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Genetic Transformation of Maize Cells by Particle Bombardment

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ABSTRACT

Intact maize cells were bombarded with microprojectiles bearing plasmid DNA coding for selectable (neomycin phosphotransferase [NPT II]) and screenable (β-glucuronidase [GUS]) marker genes. Kanamycin-resistant calli were selected from bombarded cells, and these calli carried copies of the NPT II and GUS genes as determined by Southern blot analysis. All such calli expressed GUS although the level of expression varied greatly between transformed cell lines. These results show that intact cells of important monocot species can be stably transformed by microprojectiles.

Bombardment of intact cells and tissues with DNA-coated microprojectiles (13) may represent a general scheme for the transformation of plants and should circumvent some of the inherent constraints of methods that utilize protoplasts (5) or *Agrobacterium* (12). Previous work based on the analysis of transient gene expression has shown that microprojectiles can be used for the delivery of DNA to a wide range of intact plant cells and tissues including those from onion, maize, tobacco (13–16), rice, wheat, and soybean (22). Stable transformation by particle bombardment has been shown in tobacco (16) and soybean (18). In this report we demonstrate that stably transformed cells of maize can be recovered from intact cells bombarded with microprojectiles coated with plasmid DNA containing a kanamycin resistance marker. We also show that the β-glucuronidase (GUS) marker (11) can be used in maize cells to visually screen kanamycin-resistant calli to verify their transformed nature.

MATERIALS AND METHODS

Plasmids

The plasmid pNGI (8.2 kb) contains both a neomycin phosphotransferase II (NPT II) and a β-glucuronidase (GUS) gene cloned in pUC8 (Fig. 1). The GUS gene comprises the promoter and intron 1 (nucleotides 1–1775) from the Alcohol dehydrogenase 1 (*Adh*1) gene of maize (6), a GUS coding region consisting of the *Pst*I fragment from pRAJ260 (10), and a modified 3′ end from the nopaline synthase gene (8). The NPT II gene in pNGI was derived from pCaMVNEO (9) and is composed of the 35S promoter from cauliflower mosaic virus, the NPT II coding region, and the nopaline synthase 3′ end. pCaMVINEO (2) is similar to pCaMVNEO except it contains the *Adh*1 intron 1 between the 35S promoter and NPT II coding region.

Bombardment of Cells

The suspension culture of *Z. mays* cv Black Mexican Sweet (BMS; ATCC No 54022) was maintained in MS media as previously described (8). The bombardment (15) and subsequent selection (9) of kanamycin-resistant clones was performed as detailed previously. Briefly, 2 mL of suspension culture (about 2×10^5 cells) was distributed over the surface of a filter paper (Whatman No. 4, 5.5 cm in diameter). The filter paper bearing the cells was placed over three layers of filter paper to which 2.5 mL of MS media had been added. The cells were then bombarded under a partial vacuum with tungsten particles (average diameter 1.2 μm) to which DNA was adsorbed using a calcium-spermidine precipitation procedure (15). Following bombardment, the cells were washed from the filter with 6 mL of MS medium into a 10 cm Petri dish. The Petri dish was sealed and then incubated in the dark at 26°C. After 2 d, 6 mL of fresh medium containing 300 μg of kanamycin per mL was added to each Petri dish and half of the volume was transferred to a fresh dish. After 1 week the culture was again diluted twofold with fresh medium containing 150 μg of kanamycin per mL and half the culture transferred to a new Petri dish. Subsequent additions of fresh medium containing kanamycin were made at 2 week intervals until the majority of cells in the culture clearly ceased to grow. This generally occurred after 4 to 6 weeks of selection. The cells were then transferred from the liquid medium to a membrane filter supported on a cellulose adsorbent pad (Gelman) that was on agarose-solidified MS medium containing 100 μg/mL kanamycin. Calli that developed on the filter were transferred to agarose-solidified medium supplemented with kanamycin after they had reached a diameter of about 0.5 cm.

GUS Assays

The presence of the GUS enzyme in the transformants was visualized using a histochemical reaction (11). A small amount of a kanamycin-resistant callus was placed into the well of a microtiter dish containing 200 μL of GUS assay solution (5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid [X-

gluc; Research Organics], 2 mM; potassium ferricyanide, 0.05 mM; potassium ferrocyanide, 0.05 mM; Triton X-100, 0.1% [v/v]; sodium phosphate buffer, 0.1 M, pH 7.0. After 12 h the calli were observed for the development of a blue color indicative of GUS expression (11). Levels of GUS activity were determined using the fluorometric enzyme assay with 4-methylumbelliferyl-β-D-glucuronide (MUG) as the substrate. The extraction buffer used was as described by Jefferson (11) except the concentration of Triton X-100 was reduced to 0.01%. Methyl umbelliferyl levels in the reaction mixture were determined after 15, 45, and 75 min of incubation. Protein in tissue extracts was quantified according to Bradford (1).

DNA Isolation and Hybridization Analysis

Genomic DNA was isolated as described (3). The isolated DNA (8 μg) was digested for 3 h using a three- to fourfold excess of restriction enzyme and electrophoresed in 0.8% agarose gels. The DNA was transferred (20) to nylon membranes (Hybond N, Amersham) which were then illuminated with UV light (254 nm) for 5 min. DNA fragments for probes were isolated from 1% low-melting agarose gel (SeaPlaque, FMC) and labeled with [^{32}P]dCTP by the random-hexamer primer method (7). For detection of the NPT II gene, genomic DNA was digested with *Bam*HI. The resulting blots were hybridized to the 1.0 kb NPT II fragment from *Bam*HI-digested pNGI. The presence of the GUS gene was detected by digesting genomic DNA with *Eco*RI and *Hin*dIII. The Southern blots were hybridized to a 1.8 kb GUS coding region derived from a *Sal*I to *Eco*RI digest of pRAJ260.

RESULTS

Maize cells from a suspension culture of BMS were bombarded with tungsten microprojectiles coated with plasmid DNA (pNGI) that contains both the NPT II and GUS genes (Fig. 1). The cells were allowed to grow for 2 d before transferring them to liquid medium containing kanamycin.

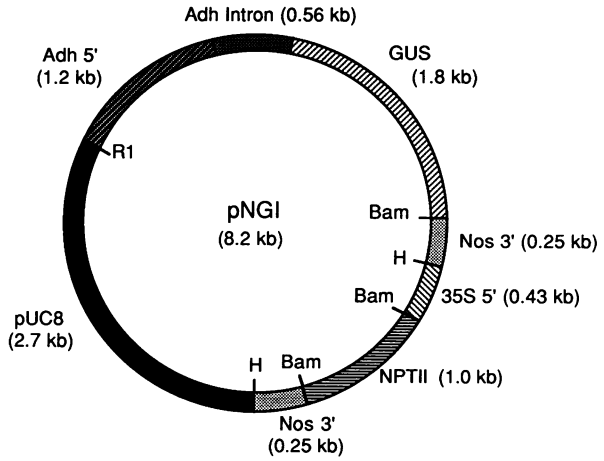

Figure 1. Structure of pNGI. H, *Hin*dIII; Bam, *Bam*HI; R1, *Eco*RI.

After 4 to 6 weeks in the liquid medium containing kanamycin, the microcalli were transferred to a membrane disc on solid medium containing kanamycin at 100 μg/mL (9). Calli resistant to kanamycin continue to proliferate under these selective conditions, while nontransformed cells stop dividing. Up to 117 kanamycin-resistant calli were recovered from a single bombardment of about 2×10^5 cells. For eight independent bombardments, the average number of kanamycin-resistant calli recovered was 56. Kanamycin-resistant calli were not recovered from unbombarded samples.

In one set of experiments a comparison was made between the number of kanamycin-resistant colonies recovered following bombardment with pCaMVINEO and pCaMVNEO, which is identical to pCaMVINEO but lacks the *Adh1* intron. The presence of the *Adh1* intron between the promoter and coding region has been shown to increase transient expression of the NPT II gene in maize (2). Similar numbers of kanamycin-resistant calli were recovered following bombardment with pCaMVINEO, pCaMVNEO, and pNGI. Apparently the level of expression of the gene that lacks the intron is sufficient to confer resistance to kanamycin. pCaMVINEO and pCaMVNEO were linearized by digestion with *Eco*RI prior to their adsorption to microprojectiles and acceleration into maize cells. We found that linearization did not increase the frequency of transformation in relation to supercoiled plasmid.

The kanamycin-resistant calli were treated with X-gluc and observed for the development of the blue color indicative of GUS expression (11). Triton X-100 was present in the substrate mixture to permit X-gluc to pass through the cell membrane. All kanamycin-resistant isolates had at least some GUS-expressing cells while blue cells were not observed in unbombarded samples (Fig. 2). The staining pattern of the calli ranged from virtually all of the cells turning blue in some samples to those samples with only a few blue cells (Fig. 2). Samples that strongly expressed GUS often exhibited a 'patchy' distribution of GUS expression with densely stained aggregates of cells interspersed with aggregates that were only faintly blue. The levels of GUS expression in the kanamycin-resistant calli as determined with X-gluc are expressed qualitatively in Table I.

The variation in GUS expression may have been the result of differences in the ability of the substrate to diffuse into the detergent-treated calli. Therefore, GUS expression in extracts from kanamycin-resistant calli was analyzed quantitatively using the fluorometric substrate, MUG (Table I). Levels of GUS expression, as assayed with the fluorometric substrate, generally corresponded with the degree of staining observed with the histochemical substrate indicating that the low levels of GUS expression as detected by the histochemical assay were not caused by a lack of penetration of the substrate into the cells.

To verify the transformed nature of the kanamycin-resistant calli, hybridization analyses were performed to probe for the presence of the NPT II coding region. Genomic DNA from the calli was digested with *Bam*HI which should release a 1.0 kb NPT II fragment from the transforming pNGI plasmid DNA (see Fig. 1). The Southern blot containing the transferred genomic DNA was hybridized with radioactive probe

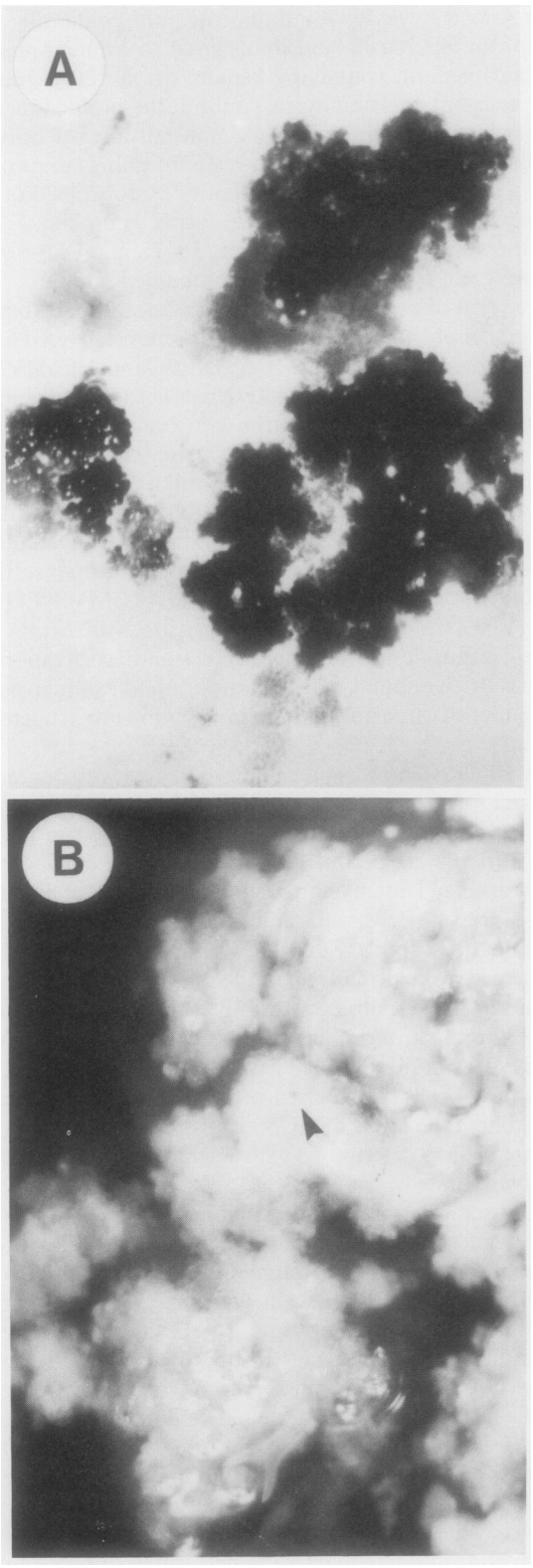

Figure 2. GUS expression in kanamycin-resistant calli that were recovered from cells bombarded with pNGI. The tissue was treated with the histochemical substrate X-gluc. A, An isolate that expressed high levels of GUS; B, an isolate that expressed little GUS activity. The arrow points to one of the few blue cells in this isolate.

Table I. *GUS Expression in Kanamycin-Resistant Calli as Determined Qualitatively by Histochemical Staining Using X-gluc or Quantitatively by an Enzyme Assay Using MUG as the Substrate*

Also given is the number of intact copies of the GUS gene present in the various isolates.

Sample	GUS Staining (qualitative estimate[a])	GUS Activity × 10^{-3}	GUS Copy No.
		pmol MU/μg protein/min	
1-1	3	104	8
1-4	4	172	2
1-5	2	18	2
1-9	2	50	8
1-14	3	98	1
1-15	2	17	10
2-1	1	5	10
2-2	1	8	5
2-3	2	14	1
2-5	2	12	1
2-8	4	94	8

[a] Calli were qualitatively evaluated for their level of GUS expression following treatment with X-gluc with a value of four representing isolates that uniformly and densely stained blue and a value of 1 representing weak expression with only a few blue cells present in the isolate. Calli that had GUS staining values of 2 or 3 had some densely stained aggregates of cells interspersed among aggregates that exhibited little or no staining. Untransformed BMS calli had no detectable GUS activity when analyzed with either X-gluc or MUG.

prepared from the NPT II coding region. All of the kanamycin-resistant calli tested yielded a 1.0 kg band that comigrated with the 1.0 kb NPT II fragment from *Bam*HI-digested pNGI DNA (Fig. 3A). Copy number reconstructions indicated that most of the transformants contained from 1 to 8 intact copies of the NPT II gene per haploid genome. Occasionally isolates with less than 1 copy of the NPT II gene were recovered (for example, isolate 2-5, Fig. 3A) indicating that some of the kanamycin-resistant calli could be chimeras containing non-transformed cells. Most of the calli analyzed had additional rearranged copies integrated into the genome. Unbombarded tissue did not contain DNA that hybridized to the NPT II probe (Fig. 3A, lane pNGI-0).

Southern blot analyses were also performed to determine the copy number and structure of the GUS gene in the kanamycin-resistant calli. Digestion of pNGI with *Eco*RI and *Hin*dIII releases the 3.8 kb GUS gene (Fig. 1). Southern blots of genomic DNA digested with *Eco*RI and *Hin*dIII were hybridized to a 1.8 kb fragment of the GUS coding region. All of the kanamycin-resistant calli tested contained both intact and rearranged copies of the GUS gene (Fig. 3B) with the number of rearranged sequences often exceeding the number of intact copies. The number of intact copies of the GUS gene varied from 1 to 10 in the transformed calli. Unbombarded calli did not contain DNA that hybridized to the GUS probe. The GUS DNA is maintained in the calli after 1 year of growth in culture as indicated by GUS expression using X-gluc as the substrate.

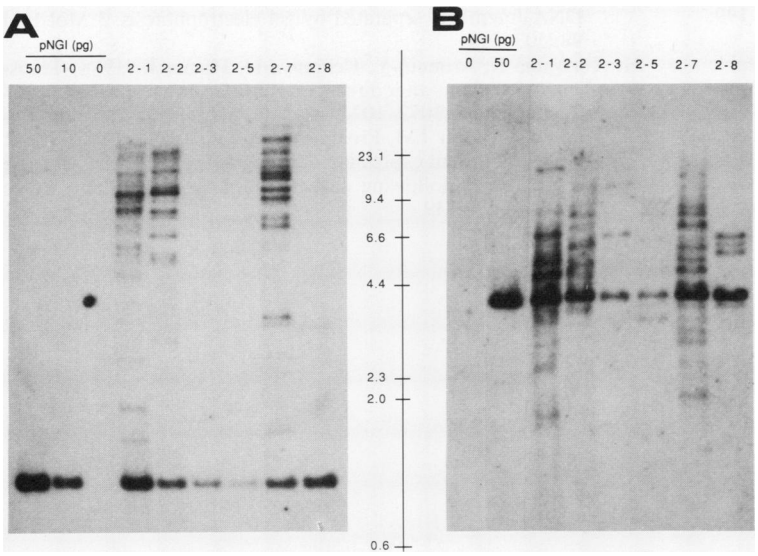

Figure 3. Analysis of the NPT II and GUS gene structures in maize calli transformed with pNGI DNA. DNA was isolated from kanamycin-resistant calli 12 weeks after bombardment. The DNA was digested with either *Bam*HI (A) or *Eco*RI and *Hind*III (B), separated by electrophoresis in a 0.8% agarose gel and transferred to nylon membranes. A radioactive NPT II (A) or GUS (B) probe was hybridized to the DNA on the membrane and the hybridizing sequences visualized by autoradiography. The position of *Hind*III digested size markers (kb) is indicated between the two panels. The lanes designated pNGI contain the indicated amount (pg) of pNGI DNA digested with either *Bam*HI (A) or *Eco*RI and *Hind*III (B) as well as 8 μg of similarly digested DNA from untransformed maize callus. Since the haploid genome of maize is 5×10^9 bp, 10 and 50 pg of pNGI represents 0.8 and 4 gene copies, respectively. Digestion of pNGI with *Bam*HI releases a 1.0 kb hybridizing fragment while digestion with *Eco*RI and *Hind*III releases a 3.8 kb GUS gene hybridizing fragment.

DISCUSSION

Stably transformed calli of maize can readily be recovered following bombardment of intact cells with DNA-bearing microprojectiles. The frequency of transformation found in this study (about 1 in 5000 of the cells treated with microprojectiles) was similar to that previously found for microprojectile-mediated transformation of intact tobacco (16) and soybean (4) cells. The frequency of transformation by microprojectiles is comparable to that observed for methods used for the transformation of plant protoplasts such as electroporation (9) or PEG-mediated delivery (17). Recently, as a result of improved methods for the culture of protoplasts, transformed plants of several cereal species have been produced by gene delivery to protoplasts. For example, transgenic rice plants have been obtained by electroporation-mediated delivery of DNA to protoplasts (21). Transgenic maize plants have also been recovered by gene transfer to protoplasts, but the regenerated plants were sterile (19). In spite of this progress, regeneration of most cereal species from protoplasts is still difficult and therefore the pursuit of alternative approaches for plant transformation is necessary. Microprojectile-mediated transfer represents a means for delivering DNA into intact cells within tissues. Therefore, with the appropriate selectable or screenable markers, it should be possible to recover transformed calli from bombarded tissues or cultures of maize and other cereals that have the potential to develop into whole plants. Improvements in transformation efficiencies as a result of further refinements in the design of microprojectiles and acceleration devices will aid in the recovery of transformed plants of recalcitrant species.

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