Mitotic Karyotype of the Primitive Red Alga *Cyanidioschyzon merolae* 10D

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Summary It is important to understand how a single circular chromosome in the prokaryotic nucleus evolved into multiple linear chromosomes in the eukaryotic nucleus. In most eukaryotic cells that have >~15 Mbp of genomic DNA, chromosomes remain condensed through all the mitotic phases. Therefore, we observed nuclei of primitive organisms in which linear chromosomes had not been observed previously using conventional methods. Cells of the primitive red alga *Cyanidioschyzon merolae*, having a genome size of 16.5 Mbp, have been used to study the division of organelles, such as mitochondria, chloroplasts, and peroxisomes. However, morphologically condensed chromosomes have never been observed during mitotic metaphase. Recently, we demonstrated that plastid nuclei are swollen and change from a spherical to a ring shape after being subjected to the glutaraldehyde-fixed-drying method. Using a modified method, we visualized mitotic chromosomes in *C. merolae* cells. Chromosomal condensation occurred just after the chloroplast division when cells enter metaphase. Thus, chromosomal separation in *C. merolae* cells likely occurs in a manner similar to that of typical eukaryotic cells. However, mitotic condensed chromosomes were not observed in the primitive green alga *Medakamo hakoo*, having a genome size of 8 Mbp. Thus, the results support the use of *C. merolae* as a model for eukaryotic cell analyses.

Keywords *Cyanidioschyzon merolae*, Glutaraldehyde-fixed-drying method, *Medakamo hakoo*, Primitive red and green algae, SYBR Green 1.

Chromosomal condensation is a basic process involved in the doubling of genomic materials in eukaryotic cells. When and how the chromosomal DNA condensation in cell nuclei began in eukaryotes is still unknown. The bacterial origin of the protein that supports the condensation of chromosomal DNA in eukaryotic cell nuclei is also unknown. Eukaryotic host cells were born from archaeobacteria as determined by analyses of proteins related to cytokinesis. The endosomal sorting complexes required for transport (ESCRT) may represent the conserved machinery for eukaryotic cytokinesis that was inherited from the archaeal ancestor. In *Sulfolobus*, a thermophile archaeon, ESCRT homologs mediate cytokinesis from the early to final stages (Liu *et al.* 2017, Lindas *et al.* 2008, Samson *et al.* 2008, 2011). Most eukaryotes diverged from archaea that con-

tain ESCRT (Zaremba-Niedzwiedzka *et al.* 2017, Yagisawa *et al.* 2020). Therefore, nuclei of host eukaryotic cells are also those of archaea *Sulfolobus* sp. (Makarova *et al.* 2010, Yagisawa *et al.* 2020) (Fig. 1A). *Sulfolobus*-nuclear genomic DNA forms a single circle as in other bacteria (Chen *et al.* 2005); however, the nuclear genomic DNA is divided into more than a few condensed chromosomes at mitotic metaphase in eukaryotic cells after endosymbiosis. It is unclear how and why a single genomic DNA developed into multiple chromosomes for mitotic segregation during the evolutionary process of endosymbiosis.

Although linear condensed chromosomes are observed at mitotic or meiotic metaphase in a wide range of animals and plants, the origins of condensed chromosomes and the mechanism of chromosomal condensation remain unclear. In eukaryotes with small genomes, mitotic chromosomes are visible as condensed chromosomes. In *Saccharomyces cerevisiae*, having

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Fig. 1. A phylogenetic tree of eukaryotes based on archaeal *Sulfolobus* sp., and the cell cycles of *C. merolae*. (A) The phylogenetic tree indicating that the cells of most eukaryotes have evolved from organisms based on the archaeal *Sulfolobus* (enclosed by the dotted line). (B) The cells during *C. merolae* cell cycle were examined according to the previous GFD method. One mitotic cycle of *C. merolae* is approximately 22 h. The chloroplast nuclei have been revised from the central (CN-type) to the circular shape (CL-type) but metaphase chromosomes are invisible. Since the metaphase chromosomes may be visible at the upper area enclosed by the dotted line if the cells are squashed slightly, we have practically examined the chromosomes in the experiments. cn, cell nuclei; mn, mitochondrial nuclei; pn, plastid nuclei, M, mitotic phase; C, cytokinesis; G₁, gap 1 phase; S, DNA synthesis phase; G₂, gap 2 phase.

a genome size of 12.2Mbp (Goffeau *et al.* 1996) or 20Mbp (Kuroiwa *et al.* 2016), 16 individual linear or condensed chromosomes, which correspond to those obtained by genomic sequencing, are observed during meiotic metaphase (Kuroiwa *et al.* 1984, Goffeau *et al.* 1996) but not during somatic mitosis. In the fission yeast *Schizosaccharomyces pombe*, having a genome size of 13.8–20Mbp, there are three condensed chromosomes during both meiosis and mitosis (Smith *et al.* 1987, Wood *et al.* 2002).

In thermophilic Cyanidiophyceae (Rhodophyta) algae, using the nuclear genome size of *C. merolae* (16.5Mbp) as the standard, the cell-nuclear genome sizes of *M. hakoo*, *Cyanidium caldarium*, and *Galdieria sulphuraria* were determined as 9.2, 20.6 and 35.9Mbp, respectively (Toda *et al.* 1995, Kuroiwa *et al.* 1989, 2012, 2016). They are multiplied during binary fission, four endospore divisions, and 16 endospore divisions, respectively. Large *G. sulphuraria* cells increase owing to mitotic chromosomes (Kuroiwa *et al.* 1984). In the primitive alga *C. merolae*, Maruyama *et al.* (2007) examined centromeres, which are universally conserved functional units in eukaryotic linear chromosomes, and identified a centromeric histone, CENH3, and visualized centromere dynamics. However, little is known about the structure and dynamics of the centromere in lower photosynthetic eukaryotes. Immunofluorescence microscopy showed that CENH3 spots increased rapidly during S phase and more than 15 spots appeared during metaphase. Condensed metaphase chromosomes were not observed af-

ter 4',6-diamidino-2-phenylindole (DAPI) staining even when 15 CENH3 spots appeared. Furthermore, localization of CENH3 adjacent to the nuclear envelope implies an interplay between the kinetochore complex and the nuclear envelope. Thus, dynamic centromere reconstitution occurs during the cell cycle, and the chromosomes do not condense at metaphase (Maruyama *et al.* 2007).

The primitive green alga *M. hakoo* has a cell nucleus containing 9.2Mbp genomic DNA (Kuroiwa *et al.* 2016), which is the most small among the free-living eukaryotes evaluated to date. The primitive algae *C. merolae* and *M. hakoo* have small genome, and their condensed metaphase chromosomes have never been observed using conventional DNA staining. Recently, we showed that the centrally located (CN-type) plastid-nuclei (pt-nuclei) in *C. merolae* changed into circular located (CL-type) pt-nuclei after glutaraldehyde-fixed-drying (GFD) treatment, which induces the swelling of cell nuclei (Kuroiwa *et al.* 2020). Thus, the GFD method is useful for allowing the detailed observation of packed-DNA/chromosomes. We expected that with a light modification of GFD method, metaphase chromosomes would be seen (enclosed by dotted line in Fig. 1B) and investigated the morphology and dynamics of nuclei of *C. merolae* and *M. hakoo* cells using the improved GFD method. We could not identify all 20 individual chromosomes in metaphase *C. merolae* cells as expected by genome sequencing data (Matsuzaki *et al.* 2004) but visualize condensed chromosomes. On the other hand, condensed chromosomes were not observed in the prim-

itive green alga *M. hakoo*. Our result suggests that the difference in chromosomal dynamics between *C. merolae* and *M. hakoo* is important for understanding how eukaryotic cells acquired the mechanism responsible for chromosomal condensation.

Materials and methods

Cell culture

Clones of *C. merolae* 10D were isolated in our laboratory (Toda *et al.* 1995) and then maintained in 2×Allen's (Allen 1959) or Misumi–Kuroiwa medium at pH 1.8–2.3 and 42°C. The Misumi–Kuroiwa medium was prepared by diluting 1 mL of a commercial nutrient solution (Hyponex, N:P:K 10:8:8; Hyponex Japan, Osaka, Japan) to 1 L with distilled or tap water. The pH levels of both media were adjusted to pH 2.2 with 1 mL concentrated HCl. Cells with unsynchronized division and cultured under continuous light were used. The mitotic index was ~10%. The green alga *M. hakoo* cells were incubated in Misumi medium (Hyponex, N:P:K 10:8:8; Hyponex Japan) to 1 L with distilled or tap water (pH 7) at 22°C.

SYBR Green I staining, fluorescence microscopy and fluorescence intensity-related photon counting

To avoid the influence of the GC content on the DAPI-staining, *C. merolae* cells were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA), which has been used previously to stain pt-nuclei in living *Chlamydomonas reinhardtii* cells (Nishimura *et al.* 1998). After resuspending a pellet of *C. merolae* culture medium, a 3-μL aliquot of the solution was placed on a slide glass. Next, 3 μL of 1% (v/v) glutaraldehyde was added to the drop, followed by 3 μL SYBR Green I. Finally, another 3 μL of SYBR Green I (1 μg mL⁻¹) was added to the edge of the coverslip. After 30 min, the samples were observed by fluorescence microscopy.

For the GFD method, a 3-μL aliquot of *C. merolae* culture medium solution and 3 μL of 0.5% glutaraldehyde were placed on a slide glass and dried for 10 min. After the air-drying, SYBR Green I was then added to produce a final dilution of 1:1000. The cover glass was put on the samples and squashed slightly. The stained samples were observed using an Olympus BHS epifluorescence microscope (Nishimura *et al.* 1998). The fluorescent intensities of the small metaphase chromosomes were measured using a conventional video-intensified microscope photon-counting system (VIMPCS) (Kuroiwa *et al.* 1989, 2020, Toda *et al.* 1995).

Results

Figure 1A shows an evolutionary tree of Bacteria and Eukaryota. Host cells of eukaryotes, such as fungi, animalia and plantae, seem to have evolved from the archaeal *Sulfolobus* order based on recent gene com-

parisons involving cell division (Lindas *et al.* 2008, Yagisawa *et al.* 2020). Soon after the eukaryotic cells developed, it is believed that genome fragmentation and metaphase chromosomal condensation in nuclei occurred in eukaryotes having small genome sizes (enclosed by the dotted line in Fig. 1A), but these processes have not been clearly observed. Recently, the GFD method was developed to observe compact pt-nuclei in the primitive red alga *C. merolae*. We expected that metaphase chromosome-like structures would be seen using slightly improved the GFD method (enclosed by the dotted line in Fig. 1B).

Figure 2 shows asynchronously dividing and proliferating *C. merolae* cells in which the DNA was stained with SYBR Green I (SYG) after the GFD treatment. The images were taken at the same visual field as the phase contrast, SYG-stained, phase contrast—SYG and auto-fluorescence images of chloroplasts. Based on the shapes and sizes of the cell nuclei and chloroplasts as reported by conventional staining (Imoto *et al.* 2010, 2011), cells in each phase of the cell cycle were marked as G₁, S, G₂, M and C. When there was a plurality of cells at the same phase, they were indicated as M-1 to 4 and S-1 to 4. These genomes, which were organized in CN-type pt-nuclei, were produced from preexisting plastids by binary division. The chloroplast nuclei showed typical CL-type pt-nuclei after the GFD treatment. Figure 3 shows the enlarged cells that are judged to be in each cell phase, such as G₁, S, G₂, M and C in Fig. 2. The ring shape of the chloroplast nucleus was observed throughout the cell cycle (pn and thin arrow in Fig. 3). Chromosome-like clusters were visualized between daughter chloroplasts just after chloroplast division during mitotic metaphase (thick arrows in Fig. 3). To confirm the condensation of metaphase chromosomes, the metaphase cells were collected from an asynchronously dividing and proliferating cell population (Figs. 4A, 5A). Figure 4B shows the enlarged cells during mitotic metaphase and a lineup. Within all the cell nuclei, the chromosomes were condensed into several knobs, although all the individual chromosomes were not observed. The chromosomal condensation occurred just after the chloroplast division (Fig. 4B). In one metaphase cell that might have been severely squashed, small chromosomes were independently observed as granules between daughter chloroplasts (Fig. 4B). Furthermore, in a severely squashed metaphase cell, several condensed chromosomes were visualized between daughter chloroplasts (arrows in Fig. 5B). In addition to small granular chromosomes (small arrows), slightly larger rod-shaped chromosomes (long arrows) were also visualized. The fluorescence intensity of this small chromosome was the equivalent of 424 kbp (Table 1). This corresponds to the smallest of the 20 chromosomes as revealed by genome sequencing and suggests that the condensation of mitotic chromosomes occurred during metaphase in *C. merolae*. However,

Fig. 2. Images of an asynchronous *C. melolae* cell population at low magnification after SYBR Green 1 DNA staining. These images are of the same field. PC, phase contrast; SYG, SYBR Green 1 DNA staining; PC+SYG, combined PC and SYG; and AUT, autofluorescence of chloroplast-chlorophyll. Representative G_1 , S, G_2 , and M phase cells are marked. Almost all of the cells contain one chloroplast containing a circular (CL-type) plastid nuclei. M, mitotic phase; G_1 , gap 1 phase; S, DNA synthesis phase; G_2 , gap 2 phase. Scale bar = $5\mu\text{m}$.

Fig. 3. Enlarged images and model of *C. melolae* cells in G_1 , S, G_2 , M and C phases. All cells contain chloroplasts with circular (CL-type) plastid nuclei (thin arrow). PC, phase contrast; SYG, SYBR Green 1 DNA staining; PC+SYG, combined PC and SYG; and AUT, autofluorescence of chloroplast-chlorophyll. During mitotic metaphase just after chloroplast division, cell nuclei appear to change from a spherical shape to uneven, as if rod-shaped chromosomes were forming (thick arrows). G_1 , gap 1 phase; S, DNA synthesis phase; G_2 , gap 2 phase. MP, prophase; ME, metaphase; MA, anaphase; C, cytokinesis; cn, cell nucleus; mn, mitochondrial nucleus; pn, plastid nucleus. Scale bar = $1\mu\text{m}$.

Fig. 4. Selected and enlarged images of model *C. melolae* cells. (A) *C. melolae* cells at low magnification, and images of meta-phase *C. melolae* cells (M-1–4). Cell nuclei appear to form condense mitotic chromosomes after chlorplast divisions. PC, phase contrast; SYG, SYBR Green 1 DNA staining; PC+SYG, combined PC and SYG; and AUT, autofluorescence of chloroplast-chlorophyll. (B) In a severely squashed cell, a small chromosome separated from the large groups of chromosomes was observed (arrow in M-4). Scale bar=1 μ m.

Fig. 5. Images of model *C. melolae* cells. (A) DNA staining and chloroplast-chlorophyll images. PC, phase contrast; SYG, SYBR Green 1 DNA staining; PC+SYG, combined PC and SYG; and AUT, autofluorescence of chloroplast-chlorophyll. (B) Selected and enlarged images from (A). These images are of the same field. In a severely squashed cell, small (thin arrow) and large (thick arrow) chromosomes, were observed between daughter chloroplasts. M, metaphase. Scale bar=1 μ m.

Table 1. The genome size of the smallest chromosome in *C. merolae* cell nucleus estimated from fluorescence intensity (photon numbers).

	Whole cell nucleus	Smallest chromosome
SYBR Green 1 (photon)	467×10^3	12×10^3
Genome size (Mbp)	16.5	0.424

The photon counts in whole area per one second after staining with SYBR Green 1 according to the conventional method (Kuroiwa *et al.* 2004). Genome size of *C. merolae* cell nucleus is obtained by genome sequence data (Matsuzaki *et al.* 2004). The genome size of the smallest chromosome was estimated from the value of the whole nucleus. Counts were average of 3–4 samples including Fig. 5.

Fig. 6. Images of *M. hakoo* cells during metaphase (Me) and cytokinesis (Cy) after SYBR Green 1 DNA staining. PC, phase contrast; SYG, SYBR Green 1 DNA staining; AUT, chloroplast-chlorophyll; and PC+SYG+AUT, combined PC, SYG and AUT. The cell nuclei are spherical, and condensed mitotic chromosomes are not visible (thick arrow). Small chloroplast nuclei were observed in chloroplasts (arrows) of daughter cells. Scale bar=1 μ m.

using the GFD-method, the chromosomal condensation during mitosis was investigated in small green alga *M. hakoo* cells but was not found, although one small CN-type pt-nucleus was visualized in chloroplasts (arrows in Fig. 6).

Discussion

Cells of plant and animal species have diverse chromosomal numbers. In addition, even primitive eukaryotes with small genome sizes have multiple numbers of cell chromosomes (Wood *et al.* 2002, Matsuzaki *et al.* 2004, Nozaki *et al.* 2007). A single chromosome of *S. cerevisiae* could be made artificially from 16 individual chromosomes by successive end-to-end chromosomal fusions (Shao *et al.* 2018). However, it is unclear why and how ring-shaped chromosomal DNA was divided into several linear chromosomes during eukaryotic evolution. We succeeded in observing condensed meta-

phase chromosomes using the GFD method. The present results did not conflict with immunoblotting and immunofluorescence microscopy in which CENH3 spots increased rapidly during S phase, reaching more than 10 spots during metaphase (Maruyama *et al.* 2007, Fujiwara *et al.* 2013). Each spot must correspond to a centromere of an individual chromosome. If we revise the GFD method, then 20 chromosomes may be visible. Based on condensed mitotic metaphase chromosomes, *C. merolae* develops into *G. sulphularia*.

However, condensed mitotic metaphase chromosomes were not visualized in the primitive green alga *M. hakoo* cells having 9.2Mbp genomic DNA (Kuroiwa *et al.* 2016). Similarly, condensed mitotic chromosomes were not observed in the cells of the randomly proliferating primitive small alga *Ostreococcus tauri* (Kuroiwa *et al.* 2004). This suggests that in eukaryotes having such small genome sizes less than 10Mbp, mitotic chromosomes may not be condense and be visible. The chloroplasts of *O. tauri* contain typical small chloroplast nuclei like those of *M. hakoo*.

The *C. merolae* cells are useful materials for elucidating the dynamics of cells and organelles. Comparative analyses of proteins responsible for chromatin architectural isoforms between *C. merolae* and *M. hakoo* are essential for identifying key factors involved in chromosomal condensation. *C. merolae* cells have the following features that make them eukaryotic models for analyses (Kuroiwa *et al.* 2018): 1) *C. merolae* cells are the smallest eukaryotic cells, being $\sim 2\mu$ m; 2) The cells contain a minimum set of double membraneous organelles: one cell nucleus, one mitochondrion, and one plastid. The order of these organelles' divisions has been determined; 3) It is possible to highly synchronize cell and organelle divisions using a 24-h light/dark cycle; 4) It is the only eukaryotic organism having a 100% sequenced genome and 5) It is amenable to many types of genetic techniques as multi-omics analyses, various gene disruption techniques and inducible and repressible gene expression system (Miyagishima and Fujiwara 2020).

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