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Direct gene transfer to plants

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Evidence for direct, gene-mediated stable genetic transformation of plant cells of *Nicotiana tabacum* is presented. A selectable hybrid gene comprising the protein coding region of the Tn5 aminoglycoside phosphotransferase type II gene under control of cauliflower mosaic virus gene VI expression signals was introduced into plant protoplasts as part of an *Escherichia coli* plasmid. The gene was stably integrated into plant genomic DNA and constitutively expressed in selected, drug-resistant, protoplast-derived cell clones. The mode of integration of the foreign gene into the plant genome resembled that observed for DNA transfection of mammalian cells. Plants regenerated from transformed cell lines were phenotypically normal and fertile, and they maintained and expressed the foreign gene throughout the development of vegetative and generative organs. Microspores, grown in anther culture, developed into resistant and sensitive haploid plantlets. Genetic crossing analysis of one of the transformed plants revealed the presence of one dominant trait for kanamycin resistance segregating in a Mendelian fashion in the F_1 generation.

Key words: selectable marker genes/plant protoplast transformation/recombinant DNA/plant tissue culture

Introduction

Direct gene transfer has been instrumental in the study of gene regulation and function in bacteria, fungi and animal cells. In plants the only available method for genetic transformation is based on the ability of the soil bacterium *Agrobacterium tumefaciens* to transfer and to integrate part of its genetic material into genomic DNA (e.g., Bevan and Chilton, 1982). This complex natural transformation process has also been exploited for integrating functional foreign genes into the plant genome (Herrera-Estrella *et al.*, 1983a, 1983b; Bevan *et al.*, 1983; Fraley *et al.*, 1983; Horsch *et al.*, 1984; Murai *et al.*, 1983). Further, direct transformation of isolated Ti plasmid into protoplasts (Davey *et al.*, 1980; Krens *et al.*, 1982) has demonstrated that foreign DNA could be taken up by plant cells and integrated into the genome, but did not exclude the possibility that Ti plasmid-specific functions are essential for this transformation process. Here we present proof for *A. tumefaciens*-independent direct gene transfer into plants and transmission of the foreign gene to the sexual offspring in a Mendelian pattern.

Results and Discussion

Construction and properties of the selectable hybrid gene

Part of the bacterial transposon Tn5 codes for the aminoglycoside phosphotransferase II [(APH3′)II] gene (Rothstein and Reznikoff, 1981). The protein encoded inactivates a related group of aminoglycoside antibiotics (neomycin, kanamycin, G-418) by phosphorylation and thereby confers resistance to them in bacteria (Rao and Rogers, 1979), fungi (Hirth *et al.*, 1982), mammalian cells (Colbère-Garapin *et al.*, 1981) and plant cells (Herrera-Estrella *et al.*, 1983a, 1983b; Bevan *et al.*, 1983; Fraley *et al.*, 1983). In order to obtain expression of the APH(3′)II gene in higher eukaryotic cells, corresponding expression signals have to be provided. We have constructed a functional hybrid marker gene by placing the protein coding sequence of the APH(3′)II gene under the control of the cauliflower mosaic virus (CaMV) gene VI expression signals because this gene is expressed in plant cells at very high levels during viral infection (Xiong *et al.*, 1982). The sequences flanking the gene VI mRNA coding region are typical eukaryotic promoter and termination signals (Hohn *et al.*, 1982).

We have constructed a pair of plasmids: pABDI with the correct orientation of the APH(3′)II gene with respect to the gene VI promoter region and polyadenylation site; and pABDII with the reverse orientation (Figure 1). Details of the construction are described in the legend of Figure 1. The protein product predicted from the hybrid gene (pABDI) will be an APH(3′)II amino-terminal fusion protein, 23 amino acid residues longer than the original APH(3′)II. Modifications of the APH(3′)II gene product (Beck *et al.*, 1982) have shown that this fusion is likely to have little effect on the phosphorylation activity. We have demonstrated biological activity of this particular hybrid gene and its use in selection by introducing it into tobacco cells *via* integration into T-DNA and standard co-cultivation treatments with *A. tumefaciens* (Paszkowski *et al.*, in preparation). The system thus seemed suitable to approach direct gene transfer into the plant genome by transformation of isolated protoplasts.

Protoplast transformation and selection of kanamycin-resistant cell lines

Nicotiana tabacum c.v. Petit Havana clone SR1 (Maliga *et al.*, 1973) protoplasts were chosen as the recipient system for gene transfer because (i) they are totipotent; (ii) their plating efficiency is high and reproducible; and (iii) they have good survival of treatments favouring DNA uptake. Freshly isolated mesophyll protoplasts were treated with the hybrid plasmids pABDI and pABDII using a DNA uptake procedure slightly modified from that developed by Krens *et al.* (1982). In three independent experiments we recovered resistant clones exclusively from the treatment involving pABDI (Table I). In all control treatments involving $>10^8$ protoplasts (including experiments verifying the selection procedure) no resistant colonies were recovered. A novel culture system (Shillito *et al.*, 1983) based on the use of agarose beads proved to be clearly superior to other selection regimes tested (i.e., liquid cultures, agar solidified cultures) (Figure 2a).

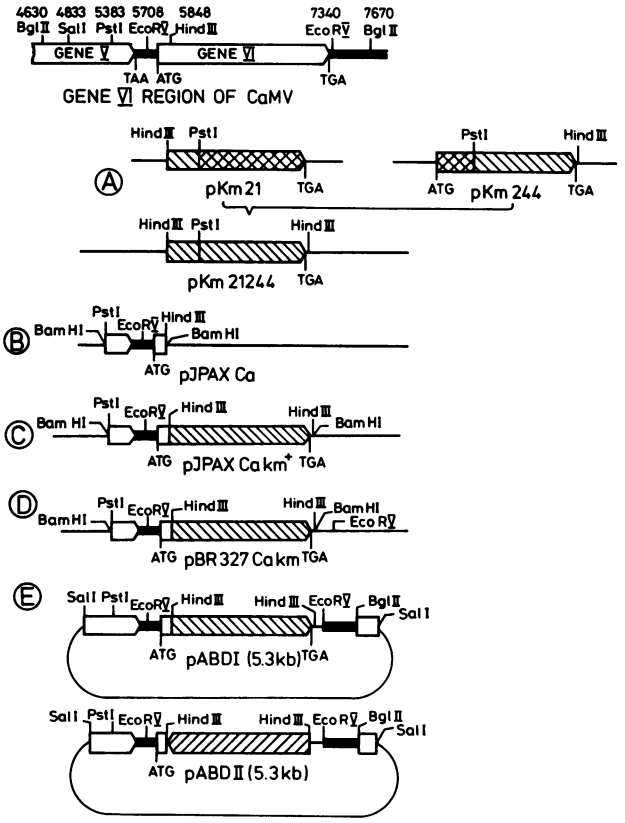

Fig. 1. Construction of the selectable hybrid gene. (**A**) Recombination of *Bal*31-deleted versions of the APH(3′)II gene. The plasmids pKm 21 and pKm 244 (Beck *et al.*, 1982) were digested by *Pst*I endonuclease, the derived fragments were purified by electrophoresis and ligated. The resulting plasmid pKm 21244 contains a combination of the 5′ and 3′ *Bal*31 deletions of the APH(3′)II gene. (**B**) and (**C**) Fusion of the CaMV gene VI promoter sequence to the APH(3′)II gene on the linker plasmid (pJPAX). pJPAX was derived from plasmids pUC8 and pUC9 (Messing and Vieira, 1982). The linker sequence of pUC9 was deleted (of 10 bp) by restriction at the *Hind*III and *Sal*I sites and the resulting cohesive ends filled in by treatment with DNA polymerase I (Klenow fragment) followed by ligation, thus restoring the *Hind*III restriction site. An 8-bp synthetic *Xho*I linker was inserted into the *Sma*I site of this deleted linker sequence. Recombination of the appropriate *Xor*II-*Hind*III fragments of pUC8 and the modified pUC9 plasmid yielded pJPAX with a partially asymmetric linker sequence containing the following sequence of restriction sites: *Eco*RI, *Sma*I, *Bam*HI, *Sal*I, *Pst*I, *Hind*III, *Bam*HI, *Xho*I, *Eco*RI. The joining of the 5′ expression signals of the CaMV gene VI and the *Hind*III fragment of APH(3′)II was carried out on pJPAX by inserting the *Pst*I-*Hind*III fragment of the CaMV gene VI promoter region between its *Pst*I and *Hind*III sites. The resulting plasmid (pJPAXCa) was restricted at its single *Hind*III site and the *Hind*III fragment of pKm 21244 was cloned into it in both orientations, yielding pJPAXCaKm$^+$ (restoring the reading frame of the fused protein) and pJPAXCaKm$^-$. (**D**) To provide an *Eco*RV site near the 3′-terminal region of the hybrid APH(3′)II gene, *Bam*HI fragments of pJPAXCaKm$^+$ and of pJPAXCaKm$^-$ were recloned into the *Bam*HI site of pBR327 (giving pBR327CaKm$^+$ or $^-$). (**E**) The *Eco*RV fragments of pBR327CaKm$^+$ and $^-$ were used to replace an *Eco*RV region of the CaMV gene VI, recloned as a *Sal*I fragment in pUC8, thereby placing the APH(3′)II protein coding region under control of both the 5′ and 3′ gene VI expresson signals. Two analogous plasmids were created: pABDI and pABDII, with correct and inverted orientation respectively of the APH(3′)II gene with respect to the gene VI promoter region. Open boxes represent open reading frames of CaMV, and hatched boxes the protein coding sequences of APH(3′)II. Thick lines represent intragenic regions of CaMV and thin lines represent bacterial vector sequences. Numbers above the restriction sites of the CaMV gene VI region indicate their positions on the CaMV map (Gardner *et al.*, 1981). Distances are not drawn to scale.

This method permits the selective medium to be replaced easily and repeatedly without disturbing the development of the cell clones in the agarose beads, thus guaranteeing a continuous and controlled selection pressure during the important early stages of protoplast culture. Resistant calli were transferred 4 weeks after exposure to DNA onto agar-solidified LS culture medium (Linsmaier and Skoog, 1965) containing 75 mg/l kanamycin and were subcultured to fresh selective medium every 4 weeks thereafter. Resistant calli proliferated under selective conditions (50 mg/l kanamycin in

Table I. Recovery of drug-resistant clones from transformation experiments

Experiment	DNA treatment	Protoplasts treated	Resistant clones recovered
1	pABDI	~2 x 10^6	2 (T_{2-1}, T_{2-2})
	Controls[a]	~10 x 10^6	0
2	pABDI	~2 x 10^6	1
	Controls[a]	~10 x 10^6	0
3	pABDI	~2 x 10^6	2
	Controls[a]	~10 x 10^6	0

[a]Every transformation experiment included the following control treatments: (i) pABDII + calf thymus DNA + PEG; (ii) pUC8 + calf thymus DNA + PEG; (iii) calf thymus DNA + PEG; (iv) PEG; (v) untreated protoplasts.

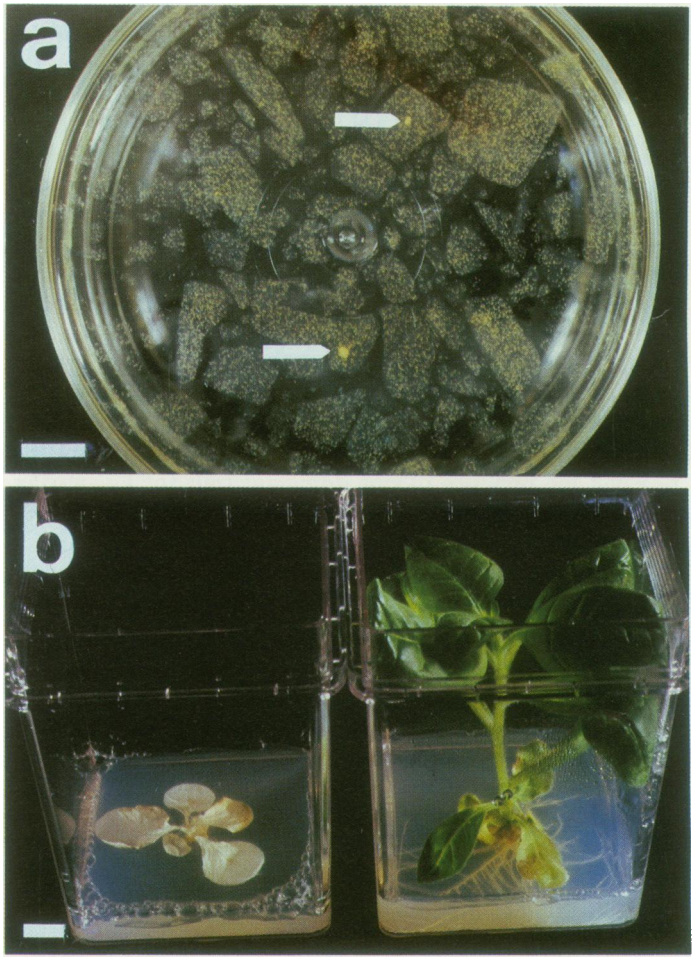

Fig. 2. (**a**) Resistant cell colonies 25 days after transformation of isolated protoplasts growing in bead type culture in 50 mg/l (0.086 mM) kanamycin sulphate. Arrows show proliferating resistant calli growing in the background of dead cell colonies. (**b**) Wild-type (kanamycin sensitive - left) and transformed (kanamycin resistant - right) protoplast-derived shoots after 4 weeks on a medium containing 150 mg/l kanamycin sulphate. The white bars represent 1 cm.

bead cultures and 75 mg/l kanamycin on plates) at the same rates as control cultures under non-selective conditions. Both kanamycin concentrations were toxic for untransformed callus colonies.

Plant regeneration from kanamycin-resistant cell lines

Two transformed clones (T_{2-1} and T_{2-2}) (Table I) were first subjected to treatments inducing plant regeneration and then to further analysis at the molecular level. Both clones regenerated numerous shoots. From these shoots ~50 plantlets were regenerated and transferred to potting compost where they continued to develop under greenhouse conditions. These plants were phenotypically identical to untransformed control plants. Plants have been regenerated in both the absence and the presence of kanamycin (150 mg/l) during the entire regeneration process. The presence of kanamycin in the regeneration medium influenced neither shoot nor root formation from the resistant clones. In contrast, non-transformed SR1 shoots, regenerated under non-selective conditions, failed to root on kanamycin-containing medium, ceased development and showed kanamycin-induced chlorophyll bleaching leading finally to complete chlorosis (Figure 2b).

Recovery of resistant cell cultures from explants and protoplasts

In order to follow the introduced trait throughout the development to the mature plant so as to record a possible loss of the kanamycin resistance at any developmental stage, explants from all parts of one selected plant from the base up to the influorescence were taken into culture, under selective (50 mg/l kanamycin) and non-selective conditions. Resistant cell cultures developed from leaves, petioles, stems, floral sepals, petals and carpels.

Protoplasts isolated from leaves proliferated under selective conditions (50 mg/l kanamycin) with the same plating efficiencies as wild-type protoplasts under non-selective conditions. Mixing experiments of protoplasts from the leaves of kanamycin-resistant and wild-type plants in ratios down to three kanamycin-resistant to 150 000 untransformed protoplasts (Figure 3), demonstrated that our selection system recovers putative transformants from large populations of sensitive protoplasts. Recovery of resistant colonies, after correction for the division frequency of the total population, varies between 50 and 100% and is lower (~50%) when the ratio of sensitive to resistant protoplasts is extremely high (>10 000).

Microspore analysis via anther culture

In tobacco, as in numerous other plants, the direct products of meiosis, the microspores, can be induced to develop into haploid plants. This provides an opportunity to follow directly transmission of the introduced trait through the male gametes. Anthers of the correct developmental stage from kanamycin-resistant and from wild-type plants were taken for anther culture (Sunderland and Dunwell, 1977) under non-selective conditions. Microspore-derived green plantlets were then transferred to selective conditions (200 mg/l kanamycin sulphate) and scored for undisturbed development or bleaching. The wild-type plantlets bleached without exception whereas ~50% (106 from 248) of microspore plantlets from T_{2-1} did not bleach and continued to develop to green haploid plants.

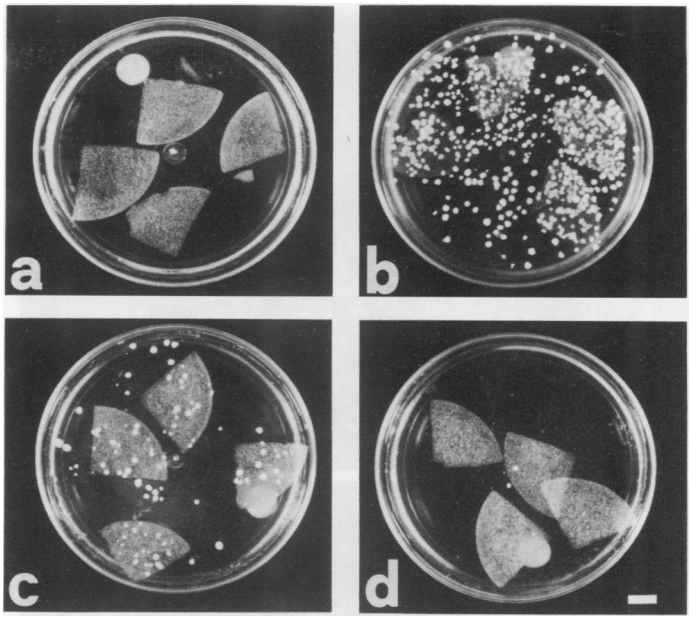

Fig. 3. Reconstruction experiment. Mesophyll protoplasts isolated from the wild-type SR1 and from the kanamycin-resistant plant T_{2-2} were mixed at different ratios and cultured under selective conditions (50 mg/l kanamycin and bead type culture technique). The figures show the plastic containers (9 cm in diameter) with the four quadrants of an agarose-protoplast gel (6 cm in diameter) after 7 weeks in culture. **(a)** 150 000 wild-type protoplasts: no resistant colony. **(b)** 148 500 wild-type protoplasts plus 1500 protoplasts from T_{2-2}: 739 resistant colonies. **(c)** 150 000 wild-type protoplasts plus 150 protoplasts from T_{2-2}: 101 resistant colonies. **(d)** 150 000 wild-type protoplasts plus 15 protoplasts from T_{2-2}: 3 resistant colonies. The white bar represents 1 cm.

Table II. Genetic crossing analysis

	T_{2-1} x self	SR1 x T_{2-1}	T_{2-2} x self	SR1 x SR1
No. of seeds tested	746	647	1731	1450
No. of seeds germinated	732	641	1684	1412
No. of kanamycin-resistant seedlings	536	308	1428	0
No. of kanamycin-sensitive seedlings	196	333	256	1412
Ratio of resistant to sensitive	2.73:1	0.92:1	5.58:1	0:1412

Genetic crossing analysis

The ultimate biological test for the fate of a foreign gene introduced into the plant genome should be provided by an analysis of the acquired trait in sexual offspring. Of the 58 plants regenerated from subclones of the two putative transformed cell lines T_{2-1} and T_{2-2} 57 were fertile. This enabled us to undertake crossing analysis of the kanamycin resistance. Data for two random plants are given (Table II). Fertility, seed set and germination were comparable with the wild-type SR1, so that segregation data could be interpreted without any additional assumptions. Controlled pollinations of emasculated flowers were done in an insect-free growth cabinet. Seed populations of individual seed capsules were sterilized and germinated on 1/10 inorganic NN culture medium (Nitsch and Nitsch, 1969) solidified with

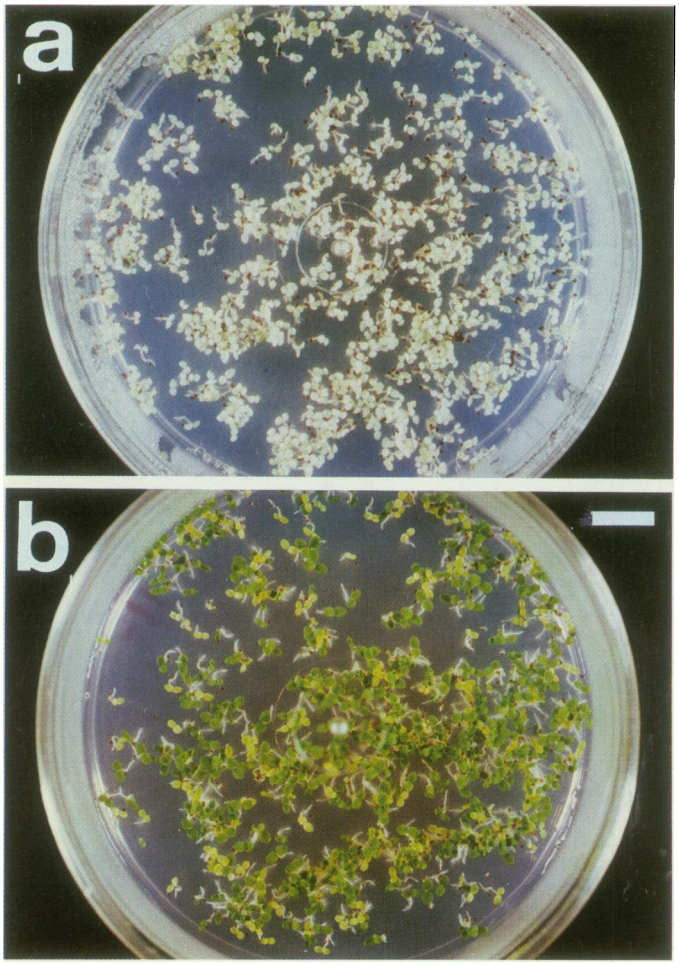

Fig. 4. Genetic crossing analysis of the transformed plant T_{2-1}. Seeds were surface sterilized and germinated on 200 mg/l kanamycin. The photographs were taken 5 weeks after germination (see also Table II). **(a)** Wild-type SR1 x self: seedlings germinate, bleach and die. **(b)** Transformant T_{2-1} x self: seedlings germinate and segregate ~3:1 viable green to bleached, dying seedlings. The white bar represents 1 cm.

0.8% agar. Approximately half of each population was germinated under selective conditions (200 mg/l kanamycin), the other half under non-selective conditions. After 2–3 weeks incubation in the light (~5000 lux, cool fluorescent tubes, 16 h per day, 27°/20°C) sensitive seedlings germinated, bleached, and died without developing a primary leaf, whereas resistant seedlings developed to green plantlets with primary and secondary leaves. The F_1 population from the self-cross of wild-type SR1 bleached without exception. The F_1 population from the self-cross of T_{2-1} segregated 3:1 resistant to sensitive and the F_1 population of the backcross of T_{2-1} with the wild-type SR1 segregated 1:1 resistant to sensitive, thus providing evidence for the presence of one dominant Mendelian factor for kanamycin resistance in T_{2-1} (Figure 4). Both microspore analysis and genetic crossing analysis of T_{2-1} demonstrate the transmission of one dominant and functional locus for kanamycin resistance.

The pattern of segregation of the kanamycin-resistant trait in plant T_{2-2} is more complex. This will be studied in the next generation. In this case there may be more than one functional copy of the gene integrated into the genomic DNA in such a way that these are genetically linked (i.e., distantly placed on the same chromosome).

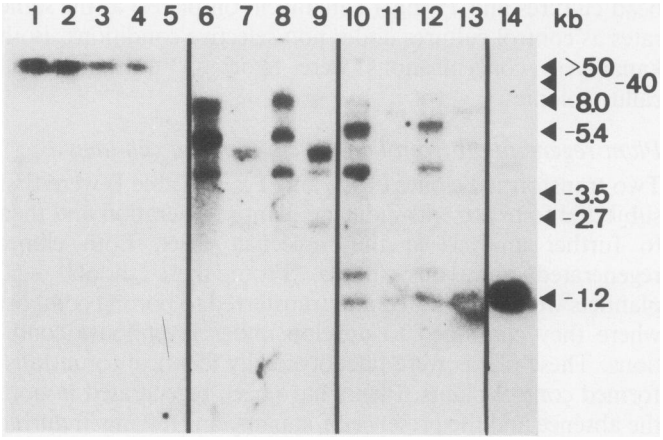

Fig. 5. Detection of APH(3′)II gene sequence in the DNA of transformed callus lines and leaves of regenerated plants. After restriction and electrophoresis of ~5 μg DNA/slot DNA was transferred onto nitrocellulose filters and hybridized with the nick translated *Hind*III fragment (5–10 x 10^8 c.p.m./μg) of pKm21244 (Figure 1). **Lanes 1–5**, unrestricted DNA; **6–9**, DNA restricted with *Bst*EII; **10–14**, DNA restricted with *Eco*RV; **lanes 1,6,10**, DNA from callus of T_{2-1}; **2,7,11**, DNA from callus T_{2-2}; **3,8,12**, DNA from leaf of T_{2-1}; **4,9,13**, DNA from leaf of T_{2-2}; **5**, DNA from wild-type SR1 callus; **14**, pABDI (2 ng, shorter exposure). The leaf DNA was isolated from plants grown in soil, regenerated from callus under non-selective conditions. Plants grown *in vitro* under continuous selective pressure (150 mg/l kanamycin) also show the cell line specific hybridization profile (data not shown).

The hybrid gene is present in selected kanamycin-resistant cell lines and leaves of regenerated plants

In order to correlate the observed kanamycin-resistant phenotype with the presence of the hybrid APH(3′)II gene, DNA of the selected kanamycin-resistant cell lines and regenerated plants was analysed. Southern blot analysis (Southern, 1975) (Figure 5) revealed in both of the lines analysed (T_{2-1}, T_{2-2}) the presence of 3–5 copies of APH(3′)II gene sequences per haploid genome, estimated from reconstruction experiments (data not shown). The APH(3′)II specific probes hybridized only to high mol. wt. (>50 kb) DNA suggesting that the APH(3′)II sequences had been integrated into the plant genome. There was no hybridization to chloroplast DNA prepared from transformed plants (data not shown). When nuclear DNA was restricted with *Bst*EII, which does not cut pABDI, we observed a transformant-specific hybridization pattern to the APH(3′)II probe. This suggests random rather than directed integration of pABDI sequences into the tobacco nuclear genome. The size of the various *Bst*EII fragments (both smaller and larger than the 5.3-kb pABDI) indicates that pieces of the pABDI rather than full length plamids were integrated. *Eco*RV restriction of DNA from both transformed lines yielded the expected 1.2-kb *Eco*RV fragment containing the intact APH(3′)II gene. In addition, fragments of different sizes were visualised which could be interpreted as representing integration of parts of the APH(3′)II coding sequence leading to the deletion of one of the flanking *Eco*RV restriction sites. The mode of integration of a non-homologous supercoiled plasmid into plant genomes will be studied in more detail, but clearly resembles non-homologous integration of foreign DNA as reported for animal cells (Colbère-Garapin *et al.*, 1981; Wigler *et al.*, 1979).

The introduced genes were not eliminated during plant development even in cases where differentiation was induced

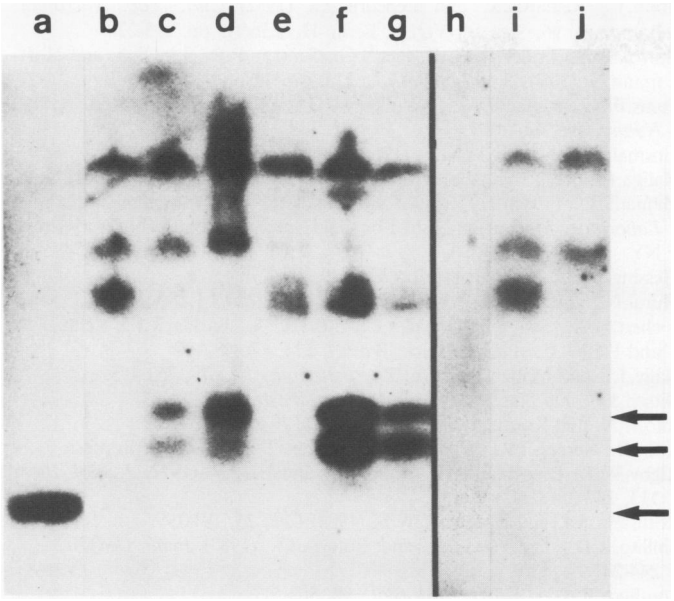

Fig. 6. Assay of APH(3')II enzyme activity in selected kanamycin-resistant cell lines and in tissues of plants derived from them. **Lanes a,h** bacterial APH(3')II enzyme from an osmotic shock extract of *E. coli* DH1 carrying plasmid pKC7 (Rao and Rogers, 1979); **b,i** extracts of wild-type leaf tissue; **c,j** extracts of leaf tissue from plants regenerated from transformant T_{2-1}; **d** extract of leaf tissue from plants regenerated from transformant T_{2-2}; **e** extract of wild type SR1 callus; **f,g** extracts of callus of the transformed lines T_{2-1} and T_{2-2}, respectively. **Lanes a–g** reacted with ^{32}P-labelled ATP in the presence of kanamycin; **h–j** reacted in the absence of kanamycin (showing background of non-specific phosphorylation). The arrows show the position of the APH(3')II activity from the bacterial enzyme and that produced in plant tissues.

on kanamycin-free medium. The pattern of integration in plants was identical to that of the corresponding undifferentiated cell lines (Figure 5). The hybrid gene is also present in the kanamycin-resistant F_1 seedlings (data not shown).

The introduced APH(3')II gene is expressed

To demonstrate that the drug-resistant phenotype was actually based on the expression of the APH(3')II gene and the synthesis of the expected protein, the APH(3')II enzyme activity was assayed according to the method of Reiss *et al.* (1984). The activity was absent in wild-type SR1 tissue and present in both transformed callus clones and in leaves of plants regenerated from them (Figure 6). These data indicate the apparently constitutive expression of the introduced gene independent both of the developmental stage of the cell and of the presence of the antibiotic in the cellular environment. The APH(3')II activity migrated in non-denaturing polyacrylamide gels as two bands. The reason for this has not been defined, but it could obviously be due to post-translational modifications, proteolysis or association with other cellular protein(s) as previously found in animal cells (Colbère-Garapin *et al.*, 1981). The two bands probably do not arise by changes in the protein coding region due to recombination within the gene, since the same protein pattern was found in the two transformed lines in spite of different patterns of integrated DNA. The introduced APH(3')II hybrid gene is also expressed in kanamycin-resistant F_1 plantlets (data not shown).

DNA-mediated transformation frequencies in our experiments were in the range of 10^{-6} if related to protoplasts treated or 10^{-5} if calculated on the basis of colonies which could have been recovered without selection, and were thus low compared with those obtainable when using the *A. tumefaciens* co-cultivation system (Herrera-Estrella *et al.*, 1983a, 1983b; Bevan *et al.*, 1983; Fraley *et al.*, 1983; Horsch *et al.*, 1984; Murai *et al.*, 1983). Experiments are now in progress to optimize the procedure for gene-mediated transformation of protoplasts.

Stable genetic transformation of plant cells by the introduction of a hybrid marker gene into protoplasts has been established on the basis of the following criteria: (i) the kanamycin-resistant phenotype of selected cell lines and the plants derived from them; (ii) the presence of DNA sequences of the introduced gene integrated into plant genomic DNA; (iii) enzymatic activity of the protein product of the introduced gene in transformed lines, plants and their progeny. These results therefore also show that neither *A. tumefaciens* nor Ti plasmid-specific functions are a prerequisite for transfer and stable integration of functional genes into the plant genome.

Further genetic analysis of the progeny obtained from several independently transformed cell lines should give an indication of the site(s) of integration of foreign gene(s) into plant genome in the process of DNA-mediated transformation. These experiments are in progress.

Direct gene transfer in plants should enlarge the scope of biological problems accessible to investigation by molecular genetics; these might include, for example, studies of DNA replication in plants, by the search for and study of autonomously replicating plant vectors, or possibly directing the integration process by providing targets on the vectors for promoting homologous recombination.

Materials and methods

Construction of hybrid gene

The steps of the construction are described in Figure 1. All enzymes used during the construction were used as recommended by suppliers (Boehringer, Bio-Labs, B.R.L.). Other DNA and *E. coli* (strain DH1) manipulation procedures were as described by Maniatis *et al.* (1982).

Protoplast isolation

Leaf mesophyll protoplasts of *N. tabacum* cv. Petit Havana line SR1 (Maliga *et al.*, 1973) were isolated from sterile shoot cultures and purified as described by Nagy and Maliga (1976) modified by addition of one volume of 0.6 M sucrose to the leaf digest before the first centrifugation.

DNA transformation of protoplasts

The transformation method was essentially that of Krens *et al.* (1982): 1 ml aliquots of 2 x 10^6 protoplasts each in K_3 medium (Nagy and Maliga, 1976) (0.1 mg/l 2,4-D, 1 mg/l NAA, 0.2 mg/l BAP) were incubated for 30 min at room temperature with 13% w/v PEG 6000, 15 μg of intact plasmid pABDI DNA (from 1 mg/ml stock) and 50 μg calf thymus DNA (from 1 mg/ml stock). The pH of the F medium was readjusted to 5.3 after autoclaving. After stepwise dilution with F medium the protoplasts were collected by sedimentation (5 min at 100 *g*) and resuspended in 30 ml of K_3 medium.

Protoplast culture, selection and culture of transformed cell lines

DNA-treated protoplasts were resuspended in 30 ml of K_3 medium and incubated in 10 cm diameter Corning Petri dishes in 10 ml aliquots (6.6 x 10^4 protoplasts/ml) in the dark at 24°C. After 3 days the culture medium was diluted with 0.3 volumes of fresh K_3 culture medium and the cultures incubated under continuous light (2000 lux, cool fluorescence SYLVANIA 'daylight') at 24°C. After a total of 7 days the protoplast-derived population of developing cell clones was embedded in medium solidified with 1% Sea Plaque LMT agarose (Marine Colloids) and further cultured under selective conditions (50 mg/l kanamycin sulphate) in the agarose bead type culture system (Shillito *et al.*, 1983) at 24°C in the dark. The selective culture medium surrounding the beads was replaced every 5 days. Following 4 weeks of proliferation under selective conditions, resistant calli (2–3 mm in diameter) were transferred onto agar-solidified LS culture medium (Linsmaier and Skoog, 1965) (0.5 mg/l 2,4-D, 2 mg/l NAA, 0.1 mg/l BAP, 0.1 mg/l kinetin) containing 75 mg/l kanamycin sulphate and subcultured every 4 weeks.

Plant regeneration

Plants were regenerated under selective (150 mg/l kanamycin sulphate) or non-selective conditions by inducing shoots on LS medium containing 0.2 mg/l BAP and rooting these shoots on T medium (Nitsch and Nitsch, 1969). Wild-type SR1 shoots were regenerated in the same way on kanamycin-free media.

DNA isolation from plant material

Plant DNA was isolated by a method modified after Thanh Huyuh (Department of Biochemistry, Stanford University; personal communication). Samples of 0.5 g of callus or leaf tissue were homogenized at 0°C in a Dounce homogenizer in 15% sucrose, 50 mM EDTA, 0.25 M NaCl, 50 mM Tris-HCl pH 8.0. Centrifugation of the homogenate for 5 min at 1000 *g* resulted in a crude nuclear pellet which was resuspended in 15% sucrose, 50 mM EDTA, 50 mM Tris-HCl pH 8.0. SDS was added to a final concentration of 0.2% w/v. Samples were heated for 10 min at 70°C. After cooling to room temperature, potassium acetate was added to a final concentration of 0.5 M. After incubation for 1 h at 0°C the precipitate formed was sedimented at 4°C by a 15 min spin in an Eppendorf microcentrifuge. The DNA from the supernatant was precipitated with 2.5 volumes of ethanol at room temperature and redissolved in 10 mM Tris-HCl pH 7.5, 5 mM EDTA containing RNase A (10 μg/ml). Afer 10 min incubation at 37°C, proteinase K was added to a final concentration of 250 μg/ml and incubation was continued for 1 h at 37°C. The proteinase K was removed by phenol and chloroform-isoamyl alcohol extractions. DNA from the aqueous phase was precipitated with 60% isopropanol, 0.6 M Na acetate and dissolved in 50 μl of 5 mM EDTA, 10 mM Tris-HCl pH 7.5. DNA concentrations were estimated spectrophotometrically. The preparations yielded high mol. wt. DNA (predominantly >50 kb) susceptible to various restriction endonucleases.

Southern blot analysis

DNA electrophoresed in 1% agarose gel was transferred to nitrocellulose membrane (Southern, 1975) and hybridized with nick-translated (Rigby *et al.*, 1977) DNA ($5-10 \times 10^8$ c.p.m./μg). Filters were washed three times for 1 h in 2 x SSC at 65°C. Blots were exposed to X-ray film with intensifying screen for 24–48 h.

APH(3')II activity test

This test was carried out essentially after the method of Reiss *et al.* (1984). Callus or leaf pieces (100–200 mg) were crushed in an Eppendorf centrifuge tube with 20 μl extraction buffer. This buffer was modified from that of Herrera-Estrella *et al.* (1983a) by omitting the bovine serum albumin and adding 0.1 M sucrose. Extracts were centrifuged for 5 min at 12 000 *g* and bromophenol blue was added to the supernatant to a final concentration of 0.004%. Proteins in 35 μl of the supernatant were separated by electrophoresis in a 10% non-denaturing polyacrylamide gel. The gel was incubated with kanamycin and γ-^{32}P-labelled ATP and then blotted onto Whatman p81 phosphocellulose paper. The paper was washed six times with deionized water at 90°C before autoradiography.

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