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Introduction of Exogenous DNA into *Chlamydomonas reinhardtii* by Electroporation

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The fate of exogenous DNA introduced into *Chlamydomonas reinhardtii* by electroporation was analyzed. With single and double electrical pulses, plasmids as large as 14 kb were introduced into cells with and without intact cell walls. Within hours after introduction, exogenous plasmid DNA was associated with nuclei isolated from cells; several weeks after introduction, exogenous DNA was stably integrated into the *Chlamydomonas* genome. These studies establish electroporation as a method for introducing DNA, and potentially other molecules, into *C. reinhardtii*.

Direct gene transfer is an ideal way to study many aspects of gene regulation. By making changes in the genes of interest in vitro and testing the effects of these changes in vivo, regions that are important in transcriptional and post-transcriptional regulation can be delineated. The unicellular green alga *Chlamydomonas reinhardtii* is particularly amenable to such studies of the interrelationships of nucleus- and chloroplast-encoded genes in photosynthetic systems and gene regulation during flagellar biogenesis. Because the chromatin organization and nucleosome composition of the relatively small *Chlamydomonas* genome are very similar to those of higher plants and animals (14), studies of the fundamental properties of eukaryotic chromatin organization and gene expression are also possible. Until recently, *Chlamydomonas* cells have been difficult to transform stably (8, 16). However, both chloroplast (2) and nuclear (5, 11) transformations have been reported with a "biolistic" delivery system, and nuclear transformation by vortexing cells in the presence of DNA and glass beads has been successful (6, 10).

We expected that transformation of *C. reinhardtii* by electroporation would also be feasible because this method has been used successfully for macromolecular transfer into a wide variety of cell types. Electroporation, or reversible electrical membrane breakdown, involves exposing cell membranes to electric field pulses of high intensity for short durations (15, 22). Electroporation has been used to induce uptake of macromolecules (including DNA, RNA, and proteins) and molecules (including nucleotides, dyes, and ions) into cell types ranging from prokaryotes and lower eukaryotes to higher plant and animal cells (3, 12, 13, 15, 19, 21). Several factors influence the success of electroporation, including field strength, pulse length, medium composition and temperature, and the membrane characteristics of the particular cell type (22). In this report, electroporation parameters that induce uptake of exogenous DNA from the medium into *C. reinhardtii* are delineated. The results represent a step in developing systems for transient and stable expression of exogenous genes within this versatile organism.

Because transformation of other cell types by electroporation is accomplished at field strength and pulse length conditions resulting in less than 50% cell viability, our initial experiments determined the electroporation conditions resulting in less than 50% survival of *C. reinhardtii*. For these experiments, mid-log-phase cells grown under a 14-h light–10-h dark cycle with aeration in liquid minimal growth medium (M.I of Sager and Granick [17]) were pelleted at 1,000 × *g* for 10 min, washed once with M.I, suspended at 2 × 10^6 cells per ml in 150 mM KHME (150 mM KCl, 30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.6], 1 mM $MgCl_2$, 0.5 mM EDTA-KOH [pH 8.0]), transferred to a sterile electroporation chamber in which aluminum electrodes were spaced 0.4 cm apart, and electroporated with exponentially decaying pulses of various lengths and field strengths with a Cell-Porator (Bethesda Research Laboratories). When given, double consecutive pulses were delivered at intervals of 10 to 15 s. Electroporated samples were incubated on ice for 5 min in their electroporation chambers and for an additional 5 min after addition of 0.5 ml of M.I. Survival was estimated by either a plating assay or a dye retention assay (Fig. 1). The range of pulse lengths and field strengths, between 17.9 ms at 750 V/cm and 26.4 ms at 1,000 V/cm, achieved 10 to 50% viability of cell wall-less, CW-92, mt^+ (4) cells (Fig. 1A and B). Electroporation of wild-type, walled cells with single or double 26.4-ms pulses at 1,000 V/cm resulted in 15 to 40% viability (Fig. 1C). Settings within these ranges were chosen for further experiments.

To examine whether these electroporation conditions also induced uptake of exogenous DNA, total nucleic acids from cells electroporated with plasmid pCaMVCAT (7) were analyzed on Southern blots. After electroporation, cells were incubated as described above and then diluted into 5 ml of M.I. After 4 h of growth, cells were washed three times in fresh M.I, lysed by addition of lysis buffer (2% sodium dodecyl sulfate, 0.3 M NaCl, 5 mM EDTA-KOH [pH 8.0], 50 mM Tris-Cl [pH 8.0], 0.04 mg of proteinase K per ml), extracted twice with phenol-chloroform and once with chloroform, and precipitated with ethanol by standard procedures (18). Samples were digested with restriction endonuclease *Bgl*I (2 U/μg of nucleic acids), RNase A (0.15 μg/ml), and RNase T_1 (50 U/ml) for 2 h at 37°C and electrophoresed on horizontal submarine gels containing 1% agarose, 40 mM Tris-acetate, and 1 mM EDTA, pH 8.0. Southern blots were prepared and hybridized under standard conditions (18). The presence of a band corresponding to the size of plasmid pCaMVCAT DNA in samples of electroporated cells indicated that the plasmid had entered both cell types (Fig. 2).

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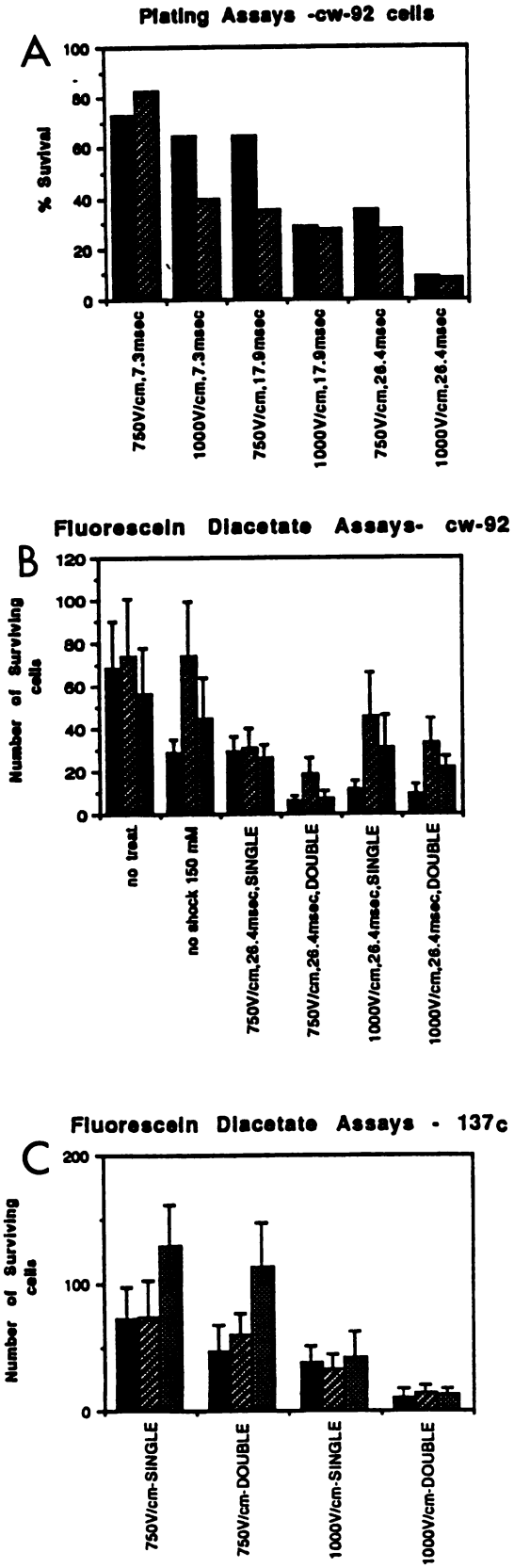

FIG. 1. Cell viability after electroporation. (A) Cell survival estimated by a plating assay, in which aliquots of the cell suspensions were either plated after inoculation into 3 ml of 0.75% top agar held at 45°C or spread directly onto 1.5% agar plates containing either M.I (assay 1) or M.I containing five times the normal phosphate buffer and 22 mM sodium acetate (assay 2). After 7 to 8 days of growth, the surviving colonies were counted and expressed as a percentage of the number of colonies formed by nonelectroporated cells. (B) Cell survival estimated by a dye retention assay (20), in which cells were electroporated, incubated on ice, and diluted as described in the text. After dilution, the cells were pelleted and resuspended in 0.25 ml of M.I; 0.25 ml of a 0.01% fluorescein diacetate solution (prepared by diluting a 5-mg/ml acetone solution [20] into M.I) was added, and the samples were incubated on ice for an additional 20 min. The total number of fluorescing cells in an aliquot of the stained cell suspension was counted under a Nikon Microphot-FX fluorescence microscope (B cube). Three aliquots were counted for each sample, and the means and standard deviations were calculated. Single and double refer to the number of consecutive pulses received by each sample. Starting numbers of cells for assays 1, 2, and 3 were 4×10^6, 3×10^7, and 6×10^7 cells per ml, respectively. (C) Dye retention assays were performed as in panel B with wild-type cells (strain 137c, mt^-) with intact cell walls. Starting cell numbers for assays 1, 2, and 3 were 3×10^7, 2×10^7, and 3×10^7 cells per ml, respectively. Bars: Solid, assay 1; hatched, assay 2; stippled, assay 3.

Single 26.4-ms pulses at 1,000 V/cm were sufficient to introduce DNA into wall-less cells (Fig. 2A and B), whereas two 26.4-ms pulses at 1,000 V/cm were required to introduce DNA into a wild-type strain of cells with intact cell walls (Fig. 2C and D). The exogenous plasmid was easily identified whether digested (Fig. 2A and B) or not digested (Fig. 2C and D) with restriction endonucleases. The band of >23,000 kb in the stained gel of Fig. 2C is *C. reinhardtii* cellular DNA. The heterogenously sized low-molecular-weight material in the stained gels and autoradiograms was not digested by RNases and is apparently degraded plasmid DNA.

To demonstrate that the electroporated plasmid DNA was inside the cells and not simply adherent to the cells in a manner resistant to our washing conditions, a different-sized plasmid, pSP64, was added directly to all of the samples shown in Fig. 2A and B after electroporation. A band corresponding to the size of plasmid pSP64 was not detected on the stained gel or autoradiograph, indicating that the washing conditions removed extracellular DNA and that the plasmid pCaMVCAT was intracellular.

C. reinhardtii wild-type cells were electroporated in the presence of plasmids of 3, 5.9, or 12 kb in order to determine whether there is an upper limit to the size of plasmid DNA that can be introduced. DNAs of all three sizes were introduced into $137c^-$ cells with intact cell walls (Fig. 3) and also into cells of the wall-less strain (not shown). In addition, a larger plasmid of 14.5 kb was also introduced by electroporation into both strains (Table 1). Cells held on ice immediately after electroporation took up slightly more exogenous DNA than cells incubated at room temperature after electroporation (Fig. 3 and other experiments not shown). Cells electroporated and diluted in the absence of plasmid DNA were capable of taking up exogenous DNA added to the medium even after 10 min of incubation on ice (not shown).

To investigate the fate of the introduced plasmid DNA, cells were grown for 3, 24, or 48 h after electroporation, and total nucleic acids were analyzed on Southern blots (Fig. 4). Each sample was treated with DNase-free RNases A and T_1,

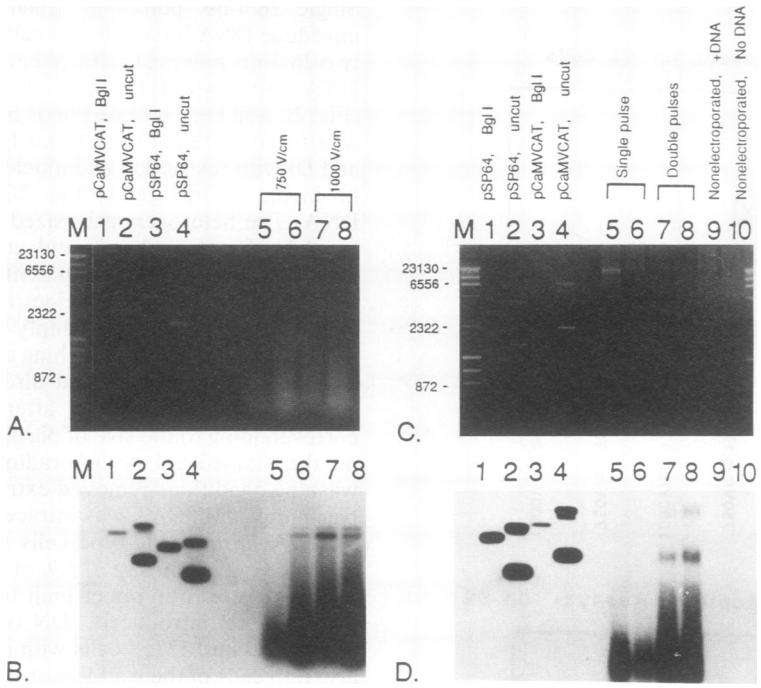

FIG. 2. Conditions for uptake of plasmid DNA. (A) Ethidium bromide-stained, 1% agarose gel shows DNA from *cw-92* cells electroporated with 10 μg of plasmid pCaMVCAT. Cells in lanes 5 and 6 were electroporated at 750 V/cm for 26.4 ms; cells in lanes 7 and 8 were electroporated at 1,000 V/cm for 26.4 ms. At 10 min after electroporation, 10 μg of a second plasmid, pSP64, was added to the medium. As markers for electrophoretic migration, lane 1 shows plasmid pCaMVCAT DNA digested with *BglI*, lane 2 shows undigested plasmid pCaMVCAT DNA, lane 3 shows plasmid pSP64 DNA digested with *BglI*, and lane 4 shows undigested plasmid pSP64 DNA. For lanes 5 through 8, 5 μg of the total sample was analyzed. Sizes are shown in bases. (B) Autoradiograph of the Southern blot of panel A was hybridized with nick-translated pCaMVCAT plasmid DNA. The presence of a band corresponding to linearized pCaMVCAT in lanes 5 to 8 shows that plasmid DNA entered the cells after electroporation. The absence of a band corresponding to linearized pSP64 shows that the washing conditions removed exogenous plasmid DNA from the medium and outside the cells. Lanes 1 to 4 show significant cross-hybridization between the pCaMVCAT and pSP64 plasmids. (C) Ethidium bromide-stained, 1% agarose gel shows DNA from cells of the $137c^-$ strain electroporated with pCaMVCAT. Conditions were as in panel A, except that no second plasmid was added to the samples in lanes 5 to 8. Samples in lanes 5 and 6 received a single 26.4-ms pulse, while those in lanes 7 and 8 received two consecutive 26.4-ms pulses at 1,000 V/cm. In lane 9, 10 μg of plasmid pSP64 was added to the medium of a nonelectroporated sample after 10 min of incubation at room temperature. Lane 10 shows a nonelectroporated sample to which no DNA was added. Total nucleic acids were isolated from the cells, and 2.4 μg of each sample was electrophoresed without digestion on a 1% agarose gel containing ethidium bromide. Lane 1 shows pSP64 digested with *BglI*, lane 2 shows undigested pSP64, lane 3 shows pCaMVCAT digested with *BglI*, and lane 4 shows undigested pCaMVCAT. (D) Autoradiograph of the Southern blot of the gel in panel C after hybridization with a mixture of nick-translated pSP64 and pCaMVCAT plasmid DNAs. Because samples were not digested with *BglI* prior to electrophoresis, plasmid bands in the samples from electroporated cells comigrated with uncut pCaMVCAT plasmid DNA. The absence of bands corresponding to plasmid pSP64 in lanes 9 and 10 shows that the washing conditions removed exogenous plasmid outside the cells.

so that hybridization intensities were proportional to the fraction of plasmid remaining in cells at the times after electroporation. Our results demonstrate that the amount of detectable plasmid DNA decreases rapidly during the first 24 h after electroporation. The "smear" of degraded DNA below the definitive plasmid bands was most apparent in the samples collected at 3 h and was diminished at the 24- and 48-h time points, indicating that much of the plasmid DNA entering the cells is quickly degraded. Of the exogenous DNA that persisted for 24 and 48 h after introduction, most had been converted from supercoiled to relaxed circular form; little of the DNA was in linear form. High-molecular-weight forms of exogenous DNA were not detected in these samples, suggesting that long concatemers of plasmid DNA did not form within 48 h after electroporation. In addition, a portion of the introduced DNA was associated with the crude nuclear fraction of the cells (Fig. 4, lanes 4 and 7).

To determine whether exogenous DNA was expressed after stable integration into the *Chlamydomonas* genome, strains of cells lacking nitrate reductase activity, the *nit1-305* mutant and *cw nit1-305* double mutants, were electroporated with plasmid pMN24 (11), which contains genomic sequences encoding the inducible *Chlamydomonas* nitrate reductase gene, used here as a selectable marker. The *nit1-305* mutant has a stable phenotype (reversion frequency, $<10^{-8}$) and requires a source of reduced nitrogen for growth. After electroporation, cells were washed with selective medium in which KNO_3 was substituted for NH_4NO_3, referred to as M.I(KNO_3) medium (17), inoculated into 0.75% top agar, and plated onto 1.5% washed agar plates containing M.I(KNO_3). Electroporated cells capable of growth under these conditions presumably express the introduced nitrate reductase gene.

Cells capable of growth on selective medium were produced by several different electroporation conditions in the presence of exogenous plasmid pMN24 (Table 1). In addition to the standard electroporation conditions described in the legend to Fig. 1 with the Cell-Porator device, other condi-

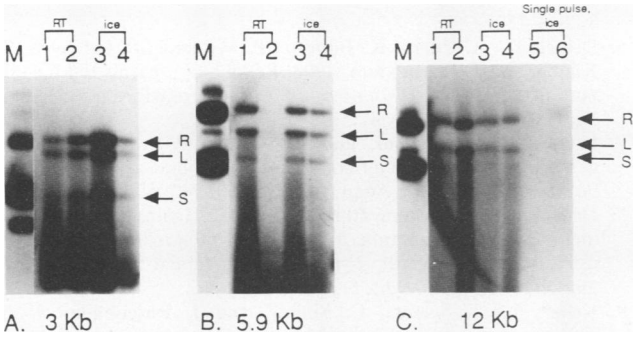

FIG. 3. Effect of plasmid size on uptake into wild-type cells. Autoradiographs of Southern blots of samples of 137c⁻ cells electroporated with a 3-kb plasmid (A), a 5.9-kb plasmid (B), and a 12-kb plasmid (C). Cells were electroporated with double pulses of 1,000 V/cm for 26.4 ms (except where indicated in panel C), diluted, and grown for 3.5 h before total nucleic acids were isolated and electrophoresed without digestion on 1% agarose gels. Southern gel transfers were hybridized with the nick-translated plasmid used in the electroporations. The marker lane in each gel contained 1 μg of the undigested plasmid used for electroporation and is from a shorter autoradiographic exposure of the same gel. Lanes 1 and 2 of each panel show total nucleic acids from samples that were held at room temperature (RT) after electroporation and dilution. Lanes 3 and 4 of each figure show total nucleic acids from cells held on ice after electroporation and dilution. Lanes 5 and 6 in panel C show total nucleic acids isolated from cells treated as in lanes 3 and 4 but given only a single pulse. Total nucleic acids from each sample were electrophoresed on the gel. Arrows indicate relaxed circular (R), linear (L), and supercoiled (S) tertiary forms of the plasmid DNA used for electroporation.

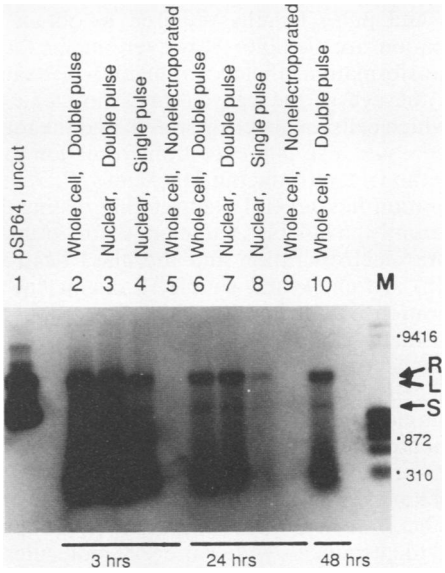

FIG. 4. Form, persistence, and localization of exogenous DNA in electroporated cells. The autoradiograph of a Southern blot shows total nucleic acids isolated from *cw-92* mutant cells electroporated with 5 μg of pSP64 plasmid DNA. Each sample received single or double pulses at 1,000 V/cm for 26.4 ms. Total nucleic acids from whole cells or from the nuclear fraction of electroporated cells were analyzed as indicated above each lane. Samples in lanes 2 to 5 were collected 3 h after electroporation; samples in lanes 6 to 9 were collected after 24 h; the sample in lane 10 was collected after 48 h. Nuclei were isolated after cell lysis with the detergent Nonidet P-40 as described before (9). Total nucleic acids from each sample were run without restriction endonuclease digestion on a 1% agarose gel containing ethidium bromide. The Southern blot was hybridized with nick-translated plasmid pSP64. Arrows indicate relaxed circular (R), linear (L), and supercoiled (S) tertiary forms of pSP64 DNA used for electroporation. Lane M, Size markers (shown in bases).

tions were tested with the Gene Pulser device (Bio-Rad Laboratories), including electroporation in M.I(KNO_3) and in sterile distilled H_2O. Southern analysis of DNA from stable transformants surviving the selective growth conditions demonstrated that most *nit*$^+$ transformants contained one or more extra restriction enzyme fragments hybridizing to the pMN24 DNA probe that were not present in genomic DNA from untransformed cells or in plasmid pMN24 itself (not shown). These bands probably represent junction fragments between integrated plasmid DNA and *C. reinhardtii* genomic DNA. Extra junction fragments were not visible in all samples, however, suggesting that either mutational reversion or integration within the endogenous nitrate reductase gene sequences may have occurred. Southern analysis of undigested DNA from transformants did not show a DNA band comigrating with undigested plasmid pMN24 DNA, suggesting that the plasmid was not maintained as an extrachromosomal element. Quantitative analysis of bands on Southern blots indicated that only one or a few copies of the introduced pMN24 DNA were integrated into the genomes of transformants generated by electroporation. These stable transformants have been maintained under selective growth conditions for 6 to 12 months.

The results summarized in Table 1 indicate that the field

TABLE 1. Electroporation conditions resulting in stable transformants[a]

Mutant strain	Initial cell no. (10^7)	Electroporation conditions			Field strength (V/cm)	Pulse length (ms)	No. of pulses	No. of transformants
		Medium	Volume (ml)	Device				
cw-15 nit1	1	KHME	0.5	Cell-Porator	1,000	26.4	1	17
nit1	1	KHME	0.5	Cell-Porator	1,000	26.4	2	1
cw-15 nit1	4	Distilled H_2O	0.8	Gene Pulser	1,000	5.0	1	7
nit1	8	M.I(KNO_3)	0.8	Gene Pulser	500	4.7	2	4
nit1	8	M.I(KNO_3)	0.8	Gene Pulser	1,000	3.8	2	12
nit1	8	M.I(KNO_3)	0.8	Gene Pulser	2,250	3.7	2	1
nit1	8	M.I(KNO_3)	0.8	Gene Pulser	500	4.8	3	3
nit1	8	M.I(KNO_3)	0.8	Gene Pulser	1,000	4.2	3	4
nit1	8	M.I(KNO_3)	0.8	Gene Pulser	2,250	3.4	3	3

[a] All samples were coporated with 5 μg of pMN24 plasmid, containing the selectable marker, and 5 μg of nonselected plasmid DNA. For all samples electroporated with the Gene Pulser (Bio-Rad) the 25-μF capacitor was used. For cells electroporated in distilled H_2O, the 200-Ω resistor in the pulse controller unit was also used. Transformants were not produced if cells were electroporated without pMN24 DNA.

strengths and pulse lengths required to obtain successful transformation are flexible. However, many factors influencing transformation efficiency remain to be examined. For example, the age of the cell culture, the stage of the cell cycle in which cells are electroporated, and the form of DNA introduced could also influence transformation rates.

Our method of introducing DNA into *C. reinhardtii* by electroporation has several features that distinguish it from other currently available techniques for transformation. While both electroporation and the glass bead method of Kindle (10) are quick and simple, a distinct advantage of electroporation is that multiple pulses introduce DNA into cells of virtually any phenotype, even those with intact cell walls. Special treatments to remove cell walls or special strains of cells lacking walls are not required. In addition, while multiple copies of the exogenous DNA are integrated after bombardment with the particle gun (11), only one or a few copies of exogenous DNA are introduced by electroporation, making feasible studies of gene expression in which copy number is critical. Most important, electroporation has been used to introduce a wide variety of molecules into other cell types. With the gene transfer conditions described here as a guide, it should be possible to use electroporation of *C. reinhardtii* for a wide range of new experimental applications.

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