The EMBO Journal vol.4 no.11 pp.2731-2737, 1985

Liposome-mediated transformation of tobacco mesophyll protoplasts by an *Escherichia coli* plasmid

Alain Deshayes, Luis Herrera-Estrella[1] and Michel Caboche

Laboratoire de Biologie cellulaire, INRA, F-78000 Versailles, France, and [1]Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Ghent, Belgium

Communicated by J.Schell

An *Escherichia coli* plasmid, pLGV23neo, carrying a kanamycin resistance gene expressed in plant cells, was encapsulated into negatively charged liposomes prepared by the reverse phase evaporation technique. These liposomes were induced to fuse with tobacco mesophyll protoplasts by polyethyleneglycol treatment. Kanamycin-resistant clones were reproducibly isolated from transfected cultures at an average frequency of 4×10^{-5}. Plants regenerated from these resistant colonies were confirmed to be transformed according to three criteria. Protoplasts isolated from their leaves were resistant to 100 μg/ml kanamycin. The enzyme aminoglycoside 3′-phosphotransferase II encoded by the plasmid pLGV23neo was detected in leaf extracts. Approximately 3 – 5 copies of the gene encoding for kanamycin resistance were inserted in the genome of at least one of the studied transformants. The restriction pattern of inserted DNA was best explained by assuming a tandem integration of the pPLGV23neo sequences, implying an homologous recombination event between these sequences during transformation. Kanamycin resistance was transmitted as a single dominant nuclear marker to the progeny of resistant plants after selfing or cross-pollination with the wild-type.

Key words: APH(3′)II/kanamycin/transformation/protoplast/tobacco

Introduction

Using the Ti plasmid of *Agrobacterium tumefaciens* as a natural vector, the transfer of foreign DNA into the genome of plants has been achieved. The expression of this transferred DNA has been detected for chimaeric genes (Herrera-Estrella *et al.*, 1983a, 1983b, 1984; Bevan *et al.*, 1983; Fraley *et al.*, 1983) encoding for antibiotic resistance as well as for plant genes of heterologous origin (Murai *et al.*, 1983; Broglie *et al.*, 1984). The T-DNA of *A. tumefaciens* carries oncogenous genes interfering with normal plant development. The inactivation of these genes (Otten *et al.*, 1981; Barton *et al.*, 1983) or their deletion (Zambryski *et al.*, 1983) from the Ti plasmid does not interfere with virulence, allowing the regeneration of transformed plants from transformed cells. Conversely, transformed plants can be directly regenerated from hairy roots induced by *A. rhizogenes* (Tepfer, 1984).

The sizes of Ti and Ri plasmids, however, make their direct manipulation for cloning experiments impossible. A binary vector strategy based on the separation of virulence and T-DNA regions on two different plasmids has been developed (Hoekema *et al.*, 1983; de Framond *et al.*, 1983), but these plasmids were also too large to be used as cloning vectors. Alternatively cloning vectors could be used for gene transfer provided efficient transfection techniques could be substituted for the *Agrobacterium*-mediated transformation.

Recently it has been proven that direct transformation of plant protoplasts by an *Escherichia coli* plasmid is feasible (Paszkowski *et al.*, 1984) using a procedure previously developed for the transformation by purified Ti plasmid (Krens *et al.*, 1982). We have developed a more efficient strategy of transfection, in which plasmid-loaded liposomes were fused with mesophyll protoplasts by polyethyleneglycol treatment. This procedure was derived from liposome-mediated techniques of transfection of mammalian cells (Papahadjopoulos *et al.*, 1980). We have previously demonstrated that, using this procedure, >70% of *Nicotiana tabacum* mesophyll protoplasts could be reproducibly infected with liposome-encapsulated tobacco mosaic virus (TMV) RNA (Rouzé *et al.*, 1983). In the present study we made use of the plasmid pLGV23neo (Herrera-Estrella *et al.*, 1983b) which carries a chimaeric gene encoding for the aminoglycoside 3′-phosphotransferase-II [APH(3′)II] from Tn5 under the control of the regulatory sequences of the nopaline synthetase gene. Kanamycin-resistant plants were regenerated from cultures transfected with this plasmid and confirmed to be transformed. A preliminary account of this work has been presented previously (Caboche and Deshayes, 1984).

Results

Reproducibility of liposome-mediated transfection experiments

Liposome-mediated transfection of tobacco mesophyll protoplasts was optimized on the basis of the recovery of transformants, starting from transfection conditions suitable for liposome-mediated TMV RNA infection (Rouzé *et al.*, 1983). Colonies resistant to 70 μg/ml kanamycin were never isolated in control experiments where protoplasts were either only treated with polyethyleneglycol (PEG), or transfected with liposomes loaded with calf thymus DNA (Table I) or with various plasmids carrying Tn5 or Tn903 transposons. On the contrary, such resistant clones were isolated in nine out of 11 experiments in which mesophyll protoplasts were transfected by liposomes loaded with pLGV23neo. A total of 276 colonies resistant to kanamycin were recovered in the nine positive experiments, at a frequency varying from 4.1×10^{-6} to 2.2×10^{-4} (average 3.9×10^{-5}). When calculated on the basis of cells surviving the transfection process, the transformation efficiency was in the range of 1 – 10 transformants per μg of encapsulated pLGV23neo DNA, a value reaching that obtained from the transfection of non-replicating sequences into yeast (Hinnen *et al.*, 1978). Calcium was required during protoplast isolation and transfection to promote the formation of resistant colonies (Table I). Resistant colonies frequency varied non-linearily with the amount of liposomes incubated with protoplasts (Table I). Using a transfection procedure similar to that of Krens *et al.* (1982) we isolated two resistant clones from pLGV23neo-transfected protoplast cultures (frequency:

Table I. Recovery of kanamycin-resistant clones according to transfection conditions

Experiment	Divalent cation concentration			Amount of encapsulated DNA incubated with 2×10^6 protoplasts (μg)	Number of transfected protoplast-derived cells submitted to selection ($\times 10^5$)	Number of resistant colonies isolated	Transformation	
	During protoplast isolation		During liposome transfection				Frequency ($\times 10^{-6}$)	Efficiency (μg)
	Ca^{2+} (mM)	Mg^{2+} (mM)						
No. 9	6	6	5	0	8.0	0	–	–
	6	6	5	1	7.8	0	–	–
	6	6	5	2.5	3.8	6	16	4.8
	6	6	5	5	6.3	43	68	10
No. 11	6	6	5	0	10	0	–	–
	6	6	5	14[a]	7	0	–	–
	6	6	5	4	8	7	9	1.75
	6	6	5	5[b]	8	0	–	–
Transfection according to Krens *et al.* (1982)				14[a]	7	2	3	0.04
No. 12	0	0	0	5	2.6	0	–	–
	0	0	1	5	2.0	1	5	0.2
	0	0	10	5	2.4	1	4	0.2
	6	6	5	5	2.4	12	50	1.2
	6	6	5	0	10	–	–	–

Transfections were performed using pLGV23neo encapsulated in liposomes as described under Materials and methods.
[a]Non-encapsulated pLGV23neo mixed directly with protoplasts before PEG treatment.
[b]Liposomes loaded with calf thymus DNA instead of pLGV23neo.

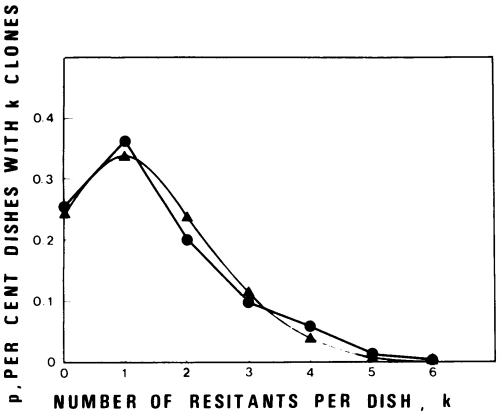

Fig. 1. Frequencies of resistant clones recovered in a transfection experiment. P-cells derived from a batch of transfected protoplasts (1.2 μg of encapsulated pLGV23neo/10^6 protoplasts) were plated at a density of 5×10^3 p-cells/ml in 124 dishes, each containing 10 ml of medium. The plating efficiency of these p-cells was 34% in the absence of selective pressure. Colonies resistant to 70 μg/ml kanamycin were isolated at an average frequency (k) of 1.41 colonies/dish. A Poisson distribution p of mode k (▲) was compared with the effective distribution of colonies among dishes (●).

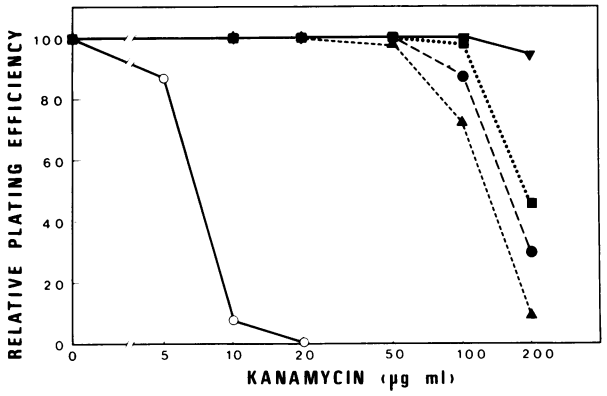

Fig. 2. Comparison of the level of resistance to kanamycin of p-cells derived from the leaves of wild-type and selected clones. Tests were performed as described in Materials and methods. Wild-type diploid cells (○), selected clones Ka4 (●), Ka50 (▲), Ka87 (■) and Ka123 (▼).

3×10^{-6}). When pLGV23neo loaded-liposomes were replaced, however, by non-encapsulated plasmid in our standard transfection conditions, no kanamycin-resistant colonies were recovered from the transfected cells (Table I). Under standard liposome preparation conditions, approximately half of the pLGV23neo molecules remained supercoiled, as tested by re-extraction of plasmid from the liposomes. When virtually all encapsulated molecules were linearized upon prolongated sonication during liposome preparation, the ability of the resulting preparations to transform protoplasts was decreased by a factor of two (results not shown). A statistical analysis of the number of resistant colonies recovered in a series of liquid cultures issued from a single transfection (Experiment 3, Figure 1) showed that their frequency varied according to a Poisson law. This is a strong indication that the event leading to resistance was not an adaptative process induced by the selection procedure, but occurred randomly in transfected cultures.

Expression of antibiotic resistance in regenerated plants

The leaves of plants regenerated from resistant colonies were used as sources of mesophyll protoplasts. Protoplast-derived cells (p-cells) obtained from these plants were still able to grow in the presence of 100 μg/ml kanamycin whereas wild-type derived cells were killed by 20 μg/ml kanamycin (Figure 2). So far the resistance of 38 out of 40 independent regenerated clones has been confirmed. This suggests that kanamycin resistance is a stable characteristic of the isolated clones and still expressed after regeneration in the absence of selective pressure. Cells derived from the kanamycin-resistant clone Ka4 were cross-resistant to neomycin, but only weakly resistant to G418 (Table III). No appreciable level of resistance of Ka4 cells towards gentamycin and tobramycin was detected when compared with the wild-type (Table II).

Table II. Susceptibility of kanamycin-resistance and sensitive cells to various aminoglycosides

Antibiotic	Concentration (μg/ml) inducing a 50% decrease of the plating efficiency of p-cells derived from		Increased tolerance
	Wild-type D8	Clone Ka4	
Kanamycin	7	150	× 20
Gentamycin	2.50	3.30	× 1.3
G418	0.35 ± 0.12	1.0 ± 0.2	× 2.8
Tobramycin	2.4	1.9	× 0.8
Neomycin	5	80	× 16

The toxicities of antibiotics were estimated under low density growth conditions as described under Materials and methods. Average values and standard deviations are given for four independent measurements of G418 toxicity.

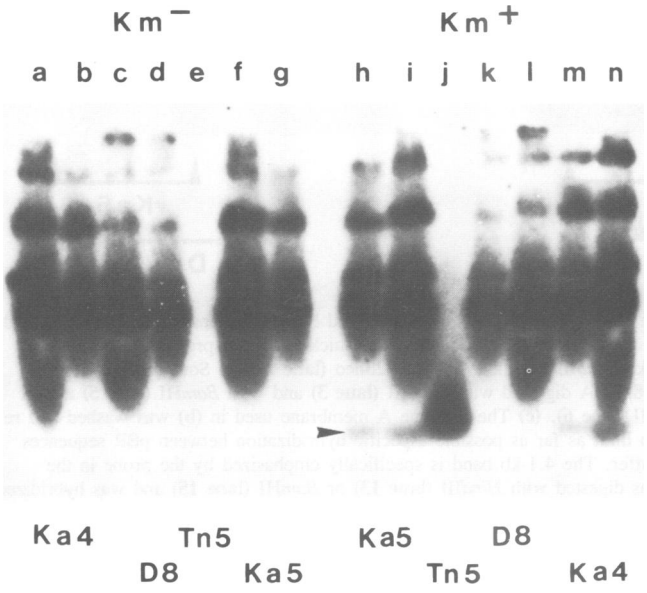

Fig. 3. Assay of APH(3')II enzyme activity in leaf extracts prepared from the kanamycin-resistant clones Ka4 and Ka5. Assays were performed as described in Materials and methods in the absence **(lanes a, b, c, d, e, f, g)** or presence **(h, i, j, k, l, m, n)** of kanamycin. Two dilutions of leaf extracts were tested for each clone. **Lanes a, b, m, n:** clone Ka4; **lanes c, d, k, l:** wild-type D8; **lanes f, g, h, i:** clone Ka5. **Lanes e** and **j:** extract of *E. coli* carrying the transposon Tn5. The arrow indicates the position of APH(3')II activity.

Detection of APH(3')II in leaf extracts

Leaf extracts from clones Ka4 and Ka5 were prepared and submitted to electrophoresis together with extracts of *E. coli* carrying the transposon Tn5 in its genome. APH(3')II was then revealed according to the procedure of Reiss *et al.* (1984) (Figure 3). A background of phosphorylated products was detected in plant extracts in the absence of kanamycin. An activity of electrophoretic mobility identical to that encoded by Tn5 was specifically detected in extracts from clones Ka4 and Ka5 incubated in the presence of kanamycin but not in the controls.

Transmission to progeny of the resistance trait

The plant development and seed production of three independent clones, Ka4, Ka5 and Ka6, were indistinguishable from wild-type plants. When wild-type seeds were germinated on rooting medium B in the presence of 100 μg/ml of kanamycin, plantlets were found to be blocked at the two cotyledons stage and became chlorotic upon prolongated incubation. Resistant plantlets, however, could develop normally under these growth conditions (Figure 4). Self-pollinated resistant plants segregated resistant and sensitive seedlings in a 3:1 proportion. Reciprocal crosses of clone Ka4 with the wild-type plant yielded resistant and sensitive seedlings segregating in the 1:1 ratio. These results suggest that the three studied transformants were all heterozygotes for a single nuclear dominant trait (Table III). This trait was transmitted to the progeny in a Mendelian mode for clones Ka4 and Ka5 but might be homozygote − lethal in the progeny of clone Ka6.

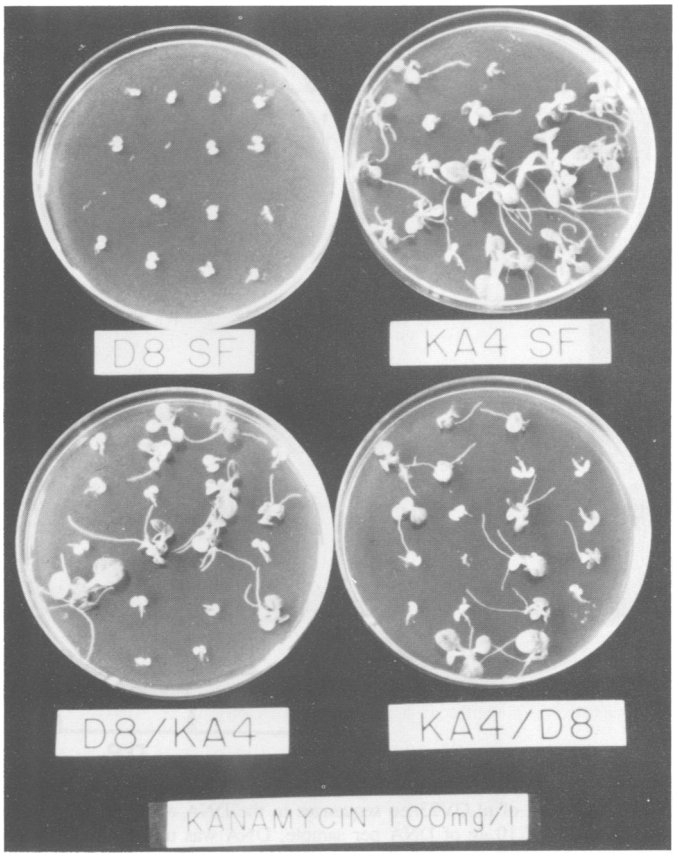

Fig. 4. Transmission to progeny of kanamycin resistance from the clone Ka4. Crosses were performed with the wild-type (D8). Seeds were surface sterilized and germinated under aseptic conditions on solid medium containing 100 μg/ml kanamycin and incubated for 1 month under light.

Table III. Transmission to progeny of kanamycin resistance

Pollination conditions	Seeds					
	Total	Non-germinated	Sensitive	Resistant	1:3	X^2 1:1
Ka4 selfed	403	14	91	298	0.53,NS	
Ka4 × D8	154	4	72	78		0.24,NS
D8 × Ka4	151	4	70	77		0.33,NS
Ka5 selfed	127	6	32	89	0.13,NS	
Ka5 × D8	93	1	48	44		0.17,NS
Ka6 selfed	98	4	30	64	2.4,NS	
D8 selfed	142	14	142	0		

Tests were performed as described in the legend of Figure 4. NS = non-significant at $P = 0.05$. Assuming a 1:2 segregation in progeny of clone Ka6 selfed, the value of X is 0.08 (NS).

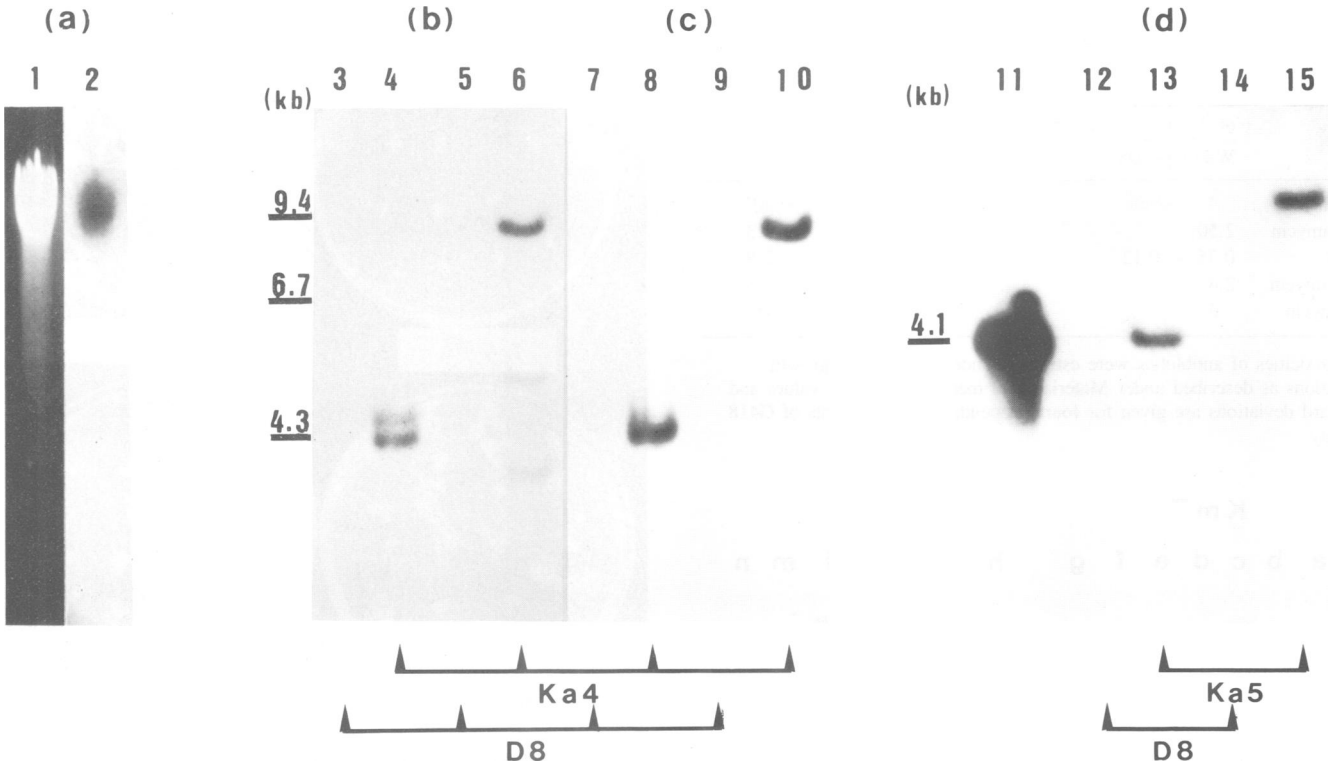

Fig. 5. Detection of pLGV23neo sequences in the DNA extracted from leaves of the kanamycin-resistant plants Ka4 and Ka5. After enzymatic restriction and electrophoresis of 10 μg of DNA per sample, DNA was transferred onto Biodyne A membrane and hybridized with a nick-translated probe ($2-4 \times 10^8$ c.p.m./μg). **(a)** Undigested DNA of Ka4 **(lane 1)** and its hybridization with the 4.1-kb *Hind*III fragment of pLGV23neo **(lane 2)**. **(b)** Southern blot hybridization, with the plasmid pLGV23neo linearized with *Bam*HI as a probe, of D8 DNA digested with *Hind*III **(lane 3)** and with *Bam*HI **(lane 5)** and of DNA from the transformed plant Ka4 digested with *Hind*III **(lane 4)** and with *Bam*HI **(lane 6)**. **(c)** The Biodyne A membrane used in **(b)** was washed and re-hybridized with the purified 4.1-kb fragment of the plasmid pLGV23neo, in order to limit as far as possible aspecific hybridization between pBR sequences and plant rDNA sequences, 20 μg/ml of pBR322 were added to the hybridization buffer. The 4.1-kb band is specifically emphasized by the probe in the *Hind*III digest of Ka4 **(lane 8)**. **(d)** In a separate experiment, the DNA from Ka5 was digested with *Hind*III **(lane 13)** or *Bam*HI **(lane 15)** and was hybridized with the 4.1-kb *Hind*III fragment showing the same result as in **(c)** for Ka4. The hybridization of the 4.1-kb fragment to itself is also shown **(lane 11)**.

pLGV23neo sequences are integrated in the genome of kanamycin-resistant clones

To correlate the kanamycin-resistant phenotype to the presence of the APH(3′)II-coding sequence, DNA from two resistant regenerated plants Ka4 and Ka5 were extracted and analysed. The probes used for Southern blot analysis were either the entire pLGV23neo linearized with *Bam*HI or the 4.1-kb *Hind*III fragment of the plasmid containing the kanamycin resistance gene. No sequence was detected by these probes in *Hind*III digests of the wild-type D8. Bands of approximate mol. wt. 4.1 and 4.4 kb were detected in *Hind*III digests of Ka4 and Ka5 DNA. A major 9-kb band was revealed by the two probes in *Bam*HI digests of clones Ka4 and Ka5 but not in D8 digests (Figure 5). Bands of weak intensity were detected in *Bam*HI digests of all clones when revealed with nick-translated pLGV23neo. These bands resulted from cross-hybridization of the pBR sequences carried by pLGV23neo to tobacco ribosomal sequences (data not shown). They are not revealed in *Hind*III digests since this enzyme does not recognize restriction sites in these repeated ribosomal sequences. A smear of sequences of high mol. wt. was revealed by hybridization of uncut Ka4 DNA with the 4.1-kb *Hind*III probe (Figure 4 lanes 2). Taken together these results allowed us to draw several conclusions. (i) pLGV23neo sequences were integrated in the plant genome. (ii) The sizes of bands detected by molecular hybridization in both *Hind*III and *Bam*HI digests were very close to that obtained by restriction of pLGV23neo. This is consistent with the hypothesis that the entire sequence of the plasmid was integrated without modification into the genome of transformed plants. (iii) No border sequences were clearly detectable in *Hind*III and *Bam*HI genomic DNA digests, thus suggesting that the corresponding border fragments were either very small or of the same size as internal fragments in two different transformants, which seems improbable. Alternatively they could be present but not detected due to a large proportion of internal fragments. If so another assumption should be made that pLGV23neo sequences could be integrated as tandem repeats.

Number of copies of the plasmid pLGV23neo integrated into the plant genome

To estimate the number of copies integrated in transformants, we carried out partial digests of DNA of transformed plant Ka4 with *Hind*III. After electrophoresis and transfer to Biodyne A filter the DNA was hybridized with the 4.1-kb *Hind*III fragment of pLGV23neo (Figure 6). Five bands could be observed in 60 min digests, the mol. wts. of which corresponded to multiples of ~4 kb. Thus, by this procedure it could be concluded that at least three copies of the pLGV23neo were integrated in tandem into the genome. In agreement with this, a reconstruction experiment (Figure 7) suggested that four to five copies of the plasmid were integrated into the genome.

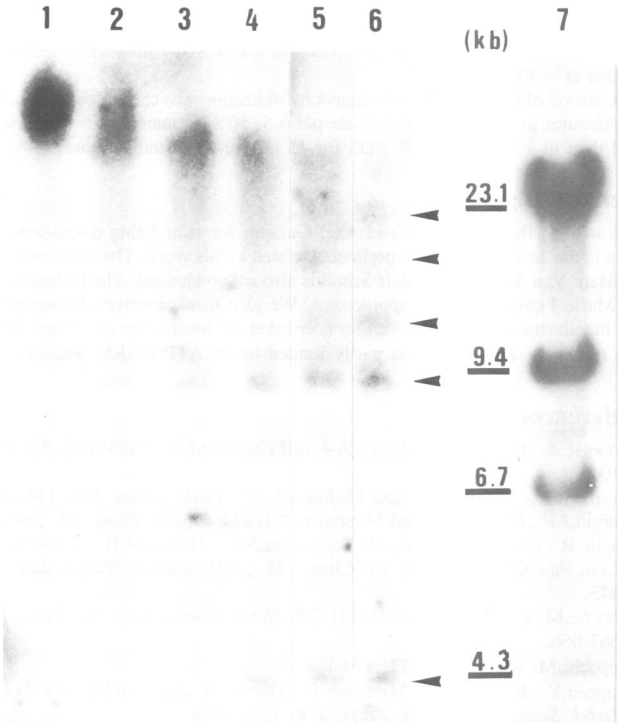

Fig. 6. Partial digestion of DNA extracted from the transformed plant Ka4. 50 μg of DNA were restricted with 30 units of *Hind*III in a final volume of 700 μl. Every 10 min, 70 μl of the reaction mixture were taken out and overnight ethanol precipitated. DNA was electrophoresed, transferred to a Biodyne A membrane and hybridized with the 4.1-kb *Hind*III fragment of pLGV23neo. **Lane 1** = undigested DNA; **lanes 2–6** = samples which have been digested for 10, 20, 30, 60 and 90 min, respectively. **Lane 7** = *Hind*III digest. Arrows indicate bands emphasized in 60-min and 90-min digests.

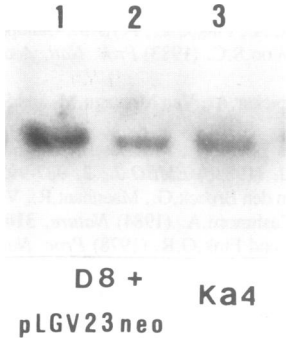

Fig. 7. Evaluation of the copy numbers of pLGV23neo integrated into the transformed plant Ka4. 10 μg of DNA extracted from clones D8 (wild-type) and Ka4 were digested with *Bam*HI before deposition on agarose gel, 2×10^{-4} μg corresponding to ten copies (**lane 1**) and 10^{-4} μg (**lane 2**) of linearized plasmid pLGV23neo were mixed with the D8 DNA Ka4 deposit (**lane 3**). After electrophoresis and transfer to Biodyne A membrane, DNA was hybridized with the linearized plasmid.

Discussion

The results presented here show that liposomes can be used to transform plant protoplasts. Resistant calli were reproducibly obtained through liposome transfection and kanamycin selection, although their frequency varied appreciably from experiment to experiment. The physiological state of cells, making them more or less competent to be transformed, could be critical in this type of experiment. As for TMV-RNA transfections, calcium is required for liposome-mediated protoplast transformation by pLGV23neo. It is presumed that transfection is promoted by the fusion of liposomes with protoplasts, a process which requires calcium and PEG. When compared with transformation frequencies observed in co-cultivation experiments between *A. tumefaciens* and tobacco protoplasts (De Block *et al.*, 1984), those obtained in liposome-mediated transfections are 100-fold lower. An accurate mesurement of the efficiency of direct transformation with kanamycin resistance genes, according to the procedure of Krens *et al.* (1982), has not been done so far. Tentatively, these frequencies are probably within the range of 10^{-6} to 10^{-5}. Liposome-mediated transfection would then be as much as 20 times more efficient than the procedure of Krens *et al.* (1982). They compare favourably with PEG-mediated transformation procedure and they reach values (4×10^{-5}) acceptable to work with. Furthermore, no carrier DNA is required for liposome-mediated transfection. This may simplify the analysis of transferred sequences in transformed plants. The major drawback of the procedure concerns the requirement of a mild sonication of samples during liposome preparation, which may create breaks in DNA fragments >25 kb.

Protoplasts of plant regenerated from resistant calli turned out to be resistant up to 200 μg/ml of kanamycin. The plasmid pLGV23neo was integrated into the plant genome and the enzyme APH(3′)II was detected in cellular extracts. Regenerated plants were normal, fully fertile and their resistance to kanamycin was transmitted to the progenies as a single Mendelian trait. More than 100 independent transformed clones are presently under study. From these data it is clear that kanamycin resistance is conferred by the expression of the APH(3′)II coding sequence. It is therefore surprising that no resistance to gentamycin and a very limited resistance to G418 is expressed by transformed cells, since APH(3′)II has a reduced but significant ability to phosphorylate gentamycins (Dowding, 1979). We presume that the phosphorylated derivatives of these molecules are still toxic for plant cells.

Southern blot analysis and genetic studies were consistent with the hypothesis of a single integration site in the plant genome. This evidence also suggests that plasmid is to be found in tandem repeats of several copies. The more satisfactory explanation for this is to assume that homologous recombination has occurred between plasmids before integration into the plant genome. Such a recombinational event has already been shown to occur, in transfection of mammalian cells, either by microinjection (Cappechi, 1980) or by fusion with bacterial sphaeroplasts (de Saint Vincent *et al.*, 1983). Similar conclusions have been drawn from yeast transformation experiments (Gaillardin *et al.*, personal communication). This homologous recombinational event between plasmids, before integration, could be very useful to realize the co-transfer of genes cloned on different plasmids, provided these plasmids share homologous sequences. Experiments are currently in progress in our laboratory to test this possibility of liposome-mediated co-transfer.

Within the plasmid pLGV23neo and upstream of the NOS promoter is found a 25-bp repeat, from the Ti plasmid, which is involved in the T-DNA integration process (Yadav *et al.*, 1982). It has also been shown that the right 25-bp terminus was required to promote T-DNA transfer (Shaw *et al.*, 1984; Wang *et al.*, 1984). Using the liposome-mediated transformation procedure, we can therefore ask two questions: (i) does this sequence have a function in the efficiency of transformation, and (ii) is it used as a border sequence at the integration site into the plant genome? Work is in progress to study these points.

Materials and methods

Encapsulation of pLGV23neo into liposomes

Liposomes were prepared by the reverse phase evaporation technique essentially as described by Rouzé *et al.* (1983). 5 μmol of phosphatidylserine (Sigma Chemicals) and 2 μmol of cholesterol were dried under vacuum and re-dissolved in 0.8 ml of diethyl ether. 200 μg of plasmid in 200 μl of liposome buffer, containing 0.44 M mannitol, 50 mM NaCl and 1 mM EDTA in 5 mM Tris-HCl buffer pH 7.6 was added. The two phases were mixed under argon by sonication for 30 s in a bath type sonicator (Bransonic 220). Ether was removed by evaporation and the resulting liposomes were stored under argon. Liposomes were separated from the non-encapsulated material by flotation onto a Ficoll gradient. The encapsulation efficiencies varied from 25 to 40% when measured as described by Fraley *et al.* (1982).

Transfection of protoplasts and selection of kanamycin-resistant clones

Protoplasts were prepared from the leaves of a diploid clone, D8, of *N. tabacum* (cv Xanthi) as described by Chupeau *et al.* (1974) and resuspended at a final density of 2×10^6 protoplasts/ml in the fusion medium (0.5 M mannitol, 5 mM $CaCl_2$, 5 mM Tris-HCl pH 7.6). In a standard experiment 50 μl of liposomes containing 3 μg of encapsulated pLGV23neo were mixed with 1 ml of protoplasts at room temperature. 5 min later 5 ml of fusion medium containing 22% PEG 6000 (w/w) were mixed with protoplasts. After incubation for 20 min, 20 ml of saline medium (0.3 M KCl, 5 mM $CaCl_2$) were added and protoplasts were sedimented at 100 *g* for 5 min. Protoplasts were plated in the dark at a density of 5×10^4 protoplasts/ml in medium To (Chupeau *et al.*, 1974). One week later protoplast-derived cells were collected by centrifugation and resuspended in medium C containing 3 mM MES pH 5.9 (Muller and Caboche, 1983) at a density of 5×10^3 cells/ml. Cultures were incubated in the presence of 70 μg/ml of filter sterilized kanamycin at 28°C under light. Resistant colonies were regenerated in the absence of kanamycin as described previously (Bourgin *et al.*, 1979).

Assay of the toxicities of aminoglycoside antibiotics

Protoplasts were prepared from the leaves of selected clones grown in the greenhouse and incubated in medium To. Four days later cultures were diluted in medium C containing 3 mM MES pH 5.9 at a density of 100 cells/ml as previously described (Muller and Caboche, 1983) and incubated for 1 month in the presence of variable concentrations of aminoglycosides. Colonies were then scored and plating efficiencies calculated.

Assay of APH(3')II activity

Two grams of leaf fragments were crushed at 4°C in a mortar with a pestle in 2 ml of extraction buffer (10 μg/ml leupeptine, 30 mM 2-mercaptoethanol, 20 mM EDTA in 0.2 M Tris-HCl buffer pH 7.5) together with 0.2 g polyclar. After two successive centrifugations in Eppendorf tubes at 12 000 *g* for 15 min, supernatants were used for electrophoresis in a 10% non-denaturing polyacrylamide gel and APH(3')II activity was revealed according to the method of Reiss *et al.* (1984).

DNA isolation

For large-scale preparations of plasmid from *E. coli* strain RR1, a Triton X-100 cleared lysate procedure was used (Kupersztock-Portnoy *et al.*, 1974). Separation of plasmid from chromosomal DNA was carried out either on CsCl gradient or on hydroxylapatite (Colman *et al.*, 1978). For small-scale preparation of plasmid, diethylpyrocarbonate (DEPC) was added to a cleared lysate and the mixture was warmed for 15 min at 65°C; after a 30 min centrifugation the supernatant was isopropanol precipitated for 1 h at −80°C and the DNA was resuspended in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

Plant DNA was isolated by a phenol extraction procedure (Fedoroff *et al.*, 1983), purified by two CsCl gradients and dialysed for 48 h against TE buffer.

Restriction enzyme, electrophoresis and purification of DNA fragments

Restriction endonucleases were from Biotec or Amersham and were used according to the instructions of the suppliers. 10 μg of plant DNA were digested with 40 units of *Bam*HI or 60 units of *Hind*III, ethanol precipitated and resuspended in 30 μl of 10 mM Tris-HCl. Electrophoresis was carried out in 1% HGT agarose gel. The 4.1-kb *Hind*III fragment of pLGV23neo was recovered directly from a 1% LGT agarose gel (Maniatis *et al.*, 1982).

Southern blot analysis

After electrophoresis, DNA was depurinated in 0.25 N HCl for 15 min at room temperature, denatured in 1.5 NaCl 0.5 N NaOH, neutralized in 3 M Na acetate pH 5.5 and then transferred to Biodyne A nylon membrane (PALL) with 6 × SSC (Southern, 1975). After baking at 80°C for 1 h in a vacuum oven, membranes were incubated in the hybridization buffer [5 × Denhardt's reagent, 5 × SSC, 50 mM Na phosphate pH 6.5, 0.1% SDS, 50% formamide, 200 μg/ml of calf thymus DNA (Boehringer Mannheim)]. Afterwards, membranes were hybridized in the same buffer with nick-translated (Rigby *et al.*, 1977) DNA ($2-4 \times 10^8$ c.p.m./μg) for 16−70 h. Membranes were washed in 2 × SSC, 0.1% SDS three times for 5 min at room temperature and then in 0.2 × SSC, 0.1% SDS twice for 15 min at 50°C. Blots were exposed to Ilford X-ray film with intensifying screen at −70°C.

Removal of the probe for subsequent hybridization was carried out by washing membranes in 10 mM Na phosphate pH 6.5, 50% formamide for 1 h at 65°C and then in 2 × SSC, 0.1% SDS for 15 min at room temperature.

Acknowledgements

We wish to thank Florence and Axel Garapin for stimulating discussions and help in the development of experiments related to this work. The encouragement of Marc Van Montagu and Jeff Schell is also acknowledged. The technical help of Marie France Dorbe is appreciated. We also thank Jocelyne Kronenberger for the illustrations, Roger Calza for correcting the manuscript and Claire Ribaut for typing it. This work was partly funded by an ATP-CNRS: 902337.

References

Barton,K.A., Binns,A.N., Matzke,A.J. and Chilton,M.D. (1983) *Cell*, **32**, 1033-1043.

Bevan,M.W., Flavell,R.B. and Chilton,M.-D. (1983) *Nature* **304**, 184-187.

Bourgin,J.P., Chupeau,Y. and Missonier,C. (1979) *Physiol. Plant.*, **45**, 288-292.

Broglie,R., Coruzzi,G., Fraley,R.T., Rogers,S.G., Horsch,R.B., Niedermeyer, J.G., Fink,C.L., Flick,J.S. and Chua,N.H. (1984) *Science (Wash.)*, **224**, 838-843.

Caboche,M. and Deshayes,A. (1984) *C.R. Hebd. Séances Acad. Sci. Paris*, **299**, 663-666.

Cappechi,M. (1980) *Cell*, **22**, 479-488.

Chupeau,Y., Bourgin,J.P., Missonier,C., Dorion,N. and Morel,G. (1974) *C.R. Hebd. Séances Acad. Sci. Paris*, **278**, 1565-1568.

Colman,A., Byers,M.J., Primrose,S.B. and Lyons,A. (1978) *Eur. J. Biochem.*, **91**, 303-310.

De Block,M., Herrera-Estrella,L., Van Montagu,M., Schell,J. and Zambryski,P. (1984) *EMBO J.*, **3**, 1681-1689.

De Framond,A.J., Barton,K.A. and Chilton,M.D. (1983) *Biotechnology*, **2**, 262-269.

De Saint Vincent,B.R. and Wahl,G.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2002-2006.

Dowding,J.E. (1979) *FEMS Microbiol. Lett.*, **6**, 95-98.

Federoff,N., Mauvais,J. and Chaleff,D. (1983) *Mol. Appl. Genet.*, **2**, 11-29.

Fraley,R.T., Dellaporta,S. and Papahadjopoulos,D. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1859-1863.

Fraley,R.T., Rogers,S.G., Horsch,R.B., Sanders,P.R., Flick,J.S., Adams,S.P., Bittner,M.L., Brand,L.A., Fink,C.L., Fry,J.S., Galluppi,G.R., Goldberg,S.B., Hoffman,N.L. and Woo,S.C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4803-4807.

Herrera-Estrella,L., Depicker,A., Van Montagu,M. and Schell,J. (1983a) *Nature*, **303**, 209-213.

Herrera-Estrella,L., De Block,M., Messens,E., Hernalsteens,J-P., Van Montagu,M. and Schell,J. (1983b) *EMBO J.*, **2**, 987-995.

Herrera-Estrella,L., Van den Broeck,G., Maenhaut,R., Van Montagu,M., Schell, J., Timko,M. and Cashmore,A. (1984) *Nature*, **310**, 115-120.

Hinnen,A., Hicks,J.B. and Fink,G.R. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1929-1933.

Hoekema,A., Hirsch,P.R., Hooykaas,P.J.J. and Schilperoort,R.A. (1983) *Nature*, **303**, 179-180.

Krens,F.A., Molendijk,L., Wullems,G.J. and Schilperoort,R.A. (1982) *Nature*, **296**, 72-74.

Kupersztoch-Portnoy,Y.M., Lovett,M.A. and Helinski,D.R. (1974) *Biochemistry (Wash.)*, **13**, 5484-5490.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.

Muller,J.F. and Caboche,M. (1983) *Physiol. Plant*, **57**, 35-41.

Murai,N., Sutton,D.W., Murray,M.G., Slightom,J.L., Merlo,D.J., Reichert, N.A., Sengupta-Gopalan,C., Stock,C.A., Barker,R.F., Kemp,J.D. and Hall, T.C. (1983) *Science (Wash.)*, **222**, 476-482.

Otten,L., De Greve,H., Hernalsteens,J.-P., Van Montagu,M., Schieder,O., Straub,J. and Schell,J. (1981) *Mol. Gen. Genet.*, **183**, 209-213.

Papahadjopoulos,D., Wilson,T. and Taber,R. (1980) in Ceis,J.E. and Loyter,A. (eds.), *Transfer of Cell Constituents into Eukaryotic Cells*, Plenum, NY, pp. 155-172.

Paszkowski,J., Shillito,R.D., Saul,M., Mandak,V., Hohn,T., Hohn,B. and Potrykus,I. (1984) *EMBO J.*, **3**, 2717-2722.

Reiss,B., Sprengel,R., Will,H. and Schaller,H. (1984) *Gene*, **30**, 217-223.

Rigby,P.W., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237-251.

Rouzé,P., Deshayes,A. and Caboche,M. (1983) *Plant Sci. Lett.*, **31**, 55-64.
Shaw,C.H., Watson,M.D., Carter,G.H. and Shaw,C.H. (1984) *Nucleic Acids Res.*, **12**, 6031-6041.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503-517.
Tepfer,D. (1984) *Cell*, **37**, 959-967.
Wang,K., Herrera-Estrella,L., Van Montagu,M. and Zambryski,P. (1984) *Cell*, **38**, 455-462.
Yadav,N.S., Vanderleyden,J., Bennett,D.R., Barnes,W.H. and Chilton,M.-D. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6322-6326.
Zambryski,P., Joos,H., Genetello,C., Leemans,J., Van Montagu,M. and Schell, J. (1983) *EMBO J.*, **2**, 2143-2150.

Received on 1 July 1985