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Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors

(gene transfer/particle gun, "biolistics"/chloroplast replicon/chloramphenicol acetyltransferase/plant organelle genetic engineering)

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ABSTRACT **Expression of chloramphenicol acetyltransferase (*cat*) by suitable vectors in chloroplasts of cultured tobacco cells, delivered by high-velocity microprojectiles, is reported here. Several chloroplast expression vectors containing bacterial *cat* genes, placed under the control of either *psbA* promoter region from pea (pHD series) or *rbcL* promoter region from maize (pAC series) have been used in this study. In addition, chloroplast expression vectors containing replicon fragments from pea, tobacco, or maize chloroplast DNA have also been tested for efficiency and duration of *cat* expression in chloroplasts of tobacco cells. Cultured NT1 tobacco cells collected on filter papers were bombarded with tungsten particles coated with pUC118 (negative control), 35S-CAT (nuclear expression vector), pHD312 (repliconless chloroplast expression vector), and pHD407, pACp18, and pACp19 (chloroplast expression vectors with replicon). Sonic extracts of cells bombarded with pUC118 showed no detectable *cat* activity in the autoradiograms. Nuclear expression of *cat* reached two-thirds of the maximal 48 hr after bombardment and the maximal at 72 hr. Cells bombarded with chloroplast expression vectors showed a low level of expression until 48 hr of incubation. A dramatic increase in the expression of *cat* was observed 24 hr after the addition of fresh medium to cultured cells in samples bombarded with pHD407; the repliconless vector pHD312 showed about 50% of this maximal activity. The expression of nuclear *cat* and the repliconless chloroplast vector decreased after 72 hr, but a high level of chloroplast *cat* expression was maintained in cells bombarded with pHD407. Organelle-specific expression of *cat* in appropriate compartments was checked by introducing various plasmid constructions into tobacco protoplasts by electroporation. Although the nuclear expression vector 35S-CAT showed expression of *cat*, no activity was observed with any chloroplast vectors.**

Most strategies for gene transfer in plants involve the introduction of foreign DNA into cells or protoplasts followed by integration into the nuclear genome (1–3). However, in plants many economically important gene products (e.g., the protein conferring atrazine sensitivity or resistance) either are chloroplast encoded (4) or, if they are nuclear encoded, are functional within the chloroplasts (e.g., 3-*enol*-pyruvoylshikimate-5-phosphate synthase, which confers sensitivity or resistance to glyphosate) (5) or mitochondria (e.g., aryl acylamidase, which confers sensitivity or resistance to propanil) (6, 7). Furthermore, the 1000-fold higher copy number of chloroplast genes over nuclear genes (8–10) makes feasible the introduction of multiple copies of foreign genes into plant cells, should the foreign gene become stably established inside the chloroplasts. Engineering foreign genes (whose products are functional within organelles) through the organelle genome instead of the nuclear genome could be energy-wise economical for the cell, since synthesis and import of precursor proteins are highly energy-consuming processes (11, 12). Currently there are techniques available to target foreign gene products into chloroplasts (13) or mitochondria (14) of higher plants by way of the nuclear genome. However, a workable system for foreign gene incorporation or replacement in chloroplasts has great merit as a tool for the study of chloroplast function, regulated gene expression, or identification of unknown chloroplast genes (open reading frames) in addition to the above-mentioned advantages while engineering foreign genes.

Recently, Sanford and coworkers (15) have developed a transformation technique that relies upon bombardment of recipient cells with high-velocity tungsten microprojectiles coated with foreign DNA. Using this delivery system, Boynton *et al.* (16) and Blowers *et al.* (17) have demonstrated stable transformation of chloroplasts of *Chlamydomonas reinhardtii*. We report here transient expression of the chloramphenicol acetyltransferase (*cat*) gene in chloroplasts of cultured tobacco cells, using appropriate vectors delivered by high-velocity microprojectiles.

MATERIALS AND METHODS

Construction of Chloroplast Expression Vectors. A series of chloroplast expression vectors has been constructed using the promoter selection vector pKK232-8 (Pharmacia), which is a pBR322 derivative containing a promoterless *cat* gene. Restriction fragments of chloroplast DNA (cpDNA) containing the entire promoter region and 5′ untranslated region of the *psbA* gene from spinach (pMP450, a gift from Wilhelm Gruissem, University of California, Berkeley) pHD306 or pea (pPPBX10218, a gift from John Mullet, Texas A & M University) pHD312 or, alternatively, the *rbcL* and *atpB* promoter region from maize (pPBI1443, a gift from Antony Gatenby, E.I. DuPont de Nemours & Co., Wilmington, DE) pHD103 have been individually inserted into the multiple cloning site (MCS) that exists 5′ proximal to the promoterless *cat* gene. The strength of each promoter has been investigated by analyzing transient expression of *cat* in cucumber etioplasts, as reported earlier by Daniell and McFadden (18).

To study *cat* expression in the cytosol, a 35S-CAT construct obtained from Abdul Chaudhury (Ethan Signer's laboratory, MIT) has been used. This is a 4.2-kilobase-pair (kbp) plasmid designated pUC8CaMVCATΔN, with a *cat* gene driven by a 35S cauliflower mosaic virus promoter and flanked by a 3′ Nos *Pst* I fragment. For negative controls, pUC118 or pUC19 has been used in all experiments (19).

***In Vitro* Replication Studies.** A replication fraction containing RNA polymerase, DNA polymerase, DNA primase, and topoisomerase I activities was isolated from pea chloroplasts

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Abbreviations: CAT, chloramphenicol acetyltransferase; MCS, multiple cloning site; cpDNA, chloroplast DNA.

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as described by Meeker *et al.* (20). The heparin-Sepharose fraction was used for *in vitro* replication reactions. Insertions of a variety of replicon fragments into chloroplast expression vectors is described in *Results*.

Bombardment of Suspension Cells with Microprojectiles. To prepare tobacco NT1 cells for bombardment with microprojectiles, about 100 mg of 4-day-old suspension cells (fresh weight) was collected on filter paper (Whatman no. 1, 5.5 cm) by vacuum filtration of 5 ml of suspension culture (1×10^6 cells per ml). Two layers of filter paper were placed inside a 5.5-cm Petri dish and moistened with 1.4 ml of MS medium (31). The single filter paper bearing the cells was then placed over the two layers of filter paper. The samples were bombarded with tungsten particles coated with DNA as described by Boynton *et al.* (16). To absorb DNA to the microprojectiles, 2.5 μl of DNA (1 μg/μl of TE buffer containing 10 mM Tris·HCl and 1 mM EDTA, pH 7.7) was added to 25 μl of a suspension of tungsten particles (0.05 mg/ml of 50% glycerol) in a 1.5-ml Eppendorf tube. After addition of the DNA, $CaCl_2$ (25 μl of a 2.5 M solution) and spermidine free base (5 μl of a 1 M solution) were added to the suspension. After 10 min of incubation, the particles were pelleted by centrifugation in a Microfuge for 30 sec, and a portion of the supernatant (25 μl) was removed. The final microprojectile preparation therefore contained 39 μg of tungsten per μl of suspension and 2 μg of DNA per μg of tungsten. The clumps of particles were dispersed by briefly (1 sec) touching the outside of the Eppendorf tube to the probe of a sonicator. After sonication, 5 μl of the tungsten/DNA suspension was placed on the front surface of a cylindrically shaped polyethylene macroprojectile. The macroprojectile was then placed into the barrel of the particle gun and accelerated. Cells were bombarded 10 cm from the end of the barrel of the particle gun. The pressure in the sample chamber was reduced to 0.1 atmosphere prior to bombardment. In all experiments, three replicate Petri plates were bombarded per treatment. Bombarded cells were shipped to the University of Idaho by Federal Express. After the addition of fresh growth medium, the cells were maintained at 26°C in the light, in plant growth chambers.

Analysis of *cat* Expression in NT1 Cells and Protoplasts. Cultured tobacco cells were transferred to Corex tubes and washed once with 10 ml of TE buffer containing 250 mM Tris·HCl and 10 mM EDTA (pH 7.8). Cells centrifuged at 8000 × *g* for 10 min were transferred to 2-ml Eppendorf tubes and resuspended in 1 ml of TE buffer (pH 7.8) containing 2 mM phenylmethylsulfonyl fluoride. The cells were sonicated twice for 20 sec each, using a probe sonicator. After a 15-min centrifugation at 4°C, the supernatants were transferred to new Eppendorf tubes and assayed for *cat* activity as reported earlier by Daniell and McFadden (18). NT1 protoplasts were prepared and electroporated as described by Paszty and Lurquin (21) except the 250 μF capacitor was charged to 250 V and 1.5 million protoplasts were used per electroporation event.

RESULTS

Expression of Foreign Genes in Isolated Chloroplasts. The cucumber etioplast *in organello* transient expression system (18, 22) was used to test the strength of chloroplast promoter fragments inserted into the MCS 5′ proximal to the promoterless *cat* gene (Fig. 1). Transient expression of *cat* in EDTA-washed etioplasts isolated from hormone-pretreated cucumber cotyledons revealed spinach or pea *psbA* promoter (pHD306, pHD312) to be the strongest among the promoter fragments tested (data not shown). Since expression studies after bombardment of foreign DNA were planned to be conducted in nonphotosynthetic cells, transient expression system in etioplasts served as an analogous comparable system to the plastids present in cultured tobacco cells.

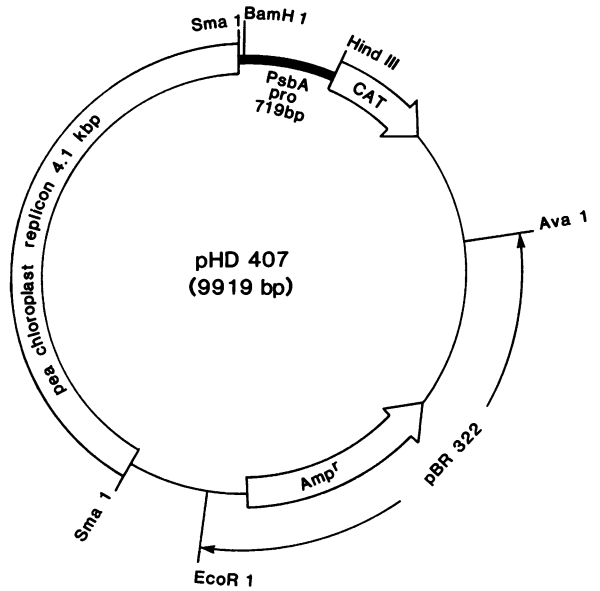

FIG. 1. The plasmid pHD407 carries a 4.1-kbp *Sma* I fragment insertion containing the origin of replication (D loop) from pea cpDNA inserted into pHD312. The plasmid pHD312 contains the entire promoter and 5′ untranslated region of the pea *psbA* gene inserted 5′ proximal to the promoterless *cat* gene present in the promoter selection vector pkk232-8.

***In Vitro* Replication of Chloroplast Expression Vectors.** Tewari and coworkers (20) have recently mapped two replication origins in pea cpDNA by electron microscopic analysis. Both of the origins of replication, identified as displacement loops (D loops), were found to be highly active in DNA synthesis when used as templates in a partially purified replication system from pea chloroplasts (20, 23). Plasmid construction pCB1-12 is a 10-kbp *Bam*HI pea cpDNA fragment in pBR322, containing both D-loop regions. The plasmid pCPH5.6 is a 5.6-kbp *Pst* I–*Hin*dIII cpDNA fragment in pUC19 containing one of the two D-loop regions. The plasmid pCS4.1 is a 4.1-kbp *Sma* I cpDNA fragment in pUC19 containing one of the two D-loop regions. The plasmid

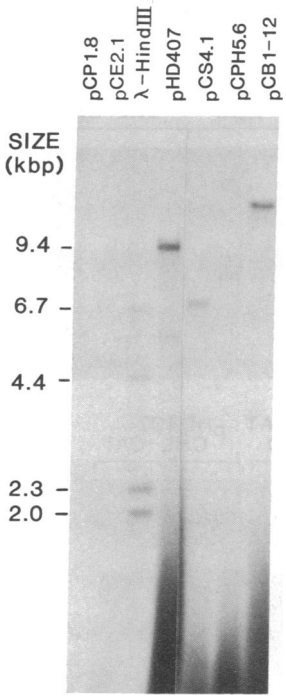

FIG. 2. Alkaline agarose gel of *in vitro* replication products. *In vitro* replication reactions were carried out as described in ref. 20. After phenol extraction and ethanol precipitation, the samples were separated in a 0.8% alkaline agarose gel, and the gel was dried and autoradiographed. Phage λ DNA markers were digested with *Hin*dIII and 5′ end-labeled with [α-^{32}P]ATP and polynucleotide kinase.

pCE2.1 is a 2.1-kbp *Eco*RI cpDNA fragment from the region between the two replication origins. Plasmid pCP1.8 is a 1.8-kbp *Pst* I clone from a distant region of the cpDNA. All of these constructions were used as templates for *in vitro* DNA synthesis using a replication fraction isolated from pea chloroplasts, and the *in vitro* replication products were analyzed on alkaline agarose denaturing gels. As seen in Fig. 2, the autoradiogram showed the lack of synthesis of any full-size DNA molecule in pCP1.8 and pCE2.1, which do not contain any D-loop region. On the other hand, the autoradiogram clearly showed *in vitro* synthesis of single-stranded DNA molecules of about 14.5 and 6.3 kbp, corresponding to full-length pCB1-12 and pCS4.1, respectively. The 4.1-kbp *Sma* I fragment in pCS4.1, found to be active in *in vitro* DNA synthesis, was inserted into the chloroplast expression vector pHD312, which contains pea *psbA* promoter 5′ proximal to the *cat* gene. The resultant construction, pHD407, when analyzed for *in vitro* DNA synthesis, appeared as a single-stranded DNA molecule of about 9.9 kbp in an alkaline agarose denaturing gel (Fig. 2).

Expression of *cat* in Tobacco NT1 Suspension Cells. Expression of *cat* was assayed in sonic extracts incubated in the presence of [*dichloroacetyl*-1-^{14}C]chloramphenicol and acetyl coenzyme A. Sonic extracts of cells bombarded with pUC118 showed no detectable *cat* activity in the autoradiograms; cells bombarded with 35S-CAT showed maximal expression 72 hr after bombardment, whereas those bombarded with pHD407 and pHD312 showed a low level of expression until 48 hr of incubation (Fig. 3*A*). A dramatic increase in the expression of *cat* was observed 24 hr after the addition of fresh medium to cultured cells in samples bombarded with pHD407. The repliconless chloroplast expression vector pHD312 showed the maximal activity at 72 hr of incubation, which is about 50% of the activity observed with pHD407 at that time point (Fig. 4). A high level of *cat* expression was maintained with subsequent incubation of cultured cells bombarded with pHD407, whereas the expression of nuclear *cat* and the repliconless chloroplast vector sharply declined. Quantitative studies confirmed the earlier visual observation (Fig. 4). These results suggest that the "biolistic" process delivered foreign DNA into chloroplasts of cultured tobacco cells and that the marker gene is expressed in appropriate compartments. The kinetics of *cat* activity, as a function of incubation time after bombardment, may be interpreted as an indication that the delivery process into the chloroplast is not as efficient as into the nucleus since

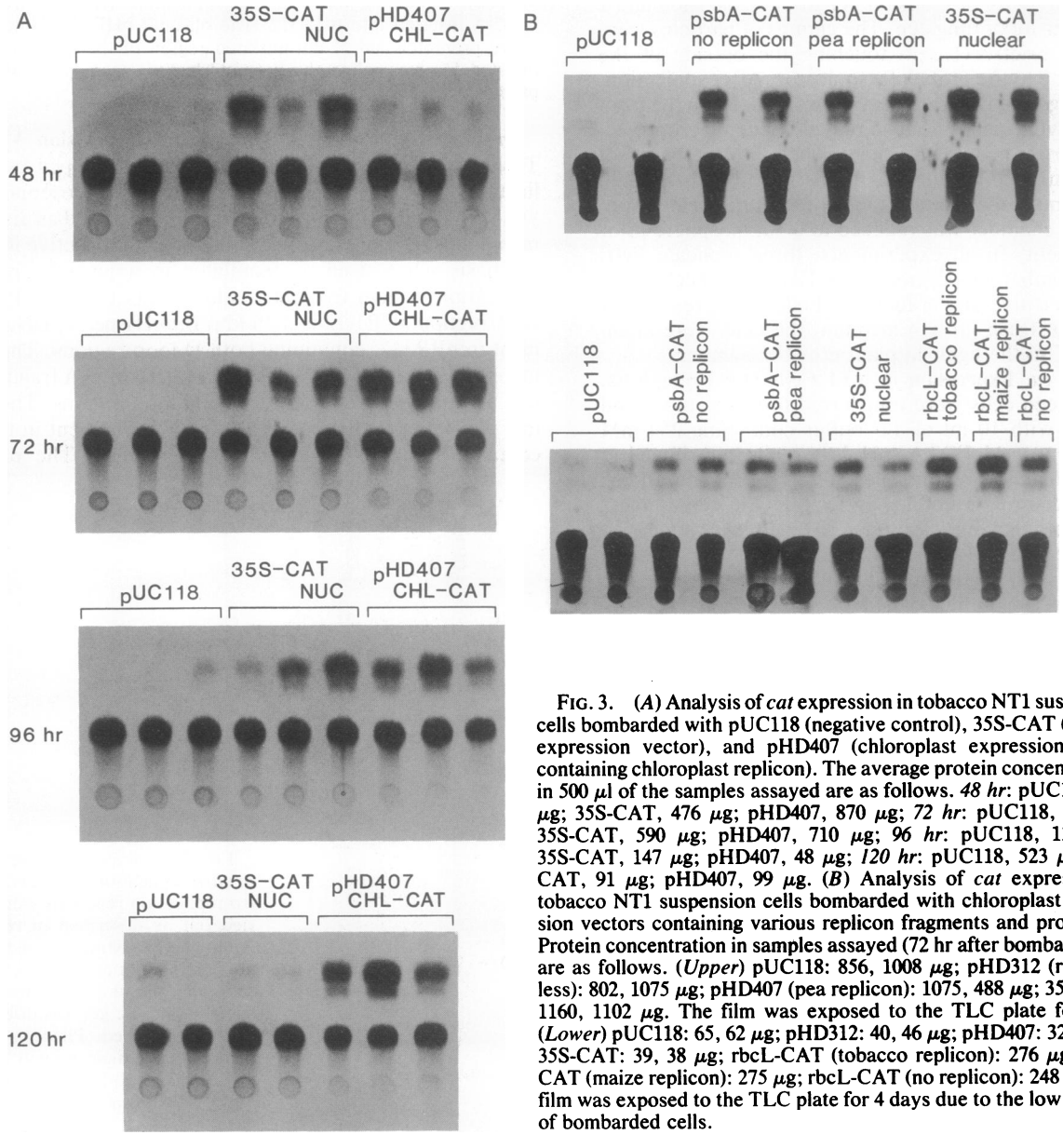

FIG. 3. (*A*) Analysis of *cat* expression in tobacco NT1 suspension cells bombarded with pUC118 (negative control), 35S-CAT (nuclear expression vector), and pHD407 (chloroplast expression vector containing chloroplast replicon). The average protein concentrations in 500 μl of the samples assayed are as follows. *48 hr*: pUC118, 900 μg; 35S-CAT, 476 μg; pHD407, 870 μg; *72 hr*: pUC118, 787 μg; 35S-CAT, 590 μg; pHD407, 710 μg; *96 hr*: pUC118, 1360 μg; 35S-CAT, 147 μg; pHD407, 48 μg; *120 hr*: pUC118, 523 μg; 35S-CAT, 91 μg; pHD407, 99 μg. (*B*) Analysis of *cat* expression in tobacco NT1 suspension cells bombarded with chloroplast expression vectors containing various replicon fragments and promoters. Protein concentration in samples assayed (72 hr after bombardment) are as follows. (*Upper*) pUC118: 856, 1008 μg; pHD312 (repliconless): 802, 1075 μg; pHD407 (pea replicon): 1075, 488 μg; 35S-CAT: 1160, 1102 μg. The film was exposed to the TLC plate for 8 hr. (*Lower*) pUC118: 65, 62 μg; pHD312: 40, 46 μg; pHD407: 32, 26 μg; 35S-CAT: 39, 38 μg; rbcL-CAT (tobacco replicon): 276 μg; rbcL-CAT (maize replicon): 275 μg; rbcL-CAT (no replicon): 248 μg. The film was exposed to the TLC plate for 4 days due to the low number of bombarded cells.

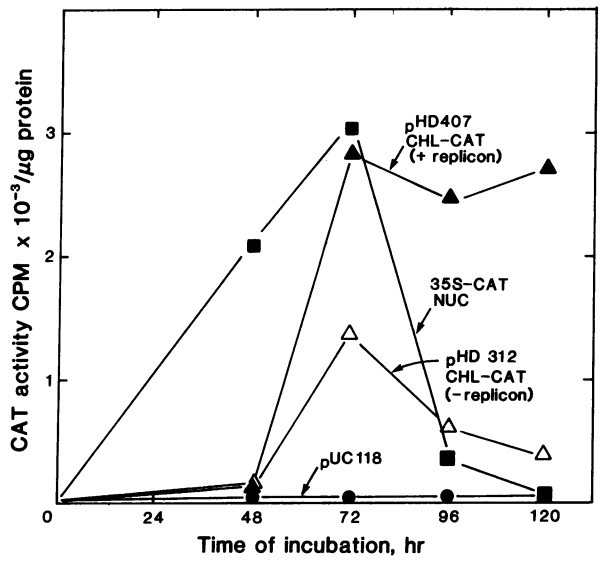

FIG. 4. Quantitative expression of *cat* in tobacco NT1 suspension cells assayed with identical protein concentrations in sonic extracts. After autoradiography of the separated acetylated chloramphenicol forms for 4 hr, spots were scraped and radioactivity was counted. Silica gel alone had a background of 2203 cpm; the ranges of cpm between different samples were as follows: pUC19, 2705–6556 cpm; 35S-CAT, 3993–220,353 cpm; pHD312, 2410–133,240 cpm; pHD407, 7484–267,364 cpm.

expression of all chloroplast vectors is delayed until 72 hr. Chloroplast vectors with the replicon (pHD407) may subsequently replicate autonomously inside the chloroplasts, resulting in a higher level of expression. However, the contribution of the upstream promoter sequences of 23S rRNA gene, present in the replicon fragment in pHD407, to enhanced transcription/translation of the *cat* gene cannot be ruled out.

These observations were subsequently confirmed by investigations using similar chloroplast expression vectors provided by L. Bogorad's laboratory. Expression of *cat* was studied in NT1 cells bombarded with vectors containing replicon inserts from tobacco and maize chloroplast genomes (Fig. 3*B*). Tobacco Bam4 cpDNA fragment was cloned into pGV825 (a Ti plasmid intermediate vector) by Alan Blowers (pACp18); this fragment cloned into pUC supports DNA synthesis *in vitro* using the replication system described by Carrillo and Bogorad (24). Maize Bam10 fragment was cloned into pGV825 by Alan Blowers (pACp19); this fragment cloned into pBR322 is not especially active in the *in vitro* DNA synthesis assay of Tewari and co-workers (25) but functions as an autonomously replicating sequence in yeast (when cloned into YIP5). The repliconless vector showed 0.74×10^3 cpm *cat* activity per μg of protein in sonic extract of cells 72 hr after bombardment; vectors containing replicon fragments from tobacco and maize showed 1.03 and 1.45×10^3 cpm per μg of protein, respectively. In all of these constructs, the bacterial *cat* gene is under the control of an *rbcL* promoter region from maize.

Sterility tests to ensure absence of microbial contamination of the cultures were performed by streaking an aliquot of cells or protoplasts on LB agar plates, for every time point, after bombardment (at Cornell University) or prior to sonication (at University of Idaho). Among the *cat* assays presented in Fig. 3*A*, two contaminants were noticed. These samples were discarded and hence fewer *cat* assays have been presented for the last time point. No bacterial contamination has been observed so far in other batches of bombarded cells. Expression studies have been performed in six different batches of cells, varying several parameters.

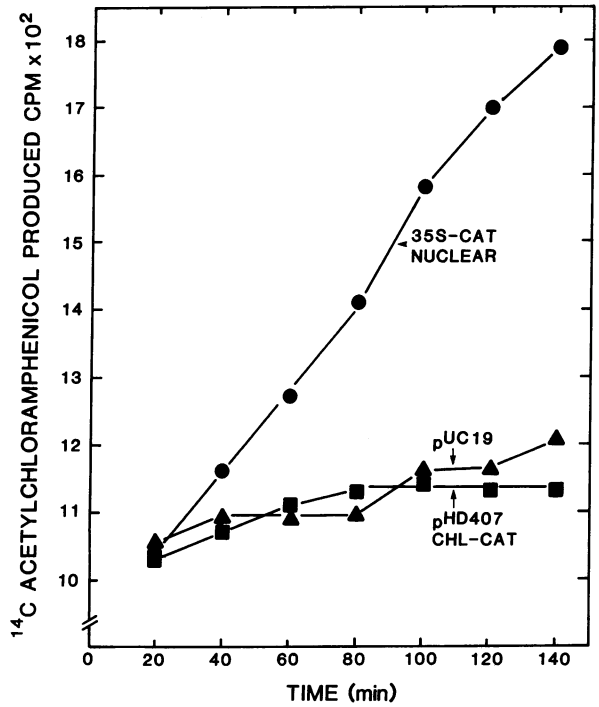

FIG. 5. Kinetics of chloramphenicol acetylation with [^{14}C]acetyl CoA, in tobacco NT1 protoplasts electroporated with foreign DNA. Pelleted protoplasts were resuspended in (400 μl) extraction buffer (5 mM EDTA/0.25 M Tris·HCl, pH 7.8, and 1.0 μg each of antipain and pepstatin per ml), sonicated for 20 sec, and centrifuged in a Microfuge for 5 min at 4°C to pellet the debris. The extract (190 μl) after heat treatment (65°C, 10 min) was mixed with [^{14}C]acetyl CoA (0.1 μCi; 1 Ci = 37 GBq) and chloramphenicol (40 μl of 8 mM stock). The reaction was carried out at 37°C using the highly quantitative two-phase assay system described by Neumann *et al.* (26). Slope values derived from data points and DNA concentrations used for electroporation were as follows: pUC19, 31.0 (15 μg); pHD407, 24.2 (50 μg); 35S-CAT, 114.1 (15 μg). The correlation coefficient varied between 0.9 and 0.99 for different sets of experiments.

Organelle-specific expression of *cat* in appropriate compartments was checked by introducing all three plasmid constructions, pUC19, 35S-CAT, and pHD407, into tobacco protoplasts by electroporation (Fig. 5) and assayed using the highly quantitative two-phase assay system (26). Although 35S-CAT showed expression of *cat*, no activity was observed with pUC19 and pHD407, establishing the fact that these two constructions do not express in the nucleus. Furthermore, recent attempts to induce tobacco *psbA* promoter to function in the nuclear compartment (to study transient expression of *bar* or *npt* II genes) revealed the absolute need to insert 35S promoter or enhancer elements 5′ proximal to the *psbA* promoter region (27). Bogorad and co-workers also observed that chloroplast genes were not transcribed from their own promoters when placed in the nuclei of trangenic tobacco plants (13). It is also known that chloroplast genes are not expressed in mitochondria because of the differences in transcriptional and translational control signals and genetic code between the two organelles (28). So far, we have not succeeded in observing the expression of *cat* gene (driven by mitochondrial *atp9* promoter) when bombarded into cultured tobacco cells (unpublished observation).

DISCUSSION

Several factors might have contributed to the continued maintenance of a relatively higher level of *cat* expression in cells bombarded with autonomously replicating chloroplast expression vectors. It has been recently reported that in

cultured tobacco cells, copy number of plastid DNA per cell increased 11-fold within 1 day after the addition of fresh medium; replication of plastids was most frequently observed on the second day (29). Therefore, addition of fresh medium to bombarded cells might have enhanced replication of introduced plasmids inside the chloroplasts. Our studies on *in vitro* replication of pHD407 support this hypothesis (see Fig. 2). In addition, cultured tobacco cells are known to lodge immature chloroplasts, which may be ideal for studies on foreign gene expression as indicated by our earlier studies (18).

In summary, the present study suggests that it is possible to introduce foreign DNA into chloroplasts using high-velocity microprojectiles and observe gene expression, based on the facts that it is neither bacterial nor nuclear as judged by sterility tests and electroporation experiments. To establish expression of foreign genes in appropriate cellular compartments, direct evidence should be provided from CAT assays performed with purified chloroplasts, mitochondria, and cytosolic fraction without the organelles. Experiments are necessary to isolate organelles from tobacco cells bombarded with foreign DNA, after protoplasting or by using a "Bead-Beater." Parallel studies are also necessary to test replication of chloroplast vectors *in vivo*. We have optimized conditions for permeabilization of tobacco cells with L-α-lysophosphatidylcholine. The permeabilized cells are capable of uptake and incorporation of deoxynucleoside triphosphates into DNA, as reported by Weissbach and coworkers (30). This may be an ideal system to study *in vivo* replication of foreign DNA because of the fact that [^{32}P]TTP gets incorporated only into organellar DNA (30). Analysis of [^{32}P]TTP-incorporated, newly synthesized DNA on alkaline agarose gels, isolated from tobacco cells bombarded with foreign DNA, should unambiguously establish foreign DNA replication in the plastids of recipient cells. The transient expression system, reported here, should facilitate studies on foreign gene expression, regulation, or DNA replication in plastids *in vivo*. Thus, an approach may be opened to major advances in genetic engineering of higher plant organelles.

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