Paternal inheritance of mitochondrial DNA in *Chlamydomonas reinhardtii* and dynamic morphological changes in mitochondria, mitochondrial nucleoids, and the cell nucleus during its life cycle

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Summary: To determine the exact mode of inheritance of mitochondrial DNA (mtDNA) in *Chlamydomonas reinhardtii*, suitable markers to determine the origin of parental mitochondrial genes are necessary. For this goal, the difference in mtDNA between a *C. reinhardtii* strain and a *C. smithii* strain was used. These two species were serially backcrossed to ensure matching of the background of their nuclear genomes. The results showed strict paternal inheritance of mtDNA. Next, the fate of mt^+ (female) and mt^- (male) mtDNA was followed using three methods: quantitative real-time PCR, Southern blotting hybridization, and fluorescence *in situ* hybridization (FISH). The disappearance of mt^+ mtDNA was observed at the beginning of meiosis. In the mutant *bp31*, in which the maternal inheritance of chloroplast DNA (cpDNA) is disrupted, the paternal inheritance of mtDNA was also found to be disrupted, indicating commonality between cpDNA and mtDNA in part of the process leading to uniparental inheritance. Mitochondrial nucleoids and mitochondria were observed in living vegetative cells. Organellar nucleoids were stained with SYBR Green I, while mitochondria were stained with DiOC₆. During the cell cycle, mitochondrial nucleoids in living cells were granular, and mitochondria formed tangled threads. When mature zygotes were exposed to the light, meiosis began. Living zygotes with thick walls was also stained by SYBR Green I and DiOC₆. At the diakinesis stage of meiosis, the total number of chromosomes was 18. When mature zygotes were exposed to the light, granular mitochondria began to form short thread-like structures and then assembled around the cell nucleus. Once assembled, the mitochondria began to scatter in the opposite hemisphere, forming long and tangled thread-like structures. Mitochondrial nucleoids were usually granular, but some of them developed bead-like structures, probably by dividing. The results from the molecular biochemical experiments suggested that mt^+ mitochondrial nucleoids disappear at the stage of assembly and the scattering of mitochondria. Then, the first and second nuclear divisions proceed, producing tetrads.

Key words: *Chlamydomonas reinhardtii*, DiOC₆, mitochondrial DNA, paternal inheritance, SYBR Green I

INTRODUCTION

Mendel's laws were established in 1866 and non-Mendelian inheritance was discovered in 1909 by Correns and Baur. Correns (1909) followed the inheritance of green, variegated, and white patterns of leaves in reciprocal crosses of *Mirabilis jalapa*. He found that the color patterns are passed on to the next generation from only the maternal parent. In the same year, Baur (1909) found that the green and variegated patterns of the leaves in *Pelargonium* are inherited biparentally. Since then, studies of cytoplasmic organelle inheritance have established that most eukaryotes inherit chloroplasts and mitochondria maternally (Tilney-Bassett 1978, Kuroiwa 1991, 2010, Gillham 1994).

The uniparental inheritance of mitochondria and chloroplasts has been explained by the difference in size between female and male gametes as female gametes contain a large number of organelles and male gametes contain only a few (Gyllenstein et al. 1991). However, the process leading to this inheritance is not simple because uniparental inheritance occurs even in isogamous unicellular green algae, *Chlamydomonas reinhardtii* (Sager 1954). Sager isolated the UV-induced mutant *sr2* that is resistant to streptomycin. When the *sr2* strain and wild-type cells were crossed in various ways, resistance in the meiotic progeny was inherited from the maternal parent (maternal inheritance). The progeny also inherited chloroplast genes from the mt^- parent at a frequency of 1–10% (paternal

inheritance). Biparental inheritance, involving the inheritance of both parental genes, also occurred at a low frequency. These maternal, biparental, and paternal types of inheritance involve inheritance that is non-Mendelian (Gillham 1994). Factors that undergo Mendelian inheritance are located on the chromosomes (Sutton 1902), while those that undergo non-Mendelian inheritance are thought to be located in the cytoplasm. Ris and Plaut (1962) revealed the presence of DNA in the chloroplasts by microscopy. This chloroplast DNA (cpDNA) was first isolated from *C. reinhardtii* cells by Sager and Ishida (1963) by centrifugation using CsCl. Liu et al. (1989) subsequently identified the *sr2* mutation in the chloroplast genome.

C. reinhardtii has chloroplasts and mitochondria. The chloroplast nucleoids contain cpDNA, which is organized into 5–10 DNA-protein complexes (Kuroiwa et al. 1981, 1982). The mitochondrial DNA (mtDNA) is a 15.8-kbp linear molecule (Grant and Chiang 1980, Ryan et al. 1978). mtDNA is organized into mitochondrial nucleoids, of which approximately 20 are contained within a cell (Nishimura et al. 1998). Via the process of fertilization, mt^+ (female) and mt^- (male) gametes form diploid zygotes. After the incubation of these zygotes in the dark for about one week, meiosis begins after exposure to light and four haploid daughter cells are usually produced (Sager 1954).

Sager and Lane (1972) reported the results of a CsCl centrifugation experiment using *C. reinhardtii*. Only male

cpDNA disappeared in zygotes 6–24 h after zygote formation. Kuroiwa et al. (1982) stained cells with the DNA-specific fluorochrome 4',6-diamidino-2-phenylindole (DAPI) and presented the first cytological evidence that male chloroplast nucleoids disappeared completely during the first 40–50 min after fusion. Kuroiwa (1985) subsequently proposed an active digestion hypothesis to explain this uniparental inheritance. This hypothesis has been applied to both mitochondrial and chloroplast inheritance in plants (Kuroiwa 2010).

Reported research on the inheritance of mtDNA in *Chlamydomonas*

Boynton et al. (1987) performed interspecific crosses of *C. smithii* mt⁺ and *C. reinhardtii* mt⁻ strains and analyzed the inheritance pattern in progeny using the physical differences in restriction fragment patterns of mtDNA. All of the meiotic progeny showed paternal inheritance.

Matagne et al. (1989) used acriflavin as a mutagen and isolated two respiration-defective mutants, *dum-1* and *dum-2*, having a 1.5-kbp deletion in the mitochondrial *cob* gene. They grew in the light, but did not divide under heterotrophic conditions in the dark. When these mt⁻ mutants were crossed with wild-type mt⁺ cells, the germination rate was low (20–50%) and the rate of paternal inheritance of mtDNA was about 85%. In the reciprocal cross, this inheritance rate recovered.

Colin et al. (1995) isolated two additional mutants, *dum-18* and *dum-19*, carrying frameshift mutations in *cox1*. They were unable to grow under heterotrophic conditions. These mutants showed the paternal inheritance of mitochondria.

Seven more mutants carrying a deletion or other mutation in the *cob* or *cox1* gene were isolated after treatment with acriflavine or ethidium bromide. These mutants were completely or almost completely unable to grow under heterotrophic (darkness + acetate) conditions (Dorthu et al. 1992). Paternal inheritance occurred when mt⁻ wild-type and mt⁺ mutant cells were crossed. However, upon reciprocal crossing, this paternal inheritance was disrupted.

Bennoun et al. (1991) isolated three mutants resistant to mucidin and myxothiazol. Diploid cells resistant to these inhibitors were first isolated and then crossed with wild-type haploid mt⁺ cells for haploidization. Finally, the isolated mutant *MUD2* showed strict paternal inheritance.

The above experiments raise questions about whether the process of paternal inheritance proceeds accurately in crosses between strains from different species (Boynton et al. 1987, Beckers et al. 1991), in crosses using respiration-deficient mutants (Matagne et al. 1989, Dorthu et al. 1992, Colin et al. 1995), or in haploid cells derived from artificially produced diploid cells (Bennoun et al. 1991).

Paternal inheritance of mitochondrial DNA in *C. reinhardtii*

To answer these questions, it is necessary to identify a suitable marker gene in the mitochondrial genome to determine parental origin. Nakamura et al. (2003) used the *cob-1* intron gene as a marker. *C. smithii* mitochondria contain this gene, while *C.*

reinhardtii mitochondria do not. These researchers successively performed interspecific backcrossing between strains of these two species in order to match their nuclear backgrounds and examined the inheritance of mtDNA at each backcross (Fig. 1). The mating efficiency increased from less than 1% to about 75% at the fourth step of backcrossing to *C. reinhardtii*. The frequency of complete tetrad production increased from less than 10% to almost 100% at the third step. An analysis of the progeny revealed that cpDNA was inherited maternally, while mtDNA was inherited strictly paternally.

Period of disappearance of maternal mtDNA in zygotes

Next, changes in the total amounts of mtDNA and cpDNA were tracked using real-time PCR and quantitative Southern blotting hybridization. As shown in Fig. 2, two periods, at about 6 h after zygote formation and just before the first meiotic division, were the candidates for the timing when maternal mtDNA disappears. To further analyze this issue, the fates of mt⁺ and mt⁻ parental *cox1* genes of mtDNA were followed. To obtain accurate data, a single zygote was picked up using a manipulator under a microscope and subjected to analysis. Between 6 and 12 h after the mature zygotes had been exposed to light, all mt⁺ parental (female) mtDNA corresponding to the lower band in the lane of *Xba* I cut disappeared (Fig. 3). This time point was confirmed by FISH analysis (Fig. 4). The results show that the female mtDNA disappeared selectively between 6 and 18 h after exposure of the mature zygotes to light, that is, just before the first meiotic cell division.

Gene expression at the early stage of germination

When female and male gametes are mixed, they fuse to form diploid zygotes. During the first 24 h after fusion, the disappearance of flagella, selective elimination of paternal chloroplast DNA, nuclear membrane fusion, and chloroplast membrane fusion occur. The regulatory mechanisms involved in the maternal inheritance of cpDNA have been partially studied in terms of the transcription products of zygote-specific genes (Armbrust et al. 1993, Kuriyama et al. 1999, Uchida et al. 1999, Kubo et al. 2001). However, there are no reports on the transcription products at the early stage of germination of mature zygotes of *C. reinhardtii*.

Analysis using a next-generation sequencer (454 GS Junior) and quantitative real-time PCR identified genes expressed after exposure of mature zygotes to light (Aoyama et al. 2014). At 6 h of light exposure, the levels of expression of genes encoding antenna proteins involved in photosynthesis and two methionine metabolism-related genes were increased compared with those at 1 h. Meanwhile, at 1 h, the expression levels of three uncharacterized genes containing an antibiotic biosynthesis monooxygenase domain and an HSP20/alpha crystallin family protein increased. Further elucidation is necessary to reveal their involvement in mtDNA inheritance.

Figure 1 Inheritance of mitochondrial and chloroplast DNA during serial backcrosses. (A) Inheritance of mtDNA. (B) Inheritance of cpDNA. T1 to T4, tetrad numbers. Asterisk, progeny used for the following backcross. #, exceptional paternal transmission. (reproduced from Nakamura et al. 2003)

Figure 2 Changes in quantities of total mtDNA and total cpDNA relative to the quantity of nuclear DNA in zygotes. Quantities of mtDNA, cpDNA, and nuclear DNA were measured by real-time PCR. (A) Relative quantities of total mtDNA and total cpDNA at 24 h and eight days after gamete fusion. Inset, quantitative Southern blot hybridization of total mtDNA (*cox1*), total cpDNA (*rbcl*), and nuclear DNA (*nit1*). (B) Relative quantities of total mtDNA (solid line) and total cpDNA (dashed line) at 24 h after exposure of mature zygotes to light. (reproduced from Aoyama et al. 2006).

Figure 3 Disappearance of female mtDNA in zygotes. Nested PCR product of the *cox1* gene of isolated single zygotes was digested by the restriction enzyme *XbaI*. Single zygotes were isolated 24 h after mixing of the two gametes and after exposure of mature zygotes to light. 1–5, number of single zygotes. 24 h, 24 h after gamete fusion. 0, 3, 6, 12, 18; Hours after exposure of mature zygotes to light. (reproduced from Aoyama et al. 2006).

Figure 4 FISH analysis of the *cox1* and *cob-1* intron genes in mtDNA and DAPI staining of mitochondrial nucleoids. The mt⁻ gamete (*cox1* gene) and the mt⁺ gamete (*cox1* and *cob-1* intron genes) were fused. The rhodamine red signal indicates the *cox1* gene and the FITC green signal indicates the *cob-1* intron gene. (A–D) A zygote 6 h after gamete fusion. (E–H) A mature zygote 6 h after light exposure. (I–L) A mature zygote 18 h after light exposure. (A, E, I) Rhodamine signal from the *cox1* gene. (B, F, J) FITC signal from the *cob-1* intron gene. (C) Merged image of A and B. (G) Merged image of E and F. (K) Merged image of I and J. Overlap of the rhodamine (red) and FITC (green) signals produced a yellow color. (D) DAPI staining of the same cell used in A and B. (H) DAPI staining of the same cell used in E and F. (L) DAPI staining of the same cell used in I and J. Note that the FITC signal derived from mt⁺ parental mtDNA disappeared between 6 and 18 h after light exposure. Bar = 5 μ m. (reproduced from Aoyama et al. 2006).

Mutant *bp31* (biparental 31) with disruption of the maternal inheritance of cpDNA

Nishimura et al. (2012) isolated a mutant *bp31*, having a deletion of about 60 kb on chromosome 2, in which the uniparental inheritance of cpDNA and mtDNA is disturbed (Fig. 5). This mutant phenotype was rescued by co-transformation with the *GSP1* and inositol monophosphatase-like 1 genes (Table 1). This evidence shows that there is commonality between cpDNA and mtDNA in the biochemical process involved in the selective disappearance leading to uniparental inheritance.

Observation of mitochondria and mitochondrial nucleoids of *C. reinhardtii* by fluorescence microscopy

Ehara et al. (1995) observed mitochondria in *C. reinhardtii* cells by electron microscopy. They also observed these mitochondria by epifluorescence microscopy after staining with a mitochondrial membrane-binding fluorescent dye, dimethylaminostyrylmethylpyridiniumiodide. The mitochondria

changed dynamically from granules to disk-shaped structures during the cell cycle. The morphology was classified into five types. The morphology of chloroplast nucleoids was also observed. The nucleoids were stained with DAPI after fixing with glutaraldehyde. The shapes of the chloroplast nucleoids changed from granular to thread-like, or in the opposite direction, but mitochondrial nucleoids were not observed.

In 1998, Nishimura et al. (1998) first stained cells with the DNA-specific fluorochrome SYBR Green I. They found that each cell contained about 60 mitochondrial granular nucleoids. SYBR Green I is better than DAPI for visualizing mitochondrial nucleoids because they are smaller than those of chloroplasts. Hiramatsu et al. (2006) stained the nucleoids of living cells cultured under a 12:12 light-dark cycle with SYBR Green I and the mitochondria with 3,3-dihexyloxacarbocyanine iodide 6 (DiOC₆) (Fig. 6). A *mtG33* mutant containing one large chloroplast nucleoid throughout the cell cycle (Misumi et al. 1999, 2001) was used to distinguish between the mitochondrial

Figure 5 The *bp31* mutant is defective in paternal inheritance of mtDNA. Wild-type *mt⁺* and *bp31 mt⁺* gametes were crossed with myxothiazol-resistant gametes. Analysis was performed using nested PCR. (A) Cells at 30 min after fusion. (B) Fused cells incubated in the dark for one week and then in the light for 8 h. Note that crossing with wild-type gametes showed only *mt⁻* mtDNA, indicating paternal inheritance, but crossing with *bp31* showed *mt⁺* and *mt⁻* parental mtDNA, indicating biparental inheritance (Nishimura et al. 2012).

Table 1 Restored paternal inheritance of mtDNA in tetrad progeny. *bp31+GSP1gu+INM1g* (#4, 13). Cotransformed strains of *bp31* mutants with the two genes *GSP1* and *INM1*. MT, Mating type. mtDNA, Presence of the *MnlI* site, indicating paternal inheritance of mtDNA from the *mt⁻* parent. (reproduced from Nishimura et al. 2012).

<i>bp31+ GSP1gu+ INM1g#4 mt+ X myx mt-</i>				
Strain	Tetrad	Progeny	MT	mtDNA
#4	1	1	-	mt-
		2	+	mt-
		3	-	mt-
		4	+	mt-
	2	1	-	mt-
		2	+	mt-
		3	-	mt-
		4	+	mt-
	3	1	-	mt-
		2	-	mt-
		3	+	mt-
		4	+	mt-
	4	1	+	mt-
		2	-	mt-
		3	+	mt-
		4	-	mt-
	5	1	+	mt-
		2	+	mt-
		3	-	mt-

<i>bp31+ GSP1gu+ INM1g#13 mt+ X myx mt-</i>				
Strain	Tetrad	Progeny	MT	mtDNA
#13	1	1	+	mt-
		2	+	mt-
		3	-	mt-
		4	-	mt-
	2	1	-	mt-
		2	-	mt-
	3	1	+	mt-
		2	-	mt-
	4	1	+	mt-
		2	+	mt-
		3	-	mt-
	5	1	+	mt-
		2	-	mt-
	6	1	-	mt-
		2	-	mt-
		3	+	mt-
		4	+	mt-

and chloroplast nucleoids (Fig. 7). At the start of the light period during the cell cycle, the new daughter cells contained about 30 mitochondrial and 10 chloroplast granular nucleoids, with diameters of 0.1 and 0.2 μm , respectively. They were dispersed across the entire cell. With time, both chloroplast and

Figure 6 Fluorescence micrographs of wild-type cells stained with SYBR Green I (A) or DiOC₆ (B). Cells were cultured under a 12:12 light-dark cycle. N, cell nucleus. Arrows, mitochondrial nucleoids. Double arrows, thread-like or bead-like structures of mt nucleoids. Arrowheads, chloroplast nucleoids. Double arrowheads, thread-like or bead-like structures of cp nucleoids. Bar = 5 μm . (reproduced from Hiramatsu et al. 2006).

Figure 7 Fluorescence micrographs of *mocG33* cells stained with SYBR Green I (A) or DiOC₆ (B). Cells were cultured under a 12:12 light-dark cycle. N, cell nucleus. Arrows, mitochondrial nucleoids. Double arrows, bead-like structures of mt nucleoids. Arrowheads, chloroplast nucleoids. Bar = 5 μm . (reproduced from Hiramatsu et al. 2006).

mitochondrial nucleoids continuously increased in number, but they then became smaller due to a decrease in the DNA content in each particle and became indistinguishable. They were usually granular, but some nucleoids formed short thread-like or bead-like structures, probably by dividing (double arrows in Fig. 7A). In the middle of the dark period, the nucleoids again formed short thread-like or bead-like structures, probably by fusing, and decreased in number. The mitochondria appeared as tangled sinuous structures that extended throughout the cytoplasm and resembled a single large mitochondrion.

Upon the maturation of zygotes, their walls became thick and solid, and they changed from green to dark black. Because of their thick walls, the zygote interior had been analyzed only using an electron microscope (Triemer & Brown 1976, Storms & Hastings 1977). Storms & Hastings (1977) suggested that the haploid chromosome number is 18 or 20. Before observing mitochondrial nucleoids in living cells, the chromosomes should be observed. Therefore, Aoyama et al. (2008) applied SYBR Green I staining to zygotes and counted a total of 18 chromosomes (Fig. 8).

Figure 8 Chromosomes at metaphase and anaphase (A-E) and at meiosis II (F). Total chromosomes spread on a glass slide (G, H). Chromosomes were stained with SYBR Green I. (reproduced from Aoyama et al. 2008).

Aoyama et al. (2006, 2009) succeeded in visualizing mitochondrial nucleoids. They stained living mature zygotes with SYBR Green I for nucleoids and with DiOC₆ for mitochondria and then traced them under an epifluorescence microscope after the exposure of mature zygotes to light (Fig. 9). Because discrimination between SYBR Green I-stained mitochondrial nucleoids and chloroplast nucleoids was impossible, as shown in Fig. 9, the zygotes were stained with SYBR Green I and DiOC₆ simultaneously. Just after light exposure, the mitochondria were granular and scattered near the surface of the zygote (Fig. 9A-F). At about 3 h after light exposure, the granular mitochondria had transformed into short, tubular structures (Fig. 9G, H). The

tubular mitochondria contained mitochondrial nucleoids (Fig. 9I). Large plastid nucleoids were observed inside the zygotes and could be discriminated from mitochondrial nucleoids by their size. At about 7 h, the tubular mitochondria formed branch-like structures and then scattered throughout the cell (Fig. 9M–R), which contained nucleoids (Fig. 9O). At about 11 h, the scattered mitochondria and most mitochondrial nucleoids assembled around the cell nucleus. Once they had assembled there, the tubular mitochondria radiated into the other hemisphere through the surface layer at about 14 h. The mitochondrial nucleoids were then distributed in the zygote. At about 16 and 20 h, the first and second cell divisions produced four daughter cells. The mitochondria in these daughter cells were mesh-like. Mitochondrial and chloroplast granular nucleoids were distributed throughout the whole zygote. The smaller nucleoids were mitochondrial and the larger ones were of chloroplasts.

Real-time PCR analysis of the changes in the total mtDNA

amount, restriction enzyme digestion, and FISH analyses previously showed the significant disappearance of female mtDNA just before the first meiotic division (Aoyama et al. 2006). This period corresponds to Fig. 9M–X. Morphological changes leading to the preferential disappearance of mt^+ mitochondrial nucleoids should be observed in this period. Because of the numerous tiny mitochondrial and plastid nucleoids, and the vigorous movements associated with the assembly and radiation of nucleoids just before meiosis, no significant disappearance of mitochondrial nucleoids was observed. Recently, Aoyama et al. (2015) observed mitochondria and their nucleoids by fluorescent double staining in living cells using a confocal fluorescence microscope and constructed a 3D model. Time lapse observation using this approach should provide definitive information on this issue. The above observations are summarized in Fig. 10.

Figure 9 Fluorescence micrographs of mature zygotes after light exposure. Fluorescence micrographs of zygotes stained with DiOC₆, SYBR Green I, and both SYBR Green I and DiOC₆ (double staining) simultaneously. Surface, images focused on the surface layer of a single zygote. Inside, images focused on the inside of a single zygote. Asterisks, cell nucleus. Arrows, organellar nucleoids. Arrowheads, mitochondrial nucleoids. Bar = 5 μ m. (reproduced from Aoyama et al. 2009).

Figure 10 Timetable of the main events during the period between zygote formation and tetrad formation. (A) At about 1 h, *mt⁻* parental chloroplast nucleoids disappeared. At about 2 h, the two cell nuclei fused. Mitochondrial nucleoids and mitochondria were scattered throughout the zygote. Numbers, hours after mixing male and female gametes. (B) When mature zygotes were exposed to light, *mt⁺* parental mtDNA selectively disappeared just before mtDNA synthesis. Some nucleoids subsequently began to form short thread-like structures. Mitochondria probably synthesize their DNA during this period, divide, and then increase in number. Then, the nucleoids become particles again. Once mitochondria and mitochondrial nucleoids had assembled around the cell nucleus, they dispersed radially to the other hemisphere. The first and second cell divisions subsequently occurred. Numbers, hours after exposure of mature zygotes to light. (reproduced from Aoyama et al. 2006).

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