Title:

Rapid transformation of *Chlamydomonas reinhardtii* without cell-wall removal

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Abstract

Chlamydomonas reinhardtii is widely used to study many biological processes including biofuel production. Here, we present a rapid transformation technique 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 by optimizing the electric conditions.

Chlamydomonas reinhardtii, a single-cell green alga, is widely used for elucidating fundamental biological processes, including photosynthesis, cell cycle regulation, and cell motility as well as metabolic processes for biofuel production because it is relatively easy to transform. Its genome sequence is available (1), and three methods of DNA-mediated transformation have been reported for this organism: 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 method and electroporation are approximately 10^3 and 10^5 transformants per μg DNA, respectively (5). However, 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, 7). Both approaches are associated with difficulties: *cw* mutants are fragile and not suitable for some experiments, such as the measurement of photosynthetic activity, and preparation of gametolysin and removal of the cell wall are time-consuming. To overcome these difficulties, we present here a rapid transformation technique of wild-type *Chlamydomonas* without cell-wall removal using a square electric pulse generating electroporator, NEPA21 (Nepa Gene, Japan).

Although NEPA21 is widely used to transfect animal cells *in vivo* and *in vitro* (8, 9), the electroporator has not been applied to land plants, fungi, and algal cells so far. 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. In summary, six parameters, voltage [V], pulse length [msec], pulse interval [msec], 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.

Exogenous DNA was prepared as follows. A 1,999-bp DNA fragment containing the hygromycin-resistant gene *aph7"* was amplified by PCR from plasmid pHyg3 (10) using PrimeSTAR GXL DNA Polymerase (TAKARA, Japan) using 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 with a forward primer (5'-GCACCCCAGGCTTTACACTTTATGCTTCC-3') and reverse primer (5'-CCATTCAGGCTGCGCAACTGTTGG-3'). The PCR product was purified using a PCR purification kit (QIAGEN, USA) and the concentration was adjusted to 200 μg mL^{-1}.

Chlamydomonas reinhardtii strain C-9 (originally provided from the IAM culture collection at the University of Tokyo and kept in our laboratory. C-9 is available from National Institute for Environmental Studies, Japan, as strain NIES-2235) was used as a representative of cell-walled wild-type cells (11). For pre-cultivation, cells were grown in 5 mL Tris-Acetate-Phosphate (TAP) medium for ~24 h with vigorous shaking under continuous illumination at 50 μmol photons m^{-2} s^{-1}. One-day before transformation, 5 mL of pre-cultured cells was transferred into 100 mL TAP medium in a 300 mL-flask and grown for ~24 h. The flask was agitated on a gyratory shaker (100 rpm) at 25°C under continuous illumination until the cell densities reached 1–2 × 10^6 cells mL^{-1}, corresponding to an optical density of 0.3–0.4 at 730 nm. The cultured

cells were collected by centrifugation at 600 × g for 5 min and re-suspended in TAP medium containing 40 mM sucrose to a final density of 1×10^8 cells mL^{-1}. Then, 2 µL of 200 µg mL^{-1} pHyg3 PCR products was added to 38 µL of the cell suspension. As a result, 4×10^6 cells and 400 ng DNA were suspended in the total volume of 40 µL. The cell suspension was placed into an electroporation cuvette with a 2 mm gap (NEPA GENE, Japan). The measured value of electrical impedance was within 500–600 Ω in the cell conditions described above. Parameters of Pp were optimized as described below and those of Tp were fixed at a ten polarity-exchanged pulse of 20 V with 50 msec pulse length, 50 ms pulse interval, and a 40% decay rate.

After electroporation, an aliquot of the cell suspension from the cuvette was transferred into 10 mL TAP medium containing 40 mM sucrose. After incubation at dim light (2–3 µmol photons m^{-2} s^{-1}) for 24 h, the cells were collected by centrifugation at 600 × g for 5 min and plated onto 1.5% agar TAP plate containing 30 µg mL^{-1} hygromycin B. The plate was incubated at 25°C under continuous illumination at 80 µmol photons m^{-2} s^{-1}. Colonies of hygromycin-resistant transformants were visible 4 days later.

To determine the optimum transformation conditions, transformation efficiency as a function of the voltage of Pp and its pulse length was evaluated. The voltage dependency of transformation with different pulse lengths of 2, 4, 6, and 8 msec were plotted (Fig. 1A). In this experiment, the number of pulses, pulse interval, and decay rates of Pp were kept constant at two pulses, 50 msec, and 40%, respectively. When the Pp was 200 V and 250 V, maximum transformation efficiency was obtained with an 8 msec pulse length. In contrast, the transformation efficiency with Pp = 300V was highest with a 6 msec pulse length and decrease at 8 msec, suggesting that cell

damage caused by excess energy with high voltage and long pulse length led to the decrease in efficiency. Next, in order to optimize the levels of energy of the Pp, we evaluated parameters of Pp (Fig. 1B). For instance, energy by two Pps with 50 msec pulse length and 40% decay rates was calculated using following equation,

$$\text{Energy} = V_{1st} \times 0.05 \times A_{1st} + V_{1st} \times (1\text{-}0.4) \times 0.05 \times A_{2nd}$$

where V_{1st}, A_{1st}, A_{2nd} are actual voltage of the first pulse, actual current of the first pulse, and actual current of the second pulse, respectively. Optimal transformation efficiency was obtained with a Pp energy of 1.0–1.5 J. In addition, transformation efficiency was evaluated as a function of the concentration of exogenous DNA (Fig. 1C). With increasing DNA concentrations from 40 to 200 ng, the number of transformants increased, reaching a maximum at a concentration of approximately 200-800 ng. Above this DNA concentration, the transformation efficiency decreased.

From these results, the final transformation conditions applicable to the *Chlamydomonas* strain C-9 are summarized in Fig. 1D. The optimal transformation efficiency was obtained by application of two Pps of 250 V with 8 msec pulse length, 50 msec pulse interval, and 40% decay rates. Using these conditions, electroporation of 4×10^6 cells (1.0×10^8 cells mL^{-1}) resulted an average of 3,880 transformants per μg DNA for strain C-9 (Table 1, Fig. 2A), which was about 26-fold higher than the 150 transformants per μg DNA for strain C-9 strain with the previous electroporation procedure without cell-wall removal.

Transformation efficiency of cell-wall removed C-9 was also examined. Under optimal electric conditions described above, electroporation of cell-wall removed C-9 resulted an average of 2,702 transformants per μg DNA. In contrast, the transformation efficiency increased 2.8 times to 7,614 transformants per μg DNA by

application of two Pps of 200 V with 5 msec pulse length, 50 msec pulse interval, and 40% decay rates, suggesting that high voltage and long pulse length led to cell damage for cell-wall removed C-9 and the decrease in efficiency.

To examine whether this method is applicable to other cell-walled wild-type *Chlamydomonas* strains, we evaluated the transformation efficiency of strains CC-124, CC-125, and CC-1690 using the same electroporation conditions (Table 1). Although the transformation efficiencies of CC-124 (2,930 ± 471 cells) and CC-1690 (3,400 ± 327 cells) were similar or slightly decreased compared to that of C-9 (3,880 ± 470 cells), the efficiency with strain CC-125 was only 10% (404 ± 37 cells) of that of C-9. However, changing the Pp to one pulse of 300 V with an 8 msec pulse length resulted in an increase in the transformation efficiency to 1,920 ± 110 cells per μg DNA. In those conditions, energy of one Pp was 1.23-1.31 J, which is in the range of optimum energy (1.0-1.5 J) of two Pps (Fig. 1B), suggesting that larger energy is needed to induce pore formation for strain CC-125 compared to other wild-type strains.

In addition, we could introduce a longer plasmid, pTT1-*LciB*-GFP (12), with the length of 7,800-bp into C-9 cells without removal of the cell wall. Although the transformation efficiency was ~500 transformants per μg DNA, LCIB-GFP localization could be observed in the transformed cells (Fig. 2B).

In previous methods, it takes at least several days for preparation of the gametolysin before the transformation. In contrast to that, cell-walled strains can be directly transformed by DNA without any preparation or cell-wall removal using this method. Additionally, by optimizing the electric conditions, the square electric pulse generating electroporator could be applied to transformation of other industrially useful algae.

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Figure legends

Fig. 1 (A) Effects of voltage of Pp and pulse length on transformation efficiency. The number of transformants per µg DNA was plotted at voltages of 200, 250, and 300 V with different pulse length; 2 msec, 4 msec, 6 msec, and 8 msec. (B) Relationship between energy of Pp and transformation efficiency. Each white circle represents the data from one experiment. (C) Effect of DNA concentration on transformation efficiency. Strain C-9 was transformed with the indicated concentrations of pHyg3 PCR product. (D) Schematic of electric pulses delivered by NEPA21. Assignable parameters are represented. Pp, Tp, and polarity-exchanged Tp are shown as black, white, and gray bars, respectively. Optimal transformation parameters applicable to strain C-9 are indicated.

Fig. 2 (A) Colonies of hygromycin-resistant transformants plated on TAP agar medium containing 30 µg mL^{-1} hygromycin B. (B) Fluorescent signal of LCIB-GFP derived from transformants with the pTT1-*LciB*-GFP plasmid using NEPA21. Obvious ring fluorescence signals are present around the pyrenoid structure, as previously shown (12). White bar is 5 µm.

Fig. 1

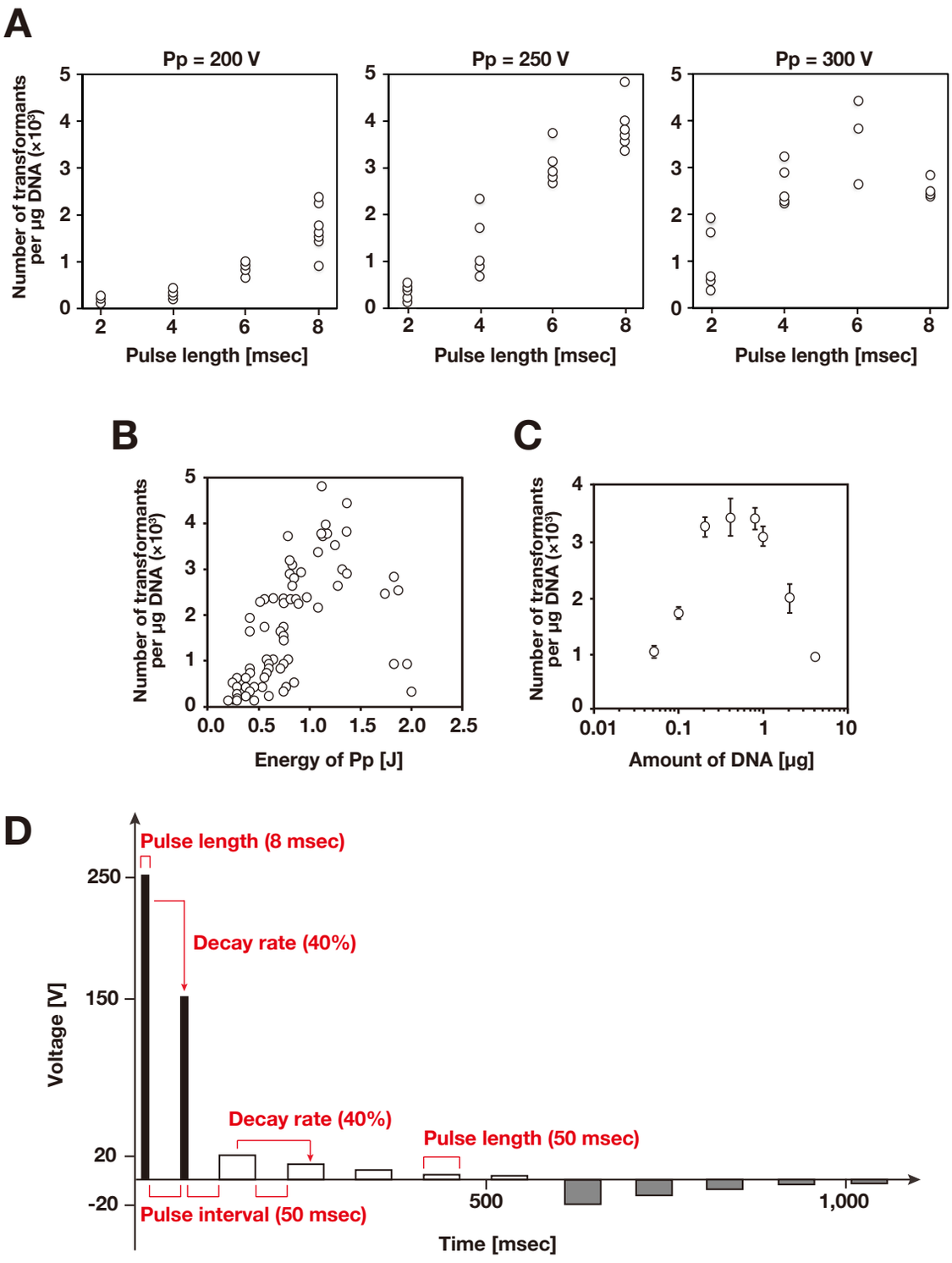
A
Pp = 200 V
Pp = 250 V
Pp = 300 V
Number of transformants per µg DNA (×10³)
Pulse length [msec]
B
Number of transformants per µg DNA (×10³)
Energy of Pp [J]
C
Number of transformants per µg DNA (×10³)
Amount of DNA [µg]
D
Pulse length (8 msec)
Decay rate (40%)
Decay rate (40%)
Pulse length (50 msec)
Pulse interval (50 msec)
Voltage [V]
Time [msec]

Fig. 2

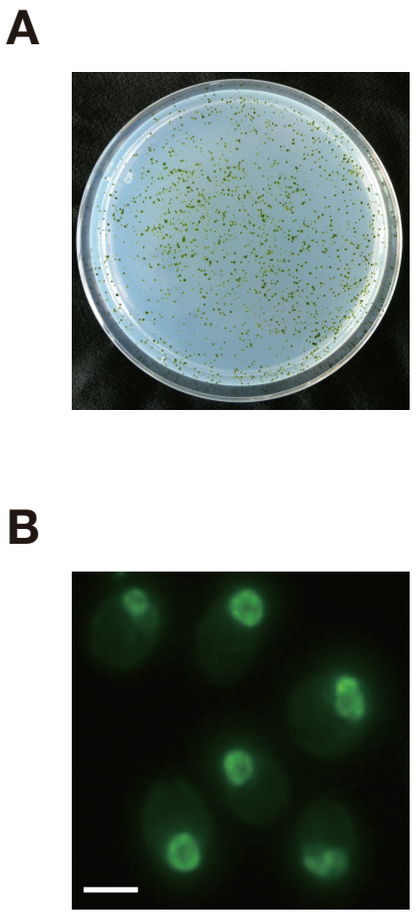

Table 1. Transformation efficiency of various *Chlamydomonas* strains using NEPA21

Device used for electroporation	Strain	Cell wall degradation by gametolysin	Electric conditions of Pp	Number of transformants per μg DNA
NEPA21 (NEPA GENE)	C-9	-	V_{1st}: 250 V for 8 msec V_{2nd}: 150 V for 8 msec	3,880 ± 470
	C-9	+	V_{1st}: 250 V for 8 msec V_{2nd}: 150 V for 8 msec	2,702 ± 303
	C-9	+	V_{1st}: 200 V for 5 msec V_{2nd}: 120 V for 5 msec	7,614 ± 693
	CC-124	-	V_{1st}: 250 V for 8 msec V_{2nd}: 150 V for 8 msec	2,930 ± 471
	CC-125	-	V_{1st}: 250 V for 8 msec V_{2nd}: 150 V for 8 msec	404 ± 37
	CC-125	-	300 V for 8 msec	1,920 ± 110
	CC-1690	-	V_{1st}: 250 V for 8 msec V_{2nd}: 150 V for 8 msec	3,400 ± 327
Gene-Pulser (Bio-Rad)	C-9	-	300 V	150 ± 25

To match the experimental conditions, carrier DNA or starch embedding methods (5) was not used for either transformation procedure. The amount of exogenous DNA and the number of cells for each transformation were constant at 400 ng and 4.0×10^6 cells, respectively. V_{1st} and V_{2nd} are first and second poring pulse, respectively.