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Genetics

Expression of genes transferred into monocot and dicot plant cells by electroporation

(maize/transfection/transient gene expression/chloramphenicol acetyltransferase)

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ABSTRACT **We have developed a general method for electrically introducing DNA into plant cells. Gene transfer occurs when a high-voltage electric pulse is applied to a solution containing protoplasts and DNA. Carrot protoplasts were used as a model system to optimize gene-transfer efficiency, which was measured 24–48 hr after electroporation by the amount of chloramphenicol acetyltransferase activity resulting from the expression of the introduced chimeric plasmids. Gene-transfer efficiency increased with the DNA concentration and was affected by the amplitude and duration of the electric pulse as well as by the composition of the electroporation medium. Our optimized gene-transfer conditions were effective when applied to tobacco and maize protoplasts, demonstrating that the method is applicable to both monocot and dicot protoplasts.**

Progress in the study of plant gene expression has been restricted by the limited availability of gene-transfer systems (1). Recent work has demonstrated DNA transformation of plant cells (2–8), but these experiments are routine only for *Agrobacterium tumefaciens* Ti-plasmid-mediated DNA transfer (4–8). Unfortunately, *A. tumefaciens* DNA transfer has been limited to dicots (8, 9) and a few non-cereal monocots (10, 11). Techniques are needed to extend gene transfer to other plant species, particularly cereal crop plants. The transformation of tobacco protoplasts by treatment with DNA and polyethylene glycol has been reported (3, 12). However, polyethylene glycol is often detrimental to protoplast viability (13, 14). In seeking alternative strategies, we have investigated the response of plant protoplasts to the electroporation gene-transfer technique used for animal cells (15, 16).

Electrical impulses of high field strength reversibly permeabilize biomembranes (17) and thus have had two important applications: the introduction of macromolecules into cells (15, 16) and the induction of cell fusions (18, 19). Conditions required to electrically transfer DNA into mouse cells have been reported (15), and recently a simple apparatus was used to transfer DNA into mouse and human cells by electroporation (16). We have used the reported conditions and apparatus as a starting point for the electroporation of DNA into plant protoplasts. We chose carrot protoplasts as a model system because of our concurrent work utilizing *A. tumefaciens* to transfer various genes into carrot cells and because viable carrot protoplasts are easily isolated in high yields (20).

We monitored the success of electroporation-mediated gene transfer into carrot protoplasts by a transient gene expression system similar to that used in mammalian cells (21–23) and petunia protoplasts (24). With this approach, a gene can be transferred into a cell and its expression analyzed within hours. We have found that the electrical introduction of genes into plant protoplasts provides the basis for such a gene expression system and is applicable to cells of both monocots and dicots.

MATERIALS AND METHODS

Plasmids and Plant Materials. pNOSCAT consists of the 3.3-kilobase (kb) *Hin*dIII fragment of the nopaline synthase (NOS) gene (25) inserted into a pBR322 derivative (nucleotides 30–2067 have been deleted). A BAL-31-generated deletion endpoint exists at approximately *NOS* nucleotide 1945 (see ref. 25 for *NOS* nucleotide numbers). This deletion removes the *NOS* ATG translation initiation site but retains the 5′ untranslated region and promoter sequences. *NOS* nucleotides from 1946 to the *Bam*HI site at nucleotide 1127 have been replaced by the 773-nucleotide *Taq* I fragment of the Tn*9* chloramphenicol acetyltransferase (CAT) gene (26) with the *Taq* I ends changed to *Bam*HI and *Bgl* II cohesive ends. pCaMVCAT is identical to pNOSCAT except that the *NOS* promoter has been replaced by the cauliflower mosaic virus (CaMV) *35S* promoter (nucleotides 7017–7437; see ref. 27 for CaMV nucleotide numbers). *Daucus carota* cell line W001C (28), *Nicotiana tabacum* cv. Wisconsin 38, and *Zea mays* cv. Black Mexican Sweet suspension cultures were generously provided by J. Ecker (Stanford University), M. Christianson (Zoecon, Palo Alto, CA), and A. Gould (Pfizer), respectively.

Protoplast Isolation. Protoplasts were isolated from rapidly growing carrot suspension cells by incubating 12 ml of packed cells in 80 ml of 1% cellulase (Cellulysin, Calbiochem-Behring) and 0.5% hemicellulase (Rhozyme, Genencor) in protoplast isolation medium (PIM: 0.2 M mannitol/50 mM $CaCl_2$/10 mM sodium acetate, pH 5.8) for 2 hr at 26°C. Protoplasts were separated from large debris by filtration through 60-μm-mesh nylon screen, centrifuged at 200 × *g* for 4 min, and washed once either in the same solution without enzymes or in Hepes-buffered saline (10 mM Hepes, pH 7.2/150 mM NaCl/5 mM $CaCl_2$) containing 0.2 M mannitol.

Electroporation. Each sample of 3×10^6 protoplasts was resuspended in 1 ml of the desired concentration of phosphate-buffered saline (1× contains 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 1.15 g of Na_2HPO_4 per liter) or of Hepes-buffered saline, containing 0.2 M mannitol with or without plasmid DNA (10 μg/ml). Electroporation then was carried out according to published procedures (16). The electrical pulse was supplied by an ISCO model 494 power supply set at 2000 V. The solution containing the protoplasts was held at 0°C for 10 min after the electric pulse. The protoplasts then were diluted into MS medium (29) containing 2% sucrose, 0.3 M mannitol, and 2,4-dichlorophenoxyacetic acid (0.1 μg/ml) and incubated at 26°C in a 10-cm plastic Petri dish. Protoplasts subjected to electroporation and then assayed in duplicate produced CAT activities that varied by

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Abbreviations: NOS, nopaline synthase; CAT, chloramphenicol acetyltransferase; CaMV, cauliflower mosaic virus; kb, kilobase(s).

<50%. Protoplast viability was determined by Evans blue exclusion (30).

CAT Assays. Extracts were prepared by sedimenting each sample of electroporated protoplasts at 200 × *g* for 5 min, resuspending them in 0.5 ml of 0.25 M Tris Cl (pH 7.8), sonicating to disrupt the cell membrane, and removing particulate debris by centrifugation in a microcentrifuge for 3 min. The supernatant was heated at 65°C for 10 min to inactivate plant substances that inhibit CAT activity, cooled to room temperature, and assayed as previously reported (23). The reaction products were extracted with ethyl acetate, separated by thin-layer chromatography, and autoradiographed. CAT activity was quantified by scanning the autoradiogram with a densitometer. Activity is expressed relative to the CAT activity of a protoplast sample that did not receive an electric pulse; this background was generally equal to 10^{-4} the total radioactivity present in a reaction.

RESULTS

Two promoters capable of expression in the recipient carrot cells were used to monitor DNA transfer by electroporation. Both were fused to the bacterial gene coding for CAT, an enzyme with a convenient and sensitive assay (23, 31). The *NOS* promoter from the Ti plasmid of *Agrobacterium* (8) functions in most dicots tested, including carrot (5). A chimeric gene consisting of the *NOS* gene 5′ and 3′ control regions and the bacterial CAT coding region is also expressed in a number of plants (6). The CaMV *35S* promoter is active in many dicots (27, 32), and we found it to be active in carrot cells after introduction by *A. tumefaciens*-mediated DNA transfer (unpublished results). The *NOS* and CaMV promoters were ligated to the CAT coding region and the *NOS* gene 3′ end to form the chimeric genes NOSCAT and CaMVCAT, respectively, which were inserted into a pBR322 derivative to form the plasmids pNOSCAT and pCaMVCAT (Fig. 1).

Carrot protoplasts were electroporated in the presence of pNOSCAT and pCaMVCAT as described in *Materials and Methods*. Forty-eight hours later, extracts were prepared from the electroporated protoplasts and were assayed for CAT activity. An autoradiogram of a chromatogram from such an assay is shown in Fig. 2. Two monoacetylated forms and a single diacetylated form of [^{14}C]chloramphenicol are produced by the CAT activity (23, 31) in extracts of *Escherichia coli* carrying the CAT gene of pBR325 (34). Neither the addition of pNOSCAT DNA to carrot protoplasts without an electric pulse nor an electric pulse applied to protoplasts without the addition of pNOSCAT DNA resulted in any detectable CAT activity (Fig. 2, lanes d and c, respectively). However, the combination of protoplasts plus pNOSCAT or pCaMVCAT DNA and an electric pulse produced a 50-fold and a 145-fold increase in CAT activity, respectively (Fig. 2, lanes e and f). pNOSCAT DNA was not contaminated by active CAT enzyme, because no detectable CAT activity was produced when DNA was added directly to the CAT assay (Fig. 2, lane b). As an additional control, aliquots of electroporated cells were incubated on nutrient plates and showed no evidence of microbial contamination. Therefore, an electrical pulse introduces DNA into carrot protoplasts in amounts adequate for detection of the transferred gene's expression.

The effects of DNA and salt concentrations and the period of incubation following electroporation on the efficiency of gene transfer were examined. The amount of CAT activity produced was proportional to DNA concentration up to 40 μg/ml for supercoiled plasmid DNA, the highest DNA concentration tested (Fig. 3*A*). A time course of gene expression after electroporation showed that CAT expression could be detected as early as 3 hr and as late as 96 hr after electroporation, with maximum expression occurring between 24 and 48 hr (data not shown). The effect of phosphate-buffered saline concentration on protoplast survival and gene-transfer efficiency is shown in Fig. 3*B*. As the salt concentration is reduced, an increase in CAT activity is observed with a concomitant decline in protoplast viability. An optimal signal is observed at 0.6× phosphate-buffered saline, a 40-fold increase in CAT activity relative to that at 1× (Fig. 3*B*).

Monitoring the amplitude and duration of the electric pulse provided an explanation for the increase in CAT activity at reduced salt concentrations. Measurement of the electric pulses with an oscilloscope showed that although the steady output of the power supply was 2000 V, the pulse delivered to the cuvette was 112 V with 1× and 181 V with 0.6× phosphate-buffered saline (Fig. 4 *A* and *B*). Our interpretation of this result is that most of the voltage is dissipated in the internal resistance of the power supply when the electric pulse is delivered to the low-resistance solution in the cuvette. The higher resistance of the 0.6× phosphate-buffered saline solution absorbs a larger fraction of the 2000-V pulse than the lower resistance of the 1× phosphate-buffered saline solution.

The effect of pulse length and amplitude were examined in detail by means of the circuit shown in Fig. 4*D*. This circuit allows the pulse length and amplitude to be varied independent of the internal characteristics of the power supply and can generate an electric pulse similar to that obtained using the ISCO power supply directly (compare Fig. 4 *B* and *C*). When this capacitor-discharge circuit was used, the reduction of the electroporation solution from 1× to 0.6× phosphate-buffered saline produced only a 2-fold increase in CAT activity (data not shown); because there is increased cell

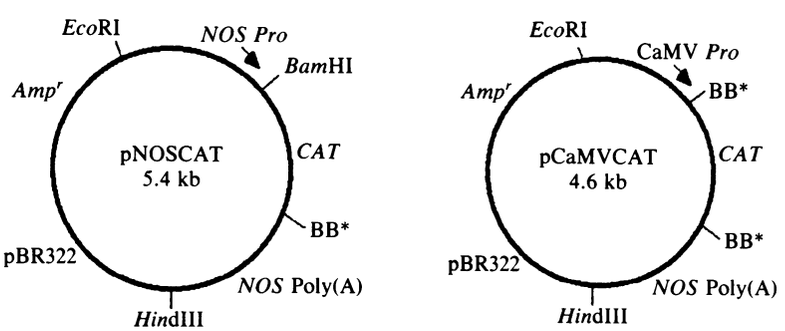

FIG. 1. Diagram of pNOSCAT and pCaMVCAT. The source of each DNA fragment and the restriction sites used to construct each plasmid are shown. A 2.3-kb derivative of pBR322 (33) containing the origin of replication and gene for ampicillin resistance (*Amp*^{r}) was used as the plasmid vector. The arrows are located near the predicted RNA 5′ ends and point towards the gene 3′ end. BB*, *Bam*HI and *Bgl* II cohesive ends were joined to make these junctions with neither restriction site retained. *Pro*, promoter.

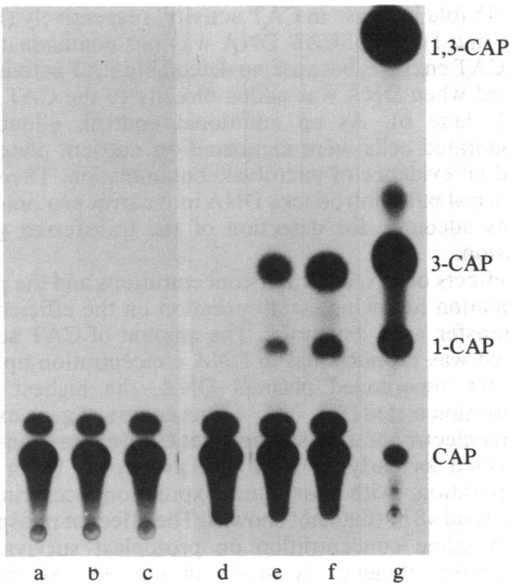

FIG. 2. Expression of CAT genes electroporated into carrot protoplasts. The CAT activities of extracts of protoplasts electroporated with an electric pulse from the ISCO power supply were measured by incubating the heat-treated lysates with 400 μM acetyl coenzyme A and 0.6 μCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol; the reaction products were separated by thin-layer chromatography and detected by autoradiography. The positions of unreacted chloramphenicol (CAP) as well as 1-acetylchloramphenicol, 3-acetylchloramphenicol, and 1,3-diacetylchloramphenicol are indicated. Lanes: a, unreacted chloramphenicol substrate; b, 10 μg of pNOSCAT DNA was added directly to the enzyme assay; c, an electric pulse was delivered to protoplasts without plasmid DNA present; d, protoplasts with 10 μg of pNOSCAT DNA present but without an electric pulse delivered; e, an electric pulse was applied to protoplasts with 10 μg of pNOSCAT DNA present; f, an electric pulse was applied to protoplasts with 10 μg of pCaMVCAT DNA present; g, enzymatic products from an extract of an *E. coli* strain containing pBR325, which carries a *CAT* gene (34).

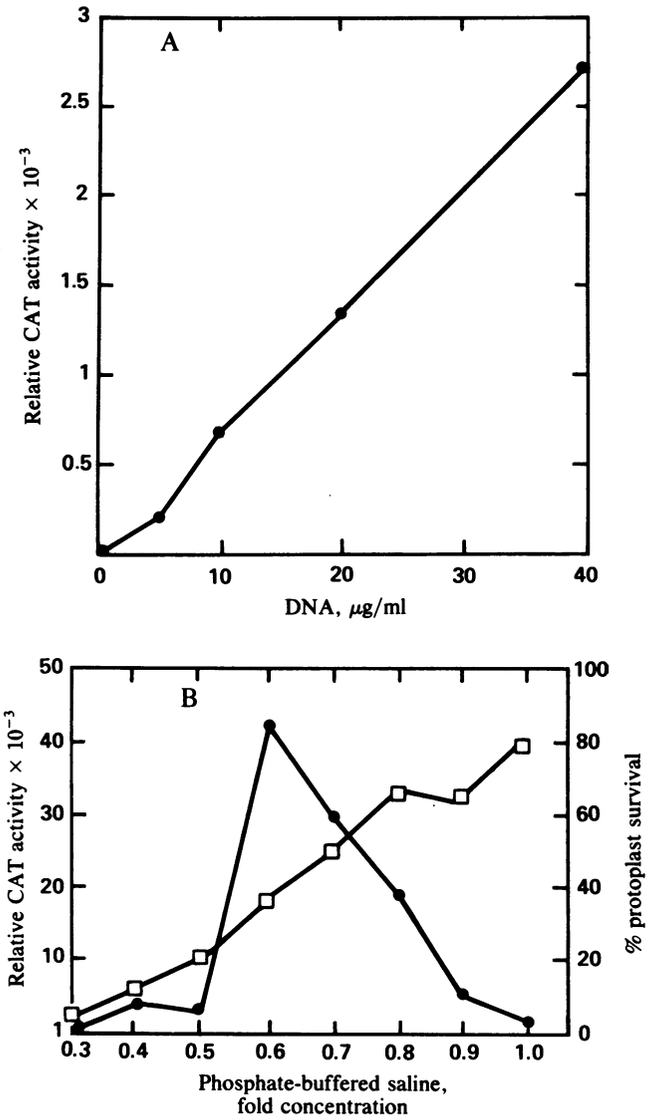

FIG. 3. Dependence of *NOS-CAT* gene expression on the DNA and phosphate-buffered saline concentration used during electroporation. Carrot protoplasts were treated with an electric pulse (from the ISCO power supply set at 2000 V) in a solution containing phosphate-buffered saline, 0.2 M mannitol, and supercoiled pNOSCAT DNA. The levels of CAT activity in extracts made from protoplasts 24 hr after electroporation are shown. (*A*) CAT activity produced by electroporation at various DNA concentrations in a solution containing 1× phosphate-buffered saline. Results are expressed relative to the CAT activity of protoplasts that received 10 μg of plasmid DNA per ml but no electric pulse. (*B*) Protoplast survival (□) and CAT activity (●) produced by electroporation at various concentrations of phosphate-buffered saline in solutions containing 10 μg of pNOSCAT DNA per ml. Results are expressed relative to the CAT activity observed with 1× phosphate-buffered saline and 10 μg of pNOSCAT DNA per ml, which was 50-fold greater than the activity of mock-electroporated (no DNA) protoplasts. Protoplast viability was measured by Evans blue exclusion (30) 24 hr after electroporation. Similar results were obtained in two separate experiments.

death after electroporation at 0.6× phosphate-buffered saline (Fig. 3*B*), 1× phosphate-buffered saline or an equivalent salt solution was used subsequently.

Various capacitors were substituted into the circuit shown in Fig. 4*D* to alter the pulse length (we define pulse length as the time required for the voltage to decrease to <5% of its initial value). Various-length pulses at 200 V and 400 V were delivered to protoplasts in a solution containing 1× phosphate-buffered saline, 0.2 M mannitol, and 10 μg of pNOSCAT DNA/ml (Fig. 5*A*). At 200 V, a 100-msec pulse length produced maximal CAT activity. Longer pulses yielded no greater CAT activity and resulted in decreased cell survival. Greater CAT activity could be recovered after shorter pulses at 400 V. Heat generation precluded 400 V pulses longer than 54 msec. Pulses of 400 msec at 100 V and of 1.7 sec at 50 V resulted in less CAT activity (data not shown). An 8000-V/cm, 15-μsec pulse similar to that described for animal cells (15) was tested, but no significant CAT activity was produced (data not shown) and we did not pursue the use of high-voltage, microsecond pulses.

A short high-voltage pulse produces approximately the same CAT activity as a longer pulse at lower voltage (Fig. 5*A*). Therefore, it seems that the pulse length can be between 30 and 230 msec and the voltage can be adjusted to achieve the maximal electroporation efficiency. The effect on CAT activity of varying the voltage at a fixed pulse length of 54 msec was examined using two 490-μF capacitors in parallel in the circuit shown in Fig. 4*D*. CAT activity was maximal at 350 V (875 V/cm, Fig. 5*B*). Higher voltages produce less CAT activity, probably because fewer protoplasts survived the treatment: 80–100% of the protoplasts survived a 200 V pulse, whereas <5% survived the 500-V pulse (data not shown).

The dependence of electroporation efficiency on Ca^{2+} was also evaluated. To prevent calcium phosphate precipitation, a Hepes buffer was substituted for the phosphate in phosphate-buffered saline. Substitution of this 10 mM Hepes, pH

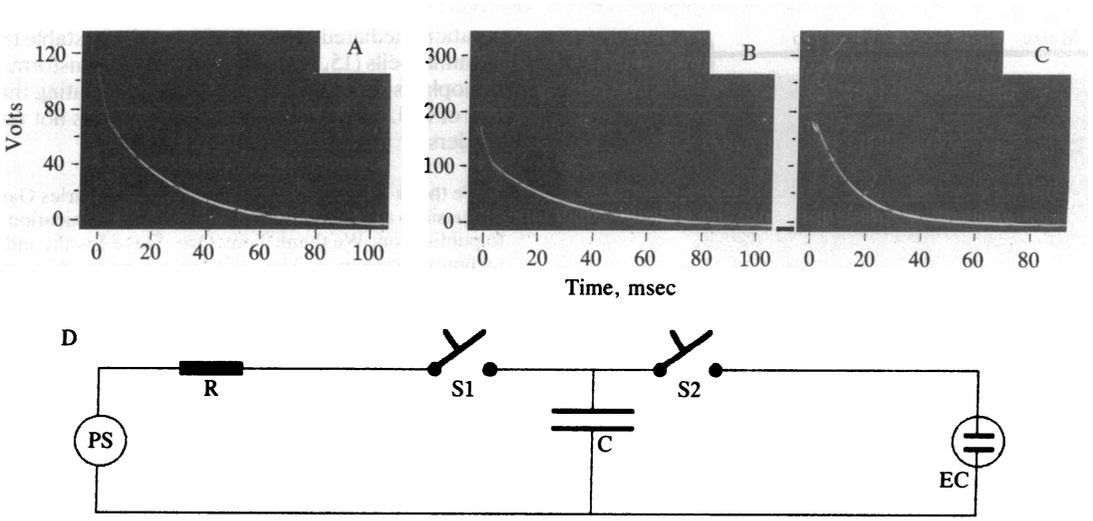

FIG. 4. Electric pulse lengths and amplitudes from the ISCO power supply and capacitor-discharge circuit. A storage oscilloscope was used to measure the electric pulses delivered to the electroporation cuvette. (*A* and *B*) The ISCO power supply, set at 2000 V, was discharged across the electroporation cuvette containing 1 ml of 1× (*A*) or 0.6× (*B*) phosphate-buffered saline/0.2 M mannitol. (*C*) A 980 μF capacitor charged to 200 V was discharged across the electroporation cuvette containing 1 ml of 1× phosphate-buffered saline/0.2 M mannitol. (*D*) The circuit used to charge and discharge the capacitor. PS, power supply; R, 50-ohm resistor; S1 and S2, switches; C, capacitor; EC, electroporation cuvette.

7.2/150 mM NaCl/0.2 M mannitol solution did not alter the electroporation efficiency (data not shown). Various concentrations of $CaCl_2$ were added to the Hepes-buffered saline-containing solution (all made to have the same final conductivity by adjusting the NaCl concentration), and protoplasts were washed and electroporated with a 300 V, 54-msec pulse in each solution (containing 10 μg of pNOSCAT DNA per ml). Maximal CAT activity was produced at a $CaCl_2$ concentration of 4 mM (Fig. 5*C*).

The electroporation technique of gene transfer was also applied to tobacco and maize protoplasts to determine whether electroporation would be generally useful for plant protoplasts. Both pNOSCAT and pCaMVCAT were tested, in combination for tobacco and separately for maize. Pulses (200, 275, and 350 V) 54 msec long were applied to tobacco protoplasts in a solution containing pNOSCAT (10 μg/ml) and pCaMVCAT (18 μg/ml). A 250 V, 54-msec pulse was applied to maize protoplasts in solutions containing either pNOSCAT (20 μg/ml) or pCaMVCAT (35 μg/ml). CAT activity was measured in extracts prepared 20 hr after electroporation (Fig. 6). High levels of CAT activity were produced in electroporated tobacco protoplasts (lanes e–g) and in maize protoplasts electroporated with pCaMVCAT (lane b), but in maize protoplasts pNOSCAT (lane c) produced CAT activities only 10-fold above background (lane a, equivalent to 0.1% of input label in these experiments).

DISCUSSION

The results show that an electric pulse is an effective method for the introduction of genes into monocot and dicot protoplasts. The advantages of the method are convenience, low cell toxicity, efficiency, and applicability to a wide range of plant protoplasts. Electroporated carrot protoplasts reform their cell wall, divide, and form callus (unpublished results) in the same manner as protoplasts electroporated for cell fusions (19). A 200 V, 54-msec electric pulse applied to carrot protoplasts in a solution of pNOSCAT plasmid DNA

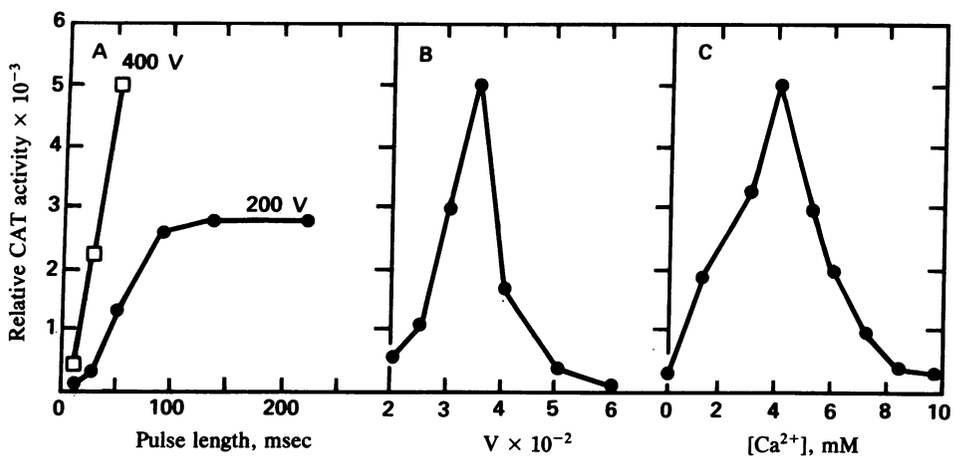

FIG. 5. CAT activity produced by electroporation with electric pulses of different duration and amplitude and at various Ca^{2+} concentrations. Carrot protoplasts were electroporated with pNOSCAT DNA (10 μg/ml) and the CAT activity in the protoplasts was measured 20 hr later. Each experiment was repeated twice with similar results. (*A*) CAT activity produced by varying the pulse length at 200 V (●) and at 400 V (□), by using different capacitance in the circuit shown in Fig. 4*D*. At 400 V, pulses longer than 54 msec developed too much heat to be useful. (*B*) CAT activity produced by varying the voltage of a 54-msec pulse. Protoplasts were suspended in 1× Hepes-buffered saline/0.2 M mannitol/5 mM $CaCl_2$/pNOSCAT DNA (10 μg/ml) and an electric pulse of the indicated voltage was delivered to the cuvette. (*C*) CAT activity produced by varying the Ca^{2+} concentration. Protoplasts were washed in 1× Hepes-buffered saline/0.2 M mannitol with various concentrations of $CaCl_2$ and were electroporated (300 V, 54-msec pulse) in the solutions of the same composition but containing pNOSCAT DNA at 10 μg/ml.

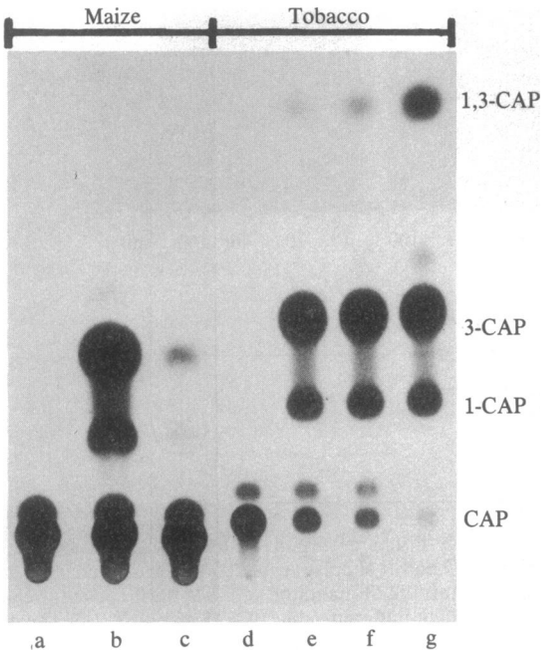

FIG. 6. CAT activity produced by tobacco and maize protoplasts electroporated with pNOSCAT and pCaMVCAT. A 54-msec pulse was delivered to the protoplasts in a solution containing plasmid DNA, 1× Hepes-buffered saline, 0.2 M mannitol, and 5 mM $CaCl_2$. Autoradiograms of the chromatographically separated products of the CAT activities from maize (48-hr exposure) and tobacco (12-hr exposure) are shown. A 250 V pulse was delivered to maize protoplasts in a solution containing pNOSCAT DNA at 20 μg/ml (lane c) or pCaMVCAT DNA at 35 μg/ml (lane b). Similar results were obtained in three separate experiments with maize protoplasts. Lane a: Background (maize protoplasts, no electroporation). Tobacco protoplasts were electroporated at various voltages in a solution containing a mixture of pNOSCAT (10 μg/ml) and pCaMVCAT (18 μg/ml) DNA. Lanes: d, 0 V; e, 250 V; f, 275 V; g, 350 V.

at 10 μg/ml gives 80–100% protoplast survival and produces levels of CAT activity that are easily detected. Higher concentrations of DNA produces more expression with no detrimental effects on protoplast viability. Higher voltages produce more CAT activity from fewer surviving protoplasts. The presence of 4 mM $CaCl_2$ in the electroporation solution increases protoplast survival and electroporation efficiency.

The most efficient gene transfer was achieved using a 54-msec, 350 V electric pulse. These values are very different from the ≈8000 V/cm, 15-μsec pulse used by Neumann *et al.* (15) but are similar to that used by Potter *et al.* (ref. 16; 60 msec, 112 V in our system). The electric field intensities we tested (500–875 V/cm) are similar to those used to induce plant cell fusions (19). The use of a capacitor allows the electric pulse length and amplitude to be optimized for each system and permits electroporation to be performed with any available power supply. When a power supply is used directly the salt concentration of the solution in the cuvette may affect the voltage delivered to the protoplasts, and adjustment of the salt concentration might increase the electroporation efficiency.

Electroporation and *Agrobacterium*-mediated (24) transient gene expression systems for plant cells are useful techniques for studying gene expression. Expression of the introduced DNA can be measured within hours as compared to the weeks or months required for stable transformation studies. The use of a transient assay system has allowed us to rapidly evaluate vectors for high-level expression in maize cells. It should also be possible to obtain stably transformed plant cells. Electroporation-mediated gene transfer results in stable transformation in animal cells (15, 16), and direct DNA transformations of plant protoplasts have been reported (12), indicating that the integration of DNA into plant chromosomes does not require T-DNA borders or associated functions (8).

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