Characterization of cell-cycle-driven and light-driven gene expression in a synchronous culture system in the unicellular rhodophyte Cyanidioschyzon merolae

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The unicellular rhodophyte Cyanidioschyzon merolae, having a single plastid and a single mitochondrion, is suitable for the analysis of the cell cycle involving the division of organelles. In conventional methods of synchronous culture of algae, light/dark cycles have been used as signals for synchronization, and the gene expression promoted by light is not separated from the gene expression related to cell cycle progression. We previously devised a novel synchronous culture system with controlled photosynthesis, which is triggered by 6 h-light/18 h-dark cycles combined with different levels of CO2. The cells do not enter S-phase and consequently do not divide after the minimum light period without CO2 supplementation, but do divide after a light period with 1% CO2. In this way, we can compare a dividing cycle and a non-dividing cycle. We examined changes in the expression of 74 genes throughout the cell cycle by quantitative RT-PCR. The expression of genes for two cyclins (cyclin C and H) and two CDKs (CDKA and CDKD) as well as metabolic enzymes was promoted by light, whereas the expression of genes for G1/S or G2/M cyclins and CDKs as well as DNA replication enzymes and proteins related to organelar division was promoted only in the dividing cycles. These results suggested that C. merolae has a checkpoint for G1/S progression, which is regulated by nutrients within the 6 h light period.

INTRODUCTION

Synchronous culture is useful in analysing the progression of the cell cycle. The strategy for synchronization is different in different organisms. Culture of plant cells has been synchronized by starvation and subsequent supply of nutrients, such as phosphate, nitrate and sucrose, or hormones (Amino et al., 1983; King et al., 1974; Kodama et al., 1991; Nishida et al., 1992; Riou-Khamlichi et al., 1999, 2000). Another method of synchronization involves application of reversible inhibitors that block the cell cycle at a specific phase (Planchais et al., 2000). The light/dark cycle is another method of synchronization that has commonly been used in photosynthetic organisms such as algae since the first publications describing synchronous culture of Chlorella (Tamiya et al., 1953) and Chlamydomonas (Bernstein, 1960).

Cyanidioschyzon merolae, a unicellular rhodophyte isolated from an Italian hot spring, has a very simple cell structure consisting of a single mitochondrion, plastid and microbody per cell (Matsuzaki et al., 2004). The cell proliferates by binary fission, and organelle division occurs synchronously in each cell division cycle. The alga’s normal habitat is warm (up to 50°C) and acidic (pH 1.5–2.5) water containing sulfuric acid. The 100% complete genome sequence (16,546,747 bp) of this organism has been determined, including telomeres and rDNA sequences (Nozaki et al., 2007), a completeness that has never been achieved in other eukaryotes. The number of predicted protein-coding genes is 4775, which is less than the number of proteins in yeasts or other unicellular organisms sequenced to date. These characteristics, as well as phylogenetic analysis (Nozaki et al., 2007), suggested that C. merolae is one of the most primitive red algae, probably
diverged from a point near the root of the eukaryotes. We also reported a unique pathway of lipid biosynthesis in this alga based on genomic and experimental analyses (Sato & Moriyama, 2007) and the DNA polymerase localized to both plastid and mitochondrion (Moriyama et al., 2008). We proposed to name this new type of organellar DNA polymerase POP (plant organellar DNA polymerase). This is an algal example of dual targeting, which has not been reported in many cases.

In the latter study, we devised a synchronous culture system with controlled photosynthesis, which is triggered by 6 h-light/18 h-dark cycles combined with different levels of CO2. The cells do not divide after the minimum light period without CO2 supplementation, but do divide after a light period with 1 % CO2 (Moriyama et al., 2008). Comparing the expression of the DNA polymerase between a dividing cycle and a non-dividing cycle, we found indications that the expression of polymerase is related to cell proliferation.

In the present study, we determined the S-phase of nuclear and organellar genomes by quantitative PCR (qPCR), and measured the level of 74 transcripts by quantitative RT-PCR (RT-qPCR) in our synchronous culture, and then classified the changes in the level of transcripts into those related to the cell division cycle and those induced by light.

METHODS

Non-synchronous culture. Cells of Cyanidioschyzon merolae strain 10D (Toda et al., 1998) were inoculated in 2 × Allen’s medium (Minoda et al., 2004) at pH 2.5, and flasks were shaken under continuous light provided by two fluorescent tubes (30 10D (Toda et al., 2004)) at pH 2.5, and flasks were shaken under continuous light provided by two fluorescent tubes (30 µE m⁻² s⁻¹) at 42 °C.

Synchronous culture. Subcultured cells (pre-culture) were grown to a density of approximately OD₇₅₀ 7–9 (stationary phase), and diluted in fresh medium to a density of OD₇₅₀ 0.25. They were then subjected to a 30 h-dark/6h-light/18 h-dark cycle at 44 °C with aeration with ordinary air, and then two 6 h-light/18 h-dark cycles with aeration by 1 % CO₂ in air. In the light period, the cells received high light provided by three 20 W krypton bulbs (300 µE m⁻² s⁻¹ at the level of growing cells). At the end of each dark period, fresh 2× Allen’s medium was added to the culture to control cell density as described previously (Moriyama et al., 2008).

Microscopic examination of C. merolae cells. Cells were fixed with 1 % (w/v) glutaraldehyde and 0.5 % (w/v) Triton X-100, and then stained with 1 µg DAPI ml⁻¹ as described previously (Moriyama et al., 2008).

Quantification of genomic DNA. DNA content was analysed by qPCRs. To estimate the absolute amount of nuclear, plastid and mitochondrial genomes, chemically fixed cells of the cyanobacterium Synechocystis sp. PCC 6803 were used as an internal standard. For this purpose, the Synechocystis cells, which had been grown as described previously (Ishikawa et al., 2009), were fixed with 5 % (w/v) phenol in ethanol, washed twice with TE 50/20 solution (50 mM Tris/HCl pH 7.5 and 20 mM EDTA), and stored frozen in TE 50/20. An aliquot of Synechocystis cell suspension was added to 400 µl C. merolae cell culture, and the mixture was frozen in liquid nitrogen and stored at −80 °C. After thawing, 10 µg lysozyme ml⁻¹ was added, and the mixture was incubated at 37 °C for 30 min to digest peptido- glycan of Synechocystis added as an internal standard. Then, 100 µg Proteinase K ml⁻¹ and 1 % (w/v) SDS were added, and the mixture was incubated at 50 °C overnight. After a series of extractions with phenol/chloroform and chloroform, DNA was isolated with the GENE CLEAN II kit (Qiogene). qPCR amplification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in a Real-time PCR System (model 7300, Applied Biosystems). The primer sets of 18S rDNA, rbcL and nad5 genes (see Supplementary Table S1, available with the online version of this paper) were used for quantitative determination of nuclear DNA, plastid DNA and mitochondrial DNA, respectively. The dnaX gene (slr0446) of Synechocystis was used as an internal standard. Purification of DNA was performed in six replicates and the mean is presented. For accurate estimation of template DNA, a dilution series of template was used for each primer set, and the amplification efficiency was monitored for each quantification (Cikos et al., 2007).

Quantification of mRNA. The genes to be analysed were selected according to the annotation given in the website of the Cyanidioschyzon merolae Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/). Transcripts were purified by using an RNasy Plant Mini kit and RNase-Free DNase set (Qiagen) and quantified by qPCR as described previously (Moriyama et al., 2008). The 18S rRNA gene was used to normalize transcript abundance. The primer pairs are listed in Supplementary Table S1. Again, a dilution series of template was used for each primer set, and the amplification efficiency was monitored for each amplification reaction to ensure accurate estimation of mRNA (Cikos et al., 2007).

Self-organizing map (SOM) analysis. Each transcript level was normalized by the mean value of 30–48 h in each gene, and each value was converted to log₂. The dataset was analysed by SOM using the Cluster 3.0 program (de Hoon et al., 2004). In this program, eight genes in which changes of expression level were low (SD less than 1.0 in log₂ for three cycles) were removed from the dataset, and the SOM analysis was done using Euclidean distance as a measure.

RESULTS

Synchronous culture of C. merolae cells

We previously developed a novel synchronization system (Fig. 1a; Moriyama et al., 2008), consisting of an initial long darkness to deplete dividing cells, a 6 h light/18 h dark regime that gives a circadian cycle but is not sufficient for driving the cell cycle, and a subsequent two cycles of 6 h light/18 h dark regime with a supply of 1 % CO₂. The last two cycles enabled the cells to accumulate enough photosynthetic products to allow progression of the cell cycle. This method gave a high rate of synchronous division. It can, therefore, discriminate the effects of light from those of the cell cycle, because the cells do not divide after the first light period. This was supported by measurement of cell size by microscopic observation of cells (Fig. 1b). Cell size in the first cycle remained small, whereas that in the third cycle increased about twofold within 6 h after the start of the light period. Similar results were obtained by size measurement using flow cytometry (see Supplementary Fig. S1, available with the online version of this paper).
S-phase in synchronous culture

We examined the changes in the level of DNA by qPCR in the synchronous culture. The 18S rDNA, the *rbcL* gene and the *nad5* gene were used for quantitative determination of copy number of nuclear DNA, plastid DNA and mitochondrial DNA, respectively. The *dnaX* gene of the cyanobacterium *Synechocystis* was used as an internal standard to estimate the absolute amount of DNA.

The amounts of nuclear and organellar DNA remained unaltered in the first cycle (see Supplementary Fig. S2). Nuclear DNA increased at or near the M-phase in the second (63 h to 67 h) and the third cycle (86 h to 90 h) (Fig. 2 and Supplementary Fig. S2). Mitochondrial DNA gradually increased from the start of the dark period in the second cycle, and doubled at or near the M-phase as did nuclear DNA (Fig. 2 and Supplementary Fig. S2). In the third cycle, mitochondrial DNA began to increase from the beginning of the light phase, and ceased to increase at the M-phase. Plastid DNA gradually increased from the start of the dark phase in the second cycle, and continued to increase even after cell division finished (Fig. 2 and Supplementary Fig. S2). At 54 h in Fig. 2, the copy numbers of plastid and mitochondrial genomes were estimated to be 24 and 9 per cell, respectively, if the copy number of the G1 nucleus was assumed to be one per cell. Similar results were obtained using other primer sets, such as those for DNA polymerase delta gene representing nuclear DNA, the *dnaB* gene representing plastid DNA, and the *cytB* gene representing mitochondrial DNA (Supplementary Fig. S2b). These results suggested that replication of nuclear and organellar DNA begins after enough nutrients have been provided, but that light alone was not sufficient as a signal to enter the S-phase. The results also suggest that the replication of the three genomes is not tightly synchronized. As judged from the results in Fig. 2 and Supplementary Fig. S2, the S-phase and the M-phase are close to each other and there is virtually no G2-phase in this red alga under the growth conditions examined.

Changes in the transcript level of various genes in synchronous culture

Transcript levels of 74 selected genes that encode important proteins involved in cell cycle regulation (cyclins, CDKs), DNA metabolism (DNA polymerases, histones, MCM helicases), organelle division proteins (FtsZ proteins, dynamins) as well as metabolic enzymes (RuBisCO, citrate synthase, etc.) were examined by quantitative real-time PCR (RT-qPCR) in the synchronous culture system. Cell-cycle-related genes of *C. merolae* are summarized in Supplementary Table S2. We obtained data over the three light/dark cycles as described in the previous section. RT-qPCR was performed in triplicate using a dilution series for each replicate, and the mean is presented. The calibration plot for the 18S rRNA (see Supplementary Fig. S3) indicated high linearity ($R^2 = 0.9994$). Similar linearity was obtained with other primer sets (data not shown). Each primer pair showed high amplification efficiency ($E$), and mean $E$ was 0.98 ± 0.08 (Supplementary Table S1). We examined ‘no-template’ controls, which would not give products with reverse transcriptase, and confirmed no amplification with all primer sets (data not shown). Each transcript level was normalized by the level of 18S rRNA. Changes in the transcript levels were analysed by SOM.
replication, repair or recombination in organelles (gyrases A and B, primase). The plastid genes encoding ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and cytochrome f as well as a nuclear gene for the mitochondrial ATPase subunit α were also included in the cluster. The increased expression of these genes may be involved in elevating photosynthetic activity or metabolism in the light period. Interestingly, the four hypothetical transcripts were all present in this cluster. These were analysed as representatives of the approximately 500 hypothetical transcripts that do not have meaningful ORFs (Matsuzaki et al., 2004). The results clearly showed that they are expressed and that the expression was regulated by light.

Cluster 2 contained genes encoding organellar division proteins (plastid and mitochondrial FtsZ proteins, plastid dynamin Dmn2_Pt and mitochondrial division protein Mda1), G1/S regulators (cyclin E, E2F and DP), mitotic-specific cell cycle regulators (cyclin A, cyclin B, CDK B, WEE1 and CKS), a putative CDK CMM138C, proteins related to nuclear DNA replication (Polz, δ and ε, histones and MCM helicases), nuclear repair (Polα and Polζ and organellar replicase CmPOP (named PolB in Moriyama et al., 2008). As the S-phase and the M-phase overlapped within a short time range in our synchronous culture system (Fig. 2), the peaks of expression of the genes related to DNA synthesis and cell division also overlapped in the current time resolution of the experiment. Therefore, the results are compatible with the assumption that the expression of the genes related to nuclear DNA replication increased in the S-phase. Accordingly, cluster 2 contained all the analysed genes that are related to G1/S transition, S-phase and M-phase.

**DISCUSSION**

**Progression of G1 to S-phase**

In the synchronous culture of *C. merolae*, it was reported that the nuclear S-phase occurred just before cell division (Itoh et al., 1996). Similarly in our synchronous culture, S-phase cells were observed 1–2 h before the peak of mitosis, and the genes related to genome replication, namely nuclear DNA polymerases (Polz, Polε and Polδ), histones (H1, H2A, H2B, H3 and H4) and MCM helicases (MCM2–7), were expressed near the peak of mitosis within the time resolution of the experiment (3 h).

In the budding yeast, the commitment to cell division cycle involves the START checkpoint that keeps the cell in the G1 phase upon nutritional limitation by inhibiting the G1/S progression (Murakami & Nurse, 2000). The checkpoint of unicellular algae such as *Chlamydomonas* is thought to inhibit G1/S progression in a similar way to that of yeast (Cross & Roberts, 2001). *C. merolae* also has a basic G1/S checkpoint machinery including Rb, E2F, DP and DEL. Although *C. merolae* has two E2F homologues (CMT067C and CMT068C), CMT068C was not expressed throughout the cell cycle of *Cyanidioschyzon merolae*.

![Figure 2. Determination of S-phase of nucleus and organelles](http://mic.sgmjournals.org)

DNA quantity was determined by qPCR using the primer sets for 18S rRNA (nuclear genome, Nuc), *rbcL* (plastid genome, Pt) and *nad5* (mitochondrial genome, Mt), and was normalized using the *dnaX* gene of *Synechocystis* as an internal standard. Grey and white regions in the panel show dark and light periods, respectively. Each value is a mean ± SD of six replicates of DNA preparations.

using the Cluster 3.0 program (de Hoon et al., 2004). We obtained nine nodes [NODE(0,0) to NODE(2,2)], and the genes were classified into the corresponding nine groups (Fig. 3a). As a result, two major clusters were identified, which were named cluster 1 and cluster 2. The change in expression of each gene is shown in Supplementary Figs S4 and S5. Fig. 3(b) shows scatter plots of expression that compare the first cycle and the second or third cycle. Cluster 1 genes (orange) did not show large differences in expression in the second or the third cycle with respect to that in the first cycle. The expression of cluster 2 genes (blue) considerably increased at 3 h after the start of the dark period (labelled 3D in Fig. 3b) in the second and the third cycles with respect to that in the first cycle. The gene expression in the second and the third cycle was almost identical (Fig. 3c). These results indicated that cluster 2 contained the genes that are related to progression of the cell cycle.

Cluster 1 included genes that are mainly induced by light, and the level of transcripts remained high until the cells divided (Fig. 3a). The principal genes that belonged to this cluster encode proteins related to cell cycle regulation (cyclin C, cyclin H and putative cyclins CMM039C and CMT109C, as well as CDKA, CDKD, and putative CDKs CMM087C and CMP090C), and proteins involved in DNA
the cell cycle. This was consistent with the result of expressed sequence tag (EST) analysis in the C. merolae genome project (http://merolae.biol.s.u-tokyo.ac.jp/). The expression of these G1/S regulators started 3 h after light-on in both dividing and non-dividing light/dark cycles. When cells were aerated with ordinary air, the transcripts decreased from the start of dark phase, and cells did not enter the S-phase. However, the gene transcripts increased...
until the start of nuclear S-phase when CO₂ was supplemented. These results suggest that nutritional state rather than light signal triggers the progression of G1/S through the synthesis of G1/S regulators in *C. merolae*.

**Association of gene expression patterns to cell cycle**

We considered that comparison of dividing and non-dividing cycles can discriminate whether the expression of genes is induced by light or by cell division. In the current setup, involvement of circadian rhythm cannot be completely separated from the light/dark cycle. However, we consider that the effect of circadian rhythm may not be large, because we obtained a preliminary result of shifting the third light period to an earlier time by shortening of the preceding dark period (see Supplementary Fig. S6).

Expression of 74 genes was measured by RT-qPCR, and categorized into two clusters (Fig. 3). The genes in cluster 1 were mainly induced by light. An additional peak or shoulder of expression was found after each cell division. This suggested that genes in this cluster are needed for maintaining cellular function throughout the cell cycle. Hypothetical transcripts that do not encode proteins and were detected by EST analysis were also included in this cluster. In *C. merolae*, about 500 hypothetical transcripts were detected, but their function remains unknown. We examined four of the hypothetical transcripts, and all belonged to cluster 1.

The expression peaks of the cluster 2 genes coincided with the peak of mitotic index or occurred just before mitosis (Fig. 3). The genes in cluster 2 were also induced by light to some degree. This cluster included the genes involved in the S- and M-phase, such as G1/S regulators, mitotic-specific cell cycle regulators, DNA replication enzymes in the cell nucleus and the organelles, and organellar division proteins. In cluster 2, the expression patterns of histone genes were unique; these genes were expressed only at the peak of mitotic index, but not induced by light. Therefore, histone genes could be a marker of cell division because the histone genes were hardly expressed during the non-dividing light/dark cycle or during the interphase of the dividing light/dark cycle, but were expressed only in the S-phase. The exact timing of the peak of gene expression in the second and the third cycles did not seem identical, most probably because the sampling was done at 3 h intervals.

**Replication cycle**

The results of the present study are summarized in Fig. 4. Using the synchronous culture, we succeeded in classifying gene expression patterns with respect to dividing and non-dividing light/dark cycles. Dividing and non-dividing cycles were caused by addition of CO₂ or no addition. Therefore, *C. merolae* cells have a checkpoint for nutrient state in the G1/S transition. If the cells have enough nutrients (Fig. 4a), such as the products of photosynthesis, G1-phase cells enter S-phase in the following dark phase (bold arrow).

It is interesting to note that the replication of the organellar genomes proceeded in different time-courses from that of the replication of nuclear genome (Fig. 4b1, b2, c). The organellar genomes remained unreplicated when cell-cycle progression was arrested at the G1/S checkpoint. Once the checkpoint was cleared (Fig. 4b1), the replication of both organellar genomes proceeded in a continuous fashion. However, the kinetics of replication was different for...
plastid and mitochondrial genomes. The replication of the mitochondrial genome continued from the start of the light phase until the end of cell division, whereas the replication of the plastid genome continued throughout the whole cell cycle. Each of the multiple copies of the plastid genome is likely to be replicated randomly at various different times. The different kinetics of replication of the two genomes could be related to the copy number of these genomes, namely, 24 copies of the plastid genome and 9 copies of the mitochondrial genome at the start of the light phase. The presence of enough copies permits relaxation of the tight coupling of replication and partition of plastid genomes (Fig. 4c), namely, the level of plastid DNA decreases below the initial level after division.

This result is different from the recently published hypothesis involving the tightly regulated sequential pathway leading from organellar replication to nuclear replication and further to cell division (Kobayashi et al., 2009). In contrast, our model points to two different types of G1 cells, one with continued synthesis of plastid genomes and the other with ceased replication of plastid genomes. In other words, the former plastid is licensed with respect to plastid DNA replication, whereas the latter is not. If and only if the cell has a licensed plastid, then the cell can go through G1/S transition. This idea is different from that of Kobayashi et al. (2009) in that those authors believed that plastid DNA synthesis is complete within a short time (about 1 h), whereas our data clearly showed that plastid DNA synthesis continues over the entire cell cycle. We have repeatedly tested the results of Kobayashi et al. (2009) under similar experimental conditions (they used a 6 h light period as we did, but they used the second light period for the analysis of DNA replication). However, we could never confirm the occurrence of DNA replication in the light period (see Supplementary Fig. S7a), nor the stimulatory effect of protoporphyrinogen IX on nuclear DNA replication (Supplementary Fig. S7b). We believe that BrdU (bromodeoxyuridine), which was used for labelling DNA replication (Supplementary Fig. S7b), does not inhibit the growth of C. merolae. Clear evidence for the lack of thymidine kinase activity is that fluorodeoxyuridine (FdU), which is activated by thymidine kinase to become an inhibitor of methylation of DUMP, does not inhibit the growth of C. merolae cells. All these are evidence against the hypothesis of Kobayashi et al. (2009) involving a tight coupling of organellar and nuclear DNA replication.

We previously examined the protein level of CmPOP (Cyanidoschyzon POP) in synchronous culture, in which the protein level increased at the time of organellar DNA replication (Fig. 4e; Moriyama et al., 2008). The level of DNA polymerase and the increase of DNA do not seem to correlate, but this is explained by the fact that the replication reaction requires only two molecules of DNA polymerase per genome. The level of POP protein is sufficient high even after its decline in the dark period. Like nuclear DNA replication, the start of organellar DNA replication is also triggered by the cellular nutritional state. We need further experiments to substantiate our model.

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