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Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process

(gene transfer/transgenic plants/microprojectiles/particle gun/*Nicotiana tabacum*)

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ABSTRACT **We show that the genetic transformation of *Nicotiana tabacum* can be achieved by bombarding intact cells and tissues with DNA-coated particles. Leaves or suspension culture cells were treated with tungsten microprojectiles carrying plasmid DNA containing a neomycin phosphotransferase gene. Callus harboring the foreign gene was recovered from the bombarded tissue by selection on medium containing kanamycin. Kanamycin-resistant plants have subsequently been regenerated from the callus derived from leaves. Transient expression of an introduced β-glucuronidase gene was used to assess the efficiency of DNA delivery by microprojectiles. The frequency of cells that were stably transformed with the neomycin phosphotransferase gene was a few percent of the cells that transiently expressed the β-glucuronidase gene. These results show that gene transfer by high-velocity microprojectiles is a rapid and direct means for transforming intact plant cells and tissues that eliminates the need for production of protoplasts or infection by *Agrobacterium*.**

Recombinant DNA technologies will be an important tool for the genetic manipulation and improvement of crop species (1, 2). The application of this technology is dependent on the availability of efficient systems for the transfer of foreign genetic material into cells capable of giving rise to fertile plants. For this reason a wide array of techniques has been developed for DNA transfer into plant cells (3). Genetic transformation can be achieved with *Agrobacterium*-based vectors (4) or by delivery of DNA to protoplasts by means of electroporation (5–7) or direct DNA uptake (8). Unfortunately, the host range of *Agrobacterium* is relatively limited and regeneration of whole plants from protoplasts is far from routine for most species. Microinjection into cells (9) and organized structures such as microspore-derived embryoids (10) may circumvent some of the problems of existing systems but the technique is technically demanding and is limited to cells with thin cell walls. Despite the variety of specialized techniques that are available, there is a need for a rapid and generally applicable approach that can deliver DNA directly to intact and regenerable cells and that therefore avoids the host range and tissue culture restrictions of existing delivery systems.

Gene transfer by bombardment with DNA-coated microprojectiles has the potential to be a rapid and simple means for transforming intact cells and tissues (11, 12). The transient expression of genes coding for the production of chloramphenicol acetyltransferase or β-glucuronidase (GUS) has been used to study the microprojectile-mediated delivery to epidermal tissue of *Allium cepa* (12), scutellar tissue of *Zea mays* (13), and cells from suspension culture of several species (13–15). In this report we employ the particle bombardment process to introduce a selectable marker gene [neomycin phosphotransferase (NPTII)] to leaves and cells from suspension culture of *Nicotiana tabacum*. We also compare the number of clones that stably express NPTII to the number of cells that transiently express an independently introduced GUS gene.

MATERIALS AND METHODS

Plasmids. Plasmid pJJ3431 [6.9 kilobases (kb)], which was provided by J. D. G. Jones (Advanced Genetic Sciences, Albany, CA), contains a GUS coding region (16), the p35S(J): Cab22L promoter at its 5′ end (17), and the octopine synthase poly(A) signal at its 3′ end (18). The chimeric gene was cloned in pUC118 (19). Plasmid pLGVneo1103, which contains the NPTII gene from Tn5, the promoter from the nopaline synthase gene, and the poly(A) site from the octopine synthase gene, has been described (20) and was provided by Mark Van Montagu (Rijksaniversiteit Ghent, Belgium).

Plant Materials and Their Bombardment. Suspension cultures of *N. tabacum* (line XD) were maintained in 250-ml flasks containing 50 ml of RMS medium comprised of MS salts (21), naphthaleneacetic acid (1 mg/liter), N^6-benzyladenine (0.1 mg/liter), dichlorophenoxyacetic acid (0.1 mg/liter), Mes (3 mM), B_5 vitamins (22), and sucrose (30 g/liter) at pH 5.8. Bombardment of cells from suspension cultures of tobacco was performed essentially as described for *Z. mays* suspension cultures (13). About 2 ml of suspension culture was evenly distributed over the surface of a Whatman no. 4 filter paper (5.5 cm in diameter) with the aid of a Buchner funnel. The filter paper with cells was placed on a Petri dish (7.5 cm in diameter) containing RMS medium solidified with agarose (7 g/liter). The cells were then bombarded in a partial vacuum with tungsten particles (average diameter of 1.2 μm) to which plasmid DNA was previously adsorbed. The device (particle gun) used to accelerate these microprojectiles has been detailed in previous papers (11, 12, 14).

For bombardment of leaf tissue of *N. tabacum* cv. Petit Havana, plants were aseptically grown from surface-sterilized seed on agar supplemented with MS salts and sucrose (30 g/liter). Leaves (3–5 cm in length) were excised from plants and placed abaxial side up on RMO medium in a Petri dish. The RMO medium consists of MS salts, indoleacetic acid (2.0 mg/liter), N^6-benzyladenine (0.5 mg/liter), thiamine (1.0 mg/liter), inositol (100 mg/liter), agarose (7 g/liter) at pH 5.8, and sucrose (30 g/liter).

Staining Tissue for Detection of GUS Expression. Following bombardment of suspension cultures, the cells were incu-

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Abbreviations: GUS, β-glucuronidase: X-GlcU, 5-bromo-4-chloro-3-indoyl β-D-glucuronic acid; NPTII, neomycin phosphotransferase.

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bated for 48 hr at 28°C. The filter paper was then transferred to a Petri dish onto which 100 μl of the GUS substrate mixture was previously spotted so that, upon transfer, the filter paper adsorbed the substrate evenly over its entire area. The substrate mixture consisted of potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), 5-bromo-4-chloro-3-indoyl β-D-glucuronic acid (X-GlcU; 0.3% wt/vol), and Triton X-100 (0.3%, vol/vol). The cells were then incubated for 24 hr at 28°C. GUS-expressing cells were detected microscopically by the distinct blue color that developed within their interior as a result of the enzymatic cleavage of X-GlcU. Occasionally, two or three adjacent cells expressed the GUS enzyme. Since it was not clear if each of these cells received the GUS gene, these were counted as a single cell event. Leaves were incubated for 24 hr with the substrate 2 days after bombardment by placing the tissue in a Petri dish with 5 ml of the X-GlcU substrate mixture.

Selection of Stable Transformants. Cells from suspension culture were incubated for 7 days at 28°C following bombardment. The cells were then suspended in RMS medium and plated on agar-solidified RMS medium containing kanamycin (100 μg/ml). Kanamycin-resistant calli were identified after 3 weeks of incubation. A fraction (10%) of the suspension was plated on medium lacking kanamycin to determine the number of colony-forming units.

Seven days after bombardment, leaf tissue was transferred to RMO medium containing kanamycin (100 μg/ml) and kanamycin-resistant calli were selected after 3 weeks. Plants were regenerated from the transformed leaf-derived callus on medium lacking kanamycin (23). The regenerated plants were subsequently rooted in medium containing 50 μg of kanamycin per ml to verify their kanamycin resistance.

Southern Hybridization Analysis. Genomic DNA was isolated and then purified on CsCl density gradients (24). *Bst*EII- and *Bcl* I-digested DNA (5 μg per lane) was probed with a riboprobe synthesized with SP6 RNA polymerase (Promega Biotec, Madison, WI) using plasmid pJJ1124, a SP64 clone containing the *Bgl* II–*Sma* I fragment from transposon Tn*5* (25).

RESULTS

Monitoring DNA Delivery by GUS Expression. The ability of microprojectiles to penetrate and deliver foreign DNA to *N. tabacum* cells was evaluated by bombardment with tungsten particles coated with plasmid pJJ3431, which contains a chimeric GUS gene (16). Suspension culture cells were dispersed over the surface of a filter (Fig. 1*A*), bombarded, and incubated to allow expression of the GUS gene. The activity of the GUS enzyme was detected within individual cells 48 hr after bombardment by treating the sample with the histochemical substrate, X-GlcU. Therefore, the number and spatial distribution of cells that received the foreign DNA were determined with this transient assay of GUS expression. Following treatment with X-GlcU many cells developed a distinctive blue color indicative of GUS expression (Fig. 1*C*). An average of 143 (SEM = 80.5) GUS-expressing cells was observed per bombardment (Fig. 2*A*). The spatial distribution of GUS-expressing cells was determined by counting blue cells in different sectors of the filter paper (Fig. 3). Typically, the large number of microprojectiles that impacts the central zone of the filter kills or dislodges cells. Therefore, relatively few blue cells are observed in the central area of the filter. This central zone is surrounded by a ring-shaped area that contains the majority of cells exhibiting GUS activity beyond which few blue cells are observed. GUS-expressing cells were found in an area ranging from 4 to 9 cm^2 (or over ≈30–60% of the total surface area of the filter).

GUS expression in bombarded leaves was observed in epidermal, mesophyll, and occasionally trichome cells (Fig. 1 *E* and *D*). An average of 83.3 (SEM = 40.3) GUS-expressing cells appeared per leaf (Fig. 2*A*). The fact that GUS expression was observed in the mesophyll indicates that microprojectiles can carry DNA through at least one cell layer of leaf tissue. In leaves, the distribution of cells expressing the GUS enzyme followed a similar pattern to that observed for the suspension culture cells (data not shown), with a necrotic spot appearing in the central zone (Fig. 1*B*). Leaves or cells from suspension culture that were bombarded with microprojectiles prepared without DNA or with microprojectiles coated with plasmid (pUC118) that did not contain the GUS gene did not exhibit GUS-expressing cells.

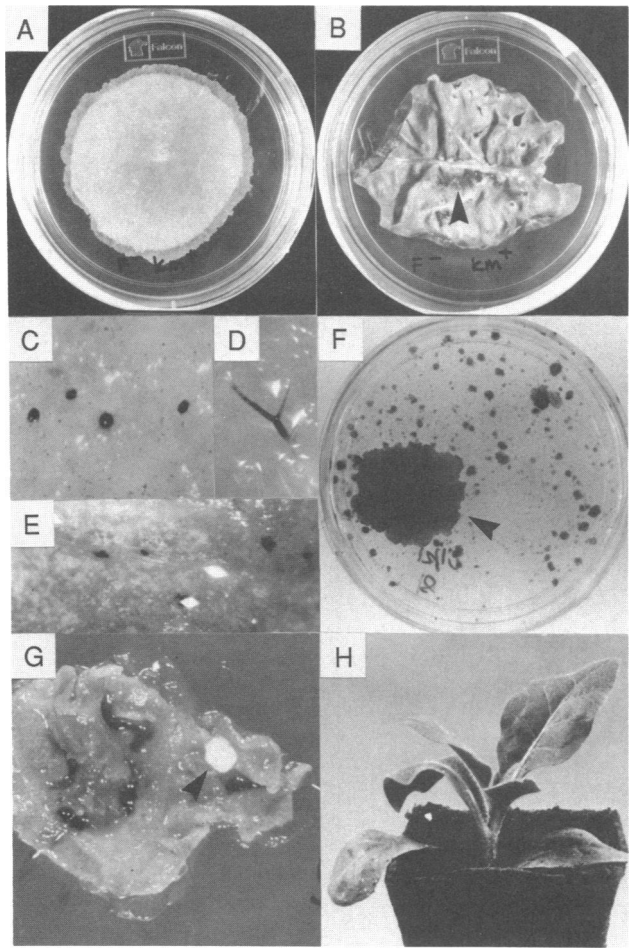

FIG. 1. Gene delivery to *N. tabacum* cells with DNA-coated microprojectiles as analyzed by transient expression of GUS or stable transformation with an NPTII gene. (*A*) Suspension cells on the filter paper support 1 week after bombardment with microprojectiles. (*B*) A leaf 1 week after bombardment with microprojectiles. The necrotic zone in the center of the leaf is caused by the impact of the tungsten-DNA preparation. (*C*) GUS-expressing cells from a bombarded suspension culture. (*D* and *E*) Cells of a trichome (*D*) and the epidermis (*E*) of a bombarded leaf expressing GUS activity. (*F* and *G*) Kanamycin-resistant calli derived from a bombarded suspension culture (*F*) and leaf (*G*). (*H*) A kanamycin-resistant plant regenerated from callus derived from bombarded leaf tissue. (*C–E*, × 9.0.)

Selection of Stable Transformants. To determine if stable transformants could be obtained from leaves or suspension cultures, tissue was bombarded with microprojectiles coated with the plasmid pLGVneo1103 (20) that contains an NPTII gene. Stable transformants (Fig. 1 *F* and *G*) were then selected by growth on kanamycin-containing medium. An average of 6.9 kanamycin-resistant clones were recovered per bombarded sample of suspension culture cells (Fig. 2*B*). An estimation of the frequency of transformation was made by comparing the total number of colonies that developed on

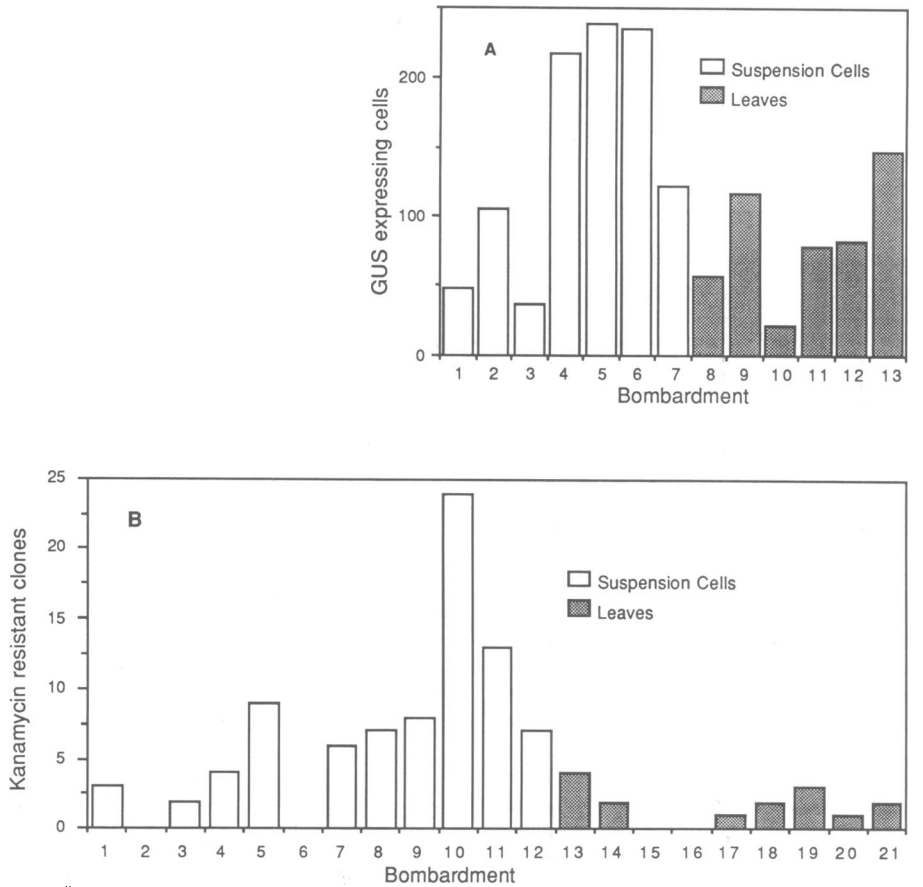

FIG. 2. The number of GUS-expressing cells (*A*) and kanamycin-resistant clones (*B*) recovered after a single bombardment. Sample numbers are given in an increasing order on the abscissa.

medium lacking kanamycin to the number of kanamycin-resistant clones. By this criterion, the frequency of stable transformation was $\approx 5 \times 10^{-4}$. In relation to the number of cells that transiently expressed GUS, the frequency of stable transformation was 4.8×10^{-2} (stable-to-transient ratio).

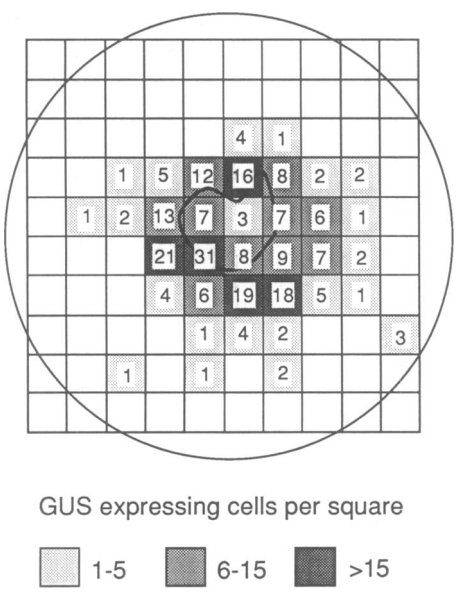

FIG. 3. Representation of the distribution of GUS-expressing suspension cells on the filter paper on which they were bombarded and subsequently treated with the X-GlcU substrate. The contour in the center represents the area from which many of the cells were dislodged during bombardment. The number of GUS-expressing cells in each division (0.25 cm^2) was determined with the aid of a dissecting microscope.

Stable transformants have also been selected by growth of kanamycin-resistant calli from bombarded leaves (Fig. 1*G*). Leaves yielded an average of 2.3 kanamycin-resistant calli per bombardment (Fig. 2*B*). In relation to the number of cells expressing GUS, the stable-to-transient ratio was found to be 1.9×10^{-2}. Plants have subsequently been regenerated from the kanamycin-resistant calli derived from bombarded leaf tissue (Fig. 1*H*). The transgenic nature of the plants was confirmed by using a rooting assay (26). The plants regenerated from the kanamycin-resistant calli readily rooted in the selective medium. Inheritance of kanamycin resistance for one of these plants was determined by germinating seed on medium containing kanamycin (200 μg/ml). Resistant seedlings on this medium are green, whereas sensitive seedlings are white (27). Segregation in the progeny of the self-pollinated plant indicated that the plant was heterozygous and carried the transforming DNA at two independent loci (96 resistant, 7 sensitive; 15:1 ratio).

The stability of the introduced NPTII gene in kanamycin-resistant calli was verified by determining the ability of the clone to grow on kanamycin-containing medium over at least five subcultures on the selective medium. To further verify that the kanamycin resistance of these clones was stably maintained, they were grown on medium lacking kanamycin for one subculture and then replated on kanamycin-containing medium. All kanamycin-resistant clones recovered from bombarded tissue maintained their kanamycin resistance over this subculturing routine. Kanamycin-resistant clones were not recovered from leaves or cells from suspension culture that were bombarded with microprojectiles

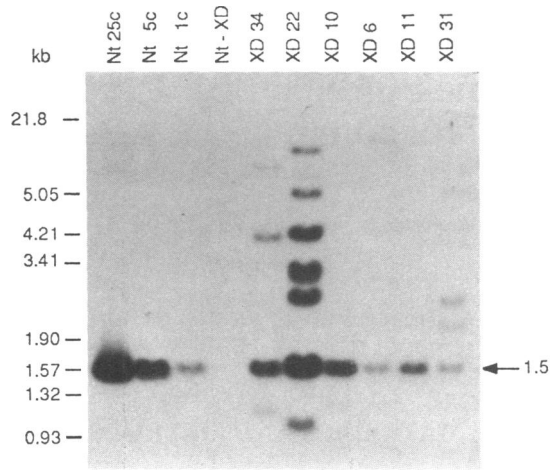

FIG. 4. Southern analysis of kanamycin-resistant calli from suspension culture cells. Lane Nt-XD, DNA from nontransformed *N. tabacum* XD cells. Lanes XD34 to XD31, DNA from kanamycin-resistant calli. Lanes Nt25c, Nt5c, and Nt1c, genomic DNA mixed with pLGVneo1103 to reconstruct the integration of 25, 5, or 1 copy of the NPTII gene per diploid nucleus.

prepared without DNA or with microprojectiles coated with plasmid (pUC118) lacking the NPTII gene.

Southern Hybridization Analysis of Kanamycin-Resistant Tissue. Southern analysis was performed on kanamycin-resistant calli from suspension culture cells (23 clones) and leaves (2 clones) using an NPTII riboprobe. Every kanamycin-resistant clone tested showed a hybridizing band of the predicted size (1.5 kb), whereas nonbombarded tissue lacked this band (Fig. 4). The number of intact NPTII gene copies present in the kanamycin-resistant clones varied from 1 to about 20 per diploid nucleus. Hybridization also revealed rearranged NPTII sequences in fragments that were larger or smaller than 1.5 kb. Such rearrangements are often observed in transformants obtained by other methods (5, 20, 27).

DISCUSSION

These results clearly show that microprojectiles can deliver DNA to cells in a form that can be stably integrated into the *N. tabacum* genome. Apparently the DNA carried into the cell desorbs from the surface of the microprojectile and is transported by some passive or active mechanism to the nucleus. Alternatively, the microprojectile may penetrate the nuclear membrane and deposit DNA directly within the nucleus. Introduction of the DNA in a precipitated form may protect it from intracellular nucleases, thus making it possible for small quantities to persist in the cytoplasm or potentially the vacuole for a time sufficient to allow uptake by the nucleus.

Previous studies that focused on the transient expression of foreign genes have permitted the optimization of parameters (such as those related to microprojectile momentum and dispersal) that influence DNA delivery by particle bombardment (13–15). Obtaining relatively high levels of transient expression has proven essential for recovery of stable transformants since only a fraction of the cells that transiently express the foreign gene incorporate it. Our data suggest that about 2–5% of the cells that transiently express a foreign gene stably integrate it.

Recent experiments using bombardment parameters similar to those described in this report have allowed the recovery of stably transformed callus from suspension culture cells of maize (unpublished data). Therefore, genetic transformation by the particle bombardment process should be widely applicable to many types of plant cells and should extend the range of plants that can be manipulated by recombinant DNA techniques.

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