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Genomic DNA can be used with cationic methods for highly efficient transformation of maize protoplasts

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Summary. Efficient delivery of genomic DNA fragments to maize protoplasts was obtained by new methods using the polycation Polybrene or Lipofectin cationic liposomes. Stable kanamycin-resistant secondary transformants were recovered after transfection with genomic DNA from a maize cell line that had previously been tagged with the bacterial gene neomycin phosphotransferase (*nptII*) in a first-round transformation. The frequency of secondary transformants with *nptII*-homologous DNA sequences was 3% or 6% of all randomly picked microcalli after Polybrene- or Lipofectin-mediated transfection, respectively. Transformation with genomic DNA by these methods may allow easy transfer of uncloned genes encoding desirable characteristics to crop species that can be regenerated from protoplasts.

Key words: Genomic DNA – Transformation – Maize protoplasts – "Black Mexican Sweet"

Introduction

The increasingly successful gene transfer systems for higher plants (Gasser and Fraley 1989) have used only cloned genes as donor DNA. However, many important genetic traits are not biochemically understood, and isolation of the underlying structural genes is therefore not feasible by conventional means. Examples of beneficial but as yet unclonable genes are those that control agronomic characteristics or resistance to pathogens (Coe et al. 1988). The development of efficient methods for direct transfer of genomic DNA could permit successful intraspecific and interspecific transfer of genes encoding molecularly uncharacterized phenotypes. This paper demonstrates for the first time that stable transformants may be recovered at high frequency after transfection[1] of protoplasts with total plant genomic DNA. Regeneration of fertile plants from protoplasts has now been accomplished for two of the world's most widely cultivated cereals, maize (Shillito et al. 1989; Prioli and Sondhal 1989) and rice (Shimamoto et al. 1989; Toriyama et al. 1988; Abdullah et al. 1986). Coupling of this regeneration capability with the cationic procedures for genomic DNA transfer described here may significantly speed up genetic engineering in these vital crop plants.

The most frequently used methods for transfer of cloned genes to cereal protoplasts involve electroporation (Fromm et al. 1986) or polyethylene glycol (PEG) treatment (Antonelli and Stadler 1989). The former technique temporarily destroys plasmalemma integrity and often causes cytoxicity (Fromm et al. 1986) and pronounced cell loss, and the latter, which allows more frequent recovery of stable transformants in maize, is also significantly damaging to protoplast membranes (Antonelli and Stadler 1989). In attempts to obtain transformation with genomic DNA fragments, we chose cationic methods rather than electroporation or PEG treatment for two reasons. First, cationic methods were previously shown to be relatively nontoxic (Antonelli and Stadler 1989). Second, it seemed reasonable to expect that polycations and cationic lipids might facilitate uptake of uncloned DNA fragments by the more natural processes of endocytosis and lipid-plasmalemma fusions. Transformation with maize genomic DNA was accomplished by using either Polybrene (PB) (Antonelli and Stadler 1989; Chaney et al. 1986) or Lipofectin (LF) (Felgner et al. 1987) methods. PB is thought to promote gene transfer

[1] Transfection is a synonym for gene transfer and is defined as the "acquisition of new genetic markers by incorporation of added DNA" (Lewin 1987)

through interactions of polycations with anionic plasmalemma to give regions of positive charge, which then react efficiently with negatively charged transforming DNA. LF-mediated transfection is carried out by mixing a suspension of small cationic liposomes with transforming DNA before presentation to protoplasts; it is proposed that this treatment creates cationic complexes (surrounding anionic DNA) which then react with anionic plasma membrane (Felgner et al. 1987). We show here that 3%–6% of randomly recovered microcalli are stably transformed after PB- or LF-mediated transfer of maize genomic DNA.

Materials and methods

Cell line

A maize "Black Mexican Sweet" suspension culture, BMS-M (Somers et al. 1987), was maintained in medium containing MS salts (GIBCO) (Murashige and Skoog 1962) supplemented with (1^{-1}) 2 mg 2.4-D, 0.5 mg thiamine, 20 g sucrose, 150 mg asparagine, and 250 mg glucose (MS2D) (Somers et al. 1987). Suspension cultures were subcultured at 7-day intervals (4 mg/ml inoculum) and grown with shaking (150 rpm) at 28 °C in the dark. Cell populations grew exponentially between 2 and 6 days after transfer and the population doubling time was 24–36 h.

Transforming DNA

Two donor plasmids were used: pCaMVI$_1$CN (Callis et al. 1987) and pCaMVNeo (Fromm et al. 1986). pCaMVI$_1$CN contains the chloramphenicol acetyl transferase gene (*CAT*), flanked by the 35S cauliflower mosaic virus promoter plus maize intron I$_1$ from *Adhl* and the nos 3′ region. pCaMVNeo contains the *nptII* gene flanked by the 35S cauliflower mosaic virus promoter and the nos 3′ region.

Genomic DNA was prepared from the BMS-M-derived Kan^R (kanamycin resistant) cell line K28 by a miniprep method (Dellaporta et al. 1983) and sheared through an 18-gauge needle. Pulsed gel electrophoresis (Schwartz and Cantor 1984) showed that most of the resulting fragments were in the 20–50 kb range.

Probe DNA

The *nptII* gene probe was prepared by BamHI digestion of pCaMVNeo to obtain a 1.0-kb fragment containing *nptII* structural regions (Fig. 2). Isolated fragments were made radioactive with ^{32}P by nick-translation.

Protoplast isolation

Protoplasts were always obtained from BMS-M cultures in log-phase growth 4 days after transfer. Growth medium was removed and the cells were plasmolyzed for 30 min at 25 rpm in MS2D with 8% mannitol (MS2D8M). Plasmolyzed cells were allowed to settle, and 1.5-ml settled cells (approximately 0.5 g) were incubated with enzyme mixture [2% cellulase plus 0.25% pectinase (Worthington) in 80 m*M* $CaCl_2$, 0.2 *M* mannitol, pH 5.0] for 3–4 h at 28 °C. The protoplast preparation was then filtered through one 70-μm nylon mesh screen (Tetko) and subsequently through three 47-μm nylon mesh screens. Enzyme mixture was removed by two 20-ml washes with MS2D6M (MS2D with 6% mannitol) and centrifugation was at 50 × *g* for 5 min. After the final wash protoplasts were resuspended in MS2D6M. Yield was determined by hemocytometer counts, viability by the fluorescein diacetate method (Widholm 1972), and whole cell contamination by the method of Chourey and Zurawaski (1981). No intact cells remain after this procedure. Protoplast viability after treatment was also determined by fluorescein diacetate staining (Widholm 1972), and at least 300 cells from each treatment were examined at every time point tested.

PB-mediated transfection

Stock solutions of PB (Aldrich; 10 mg/ml in phosphate buffered saline, pH 7.0) were freshly prepared for each experiment. The final volume of each transfection mixture was 1.0 ml, and the components were added as follows: 2×10^6 protoplasts were suspended in 0.5 ml MS2D6M. PB solution (30 μg in 0.1 ml MS2D6M) was added to the protoplast suspension, mixed gently, and protoplasts and PB were then transferred to a 60-mm petri dish where they were allowed to remain as a large unspread drop. PB and protoplasts were incubated together for 5 min. DNA suspension (desired concentration of DNA in 0.4 ml MS2D6M) was then added slowly in small drops to protoplasts and PB. The cell-Polybrene-DNA mixture was rotated on a gyrotary shaker platform at 25 rpm for 15 min and then incubated without shaking at 28 °C for 6 h. After the 6-h incubation, the mixture was diluted by adding 4.0 ml MS2D6M and incubated further at 28 °C, either for assay of transient gene expression or selection of stable transformants.

Lipofectin-mediated transfection of BMS-M

Lipofectin stock solution (1 mg liposomes/ml) was purchased from Bethesda Research Laboratories. Protoplasts (2×10^6 in 0.9 ml MS2D6M) were moved to a 60-mm dish as described above. Transfecting DNA was prepared by adding 50 μl of a 1:1 dilution of LF stock solution in water to 50 μl (desired concentration) DNA in water. This LF-DNA mixture (final concentration 25 μl LF plus DNA in 0.1 ml) was incubated for 15 min at room temperature to allow the formation of lipid/DNA complexes and then added dropwise to the protoplasts. The final protoplast-LF-DNA mixture was rotated at 25 rpm for 15 min and incubated for 6 h at 28 °C before dilution with 4.0 ml MS2D6M.

Chloramphenicol acetyl transferase (CAT) assays

Protoplasts were incubated for 40 h after transformation before assay for CAT activity (Gorman et al. 1982). Thin layer chromatography plates were exposed to X-ray film for 18 h at 25 °C. Protein concentration of each sample homogenate was determined by the method of Bradford (1976).

Selection for stable Kan^R transformants

Transfected protoplasts were diluted with 5.0 ml MS2D6M plus 25% conditioned medium (Somers et al. 1987), and were further incubated without shaking in a sealed 60-mm petri dish for 14 days at 28 °C. Microclusters of dividing cells were then recovered by gentle scraping with a rubber policeman and centrifuged at 50 × g for 5 min. Part of the supernatant was removed and 2.0-ml aliquots were plated on solid medium [MS2D with 0.3% Gelrite (Kelco) in 100-mm petri dishes] for an additional round of growth under nonselective conditions. After 7 days, large numbers of 2-mm microcalli clumps were randomly picked from all areas of the dense callus, transferred to selective medium (MS2D, 0.3% Gelrite, 200 μg/ml kanamycin), and incubated further until a subset of rapidly growing microcalli could be identified (approximately 10–14 days). These faster-growing microcalli were then picked for subculture and DNA analysis (Antonelli and Stadler 1989).

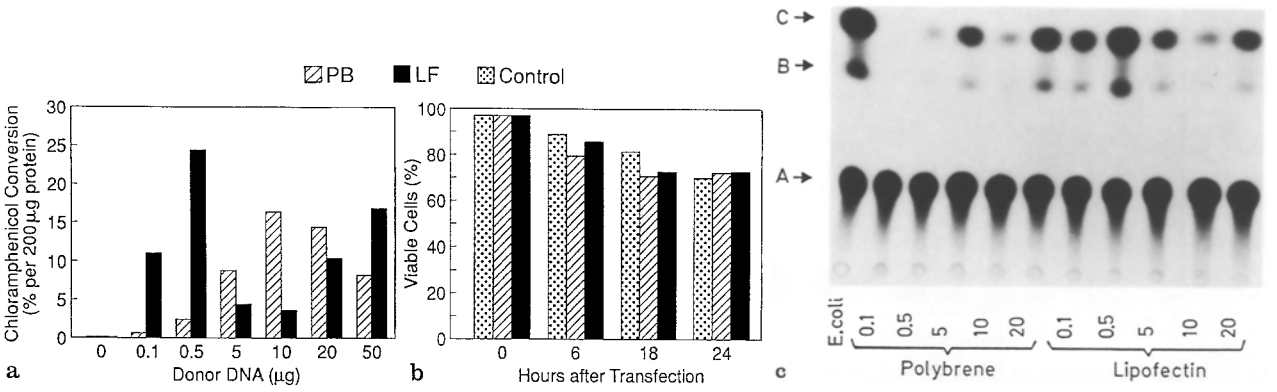

Fig. 1 a–c. Chloramphenicol acetyl transferase activity and viability of BMS-M protoplasts transfected by PB or LF methods. **a** CAT activity after transformation with various concentrations of pCaMVI$_1$CN. CAT gene activity is expressed as the percent of total ^{14}C chloramphenicol converted to acetylated derivatives per 200 µg protein. **b** Post-treatment viability of BMS-M protoplasts as determined by fluorescein diacetate staining. **c** Autoradiogram of a TLC plate showing the 1- and 3-monoacetylated products of ^{14}C chloramphenicol after transformation by several concentrations of pCaMVI$_1$CN (0.1, 0.5, 5, 10, 20 µg DNA). (*A*, unreacted chloramphenicol); *B*, 1-acetyl chloramphenicol; *C*, 3-acetyl chloramphenicol). Each data bar represents an average of 3–5 experiments

DNA isolation and Southern blot analysis

Genomic DNA was isolated from microcalli by the miniprep method of Mettler (1987). Southern blot analysis was performed as previously described (James and Stadler 1989).

Results

Experimental plan

Maize genomic DNA used in these transformation experiments was previously marked with the bacterial neomycin phosphotransferase gene (*nptII*) after PEG-mediated transfection (Antonelli and Stadler 1989) of BMS-M protoplasts with pCaMVNeo (Fromm et al. 1986). The transformed DNA donor cell line K28 has been in culture for more than 12 months and is stably resistant to 200 µg ml kanamycin (Kan). Southern analysis of K28 DNA digested with EcoRV, an enzyme that cuts at one site in the integrated pCaMVNeo plasmid and at many genomic sites, and hybridized with the BamHI *nptII* gene fragment of pCaMVNeo (Fig. 2) demonstrated that *nptII* was integrated at three sites in the K28 cell-line genome (Fig. 3 b). When unrestricted K28 DNA was similarly probed with *nptII*, hybridization occurred only in the region of high molecular weight DNA; also, a comparison of BamHI-digested, *nptII*-probed K28 genomic DNA with pCaMVNeo copy-number reconstructions indicated the presence of 9 *nptII* copies per haploid genome (data not shown).

For each experiment, one preparation of BMS-M protoplasts was subdivided and treated as follows: (1) with PB or LF but without added DNA, as controls; (2) with PB or LF and 50 µg pCaMVI$_1$CN (Callis et al. 1987) to test the efficiency of these methods by assay for transient CAT gene activity; (3) with PB or LF and 50 µg pCaMVNeo or 50 µg genomic DNA from cell line K28 for selection of stable Kan transformants.

Transient gene expression and protoplast viability after transfection of pCaMVI$_1$CN by PB or LF methods

Both PB and LF treatments mediate pCaMVI$_1$CN transfer, and good level of transient CAT gene activity can be detected over a broad range of DNA concentrations (Fig. 1 a and c). *CAT* activity is expressed as the percent of ^{14}C chloramphenicol converted to acetylated products per 200 µg protein, which is the average amount of protein recovered in cell lysates from 2×10^6 BMS-M protoplasts in each sample. Although other transformation procedures for plant protoplasts typically use 10–50 µg donor DNA, in LF-mediated transfection as little as 0.1–0.5 µg pCaMVI$_1$CN produced acetylation of 12%–25% of the chloramphenicol substrate. Success with PB transfection required the use of more donor plasmid, and best results were obtained with 10–20 µg DNA. Figure 1 b shows that the viability of treated cells 24 h after transfection, as measured by fluorescein diacetate staining, was 100% of control values. This extraordinary lack of cytotoxicity may occur because PB and LF methods exploit natural membrane functions that allow DNA uptake across the plasmalemma.

Stable transformants are recovered efficiently after transfection of genomic or plasmid DNA by PB or LB

Because BMS protoplasts do not plate efficiently at low density (Fromm et al. 1986; Somers et al. 1987), stable Kan^R transformants were recovered from transfected protoplasts grown at high density (4×10^5 per ml) in nonselective liquid medium for 14 days and for an additional 7 days on nonselective agar before small 2-mm microcalli were randomly picked to selective medium containing kanamycin (Antonelli and Stadler 1989). Recovery of stable Kan^R transformants after both PB- and LF-mediated transfection was highly efficient (Table 1; Fig. 2).

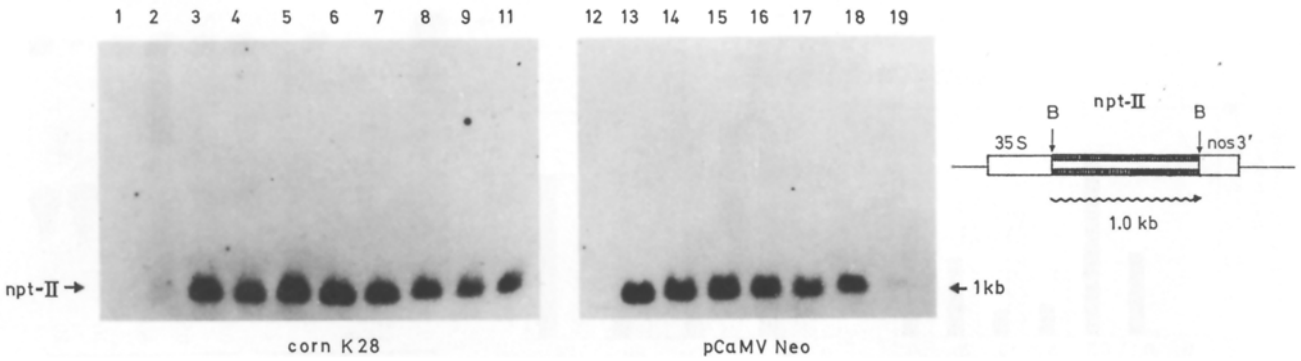

Fig. 2. Southern analysis of DNA from K28- and pCaMVNeo-transformed kanamycin-resistant microcalli. BamHI-digested genomic DNA (5 μg) was probed with the BamHI 1.0-kb fragment of pCaMVNeo that contained the entire *nptII* structural gene (see insert). *Lanes 1* and *12:* BMS-M controls treated with LF but no DNA. *Lanes 2-11:* DNA from Kan^R microcalli obtained from transformation with LF and 50 μg K28. *Lanes 13–19;* Kan^R microcalli obtained after transfection with LF and 50 μg pCaMVNeo

Table 1. Frequency of nptII-containing Kan^R transformants after transfection with pCaMVNeo or K28 genomic DNA

Treatment	Donor DNA	No. randomly picked microcalli	No. fast-growers on kanamycin	% fast-growers among randomly picked microcalli	No. analyzed by Southern	No. nptII-positive	% nptII-positive among fast growers analyzed	% nptII-positive among total randomly picked microcalli
PB	pCaMVNeo	381	27	7	nd	nd	nd	nd
	K28	578	23	4	23	16	69	2.8
LF	PCaMVNeo	260	33	13	33	21	64	8.1
	K28	631	81	13	51	38	75	6.0

Pooled data from two experiments; microcalli were obtained after PB- or LF-mediated transformations with 50 μg DNA
nd = not determined

The frequency of randomly selected microcalli that grew rapidly on selective medium 5–6 weeks after transformation with pCaMVNeo by PB or LF was 7% or 13%, respectively. The frequency of fast-growing microcalli after transfection with K28 genomic DNA by PB was 4%, and by LF 13%.

Presence of the transforming *nptII* gene in these fast growers was determined by Southern analysis. Genomic DNA from 107 independently picked, putatively Kan^R microcalli was isolated by a miniprep method (Dellaporta et al. 1983). These DNA preparations were then digested with BamHI, which produced a 1.0-kb DNA fragment containing a portion of the *nptII* gene, and hybridized with the 1.0-kb *nptII*-containing BamHI fragment from pCaMVNeo (Fig. 2, diagram). Figure 2 shows an example of the results obtained. Control genomic DNA from wild-type BMS-M (lanes 1 and 12) did not hybridize to the 1.0-kb BamHI *nptII*-DNA fragment, but the diagnostic *nptII* fragment was present in BamHI-digested DNA from eight fast-growing colonies produced by transformation with LF and 50 μg K28 genomic DNA (lanes 2–11). DNA from putative Kan^R microcalli produced by transformation with 50 μg pCaMVNeo and LF was analyzed in lanes 13–19, and all samples hybridized positively with the 1.0-kb BamHI *nptII*-DNA fragment. The number of *nptII*-hybridizing sequences varied in the microcalli lines tested, e.g., lanes 2 and 19 showed faint hybridization signals compared to lanes 3–11 and 13–18 although the amount of DNA per lane was constant. Molecular analyses of the entire sample of Kan^R putative transformants are summarized in Table 1, where it is shown that 72% of K28-transformed and 64% of the pCaMVNeo-transformed fast-growing microcalli had *nptII*-homologous insertions in genomic DNA.

The successful selection of stable Kan^R transformants relied upon positive identification of a subset of microcalli (derived from randomly picked 2-mm callus clumps) that grew rapidly on highly selective medium (200 μg/ml kanamycin). The average percent of transformants correctly identified by this technique, i.e., those fast growers with evidence of *nptII* integration, was 69%. Overall, the selection method allowed the recovery of 2.8%–8.1% *nptII*-containing Kan^R transformants from the total population of microcalli transferred from nonselective medium (Table 1).

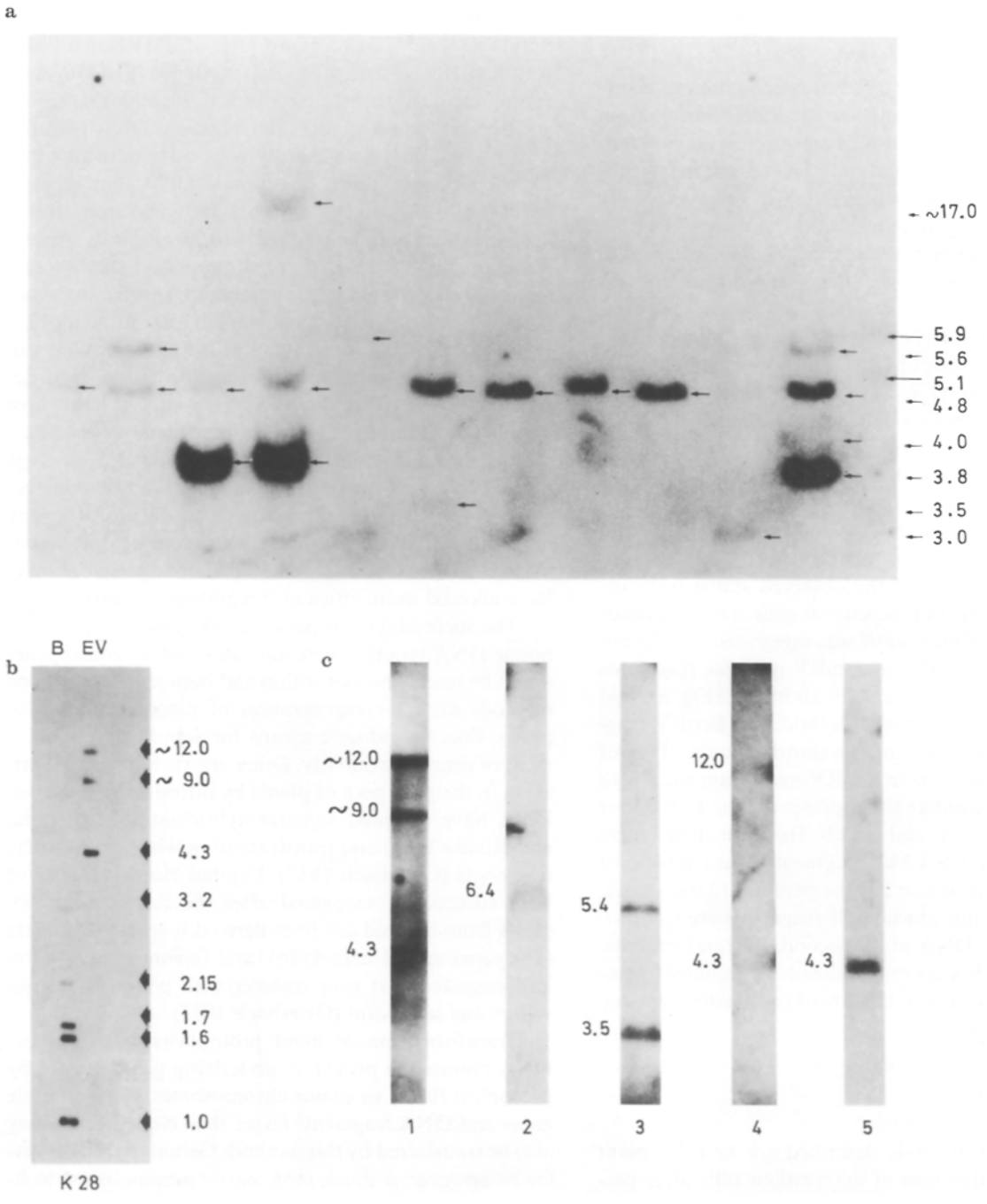

Fig. 3a–c. Southern analysis of genomic DNA from Kan^R microcalli transformed by pCaMVNeo, genomic DNA from donor cell line K28, and genomic DNA from K28-transfected secondary transformants. Genomic DNA was digested with EcoRV (which cuts at one site within the *nptII* gene and at many genomic sites) or BamHI and was hybridized to the BamHI fragment of pCaMVNeo as described in Fig. 2. **a** EcoRV-digested genomic DNA from 12 randomly picked Kan^R microcalli obtained after LF-mediated transformation with pCaMVNeo. *nptII*-hybridizing fragments in each lane are marked (→). **b** K28 genomic DNA. *Right lane* shows characteristic EcoRV(RV)-generated 4.3, 9.0, and approximately 12.0 kb bands. For comparison, the *left lane* shows the *nptII*-hybridizing fragments obtained after BamHI digestion of K28 DNA. The expected 1.0-kb band is seen, and four others that may represent rearrangement of *nptII* during insertion into genomic DNA are also present. **c** EcoRV-digested genomic DNA from K28 and four independently isolated K28 secondary transformants. *Lane 1:* K28. *Lane 2–5:* kanamycin-resistant secondary transformants selected after either PB-(*lanes 2* and *4*) or LF-mediated (*lanes 3* and *5*) transformation

High-frequency recovery of stable transformants probably occurs because of high-rate gene transfer and integration

The method used here for efficient propagation of transformed protoplasts did not allow accurate determination of the number of gene-transfer events in original treated-cell populations, since randomly picked microcalli obtained from densely grown protoplasts were probably not of single-cell origin. However, to estimate what fraction of the population of stable transformants isolated in this way might have arisen independently (or contain novel integration events), the *nptII* integration sites in 12 randomly picked fast-growing microcalli (obtained from LF-mediated transfer of pCaMVNeo) were compared by Southern analysis (Fig. 3a). Digestion with EcoRV showed that the genomes of 9 had unique *nptII*-hybridizing fragments (Fig. 3a, from left to right: approximately 4.8, 5.6, 3.8, 17.0, 5.9, 3.5, 3.0, 4.0, and 5.1 kb). This demonstrates that most of the *nptII*-containing microcalli, picked from different areas of the parent plate, did contain gene insertions of independent origin, thus implying that a majority of the observed stable transformants originated from independent gene transfer events.

As described above, *nptII* was integrated in K28 donor DNA in three different EcoRV genomic fragments approximately 4.3, 9.0, and 12.0 kb in size (Fig. 3b and c). Figure 3c shows the *nptII*-hybridizing EcoRV fragments of four K28 secondary transformants. Two of these (lanes 2 and 3) have EcoRV-*nptII* fragments with molecular lengths unlike those present in the K28 donor genome, i.e., 6.4, 5.4, and 3.5 kb. However, two others (lanes 4 and 5) have 4.3 kb fragments characteristic of K28 and one (lane 4) also has the parental 12.0 kb band. Therefore, when introduced K28 fragments are incorporated in genomic DNA of the secondary transformants, the donor EcoRV sites may either be eliminated or retained. Further studies of the fate of transfected genomic DNA are under way.

Discussion

The transfection methods described are new for plant systems. Transformation of mammalian cells after pretreatment with PB (Chaney et al. 1986) or after preincubation of LF (Felgner et al. 1987) with DNA has previously been shown to be highly successful, especially compared to the widely used calcium phosphate method for DNA transfer. Similarly, protocols developed here permit the recovery of up to 8% stable transformants, while treatment of maize protoplasts with electroporation has previouslyl yielded only 0.01%–1.0% transfectants (Fromm et al. 1986; Rhodes et al. 1988). A major benefit of these efficient transfection treatments is that they are simply performed with readily available chemical reagents and require no sophisticated equipment.

Although the incidence of recovery of *nptII*-containing stable Kan^R transformants after pCaMVNeo transfer was high for maize protoplast systems (Fromm et al. 1986), the most surprising result was the high-rate recovery of Kan^R transfectants after genomic DNA transfer. This finding contrasts sharply with early data reported for the electroporation of genomic DNA into tobacco (Saul et al. 1987). In that instance, only two stable transformants were obtained from 7×10^7 protoplasts treated. Additionally, we found that the number of stable transformants is unrelated to the number of transfecting genes per treatment (about 9×10^7 *nptII* copies in 50 µg K28 DNA and 10^{13} copies in 50 µg pCaMVNeo). What can account for the efficiency of transformation with genomic DNA by these methods? There are at least three possible explanations: (1) the cationic methods used here may have been especially suitable for transport of large molecules across the plasmalemma; (2) the flanking regions of genomic DNA surrounding the transforming marker may have protected the *nptII* gene against degradation; or (3) the long genomic flanking regions may have allowed more efficient integration of *nptII*.

The successful protoplast transfection with plant genomic DNA reported here indicates that uncloned genes might be readily moved within and between species when methods exist for regeneration of plants from protoplasts, thus providing a means for significant enhancement of genetic variability. Other efforts to increase variation in the gene pool of plants by introducing uncloned DNA have included somatic hybridization (Toriyama and Hinata 1988) and transformation with chromosome fragments (Griesbach 1987). Diploid rice hybrids have been successfully recovered after the fusion of protoplasts from haploid cell lines derived from two cultivars (Toriyama and Hinata 1988), and flavonoid expression has suggested that gene transfer with petunia chromosomes was successful (Griesbach 1987).

Transformation of plant protoplasts with genomic DNA mimics the principles underlying gene transfer by protoplast fusion or donor chromosomes, since multiple genes and DNA fragments larger than cloned genes may also be transferred by this method. Genomic DNA transfer is, however, a much more easily accomplished technique that avoids the need for development of protoplasting techniques for more than one cultivar or the necessity of difficult chromosome isolations and microinjections.

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