Metabolism, cell cycle stages, and related transcriptomes in eukaryotic algae change with the diel cycle of light availability. In the unicellular red alga *Cyanidioschyzon merolae*, the S and M phases occur at night. To examine how diel transcriptomic changes in metabolic pathways are related to the cell cycle and to identify all genes for which mRNA levels change depending on the cell cycle, we examined diel transcriptomic changes in *C. merolae*. In addition, we compared transcriptomic changes between the wild type and transgenic lines, in which the cell cycle was uncoupled from the diel cycle by the depletion of either cyclin-dependent kinase A or retinoblastoma-related protein. Of 4,775 nucleus-encoded genes, the mRNA levels of 1,979 genes exhibited diel transcriptomic changes in the wild type. Of these, the periodic expression patterns of 454 genes were abolished in the transgenic lines, suggesting that the expression of these genes is dependent on cell cycle progression. The periodic expression patterns of most metabolic genes, except those involved in starch degradation and de novo deoxyribonucleotide triphosphate synthesis, were not affected in the transgenic lines, indicating that transcriptomic changes in most metabolic pathways are independent of the cell cycle. Approximately 40% of the cell-cycle-dependent genes were of unknown function, and approximately 19% of these genes of unknown function are shared with the green alga *Chlamydomonas reinhardtii*. The data set presented in this study will facilitate further studies on the cell cycle and its relationship with metabolism in eukaryotic algae.

Photosynthetic organisms acquire energy from sunlight during the daytime. Thus, their metabolism and many of their other cellular activities are greatly affected by the diel cycle. Because of these environmental and metabolic restrictions, photosynthetic organisms possess mechanisms that adjust their internal activities according to diel changes in light availability. Many studies have demonstrated that photosynthetic eukaryotes alter the activities of most of their metabolic pathways according to the diel cycle by transcriptional and posttranslational regulation (Yakir et al., 2007; Thines and Harmon, 2011; Kinmonth-Schultz et al., 2013; Missra et al., 2015; Kim et al., 2017). However, how cell growth and division (i.e., cell cycle progression) in photosynthetic eukaryotes is coordinated with these diel changes in metabolic activities is poorly understood.

The cell cycle is a sequence of events of cell growth and cell division. Cyclins and cyclin-dependent kinase (CDK) complexes play central roles in cell cycle progression. In mammalian cells, G1 cyclin (cyclin D and E)-CDK regulates the transition from the G1 to the S phase (DNA replication phase) depending on cellular growth (Lim and Kaldis, 2013). Similarly, in plants and eukaryotic algae, the G1 cyclin (cyclin D) and A-type CDK (CDKA) complex is responsible for the G1/S phase transition. Cyclin A-CDKA is responsible for S phase progression, and the cyclin B and B-type CDK (CDKB; specific to plants and eukaryotic algae) complex regulates the G2/M transition (Vandepoele et al., 2002).

Regarding the photosynthesis-based relationship between cell cycle progression and metabolic activities, in the case of unicellular algae, the cell cycle progression of individual cells in culture can often be easily synchronized by a diel light and dark cycle (Tamiya, 1966; Miyagishima et al., 2012; Zones et al., 2020).
In most cases, the majority of genes exhibited changes with a periodicity of ~24 h at the mRNA level under 12-h-light/12-h-dark cycles (LD; Johnson et al., 2011; Ashworth et al., 2013; Zones et al., 2015; Ferrari et al., 2019; Strenkert et al., 2019). In addition, the timing of the up-regulation of various metabolic pathways at the transcript level and of cell cycle stages during the LD has been described in detail in different algal species, and correlations have thus been identified between diel metabolic changes and cell cycle progression. However, it is still unclear whether transcriptomic changes in metabolic pathways are regulated by the cell cycle or whether metabolic pathways and cell cycle progression are independently regulated in accordance with the diel cycle. That is, it is unclear whether genes that have been observed to exhibit a periodicity of ~24 h in their expression under the LD are regulated by the cell cycle or are regulated by diel/circadian rhythms independent of the cell cycle. It is important to address these questions to understand how algal coordinate cellular growth through photosynthesis during the daytime, several metabolic pathways, and cell division under a natural diel cycle.

One of the strategies to find answers to such problems is the characterization of diel transcriptomic changes in cells, in which cell cycle progression is arrested at a certain phase. In the green alga *Chlamydomonas reinhardtii*, comparative transcriptome analyses have been performed between the wild type and temperature-sensitive mutants of CDKA and CDKB to clarify transcriptional changes depending on CDKA and CDKB under the LD; as a result, the differential control of S/M phase genes by CDKA and CDKB was successfully identified (Tulin and Cross, 2015). However, whether the transcriptomic regulation of various metabolic pathways is dependent on or independent of the cell cycle was not investigated.

Among the unicellular eukaryotic algae, the molecular genetics of the cell cycle has been particularly well studied in the green alga *C. reinhardtii* (Cross and Umen, 2015; Breker et al., 2018). This is because procedures for genetic modification have been established and cell cycle progression mutants have been collected for this species. In addition, the cell cycle of this species can be synchronized under the LD (Cross and Umen, 2015). The unicellular red alga *Cyanidioschyzon merolae* is an emerging model organism that constitutes a complementary study system of eukaryotic algae. Green and red algae diverged relatively soon after the emergence of primitive eukaryotic algae (Parfrey et al., 2011); thus, a comparison of results between the two is useful for highlighting evolutionarily conserved phenomena/regulatory systems. The nuclear and organelar genomes of *C. merolae* have been completely sequenced, and its nuclear genome (16 Mb) is very small with little genetic redundancy compared with that of *C. reinhardtii* (120 Mb) and Arabidopsis (*Arabidopsis thaliana* [157 Mb]; Matsuzaki et al., 2004). The cell cycle of *C. merolae* can be synchronized under the LD with the S and M phases occurring during the dark period, as in *C. reinhardtii* (Miyagishima et al., 2014). Procedures for nuclear (Imamura et al., 2010; Fujiwara et al., 2013a) and chloroplast (Zienkiewicz et al., 2017) genome modification using homologous recombination have been established. In addition, conditional gene expression and knockdown are also feasible (Sumiya et al., 2014; Fujiwara et al., 2015).

In this study, we utilized *C. merolae* culture to investigate the relationship between the cell cycle and diel transcriptomic changes in metabolic pathways. We prepared two different transgenic lines, in which cell cycle progression was uncoupled from the diel cycle, and compared transcriptomic changes under the LD between the wild type and the two transgenic lines. The results show that most metabolic pathways, except for those for deoxyribonucleotide triphosphate (dNTP) synthesis and starch degradation, and the cell cycle are independently adjusted to the LD. In addition, the results also provide data sets of cell-cycle-dependent and -independent genes of ~24 h periodicity in a photosynthetic eukaryote with a small-genome minimal set of genes. This data set will facilitate further studies on the cell cycle and the relationship between metabolism and the cell cycle in *C. merolae* and in other photosynthetic eukaryotes by comparative analyses.

**RESULTS**

**Identification of the 1,979 Genes with a Periodicity of Approximately 24 h under the LD out of 4,775 Nucleus-Encoded Genes in *C. merolae***

To comprehensively identify cell-cycle-dependent genes in *C. merolae* and to understand the relationship between cell cycle progression and changes in the mRNA levels of metabolic genes, we first identified genes with a periodicity of approximately 24 h under the LD. Then, we extracted cell-cycle-dependent genes by comparing gene expression patterns under the LD between the wild type and transgenic lines, in which cell cycle progression was uncoupled from the LD.

To comprehensively identify nucleus-encoded genes in *C. merolae* that exhibited changes in their mRNA levels over an approximately 24-h (LD) cycle, we examined transcriptomic changes using microarray analyses. *C. merolae* cells were cultured in an inorganic phototrophic medium under the LD (100 μmol m⁻² s⁻¹ during the light period), and transcriptomic changes during the second round of the LD were analyzed. To obtain four experimental replicates, we sampled over a 28-h period from each of four identical replicate cultures, with the sampling of each occurring on different days. We used this experimental design rather than a longer 48-h time course to avoid possible transcriptomic differences between the two rounds of LD due to any decrease in inorganic nutrition due to cellular growth and any decrease in light availability.
per cell due to increased numbers of cells, both of which can be issues in 48-h culture. In addition, the G1-arrested cells (described later) continued to grow without dividing and thus reached abnormally large sizes in 48-h culture. In our previous study, in which another set of the wild-type transcriptome data under the LD was used, we only focused on changes in the mRNA level of a certain number of genes that are related to carbon fixation, glycolysis, respiration, and cell cycle progression in the wild type (Fujiwara et al., 2009; Miyagishima et al., 2019). By contrast, in this study, the wild-type culture was performed simultaneously with culture of transgenic lines, in which cell cycle progression was arrested or uncoupled from the LD, and we identified all genes with a periodicity of \( \sim 24 \text{ h} \). In addition, we changed the inorganic medium from 2\( \times \) Allen's medium (Fujiwara et al., 2009; Miyagishima et al., 2019) to MA2 medium, in which cells grow faster (Minoda et al., 2004).

Genes with a periodicity of \( \sim 24 \text{ h} \) were identified by JTK_CYCLE (Hughes et al., 2010) with a threshold of false discovery (Benjamini-Hochberg Q-value [BH.Q] \( < 0.05 \); criterion 1) and a threshold of amplitude (mRNA level changed more than twofold under the LD; criterion 2; Supplemental Fig. S1). As a result, of the 4,775 nucleus-encoded genes, we identified 1,979 genes that exhibited a change in their mRNA levels over an approximately 24-h cycle under the LD in \( C. \) \text{merolae} (Fig. 1; Supplemental Data Sets S1 and S2).

### Classification and Functional Assignments of the 1,979 Genes with a Periodicity of Approximately 24 h under the LD

Consistent with previous studies, cell-cycle-related genes such as those involved in chromosome replication and segregation exhibited a clear \( \sim 24\text{-h} \) rhythm, accumulating during 8 to 16 h (Supplemental Fig. S2). Thus, the cells were successfully synchronized under our culture conditions. Note that mRNA levels of some \( S \) phase genes such as proliferating cell nuclear antigen (PCNA) peaked at 8 h or 8 to 12 h, which corresponds to the light period, but the corresponding proteins are expressed from 12 h in \( C. \) \text{merolae}, as shown later by immunoblotting and as previously shown (Fujiwara et al., 2009, 2013b). Thus, the \( S \) phase corresponds to early in the dark period in \( C. \) \text{merolae}.

To obtain an overview of the transcriptomic changes under the LD in the wild type, the genes with a periodicity of \( \sim 24 \text{ h} \) identified above were classified based on the similarity of expression by k-means clustering (five clusters; Fig. 1B; Supplemental Data Set S2) and the genes in each cluster were functionally categorized according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Fig. 1C; Supplemental Data Set S3). Cluster 1 predominantly included genes that exhibited an mRNA level increase near 0 h (24 h) and decrease near 12 h. Clusters 2, 3, 4, and 5 predominantly included genes that exhibited mRNA level peaks at 0 to 4 h, 4 to 8 h, 8 to 12 h, and 12 to 16 h, respectively (Fig. 1B). The magnitudes of changes in mRNA levels in clusters 4 and 5 were larger than those in the other clusters (Fig. 1B).

By the KEGG classification, 13%, 31%, 31%, 18%, and 7% of genes in clusters 1, 2, 3, 4, and 5 were assigned as metabolism genes, respectively (Fig. 1C). In cluster 2, genes assigned as 1, carbohydrate metabolism, 2, energy metabolism, 8, metabolism of cofactors and vitamins, and 9, metabolism of terpenoids and polyketides were significantly enriched. In cluster 3, genes assigned as 2, energy metabolism, 6, metabolism of other amino acids, and 8, metabolism of cofactors and polyketides were significantly enriched (Fig. 1C). By contrast, in clusters 1, 4, and 5, no metabolism category was enriched, while replication and repair genes were enriched in cluster 4 (Fig. 1C). These results reasonably reflect that cell growth occurs only during the light period in photoautotrophic conditions and that nuclear DNA replication during the \( S \) phase occurs early during the dark period under LD (Miyagishima et al., 2014).

### G1 Arrest by Conditional Knockdown of CDKA in the \( HA-CDKA-eKD \) Strain

The cell cycle of many algal species is linked to the LD, and in some species, including \( C. \) \text{merolae}, it was shown that cell cycle progression is regulated by circadian rhythms (Goto and Johnson, 1995; Fang et al., 2006; Moulager et al., 2007, 2010; Johnson, 2010; Olson et al., 2010; Cross et al., 2011; Miyagishima et al., 2014). Thus, to dissect the genes that have expression patterns that depend on cell cycle progression rather than the LD or circadian rhythms, it is necessary to uncouple cell cycle progression from the LD.

We planned to knock down CDK, which is a master regulator of cell cycle progression in eukaryotes (Lim and Kaldis, 2013). Because CDK is essential for cell proliferation, we adopted a previously developed, conditional gene knockdown system, in which the expression of a transgene driven by an ammonium-repressed promoter is activated in an ammonium-depleted medium but repressed in ammonium-replete medium (Fujiwara et al., 2015). In addition to a conventional CDK (CDKA), plants and algae also possess CDKB, which is specifically expressed during the \( S/M \) phase and is involved in the \( G2/M \) transition (Nowack et al., 2012). The \( C. \) \text{merolae} genome encodes a single copy of CDKA (CME119C) and CDKB (CMH128C). In this study, we knocked down CDKA to arrest the cells in the \( G1 \) phase as described below (Fig. 2).

A 3X-HA epitope-coding sequence was added just after the start codon of the CDKA open reading frame (ORF; CME119C) to detect the protein using anti-HA antibody. The ammonium-repressed promoter of the nitrate reductase gene \( NR \) (Fujiwara et al., 2015) was fused with the \( HA-CDKA \) ORF. Then, the construct was integrated into the chromosomal CDKA locus of...
Figure 1. Overview of 1,979 genes of ~24-h periodicity under the LD out of 4,775 nucleus-encoded genes in *C. merolae*. Wild-type (WT) *C. merolae* was cultured under the LD, and transcriptomic changes were examined for 28 h from the onset of the second round of the LD (the onset of the second light period is defined as 0 h). A, Schematic illustration showing cell cycle progression under the LD based on previous studies (Imoto et al., 2011; Fujiwara et al., 2013b; Kuroiwa et al., 2018). B, Heat map showing changes in the mRNA levels of the 1,979 genes of ~24-h periodicity under the LD. Genes were grouped into five clusters on the basis of similarity of expression (k-means clustering) by using four independent LD cultures. The number of genes and the percentage in each cluster are indicated. The gene identifiers, annotations, cluster numbers, and expression values are shown in Supplemental Data Sets S1 and S2. Of the 4,775 nucleus-encoded genes examined, the mRNA levels of 1,979 genes changed more than twofold over a period of ~24 h under the LD in *C. merolae*. The number of KEGG pathway assignments (98 genes) is indicated above the heat map. C, Pathway number and term.
C. merolae so that endogenous CDKA expression could be controlled by the presence (off) or absence (on) of ammonium in the medium in the resultant HA-CDKA-cKD strain (Fig. 2A). In addition, for comparison of the level of HA-CDKA expressed by the NR promoter in the HA-CDKA-cKD strain in ammonium-depleted medium with that expressed by the endogenous CDKA promoter in the wild-type strain, we also prepared the strain HA-CDKA, in which only the HA-encoding sequence was integrated just after the start codon of the chromosomal CDKA ORF (Fig. 2A).

The HA-CDKA and HA-CDKA-cKD strains were synchronously cultured in continuous light in the ammonium-depleted inorganic medium (MA2-NO₃; NR promoter on), which contained nitrate instead of ammonium as the sole nitrogen source. HA-CDKA protein, the level of which was comparable to that in the HA-CDKA strain, was then detected using immunoblotting in the HA-CDKA-cKD strains (Fig. 2B). When the strains were transferred into MA2 medium (NR promoter off), which contained ammonium as the sole nitrogen source, HA-CDKA protein almost disappeared in the HA-CDKA-cKD strain but not in the HA-CDKA strain 2 d after the transfer (Fig. 2B). Thus, we succeeded in controlling the accumulation and depletion of HA-CDKA protein in the HA-CDKA-cKD strain by changing the nitrogen source (ammonium or nitrate) in the medium as intended.

To examine whether the depletion of CDKA in the HA-CDKA-cKD strain arrested cell cycle progression, the cells were subjected to the LD in MA2 and MA2-NO₃. Before subjecting the cultures to the LD, they were subjected to incubation in the dark for 1 d to terminate cellular growth and thereby arrest cells in the G1 phase in both the HA-CDKA and HA-CDKA-cKD cultures and to deplete HA-CDKA in the HA-CDKA-cKD culture (Fig. 2C). Protein samples were obtained every 4 h from the onset of the second LD and were subjected to immunoblotting (Fig. 2C). In the MA2-NO₃ medium, HA-CDKA protein was expressed throughout the LD, and α-tubulin, which is expressed specifically in the S and M phases in C. merolae (Nishida et al., 2005; Fujiwara et al., 2013b), was detected during 16 to 20 h (Fig. 2C). By contrast, in MA2 medium, both HA-CDKA and α-tubulin were hardly detected throughout the LD (Fig. 2C), suggesting that HA-CDKA-cKD cells were arrested in the G1 phase by the depletion of CDKA in the MA2 medium.

When wild-type and HA-CDKA-cKD cells were cultured under the LD in MA2 (NR promoter off) in the aforementioned manner, the cell number in the wild-type culture approximately doubled during the second round of the LD, whereas that in the HA-CDKA-cKD culture did not increase at all (Fig. 2D). Microscopic observation showed that both wild-type and HA-CDKA-cKD cells increased in size during the light period by photosynthetic cellular growth (0–12 h; Fig. 2D). At 12 h, cells with a dividing chloroplast (S phase; Miyagishima et al., 2012) and cells with two divided chloroplasts (M phase; Miyagishima et al., 2012) were observed in the wild-type culture but not in the HA-CDKA-cKD culture (Fig. 2D; Supplemental Fig. S3A). At 24 h, the wild-type cells returned to their original size (that at 0 h) after cell division, whereas HA-CDKA-cKD cells remained enlarged (Fig. 2D; Supplemental Fig. S3B). Because almost no dividing cells were observed and the cell number was unchanged in the HA-CDKA-cKD culture throughout the LD (Fig. 2D; Supplemental Fig. S3A), there was no cell death in the culture. The slight decrease in the HA-CDKA-cKD cell size during the dark period without cell division (Supplemental Fig. S3B) is probably due to the consumption of starch granules in the cytosol. Immunoblotting revealed that the S phase markers PCNA and FtsZ2-1, the M phase marker histone H3 phosphorylated at the Ser at the 10th position, and the S/M phase marker α-tubulin were detected in the wild-type culture specifically during the dark period. On the contrary, these proteins were barely detected in the HA-CDKA-cKD culture throughout the LD (Fig. 2D). These results indicate that HA-CDKA-cKD cells were successfully arrested in the G1 phase by replacing nitrate with ammonium as the exogenous nitrogen source in our culture conditions.

Because the difference in nitrogen source would lead to a difference in cellular metabolic state and thus a difference in the mRNA levels of metabolic genes, in the following analyses of transcriptomic changes under the LD, we compared the wild-type and HA-CDKA-cKD strains in MA2 medium rather than HA-CDKA-cKD strains between MA2-NO₃ and MA2. In the G1 phase-arrested culture of HA-CDKA-cKD, S/M phase-specific genes are not expressed under the LD (Fig. 2D), and thus the expression patterns of cell-cycle-dependent genes under the LD, but not cell-cycle-independent genes, are expected to deviate from those in the wild-type culture.
Figure 2. Conditional G1 arrest by conditional knockdown of CDKA, driven by an ammonium-repressed promoter in C. merolae.

A, Schematic diagram of the insertion of a 3×HA epitope-coding sequence, NR promoter, and URA selection marker into the chromosomal CDKA locus. The first line indicates the genomic structure of the wild type (WT). The second line indicates the genomic structure of the HA-CDKA strain in which the HA tag-encoding sequence and the URA selection marker were integrated into the CDKA locus to express HA-CDKA by the endogenous CDKA promoter. The third line indicates the genomic structure of the HA-CDKA-cKD strain in which the NR promoter, HA tag-encoding sequence, and URA selection marker were integrated into the CDKA locus to express HA-CDKA by an ammonium-repressed NR promoter.

B, Immunoblotting of total cell lysate with an anti-HA antibody comparing HA-CDKA protein expression levels between HA-CDKA and HA-CDKA-cKD strains that were cultured asynchronously for 2 d in an inorganic NO₃⁻ (NR promoter on) or NH₄⁺ (NR promoter off) medium. The wild type was
Uncoupling the G1/S Transition from the LD by Depletion of Retinoblastoma-Related Protein

As another strategy to dissect cell-cycle-dependent mRNAs from mRNAs with ~24-h periodicity under the LD, we knocked out retinoblastoma-related protein (RBR-KO) in C. merolae. RBR is an inhibitor of the G1/S transition and is widely conserved in eukaryotes, including eukaryotic algae (Fang et al., 2006; Olson et al., 2010; Cross et al., 2011; Miyagishima et al., 2014). RBR represses the G1/S transition by binding the E2 promoter-binding factor dimerization protein (E2F-DP) transcription factor heterodimer, which binds certain specific cis-elements of S phase genes, such as those encoding cyclin A and DNA polymerases (Pardée et al., 2004). During the G1 phase, G1 cyclin concentration increases with cellular growth and a G1 cyclin-CDK complex phosphorylates RBR. This phosphorylation inactivates RBR, which consequently activates the transcription of the S phase genes by E2F-DP (Malumbres and Barbacid, 2001). We previously demonstrated that the RBR-E2F-DP pathway is involved in the circadian regulation of the G1/S transition in C. merolae and that RBR depletion abolished the restriction of the G1/S transition to the night. Thus, the G1/S transition and subsequent cell division in individual cells asynchronously occur in the RBR-KO culture, even under the LD (Miyagishima et al., 2014).

The RBR-KO strain was produced by replacing a portion of the chromosomal RBR ORF with the UMP synthase (URA) selection marker in C. merolae (Supplemental Fig. S4). Immunoblotting using anti-RBR antibody demonstrated that RBR protein was depleted in the RBR-KO strain (Supplemental Fig. S4). As previously shown (Miyagishima et al., 2014), after entrainment by the LD, the RBR-KO culture was found to have a certain population of S/M phase cells throughout the LD; this was contrary to the wild-type culture, in which α-tubulin-expressing S/M phase cells were specifically detected during the dark period (Supplemental Fig. S4). Thus, in the RBR-KO strain, the expression patterns of cell-cycle-dependent genes under the LD, but not those of genes independent of cell cycle progression, are expected to deviate from those in the wild-type strain.

Identification of the 454 Cell-Cycle-Dependent Genes out of 1,979 Periodic Genes by Comparative Transcriptome Analyses of the Wild-Type, G1-Arrested HA-CDKA-cKD, and RBR-KO Strains

To extract cell-cycle-dependent genes from the 1,979 genes with a periodicity of ~24 h under the LD identified above, we performed comparative transcriptome analyses between the wild-type strain and the G1-arrested HA-CDKA-cKD and RBR-KO strains. To obtain two replicates of the data sets, the wild-type and HA-CDKA-cKD strains were cultured under the LD twice at an interval of 1 month (wild-type 1 versus HA-CDKA-cKD1 and wild-type 2 versus HA-CDKA-cKD2). In a similar manner, the wild-type and RBR-KO strains were cultured under the LD twice at an interval of 1 month (wild-type 3 versus RBR-KO1 and wild-type 4 versus RBR-KO2). Note that the transcriptome data, which were used to extract genes of approximately 24-h periodicity (Fig. 1), were obtained from the wild type (wild-type 1 and wild-type 2) cultured simultaneously with HA-CDKA-cKD and the wild type (wild-type 3 and wild-type 4) cultured simultaneously with the RBR-KO as controls.

We identified genes that exhibited altered expression patterns under the LD between the wild-type and HA-CDKA-cKD and/or RBR-KO strains (criteria are shown in Supplemental Fig. S1). The comparison was based on deviations in the expression pattern (correlation coefficient), the average level of mRNA under the LD, and the amplitude of the rhythm under the LD (Supplemental Fig. S1). For example, the mRNA level of an oxygen-evolving complex component (PSBQ/CMC133C), which encodes a component of PSII, peaked at 8 h and then was down-regulated during the dark period in the wild-type strains (Fig. 3A, WT1, WT2, WT3, and WT4). An almost similar pattern was observed in the G1-arrested HA-CDKA-cKD and RBR-KO strains (Fig. 3A). Thus, the ~24-h rhythm of the PSBQ mRNA level under the LD is independent of cell cycle progression. In a similar manner, genes encoding Thr synthase, a component of NADH dehydrogenase I in the mitochondrial respiratory chain, and a translation elongation factor exhibited almost identical ~24-h

Figure 2. (Continued.) Used as a negative control. The Coomassie Brilliant Blue-stained polyvinylidene difluoride (PVDF) membrane is shown as a loading control. C. The effect of HA-CDKA knockdown on cell cycle progression in the HA-CDKA-cKD strain was examined by immunoblotting. Before subjecting the culture to the LD, the culture was incubated in the dark for 1 d to stop cellular growth and thereby arrest the cells in the G1 phase in NO2– or NH4+ medium and deplete HA-CDKA in HA-CDKA-cKD culture in NH4+ medium. Protein samples were taken every 4 h from the onset of the second round of the LD and subjected to immunoblotting. HA-CDKA and α-tubulin, which are expressed specifically in S and M phases in C. merolae (Nishida et al., 2005), were detected with anti-HA and anti-α-tubulin antibodies, respectively. The Coomassie Brilliant Blue-stained PVDF membrane is shown as a loading control. D. Comparison of cellular growth and cell cycle progression under the LD between the wild type and HA-CDKA-cKD cultured in NH4+ medium (NR promoter off). Changes in cellular morphology examined by differential interference contrast imaging, relative cellular concentration, and levels of cell cycle marker proteins in the wild type and HA-CDKA-cKD examined by immunoblotting are shown. Error bars in the graph indicate the SD of three independent cultures. In the immunoblot analyses, PCNA (S phase), α-tubulin (S and M phases), histone H3 phosphorylated at the Ser in the 10th position (H3S10P; M phase), and FtsZ2-1 (S and M phases) were examined; culture in NO2– medium (NR promoter on) was used as a positive control. The Coomassie Brilliant Blue-stained PVDF membrane is shown as a loading control. Bar = 5 μm.
Figure 3. Assessment of cell-cycle-dependent genes and LD-dependent but cell-cycle-independent genes of ~24-h periodicity by comparative transcriptome analyses. Comparative transcriptome analyses were performed between the wild type (WT) and G1-arrested HA-CDKA-cKD or RBR-KO. To obtain two replicates of data sets, pairs of LD cultures of the wild type and HA-CDKA-cKD were performed twice (wild-type 1 versus HA-CDKA-cKD1 and wild-type 2 versus HA-CDKA-cKD2 in an inorganic NH₄Cl medium). In a similar manner, pairs of LD cultures of the wild type and RBR-KO were performed twice (wild-type 3 versus RBR-KO1 and wild-type 4 versus RBR-KO2). The comparison was based on deviations in the shape of the expression patterns (correlation coefficient), average level of mRNA under LD, and amplitude of the rhythm under LD. When the expression pattern of a gene in G1-arrested HA-CDKA-cKD and RBR-KO under the LD deviated from that in the wild type (criteria are shown...
rhythmic expression patterns in the wild-type, HA-CDKA-cKD, and RBR-KO strains (Supplemental Fig. S5). In contrast to these genes, the mRNA level of α-tubulin (CMT504C) peaked at 12 to 16 h in the wild-type strain; however, this pattern was abolished in the G1-arrested HA-CDKA-cKD and RBR-KO strains (Fig. 3A).

α-Tubulin expression was completely depressed in the G1-arrested HA-CDKA-cKD strain (Fig. 3A); the pattern of expression was altered in the RBR-KO strain, for which the timing of the peak was shifted to 0 and 24 h and the amplitude of the rhythm was markedly decreased compared with that in the wild-type strain (Fig. 3A). In addition, the S and M phase-specific expression of the S phase genes DNA replication licensing factor minichromosome maintenance6 (MCM6) and PCNA and the M phase gene cell division cycle20 (CDC20) in the wild type were also abolished or altered in HA-CDKA-cKD and RBR-KO, respectively (Supplemental Fig. S5). These results suggest that the comparison between the wild-type and G1-arrested HA-CDKA-cKD strains and between the wild-type and RBR-KO strains successfully identified genes that show expression patterns that depend on cell cycle progression rather than the LD.

Based on analyses of a total of 1,797 genes with a periodicity of approximately 24 h, we identified 695 genes that exhibited impaired expression patterns in the G1-arrested HA-CDKA-cKD strain compared with the wild type and 635 genes that exhibited impaired expression patterns in the RBR-KO strain compared with the wild type (Supplemental Data Set S4). The number of genes that exhibited impaired expression patterns in both the G1-arrested HA-CDKA-cKD and RBR-KO strains was 454 (Fig. 3B). Here, we define these 454 genes as cell-cycle-dependent genes (Supplemental Data Set S4).

To evaluate the coverage of the cell-cycle-dependent genes by the above screening, we listed the genes that have been shown to vary in their corresponding mRNA and protein levels according to cell cycle progression in C. merolae (21 genes in total; Supplemental Data Set S5; Miyagishima et al., 2003, 2014, Nishida et al., 2003, 2005, 2007, Yoshida et al., 2009, 2010; Fujiiwara et al., 2010, 2013b; Sumiya et al., 2016). This was because cell-cycle-related genes do not necessarily exhibit a cell-cycle-dependent expression pattern as in the case of CDKA (Miyagishima et al., 2014) and a dynamin-related protein involved in mitochondrial division, CmDnm1/DRP3 (Nishida et al., 2003), in C. merolae, which are constantly expressed throughout the cell cycle. As a result, all 21 of the known cell-cycle-dependent genes were successfully included in the list of 454 cell-cycle-dependent genes identified by the above screening (Supplemental Data Sets S4 and S5).

Cell-Cycle-Dependent G1 and S/M Phase Genes

We further tried to classify the 454 cell-cycle-dependent genes into S/M phase genes and G1 phase genes. The 176 and 144 (320 in total) cell-cycle-dependent genes were classified into cluster 4 and cluster 5, respectively, in which mRNA levels peaked at 12 to 20 h under the LD, which corresponds to S/M phases (Supplemental Fig. S6; Supplemental Data Set S4). Clusters 4 and 5 included genes encoding DNA replication machinery (e.g. MCM6 and PCNA), structural proteins for chromosome segregation (e.g. genes encoding components of condensin), cell cycle regulators (e.g. cyclins and mitotic kinases), and organelle division proteins (e.g. CmDnm2/DRP5B and FtsZ2; Supplemental Data Set S4).

Out of the 454 cell-cycle-dependent genes, 45, 47, and 42 genes were classified into clusters 1, 2, and 3 in which mRNA levels peaked at 0, 0 to 4, and 4 to 8 h, respectively, which correspond to the G1 phase (Supplemental Fig. S6; Supplemental Data Set S4). Compared with S/M phase genes (cell-cycle-dependent genes in clusters 4 and 5), most of the G1 phase genes (cell-cycle-dependent genes in clusters 1, 2, and 3) exhibited a lower amplitude of oscillation. Of the 320 S/M-phase genes, 171 (53%) genes exhibited an amplitude of more than 10-fold in the wild type (all of wild-type 1, 2, 3, and 4). By contrast, the G1 phase genes in clusters 1, 2, and 3 (134 genes in total), only four, two, and two genes (eight genes in total; 6%) exhibited an amplitude of more than 10-fold in the wild type. In these G1 phase genes, no specific biological functions were enriched (Supplemental Data Set S4), and the roles of these genes in the cell cycle are unclear at this point.

Cell-Cycle-Dependent Metabolic Genes

As observed in other eukaryotic algae (Zones et al., 2015; Ferrari et al., 2019), many metabolic genes had expression profiles of ~24-h periodicity under the LD and the majority of the metabolic genes peaked at 4 to 8 h, which corresponds to the light period under the LD (Fig. 1). In order to examine the relationship between the cell cycle progression and transcriptomic changes in metabolic genes and how cell cycle progression affects

Figure 3. (Continued.)
in Supplemental Fig. S1), the gene was defined as a cell-cycle-dependent gene. A, Examples of changes in mRNA level under the LD in an LD-dependent but cell-cycle-independent gene (PSBQ) and a cell-cycle-dependent gene (α-tubulin). The relative mRNA level was calculated as follows: (mRNA level of the gene/average mRNA level of all genes at the indicated time point) ∗ 100. B, Venn diagram showing the number of genes in various categories based on comparative transcriptome analyses. A list of genes in various categories with microarray data is shown in Supplemental Data Set S4.
the expression of metabolic genes, we assigned the cell-cycle-dependent and -independent genes with a periodicity of approximately 24 h to the KEGG pathway categories (Fig. 4; Supplemental Fig. S7; Supplemental Data Set S6).

As a result, 38 cell-cycle-dependent periodic genes were assigned to the metabolism category (Supplemental Fig. S7; Supplemental Data Sets S6 and S7). In the KEGG classification of the 454 cell-cycle-dependent genes, only the replication and repair category was enriched. There was no enriched metabolism category, and some metabolism categories were significantly depleted in the cell-cycle-dependent genes. Of the 38 cell-cycle-dependent metabolic genes, 15 genes were assigned to nucleotide metabolism (Supplemental Fig. S7; Supplemental Data Sets S6 and S7). Most of these nucleotide metabolism genes peaked at 8 to 12 h, as in the case of cell-cycle-dependent genes that are involved in DNA replication (Supplemental Fig. S7; Supplemental Data Sets S6 and S7). As described later, these genes are involved in dNTP synthesis for DNA replication, and the up-regulation of these genes prior to and during the S phase was almost abolished in the G1-arrested HA-CDKA-cKD strain. Of the remaining 23 metabolic genes, nine genes were assigned to carbohydrate metabolism (Supplemental Fig. S7; Supplemental Data Sets S6 and S7). Compared with the 15 nucleotide metabolism genes, the effect of G1 arrest (G1-arrested HA-CDKA-cKD) or RBR depletion (RBR-KO) on the remaining 23 metabolic genes was relatively weak (Supplemental Fig. S7; Supplemental Data Sets S6 and S7). These results suggest that most diel transcriptomic changes in metabolism, except for those for nucleotide metabolism for DNA replication during the S phase, are independent of the cell cycle.

**Cell-Cycle-Dependent Genes That Are Involved in dNTP Synthesis**

Many of the cell-cycle-dependent metabolic genes were assigned to nucleotide metabolism in the above analysis (Fig. 5; Supplemental Fig. S7; Supplemental Data Sets S6 and S7). By characterizing the details of the comparative transcriptome analyses, it was found that the mRNA levels of key enzymes involved in the de novo synthesis of dNTPs depend on the cell cycle as follows (Fig. 5). Ribonucleoside-diphosphate reductase, which is a complex of $\alpha$- and $\beta$-subunits (CMM323C, RNR-$\alpha$; CML050C, RNR-$\beta$), catalyzes the conversion of ribonucleotide diphosphate to deoxyribonucleotide diphosphate, which is a rate-limiting step for the dNTP biosynthetic pathway (Nordlund and Reichard, 2006; Fig. 5). CMP/UMP kinases (CMT083C; CMPK3) catalyze interconversions between (d)CMP and (d)CDP and between UMP and UDP (Liou et al., 2002). CTP synthase (CMP216C; CTFS2) interconverts UTP and CTP, which is utilized for dCTP and dTTP synthesis (Levitzki and Koshland, 1971; Fig. 5). dUTP pyrophosphatase (CMO126C; DUT) is involved in dTTP synthesis (Mol et al., 1996; Fig. 5). The fusion protein of dihydrofolate reductase and thymidylate synthase (CMS462C; DHFR-TS) is involved in dTTP synthesis.
The mRNA levels of these six genes peaked at 8 to 12 h in the wild type, while the up-regulation was inhibited in the G1-arrested HA-CDKA-cKD strain (Fig. 5). In addition, the mRNA expression patterns were also impaired in the RBR-KO strain (Fig. 5). Thus, the mRNA levels of these six genes are dependent on cell cycle progression. The up-regulation of RNR-α and RNR-β1 mRNA during the S phase was also observed in yeast and mammalian cells (Nordlund and Reichard, 2006), and the transcriptional regulation
together with posttranslational regulation of RNR are important to elevate the concentration of dNTP specifically during the S phase for nuclear DNA duplication (Nordlund and Reichard, 2006). On the other hand, mRNA levels of RNR-β2, which is another RNR-β gene in C. merolae, were slightly up-regulated at 4 h in the light. The oscillation of the 24-h rhythm of RNR-β2 was much less affected by the cell cycle than that of RNR-β1, and RNR-β2 was not assigned in cell-cycle-dependent genes (Fig. 5). In addition, certain levels of RNR-α, CMPK2, CTPS2, DLI1, and DHFR-TS mRNAs were detected in the LD even when CDKA was depleted (Fig. 5). Furthermore, other genes in the de novo dNTP biosynthetic pathway were up-regulated in the light independently of the cell cycle or constantly expressed (Supplemental Fig. S8). Therefore, C. merolae cells likely have a certain level of activity for dNTP synthesis even outside the S phase. RNR-β2 is probably implicated in supplying dNTPs for nuclear DNA repair in the G1 phase and chloroplast DNA replication, which occur in the daytime independently of the cell cycle (Kabeya and Miyagishima, 2013). This prediction is supported by studies on dNTP synthesis in mammals. Mammals possess two different RNR-β genes, RRM2 and p53R2/RRM2B. RRM2 is expressed only in the S phase, whereas p53R2 is transcriptionally regulated by p53 and has a crucial role in mitochondrial DNA replication in the G0 and G1 phases (Nakano et al., 2000; Bourdon et al., 2007).

**Effects of Cell Cycle Progression on Carbohydrate Metabolism**

The effects of G1 arrest or RBR depletion on most of the cell-cycle-dependent metabolic genes other than those of nucleotide metabolism were relatively weak (Supplemental Fig. S9; Supplemental Data Sets S6 and S7). However, we found that two genes involved in starch degradation were relatively strongly affected by cell cycle progression, as detailed below (Fig. 6; Supplemental Fig. S9).

Glycogen phosphorylase (CMD184C; GPL) and isomylase1 (CMI294C; ISA1) are involved in starch degradation (Rathore et al., 2009; Möller and Svensson, 2016). GPL breaks up glycogen/starch into Glc-1-P (Fig. 6) and is a major starch granule-bound protein in C. merolae (Hirabaru et al., 2010). The GPL mRNA level peaked at 8 to 12 h in the wild type (Fig. 6). By contrast, in both G1-arrested HA-CDKA-cKD and RBR-KO strains, the peak was shifted to 16 h (Fig. 6; Supplemental Fig. S9). In addition, the level of the peak was lower than that of the wild type, and the amplitude became 0.39 and 0.25 times that in the wild type in G1-arrested HA-CDKA-cKD and RBR-KO strains, respectively (Fig. 6). Isoamylase severs starch branches, thereby producing maltoextrins, which are short linear polymers of Glc (Fig. 6). The ISA1 mRNA level peaked at 12 to 16 h in the wild type under the LD, as did the GPL mRNA (Fig. 6). However, in G1-arrested HA-CDKA-cKD, the up-regulation of ISA1 mRNA was inhibited after it slightly increased from 0 to 4 h (Fig. 6). In addition, the rhythm of ISA1 mRNA was impaired in the RBR-KO strain (Fig. 6). These results suggest that GPL and ISA1 mRNA levels increase at 0 to 4 h and 0 to 16 h under the LD, even without the G1/S transition, and that the levels are further increased by the G1/S transition. A plausible explanation for the up-regulation of GPL and ISA1 mRNA levels upon the G1/S transition is that it generates more ATP to perform energy-demanding nuclear DNA replication and segregation and organelle and cell division. If this assumption is correct, there are still two possibilities: (1) the up-regulation of GPL and ISA1 is programmed by the cell cycle; or (2) the consumption of ATP by events in S and M phases secondarily up-regulates GPL and ISA1 expression by a mechanism buffering the cellular ATP pool. Another plausible explanation is that GPL and ISA1 are up-regulated in the S phase to supply Glc-6-P to the pentose phosphate pathway, which, in turn, produces ribose-5-phosphate for de novo synthesis of dNTPs (Tong et al., 2009).

**DISCUSSION**

**Relationship between the LD, Rhythmic Changes in the mRNA of Metabolic Genes, and Cell Cycle Progression**

We previously reported two transcriptome analyses of synchronized culture of C. merolae under the LD (Fujiwara et al., 2009; Miyagishima et al., 2019). The former focused on candidates for S and M phase-specific genes. The latter focused mainly on daily changes in the transcriptome that are related to energy metabolism, such as glycolysis, respiration, and photosynthesis. However, neither analysis dissected the contributions of the cell cycle and the LD to changes in the transcriptome. This situation is also applicable to other studies of diel transcriptomic changes in eukaryotic algae. Thus, except for some genes that were further examined, it was still unclear whether the approximately 24-h rhythmic change in the level of a certain mRNA resulted from a cellular response to the LD or cell cycle progression. To address this issue, in this study, we identified cell-cycle-dependent mRNAs among genes with a periodicity of approximately 24 h under the LD by comparing expression patterns between the wild-type and G1-arrested HA-CDKA-cKD strains and those between the wild-type and RBR-KO strains.

Out of 4,775 nucleus-encoded genes in total, 1,979 (41%) genes exhibited approximately 24-h periodic expression patterns in our culture conditions; these included genes involved in many metabolic pathways, most of which peaked during daytime, and genes that regulate or function in chromosome replication and cell division, which peaked in evening to night under the LD (Fig. 1). Of the 1,979 genes of ~24-h periodicity, 454 were defined as cell-cycle-dependent genes based on our comparative transcriptome analyses (Fig. 3). However, most metabolic genes, except for some genes involved in dNTP synthesis (Fig. 5) and carbohydrate metabolism
such as starch degradation (Fig. 6), exhibited normal periodic expression patterns even in G1-arrested HA-CDKA-cKD and RBR-KO strains (Supplemental Fig. S7). Thus, in C. merolae, genes of most metabolic pathways exhibit approximately 24-h periodic expression depending on the LD but independently of cell cycle progression. Taking the natural habitats into account, these cell-cycle-independent diel rhythms in most metabolic pathways appear reasonable, as follows (Fig. 7). In contrast to nutrient-rich media for laboratory culture, in natural conditions of low inorganic nutrient levels (inorganic nitrogen sources, phosphate, iron, and so on),

![Starch degradation diagram](image-url)

**Figure 6.** Effects of cell cycle progression on the mRNA levels of genes involved in starch degradation. Changes in the relative mRNA level \[(mRNA\text{ level of the gene}/\text{average mRNA level of all genes at the indicated time point}) \times 100\] are shown. The results of two biological replicates (wild-type [WT] 1 versus G1-arrested HA-CDKA-cKD1 and wild-type 2 versus G1-arrested HA-CDKA-cKD2; wild-type 3 versus RBR-KO1 and wild-type 4 versus RBR-KO2) are shown. A schematic illustration of the metabolic pathway in which the gene products are involved is also shown. Graphs of all of the genes involved in the pathway are shown in Supplemental Figure S9. Metabolic reactions (arrows) indicated in blue, orange, and gray are catalyzed by enzymes encoded by cell-cycle-dependent genes, cell-cycle-independent, ~24-h periodic genes, and nonperiodic genes (amplitude is less than twofold), respectively. One arrow in a reaction corresponds to one gene product.
cellular growth by photosynthesis during the daytime is relatively slow and only some cells in a population reach a certain size that are required for cell division (Fig. 7). Cells that have reached the threshold size enter the S/M phase and divide but others stay in the G1 phase without cellular growth during the night (Fig. 7). In addition, in our previous study, only a certain percentage of cells underwent cell division during the dark period under a dim-light LD, in contrast to cultures with sufficient light strength, in which most of the cells divided in one round of the LD as in the cultures in this study (Miyagishima et al., 2014). Thus, under such nutrient- and/or light-limited conditions, cells are subjected to diel rhythms of photosynthesis and growth regardless of the G1/S transition, which is probably a reason for the cell-cycle-independent diel rhythms in metabolic genes observed in this study (Fig. 7).

Usage of the Transcriptome Data for Further Analyses

We have identified 181 cell-cycle-dependent genes of unknown function (genes encoding hypothetical proteins in Supplemental Data Sets S1 and S8). In C. merolae, the division of the chloroplast, mitochondrion, Golgi apparatus, and peroxisome occurs at the S/M phases, and many of the organelle division genes are expressed specifically in these phases (Supplemental Data Set S4; Yoshida et al., 2010, 2017; Imoto et al., 2011, 2018; Yagisawa et al., 2013). Thus, further investigation of these cell-cycle-dependent genes of unknown function should contribute to our understanding of the mechanisms of cell cycle progression and organelle division and how the cell cycle and photosynthetic cellular growth are coordinated in photosynthetic eukaryotes.

The green and red algae separated 0.9 to 1 billion years ago, relatively soon after the emergence of primitive eukaryotic algae (Berney and Pawlowski, 2006). In C. reinhardtii, 1,719 genes out of the approximately 15,000 nucleus-encoded genes in total have been reported to be dependent on CDKA and CDKB (Tulin and Cross, 2015). Of the 454 cell-cycle-dependent genes in C. merolae, 265 genes (58%) were found in C. reinhardtii by BLASTP searches (E < 0.0001; Supplemental Data Set S1). However, only 77 of those genes were dependent on CDKA and CDKB in C. reinhardtii (Supplemental Data Sets S1 and S8). Most of these 77 genes that are dependent on the cell cycle both in C. merolae and C. reinhardtii encode well-known cell-cycle-related proteins such as cell cycle regulators and proteins involved in DNA replication, chromosome segregation, microtubule organization, and organelle division (Supplemental Data Set S8). By contrast, most of the remaining 377 C. merolae cell-cycle-dependent genes, which were not found in C. reinhardtii, encode proteins of unknown function. This large difference in the repertory of cell-cycle-dependent genes

Figure 7. The relationship between cell cycle progression and transcriptomic changes in metabolism in C. merolae. In environments in which cell growth during one round of daytime is not sufficient for cell division, some cells enter S/M phase while other cells in the same population stay in G1 during the evening and night. The cells that did not divide must wait for G1/S transition until at least the following evening and night because the G1/S transition is inhibited by circadian rhythms during the daytime (Miyagishima et al., 2014). Thus, cells are subjected to diel rhythms of photosynthesis and growth regardless of G1/S transition. This is probably a reason for the cell-cycle-independent diel rhythms of most metabolic genes, except for a few genes such as those involved in dNTP synthesis and starch degradation, as observed in this study.
between C. merolae and C. reinhardtii, except for the core cell cycle proteins, likely represents the differences in the mode of cell cycle progression (e.g. binary fission in C. merolae versus multiple fission in C. reinhardtii) and the repertory of cycle-cycle-dependent events (e.g. biogenesis of cell walls, centrosomes, and flagella in C. reinhardtii but not in C. merolae) between the two species.

Of the 181 C. merolae cell-cycle-dependent genes of unknown function, homologs of 34 genes were found in C. reinhardtii and homologs of 21 genes were found in both C. reinhardtii and the land plant Arabidopsis by BLASTP searches (E < 0.0001; Supplemental Data Sets S1 and S9). Of the 34 genes of unknown function in C. reinhardtii, mRNA levels of 14 genes exhibited ~24-h periodicity under the LD (Supplemental Data Sets S1 and S9; Zones et al., 2015) and the expression of five genes was dependent on the cell cycle (Supplemental Data Sets S1 and S9; Tulin and Cross, 2015). Any homologs of these five cell-cycle-dependent genes shared by C. merolae and C. reinhardtii were not found in cyanobacteria (E < 0.0001). Thus, investigation of these genes likely gives important insights into the cell-cycle-dependent regulation of processes that are specific to eukaryotic algae.

Because many cell-cycle-dependent genes are essential for cellular proliferation, conditional gene knockdown (Fujiwara et al., 2015) and conditional expression of dominant-negative forms (Sumiya et al., 2016) of proteins of interest in C. merolae should facilitate the analysis of cell-cycle-dependent genes. In addition, in this study, we succeeded in arresting cell-cycle-dependent genes of C. merolae cells in the G1 phase without inhibiting photosynthetic cellular growth by the conditional knockdown of CDKA (Fig. 2). The HA-CDKA-cKD strain and this method should be useful for examining cell cycle progression, cellular growth, metabolic rhythm, and the LD in further studies.

In this study, we used a microarray system because the method had been well established prior to the development of RNA-sequencing (RNA-seq) analyses. However, RNA-seq has become a powerful tool for analyzing unknown transcripts and splicing variants and ribosomal profiling (Gobet and Naef, 2017). Thus, investigation of mRNA changes depending on the diel rhythm and cell cycle by RNA-seq would give additional information for understanding the diel- and/or cell-cycle-dependent processes in photosynthetic eukaryotes.

**MATERIALS AND METHODS**

**Algal Cultures**

Wild-type *Cyanidioschyzon merolae* 10D (NIES-3357) and its transformants, except for HA-CDKA-cKD, were maintained in inorganic MA2 medium (containing 20 mM NH₄Cl as the sole nitrogen source; pH 2.3, adjusted with H₂SO₄). The HA-CDKA-cKD strain was maintained in MA2-NH₄ in which NH₄Cl was replaced with the same concentration of NO₃⁻ as the sole nitrogen source (20 mM NaNO₃, 20 mM Na₂SO₄, 8 mM KH₂PO₄, 4 mM MgSO₄, 1 mM CaCl₂, 2.8 μM ZnCl₂, 16 μM MnCl₂, 7.2 μM Na₂MoO₄, 1.28 μM CuCl₂, 0.68 μM CoCl₂, 100 μM FeCl₃, and 72 μM EDTA-2Na; pH 2.3, adjusted with H₂SO₄). The uracil-auxotrophic mutant M₄ (a derivative of C. merolae 10D, which has a mutation in the URA gene; Minoda et al., 2004) was maintained in MA2 medium supplemented with uracil (0.5 mg mL⁻¹) and 5-fluoroorotic acid monohydrate (0.8 mg mL⁻¹). All strains were maintained in 75-cm² tissue culture flasks (353136; Corning) in the light ( photon flux of 50 μmol m⁻² s⁻¹) at 42°C.

To arrest HA-CDKA-cKD cells at the G1 phase, HA-CDKA-cKD and wild-type (as a control) stock cultures, which were cultured in MA2-NH₄, were diluted with 400 mL of MA2 to give an OD₆₀₀ of 0.2. The cells were cultured in 700-mL flat glass bottles (3 cm thick) for 1 d in the dark at 42°C with aeration (3 L ambient air min⁻¹) and then maintained under LD (photon flux of 100 μmol m⁻² s⁻¹). Pairs of wild-type and HA-CDKA-cKD strains were cultured twice, 1 month apart, as biological replicates (wild-type 1 versus HA-CDKA-cKD and wild-type 2 versus HA-CDKA-cKD).

To compare changes in the transcriptome under the LD between the wild-type and RBR-KO strains, stock cultures, which were maintained in MA2, were diluted with 400 mL of MA2 to give an OD₆₀₀ of 0.2 in the flat glass bottles and then kept under the LD at 42°C with aeration (3 L ambient air min⁻¹). Pairs of wild-type and RBR-KO LD cultures were cultured twice, 1 month apart, as biological replicates (wild-type 3 versus RBR-KO1 and wild-type 4 versus RBR-KO2).

**Measurement of Cellular Density and Volume**

A total of 500 μL of culture was harvested and fixed by the addition of glutaraldehyde (final concentration, 0.35% [v/v] and 0.001% [v/v] Tween 20. Then, the cellular density and volume were measured with a Coulter Counter Z2 (Beckman Coulter).

**Preparation of Linear DNA for the Transformation of C. merolae**

The primers used in this study are listed in Supplemental Table S1. Linear DNA for the transformation of C. merolae was prepared as described below.

To produce a strain in which CDKA expression was depressed by the substitution of nitrate with ammonium in culture medium (HA-CDKA-cKD strain), an NR promoter and 3×HA epitope-coding sequences were integrated just before the start codon of the chromosomal CDKA (CME119C) ORF of C. merolae. To achieve this, we constructed the plasmid pNRp-HA-CDKA as follows. A plasmid vector backbone (pGEMT-easy [Promega]; for amplification in *Escherichia coli*), the 5′ upstream sequence of the ORF (for homologous recombination with the chromosomal DNA), the CDKA ORF, and its flanking downstream sequence (for homologous recombination with the chromosomal DNA) were amplified as linear DNA by PCR with primer set number 1/2 and pCAT-FLAG-CDKA (Fujiwara et al., 2017) as a template. The 3×HA-coding sequence was included in primer number 2. The *C. merolae* URA gene (for *C. merolae* transformant selection; Fujiwara et al., 2013a) was amplified by PCR with primer set number 3/4 and the *C. merolae* genomic DNA as a template. The NR (CMG019C) promoter (an 800-bp upstream flanking sequence of the NR ORF) was amplified by PCR with primer set number 5/6 and the *C. merolae* genomic DNA as a template. Then, the URA marker, NR promoter, and 3×HA-coding sequence were cloned between the 5′ upstream sequence of CDKA and the CDKA ORF of the above DNA amplified from pCAT-FLAG-CDKA using an In-Fusion Cloning Kit (Takara). The resultant plasmid was named pNRp-HA-CDKA. Finally, we amplified the assembled fragment containing the 5′ upstream sequence of CDKA, URA marker, NR promoter, 3×HA-coding sequence, CDKA ORF, and its flanking downstream sequence by PCR with primer set number 7/8 and pNRp-HA-CDKA as a template for the transfection of *C. merolae*.

To produce a strain in which HA-tagged CDKA was expressed by the endogenous CDKA promoter (HA-CDKA), the 3×HA epitope-coding sequence was inserted just before the start codon of the chromosomal CDKA ORF of *C. merolae*. To achieve this, we constructed the plasmid pCDKA-HA-CDKA by substituting the NR promoter of pNRp-HA-CDKA with the CDKA promoter as follows. A plasmid vector backbone (pGEMT-easy; for amplification in *E. coli*), the 5′ upstream sequence of the CDKA ORF (for homologous recombination with M₄ chromosomal DNA), the HA-CDKA ORF, and its flanking downstream sequence (for homologous recombination with the chromosomal DNA) were amplified as linear DNA by PCR with primer set number 9/10 and the pNRp-HA-CDKA as a template. The CDKA promoter (a 798-bp upstream flanking sequence of the CDKA ORF) was amplified by PCR with primer set number 11/12 and *C. merolae* genomic DNA as a template. Then, the CDKA promoter was cloned between the 5′ upstream sequence of the CDKA ORF and...
the HA-CDKA ORF of the above DNA amplified from the pVNP-HA-CDKA using the In-Fusion Cloning Kit. The resultant plasmid was named pCDKAp-HA-CDKA. Finally, we amplified the assembled fragment containing the 5’ upstream sequence of CDKA, URA marker, CDKA promoter, the HA-CDKA ORF, and its flanking downstream sequence by PCR with primer set number 7/8 and the pCDKAp-HA-CDKA as a template for the transformation of C. merolae.

The linear DNA for C. merolae transformation was purified using a QIAquick PCR Purification Kit (Qiagen). A total of 3 to 5 µg of the purified DNA was used for the transformation of C. merolae M4, a derivative of C. merolae 10D, which has a point mutation in the URA gene. The transformation and selection of transformants were performed as previously described (Fujiwara et al., 2015; Kuroiwa et al., 2018).

Antibodies and Immunoblot Analyses

Cells were harvested by centrifugation at 2,000g for 5 min at 4°C. The cell pellets were lysed with sample buffer (2% [w/v] SDS, 62 mM Tris-HCl, pH 6.8, 100 mM DTT, 10% [w/v] glycerol, and 0.01% [w/v] bromophenol blue) and then boiled for 5 min at 95°C. Then, 3 µg of total cellular protein in each lane was separated by SDS-PAGE on 10% (w/v) SDS-polyacrylamide gels and transferred onto PVDF membranes (Immobilon, Millipore). Membrane blocking, antibody reactions, and signal detection were performed as previously described (Fujiwara et al., 2015). The anti-HA monoclonal antibody (clone 16B12, BioLegend; dilution of 1:3,000) was used to detect HA-CDKA protein. Anti-α-tubulin monoclonal antibody (clone B-5-1-2, Sigma; dilution of 1:2,000), anti-PCNA polyclonal antiserum (Fujiwara et al., 2013b; dilution of 1:2,000), anti-H3S10ph polyclonal antibody (605-570, Millipore; concentration of 1 µg ml−1), anti-FtsZ2-1 polyclonal antibody (Sumiya et al., 2016; dilution of 1:10,000), and anti-RBR polyclonal antibody (Miyagishima et al., 2014; dilution of 1:10,000) were used as the primary antibodies.

Transcriptome Analyses

Cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted following the Trizol/RNeasy hybrid protocol (Trizol, Life Technologies; RNeasy Mini Kit, Qiagen). Using a customized oligo DNA microarray (8 × 15 k, 4,947 nuclear gene probes × three technical replicates; Agilent Technologies) in accordance with the manufacturer’s instructions, 100 ng of total RNA was subjected to transcriptome analyses. Fluorescent cRNA was prepared using a Low Input Quick Amp Labeling Kit (Agilent Technologies) with an oligo(dT) primer and Cy3 according to the manufacturer’s instructions. Hybridized microarrays were scanned using a SureScan Microarray Scanner (Agilent Technologies), and the signal intensities of oligo DNA spots were measured by Agilent Feature Extraction 12.0.3.1 (Agilent Technologies). Quantile normalization was utilized to obtain mRNA expression patterns under the LD (Supplemental Data Set S10). The relative mRNA level (Supplemental Data Set S1) was calculated as follows:

\[
\text{mRNA level of a gene} = \frac{\text{average mRNA level of all genes} \times 100}{\text{mRNA level of a gene}}
\]

Genes exhibiting a relative mRNA level change of more than twofold in −24 h under the LD were identified by the algorithm JTK_CYCLE (Hughes et al., 2010) as follows. To produce virtual 48-h time-course data sets, which are required to operate JTK_CYCLE, data of 0 to 24 h in wild-type 1 were concatenated with those of 4 to 24 h in wild-type 2. The mRNA level of a gene in wild-type 2 was normalized by 0 h (wild-type 2)/24 h (wild-type 1) as a coefficient. The data of wild-type 3 and wild-type 4 were also analyzed as above as a replicate (Supplemental Fig. S1). The two replicate data sets (wild-type 1+wild-type 2 and wild-type 3+wild-type 4) were used to calculate a false discovery rate (BH-Q). Genes exhibiting BH-Q < 0.05 (criterion 1) and amplitude > 2 in at least two or more LD cultures (criterion 2) were identified as approximately 24-h periodic genes (Supplemental Fig. S1). The cell-cycle-dependent genes were identified based on the criteria shown in Supplemental Figure S1.

For k-means and hierarchical clustering, data were log2 transformed [log2 (expression value at a time point/median in 0−28 h)], analyzed, and visualized by MeV 4.9 (version 4.9.0; https://en.bio-soft.net/chip/MeV.html; Saeed et al., 2003). To classify the expression patterns of 1,979 genes with approximately 24-h periodicity in the wild type, a tandemly connected transcriptome data set of wild-type 1 and wild-type 2 was analyzed. To classify those in the wild type, G1-arrested HA-CDKA-cKD, and RBR-KO, a tandemly connected transcriptome data set of wild-type 1, G1-arrested HA-CDKA-cKD1, wild-type 2, G1-arrested HA-CDKA-cKD2, wild-type 3, RBR-KO1, wild-type 4, and RBR-KO2 was analyzed.

Accession Numbers

The raw microarray data from this study before any normalization (accession no. CSEI13944) have been deposited in the National Center for Biotechnology Information, Gene Expression Omnibus. Sequence data can be found in the C. merolae Genome Project (http://czon.jp/) with the following accession numbers: CDKA (CMI219C), CDKB (CMI229C), PCNA (CMS101C), NR (CMK209C), RBR (CMF038C), E2F (CMF067C), DP (CMF061C), PSQ (CMI333C), α-tubulin (CMF304C), MCM6 (CM262C), CDC20 (CMA138C), CmDnm1/DRP3 (CME019C), CmDnm2/DRP5B (CME262C), FisZ2 (CMS004C and CMO009C), RNR-a (CMM232C), RNR-β1 (CMLO50C), RNR-β2 (CMK035C), CMPK3 (CMO383C), CTPS2 (CM216C), DUT (CMO126C), DHFR-TS (CMS462C), GPL (CMI184C), and ISAI (CMI294C).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Criteria for the identification of genes of ~24-h periodicity and cell-cycle-dependent genes.

Supplemental Figure S2. Temporal changes in the mRNA levels of some genes involved in DNA replication, construction of mitotic chromosomes, and chromosome segregation in the wild type under the LD.

Supplemental Figure S3. Comparison of cellular growth and cell cycle progression under the LD between the wild-type and HA-CDKA-cKD strains that were cultured in NH4+-medium (NR promoter off).

Supplemental Figure S4. Uncoupling the G1/S transition from the LD by depletion of RBR protein in C. merolae.

Supplemental Figure S5. Comparison of changes in the mRNA levels of cell-cycle-dependent and -independent genes under the LD in the wild-type, G1-arrested HA-CDKA-cKD, and RBR-KO strains.

Supplemental Figure S6. Heat map comparison of temporal changes in the mRNAs of ~24-h periodic cell-cycle-dependent and -independent genes in the wild-type, G1-arrested HA-CDKA-cKD, and RBR-KO strains.

Supplemental Figure S7. Heat map comparison of temporal changes in KEGG-assigned mRNAs of ~24-h periodic cell-cycle-dependent and -independent genes in the wild-type, G1-arrested HA-CDKA-cKD, and RBR-KO strains.

Supplemental Figure S8. Temporal changes in the mRNAs of genes involved in de novo synthesis of dNTPs in the wild-type, G1-arrested HA-CDKA-cKD, and RBR-KO strains.

Supplemental Figure S9. Temporal changes in the mRNAs of genes involved in starch synthesis and degradation in the wild-type, G1-arrested HA-CDKA-cKD, and RBR-KO strains.

Supplemental Table S1. Primers used in this study.

Supplemental Data Set S1. Changes in relative mRNA levels [mRNA level of the gene/average mRNA level of all genes at the indicated time point/100] of all nucleus-encoded genes under the LD in the wild-type, G1-arrested HA-CDKA-cKD, and RBR-KO strains based on microarray analysis.

Supplemental Data Set S2. K-means clustering of genes of ~24-h periodicity in the wild type under LD.

Supplemental Data Set S3. KEGG functional classification of genes of ~24-h periodicity in the wild type under LD.

Supplemental Data Set S4. List of all cell-cycle-dependent and -independent genes and their classification.

Supplemental Data Set S5. List of known cell-cycle-dependent genes in C. merolae.

Supplemental Data Set S6. KEGG functional classification of cell-cycle-dependent and -independent genes of ~24-h periodicity.
Supplemental Data Set S7. List of cell-cycle-dependent genes that were assigned to metabolism in KEGG classification.


Supplemental Data Set S10. Normalized transcriptome data before the calculation based on the average mRNA level of all genes at the indicated time point.

ACKNOWLEDGMENTS

We thank Kiyomi Hashimoto, Yoshiko Tanaka, Reiko Ujigawa, Reiko Tomita, and Uiko Sugimoto for their technical support and Dr. Yusuke Kobayashi for technical advice in the Miyagishima laboratory.

Received April 20, 2020; accepted June 1, 2020; published June 9, 2020.

LITERATURE CITED


Berenzy C, Pawlowski J (2006) A molecular time-scale for eukaryote evo-


Bourdon A, Minai L, Serre V, Jais JP, Sarzi E, Aubert S, Chrétien D, de


Breker M, Lieberman K, Cross FR (2018) Comprehensive discovery of cell-


Johnson CH (2010) Circadian clocks and cell division: What’s the pace-

maker? Cell Cycle 9: 3864–3873

Johnson CH, Stewart PL, Egli M (2011) The cyanobacterial circadian sys-


Kabeya Y, Miyagishima SY (2013) Chloroplast DNA replication is regu-

lated by the redox state independently of chloroplast division in Chla-

mydomonas reinhardtii. Plant Cell Physiol 51: 2102–2112

Kim JA, Kim HS, Choi SH, Jang JY, Jeong MJ, Lee SI (2017) The impor-

tance of the circadian clock in regulating plant metabolism. Int J Mol Sci 18: 2680


Malumbres M, Barbacid M (2001) To cycle or not to cycle: A critical de-

cision in cancer. Nat Rev Cancer 1: 222–231


oschyzon merolae 10B. Nature 428: 653–657


provement of culture conditions and evidence for nuclear transforma-

tion by homologous recombination in a red alga, Cyanidioschyzon merolae 10D. Plant Cell Physiol 45: 667–671


tive rhodophyte Cyanidioschyzon merolae contains a semiamylopectin-type, but not an amylose-type, alpha-glucan. Plant Cell Physiol 51: 1500

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