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Short Communication

Optimization of polyethylene glycol (PEG)-mediated DNA introduction conditions for transient gene expression in the unicellular red alga *Cyanidioschyzon merolae*

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Mio Ohnuma,[1,2,3] Takashi Yokoyama,[4] Takayuki Inouye,[4] Yasuhiko Sekine,[4] Tsuneyoshi Kuroiwa,[2,3] and Kan Tanaka[1,3,5,*]

[1] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113–0032, Japan*
[2] *Initiative Research Unit, College of Science, Rikkyo University, Toshima-ku, Tokyo 171–8501, Japan*
[3] *Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), 4–1–8 Honcho, Kawaguchi 332–0012, Saitama, Japan*
[4] *Department of Life Science, College of Science, Rikkyo (St. Paul's) University, Nishiikebukuro, Tokyo 171–8501, Japan*
[5] *Chemical Resources Laboratory, Tokyo Institute of Technology, Midori-ku, Yokohama 226–8503, Japan*

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Cyanidioschyzon merolae is a unicellular red alga that has an extremely simple cell structure composed of one nucleus, one mitochondrion, one plastid and a minimum set of single-membrane-bound organelles (Kuroiwa et al., 1994), and thus is an ideal model organism for the basic study of eukaryotic cell architecture. The 100% complete genome sequences of the nucleus, mitochondrion, and chloroplast have been determined (Matsuzaki et al., 2004; Nozaki et al., 2007; Ohta et al., 1998; 2003), and the development of various biological techniques for *C. merolae*, as a model organism, has become increasingly important (Misumi et al., 2008).

Previously, we succeeded in introducing DNA into *C. merolae* cells (Minoda et al., 2004; Ohnuma et al., 2008), which enabled not only localization analysis with epitope-tagged proteins and GFP-fusion proteins (Ohnuma et al., 2008; Watanabe et al., 2011), but also reverse genetics such as antisense suppression (Ohnuma et al., 2009), gene disruption (Imamura et al., 2009; 2010) and stable insertion into the nuclear genome by homologous recombination (Fujiwara et al., 2013; Watanabe et al., 2014). However, our current protocol is relatively inefficient and shows large variations in efficiency from trial to trial. To improve the experimental stability, we compared the DNA introduction efficiency under various conditions in this study by monitoring transiently introduced luciferase gene expression. Firefly luciferase is one of the most frequently used reporter genes in various organisms (DiLella et al., 1988; Ow et al., 1986; Matsuo et al., 2006) because of the easy assay procedure and high sensitivity. To make a reporter construct, we fused the promoter region of the *eEF-1α* gene (CMH226C; http://merolae.biol.s.u-tokyo.ac.jp), which is strongly expressed in *C. merolae* throughout the cell cycle (Fujiwara et al., 2009), with the firefly luciferase gene. The 1,561-bp 5′-flanking region of CMH226C was amplified from *C. merolae* DNA with the primers 5′-cccaagcttcggcgctagaactcatgacggtatcgg-3′ (*Hin*dIII site underlined) and 5′-gggccatggttttcggaacgtatgtgt gttgttcgatgcgt-3′(*Nco*I site underlined) in 25 cycles (98°C for 10 s, 60°C for 30 s, and 72°C for 2 min) using PrimeSTAR® HS DNA Polymerase (TaKaRa Bio, Otsu, Japan), and cloned into the firefly luciferase gene (*luc+*) fusion plasmid pGL3-Basic (Promega, Madison, WI) using the attached *Hin*dIII and *Nco*I sites to make the reporter plasmid pEF1.5-Luc.

The *C. merolae* M4 strain (uracil auxotrophic mutant; Minoda et al., 2004) was grown in MA2 medium (Ohnuma et al., 2008) containing uracil (0.5 mg/ml) and 5-fluoroorotic acid (0.8 mg/ml) in a glass vessel under continuous white light (50 μmol photon m^{-2} s^{-1}) at 40°C with 2% CO_2 bubbling, unless otherwise noted. The original procedure for DNA introduction was based on a previous study (Ohnuma et al., 2008). Logarithmically grown cells (OD_{750} = 1.5–3.0) were diluted to OD_{750} = 0.4 in 50 ml of MA medium containing uracil (0.5 mg/ml), and cultivated overnight (for 20–24 h). Cells were collected by centrifugation (2,000 × *g*, 5 min), washed with MA-I (Ohnuma et al., 2008) and concentrated to adjust the calculated OD_{750} to 126 in MA-I. Then, 10 nM pEF1.5-Luc (6.3 μg/150 μl) was added to the cell suspension (50 μl), which was adjusted to 150 μl with MA-I.

*Corresponding author: Dr. Kan Tanaka, Chemical Resources Laboratory, Tokyo Institute of Technology, 4259–R1–29 Nagatsuta, Midori-ku, Yokohama 226–8503, Japan.
Tel: +81–45–924–5274 Fax: +81–45–924–5274 E-mail: kntanaka@res.titech.ac.jp
Abbreviations: MA2 medium, modified Allen's medium 2; PEG, polyethylene glycol.

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Subsequently, 150 µl of 60% PEG4000 (Wako, Osaka, Japan) dissolved in MA-I was added to the sample and mixed. The mixtures were diluted to 40 ml with MA2 medium, incubated without agitation at 40°C for 24 h in an incubator containing 5% CO_2, and the luciferase activity was determined using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) and Luciferase Assay System (Promega) as follows. After centrifugation (2,000 × *g*, 5 min) of the transformed cells, the collected samples were quickly frozen in liquid nitrogen, resuspended in 50−200 µl of 1× CCLR (supplement of the Luciferase Assay System) and homogenized by vortexing at room temperature. After removing the cell debris by a brief centrifugation, an appropriate volume (5−20 µl) of the supernatant was added to 100 µl of the Luciferase Assay Reagent and mixed by pipetting. The emitted photons were counted for 10 s, and the luciferase activity was expressed as arbitrary units normalized with the OD value at 750 nm measured before the cell concentration. The experiments were performed in triplicate.

To evaluate and improve the transformation efficiency, we first examined the mixing step with 60% PEG solution. Whereas we had already optimized the concentration and molecular weight of PEG (Ohnuma et al., 2008), cell aggregation occurring at this step depends on the individual case, and thus a large deviation in the efficiency could be attributed to this step. After addition of 60% PEG solution, the mixture was gently stirred, pipetted, or repeatedly inverted until it appeared homogenous. As shown in Fig. 1A, luciferase activity was detected in every case, indicating that firefly luciferase can be used as a reporter in *C. merolae*. Among the three methods examined, gentle stirring showed the lowest luciferase activity. Pipetting resulted in the highest efficiency but also showed the greatest experimental deviation. When the solution was mixed by inversion, the average luciferase activity was about half of that obtained by pipetting but the deviation was about one seventh. Therefore, repeated inversion was the most suitable method to obtain reproducible results and was applied hereafter.

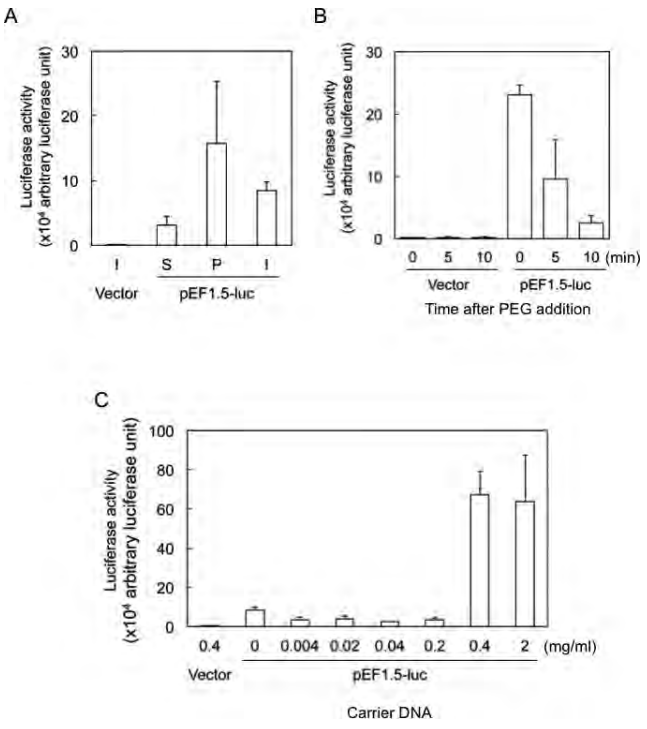

Fig. 1. Effects of PEG and DNA on DNA introduction efficiency.

(A) Effect of PEG mixing methods. The PEG was mixed by stirring (S), pipetting (P) or inverting (I) until the samples appeared homogenous. Samples were incubated for 5 min at room temperature before dilution with MA2 medium. (B) Effect of incubation period after PEG treatment. Samples were incubated at room temperature; 0.4 mg/ml carrier DNA was used. (C) Effect of carrier DNA addition. The standard errors were calculated from three independent experiments.

Next, we analyzed the effect of incubation time with PEG prior to the subsequent dilution. In yeasts and plants, samples are usually incubated for 20−30 min after PEG addition for DNA introduction (Abel et al., 1994; Schiestl and Gietz, 1989). Conversely, an incubation time of 1 min is used in *Streptomyces* (Suarez and Chater, 1980). As shown in Fig. 1B, when the cells were diluted immediately after mixing with PEG, the luciferase activity increased at least 2.4-fold compared with when the cells were incubated for longer. Thus, dilution with MA2 medium should be performed immediately to obtain high efficiency, and this method was applied hereafter.

It was reported that the inclusion of carrier DNA can also improve transformation efficiency in yeasts and plants (Abel et al., 1994; Schiestl and Gietz, 1989), which is probably because of the prevention of non-specific DNA absorption and/or degradation. Therefore, we next examined the effect of carrier DNA addition. To prepare carrier DNA, powdered salmon sperm DNA (Wako) was dissolved in sterile water at 4 mg/ml, and the solution was centrifuged for 5 min at 8,000 × *g* to remove debris. The salmon sperm DNA was not sonicated, but the DNA size was estimated at around 200−500 bp as judged by agarose gel electrophoresis (data not shown). As shown in Fig. 1C, the addition of carrier DNA at concentrations above 0.4 mg/ml (60 µg/150 µl) together with the reporter plasmid greatly increased the luciferase activity, as much as 8-fold compared with the control experiment without carrier DNA. While the possibility of insertion of carrier DNA into the recipient genome (Peerbolte et al., 1985) could be of concern here, thus far reported nuclear transformation of *C. merolae* always resulted from homologous recombination, and therefore non-homologous recombination involved the carrier DNA is unlikely to be problematic. The luciferase activity was also examined after increasing the concentration of the reporter plasmid pEF1.5-Luc in the presence of 0.4 mg/ml carrier DNA. The luciferase activity was proportionally increased up to 100 nM (63 µg/150 µl) plasmid under our conditions, and then became saturated (data not shown). The average luciferase activity at 100 nM was 18 times higher than that at 10 nM. As shown in Figs. 1B and 1C, the effects of time after PEG addition and carrier DNA were quite reproducible. However, the luciferase activity units themselves were rather variable dependent on the starting cell material used for a set of experiments. This may have resulted from the difference in the cell condition, and thus, more detailed control of the cultivation conditions would improve the fluctuation.

In this study, we estimated the DNA introduction efficiency based on the measured luciferase activity. However, this estimation is invalid if the luciferase activity is not proportional to the transformation efficiency. To evaluate this, we introduced a GFP-expressing reporter plasmid, pMtGFP (10

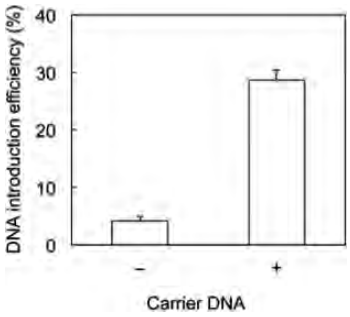

Fig. 2. Confirmation of DNA introduction efficiency.

DNA introduction efficiency with or without carrier DNA. After introducing pMtGFP to express GFP targeted to the mitochondrion, GFP-expressing cells were counted under fluorescence microscopy and the percentage of DNA introduction was calculated. At least 600 cells were counted for each condition. The standard errors were calculated from three independent experiments.

1. To prepare them for transformation, cells grown in MA2 medium (supplemented with 0.5 mg/ml uracil if necessary) to OD_{750} = 1.5−3 were diluted to OD_{750} = 0.4 in 50 ml of MA medium, and cultured overnight with aeration (2% CO_2).
2. After 20−24 h, the cells (typically OD_{750} = 0.7−1) were collected by centrifugation (2,000 × *g* for 5 min), washed once with MA-I and concentrated to adjust the OD_{750} to 126 in MA-I. Throughout the procedure, centrifugation was performed and the solution was kept at 40°C.
3. After adding 100 nM of plasmid DNA and 0.4−2 mg/ml carrier DNA to 50 µl of cell suspension (containing 4.0−4.7 × 10^8 cells) and adjusting to 150 µl with MA-I, an equal volume of PEG solution (60% w/v in MA-I) was added to the mixture to make the final PEG concentration 30%. The sample was mixed until homogeneous.
4. Immediately after the addition of PEG, the mixtures were diluted to 40 ml with MA2 medium, and incubated statically at 40°C in an incubator containing 5% CO_2.
5. The cells were collected after 24 h and analyzed.

Fig. 3. Optimized protocol for DNA introduction in *C. merolae*.

nM (4.7 µg/150 µl), Imoto et al., 2013) into *C. merolae* cells with or without carrier DNA (2 mg/ml (300 µg/150 µl)), and compared the transformation efficiency by counting the number of GFP-expressing cells using microscopy. The presence of carrier DNA resulted in an increase of GFP-expressing cells (7-fold, Fig. 2), which was comparable to the increase in luciferase activity (8-fold, Fig. 1C). Thus, we concluded that the transformation efficiency could be evaluated by measuring luciferase activity.

In conclusion, we examined various conditions for PEG-mediated transformation in *C. merolae*, and stabilized the experimental reproducibility and improved the transformation efficiency. The optimized protocol for transient gene expression is shown in Fig. 3.

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