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Selection for kanamycin resistance in transformed petunia cells leads to the co-amplification of a linked gene

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Abstract

A cell suspension culture was established from a transgenic petunia (*Petunia hybrida* L.) plant which carried genes encoding neomycin phosphotransferase II (*nptII*) and β-glucuronidase (*uidA*, GUS). Two selection experiments were performed to obtain cell lines with increased resistance to kanamycin. In the first, two independently selected cell lines grown in the presence of 350 μg/ml kanamycin were eight to ten-fold more resistant to kanamycin than unselected cells. Increased resistance was correlated with amplification of the *nptII* gene and an increase in *nptII* mRNA levels. Selection for kanamycin resistance also produced amplification of the linked GUS gene, resulting in increased GUS mRNA levels and enzyme activity. Selected cells grown in the absence of kanamycin for twelve growth cycles maintained increased copy numbers of both genes, and GUS enzyme activity was also stably overexpressed. In a second selection experiment, a cell line grown continuously in medium containing 100 μg/ml kanamycin exhibited higher *nptII* and GUS gene copy numbers and an increase in GUS enzyme activity after eleven growth cycles. In this cell line, amplification of the two genes was accompanied by DNA rearrangement.

Introduction

Gene amplification gives an organism the ability to magnify the expression of a particular gene or sets of linked genes, thereby increasing the supply of the final gene product(s). Programmed or developmentally regulated gene amplification has been found both in eukaryotes and prokaryotes [31, 32]; examples include rDNA amplification in *Xenopus* [34] and chorion gene amplification in ovarian follicle cells of *Drosophila* [30]. Amplification and subsequent gene overexpression has been observed in mammalian cell cultures in response to selective pressure by a number of drugs and other inhibitors [31]. Gene amplification has also been documented in bacteria [26], fungi [2], and yeast [36]. There are a few examples of gene amplification in plant cell cultures, and these have all involved selection for herbicide resistance. Glyphosate, L-phosphinothricin, and sulfonylureas inhibit enzymes involved in amino acid synthesis. Cell lines have been selected that are resistant to each of these herbicides, and in several cases amplification of genes encoding the target enzymes has been implicated in the mechanism of resistance [7, 11, 13, 28, 29].

Gene amplification has proven to be an effective method to increase protein levels. It is possible to select directly for amplification of the gene of interest, but for the general application of this method, coamplification systems have been developed [1, 19]. Coamplification allows for the overexpression of a gene product which cannot be directly selected. Bipartite vectors link the gene of interest to a gene which confers resistance to a specific inhibitor. Selection of transformed cells for increased resistance to the inhibitor leads to concurrent amplification of both the resistance gene and the gene of interest, and increased expression of both genes. Coamplification of linked genes has potential applications for increasing the production of antibiotics [1] and various biotherapeutic proteins [8, 16]. This technique could also be used to increase the amount of an enzyme that is the rate limiting step of a biosynthetic pathway. There has been limited research conducted in this area of plant biotechnology. The use of free-replicating geminiviruses containing heterologous genes has been proposed [15], but a high level of expression of foreign genes in plants has generally relied on the use of various promoter sequences, such as the cauliflower mosaic virus (CaMV) 35S promoter. However, transcription of some genes may not respond to the presence of the 35S enhancer [20]. Co-suppression of both the transgene and endogenous homologous genes (if present) can also result in unstable gene expression [21, 27]. In this study we have selected for amplification of a gene encoding *nptII* in response to kanamycin selection. Coamplification and increased expression of a linked reporter gene were observed in response to this selection.

Materials and methods

Cell suspension establishment, culture medium, suspension growth conditions

Cell cultures were initiated from a transgenic *Petunia hybrida* L. plant containing a single copy of the T-DNA from pBI121 (plasmid containing linked neomycin phosphotransferase II (*nptII*) and β-glucuronidase (*uidA*, GUS) genes) [17]. Stem sections from this plant were surface-sterilized for 30 min in 0.525% sodium hypochlorite with 50 μl/l of Tween 20, and rinsed 3 times with sterile water. After sterilization, 1 cm sections of stem were placed on W38 medium [14], solidified with 0.8% agar. Calli produced from these stem sections were transferred to a modified Murashige and Skoog (MS) basal liquid medium [25] containing MS mineral salts, 30 g/l sucrose, 10 mg/l myo-inositol, 1 mg/l 2,4-D, 0.1 mg/l each of nicotinic acid, pyridoxine-HCl, and thiamine-HCl. Cell suspension cultures were periodically filtered through 1 mm screens, and the filtrate transferred to fresh medium. Using these methods a suitable cell suspension culture, designated PF-OA, was obtained. All other cultures described in this paper were then derived from PF-OA. Cultures were maintained on a gyratory shaker at 120 rpm, under 16 h daily illumination of 100 μE m^{-2} s^{-1} (cool white fluorescent lights), and a constant temperature of 26 °C. Cells were subcultured every sixteen to twenty days, at a cell inoculation density of 20 mg (fresh weight)/ml. Kanamycin was sterilized by filtration and added to the medium after autoclaving.

Cell selection and characterization

Two selection experiments were performed to obtain cell lines with increased resistance to kanamycin. In the first, PF-OA cells were inoculated into medium containing 350 μg/ml of kanamycin. This concentration of kanamycin reduces growth of PF-OA cells to 20% of that observed in medium without kanamycin. Cells were transferred to fresh medium every 18 to 20 days. After approximately 14 transfers, cells became adapted to the selection conditions. Two independent cell lines, designated PF-350A and PF-350B, were obtained. From these selected cell lines, duplicate cell cultures were initiated (referred to in the text as PF-350A1, A2, B1 and B2). After approximately 30 cell culture cycles in the presence of 350 μg/ml kanamycin (ca. 25 months), the growth and kanamycin resistance of PF-350 cell lines

were assessed. Cells were inoculated into 25 ml of medium containing varied concentrations of kanamycin. Fresh weights of cells were determined after fourteen days when cells had reached the stationary phase of growth. In a second selection experiment, PF-OA cells were inoculated in medium supplemented with 100 μg/ml kanamycin. Cells were again transferred to fresh medium every 18 to 20 days. Cells that were not required as inoculum were collected and stored at −70 °C for further analysis. One cell line obtained in this second experiment, designated PF-100B, was analyzed.

GUS enzyme activity assay

Cells (0.2 g) were ground in liquid nitrogen and then in 1 to 1.5 ml of GUS extraction buffer (0.1 M potassium phosphate pH 7.8, 1 mM EDTA, 10 mM DTT, and 5% glycerol). After centrifugation, 40 μl of the supernatant was added to 960 μl of extraction buffer containing 1 mM 4-methylumbelliferyl glucuronide. Reactions were incubated at 37 °C, and aliquots were removed and the reaction stopped with 0.2 M Na_2CO_3 at 3 time points (0, 20, and 60 min) for each reaction. GUS enzyme activity was measured fluorimetrically as described [17]. Total protein in extracts was determined [4] using bovine serum albumin as a standard. At least two extracts were assayed for each data point presented in this report.

Nucleic acid analysis

Total RNA was isolated from cells as described [10]. DNA was prepared using a modification of the procedure described [6], with further purification by CsCl buoyant density centrifugation in the presence of ethidium bromide [22]. RNA denaturation, gel electrophoresis, and transfer to nitrocellulose filters were performed as described by Goldsbrough *et al.* [11] with the following modifications. RNA was crosslinked to the membrane using a UV Stratalinker (Stratagene USA). Pre-hybridization and hybridization were done at 65 °C in the absence of formamide.

Gel electrophoresis of DNA, transfer to nitrocellulose, and hybridization conditions were essentially as described [11]; DNA was crosslinked to the membranes using a UV Stratalinker (Stratagene USA) rather than baking the membranes.

The following probes were used for hybridization; pKS4, a plasmid containing a *Hin*d III-*Bam* HI fragment of the *nptII* gene of Tn5 [3], and pRAJ260, containing a *Pst* I fragment of the *uidA* (GUS) gene [17]. DNA inserts were separated from vector sequences and labeled with ^{32}P-dCTP to specific activities of approximately 1×10^8 cpm/μg using an oligonucleotide primer kit (Ambion DecaPrime) essentially as described by Feinberg and Vogelstein [9]. Radiolabelled probes were added to give 1×10^6 cpm/ml for hybridization of DNA and RNA blots.

Results

Establishment of kanamycin-resistant cell cultures

A cell suspension culture was established from a transgenic petunia plant that contained a single copy of the T-DNA from pBI121 [17]. From this initial cell culture, cell cultures with increased resistance to kanamycin were obtained by *en masse* selection. The concentration of kanamycin used in the selection procedure (350 μg/ml) initially reduced cell growth to ca. 20% of that of cells grown in kanamycin-free medium; however, the growth rate of cells under kanamycin selection gradually increased. After 30 cell culture cycles (25 months) in the presence of kanamycin, the levels of kanamycin resistance in selected cell lines (now referred to as PF-350A1, A2, B1, B2) were determined in a dose response experiment. Selected cells exhibited an eight to ten-fold increase in kanamycin resistance (based on the ID_{50}, the level of kanamycin necessary to reduce the growth of cell cultures by 50%) compared to unselected, transgenic PF-OA cells (Fig. 1). All selected cell lines showed similar levels of resistance to kanamycin. PF-OA and PF-350 cell lines had lag

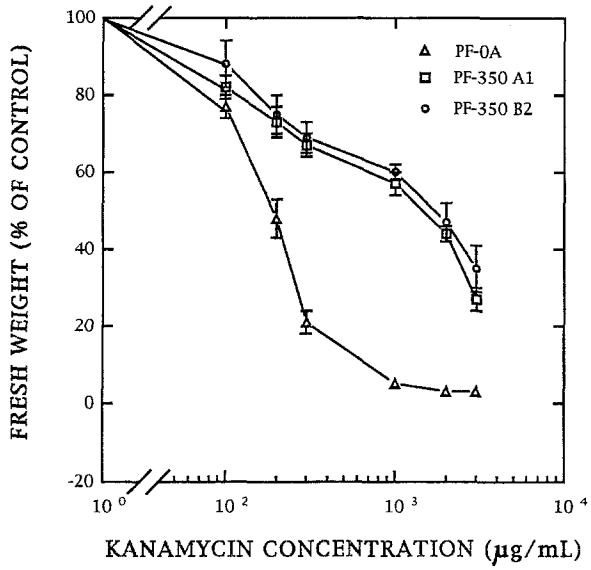

Fig. 1. Response of selected and unselected petunia cell lines to kanamycin. 25 ml aliquots of media containing increasing concentrations of kanamycin were inoculated with 500 mg of cells. Fresh weight was measured after 16 days. Fresh weights were plotted as a percentage of fresh weight of cells grown without kanamycin. Error bars represent standard error ($n = 3$).

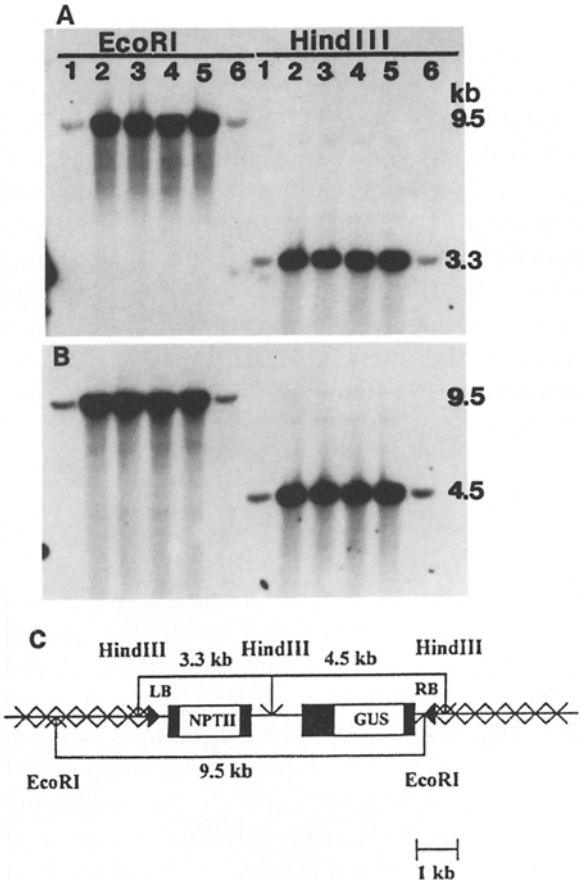

Fig. 2. Concurrent amplification of linked *nptII* and GUS genes in selected cell cultures. 5 µg of DNA was digested with either *Eco* RI or *Hin*d III as indicated. DNAs from PF-OA (1), PF-350A1 (2), PF-350A2 (3), PF-350B1 (4), PF-350B2 (5), and PF-OB (6) were used. Filters were hybridized with a probe for *nptII* (pKS4, filter A) or with a probe for GUS (pRAJ260, filter B). C is a diagram showing the location of *Eco* RI and *Hin*d III sites in the T-DNA insert and surrounding plant DNA. PF-350A1/A2 and PF-350B1/B2 are duplicate cell cultures initiated from PF-350A and PF-350B, respectively.

phases of about 4 days after subculture into fresh medium (data not shown). Maximum fresh weights were obtained after about 14 days when the cell densities reached 240 mg/ml for PF-OA and 140–160 mg/ml for PF-350 cells.

Amplification of nptII *and GUS genes*

To determine if the increase in kanamycin resistance was due to an elevated copy number for the *nptII* gene, Southern blots were performed on DNA isolated from unselected and selected cell lines. Amplification of the *nptII* gene occurred in the selected cell lines (Fig. 2A); the time at which gene amplification occurred in these cell lines was not determined, but amplification was evident in DNA isolated from all PF-350 lines after 21 cell culture cycles in the presence of kanamycin. Reconstruction experiments indicated that the *nptII* gene had been amplified ca. 10-fold in selected cultures (data not shown). This increase in *nptII* gene copy number is similar in extent to the increase in kanamycin resistance observed in the selected cell cultures.

The single *Eco* RI fragment of 9.5 kb that hybridized to the *nptII* probe in both PF-O and PF-350 DNA indicated that the entire T-DNA region, and likely the flanking DNA as well, had been amplified in PF-350 cells. This would indicate amplification of the linked GUS gene, and was confirmed by hybridizing the GUS probe to

a similar blot (Fig. 2B). The observation that fragments of the same size are detected in PF-O and PF-350 indicates that the amplified region extends beyond the T-DNA insert, without any rearrangement of the T-DNA. As we used only one transgenic plant to establish our cell suspension lines, it is unclear if the co-amplification observed in this study was dependent on integration of the T-DNA into a specific chromosomal position. Future studies will test cell lines derived from other transgenic plants to determine how frequently co-amplification can be obtained.

Expression of nptII *and GUS genes in selected cultures*

To determine if gene amplification increased levels of both *nptII* and GUS mRNA, RNA isolated from unselected and selected cell cultures was hybridized with probes for both genes (Fig. 3). Elevated steady-state levels of both *nptII* and GUS mRNA were observed in the PF-350 cell lines (*nptII* mRNA in unselected cells is detected with prolonged exposure of the filter). The abundance of *nptII* mRNA correlates with the level of gene amplification and kanamycin resistance, and presumably results in increased neomycin phosphotransferase activity, although this was not measured. GUS mRNA is more abundant than *nptII* mRNA, and this likely reflects the relative strengths of the 35S and *nos* promoters.

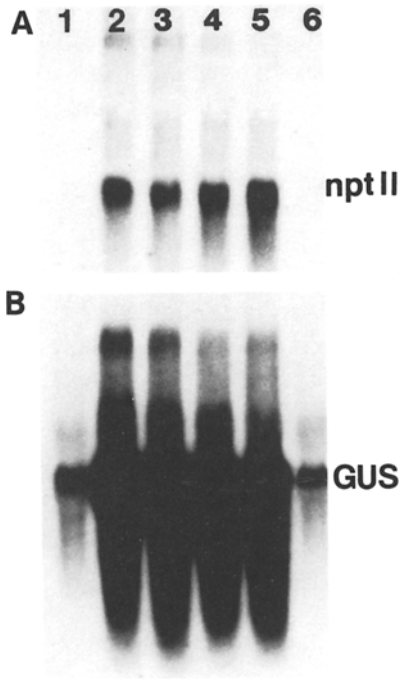

Fig. 3. NptII and GUS mRNA expression in selected cells. Total RNA (10 μg) from PF-OA (lane 1), PF-350A1 (2), PF-350A2 (3), PF-350B1 (4), PF-350B2 (5), and PF-OB (6) were hybridized with a *nptII* (filter A) or a GUS probe (filter B).

GUS enzyme activity was also enhanced in selected cells when compared to unselected cells (Table 1). The level of GUS activity correlated with the level of gene amplification observed in the selected cultures in 3 of the 4 cell lines. The reason for the higher activity observed in PF-350B1 is unknown.

Stability of DNA amplification in selected cultures

The stability of gene amplification was examined in PF-350 cells grown in the absence of kanamycin selection. There was no observable difference in the level of amplification for either gene in selected cells grown in the absence of kanamycin for up to 12 cell culture cycles when compared to PF-350A cells cultured continuously in the presence of kanamycin (Fig. 4). Removing the kanamycin selection also had little effect on GUS enzyme activity in most instances (Table 2). There was a noticeable reduction in GUS activity in cells assayed after seven and ten growth cycles without kanamycin when compared to PF-350A

Table 1. GUS activity in selected cell cultures.

Cell culture	Enzyme activity[1] (nmol per mg protein per minute)	Relative activity[2]
PF-0A	1.12 ± 0.43	1.0
PF-0B	0.98 ± 0.22	0.9
PF-350A1	8.75 ± 2.06	8.3
PF-350A2	12.53 ± 2.06	11.9
PF-350B1	21.10 ± 1.77	20.1
PF-350B2	11.10 ± 2.57	10.6

[1] Mean ± SE ($n = 3$).
[2] Compared to unselected PF-0A cells.

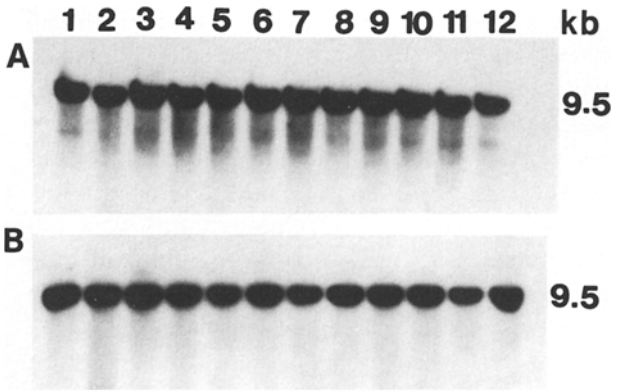

Fig. 4. Stability of the DNA amplification in a selected cell line. Cells from a PF-350A cell line, which had been grown in the presence of 350 μg/ml kanamycin for ca. 20 months, were subcultured into media containing no kanamycin. Cells were harvested at the end of each cell culture cycle and DNA isolated from this material. DNA (5 μg) was digested with *Eco* RI and separated on agarose gels. Lanes 1 and 12 are DNAs from PF-350A, lanes 2–10 are 1 to 9 cell culture cycles without kanamycin, lane 11 is DNA from cells grown 12 cycles without kanamycin. Filters were hybridized with a *nptII* (A) or a GUS (B) probe.

levels. These reductions were transient however, as GUS activity was not reduced in the following growth cycles in either case. Conversely, GUS activity was elevated in the last two growth cycles measured. Fluctuations in GUS activity observed in these experiments may be a consequence of harvesting material at the end of the culture period, with cells in the stationary phase. Alterations in protein turnover under these conditions might lead to large differences in specific activity for an enzyme that is thought to be particularly stable, such as GUS.

Progression of DNA amplification in a selected cell line

A second selection experiment was performed to determine if and when gene amplification would occur under less severe selection conditions. PF-OA cells were grown in medium containing 100 μg/ml kanamycin and cells collected for DNA and GUS activity analysis at the end of each cell culture cycle. One cell line, designated PF-100B,

Table 2. Stability of GUS activity in PF-350A cells grown without kanamycin.

Number of cycles without kanamycin	Enzyme activity[1] (nmol per mg protein per minute)	Percent of control
Control (PF-350A)	20.1 ± 3.1 (7)	100
1	19.4 ± 1.6 (3)	97
2	20.8 ± 5.1 (4)	103
3	17.6 ± 0.2 (3)	88
4	17.3 ± 2.7 (4)	86
5	20.7 ± 1.1 (3)	103
6	20.9 ± 2.8 (4)	104
7	15.4 ± 2.2 (5)	77
8	19.1 ± 0.7 (3)	95
9	22.1 ± 1.4 (3)	110
10	10.7 ± 1.9 (4)	53
11	35.6 ± 9.4 (4)	177
12	26.5 ± 5.6 (4)	132

[1] Mean ± SE (n = number of assays per data point); at least 2 separate extracts were done for each data point.

was analyzed in this experiment. Amplification of *nptII* and GUS genes was not detected until the eleventh growth cycle in 100 μg/ml kanamycin (Fig. 5A, 5B). Amplification of both genes occurred simultaneously and was accompanied by the appearance of a novel 7.7 kb *Eco* RI fragment that hybridized to both GUS and *nptII* probes. The simplest interpretation of the observed hybridization pattern is that there has been a rearrangement in the plant DNA adjacent to the *nptII* gene, although this requires further investigation. Although both the original 9.5 kb and rearranged 7.7 kb fragments were amplified, the smaller fragment was amplified to a greater degree by the end of the fifteenth passage under kanamycin selection. Other cell lines selected by this procedure also have amplified GUS genes, but, unlike PF-100B, their T-DNA inserts were not rearranged (data not shown).

The specific activity of GUS correlated with the appearance of gene amplification (Fig. 5C). GUS activity levels began to rise in the ninth growth cycle when the 7.7 kb DNA fragment was first observed (Fig. 5A, 5B, Lane 10). The level of GUS activity in cells harvested from the eleventh to the fifteenth growth cycles was approximately five- to six-fold greater than in the

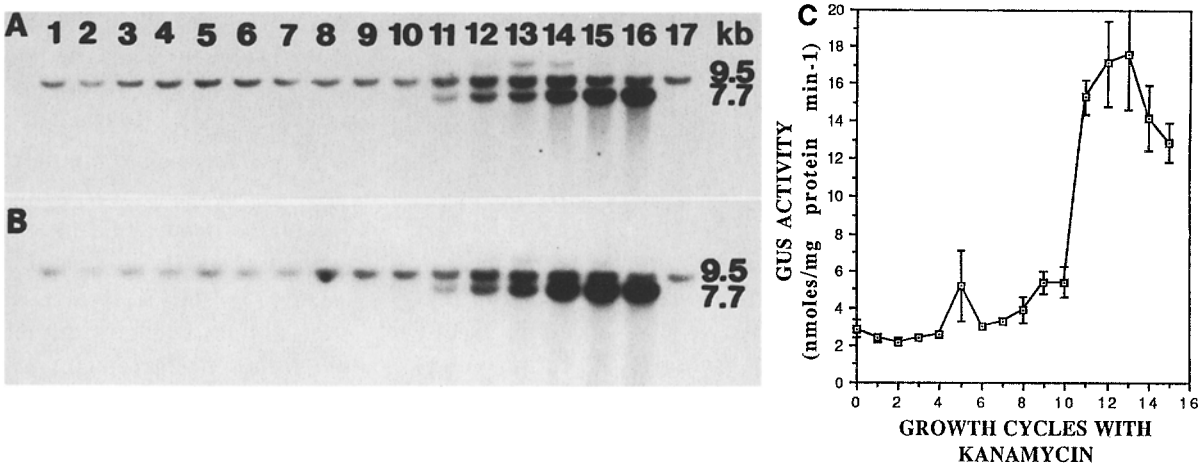

Fig. 5. Progression of DNA amplification in PF-100B. PF-OA cells were grown in media containing 100 μg/ml of kanamycin for 15 cell culture cycles. DNA was isolated from cells harvested at the end of each cell culture cycle. DNA (5 μg per lane) was digested with *Eco* RI and separated on agarose gels. Filters were hybridized with a *nptII* (A) or a GUS (B) probe. Lanes 1 and 17 are DNA from PF-OA, lanes 2–16 are DNA samples from PF-100B cultures grown with kanamycin (1 to 15 cell culture cycles). GUS activity in PF-100B cells during kanamycin selection is depicted in panel C. GUS assays were performed with cells harvested at the end of each cell culture cycle (1 through 15); at least two extracts were assayed for each data point (duplicate assays were performed for most extracts).

unselected PF-OA cell line. The DNA rearrangements that occurred during gene amplification in the PF-100B cell line did not alter GUS gene expression.

Discussion

Coamplification of genes linked to an amplified gene has been reported in mammalian cell cultures [19]. Since the region of amplification, or amplicon, is generally large, typically ranging from thirty to more than a thousand kilobases [31, 32]. it is not surprising that closely linked genes can also be amplified in response to selection. This was observed in our petunia cell cultures selected for kanamycin resistance, where increased kanamycin resistance in selected cells correlated with amplification of the *nptII* gene and elevated *nptII* mRNA levels. A concurrent increase in GUS gene copy number, mRNA level, and enzyme activity was also observed. Amplified copies of both genes were stably maintained in cell culture, and amplification occurred within eleven growth cycles in one experiment. To our knowledge, this is the first reported case of gene amplification in response to kanamycin selection [19].

The potential usefulness of amplification to increase gene expression is dependent on stable maintenance of the amplified genes in the plant genome. In the selected PF-350 cells, amplified *nptII* and GUS genes were maintained over a period of 12 growth cycles (ca. 8 months) without kanamycin selection. Although there was some fluctuation in the level of GUS gene expression observed during the course of the experiment, there was no obvious trend toward reduced GUS activity under these conditions. These results suggest that the amplified gene copies have been integrated into chromosomal DNA, rather than existing as extrachromosomal elements (double minute chromosomes) which are rapidly lost upon the removal of selection pressure [5, 31] Other studies have indicated that in plant cells gene amplification in response to selection can be quite stable. Amplification of genes encoding EPSPS in a selected tobacco cell line was maintained for three years in the absence of glyphosate [11]. However, Shyr *et al.* [29] observed a gradual decline in EPSPS enzyme activity and gene copy

number in glyphosate-tolerant carrot lines which were grown in the absence of glyphosate for two to five years. Examples of both stable and unstable gene amplification have been described in a tobacco cell line selected for resistance to a sulfonylurea herbicide [13]. Stable expression of amplified genes has been observed in several cases (including this report) [2, 11, 13, 23, 36] in the absence of selective pressure. Co-suppression of multiple genes, which has been observed in experiments utilizing the 35S promoter to increase transgene expression [21, 27], has not been observed in cases where increased gene expression is due to gene amplification.

No case of selected gene amplification at the whole plant level has yet been reported. The utility of gene amplification as a mechanism of increasing gene expression in plants would be enhanced if amplified genes could be stably maintained and sexually transmitted. Plantlets containing amplified copies of genes encoding EPSPS were regenerated from glyphosate-resistant tobacco cells [35], and the amplified genes have been maintained in vegetatively propagated plantlets for a number of years [18]. The ability to regenerate fertile plants from long-term cell suspension cultures can be greatly reduced due to increased DNA variation and/or rearrangement [24]. However, we were able to achieve a high level of amplification within 11 growth cycles in cell culture, which may be rapid enough to allow regeneration of fertile plants. This may be unnecessary if the product of increased gene expression can be obtained directly from cell suspension cultures.

The failure to regenerate fertile plants containing amplified genes has prevented the meiotic stability of amplified genes from being studied. Meiotic instability of amplified genes has been observed in *Aspergillus* [38]. In yeast [37], unequal crossing-over within the CUP1 array and gene conversion of unpaired repeat units was observed, which gave rise to variation in gene copy number in daughter cells.

The process of amplification can lead to DNA rearrangements, due to deletion of flanking DNA or insertion into another region of the chromosome [12], or to rearrangements within individual copies of the repeat unit which are then amplified further [38]. Suh *et al.* [33] recently reported that in carrot cell cultures resistant to high (35 mM) glyphosate concentrations, DNA sequences carrying amplified EPSPS genes also contained DNA rearrangements. The appearance of novel hybridizing DNA fragments in one cell line suggests that rearrangements had occurred in or around the region of amplified DNA. Rearrangements in the amplicon, due to either the amplification process or the variation inherent in plant cell culture, could have deleterious effects on the expression of the gene(s) being amplified. However, in this study, we observed no loss in GUS activity in the selected cell line (PF-100B) in which DNA rearrangements occurred. Chimeric *nptII* genes, such as that used here, are widely used in plant transformation and most procedures require prolonged selection of plant material for kanamycin resistance. The transgenic plants that are produced frequently contain multiple and/or rearranged T-DNA insertions. Some of these abnormal T-DNA structures may result from gene amplification events during selection as opposed to rearrangements during the initial transformation by *Agrobacterium*.

The coamplification system described here could be used in plants to increase the expression of any cloned gene by linking the desired gene to *nptII* in a transformation vector. Exposure of transgenic cells to elevated concentrations of kanamycin would concurrently amplify both the *nptII* and linked genes. Since *nptII* is frequently used as a selectable marker in plant transformation experiments, this technique is quite feasible. Overexpression of a gene product could result directly in increased production of specific proteins. It may also be possible to increase the productivity of a biosynthetic pathway by overexpressing a gene which encodes an enzyme which is rate limiting in the pathway. Because direct selection for increased expression of such genes is not usually possible, linkage to a selectable gene such as *nptII* may enable their expression to be increased by gene amplification in response to indirect selection pressure. This has been demonstrated previously in mammalian cell cultures

[19]. The results presented here indicate that such an approach can also be used to overexpress a variety of genes in plants.

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