Plant Cell Physiol. 45(6): 667–671 (2004)

Rapid Paper

Improvement of Culture Conditions and Evidence for Nuclear Transformation by Homologous Recombination in a Red Alga, *Cyanidioschyzon merolae* 10D

Ayumi Minoda [1], Rei Sakagami [2], Fumi Yagisawa [3,4], Tsuneyoshi Kuroiwa [3] and Kan Tanaka [1,5]

[1] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Tokyo, 113-0032 Japan*
[2] *School of Life Science, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo, 192-0392 Japan*
[3] *Department of Life Science, College of Science, Rikkyo (St. Paul's) University, Nishiikebukuro, Tokyo, 171-8501 Japan*
[4] *Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo, Tokyo, 113-0033 Japan*

Although the nuclear genome sequence of *Cyanidioschyzon merolae* 10D, a unicellular red alga, was recently determined, DNA transformation technology that is important as a model plant system has never been available thus far. In this study, improved culture conditions resulted in a faster growth rate of *C. merolae* in liquid medium (doubling time = 9.2 h), and colony formation on gellan gum plates. Using these conditions, spontaneous mutants (5-fluoroortic acid resistant) deficient in the UMP synthase gene were isolated. The lesions were then restored by introducing the wild-type UMP synthase gene into the cells suggesting DNA transformation by homologous recombination.

Keywords: Cultivation — *Cyanidioschyzon merolae* — 5-FOA resistance — Homologous recombination — Red algae — Transformation.

Abbreviations: 5-FOA, 5-fluoroorotic acid; MA medium, modified Allen medium; OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate.

Introduction

Cyanidioschyzon merolae 10D is a unicellular red alga that lives in acid hot springs rich in sulfate (pH 1.5, 45°C). This alga contains one nucleus, one mitochondrion and one plastid, and is considered one of the most primitive algae by many lines of evidence (Kuroiwa 1998, Nozaki et al. 2003, Matsuzaki et al. 2004). A sample of *C. merolae* 10D was isolated (Toda et al. 1995) from the hot spring algal collection provided from Dr. Pinto, Naples University. Because this thermophilic alga does not possess any rigid cell walls, *C. merolae* is a suitable material for studies of biochemistry, structural biology and biotechnology. Furthermore, the nuclear (Matsuzaki et al. 2004) and the organelle (Ohta et al. 1998, Ohta et al. 2003) genome sequences of *C. merolae* were recently determined by a nuclear genome project aimed at promoting an understanding of plant evolution and molecular biology. In addition, its simple life cycle also facilitates laboratory manipulations (Terui et al. 1995, Kuroiwa 1998). Despite its usefulness as a model plant system, the basic methods for cultivating *C. merolae* have not been investigated sufficiently to take advantage of it. Under conventional culture conditions with continuous illumination (35 μmol photons m^{-2} s^{-1}) at 40°C with aeration, the doubling time was reported as approximately 32 h in liquid medium. And limited use of cultures on solid plate media has been reported for *C. merolae* 10D (Toda 1996). In this study, we investigated these culture conditions to obtain a faster growth rate, and examined the possibility for nuclear transformation using exogenous DNA and the improved cultivation conditions.

Results and Discussion

To improve the cultivation conditions, the composition of the medium was examined. In our laboratory thus far, *C. merolae* has been grown in Allen's photoautotrophic medium (Allen 1959), replacing the trace elements with Arnon's A_6 solution for experimental convenience. Previously, Dr. Seckbach suggested that 2× Allen's medium; in which all ingredients are concentrated two times more than the original Allen's medium, supports better cyanidiophycea growth (Seckbach 1994). Thus, we examined *C. merolae* growth in Allen's media concentrated to various strengths. As a result, the doubling time in the double strength 2× Allen's medium was shortened from 32 to 24 h, however, increasing concentrations of the ingredients up to fivefold did not improve this growth rate further. Thus, 2× Allen's medium was used for the subsequent experiments.

When the iron ingredient of the 2× Allen's medium was mixed with the other ingredients prior to autoclaving, a significant amount of sediment appeared, which was inconvenient for the experimental manipulations. To prevent this the iron ingredient was added after autoclaving. Considering the modifications described above, a modified Allen's autotrophic medium (MA medium) was established for *C. merolae* growth (Table 1).

[5] Corresponding author: E-mail, kntanaka@iam.u-tokyo.ac.jp; Fax, +81-3-5841-8476.

Table 1 Modified Allen's (MA) autotrophic medium

$(NH_4)_2SO_4$	2.62 g liter^{-1}
KH_2PO_4	0.54 g liter^{-1}
$MgSO_4 \cdot 7H_2O$	0.5 g liter^{-1}
$CaCl_2 \cdot 2H_2O$	0.14 g liter^{-1}
$FeCl_3$ (EDTA·2Na) [a]	0.016 g liter^{-1} (0.028 g liter^{-1})
Trace elements (2× A_6 solution) [b]	
Adjust the pH to 2.5 with H_2SO_4	

[a] 250× Fe solution (filtered sterilization) is 7 g liter^{-1} EDTA·2Na and 4 g liter^{-1} $FeCl_3$.
[b] A_6 solution is 2.85 g liter^{-1} H_2BO_3, 1.8 g liter^{-1} $MnCl_2 \cdot 4H_2O$, 0.105 g liter^{-1} $ZnCl_2$, 0.39 g liter^{-1} $Na_2MoO_2 \cdot 2H_2O$, 0.04 g liter^{-1} $CoCl_2 \cdot 6H_2O$, 0.043 g liter^{-1} $CuCl_2$.
250× Fe solution was sterilized by filtration and added to the medium after the addition of all other ingredients and completion of autoclaving.

When the liquid culture was performed under higher light conditions (90 μmol photons m^{-2} s^{-1}) and bubbled with 5% (v/v) CO_2 at 40°C, doubling time was shortened further, down to 9.2 h (Fig. 1A). However, cells grown under illumination with 200 μmol photons m^{-2} s^{-1} showed a decrease in the growth rate and a yellowish color, suggesting they were suffering from high light stress at this light intensity. Under the growth conditions tested [35 μmol photons m^{-2} s^{-1}, 0.04% (v/v) CO_2, 40°C], *C. merolae* formed colonies on the MA-0.4% gellan gum plates (Fig. 1B); no colony formation has previously been observed on plates based on the original Allen's medium. The addition of 0.4% glycerol to the medium also improved the plate and liquid culture growth of *C. merolae* (data not shown), indicating that there are further possibilities for improving culture conditions.

The construction of a nuclear transformation system is important for making *C. merolae* a model experimental organism. For this purpose, it is essential to establish selectable marker genes and selection conditions. To find potentially useful drugs for marker selection, various available antibiotics and drugs generally used for genetic selection were tested, and their effect on growth was examined using MA plates. It was revealed that chloramphenicol (100 μg ml^{-1}), imazapyr (500 μg ml^{-1}, Kanto Kagaku, Japan), nalidixic acid (10 μg ml^{-1}, Itoh et al. 1997), novobiocin (100 μg ml^{-1}), and 5-fluoroorotic acid (5-FOA) (800 μg ml^{-1}) effectively prevented growth, while ampicillin (1 mg ml^{-1}), kanamycin (1 mg ml^{-1}), spectinomycin (1 mg ml^{-1}), bleomycin (20 μg ml^{-1}), and vancomycin (1 mg ml^{-1}) had no effect on growth. The lack of growth inhibition by kanamycin and bleomycin was consistent with the results of Yagisawa et al. (2004). In other organisms such as yeast (*Saccharomyces cerevisiae*), 5-FOA is used to select mutants deficient in orotidine 5′-monophosphate (OMP) decarboxylase because 5-FOA is converted to a highly toxic compound, 5-fluorouracil, by this enzyme (Boek et al. 1987). Conversely, OMP decarboxylase-deficient mutants show auxotrophy for uracil, and the structural gene for this enzyme (*URA3* in *S. cerevisiae*) can be used as a selectable marker for genetic transformation (Boek et al. 1987, Peck et al. 2000). Taking advantage of this, a gene in *C. merolae* encoding OMP decarboxylase was identified based on its nuclear genome sequence (Matsuzaki et al. 2004). In *C. merolae*, OMP decarboxylase was found to be expressed as a fused protein with orotidine-5′-phosphoribosyltransferase, which is encoded by *URA5* in *S. cerevisiae*, as in *Arabidopsis thaliana* and *Homo sapiens* (Nasr et al. 1994). Its corresponding gene was named *URA5.3* in *C. merolae*. The inhibitory concentration of 5-FOA was roughly the same as in *S. cerevisiae*; basically no colonies emerged when 10^7 cells were spread on MA-0.4% gellan gum plates containing 800 μg ml^{-1} 5-FOA (data not shown). To obtain spontaneous 5-FOA-resistant mutants, 9×10^7 cells were spread on nine MA-0.4% gellan gum plates containing 800 μg ml^{-1} 5-FOA and 500 μg ml^{-1} uracil. Eight independent clones were isolated after 2 months of plating, and designated M1–M8, respectively. 5-FOA resistance and uracil auxotrophy was a stable phenotype as shown in Fig. 2A and B. The entire DNA of three clones, M1, M3 and M4, was isolated, and the nucleotide sequences of the DNA regions around the *URA5.3* gene were directly determined after the PCR amplification using combinations of the primers listed in Table 2. As a result, we found that a dA_7 tract in the mid-portion of the *URA5.3* open reading frame (462 amino acids) had mutated to a dA_8 tract as a result of a frame

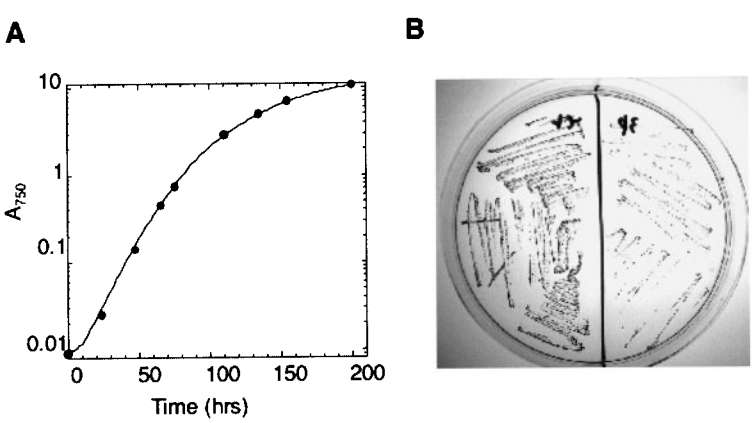

Fig. 1 *C. merolae* growth. (A) Cell growth under 70 μmol photons m^{-2} s^{-1} at 40°C with bubbling of 5% CO_2 (v/v). Values are representative of two independent experiments. (B) Formation of single *C. merolae* colonies on plates. Cells were streaked onto the MA-0.4% gellan gum plates using a toothpick, and incubated for 10 d before the photograph was taken.

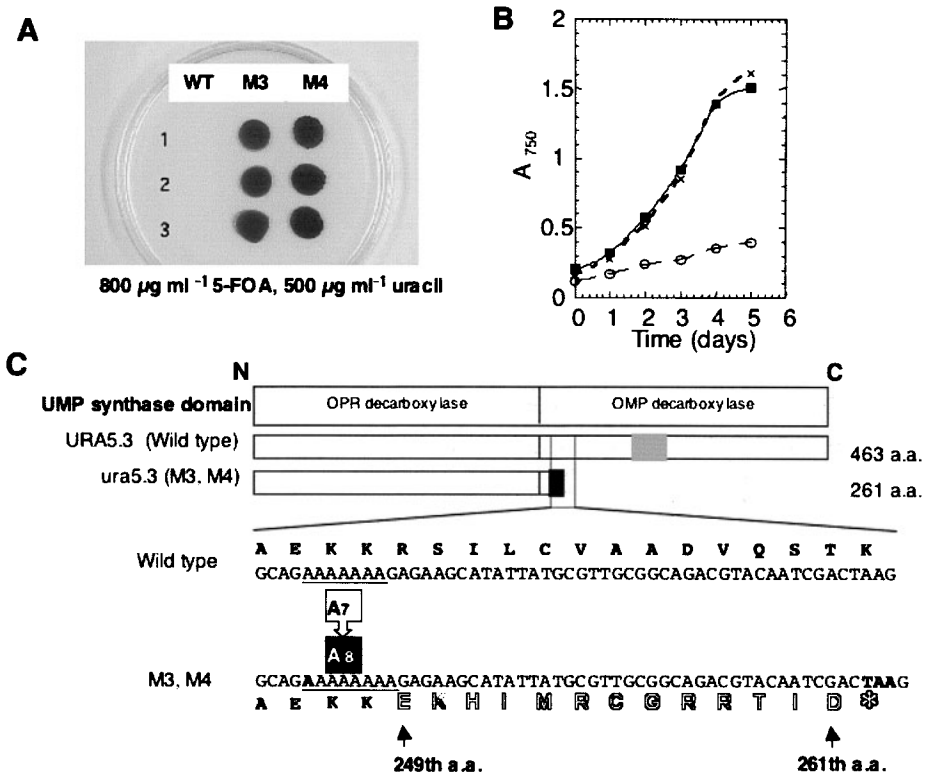

Fig. 2 Analysis of spontaneous 5-FOA-resistant *C. merolae* mutants. (A) Sensitivity to 5-FOA of the wild type and M3 and M4 mutants grown on MA-0.4% gellan gum plates containing 800 μg ml^{-1} 5-FOA and 500 μg ml^{-1} uracil. The three spots represent reproducibility. (B) Effects of uracil on 5-FOA-resistant mutant growth. The M4 mutant was grown in the presence (crosses) or absence (open circles) of 2.5 mg ml^{-1} uracil. The wild type was also grown in the absence of uracil as a control (closed squares). (C) The domain structure of UMP synthase (URA5.3) in *C. merolae*, whose amino terminal and carboxy terminal domains correspond to orotidine-5′-phosphoribosyltransferase and OMP decarboxylase, respectively. The grey area represents the catalytic site of OMP decarboxylase while the black area indicates the changed amino acid stretch of the URA5.3 protein as a result of a frameshift mutation in M3 and M4. The structure of the mutated region was also shown in detail. The nucleotide sequence of the *URA5.3* gene has been deposited in the DDBJ/EMBL/GenBank databases under Accession No. AB164641, and the depicted nucleotide sequence of the wild-type gene corresponds to positions 3,602 to 3,655.

Table 2 List of primers

Primer name	Sequence	5′-Position	3′-Position [a]
*Cla*I-*URA5.3*	5′-CCATCGATGGGAACTGAGGGGCGAACGC-3′	2012	2029
*URA5.3-Spe*I	5′-GGACTAGTCCTGGCATGCGCAGAACGCG-3′	5170	5151
primer 1	5′-GCGAGGTAGGTGCTAGTTTG-3′	2411	2430
primer 2	5′-CGAGGATACGAGACAGCAAC-3′	3030	3011
primer 3	5′-TACGGTGCAGAACACCGAAC-3′	2834	2853
primer 4	5′-GTTCAAATTCCGCAGCGTCC-3′	3448	3429
primer 5	5′-TCACACAAGGCTCTCGTTGC-3′	3225	3244
primer 6	5′-CCTGCGCTGAACTGTGCAAC-3′	3949	3830
primer 7	5′-TGCGGCAGACGTACAATCGA-3′	3632	3651
primer 8	5′-TTGCTGGCTCTTCACTCCCG-3′	4251	4232
primer 9	5′-GCGAAGCGTTTCCGTTCTGT-3′	4041	4060
primer 10	5′-CGACACTAGAGCGATTCCCT-3′	4670	4651

[a] The 5′ and 3′ positions of the primers represent the nucleotide positions in the database entry, AB164641.

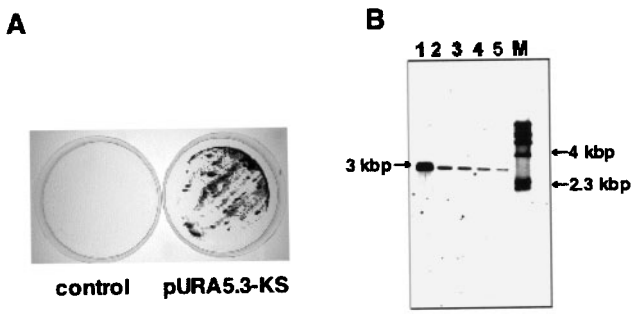

Fig. 3 Nuclear transformation of *C. merolae*. (A) pURA5.3-KS containing the wild-type *URA5.3* gene introduced into *C. merolae* cells by electroporation, and plated on MA-0.4% gellan gum plates. An example of the Effectene Transfection Reagent results is shown (right) together with a negative control with the vector plasmid (left). (B) Southern hybridization analysis of the transformants. The total DNAs were isolated from the wild type (lane 1), and the M4 mutant (lane 2) and representative transformants (lanes 3–5) were digested with *Pst*I and hybridized with the *URA5.3* probe. Each transformant was obtained by the introduction of pURA5.3-KS (lane 3) using Effectene Transfection Reagent (lane 4) or SuperFect Transfection Reagent (lane 5). Lane M is the molecular weight marker.

shift after the 259th amino acid and the production of a truncated gene product (261 amino acids, Fig. 2C). Due to this mutation, the carboxy terminal domain corresponding to OMP carboxylase activity was truncated, consistent with the 5-FOA-resistant and uracil-auxotrophic phenotype of the M3 and M4 mutants. Since the same mutation was found in two independent isolates, this dA-tract appears to be a hot spot for slippage mutations during DNA replication (Seki et al. 1999). We could not find any mutated bases in the *URA5.3* gene of the M1 strain, indicating an unknown mutation at another locus.

Next, using the isolated M4 mutant as a recipient, the possibility of nuclear transformation was examined. A DNA fragment encompassing the wild-type *URA5.3* gene was PCR amplified from the chromosome DNA, and cloned on a plasmid vector to make pURA5.3-KS. To introduce DNA into *C. merolae*, 1.5 ml cultures were harvested at the mid-exponential phase (A_{750} = 0.3–0.4), and concentrated to 40 μl by centrifugation. The cell suspension containing 3–4×10^8 cells was mixed with 1 μl of pURA5.3-KS (0.5 μg μl^{-1}), and electroporated at a field strength of 2 kV m^{-2} s^{-1}. In addition to this, 4 μg DNA was treated with Effectene Transfection Reagent (QIAGEN, Hilden, Germany) or SuperFect Transfection Reagent (QIAGEN, Hilden, Germany) as described by the supplier, mixed with the 40 μl of the cell suspension, and electroporated at a field strength of 2.5 kV m^{-2} s^{-1}. The vector plasmid, pBluescriptKS, was also electroporated using the same conditions as the controls. After electroporation, cell suspensions were inoculated with 2 ml MA medium, and incubated overnight in the dark at 40°C with shaking. The cells were spread on MA-0.4% gellan gum plates, and incubated under light conditions with 35 μmol photons m^{-2} s^{-1} at 40°C to allow selection of uracil prototrophic clones. After 1 month, a number of colonies formed only on the plates where pURA5.3-KS had been electroporated in every case, suggesting nuclear transformation by the exogenous DNA (Fig. 3A). The transformation frequency differed from time to time, but typically more than 100 colonies appeared by the introduction of 0.5 μg pURA5.3-KS plasmid DNA.

To analyze whether these clones appeared due to nuclear transformation, three uracil-prototrophic clones were collected from each transformation protocol, and their total DNAs were isolated. The results of Southern hybridization with a *URA5.3* probe indicated only one copy of the *URA5.3* gene in the nuclear genome (Fig. 3B), showing that random integration of the transformed plasmid into the nuclear genome did not occur. Sequencing analysis of the *URA5.3* region of these five clones confirmed that the frameshift mutation found in the M4 mutant was reverted to the wild-type sequence (data not shown). Thus, transformation of the nuclear genome by homologous recombination was thought to have occurred in this study. The recent isolation of cycloheximide-resistant mutants could also facilitate the establishment of gene targeting technology in the nuclear genome of *C. merolae* (Yagisawa et al. 2004).

The unicellular green alga *Chlamydomonas* is known to be a good model organism for studies of cell and molecular biology (Harris 1998). However, gene targeting of its nuclear genome by homologous recombination has never been achieved. Homologous recombination of the nuclear genome of plants has so far only been reported in the moss *Physcomitrella patens* (Schaefer 2002). Therefore gene-targeting technology using the unicellular *C. merolae* system will help solve many problems with regards to plant as well as basic eukaryotic biology.

Materials and Methods

DNA sequencing was performed with the CEQ™ 2000XL DNA analysis system (BECKMAN COULTER, CA, U.S.A.) and CEQ™ DTCS-Quick Start Kit (BECKMAN COULTER, CA, U.S.A.). To construct pURA5.3-KS, 3,158 bp DNA fragment containing the wild-type *URA5.3* gene was PCR amplified from chromosomal DNA with a primer set, *Cla*I-*URA5.3* and *URA5.3*-*Spe*I (see Table 2), and cloned into the *Cla*I–*Spe*I sites of pBluescriptKS (Stratagene, CA, U.S.A.). The identity of the cloned sequence containing the chromosomal copy was confirmed by sequencing. Electroporation was performed with Electro-gene-transfer-equipment GTE-10 equipped with Extended capacitance unit ECU-1 (Shimadzu, Japan). Southern hybridization analysis was performed as described previously (Oguchi et al. 1999). The *URA5.3* probe (625 bp) was designed to detect the 3-kbp *Pst*I fragment derived from the chromosomal *URA5.3* copy, and larger fragments were expected to be detected in case of random plasmid integration. The DIG-labeled probe was prepared by PCR amplification using primers 5 and 6 shown in Table 2.

Acknowledgments

We would like to thank Drs. Osami Misumi and Haruko Kuroiwa for helpful discussions and suggestions. This study was supported by a Grant-in-Aid for Scientific Research on Priority Area “Genome Biol-

ogy" from the Ministry of Education, Culture, Sports, Science and Technology, Japan (No. 13206011 to T.K. and K.T.).

References

Allen, M.B. (1959) Studies with *Cyanidium caldarium*, an anomalously pigmented chlorophyte. *Arch. Microbiol.* 32: 270–277.

Boek, J.D., Trueheart, J., Natsoulis, G. and Fink, G.R. (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* 154: 164–175.

Harris, E.H. (1998) Introduction to *Chlamydomonas*. *In* The molecular Biology of Chloroplast and Mitochondria in *Chlamydomonas*. Edited by Rocheix, J.D., Goldschmidt-Clermont, M. and Merchant, S. pp. 1–11. Kluwer Academic Publishers, Dordrecht.

Itoh, R., Takahashi, H., Toda, K., Kuroiwa, H. and Kuroiwa, T. (1997) DNA gyrase involvement in chloroplast-nucleoid division in *Cyanidioschyzon merolae*. *Eur. J. Cell Biol.* 73: 252–258.

Kuroiwa, T. (1998) The primitive red algae: *Cyanidium caldarium* and *Cyanidioschyzon merolae* as model system for investigating the dividing apparatus of mitochondria and plastids. *Bioessays* 20: 344–354.

Matsuzaki, M., Misumi, O., Shin-I, T., Maruyama, S., Takahara, M., Miyagishima, S., Mori, T., Nishida, K., Yagisawa, F., Nishida, K., Yoshida, Y., Nishimura, Y., Nakao, S., Kobayashi, T., Momoyama, Y., Higashiyama, T., Minoda, A., Sano, M., Nomoto, H., Oishi, K., Hayashi, H., Ohta, F., Nishizaka, S., Haga, S., Miura, S., Morishita, T., Kabeya, Y., Terasawa, K., Suzuki, Y., Ishii, Y., Asakawa, S., Takano, H., Ohta, N., Kuroiwa, H., Tanaka, K., Shimizu, N., Sugano, S., Sato, N., Nozaki, H., Ogasawara, N., Kohara., Y. and Kuroiwa, T. (2004) Genome sequence of the ultra-small unicellular red alga *Cyanidioschyzon merolae*. *Nature* 428: 653–657.

Nasr, F., Bertauche, N., Dufour, M.E., Minet, M. and Lacroute, F. (1994) Heterospecific cloning of *Arabidopsis thaliana* cDNAs by direct complementation of pyrimidine auxotrophic mutants of *Saccharomyces cerevisiae*. I. Cloning and sequence analysis of two cDNAs catalysing the second, fifth and sixth steps of the de novo pyrimidine biosynthesis pathway. *Mol. Gen. Genet.* 244: 23–32.

Nozaki, H., Matsuzaki, M., Takahara, M., Misumi, O., Kuroiwa, H., Hasegawa, M., Shin-I, T., Kohara, Y., Ogasawara, N. and Kuroiwa, T. (2003) The phylogenetic position of red algae revealed by multiple nuclear genes from mitochondria-containing eukaryotes and an alternative hypothesis on the origin of plastids. *J. Mol. Evol.* 56: 485–497.

Oguchi, K., Liu, H., Tamura, K. and Takahashi, H. (1999) Molecular cloning and characterization of *AtTERT*, a telomerase reverse transcriptase homolog in *Arabidopsis thaliana*. *FEBS Lett.* 457: 465–469.

Ohta, N., Matsuzaki, M., Misumi, O., Miyagishima, S., Nozaki, H., Tanaka, K., Shin-I, T., Kohara, Y. and Kuroiwa, T. (2003) Complete sequence and analysis of the plastid genome of the unicellular red alga *Cyanidioschyzon merolae*. *DNA Res.* 10: 67–77.

Ohta, N., Sato, N. and Kuroiwa, T. (1998) Structure and organization of the mitochondrial genome of the unicellular red alga *Cyanidioschyzon merolae* deduced from the complete nucleotide sequence. *Nucleic Acids Res.* 26: 5190–5298.

Peck, R.F., Dassarma, S. and Krebs, M.P. (2000) Homologous gene knockout in the archaeon *Halobacterium salinarum* with *ura3* as a counterselectable marker. *Mol. Microbiol.* 35: 667–676.

Schaefer, D.G. (2002) A new moss genetics: targeted mutagenesis in *Physcomitrella patens*. *Annu. Rev. Plant Biol.* 53: 477–501.

Seckbach, J. (1994) Methods for algal investigations. *In* Evolutionary Pathways and Enigmatic Algae: *Cyanidium caldarium* [Rhodophyta] and Related Cells. Edited by Seckbach, J. pp. 333–335. Kluwer Academic Publishers, Dordrecht.

Seki, M., Akiyama, M., Sugaya, Y., Ohtsubo, E. and Maki, H. (1999) Strand asymmetry of +1 frameshift mutagenesis at a homopolymeric run by DNA polymerase III holoenzyme of *Escherichia coli*. *J. Biol. Chem.* 274: 33313–33319.

Terui, S., Suzuki, K., Takahashi, H., Itoh, R. and Kuroiwa, T. (1995) High synchronization of chloroplast division in the ultramicro-alga *Cyanidioschyzon merolae* by treatment with both light and aphidicolin. *J. Phycol.* 31: 958–961.

Toda, K. (1996) Molecular cytological studies of nuclear shapes and chromosomes in an ultra-small primitive red alga, *Cyanidioschyzon merolae*. Master Thesis, The University of Tokyo, Japan.

Toda, K., Takahashi, H., Itoh, R. and Kuroiwa, T. (1995) DNA contents of cell nuclei in two *Cyanidiophycea*: *Cyanidioschyzon merolae* and *Cyanidium caldarium* Forma A. *Cytologia* 60: 183–188.

Yagisawa, F., Nishida, K., Okano, Y., Minoda, A., Tanaka, K. and Kuroiwa, T. (2004) Isolation of cycloheximide-resistant mutants of *Cyanidioschyzon merolae*. *Cytologia* 69: 97–100.

(Received March 11, 2004; Accepted April 6, 2004)