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Periodic Gene Expression Patterns during the Highly Synchronized Cell Nucleus and Organelle Division Cycles in the Unicellular Red Alga *Cyanidioschyzon merolae*

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Abstract

Previous cell cycle studies have been based on cell-nuclear proliferation only. Eukaryotic cells, however, have double membranes-bound organelles, such as the cell nucleus, mitochondrion, plastids and single-membrane-bound organelles such as ER, the Golgi body, vacuoles (lysosomes) and microbodies. Organelle proliferations, which are very important for cell functions, are poorly understood. To clarify this, we performed a microarray analysis during the cell cycle of *Cyanidioschyzon merolae*. *C. merolae* cells contain a minimum set of organelles that divide synchronously. The nuclear, mitochondrial and plastid genomes were completely sequenced. The results showed that, of 158 genes induced during the S or G2-M phase, 93 were known and contained genes related to mitochondrial division, *ftsZ1-1*, *ftsz1-2* and *mda1*, and plastid division, *ftsZ2-1*, *ftsZ2-2* and *cmdnm2*. Moreover, three genes, involved in vesicle trafficking between the single-membrane organelles such as *vps29* and the Rab family protein, were identified and might be related to partitioning of single-membrane-bound organelles. In other genes, 46 were hypothetical and 19 were hypothetical conserved. The possibility of finding novel organelle division genes from hypothetical and hypothetical conserved genes in the S and G2-M expression groups is discussed.

Key words: cell cycle; microarray; mitochondria–plastid division genes; organelle division genes; *Cyanidioschyzon merolae*

1. Introduction

Cell cycle progression is regulated and controlled by a range of mechanisms including protein modification, targeted proteolytic degradation and cell cycle-specific transcription.[1] Genes whose expression

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levels peak at a specific event of the cell cycle are often required to regulate the processes that occur at these stages.[2] In the budding yeast, *Saccharomyces cerevisiae*, of ~6000 genes, ~400–800 have transcript levels that oscillate during the cell cycle,[3,4] whereas in the fission yeast, *Schizosaccharomyces pombe*, of ~5000 genes, ~400–750 such genes have been found.[5–7] In human cancer cell line (HeLa), among a total of 22 000 genes, >1000 are regulated by the cell cycle.[8,9] These studies of periodic genes suggest that only the 112th orthologue group is cyclic and common among human cells, as well as budding and fission yeast cells.[10] Those taking part in the cell cycle progression include the *cyclin* B homologs, CDC5, SCH9, DSK2 and ZPR1. Proteins involved in DNA replication include histones, some checkpoint kinases and some proteins regulating DNA damage and repair. Many groups of genes related to translation and other metabolic processes are also cyclic in all three organisms.[10] In *Arabidopsis thaliana*, of ~25 000 genes, ~500 cell cycle regulated genes have been identified.[11] In tobacco BY-2, of the ~10 000 genes that could be analyzed by cDNA–AFLP-based gene profiling, ~1300 were regulated by the cell cycle.[12]

Cell proliferation needs division and partitioning of organelles such as mitochondria and plastids. However, there is no information on periodic gene expressions with respect to mitochondrial division in yeast or animal cells. Furthermore, in the research of the periodic expression in *A. thaliana* or tobacco BY-2, mitochondrial and plastid divisions were not shown. Mammalian, plant and yeast cells contain many organelles whose divisions occur at random, cannot be synchronized and have shapes that are very diverse and complicated.[13] Therefore, genes related to such organelles are not reflected in microarray analyses of the cell cycle in higher organisms. In previous cell cycle studies, the analysis has been based on nuclear proliferation only. Eukaryotic cells, however, have double-membrane-bound organelles, such as the nucleus, mitochondria and plastids, and single-membrane-bound organelle such as ER, the Golgi body, vacuoles (lysosomes) and microbodies. Organelle proliferation is very important for cell functions, as well as differentiation and cell division. However, there are few studies that have investigated the organelle proliferation cycle.

The unicellular red alga *Cyanidioschyzon merolae* has advantages for investigating organelle proliferation as it has a minimum set of organelles,[14–16] and organelle division can be synchronized by a light/dark cycle.[17] The mitochondrial and plastid division requires the FtsZ,[18,19] the Dynamin[20,21] and the MD/PD rings.[22,23] Northern blot analysis has shown that each transcriptional level of *ftsz1* for mitochondrial division, and *ftsZ2* for the plastid division, has a peak per cell cycle before each division.[18] Moreover, nuclear, mitochondrial and plastid genomes of *C. merolae* have completely been sequenced.[24–27] As the nucleus genome 4775 ORF's coding protein includes 27 introns only, newly designed proteins by alternative splicing are few; therefore, the functioning protein is directly identified as the same gene. Furthermore, most ORFs do not have paralogues.[24] We thought that novel organelle division-related genes like *ftsZ genes* could be found by genome-wide transcriptome of the cell cycle.

2. Materials and methods

2.1. Synchronous culture and fluorescence microscopy

Cyanidioschyzon merolae 10D-14 were synchronized according to the method discussed in Suzuki et al.[17] Cells were cultured in 2× Allen's medium at pH 2.3. Flasks were shaken under continuous light (40 W/m^2) at 42°C. The cells were sub-cultured to $<10^7$ cells/mL, and then synchronized by subjecting them to a 12 h light/12 h dark cycle at 42°C while the medium was aerated. For the observation of DNA, the cells were fixed in 1% glutaraldehyde diluted with 2× Allen's medium and stained with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole phosphate). Images were viewed using an epifluorescence microscope (BX51; Olympus, Tokyo, Japan) with 3CCD digital camera (C77780; Hamamatsu Photonics, Tokyo, Japan) under ultraviolet excitation. The cultures were harvested every 2 h, and indexes of organelle division were counted.

2.2. Reverse transcriptase–polymerase chain reaction

Cells were collected by centrifuging at 1400*g* for 3 min and immediately frozen in liquid nitrogen. Nuclear acid isolation buffer (50 mM Tris–HCl, pH7.6, 100 mM EDTA, 300 mM NaCl, 4% SDS, 2% *N*-lauroylsarcosine sodium salt) pre-warmed at 60°C was added to the frozen cell pellets. The lysate was added to an equal volume of PCI (phenol:chloroform:isoamylalcohol = 25:24:1). The aqueous phase was recovered by centrifuging at 15 000*g* for 5 min at 4°C and re-extracted using PCI. Total nucleus acid was precipitated by adding an equal volume of isopropanol and recovered by centrifugation at 15 000*g* for 15 min at 4°C. The pellet was melted in DNase I solution (0.1 U/μL DNase I, RNase Free; Roshe, 0.4 U/μL RNase Inhibitor; Sigma, 10 mM DTT, 10 mM $MgCl_2$) and incubated for 45 min at 37°C. Total RNA was precipitated by adding an equal volume of isopropanol and recovered by centrifugation at 15 000*g* for 15 min at 4°C.

The RNA samples were reverse-transcribed in 20 μL of the reaction mix comprising 1 μL of Reverse Transcriptase XL (AMV; TaKaRa Bio Inc.), 50 ng/μL of oligo(dT) primer (Novagen), 2 U/μL of RNase inhibitor and 0.5 mM dNTP mixture (TaKaRa Bio Inc.). The reaction conditions were as follows: 10 min at 25°C, 45 min at 42°C and 10 min at 70°C. Absence of genomic DNA contamination was confirmed by PCR in all the total RNA samples. In the RT–PCR assay, cDNA of *ftsZ2-1, ftsZ2-2* and the housekeeping gene *ef-1a* were amplified by 22 PCR cycles. The quantity of PCR products was analyzed by electrophoresis in 2% agarose gel. The primers used in PCR were described in Supplementary Table S1.

All real-time PCR assay kits were purchased from Applied Biosystems, and utilized according to the manufacturer's instructions. PCR amplification of each sample was carried out using 7.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems), 5.5 μL of distilled water, 0.3 μL of forward and reverse primer solutions (10 μM each) and 1.7 μL of cDNA template (25 ng/mL). For signal detection, an ABI Prism 7500 fast sequence detector was programmed to an initial step of 10 min at 95°C, followed by 50 thermal cycles of 10 s at 95°C and 30 s at 60°C. The mean Quantity values (Qty) were calculated from the cycle threshold (Ct) values using the SDS 3.1 software by the standard curve method (Applied Biosystems). The expression of each target gene was normalized by that of *ef-1a*. From the mean Qty values of the target gene and *ef-1a*, we calculated the relative values to show the changes in gene expression with the multiplication mean as standard 1 as follows:

$$\text{relative value}_i = \frac{a_i}{\sqrt[6]{a_1 \cdot a_2 \cdots a_6}}, \quad 1 \le i \le 6$$

$$a = \frac{\text{mean Qty(gene)}}{\text{mean Qty(ef} - \text{1a)}}$$

All reactions were performed in triplicate for measurement of the mean Qty in each sample and in duplicate for the standard curve. The PCR primer sequences are described in Supplementary Table S1.

2.3. Microarray manufacture

On the basis of the annotated *C. merolae* genome, we ordered synthesis of the ORF-specific oligonucleotides for Sigam Genosys and searched a unique 50-mer sequence for each of the 4586 ORF-coding protein genes (>96% of the ORFs in the nucleus genome). In brief, 50-mer sequences were first selected from ORF sequences based on a set of criteria that included Tm of 75 ± 10°C. Ninety-eight percent of the ORFs-specific oligonucleotides were selected among 1.5 kb from the 3′ end of the ORF. All the oligonucleotides were synthesized (Sigma Genosys), resuspended into 30% (v/v) DMSO and spotted onto poly-lysine–coated glass slides, using a spotting machine (SPBIO, Hitachi Software Engineering Co.). Methods of spotting were referred to the protocol of the Brown lab in Stanford University (http://cmgm.stanford.edu/pbrown/). Spotted microarray glasses were stored at room temperature in a dry cabinet for later use.

2.4. aRNA fluorescence labeling

Amino-allyl aRNA was synthesized using an Amino-Allyl MessageAmp aRNA Kit (Ambion, TX, USA) according to the manufacturer's instructions. It was transcribed in the presence of amino-allyl dUTP by templating double-strand cDNA synthesized using T7 oligo(dT) primer. Cy3- or Cy5-congugated aRNA was prepared by mixing Cy (GE Healthcare 1vial/45ul dissolved in DMSO) and 5 μg amino-allyl aRNA in coupling buffer (0.1 M $NaHCO_3$, pH 9.0), incubating for 60 min at 40°C and then purified by a Bio Spin-Column (Bio-Rad) and microcon-YM30 spin-column (Millipore).

2.5. Microarray hybridization

Hybridization solution (5× SSC, 0.5% SDS, 4× Denhalt's solution, 10% formamid, 100 ng/μL Salmon Sperm DNA) and Cy3-congugated aRNA was hybridized to spotted microarray slides covered with a coverglass (Matsunami) in a slide hybridization chamber (Sigma) for 18 h at 55°C. Hybridized slides were washed in 1× SSC/0.03% SDS for 6 min at 45°C, followed by 0.2× SSC for 5 min and 0.05× SSC for 4 min, and then spin-dried before scanning.

2.6. Microarray data mining

Microarray slides were scanned using an FLA-8000 scanner (FujiFilm) at a wavelength of 532 and 635 nm in the 5-μm resolution. Gene Spot signals on microarray images were measured by the microarray analyzing software ArrayGauge Ver.2 (FujiFilm). As for data at each time point, it was subtracted the average value from 2 to 50 h. Data were analyzed by microarray data mining software AVADIS (Hitachi software). To collect cell cycle expression genes, hierarchical clustering was performed. In hierarchical clustering analysis for gene direction, the distance metric and linkage rule were set for Euclidean and Complete, respectively. Cell cycle regulated genes collected by hierarchical clustering analysis were separated into four groups by K-means clustering. In K-Means clustering analysis for gene direction, distance metric and four number of clusters and numbers of iterations were set for Euclidean, 4 and 50, respectively.

3. Results and discussion

3.1. *Synchronization of the cell nucleus, mitochondrial and plastid divisions in C. merolae*

Organelle division in *C. merolae* can be synchronized by a light dark cycle.[17] Fig. 1A shows typical interphase and mitotic cells fixed at 8 and 18 h after the start of the synchronous culture. Interphase cells had one cell nucleus, a mitochondrial nucleus (nucleoid) and one spherical plastid, whereas mitotic cells contained a dividing cell nucleus, mitochondrion and plastid. On the basis of the previous and present observations, these divisions are illustrated relative to the cell cycle in Fig. 1B. The type I cell is in the G1-phase in which the nucleus is spherical, the mitochondrion flat and the plastid (chloroplast) cup-shaped (Fig. 1BI). Type II

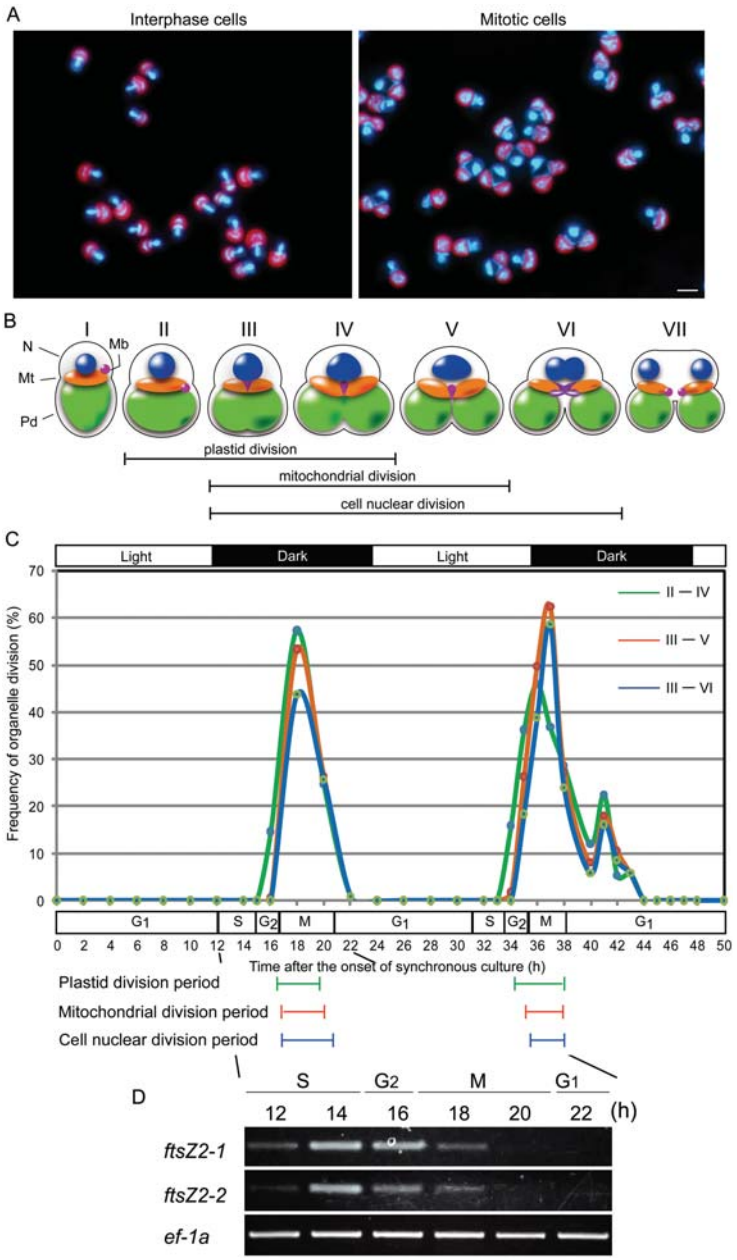

Figure 1. Synchronous culture of *C. merolae*. (**A**) Images of typical interphase and mitotic cells. The DNA of a cell nucleus, a mitochondrion and a plastid emit white-blue fluorescence stained with DAPI and plastids emit red-autofluorescent. Bar: 2 μm. (**B**) Schematic models for organelle division during the cell cycle. Plastid division is shown between II and IV. Mitochondrial division is shown between III and V. Chromosome segregation is shown between III and VI. N, nucleus; Mt, mitochondrion; Pd, plastid; Mb, microbody. (**C**) Frequencies of organelle division. The top bar indicates the 12-h light/12-h dark photoperiods and the lower bar indicates cell cycle phase. Green, orange and blue carve show the index of plastid division indicated II–IV of (B), index of mitochondrial division indicated III–V of (B) or mitotic index indicated III–VI of (B), respectively. Color bars under the graph show major periods of organelle division, green, orange and blue indicates plastid, mitochondrion and nucleus, respectively. (**D**) RT–PCR assay during the first synchronized organelle division.

cells in the G2-phase contain a cell nucleus that is larger than type I, and a plastid whose volume is increased, indicating that division has been initiated (Fig. 1BII). In type III cells, the M-phase is initiated and the nucleus begins to form a football-like structure. Mitochondrial division begins and plastid division is more advanced. The microbody has moved to the region of mitochondrial division (Fig. 1BIII). In type IV cells, organelle divisions are more advanced (Fig. 1BIV). In type V cells, the mitochondrion remains as a single body and its division is still not finished, while the plastid has divided in two (Fig. 1BV). In type VI cells, the cell nucleus is dividing without chromosome condensation, the mitochondrion dividing prior to cell nuclear separation, and the microbody is dividing (Fig. 1BVI). In type VII, cytokinesis has been initiated after cell nuclear separation and microbody division (Fig. 1AVII). Therefore, types II–IV, III–V and III–VI cells show plastid, mitochondrial and cell nuclear division, respectively.

In synchronized culture, the cells were taken at 2 h intervals for a period of 50 h to ensure that at least two complete cell cycles were covered. Fig. 1C shows the index of cell nuclear, mitochondrial and plastid division, counted according to shape of the cell in Fig 1B. The main period of plastid division took 4 h, from 16 to 20 h and from 34 to 38 h (Fig. 1C). Mitochondrial division took place about 3.5 h, from 17 to 20.5 h and from 35 to 38 h, and nuclear division took place about 3.5 h, from 17 to 21 h and 35.5 to 38 h (Fig. 1C). The mitotic cycle phase was defined by observing the change in nuclear morphology. An increase in cell nuclear volume in the S-phase occurred from 12 to 15 h and from 31 to 34 h, and the cell nuclear division period (M-phase) occurred from 17 to 21 h and 35.5 to 38 h (Fig. 2C).

To confirm whether organelle division genes were induced in the synchronous culture, the change of transcriptional level *ftsZ2* was analyzed by the RT–PCR assay at 12, 14, 16, 18, 20 and 22 h during the first cell division. *ftsZ2*, which is involved in plastid division, is an excellent marker for timing-specific gene expression.[18] There are two paralogues of *ftsZ2*, *ftsZ2-1*/CMS361C and *ftsZ2-2*/CMS004C, each transcriptional level of *ftsZ2-1* and *ftsZ2-2* had a peak at 14 h, and then was lowest at 22 h (Fig. 1D). This result showed that most genes related to organelle division were expressed synchronously at 14 h in late S-phase. Therefore, microarray analysis was performed in the synchronous culture.

3.2. *Detection of organelle division genes by microarray analysis*

Microarray of *C. merolae* can identify 4586 genes for >96% of all known and predicted *C. merolae* gene-coding proteins. We tested whether the microarray could analyze the induction of organelle division genes. Microarray analysis between 14 and 22 h showed that *ftsZ2-1* and *ftsZ2-2* were strongly induced at 14 h (Fig. 2). Moreover, expressions of *cmdnm2* involved in plastid division, and *ftsZ1-1* involved in mitochondrial division, were stronger at 14 h compared with 22 h. *ftsZ1-2* did not show a remarkable induction, but its expression was slightly stronger at 14 h compared with 22 h. Expression of *cmdnm1* that was constantly expressed during the cell cycle[20] showed a constant expression and was found to be near the diagonal showing constant expression. These results were consistent with previous experiments and showed that the microarray could analyze time-specific gene expression.

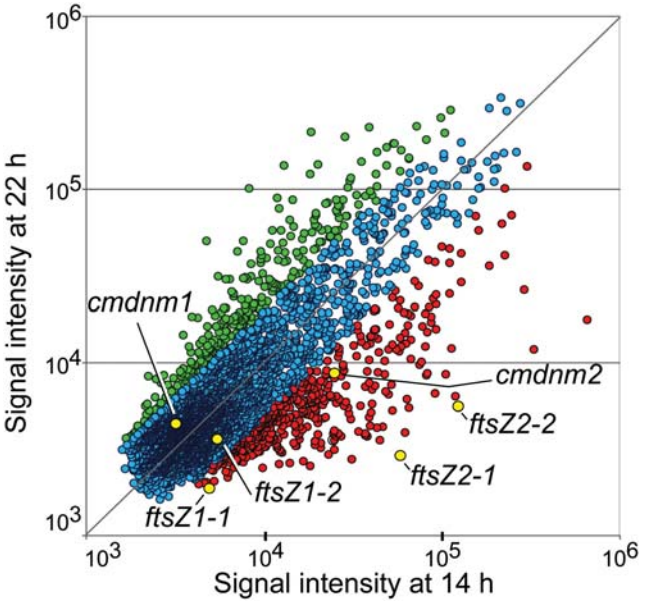

Figure 2. Scattered plot of transcriptional levels at 14 and 22 h in microarray analysis. The color circle indicated green <0.5-fold ≤ blue < 2-fold ≤ red. The values, 0.5 and 2, is the ratio of (signal at 14 h/signal at 22 h). Red, blue and green circles showed the genes were induced, constantly expressed and suppressed at 14 h, respectively. The yellow circles indicated *ftsZ1-1*, *ftsZ1-2*, *ftsZ2-1*, *ftsZ2-2*, *cmdnm1* and *cmdnm2*. In addition to *ftsZ2-1and ftsZ2-2*, *cmdnm2* and *ftsZ1-1* were confirmed to be induced at 14 h. *ftsZ1-2* and *cmdnm1* did not show changes remarkably.

3.3. *Identification of genes induced during S-, G2- and M-phases by microarray analysis*

The microarray analysis of two cell cycles from 2 to 50 hrs, in addition to the previously known periodic genes such as *ftsZ1* and *ftsZ2*, identified 358 cell cycle-regulated genes by hierarchical clustering. They were classified by K-means clustering analysis into four expression patterns, early G1 group, late G1 group, S group and G2-M group. This grouping

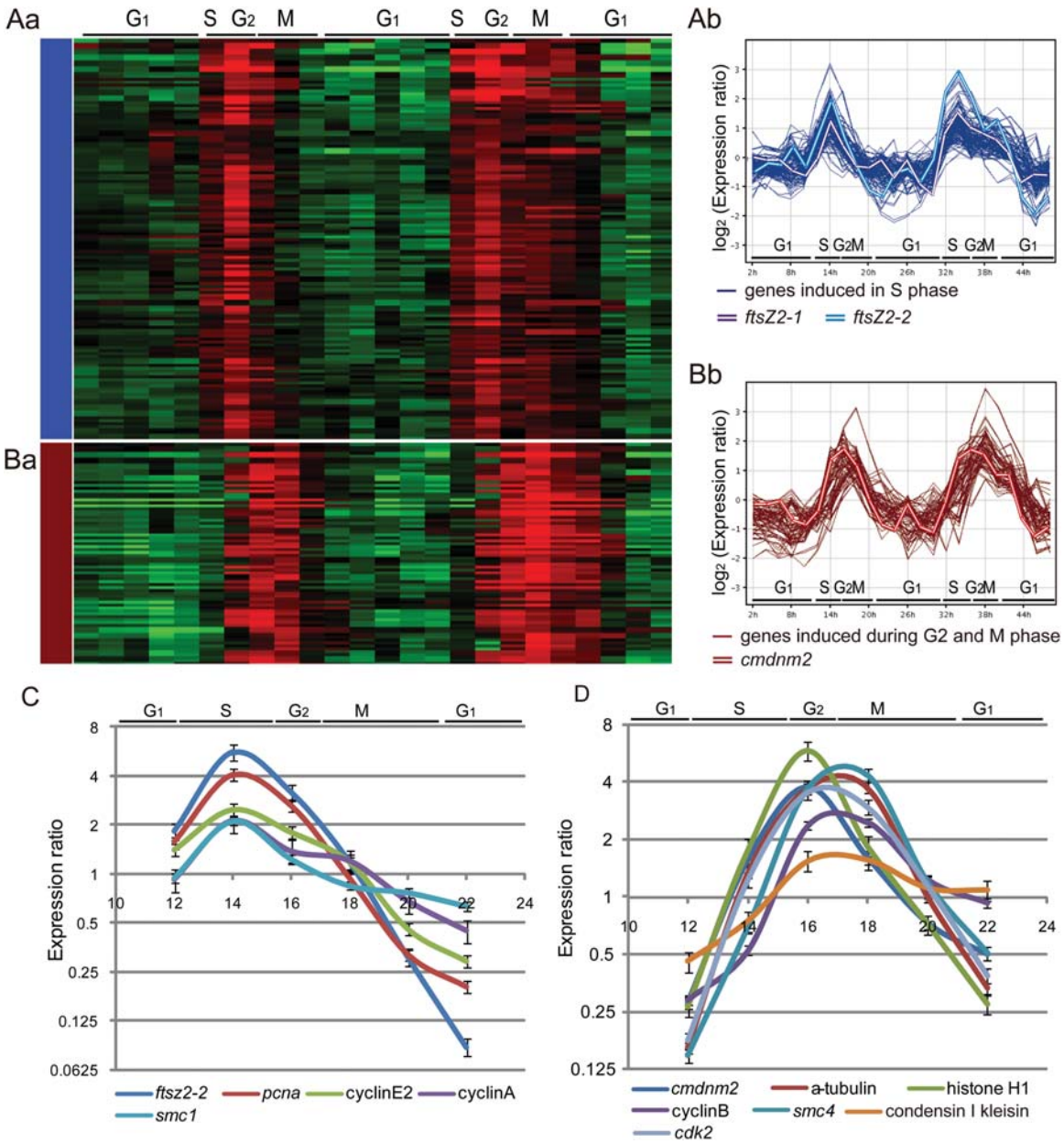

Figure 3. Heatmaps and oscillations of genes in S or G2-M groups. (**Aa**) The heatmap of transcriptional levels of genes in the S-phase group. Genes correspond to the rows, and the time points form the columns. Red and green indicate induction and suppression of genes expression, respectively. (**Ab**) The oscillations of transcriptional levels in the S-phase group. This group contained *ftsZ2-1* and *ftsZ2-2*. (**Ba**) The heatmap of transcriptional levels of genes in the G2-M-phase group. (**Bb**) The oscillations of transcriptional levels in the G2-M-phase group. This group contained *cmdnm2*. (**C**) Real-time RT–PCR assay of some genes in the S-phase group. *ftsZ2-2, pcna*, cyclin E2, cyclin A and *smc1*, which were assigned in the S-phase, were confirmed to have peak in S phase. (**D**) Real-time RT–PCR assay of some genes in the G2-M phase group. *cmdnm2*, a-tubulin, histone H1, cyclin B, *smc4*, condensing I kleisin subunit, and *cdk2*, which were assigned in G2-M phase, were confirmed to have peak in G2-M phase.

showed that *ftsZ2* and *cmdnm2* were strongly induced in the S- and G2-M phases, respectively (Fig. 3A and B). Therefore, in the present study, as the genes related to cell nuclear and organelle division were induced during the S- and G2-M phases, we focused on the genes grouped into these category.

The S- and G2-M phase groups comprised a total of 158 genes including 95 genes (Fig. 3A) in the S-phase and 63 genes in the G2-M phase (Fig. 3B) (Table 1). Twelve genes were related to cell cycle progression such as cyclin E2/CML219C, *cdk2*/CMH128C and ubiquitin-conjugating enzyme E2/CMS304C. 51 genes were related to cell nuclear function such as DNA replication, and chromosome segregation and organization. The genes characteristically induced were: DNA replication; *pcna*/CMS101C, *mcm3*/CMH264C

Table 1. List of genes induced during S or G2-M phase

Gene ID	Annotation	Phase	Fold
Nucleus 51			
DNA replication, repair and nucleotides metabolism 20			
CMC123C	Probable replication protein A (RPA)	S	2.1
CMF110C	Probable replication factor C/activator 1 subunit	S	1.7
CMG028C	Similar to meiotic nuclear division protein Mnd1	S	2.8
CMG106C	Probable flap structure-specific endonuclease 1	S	1.9
CMH264C	DNA replication licensing factor MCM3	S	3.5
CMI291C	Similar to replication protein A 30kDa	S	3.4
CMJ261C	DNA replication licensing factor MCM6	S	3.1
CML050C	Ribonucleoside-diphosphate reductase beta chain	S	3.3
CMM105C	Similar to mutT (8-oxo-dGTPase)	S	1.6
CMO126C	dUTP pyrophosphatase	S	2.5
CMP045C	Probable replication factor C	S	1.5
CMP311C	TBP-1 interacting protein, Hop2 homologue (Ev = 2e − 14)	S	3.7
CMQ381C	Hypothetical protein, conserved. Psf2, GINS complex component (Ev = 5e − 30)	S	2.2
CMR406C	Hypothetical protein, conserved. Sld5, GINS complex component (Ev = 2e − 14)	S	2.6
CMS101C	Probable proliferating cell nuclear antigen	S	5.3
CMS112C	Similar to origin recognition complex subunit 1	S	1.0
CMS462C	Fusion protein of dihydrofolate reductase and thymidylate synthase	S	1.6
CMT492C	Similar to 3-methyladenine DNA glycosylase alkA	S	1.7
CMN054C	Similar to cell cycle checkpoint and DNA repair exonuclease RAD1	G2 > M	1.5
CMS368C	DNA repair protein RAD52	G2 < M	1.8
Chromosome segregation 12			
CMN304C	γ-Tubulin	S	1.7
CMA036C	Similar to tubulin-folding cofactor B	S	2.7
CMS347C	Probable mitotic spindle assembly checkpoint protein MAD2	S	1.5
CMT504C	α-Tubulin	G2 > M	4.4
CMN263C	β-tubulin	G2 > M	4.1
CMO070C	Kinesin-related protein	G2 > M	1.4
CMR497C	Kinesin-related protein	G2 > M	2.0
CMO196C	Probable microtubule organization 1 protein	G2 > M	1.7
CMJ156C	Similar to microtubule-associated protein, RP/EB family	G2 > M	3.5
CMI101C	Hypothetical protein, conserved. SpC25 Kinetochore protein (Ev = 1e − 8)	G2 > M	2.2
CMG078C	Hypothetical protein, conserved. HEC/Ndc80p family (Ev = 9e − 31)	G2 < M	3.5
CMB012C	Similar to coiled-coil protein; putative spindle pole body associated protein	G2 < M	2.3
Chromosome organization 13			
CMI192C	14S cohesion SMC1 subunit; SMC protein	S	2.8
CMG028C	Similar to meiotic nuclear division protein Mnd1	S	2.8
CMQ181C	Hypothetical protein, conserved. Nse4, component of Smc5/6 (Ev = 2e − 14)	S	1.8
CMR457C	Histone H2A.Z variant	G2 > M	2.7
CMN183C	Similar to histone H1	G2 > M	3.5
CMN173C	Histone H2B	G2 > M	3.6
CMN165C	Histone H3	G2 > M	3.0
CMN176C	Histone H3	G2 > M	3.8
CMN169C	Histone H4	G2 > M	3.6
CMN166C	Histone H4	G2 > M	3.5

Continued

Table 1. Continued

Gene ID	Annotation	Phase	Fold
CMR484C	Similar to 13S condensin XCAP-D2 subunit	G2 < M	2.3
CMF069C	Condensin complex subunit 2	G2 < M	1.4
CME029C	Chromosome condensation protein SMC4	G2 < M	2.7
Other 6			
CMQ111C	Meiotic DNA topoisomerase VI subunit A	S	2.0
CMG143C	Similar to anti-silencing factor	S	1.6
CME102C	Similar to GATA transcription factor	S	1.6
CMH135C	Unknown transcriptional coactivator	S	2.5
CMH122C	Similar to DNA invertase	G2 > M	2.2
CMP037C	Unknown transcriptional regulator	G2 < M	2.3
Mitochondria 5			
CMS361C	Organelle division protein FtsZ (AB032071)	S	1.3
CMR185C	Mitochondria division protein mda1	S	1.6
CMC099C	NADH dehydrogenase I (Complex I) beta subcomplex 7 (B18)	S	1.8
CMT493C	Organelle division protein FtsZ	G2 > M	1.3
CMO111C	Outer mitochondrial membrane protein porin	G2 > M	4.6
Chloroplast 5			
CMS004C	Plastid division protein FtsZ (AB032072)	S	2.8
CMO089C	Plastid division protein FtsZ	S	4.8
CMI243C	Plastid terminal oxidase	S	2.6
CMI244C	Plastid terminal oxidase	S	1.8
CMN262C	Dynamin-related protein involved in chloroplast division CmDnm2/DRP5	G2 > M	3.0
Other organelle (ER, Golgi, endosome) vesicle trafficking 3			
CMR405C	Similar to retromer component VPS29	S	2.8
CMQ189C	Small GTP-binding protein of Rab family	S	1.4
CMF181C	Small GTP-binding protein of Rab family	G2 > M	1.0
Cell cycle progression 12			
Cyclin, cdk 7			
CML219C	Similar to cyclin E2	S	2.6
CMI203C	Probable mitotic cyclin a2-type	S	2.6
CMT038C	Similar to retinoblastoma-family protein	S	2.3
CMH128C	Cyclin-dependent kinase, B-type	G2 > M	3.3
CMA127C	Cyclin-dependent kinase regulatory subunit	G2 > M	1.5
CMI227C	Probable G2/mitotic-specific cyclin 1	G2 < M	2.2
CMR407C	Cell division cycle protein cdc27	G2 < M	0.9
Ubiquitin-proteasome 5			
CMN138C	Probable AAA protein spastin	S	2.2
CMB015C	Probable ubiquitin-conjugating enzyme E2	S	2.3
CMI156C	26S proteasome ATP-dependent regulatory subunit	G2 > M	1.2
CMS304C	Probable ubiquitin conjugating enzyme E2	G2 < M	1.4
CMP181C	Similar to ubiquitin conjugating enzyme E2	G2 < M	3.2
The others 17			
CMD113C	Glycerol-3-phosphate dehydrogenase	S	2.5
CMF101C	Similar to Muconate cycloisomerase related protein	S	2.4
CMH183C	Small GTP-binding protein of Rho family	S	2.5
CMI088C	ATP-binding cassette, sub-family G	S	1.5

Continued

Table 1. Continued

Gene ID	Annotation	Phase	Fold
CMK197C	Probable dolichyl-phosphate glucosyltransferase	S	1.7
CMN011C	Similar to ubiquinone/menaquinone biosynthesis methyltransferase family protein	S	3.5
CMN082C	Similar to GTP pyrophosphokinase	S	1.4
CMN212C	Similar to diaphanous-related formin	S	2.0
CMP183C	Probable 2-epi-valiolone synthase	S	1.1
CMQ134C	Similar to ubiquinone/menaquinone biosynthesis methyltransferase	S	1.6
CMQ301C	Probable alcohol dehydrogenase	S	2.3
CMR500C	Membrane-associated calcium-independent phospholipase A2	S	2.0
CMT454C	Similar to chondroitin 4-sulfotransferase	S	2.5
CMP119C	GTP cyclohydrolase II/	G2 > M	2.1
CME172C	p21-activated protein kinase	G2 < M	3.1
CML153C	Hypothetical protein. Snf7 (Ev = 7e − 8)	G2 < M	3.7
CMQ042C	UDP-GlcNAc:dolichol phosphate *N*-acetylglucosamine-1-phosphate transferase	G2 < M	3.4
Hypothetical protein 46			
CMB086C	Hypothetical protein	S	2.7
CMB114C	Hypothetical protein	S	2.4
CMC108C	Hypothetical protein	S	1.9
CMH165C	Hypothetical protein	S	1.4
CMH172C	Hypothetical protein	S	1.9
CMI108C	Hypothetical protein	S	2.7
CMJ103C	Hypothetical protein	S	3.3
CML148C	Hypothetical protein	S	2.6
CMM099C	Hypothetical protein	S	1.4
CMN081C	Hypothetical protein	S	2.0
CMO040C	Hypothetical protein	S	2.7
CMO045C	Hypothetical protein	S	2.2
CMO116C	Hypothetical protein	S	2.1
CMO238C	Hypothetical protein	S	2.5
CMP040C	Hypothetical protein	S	2.1
CMP287C	Hypothetical protein	S	2.8
CMR323C	Hypothetical protein	S	2.1
CMR324C	Hypothetical protein	S	2.7
CMS015C	Hypothetical protein	S	1.7
CMS123C	Hypothetical protein	S	2.1
CMS183C	Hypothetical protein	S	2.4
CMT135C	Hypothetical protein	S	1.4
CMT447C	Hypothetical protein	S	2.7
CMB057C	Hypothetical protein	G2 > M	2.0
CMD161C	Hypothetical protein	G2 > M	3.4
CMG015C	Hypothetical protein	G2 > M	2.5
CMG071C	Hypothetical protein	G2 > M	2.4
CMI102C	Hypothetical protein	G2 > M	2.3
CMI242C	Hypothetical protein	G2 > M	3.7
CMJ298C	Hypothetical protein	G2 > M	2.7
CMK062C	Hypothetical protein	G2 > M	2.8
CML181C	Hypothetical protein	G2 > M	3.6

Continued

Table 1. Continued

Gene ID	Annotation	Phase	Fold
CMQ048C	Hypothetical protein	G2 > M	2.3
CMQ329C	Hypothetical protein	G2 > M	1.2
CMR047C	Hypothetical protein	G2 > M	2.7
CMH048C	Hypothetical protein	G2 < M	2.0
CMH199C	Hypothetical protein	G2 < M	2.9
CMJ065C	Hypothetical protein	G2 < M	3.3
CMJ073C	Hypothetical protein	G2 < M	3.3
CMJ159C	Hypothetical protein	G2 < M	4.6
CMJ281C	Hypothetical protein	G2 < M	3.4
CML063C	Hypothetical protein	G2 < M	3.4
CMM037C	Hypothetical protein	G2 < M	1.9
CMN064C	Hypothetical protein	G2 < M	2.1
CMP074C	Hypothetical protein	G2 < M	2.0
CMQ434C	Hypothetical protein	G2 < M	2.0
Hypothetical protein, conserved. 19	Domain searched by SMART and CDs		
CMB107C	Hypothetical protein, conserved. Phosducin (Phd)-like family (Ev = 1e − 12)	S	2.0
CMM173C	Hypothetical protein, conserved. PcbC (Ev = 5e − 11)	S	1.8
CMN066C	Hypothetical protein, conserved. RhoGAP (Ev = 4e − 18)	S	2.7
CMN156C	Hypothetical protein, conserved. PLATZ transcription factor (Ev = 2e − 15)	S	2.3
CMN330C	Hypothetical protein, conserved. Not identified	S	3.5
CMP288C	Hypothetical protein, conserved. WD repeat (Ev = 2e − 10, 2e − 10)	S	1.4
CMQ062C	Hypothetical protein, conserved. GRX PICOT like (Ev = 1e-36), TRX PICOT (Ev = 8e − 3)	S	2.1
CMR358C	Hypothetical protein, conserved. Glycosyl transferase family 8 (Ev = 2e − 26)	S	2.6
CMS301C	Hypothetical protein, conserved. COG1259 (Ev = 2e − 19)	S	2.6
CMS407C	Hypothetical protein, conserved. DUF423 (Ev = 1e − 19)	S	1.4
CMT288C	Hypothetical protein, conserved. Not identified	S	2.1
CMC115C	Hypothetical protein, conserved. ERCC4, nuclease domain (Ev = 1e − 12)	G2 > M	1.5
CMO005C	Hypothetical protein, conserved. Not identified	G2 > M	3.1
CMR096C	Hypothetical protein, conserved. DUF1242 (Ev = 9e − 19)	G2 > M	2.8
CMI022C	Hypothetical protein, conserved. WD40 (Ev = 7.70e − 01)	G2 < M	2.5
CMK023C	Hypothetical protein, conserved. Not identified	G2 < M	1.5
CML211C	Hypothetical protein, conserved. Nudix hydrolase 38 (Ev = 5e − 19)	G2 < M	1.8
CMN061C	Hypothetical protein, conserved. LNS2, LNS2 (Ev = 2e − 44), Lipin N (Ev = 2e − 17)	G2 < M	2.7
CMS022C	Hypothetical protein, conserved. Not identified	G2 < M	6.1

Gene ID and Annotation were shown by the *C. merolae* genome project. Phase refers to phase when transcriptional level had peaks in microarray analysis, Fold describes $\log_2$(max − min of transcriptional levels); Ev, expect value by SMART or Conserved domain search (CDs).

and *mcm6*/CMJ261C, chromosome segregation; α-tubulin /CMT504C,[28] β-tubulin /CMN263C and γ-tubulin /CMN304C, chromosome organization; histone gene set and smc4/CME029C. In addition to the nucleus, genes related to mitochondria and plastids could be identified. 5 genes were related to mitochondrial function. *ftsZ1-1*, *ftsZ1-2* and *mda1*[29] were involved in mitochondrial division; moreover, the outer mitochondrial membrane protein, porin/CMO111C and NADH dehydrogenase I (Complex I) beta subcomplex 7/CMC099C, were also induced. 5 genes were related to plastid function. *ftsZ2-1*, *ftsZ2-2* and *cmdnm2*/CMN262C were involved in chloroplast division. 2 plastid terminal oxidases/CMI243C and CMI244C were also induced. 3 genes, including *vps29*/CMR405, Rab family protein/CMF181C and CMQ189C, related to vesicle trafficking were induced, and they might be involved in the partitioning of the ER, the Golgi body and endosome.

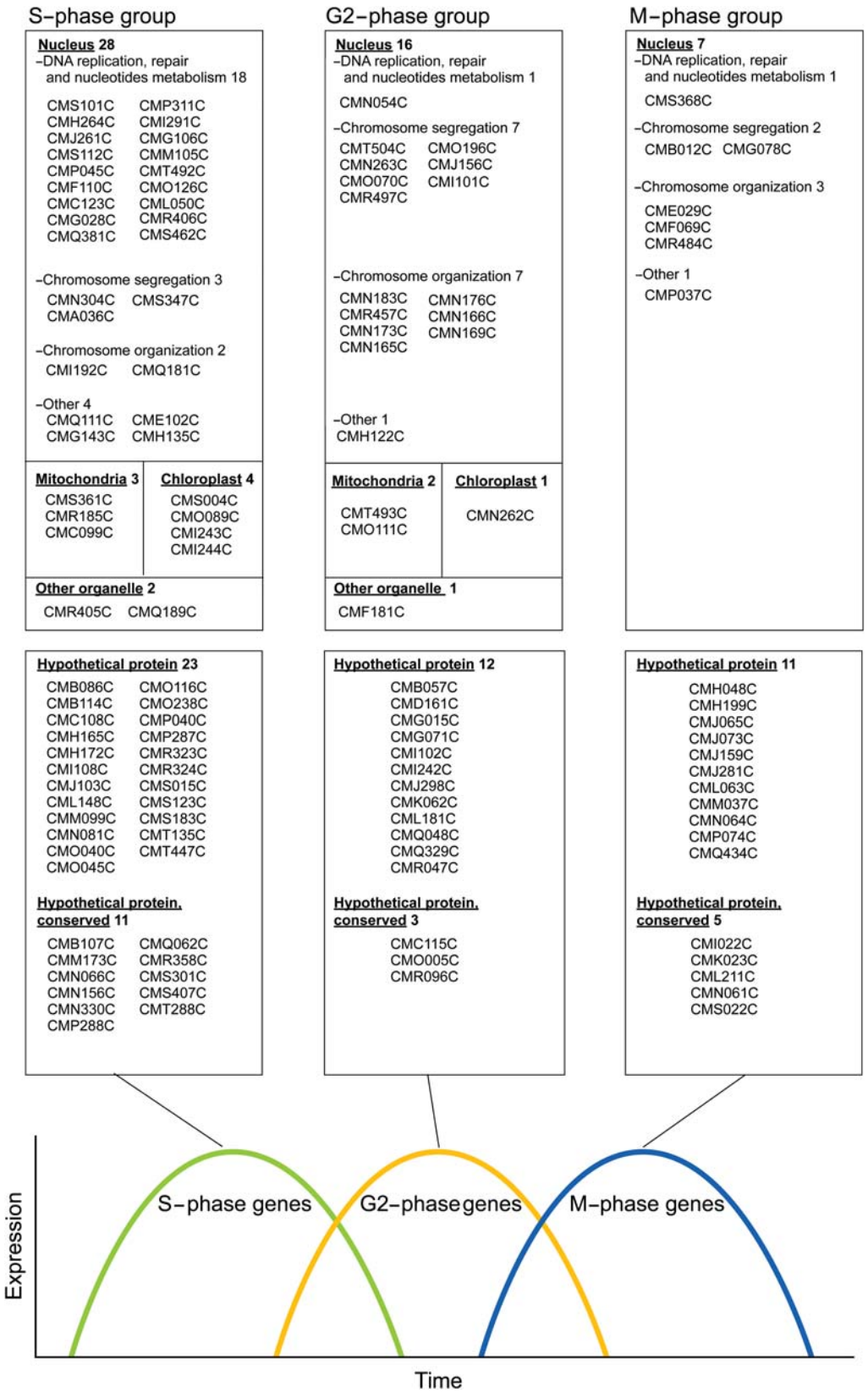

Figure 4. The list of genes related to the function in cell nucleus and organelle, and unknown genes in the S-, G2- or M-phase groups. Left, center and right column shows genes in the S-, G2- and M-phase groups, respectively. Functions of genes surrounded in upper squares were known, whereas those of hypothetical and hypothetical conserved genes surrounded in below squares were poorly known or entirely unknown.

Table 2. Predicted localization of unknown genes by PSORT

Gene ID	Cell nucleus	Mitochondria				Plastid			Microbody (peroxisome)	Golgi body	ER		Cytoplasm	Plasma membrane
		Outer membrane	Intermembrane space	Inner membrane	Matrix space	Stroma	Thylakoid membrane	Thylakoid space			Membrane	Lumen		
CMB107C	0.300					**0.834**	0.337	0.337						
CMM173C						**0.530**	0.295	0.217					0.450	
CMN066C									0.300	0.300	0.200			**0.790**
CMN330C					0.100								**0.650**	
CMP288C	0.490				**0.670**	0.468								0.600
CMQ062C					0.100				0.300				0.450	
CMR358C						0.200	0.280	0.200	**0.653**					
CMS301C		0.600	0.600	0.600	**0.903**									
CMS407C			**0.783**		0.444				0.640				0.450	
CMT288C				0.383	0.697	**0.845**	0.441							
CMC115C	0.300								0.300		**0.850**			0.440
CMO005C	**0.600**					0.522	0.283		0.300					
CMR096C										0.460	**0.685**	0.100		0.640
CMI022C	**0.600**				0.100		0.100		0.300					
CMK023C	0.606		0.361	**0.860**										0.440
CML211C	0.300				0.100		0.100		0.300					
CMN061C	0.300				0.100		0.100		**0.387**					
CMS022C					0.100				0.300				**0.450**	

Psort server; http://psort.ims.u-tokyo.ac.jp/. The numbers show certainty value in Psort server. The numbers emphasized by bold show highest certainty value among searched organelles.

The changes in *ftsZ2-1* and *ftsZ2-2* transcription levels in the microarray analysis were consistent with those in the RT–PCR assay, supporting effective microarray detection of the change of gene expressions. The expression pattern of some genes, strongly induced in the S or G2-M phases, were analyzed by real-time RT–PCR assay. *ftsz2-2*, *pcna*, cyclin E2, cyclinA/CMI203C and *smc1*/CMI192C, which were assigned to the S-phase by microarray analysis, peaked in the late S-phase (Fig. 3C). *cmdnm2*, α-tubulin, histone H1/CMN183C, cyclinB/CMI227C, *cdk2*/CMH128C, *smc4*/CME029C, condensin kleisin I subunit/CMF069C, which were assigned to the G2-M phase, peaked in the G2- or M-phase (Fig. 3D). These results also supported the accuracy of microarray data in the present analysis.

In the division of the mitochondria and plastids, it is very important that *ftsZ1-1*, *ftsZ1-2*, *mda1*, *ftsZ2-1*, *ftsZ2-2* and *cmdnm2* have been identified in the S- or G2/M-phase groups. *mdv*1 and *caf4*, *mda1* homologue in *S. cerevisiae*, do not show periodic expression in the cell cycle (*Saccharomyces* Genome Database: http://www.yeastgenome.org/). Moreover, transcriptions of *ftsZ2* and *cmdnm2* homologues in *A. thaliana* are also not considered periodic in microarray analysis[11] because mitochondria in yeast and animal cells take various shapes and perform fission and fusion randomly. Similarly, synchronous division of mitochondria and plastids do not occur in higher plants. In *C. merolae*, as organelles divide synchronously, microarray analysis could be utilized to investigate organelle divisions. It was thought that gene expression profiles during the S- and G2-M-phases were important clues for finding novel genes related to organelle division.

3.4. *Candidates for novel genes involved in organelle division*

Unknown genes periodically expressed during the S- and G2-M-phase groups can be candidates for novel genes involved in cell nucleus and organelle division. The S and G2-M groups comprised a total of 158 genes including 93 known, 46 hypothetical and 19 hypothetical conserved genes. The hypothetical and hypothetical conserved genes were unknown, and searched for using BlastP, CDs: http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi and SMART: http://smart.embl-heidelberg.de/ (Table 1). The hypothetical genes were specific to *C. merolae*, the hypothetical conserved had a conserved domain that had significant hits on genes in other organisms. Therefore, the 19 hypothetical conserved genes were good candidates for novel organelle division genes because they are possible for analyzing organelle division in eukaryotes. We grouped candidate genes with known genes into the S, G2 and M phases to show a tendency of function and localization by the phase of expression peaks (Fig. 4). Genes involved in the cell nucleus were widely found among the S-, G2- and M-phase groups. The S-phase group included genes related to DNA replication, and G2- and M-phase groups included genes related to chromosome segregation and organization. The genes involved in mitochondria and plastids were mainly contained in the S group. This suggested that candidate genes contained in the S group were more related to division of organelles such as mitochondria and plastids. In the candidate, 11, 3 and 5 genes were contained in the S, G2 and M groups, respectively. Therefore, 11 genes in the S group might be more related to organelle division, while the 3 and 5 genes in the G2 and M groups might be involved in cell nucleus division.

Localization of the 19 candidate genes were predicted by Psort server: http://psort.ims.u-tokyo.ac.jp/ (Table 2). The predictions of the S genes were, as expected, occupied by mitochondria and plastids. The G2 genes were predicted to relate to the cell nucleus and ER. The M genes were involved in cell nucleus and microbody and mitochondria. These predictions were consistent with the order of organelle division in *C. merolae*, i.e. plastid, mitochondrion, finally cell nucleus and microbody (Fig. 1B). These results must provide a tool for the analyzing the genes for organelle division.

4. Availability

The microarray data for the cell cycle in *C. merolae* have been deposited in the National Center for Biotechnology Information, Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/. Accession number is GPL5399.

Supplementary data: Supplementary data are available online at www.dnaresearch.oxfordjournals.org.

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