Title: Transformation of a model microalga, *Chlamydomonas reinhardtii*, without cell-wall removal

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Abstract

A green alga, *Chlamydomonas reinhardtii,* has been widely used to study many biological processes including photosynthesis, flagellar motility, sexual reproduction, and metabolism and genetics. Here, we describe a step-by-step protocol of rapid and efficient transformation method for cell-walled *Chlamydomonas* strains without cell-wall removal using a square electric pulses-generating electroporator. This method could be applied to transformation of other industrially useful algae including diatom by optimizing the electric conditions.

Keywords: *Chlamydomonas reinhardtii*, Electroporation, Photosynthesis, Protist, Square electric pulse.

1. Introduction

With the growing emphasis on biofuel production by algae, it is increasingly important to transform algae rapidly and efficiently. In particular, the single cell green alga *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*) is widely used to investigate the fundamental biological processes of photosynthesis, cell cycle, organelle function/interaction, and cell motility as well as of biofuel production. So far, genome sequences of nucleus, chloroplast and mitochondrion in *Chlamydomonas* are available (1) and three methods of DNA-mediated transformation have been reported: bombardment with DNA-coated microprojectiles (2, 3), vortexing with glass beads (4), and electroporation (5). For nuclear genome transformation, the transformation efficiencies of the glass beads and electroporation are approximately 10^3 and 10^5 transformants per mg DNA, respectively (5). Although electroporation-based transformation has been widely used, these procedures require the use of cell-wall-less (*cw*) mutant strains or the removal of cell wall from wild-type cells by treatment with the zinc-containing metallo-protease gametolysin for cell wall degradation (6). However, both options are associated with technical difficulties because *cw* mutants are fragile and cell-wall removal is time-consuming process. To overcome these difficulties, we developed a rapid transformation method without cell-wall removal using a square electric pulses-generating electroporator (7), providing

a more convenient method for *Chlamydomonas* transformation and enhancing its usability as a model organism.

2. Materials

2.1 Preparation of *aph7"* DNA cassette

1. PrimeSTAR GXL DNA Polymerase (Takara, cat. no. R050A). 5×PCR buffer and 2.5 mM dNTPs mixture are packaged together.
2. pHyg3 plasmid (1 ng/μL): This plasmid harbors the *aph7"* expression cassette (8), which confers hygromycin B resistance. pHyg3 plasmid is available from the *Chlamydomonas* Resource Center (http://www.chlamycollection.org/).
3. Primers for amplification of the *aph7"* expression cassette: pHyg3-F1 primer (5'-GCACCCCAGGCTTTACACTTTATGCTTCC-3') and pHyg3-R1 primer (5'-CCATTCAGGCTGCGCAACTGTTGG-3').
4. Thermal Cycler (C1000, Bio-Rad).
5. PCR tube (NIPPON Genetics, cat. no. FG-028DC).
6. QIAquick PCR Purification Kit (QIAGEN, cat. no. 28104).
7. 1% agarose/TAE gel.

2.2 Culture media

1. Nutrient solution: To prepare 1:100 stock solution, weigh 242 g of Tris, 40 g of NH_4Cl, 10 g of $MgSO_4 \cdot 7H_2O$, 5.0 g of $CaCl_2 \cdot 2H_2O$ and dissolve in 900 mL deionized water. Add 100 mL of glacial acetic acid. Sterilize the solution by autoclaving for 20 min and store at 4°C.

2. Hutner's trace elements: To prepare 1:100 stock solution, weigh 5.0 g of Na_2·EDTA, 2.2 g of $ZnSO_4 \cdot 7H_2O$, 1.14 g of H_3BO_3, 0.51 g of $MnCl_2 \cdot 4H_2O$, 0.16 g of $CoCl_2 \cdot 6H_2O$, 0.16 g of $CuSO_4 \cdot 5H_2O$, 0.11 g of $(NH_4)6MO_7O_{24} \cdot 4H_2O$, and 0.50 g of $FeSO_4 \cdot 7H_2O$ and dissolve in this order in 1 L deionized water. Sterilize the solution by autoclaving for 20 min and store at 4 °C. The color of mixture will turn purple from green.
3. Phosphate solution: To prepare 1:1,000 stock solution, weigh 108 g of K_2HPO_4 and 56 g of KH_2PO_4 and dissolve in 1 L deionized water. Sterilize the solution by autoclaving for 20 min and store at 4°C.
4. TAP (Tris-acetate phosphate) medium: Mix 10 mL of nutrient solution, 10 mL of Hutner's trace elements, and 1 mL of phosphate solution in 900 mL deionized water. Adjust pH to 7.0 with HCl, and add deionized water to a final volume of 1 L. Sterilize by autoclaving for 20 min and store at room temperature. When preparing TAP agar plate, add 1.5% agar powder prior to autoclaving of the medium.
5. TAP agar (1.5%) plate.
6. Gyratory shaker.

2.3 Transformation

1. Hygromycin B (Nacalai Tesque INC., cat. no. 07296-24).
2. TAP agar (1.5%) plate containing 30 μg/mL of hygromycin B.
3. Transformation buffer: TAP medium with 40 mM Sucrose. Store the buffer at 4°C.

4. Recovery buffer: TAP medium with 40 mM Sucrose. Store the room temperature.
5. Cell fix solution: Mix 178 µL of TAP medium and 20 µL of 10% glutaraldehyde in 1.5 mL tube.
6. 2 mm gap electroporation cuvette (NEPAGENE, cat. no. EC-002): Prechill the cuvette at 4°C.
7. Electroporator, NEPA21 (NEPAGENE).
8. Rotary shaker.
9. Hemocytometer.
10. 14 mL culture tube (sterile).
11. 300 mL flask (sterile).
12. 1.5 mL tube (sterile).
13. 15 mL conical tube (sterile).
14. 50 mL conical tube (sterile).
15. Swing rotor centrifuge.
16. *Chlamydomonas* cell-walled wild-type strain (Note 1).

1. Methods

3.1 Preparation of *aph7"* DNA cassette

1. Mix 10 µL of 5×PrimeSTAR GXL buffer, 4 µL of dNTPs mixture (2.5 mM each), 1.5 µL of pHyg3-F1 primer (10 µM), 1.5 µL of pHyg3-R1 primer (10 µM), 1 µL of pHyg3 plasmid, 0.5 µL of PrimeSTAR GXL DNA Polymerase, and 31.5 µL of distilled water in the PCR tube.

2. Amplify the *aph7''* DNA cassette by 35 cycles of denaturation for 10 sec at 98°C, annealing for 15 sec at 60°C, and extension for 2 min at 68°C.
3. Check the PCR product (1,999 bp) by 1% agarose/TAE gel electrophoresis.
4. Purify the PCR product by QIAquick PCR Purification Kit and adjust the concentration to 25 ng/μL.

3.2 Preparation of cells

1. Streak *Chlamydomonas* cells onto TAP agar plate and grow until the plates are moderately green with cells.
2. Inoculate cells into 5 mL TAP medium in 14 mL culture tube and vortex briefly to dissociate the cells. Preculture the cells for 24 h with vigorous shaking under continuous illumination at 50 μmol photons m^{-2} s^{-1}.
3. Place the precultured cells in the clean bench for 5 min, transfer 2 mL of the precultured cells into 100 mL TAP medium in a 300 mL flask. Grow them on a gyratory shaker (100 rpm) under continuous illumination at 100 μmol photons m^{-2} s^{-1} until the cell densities reach 1–2 × 10^6 cells/mL, corresponding to an optical density of 0.3–0.4 at 730 nm.

3.3 Transformation

Before electroporation, arrange all materials and supplies other than cells in the clean bench.

1. Turn on electroporator NEPA21 (Note 2) and set parameters (Note 3) as described in Table 1.
2. Collect cells by centrifugation at 600 × g for 5 min using 50 mL conical tube.

3. Decant and discard the TAP medium, and gently resuspend the cell pellet in 20 mL of prechilled Transformation buffer.
4. Centrifuge at 600 × g for 5 min and discard the Transformation buffer.
5. Gently resuspend the pellet in 1 mL of prechilled Transformation buffer.
6. Add 2 µL of the cell suspension to the cell fix solution and count cells using hemocytometer. Adjust the cell density at 1×10^8 cells mL^{-1} by adding prechilled Transformation buffer. During the counting, place the cells at 4°C.
7. Transfer 120 µL aliquot of the cell suspension to an electroporation cuvette for negative control.
8. Add 480 µL of the cell suspension and 12.3 µL of the *aph7″* DNA fragment solution in 1.5 mL tube, and mix well by gentle pipetting.
9. Dispense 120 µL of the cell suspension into four electroporation cuvettes (if you want more transformants, you can increase the volume of culture and number of cuvettes for electroporation).
10. Chill the electroporation cuvette at 4°C for 5min.
11. Measure electrical impedance (Ω) (Note 4) and perform electroporation.
12. Transfer the electroporated cells from the individual cuvettes into 10 mL of Recovery buffer in 15 mL individual conical tubes.
13. Incubate the cells for 16–20 h under dim light (2–3 µmol photons m^{-2} s^{-1}) to allow for the expression of *aph7″*.
14. Centrifuge the tubes at 600 × g for 5 min, discard the Recovery buffer by decanting, and suspend the cells using the remaining medium.

15. Plate the cell suspension onto TAP agar plate containing hygromycin B. Each suspension originating from a single electroporation cuvette is dispensed onto four selective plates.
16. Place the plates under continuous light at 100 μmol photons m^{-2} s^{-1} for 7–10 days (Note 5).

4. Notes

1. In this method, strain C-9 is used as a representative of cell-walled wild-type cells. C-9 is available from National Institute for Environmental Studies, Japan, as strain NIES-2235.
2. In contrast to other electroporators, such as Gene-Pulser series (Bio-Rad, USA) or ECM series (BTX, USA), NEPA21 has three-step multiple electroporation pulses, resulting in higher transformation efficiency and lower damage to the cells. The first pulse is a poring pulse (Pp) with high voltage and short pulse length. The second pulse consists of multiple transfer pulses (Tp) with low voltage and long pulse length for delivering exogenous DNA into cells. The third pulse is polarity-exchanged Tp for efficient delivery of DNA molecules into cells.
3. Six parameters, voltage (V), pulse length (ms), pulse interval (ms), number of pulses, decay rates (%), and polarity, are set for respective Pp and Tp. In addition, values of electrical impedance between the electrodes (Ω), actual voltage (V), current (A), and energy (J) can be measured.
4. Measured value of electrical impedance is approximately 0.2 kΩ in the cell conditions described above.

5. Typically, 500–1,000 hygromycin-resistant colonies appear per plate. With increasing DNA concentrations, the number of transformants increased. However, inserted copy number of *aph7"* cassette could be increased.
6.

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5. References

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Table 1. Settings for NEPA21 electroporation.

	Voltage (V)	Pulse length (ms)	Pulse interval (ms)	Number of pulses	Decay rate (%)	Polarity
Poring pulse (Pp)	300	8	50	2	40	+
Transfer pulse (Tp)	20	50	50	1	–	+/–

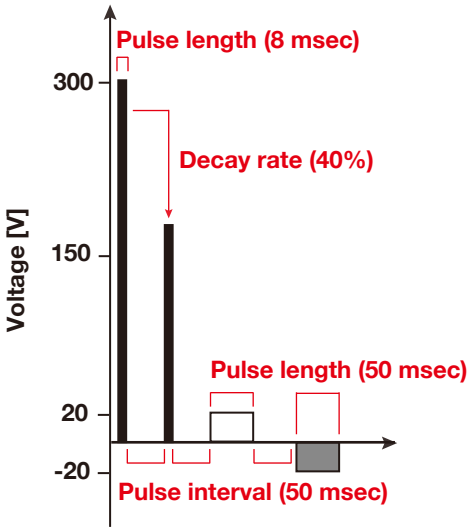

Fig. 1 Schematic of electric pulses delivered by NEPA21. Assignable parameters in this protocol are represented. Poring pulse (Pp), transfer pulse (Tp), and polarity-exchanged Tp are shown as black, white, and gray bars, respectively. Optimal transformation parameters applicable to strain C-9 are indicated in parentheses.