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Expression and inheritance pattern of two foreign genes in petunia

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Abstract Transgenic petunia (*Petunia hybrida* Vilm.) plants were obtained from *Agrobacterium*-mediated shoot apex transformation. Studies at the phenotypic as well as molecular level established both the presence of the NPT II (neomycin phosphotransferase II) and GUS (β-glucuronidase) genes and their level of activity. Twenty-nine primary transformed plants showed varying patterns of phenotype expression of both genes. NPT II and GUS expression in 7 primary plants over a 4-month interval showed varying levels of gene expression within and among individual plants. All primary transgenic plants were self-pollinated and backcrossed to establish the inheritance patterns of both genes. Mendelian and non-Mendelian inheritance patterns for both genes were observed. Analysis of the progeny showed poor transmission of the foreign genes through the pollen especially when two or more bands were present in the Southern hybridization. Most plants whose progeny segregated in Mendelian ratios for either the NPT II or GUS gene had just one copy of the gene. In this study where both foreign genes were examined in both self and test crosses, no transgenic plant showed Mendelian patterns of inheritance for both foreign traits.

Key words *Agrobacterium* · Transformation · Gene expression · Petunia

Introduction

In order to improve crop plants through genetic engineering it is important that the inheritance patterns and expression of foreign genes be predictable. Some studies have supported the idea that foreign genes are inherited in a normal Mendelian pattern (Budar et al. 1986; Ulian et al. 1988). The number of copies of a foreign gene inserted into the plant cell genome by *Agrobacterium*-mediated transformation can vary from single to multiple inserts. Several recent reports show that there is variability in the transmission of foreign genes. Also, the expression of foreign genes has been linked to the presence of extra copies inserted into the genome, methylation, environmental factors, gene interaction, and gene loss (Deroles and Gardner 1988; Feldman and Marks 1987; Hobbs et al. 1990; Kilby et al. 1992; Linn et al. 1990; Matzke and Matzke 1991; Meyer et al. 1992; Ottaviani et al. 1983; Umbeck et al. 1989).

Genetic analysis of 44 transgenic tobacco plants by Budar et al. (1986) indicated normal Mendelian inheritance of the neomycin phosphotransferase II (NPT II) gene in 35 plants that had a single gene insert. However, 4 of the plants produced progeny that did not fit expected Mendelian ratios, and 5 others segregated as expected for two gene copies. Unfortunately, the number of gene inserts was not verified by molecular data. In a similar study, Deroles and Gardner (1988) showed that all of the 104 primary transgenic petunia plants could grow and root on kanamycin (suggesting the NPT II phenotype), but when adult leaf tissue was cultured on kanamycin, 40% of these plants failed to express the kanamycin resistance phenotype. Southern hybridizations detected intact T-DNA present in plants lacking kanamycin resistance, and it was concluded that the loss of gene expression was not caused by loss of the gene. Progeny analysis showed that only half of these plants with one or two inserts transmitted the trait in a predictable inheritance pattern.

Variability in the transmission and expression of the GUS (β-glucuronidase) gene has recently been shown. Hobbs et al. (1990) reported that low levels of GUS expression seen in tobacco plants transformed with GUS were due to multiple insertions that tended to have increased methylation. The highest expression of GUS

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was associated with single T-DNA inserts. Ottaviani et al. (1983) reported a correlation between methylation and the differential expression of GUS and NPT II genes in potato. The loss of gene expression over four generations has been attributed to methylation in *Arabidopsis* (Kilby et al. 1992).

In plants transformed with two separate genes either simultaneously or sequentially, one gene was often lost and/or not expressed in the progeny. Umbeck et al. (1989) showed normal Mendelian inheritance for one gene, but the second was absent in two of the three transgenic cotton plants investigated. The third plant expressed both genes, and backcrosses showed Mendelian inheritance. Matzke and Matzke (1991) reported that foreign gene expression following a second transformation can be affected by the presence of the original T-DNA. In their study the first genes were often not expressed following a second transformation event, probably due to methylation of the promoter regions of the first genes.

Most recently, Meyer et al. (1992) examined 30,000 transgenic petunia plants in the field that expressed a single copy of the maize *Al* gene, which results in a salmon-red flower color. Methylation of the 35S promoter resulted in some white flowers as well as a 60% reduction of the red color in flowers produced later in the growing season. A small number of white flowers were due to deletion of part of the *Al* gene.

In the study reported here 29 transgenic petunia plants transformed with NPT II and GUS genes in the same construct were examined for number of inserts as well as expression and transmission of both genes. Most earlier studies have examined the inheritance of only one foreign gene. Our investigation revealed that there is much more variability in the transmission and expression of foreign genes than has been previously reported for tobacco and petunia transformed with *Agrobacterium*, particularly when transgenic plants are evaluated for the expression of both foreign genes.

Materials and methods

Transformation and initial screening

The shoot apex/*Agrobacterium* co-cultivation technique was used to obtain transgenic petunias (Ulian et al. 1988). Briefly, aseptically germinated *Petunia hybrida* Vilm. var 'Rose Flash' (Ball Seed Co, Chicago, Il.) seeds, 7 days old, were used as a source of shoot apex explants. The shoot apex (meristem plus two primordial leaves) was cultured on a MS medium (Murashige and Skoog 1962) supplemented with 30,000 mg/l sucrose, 0.1 mg/l kinetin, and 2,000 mg/l Gel-rite. After 2 days in culture a 5 µl drop of an *Agrobacterium tumefaciens* LBA 4404 containing NPT II and GUS genes (Ulian et al. 1988) was applied to each shoot apex. Figure 1 is a cartoon of the plasmid construct. After 15 min, the shoot apices were transferred to fresh medium for 2 days, and they were then recultured on the same medium as above only supplemented with 200 mg/l kanamycin and 500 mg/l carbenicillin. Shoots that developed were transferred to the same medium without kinetin and with 100 mg/l kanamycin for rooting. Shoots not able to root on kanamycin after 2 weeks were transferred to kanamycin-free medium for rooting. Rooted plants were planted in pots containing a commercial potting mix and grown to adult flowering plants under fluorescent lights with a 12-h daylength.

Plant DNA extraction and Southern blot analysis

Plant DNA was extracted utilizing the method of Dellaporta et al. (1985). Three microgram plant DNA samples were digested overnight according to manufacturer's instructions with *Eco*RI (Promega, Wis.) for studies on the presence of both genes and their copy numbers or with *Hin*dIII and *Sst*II (Gibco-BRL, N. Y.) for methylation studies. The samples were subsequently run overnight on a 0.8% agarose gel at 30 V and the DNA transferred to Genescreen Plus (Nen[R], Boston, Mass.) according to the manufacturer's instructions. After electrophoresis was completed, the gel was incubated at room temperature, with gentle shaking, in a 0.4 *N* HCl, 0.6 *M* NaCl solution for 30 min. The treatment was repeated with a 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.5 solution. The membrane was labeled, cut to the exact size of the gel, wet with dd H_2O and laid onto a 10 × SSC solution (1.5 *M* NaCl, 0.15 *M* sodium citrate). The gel was placed on top of a 3 MM Whatman paper bridge on a glass tray containing 10 × SSC, and the membrane was placed on top of the gel, with careful removal of air. A dry piece of filter paper was laid on top of the gel, and a stack of paper towels laid on top of the filter paper. A weight was placed on top of the paper towels. Transfer was performed overnight. The towels were then carefully removed and the membrane placed in an excess of 0.4 *N* NaOH for one min, after which it was neutralized in 0.2 *M* Tris-HCl, pH 7.5, 2 × SSC, and allowed to dry at room temperature.

Hybridization and rehybridization of DNA

The membrane was prehybridized at 42 °C in 30 ml of 50% formamide, 1% SDS, 1 *M* NaCl, and 10% dextran sulfate overnight. The radioactive probe solution (usually 50 µl) was added to 500 µl of a 10 mg/ml solution of denatured herring sperm DNA and injected into the bag using a 1-ml hypodermic syringe and needle. The small hole was heat sealed, and the hybridization was performed overnight at 42 °C. The membrane was then removed from the bag and washed as follows: (1) Two 5-min washes in 2 × SSC at room temperature; (2) two 30-min washes in a solution containing 2 × SSC and 1% SDS at 65 °C; (3) two 30-min washes in 0.1 × SSC at room temperature. All washes were performed with constant agitation. After washing, the membrane was placed in a plastic bag, sealed, and exposed to Kodak X-OMAT autoradiographic film. Rehybridization was performed by boiling the membrane for approximately 20 min in a solution containing 0.1 × SSC, 1% SDS, drying the membrane, prehybridizing, and hybridizing, as before.

Production of probes

Two different probes were used for identification of the GUS and NPT II genes. These were obtained by amplification of a segment of

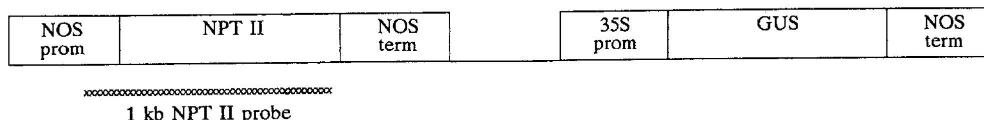

Fig. 1 A simplified map of the region of the pGUS2 plasmid containing the NPT-GUS chimeric construct. The 1-kb NPT II probe region is shown

the gene sequence by means of the polymerase chain reaction technique. In both cases this reaction contained 100 ng of total DNA from LBA 4404 containing pGUS2 as template, 10% DMSO, 200 mM solution of dNTPs, 1 µl *Taq* polymerase, 0.1 mM of each primer, and 10 × buffer. The volume was adjusted to 100 µl with dd sterile water, and 50 µl of mineral oil was added to each tube. The reaction conditions for both the GUS and NPT II amplifications were 94 °C during 1 min for denaturation of the duplex, followed by 1 min at 55 °C for annealing of the primers to their complementary sequences, and 2 min at 72 °C for extension of the annealed primers with the *Taq* polymerase. All reactions were performed in a Ericomp Thermal Cycler for 35 cycles.

The GUS probe was a 700-bp fragment that extends from position 60 to position 760 in the coding region of the GUS gene. The two primers consisted of a 24-base (5′-CTCGACGGCCTGTGGGCATTCAGT-3′) sequence starting at position 60 and a 24- base (3′-TAACCTTCACCCGGTTGCCAGAGG-5′) sequence starting at position 760 and ending at position 737.

The NPT II probe was a 1-kb region of the chimeric NOS promoter-NPT II sequence gene. The first primer contained 21 bases (5′-CCCCTCGGTCTCCAATTAGAG-3′) and was located 33 bp 5′ of the translation initiation site (ATG) in the NOS promoter. The second primer had 24 bases (5′-CCCGGGGTGGGCGAAGAACTCCAG-3′) and was located in the 3′-flanking region of the NPT II gene, 150 bp 3′ of the translation stop signal.

The synthesized sequences were recovered by running the reaction product in a 1.2% low-melt agarose gel in 1 × TAE buffer. The specific band was visualized under a UV box and removed from the gel, and the product was utilized in subsequent hybridizations.

Probe labeling was performed using the Prime-a-Gene (Promega, Wis.) labeling system. Low-melt agarose containing probe DNA was heated at 55 °C in a water-bath, and a 25-ng aliquot was transferred to a microcentrifuge tube. The DNA was heated at 95 °C for 2 min and then rapidly chilled in an ice-bath. The following reagents were then added 10 µl 5 × labeling buffer, 2 µl of a mixture of nonlabeled dNTPs (dCTP, dGTP, dTTP), 2 µl nuclease-free BSA, 5 µl [α-^{32}P] dATP (111GBq/mmole), 5 units Klenow enzyme, and nuclease-free H_2O to a final volume of 50 µl. After gentle mixing, the reaction was incubated at room temperature for 60 min and terminated by heating at 95 °C for 2 min. After the reaction was terminated, unincorporated label was removed by spinning the sample in a G-50 Sephadex column for 4 min according to the manufacturer's instructions.

Determination of GUS and NPT II activity in transformed plants

Twenty milligrams of leaf material was collected from each plant and immediately extracted in 200 µl of ice-cold GUS lysis buffer (Jefferson 1987) containing 50 mM $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100. The homogenates were centrifuged at 4 °C for 15 min in an Eppendorf 5415 microfuge, and the supernatant was then transferred to fresh tubes and stored at −80 °C.

The total protein content of each sample was determined by the Bradford method. One microliter of the above sample was added to 959 µl of dd H_2O, and the volume was adjusted to 1 ml by the addition of 40 µl of Bio-Rad assay reagent. The blank was prepared by substituting dd H_2O for the sample. A standard curve was established using BSA (1 mg/ml) from Promega at concentrations of 2.5, 5.0, 7.5, and 10.0 mg/ml. Measurements were made on a Perkin-Elmer spectrophotometer at 600 nm. The NPT enzyme produced by transformed plants was quantitatively analyzed following the technique of Staebell et al. (1990). The X-Gluc assay was utilized to identify primary transformants and progeny seedlings expressing the GUS gene, and also for histological studies of the transformed shoot apex. The assays were performed as described by Jefferson (1987). Transformed tissue was identified by the development of a blue precipitate inside the cells. The β-glucuronidase fluorimetric assay was performed according to Jefferson (1987).

Crosses and detection of NPT II-resistant progeny

The transformed plants were self-pollinated by the removal of mature stamens with a pair of tweezers and subsequent placement of their pollen on the receptive stigma. All testcrosses were performed by the touching mature stamen from the transformed plant to the stigma of the emasculated flowers of nontransformed plants. Each cross was labeled identifying the type of cross, the plants involved, and the date performed. Seeds were harvested, dried, and stored at 4 °C. Resistance to kanamycin in the progeny was assayed by germinating the seeds on MS medium containing 300 mg/l kanamycin. Seedlings that were able to develop leaves, thus overcoming the cotyledonary stage, were considered to be positive and labeled as NPT II$^+$; seedlings that stopped development at the cotyledonary stage were considered to be nontransformed and labeled as NPT II$^-$.

Results

Initial phenotype screening of primary transgenic plants

Two initial indications of the incorporation of the NPT II gene were the ability of the shoot to root on a kanamycin-containing medium (100 mg/l) and subsequent callus formation from leaf explants of plants established in soil on a medium containing kanamycin (300 mg/l). Additionally, GUS activity from leaf tissue was examined using the X-Gluc assay. Leaf samples from different locations on the plant were assayed to check for the presence of chimeric plants. Table 1 shows the results of these assays. It is interesting to note that 6 plants (R2, B3, B91, B102, W111, and R122) initially rooted on kanamycin, but later leaf disc explants did not form callus on kanamycin. Two plants (R104 and R105) initially did not root on kanamycin, but leaf disc explants formed callus on kanamycin. One plant, GC1, did not root on kanamycin nor did leaf explants form callus on kanamycin; it did, however, show GUS activity. Of the plants that initially rooted and formed callus on kanamycin 17 were also GUS positive. Only 3 plants (G82, R103 and B111) were positive for both tests of NPT II expression but GUS negative.

Table 1 Expression of NPT II and GUS by primary transformants. Shoots that developed on 300 mg/l kanamycin were rooted in medium containing this antibotic. Their leaves were tested for callus growth on kanamycin and GUS expression in the X-Gluc assay

Plant	Root initiation on kanamycin	Callus growth on kanamycin	GUS activity in leaves
R1, B1, B2, RC1, RO13, GO9, G3, W5, G81, R101, R102, B101, B112, R111, R121, B121, B122	+	+	+
R2, B3, W111	+	−	+
GC1	−	−	+
G82, R103, B111	+	+	−
B91, R122, B102	+	−	−
R104, R105	−	+	+

Southern blot analysis of primary transformed plants

Figure 2A, B shows autoradiographs of genomic DNA of the 29 primary transformed petunia plants. The DNA was digested with *Eco*RI and probed with the 1-kb NPT II probe. Each band indicates a single insert since the only *Eco*RI site in the foreign DNA is on the right border, and the probe has no *Eco*RI sites. Autoradiographs using the GUS probe produced the same pattern. Only plant B3 did not contain a DNA sequence capable of hybridizing to either probe; subsequent autoradiographs with both non-transformed and transformed controls verified this. Subsequent Southern blots confirmed the presence of the NPT II gene in plants B1 and R104.

Quantitative levels of NPT II and GUS enzyme activity

To determine whether the levels of enzyme activity varied among the primary transgenic plants, quantitative evaluations of NPT II and GUS were conducted. NPT II levels were evaluated by determining the amount (in pg) of enzyme produced in 1 h per microgram of total protein. GUS activity was determined in pmoles of MU (4-methyl umbelliferone) produced by the enzyme per hour per microgram of total protein. Fig. 3A, B and Table 2 illustrate these results. Plants did show a dramatic variation in their capacity to produce either enzyme. While plants G09, B2, and R104 had high levels of expression for both genes, others such as R101 and R102 expressed more GUS than NPT II. In general, the total lack of enzyme production was more pronounced for NPT II perhaps due to differences in the sensitivity between the NPT II assay and the GUS assay.

Table 2 Production of MU and the NPT II enzyme by transformed plants. Leaf tissue was extracted in GUS lysis buffer and assays performed as described in Materials and methods. The results for MU are in pmoles/h per microgram total protein, and the results for NPT II are in pg/h per microgram total protein

Plant	MU	NPT II
R1	0.17	1.52
R2, B3	0.00	0.00
B1	6.91	0.00
B2	31.84	21.82
RC1	5.14	0.00
R013	12.48	1.65
GC1	0.19	0.00
G09	78.49	39.77
G3	8.26	5.52
W5	13.35	0.91
G81	11.21	1.43
G82	0.07	0.34
B91	0.21	1.96
R101	18.88	1.97
R102	15.50	1.20
R103	0.68	0.00
R104	65.43	6.99
B101	9.94	15.57
B102	0.05	0.38
B111	0.25	0.00
B112	0.26	0.81
W111	11.21	0.02
R111	20.07	1.14
R121	13.43	7.91
R122	0.41	0.00
B121	21.89	4.14
B122	9.31	4.93

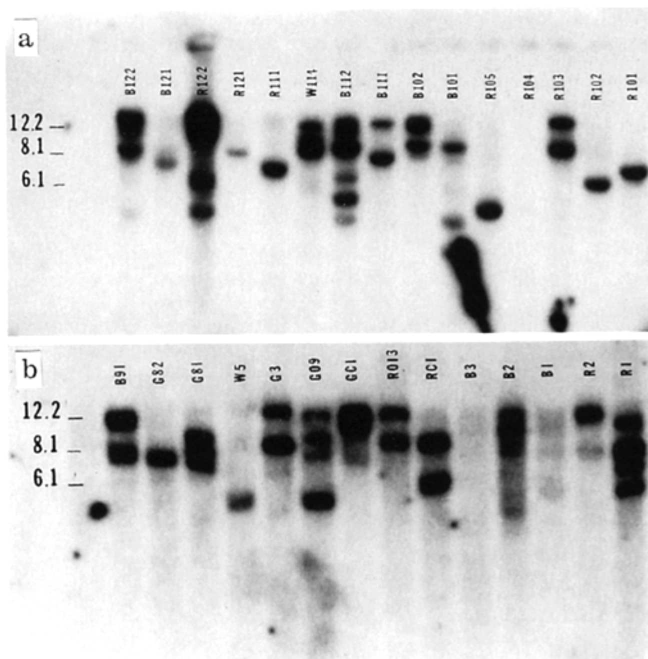

Fig. 2A, B Autoradiographs of genomic DNA of 29 petunia plants digested with *Eco*RI and probed with the 1-kb NPT II probe. Each band reflects a single insert since the only *Eco*RI restriction site in the foreign DNA is on the right border and the probe has no *Eco*RI sites

When the amount of NPT II enzyme activity present in mature plants was compared to the levels found earlier in vitro screening (Table 1), it was found that most of the plants unable to callus on kanamycin also did not produce NPT II at this later stage. Examples of this are plants R2, B3, GC1, W111, and R122. However, another situation occurred where plants (B91 and B102) that could not produce callus from leaf explants after rooting on kanamycin were able to produce measurable amounts of NPT II. Perhaps these 2 plants did not form callus on 300 mg/l kanamycin not because the amount of enzyme produced was too low but as a result of other culture-dependent reasons. Other plants producing lower amounts of the enzyme (W5 and R102) were able to callus at the same kanamycin level. Moreover, some plants after rooting in vitro and producing callus on kanamycin could not produce NPT II when mature (RC1, R103 and B111).

The results of GUS expression in these plants are also presented in Table 2. Only 1 plant which had the R2 gene did not express GUS enzyme activity. This plant gave positive results in the in vitro X-Gluc assay. Other

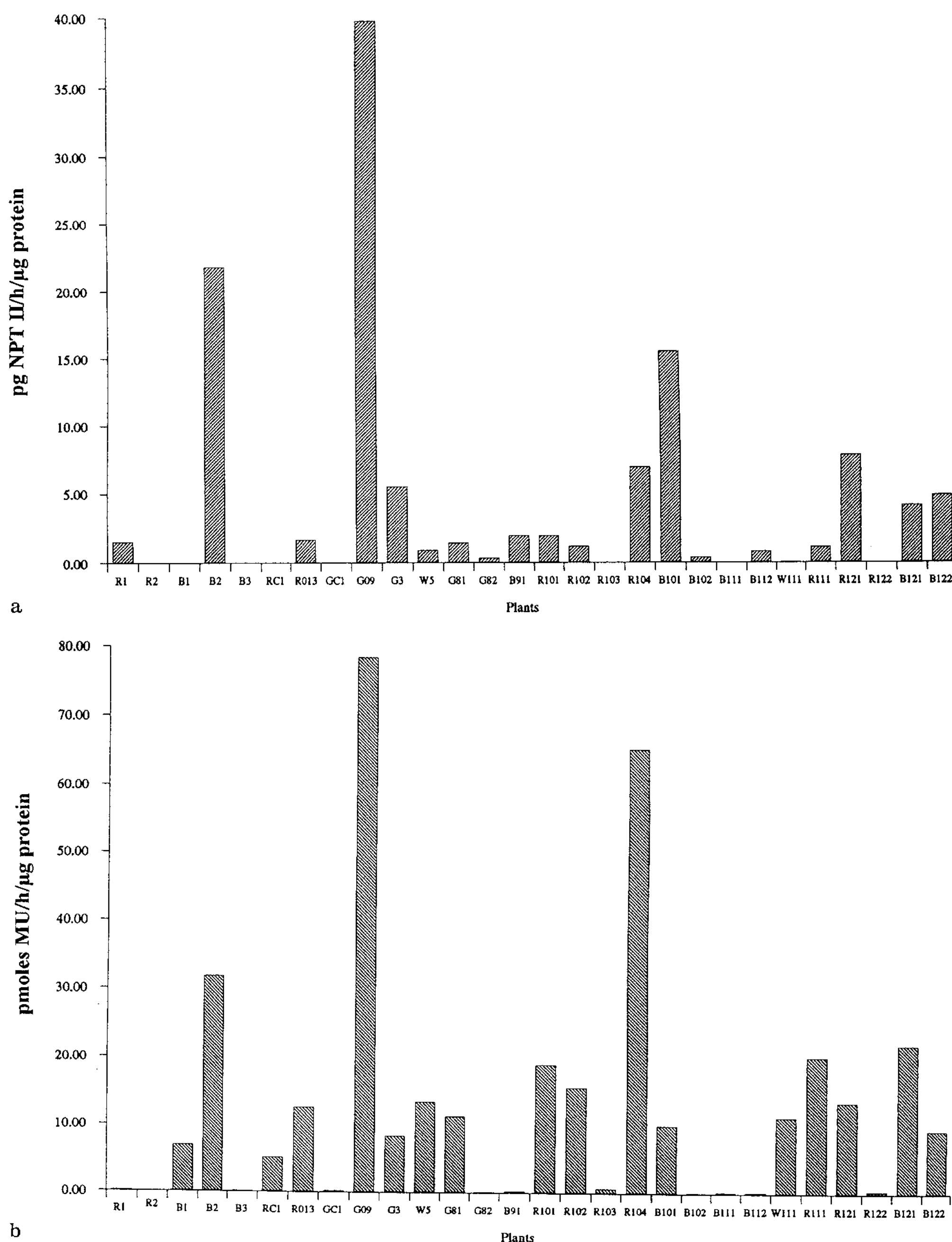

Fig. 3A, B **A** *Shaded* histograms indicate activity of the NPT II gene in the different plants in this study. The values are in pg NPT II/h per microgram protein. **B** *Shaded* histograms indicate activity of the GUS gene in the different plants in this study. The values are in pmoles MU/h per microgram protein

plants produced very low amounts of MU, and most of these plants (G82, B91, R103, B102, B111, and R122) showed the same lack of expression in vitro as indicated by the X-Gluc assay.

Variation in gene expression over 4 months

Seven mature plants (R2, B2, RC1, RO13, GO9, G81, and B111) were selected to evaluate GUS and NPT II gene expression over a 4-month period. Quantitative measurements of enzyme activity were recorded at 30-day intervals. The results are given in Table 3. Five plants did not have major shifts in gene expression over time. However, plants G81 and B111, while not changing in GUS gene expression, showed a change in NPT II gene expression. Plant B111 started to produce NPT II after 30 days which it had not done since the initial transformation. Plant G81 stopped NPT II gene expression at 90 days.

Inheritance studies for NPT II and GUS

Crosses to establish the inheritance patterns of the two foreign genes were performed using the transgenic plants in selfs and in testcrosses where the transgenic plant was always the pollen parent. Table 4 shows the

Table 3 Monthly variation in expression of the NPT II and GUS genes in mature, transformed plants. Some transformed plants were selected and their expression of the genes was followed on a monthly basis. Assays were performed as described in Materials and methods. Results for GUS are in pmoles MU/h per microgram protein and for NPT in pg NPT II/h per microgram protein

	Plant	Days				
		0	30	60	90	120
NPTII	R2	0.00	0.00	0.00	0.00	0.00
	B2	21.82	25.43	27.30	22.40	20.10
	RC1	0.00	0.00	0.00	0.00	0.00
	R013	1.65	1.85	2.13	2.82	2.36
	G09	39.77	42.80	40.60	38.40	35.60
	G81	1.43	1.23	1.05	0.00	0.00
	B111	0.00	0.60	1.20	1.00	0.65
GUS	R2	0.00	0.00	0.00	0.00	0.00
	B2	31.84	33.50	36.30	38.20	36.40
	RC1	5.14	3.85	3.62	3.42	3.95
	R013	12.48	11.50	10.60	10.80	10.85
	G09	78.49	75.30	76.20	70.20	72.40
	G81	11.21	10.80	10.35	10.25	10.80
	B111	0.25	0.30	0.30	0.35	0.40

segregation of GUS^+ and NPT II^+ in progeny plants. None of the primary transgenic plants produced progeny in the expected Mendelian ratios for either of the foreign genes in both the selfs and testcrosses. However, if the transmission of each foreign gene is considered separately, the GUS gene segregated in Mendelian ratios in progeny from both selfs and testcrosses in 4 of the transgenic plants, R121, R111, B122, and B101. When the inheritance patterns of the NPT II gene are considered alone, progeny from only 2 of the 23 plants (R102 and W5) fit the 3:1 ratio for selfs and 1:1 ratios for testcrosses. An analysis of progeny from crosses of primary transgenic plants having two or more bands showed that in these plants there was generally a poorer transmission of the foreign genes. Of the 14 transgenic plants with two or more bands 6 failed to transmit one of the foreign genes to any progeny, and 2 transgenic plants (B1 and R2) showed no transmission of either foreign gene even though the Southern hybridizations clearly indicated the presence of both genes in each plant.

In determining the expected ratios for the transmission of foreign traits, it was assumed that each plant behaved as a monohybrid for each gene and that the gene loci were unlinked. One transgenic plant, G81, gave progeny from selfs and testcrosses which fit segregation patterns for GUS^+ that would be expected for a dihybrid cross between unlinked genes (self, 15 GUS^+: 1 GUS^- and testcross, 3 GUS^+: 1 GUS^-). Since plant G81 had at least three copies of the GUS gene (or portions of it), perhaps there were multiple insertions, and two active copies behaved independently in the selfs and testcrosses.

The testcross results indicate that there was a poor transmission of foreign genes through the pollen parent, especially when Southern hybridization showed that the primary transgenic parent had two or more bands. Of the 14 transgenic plants with more than one band, 3 failed to transmit either the GUS or the NPT II genes through the pollen parent; 4 other plants showed little or no transmission of the GUS gene; and 6 plants showed little or no transmission of the NPT II gene. Only 1 plant, G82, of the 6 plants with one band on Southern hybridization failed to transmit a foreign gene through the testcrosses.

Discussion

Initial phenotypic screening of primary transgenic plants versus molecular studies

Table 1 indicates some inconsistencies in the initial screening for expression of the NPT II and GUS genes. If kanamycin screening had been used to eliminate plants not expressing kanamycin, 3 plants would have been eliminated on the basis of a lack of root formation on kanamycin, 6 more would have been eliminated on the basis of lack of leaf callus formation on kanamycin, and 3 more would have been eliminated based on a lack of GUS activity. Out of 29 plants, 12 (41%) potentially transgenic plants would have been eliminated. These same 29 plants, however, when examined for incorporation of the genes in the plant's nuclear genome were all positive, with the exception of B3. Since B3 was positive for both rooting on kanamycin and GUS expression in leaf tissue, one can speculate that it may have had a systemic *Agrobacterium* infection, thereby resulting in initial false positive screening results, or that the DNA sequence was lost at some point during plant development. The presence of seemingly intact T-DNAs in all of the transformed plants (except B3) does not agree with the results from Table 1 and shows that the interpretation of transformation results by simple observation of the expected phenotype can be misleading since there may be many false negatives. There are many reasons that could account for the lack of expression of one or both genes in the studied plants.

Subsequent studies on plants G09, RC1, G81, B111 and their progeny showed that methylation of the regulatory region for NPT II accounted for the lack of NPT II expression. However, a few plants which were not methylated did not express NPT II activity, indicating that other factors are also involved in gene regulation. Perhaps the site of gene insertion (positional effects) is also involved in gene expression Meyer et al. (1992).

Deroles and Gardner (1988) implied that a large number of copies of the NPT II gene in transformed petunias seem to be related to the lack of gene expression and non-Mendelian inheritance of the trait. In this study, there does not seem to be any correlation between copy number and gene expression in the primary transformants, though Mendelian inheritance of one gene is more often seen in progeny of plants with a single insert.

Table 4 Segregation of GUS and NPT II in progeny from selfs of primary transgenic plants and testcrosses using pollen from primary transgenic plants to fertilize untransformed plants (– No data available)

a. Plants with one band on Southern hybridizations with GUS and NPT II probes

Plant	Self, 3:1 expected						Testcross, 1:1 Expected					
	Observed GUS$^+$	GUS$^-$	χ^2	Observed NPT II$^+$	NPT II$^-$	χ^2	Observed GUS$^+$	GUS$^-$	χ^2	Observed NPT II$^+$	NPT II$^-$	χ^2
B121	62	34	5.5[a]	3	97	2.76[b]	27	21	0.75	27	98	40.3[b]
R121	70	26	0.21	100	52	6.36[a]	56	40	2.66	90	86	0.09
R111	68	16	1.4	75	54	86.6[b]	62	46	2.37	50	68	0.6
R102	50	46	27[a]	114	55	4.54	48	48	0	78	86	0.39
G82	0	96	738[b]	–	–	–	0	96	96[b]	–	–	–
W5	75	24	0	115	50	2.45	88	8	66.6[b]	60	65	0.2

b. Plants with two bands on Southern hybridizations with GUS and NPT II probes

Plant	Self, 3:1 expected						Testcross, 1:1 expected					
	Observed GUS$^+$	GUS$^-$	χ^2	Observed NPT II$^+$	NPT II$^-$	χ^2	Observed GUS$^+$	GUS$^-$	χ^2	Observed NPT II$^+$	NPT II$^-$	χ^2
R103	0	96	96[b]	70	60	30.0[b]	0	96	96[b]	70	76	0.24
B91	4	92	91[b]	99	64	163.0[b]	4	92	91[b]	74	64	0.72
RC1	16	48	16[b]	24	108	227.25[b]	16	48	16[b]	0	90	90[b]
R2	0	96	96[b]	0	90	269.5[b]	0	96	96[b]	0	90	90[b]
B101	52	44	0.67	96	0	738[b]	52	44	0.67	112	39	36[b]

c. Plants with three or more bands on Southern hybridizations with GUS and NPT II probes.

Plant	Self, 3:1 expected						Testcross, 1:1 Expected					
	Observed GUS$^+$	GUS$^-$	χ^2	Observed NPT II$^+$	NPT II$^-$	χ^2	Observed GUS$^+$	GUS$^-$	χ^2	Observed NPT II$^+$	NPT II$^-$	χ^2
G3	60	36	8.0[b]	90	28	0.925	64	32	10.6[b]	60	85	4.3[a]
B122	80	16	3.6	96	84	45.07[b]	46	50	0.17	88	102	1.3
R122	4	92	256.9[b]	0	100	300[b]	0	96	96[b]	0	100	100[b]
W111	30	68	98.0[b]	0	100	300[b]	42	54	1.5	0	100	100[b]
G81	60	4	12.0[b,c]	0	90	270[b]	49	15	18[b,d]	0	90	90[b]
G09	34	30	16.25[b]	73	57	27.3[b]	32	32	0	49	68	3.84[a]
B2	64	0	21.3[b]	94	30	0.04	64	0	64[b]	136	16	94.8[b]
B1	0	64	192.0[b]	0	90	270[b]	0	64	64[b]	0	90	90[b]
R1	4	64	173.3[b]	36	55	60.95[b]	14	82	49[b]	25	75	25[b]

[a] Significant deviation from expected ratios
[b] Highly significant deviation from expected ratios
[c] Fits 15 GUS$^+$: 1 GUS$^-$ ratio for two GUS$^+$ genes segregating independently
[d] Fits 3 GUS$^+$: 1 GUS$^-$ ratio for two GUS$^+$ genes segregating independently

Quantitative levels of NPT II and GUS enzyme activity

It was evident that levels of gene expression can be very different among primary transgenic plants. This was true for both genes. Even though quantitative studies were not conducted at the early stages of plant growth, it was also apparent that there was a difference in the levels of gene expression at different developmental phases of the plant. Differences between the two genes in levels of gene expression could probably be related to the different promoters for each of the two genes. Additionally, the number of methylation events (Meyer et al. 1992) can increase as the plant ages, decreasing the level of gene expression, or as Ottaviani et al. (1983) have observed, demethylation and reactivation of the gene can occur as the plant material ages.

Variation gene expression over 4 months

Since the phenotype (Table 1) and quantitative (Table 2) measures of gene expression indicated inconsistencies in gene expression over time, 7 mature plants were examined at 30-day intervals for 4 months to see if there were significant differences in gene expression during a 4-month period. Expression of the GUS gene was consistent over the 4-month period. NPT II gene expression was consistent in 5 plants but was apparently turned off

in G81 at 90 days. Plant B111, which was initially negative for NPT II expression, started to express the gene at 30 days. Subsequent studies on these plants (E. C. Ulian et al. submitted) verified that methylation events in the NOS promoter region are correlated with a lack of gene expression. Meyer et al. (1992) also observed phenotype variation in transgenic petunias and suggested that methylation can occur at any time during the plant's life cycle. They did observe decreased flower pigmentation and increased methylation as the growing season progressed.

Inheritance studies for NPT II and GUS

A significant difference between previous studies and the results shown in this paper is that in previous studies, (Budar et al. 1986; Hobbs et al. 1990; Linn et al. 1990; Meyer et al. 1992; Ulian et al. 1988) the segregation patterns of only one foreign gene had been followed, whereas in our study two foreign genes used in the same construct to transform petunia plants were followed through selfs and testcrosses. Another significant difference is that in our study all of the transgenic plants, even those which gave negative results for either the NPT II or GUS positive phenotype but showed the presence of both genes on Southern hybridizations, were used to determine inheritance patterns in crosses. In other studies, plants which failed to express the foreign gene in the selection process were eliminated.

The results given in this study show much more variability in the expression and transmission of both foreign genes in transgenic petunia plants than has been suggested in other studies looking at one gene. Expression of the GUS^+ phenotype does not guarantee expression of the NPT II^+ phenotype, even though the two genes were linked in the same construct to transform the petunia plants. The variability in the inheritance of foreign genes is shown in the absence of expected Mendelian ratios for both genes in the progeny of selfs and testcrosses. In addition, the poor transmission of foreign genes through the pollen may suggest that insertion of foreign genes causes some de-stabilization of the chromosome structure, especially when multiple insertions occur. This may help explain the decreased transmission of foreign traits in general by transgenic plants with two or more copies of the genes inserted into the chromosomes.

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