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***Short Communication***

# Polyethylene Glycol (PEG)-Mediated Transient Gene Expression in a Red Alga, *Cyanidioschyzon merolae* 10D

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**DNA introduction into cells is an essential technique for molecular genetic analysis. Here, we show that DNA is easily introduced into cells of the unicellular red alga *Cyanidioschyzon merolae* by a polyethylene glycol (PEG)-mediated protocol. In this study, the β-tubulin gene of *C. merolae* was cloned on a plasmid and a hemagglutinin (HA) tag then added at the C-terminus. This plasmid was then introduced into *C. merolae* cells by a PEG-mediated transformation protocol. At 24 h after PEG-mediated transformation, intracellular localization of the tagged protein was detected by anti-HA immunocytochemistry, indicating the utility of this transient expression system for molecular genetic analyses.**

**Keywords:** *Cyanidioschyzon merolae* — Hemagglutinin — Polyethylene glycol — Transformation — Transient expression system.

Abbreviations: DAPI,4′, 6-diamidino-2-phenylindole; 5-FOA, 5-fluoroorotic acid; HA, hemagglutinin; MA medium, modified Allen's medium; PEG, polyethylene glycol.

*Cyanidioschyzon merolae* 10D is a unicellular red alga that lives in sulfate-rich acid hot springs. It has one of the simplest cellular structures among eukaryotes, which consists of a minimum set of organelles: one nucleus, one mitochondrion and one plastid. *Cyanidioschyzon merolae* has, therefore, been developed as a model organism for the investigation of the basic architecture of eukaryotes, such as organelle division machinery (Kuroiwa et al. 1994, Suzuki et al. 1994). Phylogenetic analyses also support *C. merolae* as having diverged very early in the eukaryotic lineage (Nozaki et al. 2003), and primitive characteristics appear to have been conserved throughout evolution. The 100% complete sequences of each of the three genomes, which are contained in the nucleus, mitochondrion and plastid, have been determined for the first time for a eukaryotic organism (Ohta et al. 1998, Ohta et al. 2003, Matsuzaki et al. 2004, Nozaki et al. 2007). The number of protein-coding genes in the nuclear genome is estimated as 4,775, representing the simplest genome of a photosynthetic eukaryote. Striking features are, for example, only three rRNA gene clusters, the smallest known histone gene cluster, and very few introns and transposable elements (Matsuzaki et al. 2004, Nozaki et al. 2007).

Molecular genetic analysis has the power to understand cellular mechanisms at the molecular level, and techniques for DNA introduction into cells are an essential requirement for the methodology. However, no method to introduce DNA into *C. merolae* had been developed before the genome project; therefore, we have been trying to establish such a protocol. In a previous study, we isolated a uracil auxotrophic mutant of *C. merolae*, by choosing clones resistant to 5-fluoroorotic acid (5-FOA), that were determined to have a lesion (a one base insertion in an A tract) in the chromosomal *URA5.3* gene. Using this mutant as a recipient, the wild-type *URA5.3* gene fragment was introduced into cells by electroporation, and uracil prototrophic transformants were selected on gelan-gum plates. Transformants appeared at a significant frequency, indicating successful introduction of DNA and homologous recombination with the chromosomal mutant gene (Minoda et al. 2004).

Chromosomal transformation experiments take a long time, typically >1 month; therefore, transient introduction and expression of exogeneous DNA is a useful technique for various analyses. In the present study, we successfully introduced DNA containing a hemagglutinin (HA)-tagged β-tubulin gene by a polyethylene glycol (PEG)-mediated protocol, and confirmed the expression and the localization of the gene product by immunocytochemistry.

Transient DNA introduction into cells and gene expression assays are versatile tools for molecular genetic applications. To establish the experimental conditions for optimized DNA introduction, it is useful to construct a vector from which epitope-tagged protein is expressed and which can easily be detected. For this purpose,

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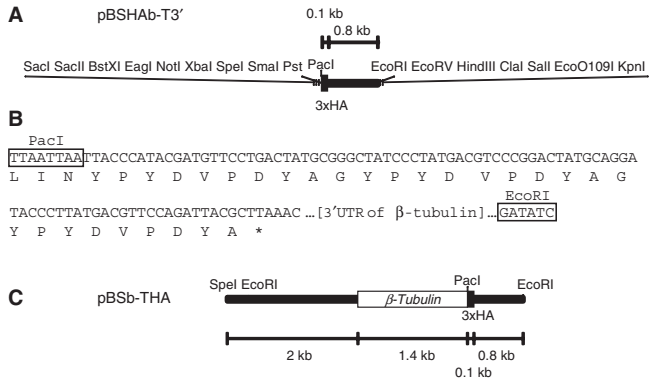


**Fig. 1** Structure of plasmids constructed in this study. Thick lines represent upstream and downstream regions of CMN263C encoding β-tubulin, and filled and open boxes show coding sequences for the triple HA tag and β-tubulin, respectively. (A) pBSHAb-T3′: the multiple cloning site used in this study, and potentially usable for other constructions, is shown. (B) Structure of the *Pac*I–*Eco*RI region containing the triple HA tag and the 3′-downstream sequence of β-tubulin. (C) pBSb-THA: the region related to the HA-tagged β-tubulin construct is shown.

we cloned a nuclear gene CMN263C (http://merolae.biol.s.u-tokyo.ac.jp) encoding β-tubulin, which was subsequently modified so as to express a protein fused with a triple HA tag peptide (YPYDVPDYAGYPYDVPDYAGYPYDVPDYA; Chen et al. 1993, Forsburg and Sherman 1997) at the C-terminus. At first, the 3′-flanking region of CMN263C was PCR amplified with DNA sequence for the HA tags in a primer, and cloned in pBluescript SK using *Eco*RI and *Pst*I sites. The resultant plasmid, pBSHAb-T3′, has a polylinker sequence in front of the HA tag sequence and, therefore, any protein of interest could be cloned and expressed as an HA-tagged protein (Fig. 1A). Based on this plasmid, a DNA fragment containing the 5′-flanking region of CMN263C probably including the promoter element and the β-tubulin coding region was PCR amplified, and introduced into the polylinker site of pBSHAb-T3′ using the attached *Spe*I and *Pac*I sites, to make pBSb-THA (Fig. 1B). Using this plasmid, an HA-tagged β-tubulin protein was expected to be expressed under the control of its native promoter.

Microscopic and other analyses indicated that *C. merolae* does not have a rigid cell wall structure, and the cell can be easily disrupted even by gentle detergent treatment in our hands. These observations indicate the possibility that *C. merolae* cells can be manipulated just as protoplasts of other plant species can be manipulated. In fact, we have previously succeeded in introducing exogeneous DNA by electroporation using standard cell culture (Minoda et al. 2004). Since PEG-mediated transformation is a standard method of introducing DNA into protoplasts (O'Neill et al. 1993, Koop et al. 1996, Datta and Datta 1999, Kim et al. 2002, Baur et al. 2005, Davey et al. 2005), we have examined this approach in the present study. Using logarithmically growing cells as the recipient, pBSb-THA was introduced into *C. merolae* cells under standard conditions used for plant protoplasts (20% PEG4000 at the time of DNA introduction). After the PEG treatment, the cell suspension was sufficiently diluted to avoid the cytotoxicity of PEG, and the expression of the HA-tagged β-tubulin was monitored by immunoblot analysis. As shown in Fig. 2A, no expression of the HA-tagged protein was detected by the introduction of pBluescript SK (vector plasmid). However, expression of the HA-tagged protein at the expected size (55 kDa) was clearly detected after the 24 h incubation. Thus, we concluded that the PEG-mediated DNA introduction and the transient protein expression from the introduced plasmid was successful. To examine which concentration of PEG4000 is optimum for the transformation, we changed the working concentration of PEG4000 and analyzed the expression of the HA-tagged protein, which is expected to be proportional to the transformation frequency. As the result, we found that 30% gave the best result (Fig. 2B). Similarly, we examined the effect of PEG average molecular weights: PEG1000, PEG4000, PEG6000 and PEG8000, and found that PEG4000 gave the best result (data not shown). This appeared to be because transformation efficiency by PEG1000 was relatively low, and the cytotoxic effect was more severe when PEG6000 or PEG8000 was used.

Next we performed indirect immunofluorescence microscopy analysis to examine the cellular localization of HA-tagged β-tubulin after the transformation of *C. merolae* cells. It is well known that α- and β-tubulin are microtubule components, and co-localize with the spindle structure during the cell mitosis. The expression and the spindle localization of α-tubulin have been confirmed in *C. merolae* (Nishida et al. 2005, Maruyama et al. 2007). As shown in Fig. 3, HA-tagged β-tubulin was detected predominantly at the spindle structure during mitotic phase in a similar manner to the localization of α-tubulin. However, the fluorescence tends to be weak and not localized in cells of other cell cycle phases (Fig. 3B). This may indicate that the amount of protein is less in these phases or that the protein is only condensed during the mitotic phase. The number of apparently fluorescent cells was counted using microscopy, and the transformation frequency was calculated as 2–10% based on the number of 4′,6-diamidino-2-phenylindole (DAPI)-stained cells.

In this study, we have demonstrated that PEG-mediated transformation and transient expression is a practical technique in *C. merolae*. Expression of epitope-tagged protein could facilitate localization analysis, as shown in this study, and expression of specifically modified proteins may help to elucidate protein function. The extensive possibilities of the present technique will further the emergence of *C. merolae* as a model eukaryotic cell.

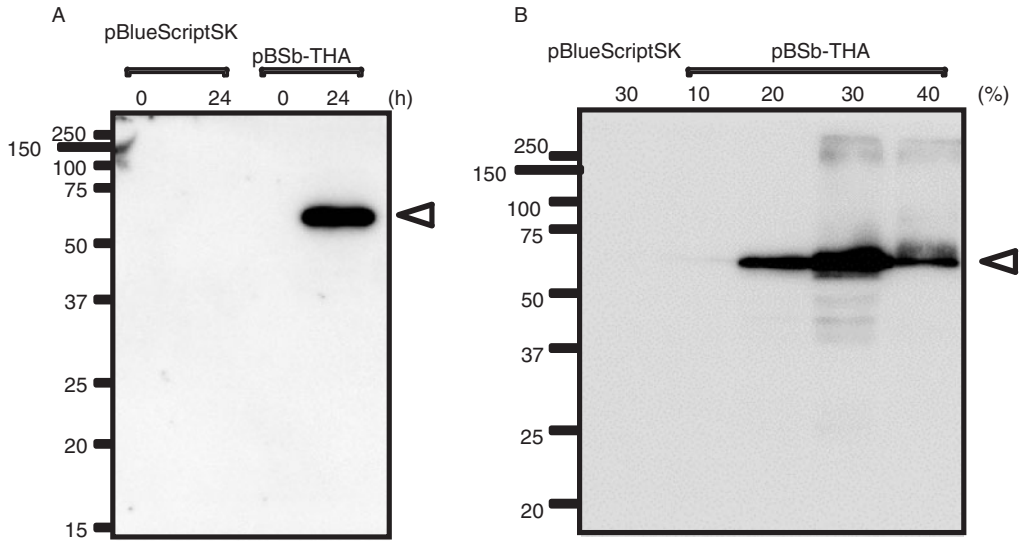


**Fig. 2** Expression of HA-tagged β-tubulin in *C. merolae*. pBlueScript SK (vector) or pBSb-THA was introduced into *C. merolae* cells by the PEG-mediated method, and the expression of the HA-tagged β-tubulin was examined by immunoblot analysis with anti-HA antibody after incubation for the indicated period. (A) Plasmids were introduced by 20% PEG4000, and samples were taken at 0 or 24 h after the transformation. (B) Plasmids were introduced with the indicated final concentration of PEG4000, and analyzed after 24 h. Arrowheads indicate positions of the HA-tagged β-tubulin. Precision Plus Protein dual color standard (Bio-Rad Laboratories, Hercules, CA, USA) was used to estimate the molecular mass.

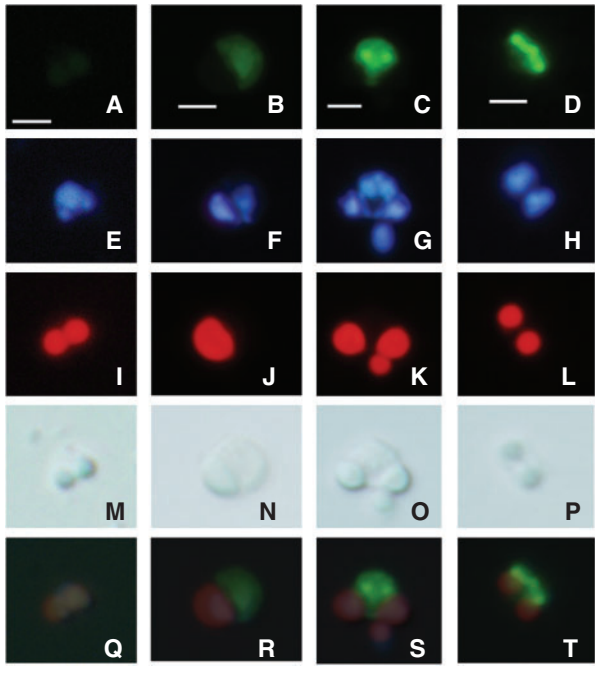


**Fig. 3** Subcellular localization of the HA-tagged β-tubulin. pBlueScript SK (left column) or pBSb-THA (the other columns) was introduced into *C. merolae* by 30% PEG4000, and incubated for 24 h. Localization of the HA-tagged β-tubulin was examined by immunofluorescent microscopy. HA-tagged β-tubulin (green, A–D), DAPI-stained DNA (blue, E–H), intrinsic chlorophyll fluorescence (red, I–L) and the merged images (Q–T) are shown together with the bright field images (M–P). Bars correspond to 2 μm.

## Materials and Methods

*Cyanidioschyzon merolae* 10D was used in this study. Cells were grown in MA2 medium [40 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 8 mM $KH_2PO_4$, 1 mM $CaCl_2$, 0.1 mM $FeCl_3$ (EDTA·2Na), 2-fold concentration of trace elements described in Minoda et al. (2004), pH 2.5] in a glass vessel under continuous white light (50 μmol photon $m^{-2}$ $s^{-1}$) at 40°C with 2% $CO_2$, unless otherwise noted.

The 3′-flanking region of the β-tubulin gene with a triple HA tag sequence for the C-terminal protein fusion was amplified from *C. merolae* total DNA with primers 5′-aactgcagttaattaattacccatac gatgttcctgactatgcgggctatccctatgacgtcccggactatgcaggataccttatgacgt tccagattacgcttaaactagctatttatctggtacatatc-3′ (*Pst*I and *Pac*I sites underlined) and 5′-ctgaattccgatcagtacggttaagtagtc-3′ (*Eco*RI site underlined) in 25 cycles (98°C for 10 s, 60°C for 30 s and 72°C for 1 min). The amplified fragment (904 bp) was digested with *Pst*I and *Eco*RI, and cloned into pBluescript SK to make pBSHAb-T3′. Subsequently, the 5′-flanking region (about 2 kb) and the coding region of the β-tubulin gene were amplified from *C. merolae* total DNA with primers 5′-ccactagtatccgtactgatgcaga-3′ (*Spe*I site underlined) and 5′-gcttaattaaactcatgatcgagctttcgata-3′ (*Pac*I site underlined) in 25 cycles (98°C for 10 s, 61°C for 30 s and 72°C for 4 min). The amplified fragment (3,335 bp) was digested with *Spe*I and *Pac*I, and cloned into pBSHAb-T3′ to make pBSb-THA. PCRs were performed using PrimeSTAR® HS DNA Polymerase (TAKARA BIO INC., Otsu, Japan). Restriction enzymes were from TAKARA BIO INC. and New England Biolabs (Ipswich, MA, USA).

To prepare cells for transformation, cells grown in MA2 medium to $OD_{750} = 1.5–3$ were diluted to $OD_{750} = 0.4$ in 100 ml of MA medium (Minoda et al. 2004), and cultured overnight. After 20–24 h, cells were collected by centrifugation (2,000 × *g* for 5 min), washed once with MA-I [20 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1 × trace elements] and resuspended in MA-I to concentrate to

150- to 200-fold. Throughout the procedure, centrifugation was performed at 40°C. After adding 20 μg of plasmid DNA in 400 μl of MA-I to 100 μl of cell suspension (containing $1.5–3.3 \times 10^7$ cells), 500 μl of PEG solution [20–60% (w/v) in MA-I] was added to the mixture to make the final PEG concentration 10–30%. To make the final concentration 40%, 20 μg of plasmid DNA in 233 μl of MA-I was mixed with the 100 μl cell suspension, and 667 μl of 60% PEG solution was added to the mixture. After leaving for 5 min at room temperature, the mixtures were diluted to 50 ml with MA2 medium, and incubated at 40°C in an incubator containing 5% $CO_2$. Cells were collected after 24 h and analyzed by immunoblotting and immunofluorescence microscopy.

A 16 ml aliquot of culture was sampled after transformation, and cells were collected by centrifugation ($3,000 \times g$ for 5 min at 4°C). Cell pellets were resuspended in 250 μl of lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol and 10% glycerol, containing a protease inhibitor cocktail (1× Complete Mini protease inhibitor, EDTA-free, Roche, Basel, Switzerland)]. After adding 150 mg of glass beads (ϕ106 μm, Sigma-Aldrich, St Louis, MO, USA), the mixture was vortexed for 5 min, and debris was removed by centrifugation. Protein concentration was estimated by the BCA™ Protein Assay Kit (Pierce), and 20 μg of the protein was subjected to SDS–PAGE. Immunoblot analysis was performed as described previously (Terashita et al. 2006). HA-tagged protein was first reacted with mouse anti-HA antibody (Sigma-Aldrich, diluted 1 : 3,000), and subsequently reacted with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Promega, Madison, WI, USA; diluted 1 : 5,000). The ECL-Plus Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK) was used for detection.

Indirect immunofluorescence staining for HA-tagged β-tubulin was performed as described by Nishida et al. (2005) with a few modifications, as follows. Cells were collected by centrifugation, and directly immersed in 10× the packed cell volume of fixation buffer (1% paraformaldehyde and 10% dimethylsulfoxide in methanol, pre-cooled at –80°C) for 5 min at –20°C. Alexa Fluor 488-conjugated mouse monoclonal anti-HA (Invitrogen, Carlsbad, CA, USA) was diluted 1: 500 before the reaction. The blocking and labeling steps were performed on ice. Cells were examined using an Olympus BX50 microscope (Olympus). Images were captured with an Olympus DP70 digital camera and processed by Adobe Photoshop plug-in software.

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