Expression of bacterial genes in plant cells

(plant protoplasts/transformation/foreign DNA/antibiotic resistance/selectable markers)

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ABSTRACT   Chimeric bacterial genes conferring resistance to aminoglycoside antibiotics have been inserted into the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid and introduced into plant cells by *in vitro* transformation techniques. The chimeric genes contain the nopaline synthase 5' and 3' regulatory regions joined to the genes for neomycin phosphotransferase type I or type II. The chimeric genes were cloned into an intermediate vector, pMON120, and inserted into pTiB6S3 by recombination and then introduced into petunia and tobacco cells by cocultivating *A. tumefaciens* cells with protoplast-derived cells. Southern hybridization was used to confirm the presence of the chimeric genes in the transformed plant tissues. Expression of the chimeric genes was determined by the ability of the transformed cells to proliferate on medium containing normally inhibitory levels of kanamycin (50 μg/ml) or other aminoglycoside antibiotics. Plant cells transformed by wild-type pTiB6S3 or derivatives carrying the bacterial neomycin phosphotransferase genes with their own promoters failed to grow under these conditions. The significance of these results for plant genetic engineering is discussed.

The transformation of plant cells by virulent strains of *Agrobacterium tumefaciens* has been studied extensively by several laboratories (1–4). A small fragment of the tumor-inducing (Ti) plasmid, called transferred DNA (T-DNA), is known to be transferred to and stably incorporated in the nuclear DNA of transformed plant cells (5–7). The T-DNA is actively transcribed in plant cells (8–10) and specific gene products have been shown to be responsible for the observed phytohormone-independent growth characteristics (11, 12) and novel metabolic capacities (13) exhibited by crown gall tumor cells. The transfer and insertion of T-DNA into plant DNA is thought to involve repeated nucleotide sequences present near the T-DNA "borders" (14, 15) as well as other genes of unknown function located in specific virulence regions outside of T-DNA (16, 17).

In spite of our considerable understanding of the *A. tumefaciens*-Ti plasmid system, several problems remain which limit its use as a vector for genetically modifying higher plants. Because of the high levels of phytohormones produced by crown gall tumor cells (18) they have generally proven recalcitrant to attempts to induce regeneration into whole plants (19, 20). Exceptions to this are cases in which, as a result of aberrant integration or spontaneous deletion events, transformed cells have lost all or part of the Ti plasmid tumor genes and can now be regenerated (21, 22). In addition, transformation of cells by weakly virulent, mutant Ti plasmids (23) and transformation by root-inducing (Ri) plasmids (24, 25) have been shown to produce callus that can be regenerated into whole plants. However, these plants often display morphological aberrations and

may retain certain tumorous properties (26). Another obstacle has been the failure to obtain expression from a variety of foreign genes that have been introduced into plants (23, 27). Reasons for this include the fact that, up to now, most studies have utilized either heterologous genes from bacteria, fungi, and mammalian cells whose regulatory regions may not be recognized by the plant RNA polymerases or highly regulated plant genes which are normally expressed in specialized tissues and which may not be transcribed in undifferentiated crown gall tumor tissue.

To bypass the dependence on tumor genes for identifying transformed plant cells and to overcome the barriers to gene expression in plants, chimeric genes that function as dominant selectable markers have been assembled. These contain the neomycin phosphotransferase (NPTase) coding sequences from the bacterial transposons Tn5 (type II) or Tn601 (type I) joined to the 5' and 3' regulatory regions of the nopaline synthase gene from the Ti plasmid. This paper describes the construction of these chimeric genes and their introduction and expression in plant cells.

MATERIALS AND METHODS

DNA Preparation. Plasmid pBR322 and its derivatives or M13 replicative form DNAs were purified by using either a Triton-X-100/CsCl procedure (28) or a large-scale alkaline lysis procedure (29), followed by purification on hydroxylapatite (30).

DNA fragments were isolated by electroelution into dialysis bags after polyacrylamide gel electrophoresis and band excision or by adsorption onto NA-45 DEAE membrane (Schleicher & Schuell) after agarose gel electrophoresis (31).

The *Bam*HI synthetic DNA linkers (5' C-C-G-G-A-T-C-C-G-G) were purchased from Collaborative Research (Waltham, MA). Other synthetic DNAs were synthesized by using a modification of the phosphite procedure (32).

Enzymes. All restriction endonucleases and the large Klenow fragment of DNA polymerase I were obtained from New England Biolabs or Bethesda Research Laboratories and were used according to the instructions of the supplier. Phage T4 DNA ligase was prepared as in ref. 33. DNA fragment assembly was carried out as described (31).

Transformation of *Escherichia coli* Cells. Plasmid DNAs were introduced into *E. coli* cells by using CaCl$_2$-treated or RuCl$_2$-treated cells (31). The recipient *E. coli* K-12 strains were SR200 = C600 *thr pro recA56 hsdR(r⁻m⁺)* (34); LE392 = ED8554 *hsdR (r⁻m⁺)* (31); SR20 = GM42 = *his dam*-3 (35); and the M13

Abbreviations: bp, base pair(s); kb, kilobase(s); NPTase I and NPTase II, neomycin phosphotransferase, types I and II, respectively; Ti plasmid, tumor-inducing plasmid; T-DNA, transferred DNA; Ri plasmid, root-inducing plasmid.

phage host, JM101 (36). Cells carrying recombinant plasmids were selected or grown (or both) on Luria medium plates or broth at 37°C containing appropriate antibiotics (ampicillin, 200 μg/ml; spectinomycin, 50 μg/ml; and kanamycin, 40 μg/ml).

Introduction of pMON120 Derivatives into *A. tumefaciens.* Plasmid pMON120 or its derivatives were transferred to a chloramhpenicol-resistant *A. tumefaciens* strain GV3111 = C58C1 Cm^R carrying pTiB6S3tra^c (37) by using a triparental plate mating procedure (ref. 38; R. Riedel, personal communication). Briefly, 0.2 ml of a fresh overnight culture of LE392 carrying pMON120 or its derivative was mixed with 0.2 ml of an overnight culture of HB101 (31) carrying the pRK2013 (38) plasmid and 0.2 ml of an overnight culture of GV3111 cells. The mixture of cells was spread on an LB plate and incubated for 16–24 hr at 30°C to allow plasmid transfer and recombination. The cells were resuspended in 3 ml of 10 mM MgSO$_4$ and a 0.2-ml aliquot was then spread on an LB plate containing 25 μg of chloramphenicol per ml and 100 μg each of spectinomycin and streptomycin per ml to select *A. tumefaciens* carrying the pMON120 derivatives. After incubation for 48 hr at 30°C, ≈10 colonies per plate were obtained. Control matings between HB101/pRK2013 cells and GV3111 cells never gave rise to colonies after this selection. Typically, one colony was chosen and grown at 30°C in LB medium containing chloramphenicol, spectinomycin, and streptomycin at the same concentrations given above.

Protoplast Isolation and Culture. "Mitchell" petunia plants were grown in environmental chambers under fluorescent and incandescent illumination (≈5,000 lux, 12 hr/day) at 21°C in a 50:50 mixture of vermiculite and Pro-mix BX (Premier Brands, Quebec, PQ, Canada). Leaves were surface sterilized, cut into 2-mm strips, and enzymatically digested as described (39). The resulting protoplasts were purified by passage through stainless steel meshes and by density floatation as described (39). The protoplasts were plated in tissue culture flasks (T75, Falcon; 6 ml per flask) at a cell density of 10^5 cells per ml in culture medium [MS salts (GIBCO), B-5 vitamins, 3% (wt/vol) sucrose, 9% (wt/vol) mannitol, 1 μg of 2,4-D per ml, and 0.5 μg of benzyl adenine per ml, pH 5.7].

Cocultivation of *A. tumefaciens* Cells with Plant Protoplasts. On day 2 after protoplast isolation, aliquots (10–50 μl) of an overnight culture of *A. tumefaciens* cells were added to each flask (final bacterial cell density = 10^8 cells per ml) and cocultivation with plant cells was carried out for 24–30 hr essentially as described (40). On day 3, 6 ml of culture medium (lacking phytohormones and mannitol) containing carbenicillin (1.5 mg/ml) was added to each flask (final concentration = 500 μg/ml) to prevent further bacterial growth. On day 4, an additional 6 ml of the above medium (containing carbenicillin at 500 μg/ml) was added. On day 6, 0.5 ml of the cell mixture was transferred to and spread in a thin layer on the surface of double-filter feeder plates (41). These consisted of agar medium (MS salts, B-5 vitamins, 3% sucrose, 3% mannitol, 0.1 μg of indole acetic acid per ml, and 500 μg of carbenicillin per ml at pH 5.7), a layer of *Nicotinia tabacum* suspension cells, a tight fitting 8.5-cm Whatman filter paper disc (guard disc), and a 7.0-cm Whatman filter paper disc (transfer disc). After 7–10 days, microcolonies (≈0.5 mm) were observable on the feeder plates and the transfer disc was removed and placed on selection medium (MS salts, B-5 vitamins, 3% sucrose, 500 μg of carbenicillin per ml at pH 5.7) lacking phytohormones. Within 2 wk, hormone-independent transformants could be readily distinguished as green colonies against a background of dying, brown nontransformed cells. The transformation frequency in these experiments was ≈10^{-1}. The hormone-independent transformants were then transferred to medium (MS salts, B-5 vitamins,

3% sucrose, 500 μg of carbenicillin per ml at pH 5.7) containing kanamycin (50 μg/ml).

Analysis of Transformants. Octopine and nopaline synthase activities were determined as in ref. 42 with the substitution of [^{14}C]arginine (Amersham, 0.5 μCi/2.5-μl assay; 1 Ci = 3.7 × 10^{10} Bq) for the unlabeled arginine in the assay buffer. The conditions for electrophoresis were as described (42) and the resulting electrophoretograms were exposed to x-ray film (Kodak, XAR-5) for 16–24 hr. The positions of octopine, nopaline, and arginine were established by their comigration with authentic standards.

Callus for NPTase assays were frozen in liquid N$_2$ and extracted by using a mortar and pestle in a minimal volume of buffer (0.2 M Tris·HCl/2 mM EDTA/7.5% polyvinylpolypyrrolidone). The crude extract was clarified by centrifugation (Eppendorf; Brinkmann) and assays were performed as described (43).

RESULTS

NPTase coding sequences were used in the initial chimeric gene constructions described in this study because plant cells were determined to be sensitive to various aminoglycoside antibiotics (unpublished data), and the expression of NPTase in yeast (43) and mammalian cells (44, 45) has been previously shown to confer resistance to the antibiotic, G418. The nopaline synthase gene promoter and 3'-nontranslated regions were selected because this gene has been well characterized (9, 46) and it is known to be expressed constitutively in most plant tissues transformed with the *A. tumefaciens* Ti plasmid (47).

Construction of Chimeric Genes. The nopaline synthase promoter region, obtained on a 350-base-pair (bp) *Sau*3A fragment from the *Hind*III-23 fragment of pTiT37 (Fig. 1; ref. 46), was engineered to remove the entire nopaline synthase coding sequence. The resulting promoter fragment that extends from base −264 to base 35 of the nopaline synthase sequence (46) was positioned next to the *Bgl* II site located just outside the NPTase II coding sequence (49). In addition, a 260-bp *Mbo* I fragment, extending from base 1,297 to base 1,554 of the published nopaline synthase sequence (46), was isolated from the *Hind*III-23 fragment. This *Mbo* I fragment contains the nopaline synthase 3'-nontranslated region and polyadenylylation site. This fragment was ligated together with the *Eco*RI–*Bam*HI fragment that contained the nopaline synthase promoter and NPTase II structural gene to yield the intact chimeric gene on a 1.5-kilobase (kb) *Eco*RI fragment (Fig. 1). A second chimeric gene, containing the nopaline synthase promoter and 3'-nontranslated region joined to the NPTase I coding sequence (Fig. 2), was constructed in a similar fashion. As controls, plasmids were constructed that contained an intact NPTase II promoter and structural sequence with the nopaline synthase 3'-nontranslated region (pMON139 and pMON140; Fig. 2).

Introduction of Chimeric Genes into the Ti Plasmid. The vector pMON120 used for the transfer of the chimeric genes into *A. tumefaciens* cells is shown in Fig. 2. Its essential features include (*i*) a segment of pBR322 DNA for replication in *E. coli*, (*ii*) a segment from pTiT37 that contains a functional nopaline synthase gene to facilitate the rapid identification of transformants, (*iii*) a segment of Tn7 carrying the spectinomycin/streptomycin-resistance determinant for selection in *A. tumefaciens*, (*iv*) a DNA segment obtained from the pTiA6 T-DNA fragment *Hind*III-18c (see T-DNA map, ref. 11), which is included to provide homology for recombination with a resident octopine-type Ti plasmid in *A. tumefaciens*, and (*v*) unique restriction sites (*Eco*RI and *Hind*III) for insertion of the chimeric genes. The pMON120 plasmid and derivatives were in-
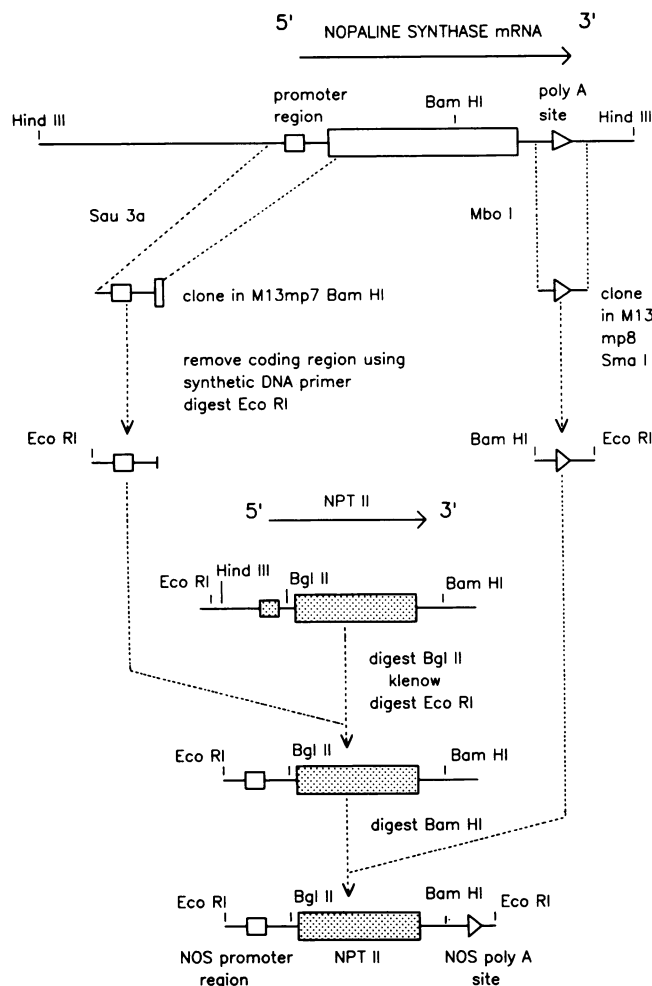
FIG. 1. Isolation and assembly of a chimeric gene containing no-paline synthase (NOS) promoter-NPTase II (NPT II) coding sequence-nopaline synthase 3'-nontranslated region. The nopaline synthase pro-moter was isolated on a 350-bp *Sau*3A fragment that also contained the first 44 bp of the nopaline synthase coding sequence. The sense strand of this fragment was cloned into the *Bam*HI site in M13 mp7 (36), the 44 bp was removed by using a modification of a published synthetic primer procedure (48) with a primer complementary to bases 22–35 of the published nopaline synthase sequence (46), and a 308-bp promoter fragment was obtained after digestion with *Eco*RI. The flush-end of the promoter fragment was joined to a 1-kb *Bgl* II–*Bam*HI fragment car-rying the NPTase II coding sequence (a *Bam*HI linker had been in-serted at the *Sma* I site) at the filled-in *Bgl* II site (49). This fusion re-generates the *Bgl* II site. The chimeric gene was completed by the addition of a 260-bp *Mbo* I fragment that contained the nopaline synthase 3'-nontranslated region. This fragment, which contains a polyadenylyl-ation signal (46), was converted to a flush-ended fragment with Klenow polymerase and cloned into the *Sma* I site of a M13 mp8 (50) to intro-duce *Bam*HI and *Eco*RI sites at the 5' and 3' ends, respectively. The resulting 280-bp fragment was joined to the 1,300-bp *Eco*RI–*Bam*HI nopaline synthase promoter-NPTase II coding sequence fragment to generate the complete chimeric gene.

troduced into *A. tumefaciens* as described in *Materials and Methods.*

Selection of Kanamycin-Resistant Petunia Transformants. Several hundred hormone-independent calli (1–2 mm in di-ameter) obtained from cocultivation experiments with *A. tu-mefaciens* strains carrying pTiB6S3::pMON120 (or derivatives) recombinant plasmids were pooled and analyzed by DNA blot hybridization for the presence of the chimeric genes (Fig. 3). The results confirm the presence of the expected 1.6-kb *Eco*RI fragment, which carries the chimeric nopaline synthase-NPTase II-nopaline synthase gene in pMON128 and pMON129 trans-
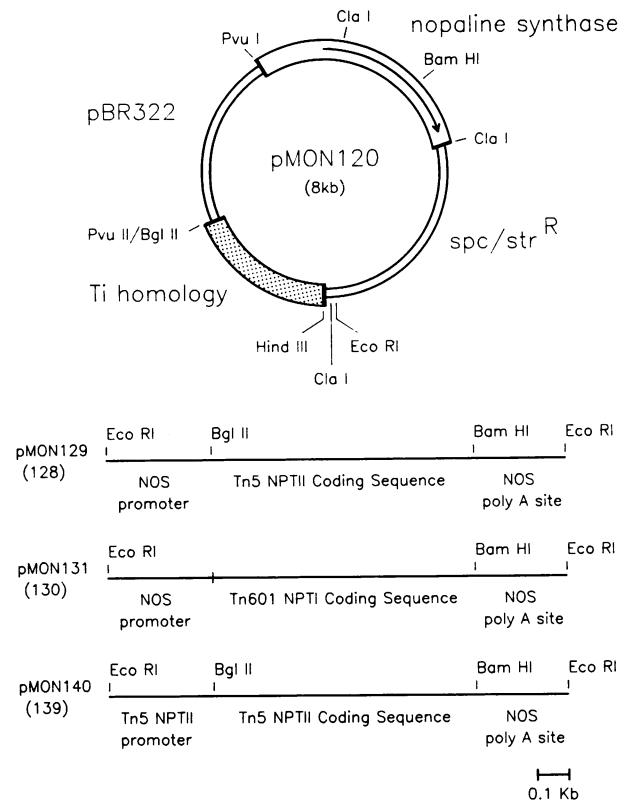
FIG. 2. Structures of the pMON120 intermediate vector and chi-meric genes introduced into plant cells. Plasmid pMON120 contains the following segments of DNA: the 1.7-kb pBR322 *Pvu* II to *Pvu* I frag-ment that carries the origin of replication and *bom* site (51), a 2.2-kb partial *Cla* I to *Pvu* I fragment of pTiT37 DNA that encodes an intact nopaline synthase (NOS) gene, a 2.7-kb *Cla* I-*Eco*RI fragment of Tn7 (37) DNA carrying the determinant for spectinomycin/streptomycin resistance, and the 1.6-kb *Hind*III–*Bgl* II fragment from the *Hind*III-18c fragment of the pTiA6 plasmid. This T-DNA fragment is known to specify two transcripts that are not essential for tumorous growth (8, 12). At the bottom are three chimeric genes inserted at the unique *Eco*RI site of pMON120. The chimeric nopaline synthase-NPTase II-nopaline synthase gene was inserted to give pMON129 and pMON128. In all of these examples, the first plasmid carries the inserted gene as it is drawn in the figure. The second plasmid carries the insert in the opposite ori-entation to that drawn. Plasmids pMON131 and pMON130 carry a chi-meric nopaline synthase-NPTase I-nopaline synthase gene. The final chimeric gene is carried in plasmids pMON140 and pMON139. The bac-terial NPTase II promoter and coding sequence have been joined to the nopaline synthase 3'-nontranslated region.

formants, and the control NPTase II-NPTase II-nopaline syn-thase construct in pMON139 and pMON140 transformants (Fig. 3a).

Similar results were obtained for pMON130 and pMON131 transformants, which contain the chimeric nopaline synthase-NPTase I-nopaline synthase gene on a 1.5-kb *Eco*RI fragment (Fig. 3b). No hybridization with either the Tn5- or Tn601-spe-cific probe was detected in transformants containing only the pMON120 vector. Other minor bands of hybridization are present in the pMON129 and pMON140 transformants; these may be attributable to partial digestion or aberrant integration events and their assignment awaits further analysis of clonal tissue. Blot hybridization analysis of DNA from these trans-formants using T-DNA-specific probes confirmed the presence of the expected internal T-DNA fragments in the transformed tissues and ruled out any possibility that the plant tissue was contaminated by *A. tumefaciens* cells (data not shown).

Other transformed, hormone-independent calli from these experiments were transferred to agar medium containing kana-
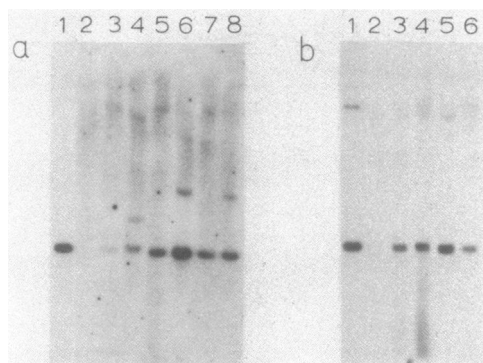
FIG. 3. DNA blot hybridization analysis of *in vitro* transformants. Several hundred hormone-independent *in vitro* transformants from each experiment were pooled and total DNA was extracted (52). The DNAs were digested with *Eco*RI and the fragments were separated by electrophoresis and transferred to nitrocellulose (53). (*a*) Hybridization with NPTase II-specific probe. A gel-purified 3.3-kb *Hind*III fragment from Tn*5* (54) was used as probe. Lane 1, pMON128::Ti plasmid marker; lane 2, pMON120 transformants; lane 3, pMON139 transformants; lane 4, pMON140 transformants; lane 5, pMON128 transformants; lane 6, pMON129 transformants; lane 7, pMON128 transformants; and lane 8, pMON129 transformants. Lanes 2–6 represent transformants selected for hormone-independent growth prior to scoring for kanamycin resistance; lanes 7 and 8 represent transformants selected only for kanamycin resistance on medium containing phytohormones. (*b*) Hybridization with NPTase I-specific probe. A gel-purified 1.2-kb *Ava* II fragment from Tn*601* (55) was used as a probe. Lane 1, pMON130::Ti plasmid marker; lane 2, pMON120 transformants; lane 3, pMON130 transformants; lane 4, pMON131 transformants; lane 5, pMON130 transformants; and lane 6, pMON131 transformants. Lanes 2–4 represent transformants selected for hormone-independent growth prior to scoring for kanamycin resistance; lanes 5 and 6 represent transformants selected only for kanamycin resistance on medium containing phytohormones.

mycin (50 μg/ml) and these were scored after 2–3 wk for resistance to the antibiotic. All transformants obtained from experiments utilizing pMON120, pMON139, or pMON140 failed to grow on medium supplemented with kanamycin, whereas all the transformants from experiments utilizing pMON128, pMON129, pMON130, or pMON131 grew on medium containing the antibiotic at rates comparable to growth on normal medium. A quantitative assessment of the level of resistance conferred by the chimeric genes is shown for pMON120,
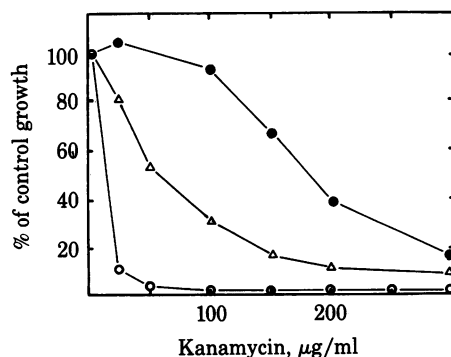

FIG. 4. Growth of transformants at various antibiotic concentrations. *In vitro* transformants were obtained after cocultivation with *A. tumefaciens* strains carrying cointegrate pMON120, pMON129, or pMON131. Hormone-independent calli (1- to 2-mm diameter) from each experiment were transferred to plates (16 calli per plate) containing the antibiotic concentration shown. After 3 wk, the net growth (wet weight) at each antibiotic concentration was determined and the results were expressed as the % of control growth (growth in the absence of antibiotics). ●, pMON129 transformants; △, pMON131 transformants; and ○, pMON120 transformants.

pMON129, and pMON131 (Fig. 4). The results are based on the net growth of independent transformants on medium containing the levels of antibiotic shown in the figure, compared to growth in the absence of antibiotics. It is apparent that transformants containing the chimeric nopaline synthase-NPTase II-nopaline synthase gene (pMON129) require ≈20-fold higher levels of kanamycin to depress net growth by 50% in comparison to transformants lacking the chimeric gene (pMON120). Similar results were obtained for pMON128, which contains the chimeric gene in the opposite orientation in the pMON120 vector (not shown). Transformants containing pMON139 and pMON140 have dose responses identical to pMON120. Transformants containing pMON130 or pMON131 (chimeric nopaline synthase-NPTase I-nopaline synthase gene) are less resistant to kanamycin than those containing pMON128 or pMON129 (results shown for pMON130). However, this level of resistance (≈3-fold greater than control cells) is still quite adequate for selection (see below).

Additional cocultivation experiments were carried out without hormone-independent selection (i.e., medium supplemented with phytohormones which support the growth of nontransformed cells). The resulting microcolonies (≈1 mm) were transferred to phytohormone-supplemented medium containing kanamycin (50 μg/ml) and within 2–3 wk, growing colonies were readily observable on plates containing cells that were transformed with pMON128, pMON129, pMON130, or pMON131. The frequency of transformation obtained by using antibiotic selection was comparable to that obtained by using hormone-independent selection. Opine (data not shown) and Southern hybridization analysis (Fig. 3*a*, lanes 7 and 8; Fig. 3*b*, lanes 5 and 6) of the kanamycin-resistant colonies confirmed that they were indeed transformants. No growing colonies were observable on plates containing cells transformed by pMON120, pMON139, or pMON140 plasmids.

DISCUSSION

The expression of the prokaryotic NPTase I and NPTase II enzymes in plant cells by using the intermediate vector pMON120 probably depends on transcription from the nopaline synthase promoter. Support for this comes from the facts that (*i*) the prokaryotic genes with their own promoters do not confer antibiotic resistance to petunia cells (Figs. 2 and 4) and (*ii*) all of the constructions function identically in either orientation in the pMON120 vector, suggesting that transcription does not initiate elsewhere in the vector. RNA blot hybridization experiments have confirmed the presence of NPTase II-specific mRNA in the transformed tissues and nuclease S1 mapping experiments demonstrate the expected 5′ and 3′ ends for the chimeric NPTase II mRNA (data not shown). In addition, low levels of neomycin-dependent NPTase II activity have been reproducibly observed in crude cell extracts from tissues transformed with pMON128 or pMON129 (no activity has been detected in extracts from control cells or cells transformed with pMON120, pMON139, or pMON140).

The useful range of these chimeric antibiotic resistance genes appears to be quite broad. In addition to the results presented for petunia, successful selection of aminoglycoside-resistant transformants has also been demonstrated for tobacco, sunflower, and carrot (results not shown). It seems likely that most plants within the host range of *A. tumefaciens* could be transformed and identified in this manner. Those plant cells that are not particularly sensitive to kanamycin may be killed by other aminoglycoside antibiotics. In this respect pMON128 (or pMON129) and pMON130 (or pMON131) also function to confer resistance to G418 and neomycin on petunia, carrot, sun-

flower, and tobacco (unpublished data).

The availability of dominant selectable markers on small plasmids such as pMON120 should facilitate the development of alternate, non-*A. tumefaciens*-mediated methods for transforming plant cells such as spheroplast fusion (56) or the use of liposomes (57) or calcium-phosphate (58) techniques. These chimeric genes should also prove useful as markers in somatic hybridization experiments or as sensitive probes for studying promoter function. Finally, two obvious but significant aspects of the results presented in this paper are (*i*) it should now be possible, by using Ti plasmids that have the tumor genes (i.e., tms and tmr loci, 12) deleted, to obtain kanamycin-resistant transformants that can be readily and reproducibly regenerated into phenotypically normal plants, and (*ii*) there is no reason to believe that NPTase I and NPTase II are unique in their ability to be expressed in plant cells and it is quite likely that other bacterial, fungal, or mammalian genes, including those whose products could be expected to modify plant properties in a useful manner, could also be successfully engineered and expressed.

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