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Expression of a foreign gene linked to either a plant-virus or a *Drosophila* promoter, after electroporation of protoplasts of rice, wheat, and sorghum

(chloramphenicol acetyltransferase/transformation/chimeric gene)

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ABSTRACT **The bacterial chloramphenicol acetyltransferase (CAT) gene was expressed in protoplasts of three important graminaceous plant species after introduction of the gene by electroporation. Gene transfer occurred when high-voltage electric pulses were applied either directly or indirectly (without anode contact) to a solution containing plasmid DNA and protoplasts of rice, wheat, or sorghum. The indirect method was more rapid, resulted in higher protoplast viability, and was less subject to contamination than the direct-contact method. Gene expression of approximately equal magnitude resulted when the CAT gene was fused to either the 35S promoter of cauliflower mosaic virus or the *copia* long terminal repeat promoter of *Drosophila*. Together with recent advances in regeneration of callus and whole plants from protoplasts, this system makes it possible to study inheritance and expression of genes introduced into graminaceous monocotyledonous plants.**

Isolated genes have been introduced into eukaryotic cells to define the requirements for their expression *in vivo* and to develop desired genetic recombinants (1, 2). In plants, infection with *Agrobacterium tumefaciens* has been established as the most effective method of transforming dicot species (3–5). Monocot transformation has recently been successful using polyethylene glycol (PEG)-facilitated DNA uptake (6, 7), brome mosaic virus infection (8), and electroporation (9–11). Electroporation, subjecting cells to brief pulses of electric current to permeabilize biomembranes, was developed for use in animal cell hybridization and transformation (12–14). This approach is especially promising because it is applicable to a wide variety of species.

The chloramphenicol acetyltransferase (CAT) assay (15) offers a sensitive and rapid method to detect the functional activity of different promoter sequences. This paper reports experiments on the introduction and expression of the CAT gene with the 35S promoter of cauliflower mosaic virus (CaMV) into protoplasts of three important cereal crop plants by electroporation. We also report that high levels of expression were obtained when the CAT gene was fused with the heterologous *copia* long terminal repeat (LTR) promoter of *Drosophila*.

MATERIALS AND METHODS

Plant Material and Protoplast Isolation. *Triticum monococcum* cell line R-TM 1066 and *Sorghum bicolor* callus were kindly provided by E. Earle (Cornell University). *Oryza sativa* (cv. Tai-nung 67) seeds and anther-derived culture were generously supplied by S. C. Wu (Academia Sinica, Taipei, Taiwan).

Protoplasts were isolated from sterile leaves of 20- to 30-day-old plants grown on agar-solidified Murashige and Skoog (MS) medium (16) or from exponentially growing suspension cells grown on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (5 mg/liter). Plant material (0.8–1.0 g wet weight) was incubated in 8 ml of enzyme solution on a rotary shaker at 50 rpm and 30–32°C for 3–6 hr. The enzyme solution contained 2% (wt/vol) cellulase "Onozuka" RS and 1% (wt/vol) Macerozyme (both from Yakult Honsha, Nishinomiya, Japan), 0.01% (wt/vol) pectolyase (Sigma), 0.4 M mannitol, 40 mM $CaCl_2$, and 10 mM Mes buffer (pH 5.5). Released protoplasts were separated from debris by filtration through 80-μm mesh-size nylon screen and rinsed with an equal amount of electroporation buffer containing 8 g of NaCl, 0.2 g of KCl, 0.1 g of KH_2PO_4, 0.6 g of Na_2HPO_4, 5 mmol of $CaCl_2$, and 0.4 mol of mannitol per liter. Protoplasts were washed twice by centrifugation at 200 × *g* for 5 min and the pellet was resuspended in electroporation buffer. If present, debris was removed by overlaying on 0.5 M sucrose and recollecting the floating layer of protoplasts.

For determination of cell viability, total cell number was counted by phase-contrast microscopy and viable cells were counted by fluorescence microscopy (Olympus, BH2-RFL) following staining with 6-carboxyfluorescein diacetate (Calbiochem) (17). Cell counts were made with a Fuchs–Rosenthal hemocytometer; results are expressed as the mean of two samples of 80–100 cells counted over four grid squares.

Plasmids. Plasmid p35S-CAT (Fig. 1 *Left*) (18) is 5.3 kilobases (kb) long and consists of (*a*) a 941-base-pair (bp) CaMV 35S promoter (nucleotides 6504–7444) (19), (*b*) a 900-bp region coding for the CAT gene (20), (*c*) a 700-bp region containing 3′ noncoding sequences and transcription terminator for the gene encoding the small subunit of ribulose 1,5-bisphosphate carboxylase (rbcS), and (*d*) a derivative of plasmid pUC13 in which the *Hin*cII site was changed to a *Cla* I site. This plasmid was kindly provided by N.-H. Chua (Rockefeller University).

Plasmid pCopia-CAT (5.5 kb) consists of (*a*) a *copia* LTR promoter and upstream 5′ flanking region from *Drosophila* (21) and (*b*) a promoterless derivative of pSV2cat[s] carrying the small tumor antigen intron and the early region polyadenylylation site from simian virus 40 (15) cloned into the pBR322 vector (Fig. 1 *Right*). The recombinant plasmid was constructed by P. P. DiNocera and I. B. Dawid (22) and provided by J. Lis (Cornell University).

Recombinant plasmids were grown in *Escherichia coli*, isolated by phenol extraction, and purified by CsCl/ethidium

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Abbreviations: CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; CaMV, cauliflower mosaic virus; kb, kilobase(s); bp, base pair(s).

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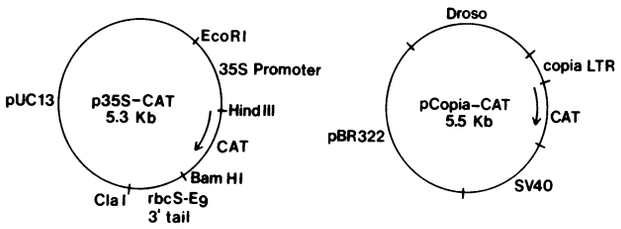

FIG. 1. Diagram of p35S-CAT and pCopia-CAT plasmids. p35S-CAT consists of a CaMV 35S promoter region, a region coding for CAT, a 3′ noncoding region from the gene for ribulose bisphosphate carboxylase small subunit (rbcS), and a derivative of pUC13. pCopia-CAT consists of a *Drosophila* DNA segment (Droso), a *copia* LTR promoter, a CAT coding region, a 3′ noncoding region from simian virus 40 (SV40), and a derivative of pBR322. The arrows start near the 5′ ends and point toward the 3′ ends of the CAT gene.

bromide density gradient centrifugation (23). The identity and purity of plasmids were checked by restriction digestion and gel electrophoresis. Experiments were carried out in P1 facilities.

Electroporation System and Procedures. We compared two different systems. One was a Pulsar (Frederick Haer, Brunswick, ME) pulse generator connected to a helical fusion chamber (GCA, Chicago). Variable parameters were pulse length ($\geq$1 μsec), voltage (1–150 V), and pulse number. The reaction chamber had two platinum electrodes wound in a double helix with a 200-μm gap and had a maximum sample volume of 200 μl. The electrodes were immersed in the sample. This electroporation system, referred to as FH-GCA, could generate a maximum field strength of 7.5 kV/cm.

The other electroporation system was a Baekon 2000 Advanced Gene Transfer system (Baekon, Saratoga, CA). The pulse generator had a fixed pulse length of 62.5 μsec. Variable parameters were voltage (0.1–10 kV) and pulse number. The reaction chamber, known as a receptacle, had a 1.0-ml maximum volume and was disposable. A steel ball embedded in the bottom of the receptacle was connected to the cathode. The system can be operated in either the non-contact or the contact mode. To avoid cross-contamination, we have used only the former. In this mode, the non-contact electrode, the anode, was positioned $\approx$1 mm above the sample solution surface. Pulsed current was discharged across the gap between the anode and solution surface.

Isolated protoplasts were resuspended in electroporation buffer at a population density of 1.5–2.0 $\times$ 10^6 per ml counted with a hemocytometer. They were mixed with plasmid DNA (20 μg/ml) and carrier (calf thymus) DNA (50 μg/ml) in a total volume of 200 μl. This mixture was incubated at 0°C for 5 min and mixed well by gentle inversion before electroporation. After the electric pulse, the solution containing the protoplasts was held at 0°C for 10 min to prolong the open-pore status (13). Then the protoplasts were diluted into an equal volume of MS medium containing 4% sucrose and 0.2 mg of 2,4-dichlorophenoxyacetic acid and 0.1 mg of kinetin per liter at 25°C. CAT enzyme activity in protoplasts was assayed 40–48 hr after electroporation.

CAT Assay. Cell extracts were prepared from protoplasts by sonication and centrifugation in 100 μl of 0.25 M Tris·HCl buffer (pH 7.8) contained in 1.5-ml Eppendorf tubes. CAT activity was assayed as previously reported (15), except that the amount of [^{14}C]chloramphenicol was decreased to 0.1 μCi (1 Ci = 37 GBq) per sample and recovery was improved by extracting with ethyl acetate twice. All the radioactivity in the centrifuge tube ($\approx$170,000 cpm in each experiment) was used for spotting the TLC plate (Chromagram; Kodak). After resolution of chloramphenicol and its acetylated derivatives by chromatography, an autoradiograph was made using an intensifier screen. Quantitative results were obtained by scintillation counting of separated spots of chloramphenicol and its acetylated products, and the percent conversion was calculated.

RESULTS

The percent survival rate of protoplasts after electroporation depended on the tissue of origin. Leaf protoplasts lost viability more rapidly than protoplasts from suspension cultures of rice (Fig. 2) or tobacco (data not shown). Electric pulses decreased the viability of leaf protoplasts, but not protoplasts from suspension culture cells, in the early period after electroporation ($\leq$13 hr). Protoplast survival with the Baekon electroporator (47%) was higher than with the FH-GCA system (30%) at 36 hr after electroporation. The control value without electroporation was 56% survival.

Electroporation of protoplasts from rice leaves, sorghum, and wheat in the presence of plasmid DNA (p35S-CAT) resulted in expression of the introduced CAT gene (Fig. 3). Protoplasts from various sources were electroporated with plasmid DNA as described in *Materials and Methods*. Forty to forty-eight hours later, protoplast extracts were assayed for CAT activity. Autoradiographs made from chromatograms of CAT reaction products are shown in Fig. 3. Two monoacetylated forms (1-AcCAP, 3-AcCAP) and a single diacetylated form (1,3-AcCAP) were produced in *E. coli* carrying the CAT gene of pBR325 (24). In our experiments, using 3–4 $\times$ 10^5 protoplasts per sample and a 30-min incubation for enzyme assay, we did not see the diacetylated form. Thus, only the two monoacetylated spots from the TLC plates were analyzed by scintillation counting.

Rice protoplasts incubated with p35S-CAT without electroporation showed a low level of CAT expression. The amount of reaction product was proportional to the number of protoplasts added (Fig. 3*A* and Table 1, Exp. A, lanes a–c). Protoplasts electroporated with p35S-CAT showed much higher levels of CAT expression. The amount of reaction product was, again, proportional to the number of protoplasts (Fig. 3*A* and Table 1, Exp. A, lanes d–f). Expression of the CAT gene in sorghum was compared using two electroporation systems: the non-contact Baekon system and the contact FH-GCA system. The optimum condition for the non-contact system was 10 kV, 20 pulses (2^2 pulses for 5 cycles), and for the contact system the optimum was 50 V (2.5 kV/cm), 2 pulses (Fig. 3*B* and Table 1, Exp. B, lanes c and f). Since the non-contact system resulted in higher viability of protoplasts,

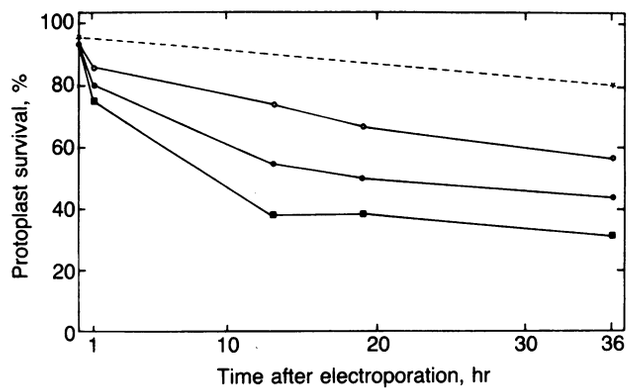

FIG. 2. Rice protoplast viability. Percent protoplast survival was determined at various times after electroporation, using 6-carboxyfluorescein diacetate. Protoplasts from rice suspension culture were suspended in buffer without electric pulses (★). Protoplasts from rice leaves were treated with 20 pulses (7.5 kV, 62.5 μsec) by the Baekon system (●) or 2 pulses (0.05 kV, 100 μsec) by the FH-GCA system (■) or were not pulsed (○).

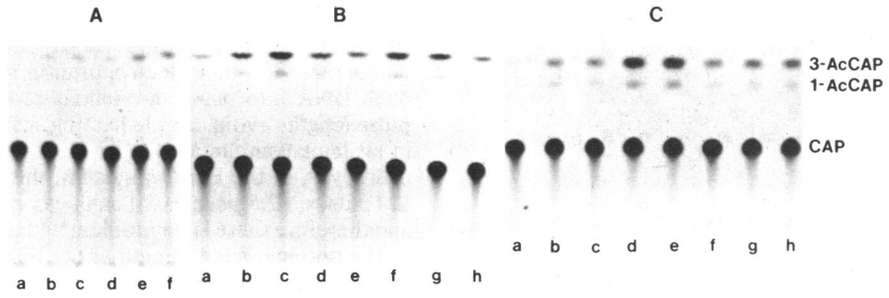

FIG. 3. Expression of the CAT gene introduced into rice leaf, sorghum, and wheat protoplasts by electroporation. Baekon and FH-GCA pulse generators were used to electroporate protoplasts in the presence of plasmid p35S-CAT. After incubating electroporated protoplasts for 40–48 hr at 20–21°C, the CAT activities of protoplast extracts were measured. Autoradiographs were exposed with an intensifier screen for 48 hr (*A*), 24 hr (*B*), and 12 hr (*C*). CAP, chloramphenicol; 3-AcCAP and 1-AcCAP, monoacetylated forms of chloramphenicol. For description of experiments and quantitation of results see Table 1.

it was used in all other experiments to be described. In wheat, CAT gene expression was optimal at 20 pulses (Fig. 3*C* and Table 1, Exp. C, lanes d and e). Duplicate samples electroporated separately for 10 or 20 pulses demonstrated that the level of CAT gene expression was reproducible (lanes b–e).

Table 1. Expression of the CAT gene introduced into rice leaf, sorghum, and wheat protoplasts by electroporation with p35S-CAT (see Fig. 3)

	Electroporation				CAT assay		
			Pulses			Products*	
Lane	System	Voltage, kV	No.	Length, μsec	Extract, μl	cpm	%
		Exp. A. Rice leaf protoplasts					
a	None	—	—	—	10	716	0.4
b		0	0	0	50	2,117	1.2
c		0	0	0	100	3,175	1.8
d	FH-GCA†	0.05	2	100	10	2,116	1.2
e		0.05	2	100	50	8,817	5.0
f		0.05	2	100	100	11,285	6.5
		Exp. B. Sorghum protoplasts					
a	Baekon	10	20	62.5	200	1,411	0.8
b		5	20	62.5		8,996	5.1
c		10	20	62.5		25,049	14.2
d		10	120	62.5		10,231	5.8
e	FH-GCA	0.01	2	100		6,174	3.5
f		0.05	2	100		16,229	9.2
g		0.10	2	100		14,465	8.2
h		0.15	2	100		6,174	3.5
		Exp. C. Wheat protoplasts					
a	None	—	—	—	200	429	0.3
b	Baekon	10	10	62.5		9,484	6.0
c		10	10	62.5		10,089	6.4
d		10	20	62.5		45,148	28.8
e		10	20	62.5		51,536	32.9
f		10	40	62.5		14,505	9.3
g		10	80	62.5		25,806	16.5
h	None	—	—	—	10‡	26,941	17.2

Protoplasts ($3–4 \times 10^5$) in 200 μl were subjected to electroporation. The amount of plasmid used was 20 μg/ml in all experiments, except in Exp. B, lane a, and Exp. C, lanes a and h, in which no plasmid was used.

*[^{14}C]Chloramphenicol and its acetylated products were separated by TLC (see Fig. 3). Radioactivity in the two monoacetylated forms of chloramphenicol was measured by scintillation counting, and % conversion was calculated.

†For FH-GCA system, 0.05 kV produces a field strength of 2.5 kV/cm.

‡One colony of *E. coli* carrying pBR325 was sonicated in 1 ml of buffer, the homogenate was centrifuged at $10,000 \times g$ for 5 min, and the supernatant (10 μl) was used for the CAT assay, which serves as a positive control.

Sorghum and rice protoplasts were transformed with plasmids carrying the CAT gene fused to either the CaMV 35S promoter or the *Drosophila* transposable element (*copia*) promoter (Fig. 4; Table 2). A high level of CAT gene expression in electroporated sorghum protoplasts was observed when plasmids containing either CaMV promoter or *copia* promoter were used (Fig. 4*A* and Table 2, Exp. A, lanes c and d). Both chimeric plasmids (p35S-CAT and pCopia-CAT) were also expressed in rice leaf protoplasts electroporated at 7.5 kV, 62.5 μsec for 10 or 30 pulses (Fig. 4*B* and Table 2, Exp. B). In protoplasts from rice suspension culture, both CaMV and *Drosophila* promoters showed high levels of CAT gene expression (Fig. 4*C* and Table 2, Exp. C). Duplicate electroporated samples at 10 kV, 20 pulses showed repeatable levels of expression (lanes d and e). No CAT activity was expressed using either *copia* plasmid without the CAT gene or pBR325 plasmid containing the CAT gene but without a eukaryotic promoter (lanes b and a).

Although cultured cells of *Drosophila* did not have endogenous background (data not shown), all the plant species that we have tested (rice, wheat, sorghum, and tobacco) contained low levels of endogenous CAT activity without foreign gene introduction (Table 1; data for rice and tobacco not shown). Thus, in CAT assays with plant tissues, it is critical to include tests of endogenous CAT activity. In a previous report (10), CAT activity was enhanced by heating extracts to 65°C for 10 min. Heating presumably inactivated an inhibitor. We found, by mixing protoplast extracts with extracts of *E. coli* harboring pBR325, that protoplast extracts inhibited CAT activity about 4–13%. However, heat treatment (65°C, 5 min) partially inactivated CAT activity as well as the inhibitor and thus was not used in our routine assay procedure.

DISCUSSION

Our results show that electric pulse-mediated DNA uptake and expression can be applied to several important graminaceous species. The ease and convenience of this system will allow rapid evaluation of potential vectors for monocot transformation and for delineating important regions of plant promoters. It is interesting that, even though *Drosophila* and plants diverged some 900 million years ago (25), the *Drosophila* promoter (*copia*) not only directs synthesis of functional CAT enzyme in plant cells but is as effective as one of the strongest promoters (CaMV 35S) known to function in plants. The DNA sequences required for the functioning of *Drosophila* promoter in plants is not known. Although we found (19, 21) two short homologous sequences (TATAA and GTTGAA) in the 5′ noncoding regions of both the CaMV

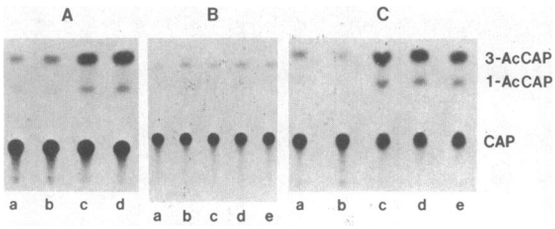

FIG. 4. Expression of the CAT gene linked to different promoters. Electroporation was carried out with a Baekon pulse generator with pulse duration of 62.5 μsec. Protoplast extract volume was 200 μl in all cases. Incubation and CAT assays were the same as described for Fig. 3; autoradiographs were exposed for 12 hr (*A*), 24 hr (*B*), and 12 hr (*C*). For description of experiments and quantitation of results see Table 2.

(35S) and *Drosophila* (*copia*) promoters, their significance remains to be determined.

A previous report showed that either the *Drosophila hsp70* gene or its promoter inserted into a chimeric gene could be expressed in tobacco (26). However, in the heat shock system the promoter regions in *Drosophila* and plants were known to have similar consensus sequences. Thus, the present results show that two widely different promoters (35S and *copia*) are both functional in plant systems.

We found that, to obtain high expression levels, protoplasts must be prepared from rapidly growing suspension culture cells. Incubation of tissues with protoplast-releasing enzymes for longer than 6 hr seldom yielded successful transformations.

Both FH-GCA and Baekon electroporators generate square-wave electrical pulses of defined duration (pulse length), thus allowing experiments to be repeated precisely. In contrast, pulse length of the capacitor-discharging system (10) is more difficult to define because the discharge curve changes with the concentration of electrolytes in the buffer and with the voltage. The pulse length of the FH-GCA and Baekon system is in the range 50–100 μsec, which is comparable to other electroporation systems used to introduce DNA into plant and animal cells (27–29). Such short pulse lengths avoid sample heating, which has been reported in systems using much longer (100 msec) pulse length (10). Moreover, in the Baekon system, the electroporation (e.g., 120 pulses, 62.5 μsec each) can be completed within 0.2 sec, and therefore there is no problem with settling of protoplasts.

The optimal field strength of the FH-GCA system was 2.5 kV/cm. This is comparable to field strengths used in other plant cell transformation systems (7, 27, 28, 30). However, animal cells, which usually are smaller than plant cells, required slightly higher field strengths (12, 13, 31). The intensity of electrical pulse (voltage, pulse length) and pulse number required for transformation of protoplasts were inversely proportional to protoplast diameter (32). Consistent with this, we found that rice protoplasts (17–25 μm) required a higher electrical intensity than sorghum protoplasts (30–50 μm) (data not shown).

In this paper, we describe successful transformation of rice protoplasts. Since rice is an important crop plant, we believe that this is a significant finding. Using this simple transformation procedure, it is now possible to study in depth the expression of rice genes in a homologous system. For example, one can modify the noncoding regions of important rice genes, ligate them to the CAT gene, and use them to transform rice protoplasts. This will allow the precise identification of regulatory sequences. It is possible to regenerate stable cell lines and calli from rice protoplasts (33–35), which provides us with the opportunity to study the stable integration and long-term expression of genes introduced into rice. Successful plant regeneration from protoplast-derived callus of rice has been achieved (36). Thus, it may now be possible to study, at the whole plant level, the stable inheritance and expression of genes, including those of agronomic interest, introduced into rice protoplasts.

Table 2. CAT gene expression by p35S-CAT or pCopia-CAT in sorghum and rice protoplasts (see Fig. 4)

Lane	Plasmid	Voltage, kV	No. of pulses	CAT assay products* cpm	%
	Exp. A. Sorghum suspension-cell protoplasts				
a	None	10	20	1,487	0.9
b	pCopia (no CAT)			1,916	1.2
c	p35S-CAT			44,022	26.6
d	pCopia-CAT			58,116	35.1
	Exp. B. Rice leaf protoplasts				
a	pCopia (no CAT)	7.5	10	1,058	0.6
b	p35S-CAT	7.5	10	10,584	6.0
c	p35S-CAT	7.5	30	6,526	3.7
d	pCopia-CAT	7.5	10	10,584	6.0
e	pCopia-CAT	7.5	30	5,997	3.4
	Exp. C. Rice callus protoplasts				
a	pBR325	10	20	2,116	1.2
b	pCopia (no CAT)			1,058	0.6
c	p35S-CAT			67,384	38.2
d	pCopia-CAT			80,262	45.5
e	pCopia-CAT			71,916	40.8

The Baekon pulse generator (62.5-μsec pulse length) was used for electroporation. The amount of plasmid used was 20 μg/ml in all experiments, except in Exp. A, lane a, in which no plasmid was used. See legend to Fig. 4 for other conditions.

*See Fig. 4 for autoradiographs. Radioactivity in the two monoacetylated forms of chloramphenicol was measured by scintillation counting, and % conversion was calculated.

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