2 weeks, and then placed at 17 to 20 µmol·m⁻²·s⁻¹ 16 h day at 22C. Leaf pieces were transferred to fresh medium every 3 weeks. Regeneration was measured as the percent of explants (leaf pieces) forming one or more buds or shoots. Since regeneration rates varied between experiments (range: 0.13 to 0.47 at 0 µg antibiotic/ml), regeneration was expressed as a percent of amount of regeneration that occurred in the absence of antibiotics. Tobacco (*Nicotiana tabacum* L.) cell suspensions of line NT1 (Paszty and Lurquin, 1987) were grown in the modified Murashige-Skoog (MS) (Murashige and Skoog, 1962) medium described by Russell et al. (1992) and transferred weekly. Nurse cultures used for *Agrobacterium* transformation consisted of 1-week-old NT1 suspension cultures washed in modified MS medium without 2,4-D and adjusted to a density of 20% (v:v); 10 ml of culture was vacuum filtered onto a 7-cm filter paper disc that was then inverted on cocultivation medium.

Plant transformation with *A. tumefaciens*. The *A. tumefaciens* strains used for transformation in this study were all derived from strain C58. C58sZ707 is a spectinomycin-resistant disarmed (T-DNA removed) strain (Angus Hepburn, personal communication) and NT1 (Watson et al., 1975) is a nontumorigenic *A. tumefaciens* strain that is lacking the pTiC58 plasmid (both T-DNA and vir genes removed). Two binary vector plasmids, pBI121 and p35SGUS-INT, were used, both derived from pBIN19. pBI121 has a b-glucuronidase gene and *nptII* within T-DNA borders for transfer to plants (Jefferson et al., 1987), while p35SGUS-INT has a chimeric b-glucuronidase gene containing a plant intron and *nptII* within T-DNA borders (Vancanneyt et al., 1990). C58sZ707 (p35SGUS-INT) was used for all studies on the effect of antibiotic concentrations on the selection of transformed cells.

***Agrobacterium tumefaciens* inoculum** consisted of a 48 h culture grown at 28C on Luria Bertani solid medium (Maniatis et al., 1982) containing 50 µg kanamycin/ml and suspended in 0.5X MS salt liquid medium, pH 5.4, containing 100 µM acetosyringone. Inoculum was adjusted to a density of 2×10^9 cfu/ml by measuring absorbance at 600 nm. Before inoculation, leaves were wounded by crushing the entire leaf surface between the blades of nontraumatic dissecting forceps (Aesculap, Burlingame, Calif.). Leaf pieces were placed in *A. tumefaciens* inoculum for 5 min, blotted dry, and placed abaxial side down on cocultivation medium containing no antibiotics. Cocultivation medium consisted of regeneration medium containing 100 µM acetosyringone overlaid with tobacco cell nurse culture. Plates were incubated in darkness for 48 h at room temperature to allow for infection and transformation by *A. tumefaciens*, and then leaf segments were transferred abaxial side up to regeneration medium containing 250 µg·ml⁻¹ of cefotaxime and 100 µg carbenicillin/ml to inhibit further growth of *A. tumefaciens*.

GUS assays. The quantity of transformed cells present in apple leaf and callus tissue was quantified by determining the rate of GUS activity using a fluorometric assay based on the cleavage of MUG to MU as described by Jefferson et al. (1987), except that 20-µl aliquots were removed at sample times, four sample times were used to calculate rates of activity, and assays were run for up to 200

min. The accumulation of MU over time was linear and did not approach an asymptote or depart from linearity during the assay period. Fluorescence was measured in a fluorometer (Model TKO 100, Hoefer Scientific Instruments, San Francisco) calibrated with freshly prepared 100 nM MU standard. Activity of blank MUG controls ranged from 0.05 to 0.5 h mole/min.

Data analysis. All experiments were repeated at least once; however, only one set of data is presented for each experiment. Trends, treatment effects, and conclusions of all data sets were consistent between experiment repetitions unless noted otherwise.

Rates of GUS activity were calculated using Minitab (Minitab, State College, Pa.) regression analysis. The unit of replication for in planta GUS activity and growth data was individual leaf pieces, and the number of replications in individual experiments is described in table and figure legends. Box-Cox transformation analysis (Box and Cox, 1964) determined that a log transformation was appropriate to normalize GUS data. Reported mean values were calculated from nontransformed data. Individual treatment effects were determined from log transformed data using SAS (SAS Institute, Cary, N.C.) general linear model (GLM) procedure. Figure graphs were produced using Cricket Graph (Cricket Software, Malvern, Pa.).

The unit of replication in regeneration experiments was a single petri dish containing six leaf pieces. Due to contamination of plates, the actual number of replicates varied and is reported as a mean for each experiment (Fig 1). Standard errors were calculated based on standard deviations of specific treatment means. Specific mean comparisons between treatments were performed by single degree of freedom contrasts using SAS GLM procedure.

Results

Effect of antibiotics on regeneration of M.26 and T1. Regeneration from M.26 apple leaf tissue was completely inhibited at low concentrations of geneticin (1 µg·ml⁻¹) and kanamycin (5 µg·ml⁻¹) after 6, 9, or 12 weeks of cultivation on regeneration medium (Fig 1). In one experiment, 10 µg paromomycin/ml completely inhibited regeneration from leaf tissue; in another experiment, regeneration was reduced at 10 µg paromomycin/ml (20% of amount observed at 0 µg·ml⁻¹) and was completely inhibited at 25 µg·ml⁻¹. M.26 leaf tissue was more tolerant of neomycin than of other aminoglycosides (Fig 1); regeneration was completely inhibited at 100 µg neomycin/ml.

T1 has a higher tolerance to all four of the antibiotics tested compared to M.26 (Fig 1). Regeneration from T1 tissue was completely inhibited at 2.5 µg geneticin/ml (2.5X increase over M.26), 375 µg paromomycin/ml (15 to 38X increase), and 375 µg neomycin/ml (3.8X increase). Regenerants began to develop at 2.5 µg geneticin/ml at 6 and 9 weeks but became necrotic by 12 weeks. Results obtained on the effect of kanamycin on T1 regeneration were inconsistent; regeneration was completely inhibited at 10 µg kanamycin/ml (2X increase over M.26) in one experiment and at 100 µg·ml⁻¹ (20X increase) in another.

Below inhibitory concentrations, neomycin and paromomycin had stimulated the amount of regeneration from T1 tissue (Fig 1). Regeneration from T1 tissue was significantly greater at 100 and 250 µg paromomycin/ml than at 0 µg·ml⁻¹ ($F = 23.38$, $df = 1, 49$), and at 250 µg neomycin/ml than at 0 µg·ml⁻¹ ($F = 29.11$, $df = 1, 42$).

GUS assay. The use of the fluorometric assay for quantifying the quantity of transformed cells present in leaf tissue was evaluated by varying fresh weight amounts of M.26 and T1 leaf tissue, mixed to give proportions (T1/M.26 + T1) of 0, 0.13, 0.25, 0.36, 0.46, 0.56, 0.66, 0.75, 0.84, and 0.9. Regression analysis indicated

Fig. 1. Effect of various aminoglycoside antibiotics on the regeneration of M.26 apple (open squares) and T1 (nptII-transgenic M.26; closed circles) leaf tissue. Regeneration was measured as the percentage of leaf pieces forming ≥ 1 bud or shoot and was determined after 12 weeks of cultivation on regeneration media. Since regeneration rates varied between experiments (range: 0.13 to 0.47 at 0 $\mu\text{g}\cdot\text{ml}^{-1}$ antibiotic), regeneration was expressed as a percentage of the amount of regeneration that occurred in the absence of antibiotics. Vertical bars indicate the SE. Antibiotic concentrations were 1, 2.5, 5, 10, 25, 50, 100, 250, 375, 500, and 1000 $\mu\text{g}\cdot\text{ml}^{-1}$. The mean number of replicates (petri plates with six leaf pieces per plate) per experiment were: geneticin/M.26, 6; genetic/T1, 6.2; kanamycin/M.26, 5.3; kanamycin/T1, 6; paromomycin/M.26, 3.8; paromomycin/T1, 5.9; neomycin/M.26, 5.6; and neomycin/T1, 5.25.

a highly significant relationship ($F = 764.1$, $df = 1, 25$) between the rate of GUS activity present in tissue extracts as determined by the fluorometric assay and the percentage of transgenic tissue in the mixtures with an R^2 value of 96.7%.

The usefulness of p35SGUS-INT for evaluating the amount of β -glucuronidase gene expression in plant tissue as opposed to that produced by *A. tumefaciens* was tested in apple by comparing the GUS activity of *A. tumefaciens* strains in vitro with the GUS activity observed in leaf pieces following inoculation with different *A. tumefaciens* strains. High levels of GUS activity were obtained in vitro and in leaf pieces 4 days following inoculation with strains C58sZ707 and NT1 containing pBI121, a binary vector plasmid containing a native β -glucuronidase gene under the regulation of the 35S promoter (Jefferson et al., 1987) (Table 1). The in vitro GUS activity of both of these strains is due to transcription from the 35S promoter in *A. tumefaciens*. GUS activity in leaf pieces 4 days after inoculation with C58sZ707 (pBI121) could be due to β -glucuronidase gene expression in transgenic plant cells and/or the bacteria; GUS activity of leaf pieces inoculated with NT1 (pBI121) must be due solely to transcription from the 35S promoter in *A. tumefaciens*, since NT1 lacks a Ti plasmid and, therefore, cannot transfer T-DNA to plant cells. Six weeks after cultivation on selection medium containing cefotaxime and carbenicillin to inhibit growth of *A. tumefaciens* and neomycin to select for the growth of transformed cells, significant GUS activity from pBI121 was detected in leaf pieces inoculated with C58sZ707 and with NT1 (Table 1).

No significant level of GUS activity was observed in vitro or 4

days after inoculation with strains C58sZ707 or NT1 containing p35SGUS-INT, the binary vector plasmid containing the β -glucuronidase-intron chimeric gene under the regulation of the 35S promoter (Table 1). However, after 44 days of cultivation on selective medium, leaf pieces inoculated with C58sZ707 (p35SGUS-INT) but not NT1 (p35SGUS-INT) exhibited significant levels of GUS activity, suggesting that GUS activity resulting from inoculation with this plasmid was due to β -glucuronidase gene expression in transformed plant cells and not *A. tumefaciens*.

Effect of antibiotics on selection of nptII-transgenic cells. To evaluate the ability of various aminoglycoside antibiotics to select for nptII-transgenic cells following transformation, M.26 leaf pieces were inoculated with *A. tumefaciens* strain C58sZ707 (p35SGUS-INT) and placed on regeneration medium with various concentrations of kanamycin, neomycin, and paromomycin. Inhibition of nontransgenic cells was evaluated by comparing the growth of leaf pieces (primarily callus), determined by weight, at various concentrations of the different antibiotics with growth in the absence of antibiotics. The growth of nptII-transgenic cells relative to nontransgenic cells was estimated by comparing the rate of GUS activity (η moles/minute) in leaf pieces on various concentrations of the different antibiotics with GUS activity in the absence of selection.

On regeneration medium containing $\geq 1.6 \mu\text{g}$ kanamycin/ml the amount of leaf piece growth decreased after 6 weeks of cultivation (Fig 2). Increases in the rate of GUS activity (η moles/minute) occurred on medium containing $\geq 2.5 \mu\text{g}\cdot\text{ml}^{-1}$ and $< 16 \mu\text{g}$ kanamycin/ml (Fig 2). When observed after 3 weeks on regeneration

Fig. 2. Effect of concentration of three aminoglycoside antibiotics on selection of nptII-transgenic cells 44 days after M.26 leaf pieces were inoculated with *A. tumefaciens* strain C58sZ707 (p35SGUS-INT) and placed on regeneration medium containing antibiotics. Growth (open squares) was a measure of the inhibition of nontransgenic cells and is the leaf piece plus callus weight expressed as the proportion of the weight in the absence of antibiotics. The rate of GUS activity (closed circles) was used as a measure of growth of nptII-transgenic cells and is expressed as the proportion of the rate (η moles/minute) that occurred in the absence of antibiotics. Antibiotic concentrations were 2.5, 4, 6.3, 10, 25, 40, 63, 100, 250, and 400 $\mu\text{g}\cdot\text{ml}^{-1}$. There were six replicated leaf samples per treatment mean.

medium, the effect of kanamycin on leaf piece weight was the same (data not shown). However, after 3 weeks only leaf pieces on 4 μg kanamycin/ml had a higher rate of GUS activity than pieces on 0 $\mu\text{g}\cdot\text{ml}^{-1}$.

The weight-adjusted rate of GUS activity in noninoculated M.26 leaf pieces (0.3 η moles/minute per milligrams fresh weight) was not significantly different from the rate in inoculated M.26 on medium without kanamycin (1.4 η moles/minute per milligrams fresh weight) ($F = 0.01$, $df = 1, 12$), while the rate in T1 leaf pieces (76.6 η moles/minute per milligrams fresh weight) was significantly greater than the rate in inoculated M.26 on medium without kanamycin ($F = 23.13$, $df = 1, 12$). The same results were obtained for M.26 and T1 controls in neomycin and paromomycin tests.

On medium containing ≥ 63 μg neomycin/ml there was a reduction in leaf piece growth and an increase in the rate of GUS activity after 6 weeks (Fig 2). The rate of GUS activity decreased on medium containing ≥ 160 $\mu\text{g}\cdot\text{ml}^{-1}$. The weight-adjusted rate of GUS activity (η moles/minute per milligrams fresh weight) increased continuously from 40 to 160 μg neomycin/ml (data not shown); however, this trend was due largely to low average leaf piece weights at 100 and at 160 μg neomycin/ μl (130 and 32 mg, respectively, compared with 216 mg without selection). Similar trends were observed after 3 weeks on medium with neomycin (data not shown), except that leafpiece weights and the rate of GUS activity were affected by lower concentrations of neomycin. Reductions in leaf piece weight began at 40 μg neomycin/ml rather than at 63 $\mu\text{g}\cdot\text{ml}^{-1}$; the level of GUS activity was highest at 63 $\mu\text{g}\cdot\text{ml}^{-1}$ and decreased on media containing ≥ 100 $\mu\text{g}\cdot\text{ml}^{-1}$.

On medium containing ≥ 25 μg paromomycin/ml there was a reduction in leaf piece weight and an increase in the level of GUS activity after 6 weeks (Fig 2). As with neomycin, leaf piece growth and the rate of GUS activity were affected by lower concentrations of paromomycin when observed after 3 weeks of cultivation (data not shown). After 3 weeks, there was a reduction in leaf piece weight on medium containing ≥ 10 $\mu\text{g}\cdot\text{ml}^{-1}$ paromomycin rather than 25 $\mu\text{g}\cdot\text{ml}^{-1}$. After 3 weeks, the rate of GUS activity at 10 $\mu\text{g}\cdot\text{ml}^{-1}$ was greater than the rate at 0 $\mu\text{g}\cdot\text{ml}^{-1}$, but the rate of GUS activity at 25, 40, and 63 $\mu\text{g}\cdot\text{ml}^{-1}$ did not differ greatly from the rate at 0 $\mu\text{g}\cdot\text{ml}^{-1}$.

Discussion

The potential benefits of genetic engineering are presently not applicable to many important crop plants that are either recalcitrant to transformation or have exceedingly low transformation frequencies. Although apple has been successfully transformed (James et al., 1989), frequencies of transformation are generally too low to allow for the development of new cultivars by genetic engineering. Attempts to study the effect of diverse factors on transformation by observing plant transformation directly have been unsuccessful because too few transformants are produced to observe or analyze differences between treatments. In these studies we overcame this problem by analyzing the effect of various factors on important steps in the transformation process.

Although the mode of action of nptII is highly specific and the four antibiotics tested were all aminoglycosides, the response of apple tissue to them was very diverse. Paromomycin and neomycin clearly stimulated regeneration from T1 tissue and appeared to have effects similar to growth regulators (Fig 1). The antibiotic cefotaxime has stimulatory effects on wheat (*Triticum aestivum* L.) regeneration (Mathias and Boyd, 1986) and increases the frequency of apple regeneration (James et al., 1990, Norelli, personal observation). Neomycin also appeared stipulatory to nontransgenic M.26 regeneration at lower concentrations, whereas

Table 1. GUS activity^z of *A. tumefaciens* strains in vitro and in leaf pieces of M.26 following inoculation.

Strain	Plasmid	In vitro ^x	In planta ^y	
			4 days	44 days
None		0.5 a ^w	1.5 a	0.4 a
C58sZ707	none	1.0 a	2.0 a	1.0 a
NT1	none	0.6 a	2.0 a	0.6 a
C58sZ707	pBI121	398.0 b	48.3 b	71.0 c
NT1	pBI121	477.4 b	34.8 b	2.8 b
C58sZ707	p35SGUS-INT	0.9 a	1.3 a	196.9 d
NT1	p35SGUS-INT	0.6 a	1.7 a	0.9 a

^zRate of MUG to MU conversion (η moles MU/minute) determined by fluorometric assay (Jefferson et al., 1987).

^y η moles MU/minute per milligram tissue in M.26 leafpieces after inoculation with *A. tumefaciens* strains, co-cultivation for 2 days on permissive regeneration medium, and cultivation on regeneration medium containing ($\mu\text{L}\cdot\text{mL}^{-1}$) 250 cefotaxime, 100 carbenicillin, and 75 neomycin for 2 days and 6 weeks. Mean of six replicated leaf samples.

^x η moles MU/minute/ 2×10^9 cfu. Bacteria were grown for 18 h at 28C in Kado 523 broth, resuspended in GUS assay buffer, and sonicated for 1 min at 50% pulsed cycle with 10W power output using a 500W Vibra Cell Sonicator (Sonics & Materials, Danbury, Conn.). Mean of five replicated samples.

^wTreatments within a column followed by the same letter did not differ significantly at $P = 0.05$ as determined by a Waller-Duncan K-ratio T test.

paromomycin was inhibitory at low concentrations (Fig 1).

Paromomycin has been used to select nptII-transgenic Citrus (Vardi et al., 1990), rapeseed (*Brassica napus* L.) (Guerche et al., 1987), sunflower callus (*Helianthus annuus* L.) (Escandon and Hahne, 1991), and tobacco (*Nicotiana tabacum* L.) (Bellini et al., 1989). In general, paromomycin has been superior to other aminoglycosides for the selection of nptII-transgenics.

Although T1 had an increased tolerance to geneticin, it was too toxic to apple to be useful for selection of transformed cells. Its high toxicity prevented statistical analysis of its effects. Several plant species, such as rice (*Oryza sativa* L.) (Dekeyser et al., 1989) and sugar beet (*Beta vulgaris* L.) (Catlin, 1990), have demonstrated much greater sensitivity to geneticin than to kanamycin. However, geneticin has proven to be useful for selecting nptII-transformants of several plant species including tobacco (Iida et al., 1990) and *Arabidopsis thaliana* *thaliana* (L.) Heyhn. Similar concentrations of geneticin ($20 \mu\text{g}\cdot\text{mL}^{-1}$) and kanamycin ($25 \mu\text{g}\cdot\text{mL}^{-1}$) are used for the selection of nptII-transgenic *Arabidopsis* (Schmidt and Willmitzer, 1988).

The response of T1 tissue to kanamycin was variable in our studies. In one test it appeared quite intolerant, with regeneration being inhibited at $10 \mu\text{g}$ kanamycin/mL, and in another test, regeneration was not inhibited until $100 \mu\text{g}\cdot\text{mL}^{-1}$. However, kanamycin did not demonstrate the stimulatory effects on regeneration observed with paromomycin and neomycin in either test. High doses of kanamycin caused apple leaf tissue to turn pale yellow or white, whereas high doses of geneticin, paromomycin, and neomycin resulted in necrosis. This feature may make kanamycin useful for the detection of certain types of chimeras resulting from nptII transformation.

The GUS activity observed in *A. tumefaciens* strains grown in vitro and in leaf pieces following inoculation with *A. tumefaciens* strains that were either transformation competent (C58sZ707) or incapable of plant transformation (NT1) demonstrated that p35SGUS-INT was a useful tool for studying the early events of transformation in apple (Table 1). This plasmid allowed us to use the amount of GUS activity as a measure of the ability of specific antibiotic concentrations to select for the growth of transformed cells. Six weeks of cultivation on selective medium was used to

evaluate p35SGUS-INT because this was the period after which we observed a stabilization in the effect of antibiotics on the selection of transformed cells and when we normally begin to observe regeneration from leaf pieces. Regeneration was absent when we used p35SGUS-INT (Table 1) or in determining optimal aminoglycoside concentrations for the selection of nptII-transgenic cells (Fig 2), because the presence of carbenicillin in the medium inhibited regeneration from apple leaf pieces.

GUS activity was never present in leaf pieces unless they were cultivated on media containing aminoglycoside antibiotics sensitive to nptII activity. This result indicates that probably very few cells of apple are transformed following co-cultivation with *A. tumefaciens*, and that the use of antibiotic selection will be necessary for the efficient selection of apple transformants.

Since relatively few apple leaf cells appear to be transformed following inoculation with *A. tumefaciens*, and the vast majority of leaf tissue is not transformed, we were able to use leaf piece weight following cultivation on medium with antibiotics as a measure of the ability of antibiotics to inhibit the growth of nontransformed cells. Due to the sensitivity of GUS assay methods (Jefferson et al., 1987), we were able to use the rate of GUS activity in the same tissue to measure the ability of the antibiotics to select for growth of nptII-transgenic cells. We used the total rate of GUS activity (η mole/minute) rather than a weight adjusted rate (η moles/minute/milligram fresh weight), since leaf pieces were of uniform weight and tissue type at the start of the experiment and since antibiotics had effects on leaf piece weights that compounded their effect in weight adjusted rates. Unless noted otherwise in the results, the trends observed with total GUS rates were the same as those observed with weight adjusted rates. Concentrations of aminoglycosides considered optimal for the selection of nptII-transgenic M.26 cells were those that caused a significant decrease in the growth of nontransgenic cells and a significant increase in the growth of transgenic cells.

Our results indicate that neomycin and paromomycin likely will be more useful aminoglycosides for the selection of nptII-transgenic apple plants than either kanamycin or geneticin. Paromomycin elicited a much greater differential between the sensitivity of T1 and M.26 tissue than neomycin. This difference

suggests that paromomycin may be of greater use for apple transformation. However, concentrations of paromomycin necessary for the selection of nptII-transgenic cells (Fig 2) result in the necrosis of nontransgenic M.26 tissue, which may be inhibitory to regeneration. Research on the effect of these and other factors on apple transformation need to be continued so that the full benefits of genetic engineering may be extended to this economically important crop plant.

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