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Transient expression of electroporated DNA in monocotyledonous and dicotyledonous species

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ABSTRACT

Transient expression of electroporated DNA was monitored in protoplasts of several monocot and dicot species by assaying for expression of chimeric chloramphenicol acetyltransferase (CAT) gene constructions. Expression was obtained in the dicot species of Daucus carota, Glycine max and Petunia hybrida and the monocot species of Triticum monococcum, Pennisetum purpureum, Panicum maximum, Saccharum officinarum, and a double cross, tri-specific hybrid between Pennisetum purpureum, P. americanum, and P. squamulatum. Recovery and viability of protoplasts after electroporation decreased with increasing voltages and capacitance while CAT activity increased up to a critical combination of voltage and capacitance beyond which the activity dramatically decreased. The optimal compromise between DNA uptake and expression versus cell survival was determined for D. carota and applied successfully to the other species. Maximum transient expression occurred 36 hours after electroporation of D. carota. The potential for using this procedure to rapidly assay gene function in dicot and monocot cells and application of this technique to obtain transformed cereals is discussed.

INTRODUCTION

Electroporation stimulates uptake of DNA into plant protoplasts permitting transient expression of foreign genes and production of stable transformants in several species (Fromm et al., 1986; Langridge et al., 1985; Ou-Lee et al., 1986; Riggs and Bates, 1986; Shillito et al., 1985). Alternative methods for introduction of DNA into dicots involve either chemical treatments such as PEG/$CaPO_4$ (Davey et al., 1980;, Krens et al., 1982; Paszkowski et al., 1984) or Agrobacterium-mediated transformation (Fraley et al., 1986). There are, however, apparent limitations in the host range of Agrobacterium, with transformation efficiency being reduced in some dicot species and limited in the gramineous monocots. Chemical methods of DNA delivery have been successfully used in monocot systems (Lorz et al., 1985; Potrykus et al., 1985; Uchimiya et al., 1986; Werr and Lorz, 1986) but the demonstrated toxicity of PEG can be a problem (Mercer and Baserga, 1981). Electroporation has the potential to be a universal and high frequency method of DNA delivery as further demonstrated in mammalian systems (Neumann et al., 1982; Potter et al., 1984; Wong and Neumann, 1982).

Free DNA delivery methods have several uses in transient gene expression studies. Non-integrated DNA is free from position effects which have been observed in stable transformants and require the pooling of many independent events to elucidate promoter strength or other regulatory factors involved in the control of gene expression (Horsch et al., 1985; Jones et al., 1985). In addition, the functionality of gene constructions can be evaluated rapidly using electroporated DNA.

In this report, we evaluate electroporation as a universal method for delivering DNA to protoplasts of dicots and monocots as determined by monitoring transient expression of several chimeric gene constructs.

MATERIALS AND METHODS

Protoplast Isolation and Culture in Dicot Species

The dicot suspension culture strains used were Glycine max (GM), Petunia hybrida Mitchell (MP4) and Daucus carota (TC) (Widholm, 1977). They were grown in 50 ml of MS culture medium (Murashige and Skoog, 1962) containing 0.4 mg/l 2,4-D for TC and GM or 0.2 mg/l p-chlorophenoxyacetic acid for MP4 in 250 ml Erlenmeyer flasks on gyrotory shakers at 135 rpm at 27° to 28°C.

Protoplasts were isolated by incubating 10 ml packed cell volume of exponentially growing suspension culture cells in 40 ml of enzyme dissolved in 10% mannitol and 0.1% $CaCl_2.2H_2O$, pH 5.7 for approximately 17 hours. The enzyme mixture consisted of 2% Cellulase R-10 (Yakult Honsha Co., LTD.), 0.1% Macerozyme R-10 (Yakult Honsha), and 0.05% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Noda, Chiba, Japan). The resulting protoplasts were isolated, purified, and cultured in MS culture medium containing 5% filter sterilized glucose at a density of 7×10^4 protoplasts/ml as previously described (Hauptmann and Widholm, 1982).

Mesophyll protoplasts from Petunia hybrida were isolated and cultured as previously described (Fraley et al., 1984) except that the enzyme mixture was the same as that used for the suspension cultures.

Protoplast Isolation and Culture in Monocot Species

The monocot cell lines used were Triticum monococcum (TM), Pennisetum purpureum (PP) (Vasil et al., 1983; Karlsson and Vasil, 1986), Panicum maximum (PM) (Karlsson and Vasil, 1986; Lu and Vasil, 1981), Saccharum officinarum (SC) (Ho and Vasil, 1983; Srinivasan and Vasil, 1986) and a double cross tri-

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specific hybrid between Pennisetum americanum, P. purpureum, and P. squamulatum (PAPS) (Dujardin and Hanna, 1984; Ozias-Akins, Hanna, and Vasil, unpublished). Suspension cultures of PM and PAPS were grown in a modified MS medium (Vasil and Vasil, 1981) containing 5% coconut milk and 2 mg/l 2,4-D. PP was grown in the same medium but with 2.5 mg/l 2,4-D; the medium for SC contained an additional 500 mg/l casein hydrolysate. The TM suspension culture was grown in liquid C_8 (Dudits et al., 1977). All suspension cultures were grown on a 7 day subculture regimen, except TM and PAPS which were subcultured twice weekly, with a 2-8 ml inoculum in 25-35 ml medium. Prior to protoplast isolation the suspensions were subcultured on the fourth to fifth day with a 5-8 ml inoculum in 25-35 ml medium.

Protoplasts were isolated using various enzyme mixtures dissolved in 3 mM MES, 0.45 M mannitol, 7 mM $CaCl_2.2H_2O$, and 0.7 mM NaH_2PO_4, pH 5.6 as previously described (Vasil and Vasil, 1980). The enzyme mixtures consisted of 1.0% Cellulase RS (Yakult Honsha), and 0.8% pectinase (Sigma) for TM and PM; 2% Cellulase RS and 0.7% pectinase for SC; 3% Cellulase R-10 and 0.7% pectinase for PP; and 2.5% Cellulase R-10 and 0.75% Pectinase for PAPS.

The monocot protoplasts were cultured in Kao and Michayluk's (1975) modified 8p medium (Vasil and Vasil, 1980) containing 0.4-0.5 M glucose, 0.5-1.0 mg/l 2,4-D and 0.2 mg/l zeatin and diluted 1:2.3 with protoplast culture medium after 1 week. To determine plating efficiency of PAPS and TM, the equivalent of 2 ml of the original protoplast culture was diluted to 36 ml with suspension culture medium containing 0.4% Seaplaque agarose (FMC) after 2-3 weeks. Three ml of the diluted culture was then plated over a layer of the same medium containing 0.6% agarose in a 10 cm petri dish.

Free DNA Delivery by Electroporation

Protoplasts were electroporated using the Zimmerman Cell Fusion System (GCA Precision) or a capacitor discharge bank (Fromm et al., 1985).

Electroporation with the Zimmerman Cell Fusion System was performed using the Zimmerman Helical Fusion Chamber or in an electroporation chamber constructed out of cuvettes containing platinum or aluminum electrodes with a 0.5 cm path length (Potter et al., 1984). Homogeneous field strengths (V/cm) would be equal to twice the voltage applied in electroporation cuvettes as compared to the Zimmerman helical chamber which produces an inhomogeneous field strength of approximately 50x the applied voltage (200 µm electrode distance). Pulses were delivered at 999.9 µsec, 240 V DC in batteries of 9 pulses each. Each battery of pulses was delivered 1, 10, 50, and 100 times using 14 µg of plasmid DNA with and without 50 µg of calf thymus DNA in the protoplast wash solution.

A capacitor bank was constructed of 4 each of 40, 110, 240, 340, and one each of 1000 and 2400 µF capacitors (Mallory) which could be charged or discharged individually or in parallel. The single pulse discharge was monitored using a Tectronics model 584B dual channel recording oscilloscope. Amperage was determined by measuring the discharge across a 1 ohm resistor during electroporation.

Prior to electroporation the protoplasts were washed once in 10 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2.2H_2O$, and 0.2 M mannitol, pH 7.2 and brought to a density of approximately $3x10^6$ protoplasts/ml using the same buffer (Fromm et al., 1985) and put in an ice bath. To 1 ml of resuspended protoplasts, 20 µg of plasmid DNA was added, mixed and pipetted into individual electroporation cuvettes. The protoplasts were electroporated with the capacitor bank using various voltages and capacitances. The protoplasts were maintained on ice for approximately 10 minutes until plating in liquid culture medium.

To estimate the number of dicot protoplasts that were lysed by various electroporation treatments, the density of TC protoplasts were determined prior to, and immediately after, delivering the pulse discharge. Values were expressed as percent survival. Viability determination was based on phenosafranin dye exclusion (Widholm, 1972) 2 days after electroporation and expressed as percent viability as compared to a non-electroporated control. All data are means from at least 2 separate experiments.

Plating efficiency of electroporated monocot protoplasts was obtained by counting the number of colonies formed after 3-4 weeks of culture and expressed as percent of non-electroporated control.

Preparation and Characteristics of Plasmids Used

E. coli plasmid DNA was isolated using a Triton/sodium dodecyl sulfate (Cannon et al., 1984) or alkaline lysis procedure (Maniatis et al., 1982) and purified on a CsCl gradient. The DNA was quantified either spectrophotometrically or by ethidium bromide staining on agarose gels.

E. coli plasmid pMON145' contained the chloramphenicol acetyl transferase gene (CAT) expressed by the CaMV 35S promoter (Odell et al., 1985) derived from pMON145 (Broglie et al., 1984). Two additional plasmids that contain the CAT gene, pMON243 and pMON244, were also used. These plasmids were constructed by insertion of the pBR320 1100 bp Sau 3A fragment carrying the CAT coding sequence (Soberon et al., 1980) into the Bgl II site of pMON237 (Rogers et al., 1986), a derivative of pMON200 (Fraley et al., 1985) carrying a CaMV 19S promoter-nopaline synthase 3' poly-adenylation signal expression cassette. In the case of pMON243, the CAT gene is in the sense orientation and in pMON244 in anti-sense orientation as a negative control. Other plasmids containing neomycin phosphotransferase II (NPT II) were driven either by the nopaline synthase promoter (NOS) in pMON200 (Fraley et al., 1985) or by the CaMV 35S promoter, pMON273 (Rogers et al., 1987).

Transient Expression Assays

CAT assays were performed (Fromm et al., 1986; Gorman et al., 1982) with the acetylated ^{14}C chloramphenicol quantitated using a TLC plate scanner (Bioscan) and expressed as the percent acetylated chloramphenicol/mg protein/hr under linear assay conditions. Total cellular protein was determined using the Bio-Rad Protein Assay following electroporation and protoplast culture. Quantitative electroporation experiments were based on the mean value from at least two replicates each of $3x10^6$ electroporated protoplasts.

Nopaline production was assayed by paper electrophoresis (Rogers et al., 1987) and NPT II activity was assayed using the procedure of Reiss et al. (1984) as modified by Rogers et al. (1985).

RESULTS

We compared the sensitivity of assays for CAT, NPT II, and nopaline synthase activities by examining protoplasts from transgenic plants containing chromosomal copies of each of the marker genes. The plants contained the same gene constructions used in the electroporation experiments, but were produced by leaf disc transformation using Agrobacterium tumefaciens.

Nopaline production could be detected in a minimum number of 1.5×10^4 protoplasts using petunia

plants transformed with pMON200 or pMON273 (Table 1).

	Number of protoplasts	ug protein
Nopaline production		
pMON200 (NOS/NOS)	1.5×10^4	---
Neomycin phosphotransferase		
pMON200 (NOS/NPT II)	1.0×10^5	100
pMON273 (35S/NPT II)	1.0×10^4	10
Chloramphenicol acetyltransferase		
pMON145' (35S/CAT)	5.0×10^3	5

Table 1: Relative sensitivities of transient expression assays. Protoplasts derived from transformed Petunia plants were assayed for marker gene activity and the minimum number needed for positive detection was determined. Plant transformation, protoplast isolation and enzyme assays were performed as described in the 'Materials and Methods'.

NPT II activity could be detected in approximately the same amount of material when expressed from the CaMV 35S promoter but at least 10 times more protoplasts were required to detect NPT II activity with the gene expressed from the NOS promoter. Sensitivity of the CAT assay could be enhanced by using longer exposures while in the case of NPT II, longer exposure generally resulted in a masking of activity due to background radioactivity.

Attempts were made initially to measure transient expression of free DNA delivered by electroporation using the DC pulse generator in the Zimmerman Cell Fusion System. In carrot protoplasts, weak NPT II activity was detected 48 hours after electroporation using 14 µg of pMON273 plasmid DNA only when multiple pulses of 10 to 100 each at 240 V DC, 999.9 µsec were used. Single pulses or multiple pulses using calf thymus DNA alone gave no detectable NPT II activity. No NPT II activity was detected in electroporated petunia mesophyll or soybean suspension culture protoplasts using single or multiple pulses with the Zimmerman apparatus.

A capacitor discharge circuit and electroporation cuvettes were constructed to increase delivery of plasmid DNA and to further optimize the voltage and pulse length parameters. Using petunia mesophyll protoplasts, expression of CAT activity was detectable using a single 1000 µF and 200 V pulse, 2 days after electroporation (Figure 1). Non-electroporated leaf tissue as well as petunia protoplasts electroporated without DNA showed no CAT activity. Transgenic petunia plants transformed with pMON243 (CaMV 19S/CAT) showed high levels of CAT activity while pMON244 (CaMV 19S/CAT with CAT in inverse orientation behind the promoter) showed no CAT activity.

To examine the kinetics of expression of electroporated DNA, carrot protoplasts were electroporated at 200 V, 1000 µF and assayed for CAT activity at 12 hour intervals for 132 hours. Detectable CAT activity was observed 12 hours after electroporation which was the first time point sampled. Maximal expression was observed 36 hours after electroporation and after that expression decayed in an exponential fashion (Figure 2).

To optimize parameters for transient expression of electroporated DNA and the subsequent recovery of viable cells, various voltages and capacitances were tested in combination. The number of protoplasts which lysed after electroporation increased with increasing voltages and capacitance (Figure 3). There was a similar trend in regard to viability as determined by phenosafranin dye exclusion 2 days after electroporation (Figure 4).

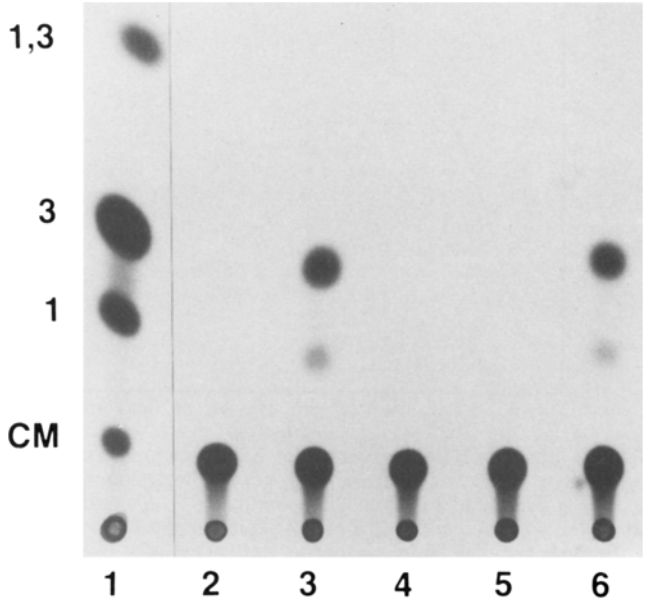

Figure 1: CAT activity in transformed and electroporated Petunia. Petunia protoplasts were isolated, electroporated, and CAT assays performed as described in 'Materials and Methods'. Chloramphenicol (CM), 1-acetate chloramphenicol (1), 3-acetate chloramphenicol (3), and 1,3-diacetate chloramphenicol (1,3) show increasing mobilities as separated on silica gel TLC plates. Lanes 1: bacterial standard; 2: protoplasts electroporated without DNA at 1000 µF, 200V; 3: protoplasts electroporated with 20 µg pMON145' plasmid DNA (containing CAT driven by the CaMV 35S promoter). Lanes 4-6: Petunia leaf disks were transformed using pMON243 (containing CAT driven by the CaMV 19S promoter) and pMON244 (containing CAT in reverse orientation behind the nopaline synthase promoter) and plants regenerated using the leaf disk transformation procedure. Lanes 4: non-transformed leaf; 5: pMON244 transformed leaf; 6: pMON243 transformed leaf.

Variation in CAT activity was observed at different capacitances for each voltage tested (Figure 5) with maximal activity at 110 µF for 400 V, 510 µF for 300 V, and 1000 µF for 200 V. A deviation from these conditions resulted in a dramatic loss of CAT activity. The higher capacitances, which reflect longer pulse length, combined with higher voltages resulted in greatly reduced viability with a large amount of heating observed in the electroporation chamber. In addition, there also appears to be a lower voltage threshold since the 100 V, 2000 µF pulse did not give appreciable CAT activity.

For evaluation of DNA delivery by electroporation to other species, the 200 V series of pulses were generally used due to good CAT expression over a variety of conditions. The trend of decreased viability at higher charges was also observed in the other dicot species examined including Glycine max suspension culture protoplasts and petunia mesophyll and suspension culture protoplasts. CAT expression was obtained in these species using a 200 V, 1000 µF pulse.

Expression of electroporated DNA was similarly examined in several monocot protoplast systems. High levels of CAT expression were seen in TM, PM, and PAPS

electroporated protoplasts (Figure 6) while the controls which were electroporated without DNA showed little or no CAT activity.

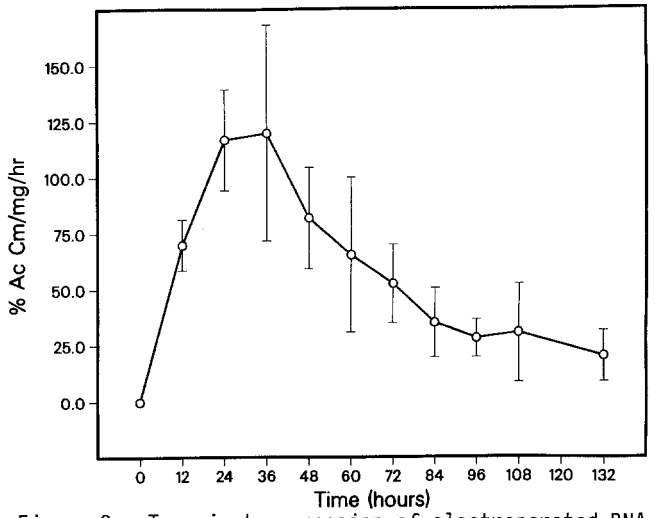

Figure 2: Transient expression of electroporated DNA in carrot. Carrot protoplasts were electroporated using 200V, 1000 µF with 20 µg pMON145' DNA and assayed for CAT activity at 12 hour intervals. Each data point represents specific activity (% chloramphenicol acetylated/mg/hr) from three replicates of $3x10^6$ protoplasts.

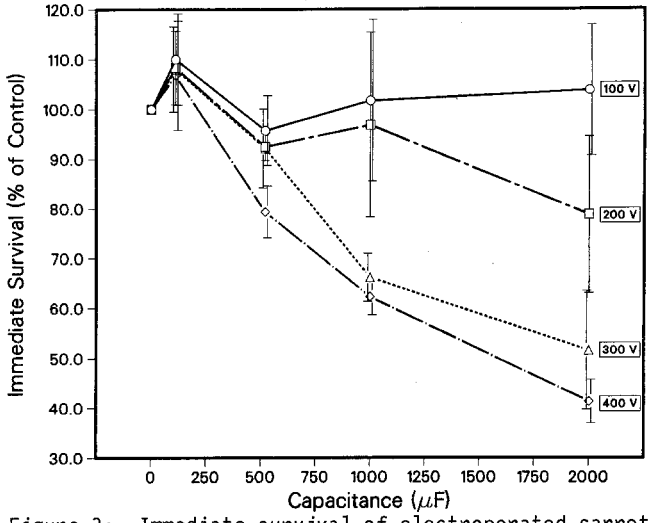

Figure 3: Immediate survival of electroporated carrot protoplasts. One ml of $3x10^6$ carrot protoplasts were electroporated at various voltages and capacitances. Percent recovery is expressed as the number of protoplasts which remained intact immediately following the pulse discharge compared to the initial protoplast density. Each data point represents the mean value from 3 separate experiments.

Maximal expression was found to occur at 400 V, 510 µF in the cell strains of TM and PM which were the only ones tested under these conditions. Transient expression of CAT activity in the monocot lines increased with capacitance when electroporated at 200 V (Figure 7), but levels of expression were consistently 10 to 100 fold lower than in carrot protoplasts electroporated under identical conditions. The monocot protoplasts underwent sustained divisions following electroporation. SC and PP protoplasts expressed weak CAT activity following electroporation; however, culture conditions were suboptimal during these experiments.

The monocot cell lines tested, TM and PM, showed little or no reduction in viability following electroporation as determined visually or by plating efficiency (Table 2). TM and PM protoplasts are densely cytoplasmic and ranged in size from 15-20 µm, while the dicot protoplasts TC, GM and petunia are more vacuolated and are larger in size.

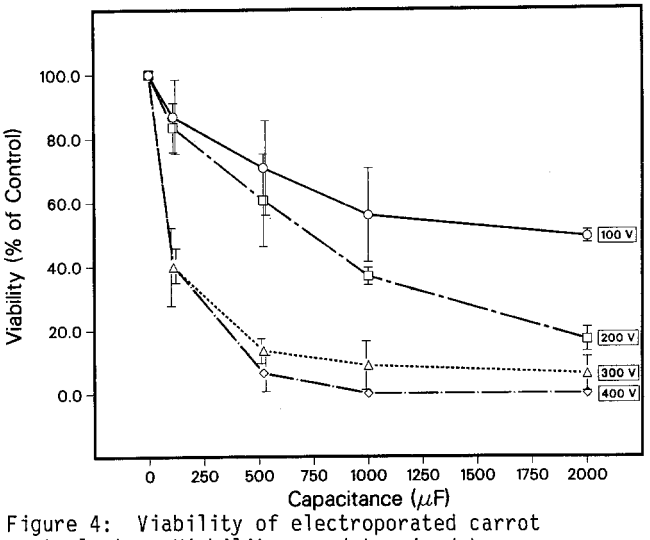

Figure 4: Viability of electroporated carrot protoplasts. Viability was determined by phenosafranin dye exclusion 2 days after electroporation using various voltages and capacitances. Each data point is based on mean values of at least 200 protoplasts from 3 separate experiments.

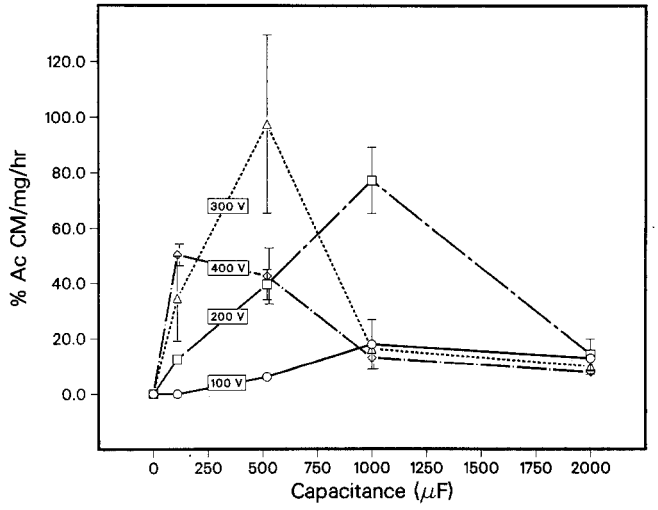

Figure 5: Expression of electroporated DNA in carrot protoplasts. Protoplasts were electroporated using various voltages and capacitances with 20 µg pMON145' DNA as described in 'Materials and Methods'. CAT activity was assayed after a 2 day culture period and mean values determined from at least 2 separate experiments.

DISCUSSION

The high level expression of electroporated DNA in carrot was found to increase up to a critical combination of voltage and capacitance beyond which the activity dramatically decreased. Similar levels of CAT activity could be obtained using specific combinations of voltage and capacitance, with the capacitance used being related to pulse length. Fromm et al. (1985) showed that similar CAT activity could be obtained with either a short high-voltage pulse or a long low-voltage pulse in transient expression experiments with carrot protoplasts. Under extremes in capacitance and voltage other factors, such as

production of excessive heat, may play a significant role.

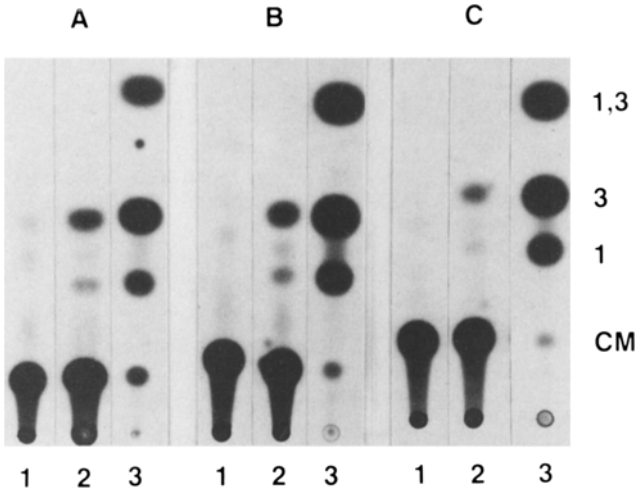

Figure 6: Expression of electroporated DNA in monocotyledonous protoplasts. Monocot protoplasts were isolated, electroporated, and CAT assays performed as described in 'Materials and Methods'. Chloramphenicol (CM), 1-acetate chloramphenicol (1), 3-acetate chloramphenicol (3) and 1,3-diacetate chloramphenicol (1,3) show increasing mobilities as separated ascendingly on TLC plates. A: Triticum monococcum, B: Tri-specific hybrid between Pennisetum americanum, P. purpureum, and P. squamulatum, C: Panicum maximum. Lanes 1: Protoplasts electroporated at 200 V, 1000 µF without DNA; 2: Protoplasts electroporated at 400 V, 510 µF with 20 µg pMON145' plasmid DNA; 3: Bacterial standard.

		Plating Efficiency (% of control)	
		Triticum monococcum	Panicum maximum
µF	V		
0	0	100	100
1000	200	76	58
760	200	74	74
520	200	77	105
350	200	77	--
3900	50	--	79
2400	100	64	74
520	400	81	16

Table 2: Effect of electroporation on plating efficiency of Triticum monococcum and Panicum maximum. Protoplasts were electroporated at various capacitances and voltages in the presence of 20 µg of pMON145' plasmid DNA as described in the 'Materials and Methods'. Plating efficiency is expressed as % of a non-electroporated control.

In carrot protoplasts, maximal expression occurs using a 200 V, 1000 µF or a 300 V, 510 µF pulse with a drop in expression resulting from a slight deviation from these conditions. We do not know whether the induction of high levels of expression is due to introduction of more copies of plasmid DNA per cell or introduction of plasmid DNA into more protoplasts.

There was an optimal compromise between DNA uptake and expression and survival of the protoplasts as charge (voltage and capacitance) was increased. The sensitivity of cells to electrical discharge may be influenced by degree of vacuolation and cell size as well as membrane composition or other factors. For example, the highly vacuolated petunia mesophyll protoplasts were very sensitive to electrical discharge, while the densely cytoplasmic monocot protoplasts were very resistant. Protoplasts from carrot suspension cultures were intermediate in vacuolation and in their sensitivity to electroporation. While our data does not prove that such differences in sensitivity are due to these particular factors, they demonstrate the importance of optimizing conditions for any particular system. Fortunately, several species seem to respond reasonably well within a range of voltage and capacitance which gives high levels of expression of delivered DNA.

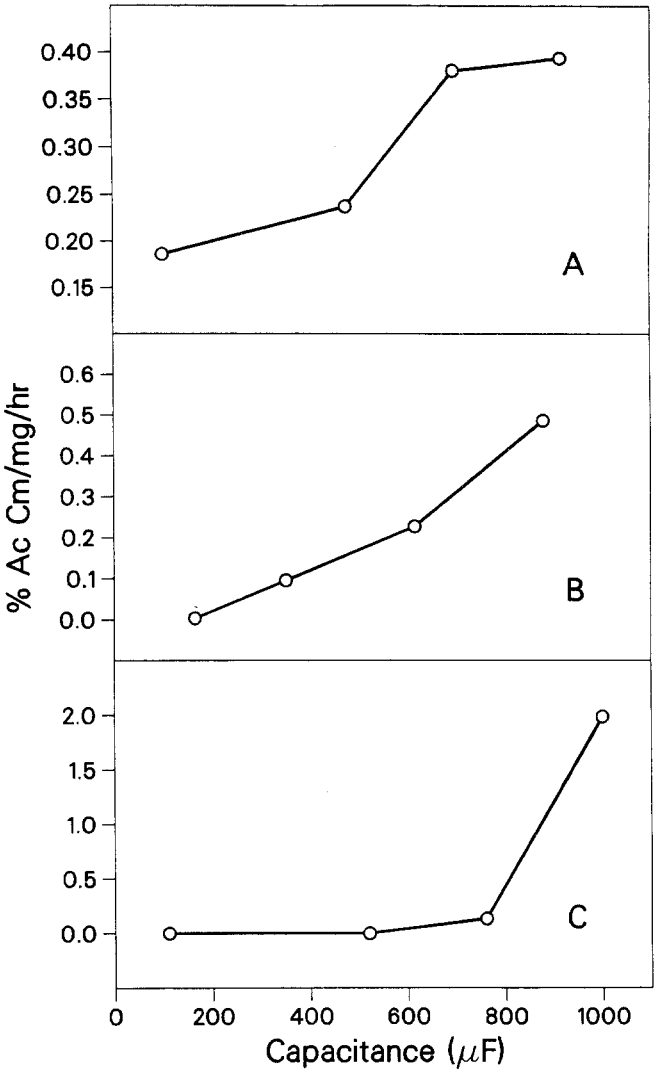

Figure 7: Expression of electroporated DNA in monocotyledonous protoplasts. Protoplasts were electroporated using various voltages and capacitances with 20 µg pMON145' DNA as described in 'Materials and Methods'. CAT activity was assayed 2 days after electroporation of protoplasts and based on values from at least 2 separate experiments. A: Panicum maximum, B: Triticum monococcum, C: Tri-specific Pennisetum hybrid.

The kinetics of delivery of DNA to monocots was similar to those for dicot protoplasts except that the expression was generally 10 to 100 times lower in the monocots. A similar result has been observed in electroporated maize protoplasts (Fromm et al., 1985). The CaMV 35S promoter fusions gave higher levels of expression than the NOS fusions (data not presented here) which have been consistently observed in a number of dicot (Rogers et al., 1985; Sanders, in preparation) and monocot (Fromm et al., 1985) systems.

In initial attempts to select stable monocot transformants, selection was based on the ability of the NPT II gene to confer kanamycin resistance. In the monocot cell lines PP, PM, SC and PAPS, sustained growth could be obtained on as high as 1000-3000 µg/ml kanamycin in suspension culture or protoplast derived colonies. In TM, growth was inhibited only 40% as compared to the control on 500 µg/ml kanamycin (data not presented here). Since kanamycin resistance is demonstrated by a wide variety of wild-type monocot species, it potentially has limited application for the routine selection of stable monocot transformants. Studies are in progress to evaluate other combinations of drugs and drug resistance genes for use in selection of transformed monocot protoplasts.

The importance of transient expression of DNA delivered to protoplasts is that it permits evaluation of gene expression and protein compatibility/function prior to attempts to obtain stable transformants in a new species. It also permits optimization of DNA delivery conditions to maximize chances of obtaining stable transformants. The introduction of DNAs with more efficient selectable markers into monocot cell lines with the capacity to regenerate such, as those used in this study, makes the prospect of producing transgenic cereals an increasingly approachable goal.

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