High-Efficiency Transformation of Chlamydomonas reinhardtii by Electroporation

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ABSTRACT

We have established a high-efficiency method for transforming the unicellular, green alga Chlamydomonas reinhardtii by electroporation. Electroporation of strains CC3395 and CC425, cell wall-less mutants devoid of argininosuccinate lyase (encoded by ARG7), in the presence of the plasmid pJD67 (which contains ARG7) was used to optimize conditions for the introduction of exogenous DNA. The conditions that were varied included osmolarity, temperature, concentration of exogenous DNA, voltage and capacitance. Following optimization, the maximum transformation frequency obtained was 2 \times 10^7 transformants per \mu g of DNA; this frequency is two orders of magnitude higher than obtained with the current standard method using glass beads to introduce exogenous DNA. The electroporation procedure described in this article is of general utility, and makes it feasible to isolate genes by direct complementation of Chlamydomonas reinhardtii mutants.

CHLAMYDOMonas reinhardtii is a unicellular, eukaryotic green alga that has been used to elucidate aspects of photosynthesis, phototaxis, flagella assembly, cell wall biogenesis, gametogenesis, cell cycle events, mating processes, and nuclear/chloroplast interactions (for review, see Rochaix 1995). There are many advantages of using C. reinhardtii as a model eukaryotic, photosynthetic organism: (1) It grows rapidly (doubling time, 6–8 hr) and is easy and inexpensive to culture, (2) it can grow photoautotrophically or heterotrophically, which permits the isolation of mutants unable to perform photosynthesis, (3) it is amenable to classical genetic analysis, (4) it is haploid during vegetative growth, allowing any mutation to be immediately expressed, (5) characterized mutants are available at the C. reinhardtii stock center (Harris 1989), (6) exogenous DNA can be introduced into the nuclear, chloroplast, and mitochondria genomes (Boytont et al. 1988; Kindl et al. 1989; Newman et al. 1991; Sodeinde and Kindle 1993; Schnell and Lefebvre 1993; Randolf-Anderson et al. 1993; Davies et al. 1994, 1996), (7) a reporter gene has been developed and used to dissect the regulation of various promoters (Davies et al. 1992; Davies and Grossman 1994; Quinn and Merchant 1995), and (8) cosmid and yeast artificial chromosome libraries have been constructed (Purton and Rochaix 1994; Zhang et al. 1994; Fant et al. 1995), including an indexed cosmid library (Zhang et al. 1994) for the complementation of mutants (Sodeinde and Kindle 1993; Tam and Lefebvre 1993; Zhang et al. 1994; Davies et al. 1996; Vashishtha et al. 1996; Funke et al. 1997).

One of the recent technological advances that has helped to establish C. reinhardtii as a model photosynthetic organism for the analysis of biological processes has been the development of a transformation system for the stable introduction of DNA into the nuclear genome (Kindle 1990). This method of transformation, superior to other methods that have been used (Kindle et al. 1989; Brown et al. 1991; Dunahay 1993; Butanayev 1994; Tang et al. 1995), involves vortexing cells in the presence of exogenous DNA, acid washed glass beads, and polyethylene glycol. This procedure can yield up to 1000 transformants per \mu g DNA. However, this frequency is still lower than that observed for various unicellular organisms (e.g., yeast, E. coli, and cyanobacteria) where other methods of introducing the exogenous DNA were used. The glass bead transformation procedure was originally developed to introduce DNA into yeast (Cosmanzo and Fox 1988), and although it is convenient, it generally results in low efficiencies of transformation (~200 transformants/ \mu g DNA). Either electroporation (Manivasakam and Schiestl 1993) or the lithium acetate/single-stranded DNA/polyethylene glycol methods (Gietz et al. 1995) can yield transformation frequencies of ~10^4 transformants/ \mu g DNA in yeast. An electroporation procedure was optimized in the studies presented in this article to yield transformation frequencies far better than those that have been reported previously for C. reinhardtii.

MATERIALS AND METHODS

C. reinhardtii cultures and growth conditions: C. reinhardtii strains CC125 (wild-type mt+) and CC425 (arg7-8 cw15 mt+ sr-u-2-60) were obtained from Dr. J. Davies at the Carnegie Institution of Washington (Stanford, CA), and strain CC3395.
(arg7-8 cwd mt+) was obtained from Dr. R. Funke at the University of Nebraska, Lincoln. The last strain was originally isolated by Dr. R. Matagne of Liège University (Liège, Belgium). Cells were grown in 100 ml TAP culture medium (Harris 1989) and supplemented with 200 μg/ml arginine when appropriate. Illumination was continuous at 50 μmol photons m⁻² sec⁻¹ from fluorescent tubes. The flasks were agitated on a gyratory shaker (120 rpm) at 27°C without aeration.

**Preparation of exogenous DNA:** The plasmid pJD67, carrying the ARG7 gene, was obtained from Dr. J. Davies at the Carnegie Institution (Davies et al. 1994). The cosmid clone 42G2, which contains the ARG7 gene, was obtained from Dr. D. Weeks of the University of Nebraska, Lincoln (Zhang et al. 1994). These DNAs were amplified in E. coli strain JM109, and purified by a standard alkaline lysis extraction procedure followed by centrifugation in a cesium chloride gradient (Sambrook et al. 1989). Salmon sperm DNA (Sigma, St. Louis, MO) was used as a carrier during transformation; the carrier DNA was dissolved in water at 10 mg/ml, sheared by sonication and then denatured by boiling for 5 min. All of the DNA samples were quantified by measuring the absorbance at 260 nm.

**Electroporation protocol:** The cell cultures were chilled on ice prior to the addition of a 10% Tween-20 solution at 1/2000 (v/v); this facilitates pelleting of the *C. reinhardtii* cells. The cells were collected by centrifugation at 800×g for 5 min at 4°C and resuspended in Tris acetate phosphate (TAP) medium containing indicated concentrations (typically 40 mM) of sucrose to a final density of between 1×10⁸ and 4×10⁹ cells per ml. Under standard conditions, 10 μg/ml of the plasmid pJD67 (linearized by HindIII digestion) and 200 μg/ml of carrier DNA were added. The cell suspension of 250 μl was placed into a disposable electroporation cuvette with a 4-mm gap (Bio-Rad Labs., Hercules, CA), which was then immersed in a water bath to maintain specific temperatures. An exponential electric pulse (typically between 1900 and 2400 V/cm) was applied to the sample using the model GTE-10 (SHIMADZU, Kyoto, Japan) electroporation apparatus. Unless otherwise noted, the capacitance was set at 10 μF and no shunt resistor was used. It was crucial that less than 1 hr elapse between the time of harvesting the cells and the application of the electric field. Following electroporation, the cuvette was removed from the electroporation apparatus and incubated in a 25° C water bath for at least 5 min (and no more than 60 min), and an aliquot of the cell suspension was plated onto solid medium (TAP-0.5% agarose) by a starch embedding method. Five hundred cells of the indicated strains were plated onto solid TAP medium with or without being embedded in starch. The colonies that grew are presented as a percentage of the number of cells spread onto the medium. Error bars indicate standard deviation (n = 2).

**RESULTS**

**Enhancement of plating efficiency using the starch embedding method:** Cell wall-less strains of *C. reinhardtii* have considerably lower plating efficiencies than those that synthesize a cell wall (Harris 1989). Examination of wall-less cells spread onto the surface of solid medium revealed that most of the cells became flattened and lysed as the surface dried. The loss of moisture around the cells probably caused cell death, which could be prevented by plating the cellsin an appropriate supporting medium. Many different supporting media (e.g., sodium alginate, mineral oil, soft agar, various water soluble or insoluble polymers, and starches of various grains) were tested. While all were effective to some extent, the best results were obtained by embedding the cells in a suspension of corn starch. Changes in plating efficiencies for both the walled and wall-less strains as a consequence of corn starch embedding are shown in Figure 1. Without the starch, the plating efficiency of strain CC3395 was about 15% while that of CC425 was approximately 0.2%. Embedding the cells in the starch improved the plating efficiencies to 60% and 40% for CC3395 and CC425, respectively. The plating efficiency of the wild-type, cell-walled strain, CC125, was high even in the absence of the supporting medium.

**Figure 1.—Improvement of plating efficiencies by the starch embedding method.** Five hundred cells of the indicated strains were plated onto solid TAP medium with or without being embedded in starch. The colonies that grew are presented as a percentage of the number of cells spread onto the medium. Error bars indicate standard deviation (n = 2).
Figure 2.—Electrical field strength dependency of transformation frequency using different capacitors. C. reinhardtii strain CC3395 cells were electroporated in a 250-µl reaction mixture at a cell density of 1 × 10^7/ml with 10 µg/ml pJD67 linearized with HindIII, and 200 µg/ml of carrier DNA in TAP supplemented with 60 mM sucrose. The cells were incubated at 10°C prior to the electroporation at three different capacitances (3, 10, and 35 µF). The relative transformation frequencies are plotted, with the maximum frequency set at 1. The time constants with capacitors of 3, 10, and 35 µF were 1–2, 5–6, and 15–20 msec, respectively. Error bars connect the values of duplicate samples.

In the transformation experiments described below, we routinely used the starch embedding method.

Optimization of transformation efficiency: We tested five parameters that appeared to contribute to the efficiency of transformation: temperature, osmolarity, electric conditions (electric field strength and time constant of discharge) and concentration of exogenous DNA.

Figure 2 shows the electric field strength dependency of transformation of CC3395 using three different capacitances (3, 10, and 35 µF). The optimal transformation frequency was nearly identical at 10 µF and 35 µF, but was attained at two different field strengths (1500 V/cm for the 35 µF capacitor and 1800 V/cm for the 10 µF capacitor). Similar results were obtained for strain CC425; however, the electric field strength that gave optimum transformation frequencies was higher (2300–2400 V/cm with 10 µF capacitor, data not shown). We used a 10 µF capacitor in the remaining experiments.

As shown in Figure 3, the addition of sucrose to between 20 and 60 mM greatly improved the transformation frequency. Identical results were obtained when the sucrose was replaced with sorbitol (data not shown).

Optimum transformation required between 20 and 40 mM sucrose for CC3395 and between 30 and 60 mM sucrose for CC425. Unless otherwise specified, the remaining experiments were performed in the presence of 40 mM sucrose, which gave near optimal results for both strains.

The effect of the concentration of heat-denatured carrier DNA on the transformation frequency is shown in Figure 4. The transformation frequency for both CC3395 and CC425 increased with increasing carrier DNA up to approximately 200 µg/ml; at concentrations
Figure 5.—Effect of temperature on transformation frequency. The electrical field strength dependency of the transformation frequency with the strain (A) CC3395 and (B) CC425 at 0, 10, 20 and 30°. The TAP medium was supplemented with 40 mm sucrose for CC3395 and 60 mm sucrose for CC425. The relative transformation frequencies are plotted, with the maximum frequency set at 1. Other conditions were the same as given in the legend of Figure 2. The broken line shows the calculated temperature increase caused by Joulian heat generated during the electroporation. Error bars connect the values of duplicate samples.

Figure 6.—Effect of DNA concentration on transformation frequency. Strain CC3395 was transformed with the indicated concentration of HindIII linearized pJD67 DNA and 200 μg/ml of carrier DNA in 40 mm sucrose-supplemented TAP. Electroporations were performed at 1800 V/cm, 20° with 10 μF capacitor and the cell density was maintained at 4 × 10⁹/ml. (A) Transformation frequency expressed as the total number of transformants per trial (1 × 10⁸ cells). (B) Transformation efficiency expressed as the number of transformants per μg DNA. Error bars connect the values of duplicate samples.

The incubation temperature was increased from 10° to 20°, the voltage required for optimal transformation was shifted down by roughly 100 V/cm.

Figure 6 shows the transformation frequency of CC3395 as a function of the concentration of linearized pJD67. The number of transformed cells increased gradually, reaching a maximum at a concentration of approximately 10 μg/ml (Figure 6A). At concentrations above this the frequency seemed to decrease to some extent. Normalized to the level of exogenous DNA, the maximum efficiency of transformation was at a DNA concentration of approximately 1 μg/ml (Figure 6B); at this concentration we obtained 1.9 × 10⁵ transformants per μg DNA.

Southern blot analysis of integrated DNA: To demonstrate that the exogenous DNA integrated into the genome of the transformants, total DNA from 18 independent transformants of CC3395 was analyzed by Southern
Figure 7—Southern blot analysis of genomic DNA of the transformants. Cells were transformed in the presence of three different concentrations of *Hind*III linearized *pJD67* DNA (2.5, 10, and 40 μg/ml, indicated at the top). Genomic DNA was prepared from individual transformants, as described in materials and methods. To visualize the introduced copies of the *ARG7* gene, a *SacI*/*Hind*III fragment of 2.9 kb from *pJD67* (which contains the 3' end of the *ARG7* gene; see the inset map) was hybridized to *SacI* digested genomic DNA from the various transformants. The right-most and left-most lanes have *SacI* digested DNA from untransformed cells. The hybridization signal corresponding to the endogenous *ARG7* gene is indicated by an arrowhead. The average number of copies of exogenously introduced *ARG7* present in the genomes of the transformants was estimated as the number of bands that hybridized to the probe. The estimated copy number of integrated DNA for each transformant is given below each of the lanes. For the lanes having doublets, as deduced from relative band intensities, the number of hybridizing bands is appended with an asterisk (the doublets were counted as two bands).

Table 1—Standard conditions for electroporation of DNA into *Chlamydomonas reinhardtii*

<table>
<thead>
<tr>
<th>Sample conditions</th>
<th>Sample size</th>
<th>Cell number</th>
<th>Exogenous DNA</th>
<th>Carrier DNA</th>
<th>Transformation medium</th>
<th>Temperature</th>
<th>Electric conditions</th>
<th>Electric field strength</th>
<th>Time constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>250 μl in 4-mm cuvette</td>
<td>0.25–1 × 10⁸ cells (1–4 × 10⁷/ml)</td>
<td>2.5 μg (10 μg/ml)</td>
<td>50 μg (200 μg/ml)</td>
<td>TAP + 40 mm sucrose</td>
<td>10–20°C</td>
<td>1800–2300 V/cm</td>
<td>5–6 msec (10 μF capacitor)</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2
Comparison of transformation efficiencies with electroporation and glass bead method

A. Electroporation

<table>
<thead>
<tr>
<th>Strain and genotype</th>
<th>Exogenous DNA</th>
<th>Electric field strength (V/cm)</th>
<th>Temp.</th>
<th>No. of cells per trial (×10⁶)</th>
<th>Starch embedding</th>
<th>Transformants per trial (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC3395 arg7-8 cwd</td>
<td>pJD67</td>
<td>1900</td>
<td>20⁰</td>
<td>1.0</td>
<td>+</td>
<td>210 ± 50</td>
</tr>
<tr>
<td>CC3395 arg7-8 cwd</td>
<td>42G2</td>
<td>1900</td>
<td>10⁰</td>
<td>1.0</td>
<td>+</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>CC425 arg7-8 cw15</td>
<td>pJD67</td>
<td>2300</td>
<td>10⁰</td>
<td>1.0</td>
<td>+</td>
<td>136 ± 20</td>
</tr>
</tbody>
</table>

B. Glass bead transformation

<table>
<thead>
<tr>
<th>Strain and genotype</th>
<th>Exogenous DNA</th>
<th>Time of vortexing (sec)</th>
<th>No. of cells per trial (×10⁶)</th>
<th>Starch embedding</th>
<th>Transformants per trial (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC3395 arg7-8 cwd</td>
<td>pJD67</td>
<td>20</td>
<td>0.25</td>
<td>−</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>CC425 arg7-8 cw15</td>
<td>pJD67</td>
<td>20</td>
<td>1.0</td>
<td>+</td>
<td>0.88 ± 0.07</td>
</tr>
</tbody>
</table>

² Mean ± SD (n = 3).
³ Performed as in Kindle (1990).

slightly less (1.4 × 10⁵/trial) than that for CC3395. For both strains the transformation frequencies declined if the cells were plated without embedding them in starch (under optimal conditions the number of transformants was reduced to 1/4 and 1/20 of the maximal levels for CC3395 and CC425, respectively). When the cosmid clone containing the ARG7 gene (clone 42G2) was used as the exogenous DNA instead of pJD67, the transformation frequencies declined by approximately one order of magnitude (2.4 × 10⁴ transformants per trial, Table 2). The results also clearly demonstrate that the frequency of transformation using electroporation was much higher (approximately two orders of magnitude) than that obtained by the glass bead procedure (compare Table 2A and 2B).

DISCUSSION

We report here detailed conditions for optimal transformation by electroporation of C. reinhardtii strains CC3395 and CC425. The final number of transformants that we obtained reflects both an increase in the survival of the cells during plating in a supporting medium plus an increase in the efficiency of introducing the exogenous DNA into the cells. While several other attempts have been made to achieve high frequency transformation of C. reinhardtii using electroporation (Brown et al. 1991; Butanova 1994), the efficiency achieved in this study was far better than those reported previously. Recently, Tang et al. (1995) reported a procedure in which they were able to successfully electroporate DNA into C. reinhardtii. While relatively high numbers of transformants were obtained (500 transformants from 1.8 × 10⁶ cells using 2 µg exogenous DNA), the transformation efficiency (250 transformants per µg DNA) was much lower than reported in this article (1.9 × 10⁶ transformants per µg DNA; Figure 6B). Furthermore, Tang et al. (1995) used specialized equipment for imposing the electric field.

The absolute transformation frequency that we observed was between 1.5- and 2-fold higher for CC3395 than for CC425, and the conditions required for optimal transformation were also somewhat different between the strains. Variations in transformation characteristics among strains may reflect differences in the average diameter of the cells, the rigidity and composition of the cytoplasmic membranes and the metabolism of the cells. Metabolic variation between CC3395 and CC425 is reflected in the different growth rates of the two strains; it takes 3 days for CC3395 to form detectable colonies on solid medium while colonies of CC425 are observed only after a week.

Starch embedding of the cells prior to spreading them onto solid medium resulted in a great increase in the final number of transformants for both CC425 and CC3395 following electroporation (Table 2). This reflects improved plating efficiency (viability) on the solid medium (Figure 1). Interestingly, the starch embedding procedure did not result in increased numbers of transformants if the DNA was introduced by the glass bead
method (Table 2). For the glass bead method, the entire transformation mixture is spread on a single plate because of the lower transformation frequency. Therefore the density of cells placed on the plate following glass bead transformation is far greater than that used following electroporation. The high density of untransformed cells may act as a protective medium similar to the starch granule embedding medium.

To attain the highest transformation frequency, a number of different parameters were critical; these included electric conditions (electric field strength and time constant), temperature and osmolarity. High efficiencies of transformation occurred over a narrow range of temperatures (10-20°C) and osmolarities (30–60 mm of added sucrose). The reason for the pronounced temperature effect is not clear, although decreasing the temperature would make the membranes more rigid, which in turn might make it more difficult for the cells to resel following the electric pulse. The final number of transformants would be determined by the facility with which the DNA enters the cell and integrates into the genome, and the number of cells that die because of irreversible damage caused by disruption of the cell membrane; the temperature might affect the balance between these processes at any given voltage. The addition of an appropriate osmoticum to the electroporation medium may lead to an increase in the number of viable transformants by minimizing cell lysis that results from the electric pulse. This is supported by microscopic observation of the cells after electroporation; in the absence of sucrose, most of the cells appeared to be lysed immediately following the electric pulse, while little lysis was observed if electroporation was performed on cells in TAP medium containing 40 mm sucrose. Microscopic observation also revealed that pulsation of contractile vacuoles, by which permeated water is pumped out of the cell, stopped when the cells were suspended in the sucrose-enriched TAP, which indicates that the sucrose makes the medium nearly isotonic with the cytoplasm of the cell.

The concentration of exogenous DNA that resulted in the maximum number of transformants per trial at a given cell density was 10 μg/ml. However, the concentration of DNA that led to the maximum number of transformants per μg of DNA was approximately 1 μg/ml (Figure 6B). The concentration of DNA also affected the number of copies of the exogenous DNA that inserted into the nuclear genome (Figure 7). Insertion of more than one copy of DNA is not desirable for the purpose of insertional mutagenesis; it would make the identification of tagged genes more difficult. Therefore, for generating tagged mutants the exogenous DNA should be maintained at a concentration that yields approximately one integration event per transformant. When multiple copies of the inserted DNA are not a great disadvantage (e.g., complementation of mutants), one could use a relatively high concentrations of exogenous DNA.

Another factor that contributed to the improvement of the final transformation frequency was the addition of carrier DNA. With the glass bead method it was reported that the addition of carrier DNA (50 μg/assay) increased the transformation frequency moderately (1.8-fold). However, when carrier DNA was included in the electroporation mixture the transformation frequency increased by approximately one order of magnitude (Figure 4). A large enhancement of the transformation frequency by the addition of single-stranded DNA was also reported for yeast (Schiestl and Gietz 1989). Little is known about how the carrier DNA elevates the frequency of transformation. It may act as a competitive inhibitor of nucleases, thereby protecting the exogenous DNA from degradation and/or block nonspecific DNA binding to the cell surface. However, if the carrier DNA integrates into the genome of transformed cells, it may cause difficulties in experiments in which an attempt is being made to create mutants and tag the altered genes by a specific DNA sequence such as that of ARG7 (Davies et al. 1996). There are a number of ways in which this problem can be resolved. It is possible to omit the carrier DNA from the electroporation reaction mixture and still maintain a relatively high transformation frequency. Furthermore, rRNA can serve the same purpose as carrier DNA (Schiestl and Gietz 1989) and would not be able to integrate into the genome.

The frequency with which we have been able to introduce DNA into C. reinhardtii by electroporation is approaching that obtained for yeast (for yeast, 1 μg of DNA can yield as many as 10⁶ transformants) (Manivasakam and Schiestl 1993; Gietz et al. 1995). This high frequency of transformation is critical when trying to complement mutants of C. reinhardtii with recombinant phage or cosmid libraries. In C. reinhardtii, the transformation efficiency with cosmid DNA was about one order of magnitude lower than that obtained with the plasmid DNA (Table 2). 42G2 compared to pJD67), which is probably a consequence of the difference in the sizes of the DNA molecules. A similar dependency of transformation frequency on DNA molecule size was reported for E. coli (Hanahan 1983).

The complementation of mutants of C. reinhardtii with a cosmid library has become feasible given the frequency of transformation obtained in these studies. Transformation with the cosmid 42G2 yielded approximately 2.4 × 10⁵ transformants per trial (Table 2). Recombinant cosmid clones of 1 × 10⁴ would have a more than 90% chance (Zhang et al. 1994) of containing any particular C. reinhardtii gene. Therefore, with a single electroporation trial using the cosmid library, we are likely to be able to complement any recessive mutation. Indeed, we have recently used the high efficiency transformation procedure reported here to complement a mutant that does not properly acclimate to phosphorus starvation (D. D.
Wykoff, D. P. Weeks, J. L. Kovar, H. Usuda, A. R. Grossman and K. Shimogawara, unpublished results). Furthermore, the increased transformation frequency would potentially make targeted disruptions of genes easier. With C. reinhardtii it was reported that the ratio of homologous to nonhomologous recombination events was ~1/1000 with the glass bead method or ~1/24 with the microprojectile bombardment method (Sodeinde and Kindle 1993). Although we have not examined the frequency of homologous recombination with electroporation, we should be able to isolate several homologous recombinants with a single electroporation trial. Finally, the increased level of transformation would also help facilitate the use of reporter genes (Davies et al. 1992) for evaluation of expression from regulated promoters (Davies and Grossman 1994).

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