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Chloroplast nucleoids in large number and large DNA amount with regard to maternal inheritance in *Chlamydomonas reinhardtii*

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Summary. We studied the maternal chloroplast inheritance of *Chlamydomonas reinhardtii* by epifluorescence microscopy after staining with DNA specific fluorochrome DAPI and by genetic methods, using wild type cells and cells containing previously isolated mutation of cond-1 and cond-2. Wild type cells contained about 7 chloroplast (cp) nucleoids, while mutants, cond-1(+) and cond-2(+), contained about 14 and 23 cp nucleoids, respectively, after one week culture on agar plates. The total cpDNA contents were almost proportional to the numbers of cp nucleoids. When cells containing cond-1 or cond-2 mutation were used as a parental source to cross with wild type cells of the other parent, preferential digestion of cp nucleoids from male parent (mt^-) origin occurred in the zygotes, although the frequencies of the digestion were slightly lower than that in the zygotes from the cross between wild type cells. Western blot analysis of the protein of *zys1B* gene, which has been found related to preferential digestion of mt^- origin cp-nucleoids DNA, showed that a high amount of this protein was detected with the initiation of preferential digestion of mt^- cp nucleoids and disappeared with the completion of the digestion. Cp genetic markers for antibiotic resistance were maternally inherited in all crosses. These results showed that although the preferential digestion of cp nucleoids consisting of large number and large cpDNA amount requires a slightly longer period to complete, this high ploidy of the cp nucleoids does not disturb maternal inheritance.

Keywords: Chloroplast nucleoid number; Chloroplast DNA amount; Preferential digestion; Maternal inheritance; *Chlamydomonas reinhardtii*.

Introduction

The heterothallic isogamous green alga *Chlamydomonas reinhardtii* has been used as a model organism for the study of maternal inheritance of chloroplast genes (Sager 1954, Gillham 1974). With high resolution epifluorescence microscopy on the behavior of chloroplast (cp) nucleoids, Kuroiwa et al. (1982) showed that in the zygotes of *C. reinhardtii* the cp nucleoids from male parent (mating type minus, mt^-) disappear within 40–50 min after mating, while those from female parent (mating type plus, mt^+) persist. Preferential digestion of the mt^- cp nucleoids in young zygotes has also been reported in *C. moewusii* (Coleman and Maguire 1983), *Dictyosphaeria cavernosa* and *Acetabularia calyculus* (Kuroiwa et al. 1985). Thus, the preferential digestion of chloroplast nucleoids was suggested as the primary reason for the maternal inheritance of chloroplast genes (Kuroiwa et al. 1982, Kuroiwa 1985).

Biochemical and physiological studies with *C. reinhardtii* has revealed that soon after zygote formation, specific mRNAs are synthesized in the cell nucleus of mt^+ cells, which code for proteins that directly or indirectly activate Ca^{2+} dependent nuclease to digest mt^- cp nucleoids in zygotes (Kuroiwa 1985). A zygote specific gene coding 20 kDa protein, *zys1B*, was isolated by Uchida et al. (1993). The antibody, raised against its fusion protein with MBS, recognized a 22 kDa protein in Western blotting. 22 kDa protein was detected only in the stage of the occurrence of preferential digestion, as was the transcript of *zys1B* gene. Analysis of this protein by physiological treatments to block preferential digestion, revealed that *zys1B* gene tightly relates to preferential digestion of

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male origin cp-nucleoids DNA (Uchida et al. unpubl. data). Several other zygote specific genes were identified (Ferris and Goodenough 1987, Wegener and Beck 1991, Matters and Goodenough 1992, Armbrust et al. 1993). Further investigations elucidated the roles of their genes.

To understand the preliminal feature of maternal inheritance, diploid cells having a higher amount of cpDNA molecules were crossed with normal haploid cells. The proportion of zygotes transmitting the allele from a diploid parent to its meiotic progeny increased (Matagne and Mathieu 1983). When the parent cells are treated with 5-fluorodeoxyuridine (FUdR) to reduce cpDNA and the number of cp nucleoids and mated with untreated cells of the opposite mating type, the proportion of zygotes transmitting cp alleles from the FUdR-treated parent to their meiotic progeny dramatically decreased (Wurtz et al. 1977, Matagne and Beckers 1983). However, because of artificial diploid cells or physiological inhibitor treatment, it is not clear whether chloroplast DNA amount and the number of cp nucleoids disturb maternal inheritance.

In the present experiment, we studied whether higher number and higher DNA amount of cp nucleoids disturb the process of the preferential digestion and maternal inheritance. Changes in amount of Zys1B protein in zygotes during the process of preferential digestion were also analyzed. The results showed that preferential digestion occurred in all crosses with the appearance of Zys1B protein, although a slightly longer period was needed to complete the digestion of cp nucleoids in high number. The cp genes were maternally inherited.

Material and methods

Wild type cells and other cells used in the present experiments are listed in Table 1. Mutants, cond-1 and cond-2, were isolated previously (Nakamura et al. 1994). Mutants, cond-1(–) and cond-2(–), were progeny having mutant phenotype after the cross of cond-1(+) and cond-2(+) cells with wild type mt^- cells, respectively. Cells of cc-118 and cc-119 were obtained from the *Chlamydomonas* Genetic Center, Department of Zoology, Duke University, Durham, NC. Cells were cultured on agar culture plates as described previously (Nakamura et al. 1986).

For gametogenesis, cells which had been cultured for one week were harvested from the agar plates, suspended in a nitrogen-free medium, and then incubated for 8 h with gentle shaking under constant illumination of 5000 lux. Zygotes were formed by mixing approximately equal number of mt^+ and mt^- gametes.

To observe the preferential digestion of mt^- cp nucleoids, the zygotes were fixed and stained with a DNA specific fluorochrome, DAPI as described previously (Kuroiwa et al. 1982). Frequency of the preferential digestion was calculated as follows:

Preferential digestion (%) = 100 × (number of zygotes lacking mt^- cp nucleoids)/(total number of zygotes observed).

Total DNA amount was measured by VIMPCS (Hamamatsu Photonics Ltd., Hamamatsu, Japan) including a high-sensitivity videocamera SIT, which was connected to the Olympus epifluorescence microscope, as described previously (Kuroiwa and Nakamura 1986). Prior to the staining, cells were treated with 80% acetone solution to eliminate chlorophyll.

Preparation of anti-Zys1B protein antibody and Western blot analysis were done as described in a paper (Uchida et al. unpubl). A 609 bp Bsp MI fragment of cDNA clone pZS102-69 (Uchida et al. 1993) was cloned into the Stu I site of expression vector pMALc (England Biolabs, Beverly, MA, U.S.A.), and used to transformed *E. coli*, XL1 blue. The fusion protein was purified and used to generate polyclonal antibodies in a rabbit. For protein analysis of *Chlamydomonas*, 100 ml of 1×10^6 cells per ml were mixed to mating, collected, frozen with liquid nitrogen, and suspended in 1 ml of cold extraction buffer. The cells were disrupted, and spun for 15 min at 9000 **g**. Protein content of the supernatant was measured by Bio-Rad Protein Assay Kit. The supernatant was analyzed by SDS-PAGE on 15% gel. 40 μg protein was applied in each lane. The protein was transferred to PVDF membrane (Bio-Rad) using a mini-trans-blot (Bio-Rad). Western blot analysis was performed using an immune-blot assay kit (Bio-Rad). The PVDF membrane was blocked for 1 to 3 h, washed, then incubated over night with anti-Zys1B protein antibody. After washing two times, the membrane was incubated for 2 h with goat anti-rabbit IgG (H+L) AP conjugate, washed three times, then incubated for 20 min in AP color reagents, and again washed.

Genetic analysis was done using streptomycin resistant (str) and kanamycin resistant (km) strains as markers for the transmission of chloroplast genes, as described previously (Nakamura et al. 1991).

Table 1. Various cell types of *Chlamydomonas reinhardtii* used in the present experiments

Cells	Mating types	Chloroplast markers[a]
W (wild, 137c)	mt^+	no
W (wild, 137c)	mt^-	no
Cond-1(+)[b]	mt^+	Km
Cond-1(–)	mt^-	Km
Cond-2(+)[b]	mt^+	Km
Cond-2(–)	mt^-	Km
Cc-118	mt^+	Str
Cc-119	mt^-	Str
Km-2	mt^-	Km

[a] *Km* kanamycin resistant; *Str* streptomycin resistant

[b] Previously isolated mutants of cond-1 and cond-2, respectively (Nakamura et al. 1994)

Results

Each wild type gamete contains a cell nucleus and a cup-shaped chloroplast. Under epifluorescence microscope, each gamete was observed to contain a cell nucleus and small spherical chloroplast (cp) nucle-

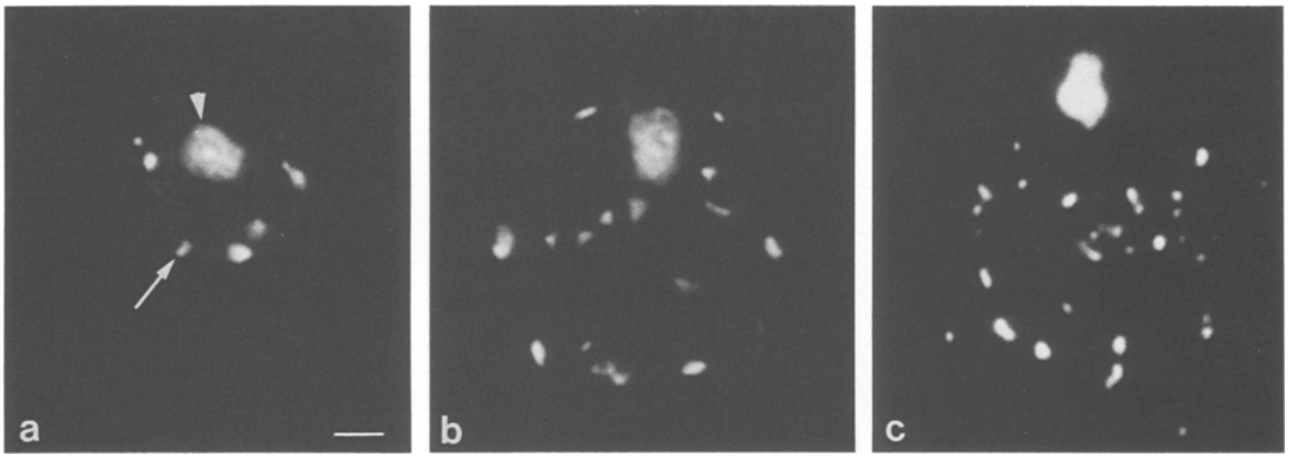

Fig. 1 a–c. Epifluorescence photomicrographs of cp nucleoids and cell nuclei in gametes of wild type, cond-1(+) and cond-2(+) cells. The cells were harvested from the agar plate after one week of culture, and then suspended in nitrogen-free medium for 8 h. **a** An mt^+ wild type gamete; **b** a cond-1 gamete; **c** a cond-2 gamete. Arrowhead and arrow indicate a cell nucleus and a cp nucleoid, respectively. Bar: 2 μm

Table 2. Number of chloroplast nucleoids in various gametes

W (mt^+)	7.1 ± 2.6
Cond-1(+)	13.3 ± 3.5
Cond-1(–)	13.7 ± 4.2
Cond-2(+)	23.7 ± 6.8
Cond-2(–)	23.2 ± 6.7

Cells were harvested after one week of culture and induced into gametes. In each experiment, about 100 cells were analyzed

oids in a chloroplast, after staining with DAPI. The numbers of cp nucleoids per chloroplast of gametes of wild type, and mutant cells cond-1(+) and cond-2(+) were about 7, 14, and 23, respectively (Fig. 1 and Table 2). Total amounts of cpDNA in three cell types were measured with VIMPCS (Table 3). We concluded that the amounts of three cell types were almost proportional to the numbers of cp nucleoids as reported previously (Nakamura et al. 1994).

10 min after the mating of wild type female and male gametes, the newly formed zygotes were spherical containing two cell nuclei and two chloroplasts (Fig. 2 a). 1 h after mating, cp nucleoids from male parents disappeared completely, but those from mt^+ parent remained unchanged (Fig. 2 b). After 5 h, two cell nuclei had already fused (Fig. 2 c).

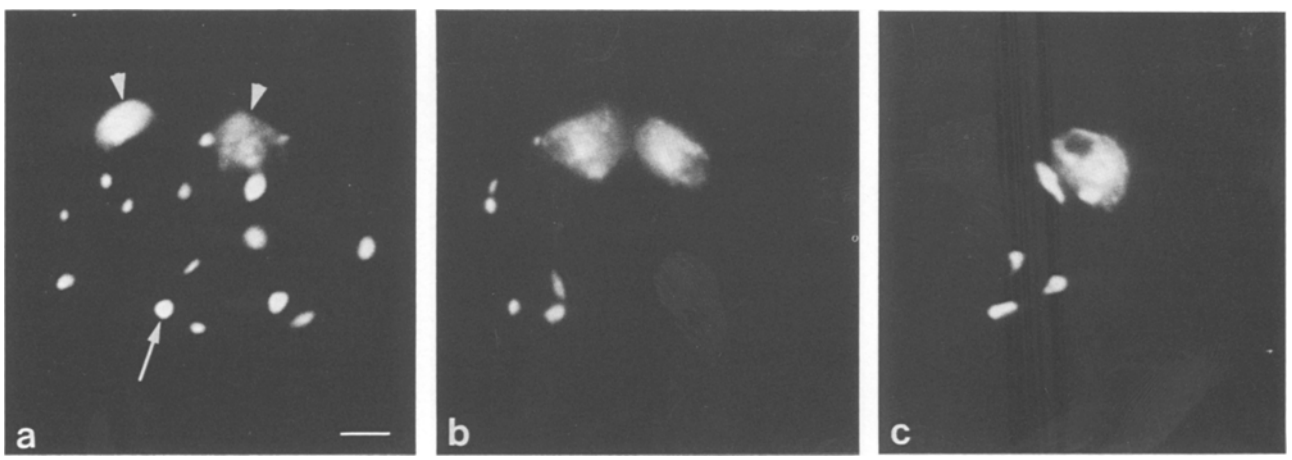

Fig. 2 a–c. Epifluorescence photomicrographs of cp nucleoids and cell nuclei in young zygotes after mating between wild type cells. **a** A zygote 30 min after mixing of wild type mt^+ and mt^- gametes, showing two discrete cell nuclei and cp nucleoids of both gamete origins; **b** a zygote 2 h after mixing, showing cp nucleoids of mt^+ origin (left half), only mt^- cp nucleoids (right half) disappeared; **c** a zygote 4 h after mixing, showing a fused cell nucleus and cp nucleoids of mt^+ origin (left half). Arrowhead and arrow indicate a cell nucleus and a cp nucleoid, respectively. Bar: 2 μm

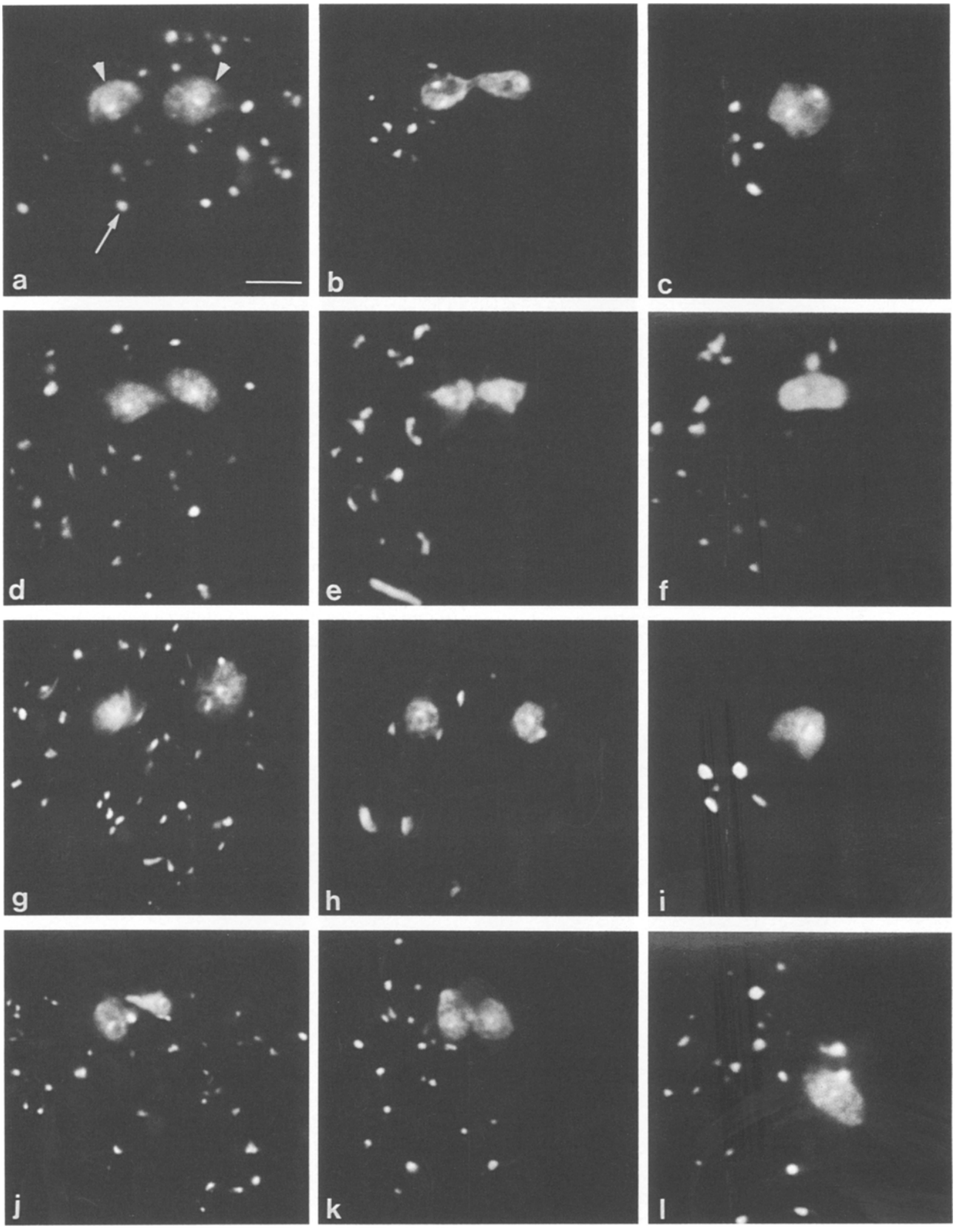
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Table 3. Relative fluorescence intensities of total chloroplast DNA ($\times 10^6$ counts) in various cell types measured with VIMPCS

W (mt^+)	3.1 ± 0.7
Cond-1(+)	7.8 ± 4.1
Cond-1(–)	7.5 ± 1.4
Cond-2(+)	16.5 ± 10.1
Cond-2(–)	19.6 ± 10.9

In each experiment, 10–17 cells were analyzed

Table 4. Frequencies of preferential digestion of mt^- chloroplast nucleoids in various zygotes

Crosses	Preferential digestion of cp nucleoids (%)		
	1 h	3 h	5 h
W (mt^+) × W (mt^-)	6.2	78.0	95.2
W (mt^+) × cond-1(–)	0	72.8	87.0
Cond-1(+) × W (mt^-)	13.8	79.6	82.9
W (mt^+) × cond-2(–)	2.8	69.2	83.4
Cond-2(+) × W (mt^-)	1.9	43.7	63.2

In each experiment, about 200 zygotes were analyzed

Next, we used the mutant cells as one of the parents and mated them with wild type cells. When wild type mt^+ cells were mated with cond-1(–) cells, cp nucleoids from cond-1(–) cells were digested in most zygotes (Fig. 3 a–c). Alternately when cond-1(+) cells were mated with wild type mt^- cells, preferential digestion occurred in the zygotes (Fig. 3 d–f). Similarly, when cells containing cond-2 mutation were mated with wild type cells, preferential digestion occurred in most zygotes (Fig. 3 g–l), although exceptions were observed.

We summarized the frequencies of preferential digestion in all crosses in Table 4. Frequencies were counted 1, 3, and 5 h after the mixing of two gametes. In the cross between two wild type cells, the frequency was almost 95% 5 h after the mixing. But when mutant cells were used as one of the parents, the frequency was slightly lower than that of wild type zygotes. Especially, when cond-2(+) cells were mated with wild type mt^- cells, the frequency was 63%. In this cross, we cannot exactly recognize the very narrow space derived from the wild type cell in the zygotes, because the mt^- cell is much smaller than the mt^+ cell (Fig. 1).

We examined the accumulation of the protein coded by *zys1B* gene through the preferential digestion. The purified antibody detected a 22 kDa band in Western blotting. High amount of Zys1B protein was detected at 40 min after the mixing of wild type female and male gametes, and then decreased to an undetectable amount (Fig. 4 a). When the mutant cells were mated with wild type cells, the changes of the amount of

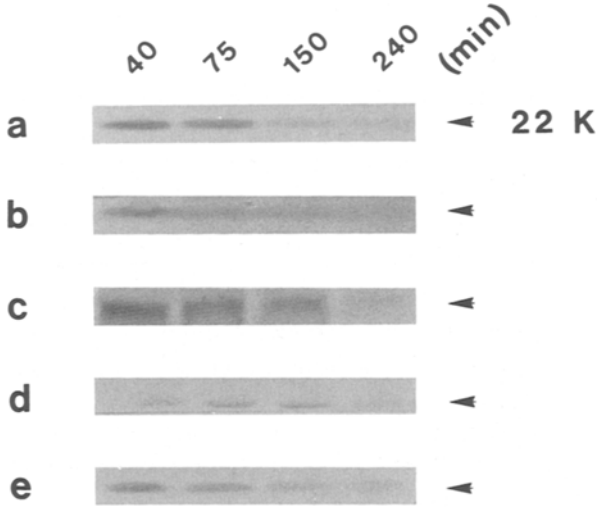

Fig. 4 a–e. Accumulation of Zys1B protein after mixing. Total proteins were extracted from zygotes after the mating between wild type cells or wild type and mutant cells. Zys1B protein was detected by immunoblotting. The cross between wild type cells (**a**), wild type mt^+ and cond-1(–) cells (**b**), cond-1 mt^+ and wild type mt^- (**c**), wild type mt^+ and cond-2(–) cells (**d**), cond-2(+) and wild type mt^- cells (**e**). Proteins were extracted at 40 min, 75 min, 150 min, 240 min after mixing, respectively

Zys1B protein were similar to that of zygotes of wild type cells (Fig. 4 b–e).

Table 5 shows the frequencies of maternal inheritance. Antibiotic resistance was used as marker for

Fig. 3 a–l. Epifluorescence photomicrographs of cp nucleoids and cell nuclei in young zygotes from the cross between mutant and wild type cells. **a–c** Zygotes from the cross between wild type mt^+ (left half) and cond-1(–) (right half) gametes; **d–f** the zygotes from the cross between cond-1(+) (left half) and wild type mt^- (right half) gametes; **g–i** the zygotes from the cross between wild type mt^+ (left half) and cond-2(–) (right half) gametes; **j–l** the zygotes after the cross of cond-2(+) (left half) and wild type mt^- (right half) gametes. a, d, g, and j 1 h after the mixing of two gametes; b, e, h, and k 3 h; c, f, i, and l 5 h. Arrowhead and arrow indicate a cell nucleus and a cp nucleoid, respectively. Bar: 2 μm

Table 5. Frequencies of maternal inheritance of antibiotic resistance in various crosses

Crosses	No. zygotes analyzed	% maternal inheritance
Cc-118 (mt^+) × cond-1(–)	643	98.9
Cond-1(+) × cc-119 (mt^-)	405	98.3
Cc-118 (mt^+) × cond-2(–)	501	99.6
Cond-2(+) × cc-119 (mt^-)	251	98.8
Cc-118 (mt^+) × Km-2 (mt^-)	427	98.6

the chloroplast inheritance. In all crosses, the cp genetic markers showed a maternal inheritance.

Discussion

Two mutant cells having higher amount of chloroplast (cp) DNA and higher number of the nucleoids, cond-1 and cond-2 were isolated previously (Nakamura et al. 1994). We studied the maternal chloroplast inheritance of *C. reinhardtii* using these mutant cells. The preferential digestion of mt^- (male) cp nucleoids occurred in the young zygotes, although the frequencies of preferential digestion were slightly lower than in the normal zygotes from the cross between two wild type cells. The protein coded on *zys1B* gene expressed during the very early stage of zygotes (Uchida et al. 1993) was detected in higher amount at 40 min in all crosses (Fig. 4 a–e). Cp markers for antibiotic resistance were maternally inherited to the meiotic progeny in all crosses (Table 5). These results indicate that higher number of cp nucleoids and higher amount of cpDNA in one of the parents had almost no effect on the completion of preferential digestion and do not have any effects on maternal inheritance. Zys1B protein was detected in early stages of the zygote formation with the occurrence of the preferential digestion, then decreased to an undetectable level with the completion of the preferential digestion. The transit accumulation of transcript of *zys1B* gene (Uchida et al. 1993) is very similar to that of the protein. The turnover of the transcript and the protein is rapid. A putative amino-acid sequence of this protein has cysteine- and glutamine-rich domain (Uchida et al. 1993), indicating that this protein would be a transcriptional regulator, which may be related to the preferential digestion of mt^- cp nucleoids.

Wurtz et al. (1977) reported that the decrease of mt^+ cpDNA by the treatment of FUdR causes a dramatical increase in the proportion of exceptional zygotes transmitting genes from the mt^- parent. In our present experiment, higher cpDNA amount of mt^- than that of mt^+ did not disturb maternal inheritance. Nakamura and Kuroiwa (1989) suggested a possibility of underestimation of frequency of maternal inheritance in the experiment of FUdR treatment, from the observation of young zygotes containing no cp nucleoid in high frequency. Otherwise, FUdR probably inhibited the process of maternal inheritance, because FUdR has been reported to cause point mutation, deletion, or rearrangement for chloroplast genes (Wurtz et al. 1979, Shepherd et al. 1979, Myers et al. 1982). Matagne and Mathieu (1983) studied the role of mating type gene dosage and cpDNA content after crossing the diploid cells with normal cells. Double the number of cpDNA copies and nucleoids in one parent increase the proportion of zygotes transmitting the allele from this parent to its meiotic progeny. Matagne and Beckers (1983) supported the above result by treating diploid cells with FUdR and suggested the combined action of preferential digestion and random elimination of both parental cpDNA for maternal inheritance. However, their results are not easily explained because diploid cells used as one of the parents contribute two-fold mating type gene dosage and two-fold cpDNA amount simultaneously to the zygotes. Furthermore, normal meiosis does not occur in the zygotes from haploid × diploid crosses and many progeny die or abort after a few generations of growth because of aneuploid progeny (Gillham et al. 1987).

In the present experiment, the frequency of the preferential digestion observed under the microscope was in accordance with that of maternal inheritance of cp markers. Immunogold electron microscopy suggests that the preferential digestion would be caused by the digestion of the cp molecules, not by a reorganization of cpDNA from the tightly packed nuclei into a looser structure that is no longer visible by DAPI staining (Uchida et al. 1992). These observations indicate that the fundamental cause of the maternal inheritance is the preferential digestion of mt^- cp nucleoids as reported previously (Kuroiwa et al. 1984, Kuroiwa 1985). Regardless of five-fold cpDNA amount of mt^- parent, almost no time lag for the completion of the preferential digestion of mt^- cp nucleoids was observed. There is probably a mechanism for mt^- nucleoids to be quickly digested in the mt^- cells coordinately with the active initiation of the preferential digestion in mt^+ cells (Kuroiwa 1985), or for proteins leading to the initiation of the digestion to be excessively produced during mating.

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