Running Head: Redox control of chloroplast DNA replication

Research category: Cell Biology and Signal Transduction

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Title: Chloroplast DNA replication is regulated by the redox state independently of chloroplast division in Chlamydomonas reinhardtii.

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One-sentence summary
This study elucidates that chloroplast DNA replication is regulated by the redox state in the cell, which is sensed by the chloroplast nucleoids in Chlamydomonas reinhardtii.
Footnotes

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Abstract

Chloroplasts arose from a cyanobacterial endosymbiont and multiply by division. In algal cells, chloroplast division is regulated by the cell cycle so as to occur only once, in the S-phase. Chloroplasts possess multiple copies of their own genome which must be replicated during chloroplast proliferation. In order to examine how chloroplast DNA replication is regulated in the green alga *Chlamydomonas reinhardtii*, we first asked whether it is regulated by the cell cycle, as is the case for chloroplast division. Chloroplast DNA is replicated in the light and not the dark phase, independent of the cell cycle or the timing of chloroplast division in photoautotrophic culture. Inhibition of photosynthetic electron transfer blocked chloroplast DNA replication. However, chloroplast DNA was replicated when the cells were grown heterotrophically in the dark, raising the possibility that chloroplast DNA replication is coupled with the reducing power supplied by photosynthesis or the uptake of acetate. When dimethylthiourea (DMTU), a reactive oxygen species (ROS) scavenger was added to the photoautotrophic culture, chloroplast DNA was replicated even in the dark. In contrast, when methylviologen, a ROS inducer, was added, chloroplast DNA was not replicated in the light. Moreover, the chloroplast DNA replication activity in both the isolated chloroplasts and nucleoids was increased by dithiothreitol (DTT), while it was repressed by diamide, a specific thiol-oxidizing reagent. These results suggest that chloroplast DNA replication is regulated by the redox state that is sensed by the nucleoids, and that the disulfide bonds in nucleoid-associated proteins are involved in this regulatory activity.
Introduction

Chloroplasts are semi-autonomous organelles that possess their own genome, which is complexed with proteins to form nucleoids and also certain machinery needed for protein synthesis, as is the case in prokaryotes. It is generally accepted that chloroplasts arose from a bacterial endosymbiont closely related to the currently extant cyanobacteria (Archibald, 2009; Keeling, 2010). In a manner reminiscent of their free-living ancestor, chloroplasts proliferate by the division of preexisting organelles that are coupled to the duplication and segregation of the nucleoids (Kuroiwa, 1991) and have retained the bulk of their bacterial biochemistry. However, chloroplasts have subsequently been substantially remodeled by the host cell so as to function as complementary organelles within the eukaryotic host cell (Rodriguez-Ezpeleta and Philippe, 2006; Archibald, 2009; Keeling, 2010). For example, most of the genes that were once in the original endosymbiont genome have been either lost or transferred into the host nuclear genome. As a result, the size of the chloroplast genome has been reduced to less than one tenth that of the free-living cyanobacterial genome. Thus, the bulk of the chloroplast proteome consists of nucleus-encoded proteins that are translated on cytoplasmic ribosomes and translocated into chloroplasts. In addition, chloroplast division ultimately came to be a process tightly regulated by the host cell, which ensured permanent inheritance of the chloroplasts during the course of cell division and from generation to generation (Rodriguez-Ezpeleta and Philippe, 2006; Archibald, 2009; Keeling, 2010).
Chloroplast division is performed by constriction of the ring structures at the division site, encompassing both the inside and the outside of the two envelopes (Yang et al., 2008; Maple and Moller, 2010; Miyagishima, 2011; Pyke, 2012). One part of the division machinery is derived from the cyanobacterial cytokinetic machinery that is based on the FtsZ protein. In contrast, other parts of the division machinery involve proteins specific to eukaryotes, including one member of the dynamin family. The majority of algae (both unicellular and multicellular), which diverged early within the Plantae, have just one or at most only a few chloroplasts per cell. In algae, the chloroplast divides once per cell cycle before the host cell completes cytokinesis (Suzuki et al., 1994; Miyagishima et al., 2012). In contrast, land plants and certain algal species contain dozens of chloroplasts per cell that divide non-synchronously, even within the same cell (Boffey and Lloyd, 1988). Because land plants evolved from algae, there is likely to have been a linkage between the cell cycle and chloroplast division in their algal ancestor that was subsequently lost during land plant evolution. Our recent study showed that the timing of chloroplast division in algae is restricted to the S-phase by S-phase-specific formation of the chloroplast division machinery, which is based on the cell cycle-regulated expression of the components of the chloroplast division machinery (Miyagishima et al., 2012).

Because chloroplasts possess their own genome, chloroplast DNA must be duplicated so that each daughter chloroplast inherits the required DNA after division. However, it is still unclear how the replication of chloroplast DNA is regulated and
whether the replication is coupled with the timing of chloroplast division, even though
certain studies have addressed this issue, as described below.

Bacteria such as *Escherichia coli* and *Bacillus subtilis* possess a single circular
chromosome. In these bacteria, the process of DNA replication is tightly coupled with
cell division (Boye et al., 2000; Zakrzewska-Czerwinska et al., 2007)), in which the
initiation of replication is regulated such that it occurs only once per cell division cycle
(Boye et al., 2000). In contrast, cyanobacteria contain multiple copies of their DNA (e.g.
3-5 copies in *Synechococcus elongatus* PCC 7942) (Mann and Carr, 1974; Griese et al.,
2011). In some obligate photoautotrophic cyanobacterial species, replication is initiated
only when light is available (Binder and Chisholm, 1990; Mori et al., 1996; Watanabe et
al., 2012). Replication is initiated asynchronously among the multiple copies of the
DNA. Although the regulation of the initiation of DNA replication is less stringent than
that in *E. coli* and *B. subtilis*, as described above, a recent study using *S. elongatus* 7942
showed that this replication peaks prior to cell division, as in other bacteria.

Chloroplasts also contain multiple copies of DNA (~1,000 copies) (Boffey
and Leech, 1982; Miyamura et al., 1986; Baumgartner et al., 1989; Oldenburg and
Bendich, 2004; Oldenburg et al., 2006; Shaver et al., 2008). In algae, chloroplast DNA
is replicated in a manner which keeps pace with chloroplast and cell division in order to
maintain the proper DNA content per chloroplast (i.e. per cell). In contrast, in land
plants, the copy number of DNA in each chloroplast (plastid) changes during the course
of development and differentiation, although contradictory results were reported about
leaf development (Lamppa and Bendich, 1979; Boffey and Leech, 1982; Hashimoto and
Previous studies which synchronized the algal cell cycle by means of a 24-h light–dark cycle showed that chloroplast DNA is replicated only during the G1 phase, after which it is separated into daughter chloroplasts during the S-phase by chloroplast division, implying that chloroplast DNA replication and division are temporally separated (Chiang and Sueoka, 1967; Grant et al., 1978; Suzuki et al., 1994). However, under these experimental conditions, G1 cells grow and the chloroplast DNA level increases during the light period. Cells enter into the S phase, chloroplast DNA replication ceases, and the chloroplasts divide at beginning of the dark period. Thus, it is still unclear whether chloroplast DNA replication is directly controlled by the cell cycle as is the case chloroplast division, or that chloroplast DNA replication simply occurs merely when light energy is available.

We addressed this issue using a synchronous culture as well as heterotrophic culture of the mixotrophic green alga *C. reinhardtii*. The results show that chloroplast DNA replication occurs independently of either the cell cycle or the timing of chloroplast division. Instead, it is shown that chloroplast DNA replication occurs when light is available in photoautotrophic culture and even under darkness in heterotrophic culture. Further experimental results suggest that chloroplast DNA replication is regulated by the redox state in the cell, which is sensed by the chloroplast nucleoids.

**Results**
The relationship between chloroplast DNA replication and the timing of chloroplast division

In order to determine the changes in the chloroplast DNA level that take place during the cell cycle by means of quantitative real-time PCR (qPCR), the green alga *C. reinhardtii* was used in this study for the following reasons. The cell cycle in this alga is synchronized by a light and dark cycle (Surzycki, 1971). Because the cell cycle is linked to circadian rhythms, cell-cycle synchrony is maintained even under continuous light after initial entrainment by a light and dark cycle (Goto and Johnson, 1995). *C. reinhardtii* cells contain a single chloroplast and this chloroplast divides during the S/M phase. These features allow an examination of the relationship between chloroplast DNA replication and the cell/chloroplast division cycle. In addition, cells grow in size by many fold during the G1 phase and then enter into 1-4 rounds of the S/M phases to produce 2-16 daughter cells during a single 24-h light and dark cycle. Thus, the levels of the nuclear and chloroplast DNA increase many fold in a single cycle, which makes it easier to detect any change in the DNA level by qPCR.

Previous studies using a 24-h light/dark synchronous culture of the red alga *Cyanidioschyzon merolae* (Suzuki et al., 1994) and the green alga *Scenedesmus quadricauda* (Zachleder et al., 1995) showed that the level of chloroplast DNA increases only during the light period (the G1 phase) and chloroplasts divide early in the dark period (the S phase). Thus, it has remained unclear whether the timing of chloroplast DNA replication is defined by the cell/chloroplast division cycle or is coupled with the availability of light.
In order to address this issue, we synchronized two populations of *C. reinhardtii* using a light and dark cycle, and then one population was cultured in the same light and dark cycle while the other was cultured under continuous light. The nuclear and chloroplast DNA levels were examined by qPCR of the *RBCS* gene (the nuclear genome) and the *rbcL* gene (the chloroplast genome), respectively.

Under the light/dark cycle (L/D/L in Fig. 1B), the chloroplast DNA level increased only during the light period (corresponding to the G1 phase; 0-12 h and 24-36 h) prior to the increase of the nuclear DNA level and cell division (corresponding to the S/M phase when the chloroplast divide; 8-20 h, Figs. 1A and B) as observed in previous studies on other algae (Chiang and Sueoka, 1967; Suzuki et al., 1994; Zachleder et al., 1995). In contrast, when cells were cultured under continuous light (L/L/L in Fig. 1B) after the entrainment by a light/dark cycle, the level of chloroplast DNA kept increasing, even though nuclear DNA replication (8-24 h) and cell division (8-24 h) were restricted to subjective night. These results indicate that chloroplast DNA replication is linked to the availability of light and not the cell/chloroplast division cycle.

Although the chloroplast DNA level kept increasing under continuous light, there was little difference in the ratio of chloroplast DNA to nuclear DNA between the light/dark culture and the continuous light culture (Fig. 1C). This is because both the nuclear DNA level and the cell number increased more under continuous light than under the light/dark cycle (Fig. 1A). In addition, it is known that both the cell and chloroplast size increase, even in the S/M phase, when light is available (Fig. 1A).
(Rollins et al., 1983). Thus, it appears that chloroplast DNA replication is linked to increase of the cell and chloroplast size.

**The relationship between chloroplast DNA replication and photosynthesis**

The obtained results indicate that the chloroplast DNA is replicated when light is available during photoautotrophic growth. In order to investigate the relationship between photosynthesis and chloroplast DNA replication, 3-(3’, 4’-dichlorophenyl)-1,1-dimethylurea, which blocks electron transfer between photosystem II and plastoquinone (Taiz and Zeiger, 2010), was added to a non-synchronous log-phase culture that was grown under continuous light. After the addition of DCMU, cells were cultured for 12 h under continuous light and the change in the chloroplast and nuclear DNA levels over 12 h were examined by qPCR (Fig. 2A). After the addition of DCMU, the increases in the chloroplast and nuclear DNA levels were blocked (Fig. 2A). When DCMU was removed by replacing the medium with a fresh one lacking DCMU, the increase in the chloroplast DNA level was resumed (Fig. 2B). These results indicate that chloroplast DNA replication requires photosynthesis during photoautotrophic growth.

In order to examine whether there are factors specific to photosynthetic electron transfer that are required for chloroplast DNA replication, we cultured the cells heterotrophically under darkness. *C. reinhardtii* cells are capable of efficient heterotrophic growth in the presence of acetate (Harris, 1989). qPCR analyses showed that the chloroplast DNA level keep increasing in accordance with replication of the nuclear DNA and cell division (Fig. 3). Given these results, it is suggested that
chloroplast DNA replication is coupled with cell growth by photosynthesis or the uptake of acetate, which supply a carbon source and reducing power to the cell.

**Relationship between chloroplast DNA replication and redox state**

One possibility raised by the above results is that chloroplast DNA replication is linked to the cellular redox state, because photosynthesis and acetate uptake metabolism supply reducing power to the cell. Therefore, we examined the effects of the oxidative and reductive states on chloroplast DNA replication using membrane-permeable redox reagents. Methylviologen accepts electrons from photosystem I and transfers them to oxygen molecules, resulting in the production of ROS in chloroplasts (Taiz and Zeiger, 2010). DMTU quenches ROS and reduces oxidative stress (Levine et al., 1994). Under the culturing conditions we used, treatment with DMTU for more than 4 h perturbed the cell shape and decreased both the chloroplast and nuclear DNA levels, probably as the result of cell death. Therefore, we examined the effects of methylviologen and DMTU on DNA replication 4 h after the addition of these reagents (Fig. 4, Table I).

First, we examined the effect of redox reagents on cellular redox state by quantifying cellular glutathione level (Table I). When methylviologen was added to a nonsynchronous log-phase culture that was grown photoautotrophically under continuous light, the level of reduced glutathione (GSH) decreased by one-fifth of that of untreated cells. In addition, the ratio of GSH to oxidized glutathione (GSSG) also decreased from 3.0 ± 0.5 to 0.7 ± 0.2, which was lower than the ratio in cells cultured in the dark without methylviologen (2.0 ± 0.1). In contrast, when DMTU was added to
photoautotrophic synchronous culture at the end of a 12-h dark period, the amount of GSH increased two fold and the GSH/GSSG ratio also increased from 2.2 ± 0.7 to 7.8 ± 3.2, which was higher than the ratio in cells cultured under light without DMTU (ratio = 3.3 ± 0.5). These results indicate that methylviologen and DMTU treatment changes cellular redox state as expected. Further, we confirmed that the light and dark treatment also changes redox state in chloroplasts.

When methylviologen was added to culture as described above, the replication activities of the chloroplast and nuclear DNA were blocked (Fig. 4A). In contrast, when DMTU was added to dark culture as described above, the chloroplast DNA level, but not the nuclear DNA level, increased under the dark condition to the same level as that in cells grown under light without DMTU (Fig. 4B). We also examined the effect of DMTU on chloroplast DNA replication by DAPI-staining (Fig. 4C). The DAPI fluorescent intensity in the chloroplast (the foci overlapping the red chloroplast autofluorescence) did not change under darkness without DMTU. In contrast, DMTU treatment increased the fluorescent intensity up to 2.5-fold over 4 h. These results suggest that the chloroplast DNA replication is regulated by the cellular redox state and that replication occurs when the cells are in a reduced state.

The connection between the cellular redox state and chloroplast DNA synthesis

In order to understand how chloroplast DNA replication is linked to the cellular redox state, we tested the following three hypotheses: (1) The chloroplast DNA polymerase level increases under a reducing condition, (2) The chloroplast DNA polymerase
activity is enhanced under a reducing condition by the modification of the polymerase or other regulatory proteins, and (3) The intra-chloroplast deoxynucleotide level (dNTP; the substrate of DNA replication) increases in the reduced state.

To test the first hypothesis, we examined the level of chloroplast DNA polymerase in synchronous culture. Plant and algae do not possess any DNA polymerase of cyanobacterial origin. Recent studies showed that the replication of chloroplast DNA is performed by the plant organellar DNA polymerase (POP) (Ono et al., 2007; Moriyama et al., 2008; Parent et al., 2011; Udy et al., 2012), which is similar to bacterial DNA polymerase I and is targeted to both chloroplasts and mitochondria. We performed BLAST searches in the *C. reinhardtii* database in an effort to identify POP orthologs. We observed that the *C. reinhardtii* POP gene was misannotated as two adjacent but separate genes (accession No. XM_001695976 and XM_001695977). XM_001695976 and XM_001695977 encode the 3’-5’ exonuclease domain, which is in the N-terminus, and the PolA domain, which is in the C-terminus of the POP protein in other species. By means of reverse transcription PCR, we verified that XM_001695976 and XM_001695977 are indeed transcribed as a single gene. We prepared polyclonal antibodies against CrPOP that detect a band of 130 kDa (Fig. 5), which is similar to the molecular mass of the POP proteins in other species (Ono et al., 2007; Moriyama et al., 2008). The immunoblot analysis showed that the CrPOP level was constant throughout the cell cycle when synchronized by a light and dark cycle (Fig. 5). This result indicates that chloroplast DNA is not replicated in photoautotrophic culture grown in the dark, even though the POP protein is present at the same level in culture under light.
To test the second and the third possibilities, we performed an in vitro chloroplast DNA synthesis assay using isolated chloroplasts. Intact chloroplasts were isolated from photoautotrophic synchronous culture either from at the end of the dark period or at the end of the subjective night phase of continuous light culture. After chloroplasts were osmotically permeabilized in a hypertonic buffer, an excess amount of substrates for DNA replication (dNTPs and digoxygenin-labeled dUTP (DIG-dUTP)) were added, and then the DNA synthesis activity was determined as the incorporation of DIG into the chloroplast DNA. The assay showed that the level of DIG-dUTP incorporation into the chloroplast DNA was larger in the chloroplasts isolated from culture under light than under dark (Fig. 6). The incorporation of DIG-dUTP was blocked when ethidium bromide, which blocks DNA replication, was added to the reaction mixture, indicating that this DIG incorporation is specific to DNA replication.

To examine whether the redox state has an effect on the activity of chloroplast DNA replication as observed in vivo (Fig. 4), redox reagents were added to the reaction mixture (Fig. 6). Addition of reduced thioredoxin, NADPH, and GSH, which are reducing agents in chloroplasts (Scheibe, 1991), had no significant effect on DNA synthesis. However, the addition of DTT elevated the DNA synthesis activity of chloroplasts prepared from dark-grown culture so as to be as high as that in the light-grown culture. In accordance with this result, the addition of diamide, a sulphhydryl group-specific oxidizing agent, reduced the DNA synthesis in the chloroplasts prepared from the light-grown culture to as low as that from dark-grown culture. Even though excess substrate levels were included in the reaction mixture, DNA synthesis activity
was still affected by either the culture condition (light or dark) or redox reagents. Although it cannot completely be ruled out that a change in the intra-chloroplast dNTP level might also be involved in the regulation of DNA replication, the above results suggest that replication is regulated by the redox state of a sulphhydryl group in chloroplast proteins.

Next, we asked whether chloroplast nucleoids are sufficient to sense the redox state and thus affect DNA replication activity. To this end, we performed an in vitro chloroplast DNA synthesis assay using isolated chloroplast nucleoids (Fig. 7). The isolated chloroplasts were solubilized by a non-ionic detergent Nonidet P-40 and the nucleoids were isolated. As in the case of isolated chloroplasts, DNA synthesis activity was found to be higher in the nucleoids isolated from the light-exposed culture than that from the culture under darkness. DTT increased while diamide reduced the DNA synthesis activity. These results suggest that the chloroplast nucleoid is sufficient to respond to changes in the redox state in order to regulate DNA replication.

**Discussion**

Previous studies using a 24-h light/dark synchronous culture of algae showed that the chloroplast DNA is replicated only during the light period (G1 phase), prior to nuclear DNA replication (S phase), chloroplast division and cytokinesis, which occur during the dark period (Chiang and Sueoka, 1967; Suzuki et al., 1994; Zachleder et al., 1995). Based on these results, it was concluded that the DNA replication and division phases are temporally separated in chloroplasts. However, here we have shown that the
chloroplast DNA is replicated independently of the timing of chloroplast division and the cell cycle, and that chloroplast DNA continues to be replicated as long as light energy is available to drive photosynthetic electron transfer during photoautotrophic growth (Figs. 1 and 2).

Under a continuous light condition after the entrainment with a light/dark cycle in the photoautotrophic medium, chloroplast DNA continued to be replicated and the chloroplast and cell size also continued to increase, even in the S/M phase (Figs. 1A and B). Because cells grown under continuous light undergo more rounds of the S/M phase than cells grown in a light/dark condition, the chloroplast DNA level per cell/chloroplast does not change in daughter cells in the two conditions (Fig. 1C). Given these results, it appears that chloroplast DNA replication is correlated with cell/chloroplast growth, thereby maintaining the proper DNA content per cell/chloroplast volume. The significance of chloroplast polyploidy has been much debated. Mutation in the organellar DNA polymerase in maize reduces the chloroplast DNA copy number as well as the chloroplast-encoded transcripts and proteins, suggesting that the DNA copy number is a limiting factor for the expression of chloroplast-encoded genes (Udy et al., 2012). Other recent studies revealed a reduction in the chloroplast DNA copy number under phosphate-limited condition in C. reinhardtii (Yehudai-Resheff et al., 2007) and an increase in the copy number under a phosphate-rich condition in the green alga Nannochloris bacillaris (Sumiya et al., 2008). These studies raise the possibility that polyploidal chloroplast DNA may at least serve as repository of phosphorus. Taken together, one possibility is that the chloroplast DNA
replication is correlated with cell/chloroplast growth by phosphorus that are assimilated by reducing power supplied by photosynthesis or uptake of acetate. In this point, further studies will be required.

When cells were cultured in a photoautotrophic medium, photosynthetic electron flow was required for the chloroplast DNA replication (Fig. 2). However, even under darkness, chloroplast DNA replicated in a heterotrophic medium which contained acetate as sources of carbon and reducing power (Fig. 3). These results raised a possibility that the chloroplast DNA replication is linked to cellular redox state. Consistent with this assumption, Lau et al. showed that cells treated with cadmium and cells growing in a nitrogen-replete medium, in which levels of chloroplast DNA decreased, contained low GSH levels (Lau et al., 2000). In this study, we showed that artificial alteration of cellular redox state by redox reagents changes the activity of chloroplast DNA replication (Fig. 4). In our study, chloroplast DNA replication was blocked by the addition of methylviologen under light (Fig. 4A). In contrast, replication of the chloroplast DNA, but not that of nuclear DNA, was activated by the addition of DMTU under dark (Figs. 4B and C). In addition, we confirmed that cellular redox state was changed when cells were treated with the above redox reagents (Table I). Thus, our results suggest that cellular redox state affects the level of chloroplast DNA replication.

For factors that link chloroplast DNA replication and the cellular redox state, three candidates were considered. These include (1) the level or (2) replication activity of the POP protein (chloroplast DNA polymerase) and (3) the level of dNTPs (substrates for DNA replication). The results show that the POP level is constant under
the light and dark conditions during photoautotrophic growth (Fig. 5). The results obtained showed that DNA replication activity is higher under light than under dark even when excessive levels of dNTPs were supplied *in vitro* (Figs. 6 and 7). Thus, our results suggest that the activity of the DNA polymerase (probably POP) is a rate-limiting factor in chloroplast DNA replication. However, *in vitro* DNA synthesis was still observed in chloroplasts isolated from culture grown under darkness (Figs. 6 and 7). Therefore, at this point, we can not completely rule out the possibility that change in the intra-chloroplast dNTP level might also be involved in the regulation of the chloroplast DNA replication. However, replication of the nuclear DNA occurs during the dark period in photoautotrophic synchronous culture, indicating that there is at least a sufficient dNTP level for DNA synthesis in the whole cell.

Chloroplast DNA replication *in vitro* was activated by DTT, but was inactivated by diamide, which is a sulphydryl group-specific oxidative agent (Figs. 6 and 7). These results suggest that activity of the chloroplast DNA polymerase is regulated by redox state of the sulphydryl group, at least in some of the chloroplast proteins. Besides, given that chloroplast nucleoids are sufficient to sense the redox state to change the DNA replication activity (Fig. 7), the sulphydryl group in certain chloroplast nucleoid-associated proteins, including POP itself, may link the redox state to DNA replication activity. In this regard, FrxB protein is likely a candidate. FrxB binds to the chloroplast DNA replicative origin in *C. reinhardtii*. In addition, FrxB is an iron-sulfur redox protein subunit of chloroplast NADH dehydrogenase, raising a
possibility that FrxB links redox state the chloroplast DNA replication (Wu et al., 1989; Wu et al., 1993).

For a better understanding of the regulation of chloroplast DNA replication, the identification of the redox sensing, replication-related proteins will be required. Plant and algal genomes do not encode any DNA replication-related proteins of cyanobacterial origin, such as the DNA polymerase and DnaA protein which are involved in the initiation of replication in bacteria. Therefore, biochemical approaches, including disulfide proteomics of the chloroplast nucleoids, will be needed to determine the eukaryote-specific mechanism of the redox state-based regulation of chloroplast DNA replication.

Materials and Methods

Synchronous culture

*C. reinhardtii* 137c mt+ and cw-15 cells were cultured in Sueoka’s high salt medium (HSM) (Sueoka, 1967) photoautotrophically. For synchronization, the cells in log phase were subcultured to 1x 10^6 cells mL^{-1} and were subjected to a 12-h light/12-h dark cycle (100 µmol photons m^{-2}s^{-1}) at 24°C under aeration with ambient air (Surzycki, 1971). Cells in the second and the third cycle were used for further analyses.

Non-synchronous heterotrophic culture
For heterotrophic culture, the 137c mt+ cells were cultured in Tris-acetate-phosphate (TAP) (Gorman and Levine, 1965) medium under complete darkness at 24°C and ambient aeration with ambient air (Chen and Johnson, 1996).

**Drug treatments**

For the drug treatments, 137c mt+ cells were used. For 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) treatment, a 1/1,000 volume of 50 mM DCMU stock solution in ethanol was added to nonsynchronous log-phase culture that was grown under continuous light. For methylviologen treatment, a 1/1,000 volume of 1 mM methylviologen stock solution in water was added to nonsynchronous log-phase cultures that were grown under continuous light. For dimethylthiourea (DMTU) treatment, a 1/100 volume of 2 M DMTU stock solution dissolved in HSM medium was added to the synchronous culture at the end of the second dark period.

**Quantification of nuclear and chloroplast DNA by quantitative PCR**

The DNA level was analyzed by quantitative PCR (qPCR). Cells were harvested from 0.5 mL culture by centrifugation at 1,000 g for 5 min and stored at -80°C until DNA extraction. Cells were resuspended in TES buffer (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 200 mM NaCl, 1% SDS) and then DNA was extracted by the phenol/chloroform method. The extracted DNA was dissolved in 100 µL of distilled water. qPCR amplification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in a Real-time PCR system (StepOne Plus, Applied Biosystems).
qPCR was performed using the primers 5’-gcgccgccagctgtact-3’ and 5’-
aacatgggcagctccacat-3’ for the nuclear DNA (RBCS1 gene), and 5’-
gtcctggctgcctgaa-3’ and 5’-tctggagaccattgagaag-3’ for the chloroplast DNA (rbcL gene).

**Quantification of reduced glutathione and oxidized glutathione**

Cells were harvested by centrifugation at 1,000 g for 5 min at 4°C and then washed with phosphate buffer saline (PBS). Cells were resuspended in 5% sulfosalicylic acid solution and disrupted by sonication. Supernatants were obtained by centrifugation at 15,000 g for 5 min at 4°C. Chloroplasts isolated as described below were harvested by centrifugation at 700 g for 5 min at 4°C and then were washed with 50 mM HEPES-KOH pH 7.5 containing 300 mM sorbitol. The isolated chloroplasts were resuspended in 5% sulfosalicylic acid solution and centrifuged at 15,000 g for 5 min at 4°C to remove debris. Reduced glutathione (GSH) and oxidized glutathione (GSSG) extracted into the supernatants were quantified by using the GSSG/GSH quantification kit (Dojindo) according to the manufacturer’s instructions.

**Antibody preparation**

The cDNA sequence encoding a partial fragment of CrPOP was amplified by PCR using the primers 5’- caccagccgcagcggcaagaagacctacgg -3’ and 5’-
ctacacattcatgcctacccactgtgcctgaa-3’. The PCR product was cloned into a pET100 expression vector (Invitrogen) and the 6xHis fusion polypeptide was expressed in
Rosetta (DE3) *E. coli* cells, purified using HisTrap HP column (GE healthcare).

Polypeptide was further separated electrophoretically in a preparative acrylamide gel. The antibodies against CrPOP were raised in rabbits using the gel band containing the recombinant polypeptide and then antibodies were affinity-purified from the anti-sera using a HiTrap NHS-activated HP column (GE Healthcare), to which recombinant polypeptide (eluate of the HisTrap HP column) was covalently bound.

**Immunoblot analyses**

Cells were harvested from 5 mL of culture by centrifugation and stored at -80°C. Cells were suspended in the protein extraction buffer (50 mM Tris, pH 7.5, 8 M urea, 0.1% Triton X-100) and sonicated. Protein content was determined by Bradford assay and equal amounts of the total proteins were subjected to immunoblot analyses. Immunoblotting assays were performed as previously described (Kabeya et al., 2010). Anti-CrPOP was used at a dilution of 1:1,000.

**Isolation of intact chloroplasts**

Chloroplasts were isolated using a modified version of Mason’s method (Mason et al., 2006). All manipulations were done at 4°C or on ice. The *cw-15* cells were synchronously cultured to < 1.0 x 10^7 cells mL^-1. Cells were harvested by centrifugation at 3,000 g for 10 min and resuspended in CP isolation buffer (50 mM HEPES-KOH pH 7.5, 2 mM EDTA, 1 mM MgCl2, 1% BSA, 300 mM sorbitol). The cells were then broken by a single passage through an airbrush (0.2-mm aperture...
airbrush, HP-60, OLYMPOS, Japan) at a pressure of 0.7 kg cm\(^{-2}\) according to the reported airbrush method (Nishimura et al., 2002). The lysate was centrifuged at 750 g for 2 min and the pellet was gently resuspended in 3 mL of CP isolation buffer using a paintbrush. The sample was layered on the top of a discontinuous Percoll gradient (20/45/65% Percoll in CP isolation buffer). After centrifugation at 3,220 g for 30 min, the green band that appeared at the interface between the 45% and 65% layers was collected. It was confirmed by DAPI staining that the collected fraction contained scant nuclear or mitochondrial contamination. The chloroplast-rich fraction was diluted with CP isolation buffer and then centrifuged at 670 g for 1 min to remove the Percoll. The purified chloroplasts were used for the \textit{in vitro} DNA synthesis or isolation of the nucleoids.

**Isolation of the chloroplast nucleoids**

Chloroplast nucleoids were isolated using a modified version of Sato’s method (Sato et al., 1998). All manipulations were performed at 4°C or on ice. Isolated chloroplasts were lysed with 2% Nonidet P-40 dissolved in TAN buffer (20 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 7 mM 2-mercaptoethanol, 1.2 mM spermidine, 0.5 M sucrose) for 1 h. Then the sample was centrifuged at 20,000 g for 30 min. The pellet with the enriched chloroplast nucleoids was resuspended with TAN buffer containing 2% Nonidet P-40 and centrifuged again. The isolated nucleoids were used for \textit{in vitro} DNA synthesis.
Detection of newly synthesized chloroplast DNA using the isolated chloroplasts and chloroplast nucleoids

1 x 10^7 chloroplasts were suspended in 300 µL of hypertonic replication buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 5 mM MgCl₂). Nucleoids isolated from 1 x 10^9 chloroplasts were suspended in 300 µL of replication buffer. After incubation with or without 10 mM DTT, 0.5 mM NADPH, 0.1 mM thioredoxin (#203-13041, Wako) reduced by DTT, 10 mM GSH, 1 mM diamide, or 10 µg/ml ethidium bromide for 15 min at 24°C, a one-tenth volume of DIG labeling Mix (Roche) was added and then incubated at 24°C up to 1 h. After incubation, DNA was extracted at 0, 20, and 60 min and slot-blotted onto a Hybond-N+ membrane. The signals representing newly synthesized chloroplast DNA were detected by anti-DIG-AP conjugates (#11585550910, Roche) and a CDP-Star system (Applied Biosystems).

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Figure Legends

Figure 1

The changes in the nuclear and chloroplast DNA levels in the synchronous culture. C. reinhardtii 137c mt+ cells were cultured in the photoautotrophic medium (HSM medium). A, Progression of the cell cycle in the synchronous culture. Cells were entrained by a 12-h light and 12-h dark cycle and then grown under a light/dark cycle (L/D/L) or continuous light condition (L/L/L). Scale bar = 10 µm. B, Changes in the cell number and the nuclear and chloroplast DNA levels in the synchronous culture were determined by qPCR analyses using the primer sets for rbcL (chloroplast DNA, cpDNA), RBCS1 (nuclear DNA, nuDNA). The gray and white regions in the graph indicate the subjective night and light periods, respectively. The error bars represent the standard error of 3 technical repeats of qPCR. C, The ratio of the chloroplast DNA relative to the nuclear DNA in cells grown under the L/D/L or L/L/L conditions. The ratio was calculated from the DNA levels in B. The error bars represent the standard error. Two independent experiments showed similar results.

Figure 2

The effect of an inhibition of photosynthetic electron transfer on chloroplast DNA replication. C. reinhardtii 137c mt+ cells were cultured in the photoautotrophic medium.
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**Figure 3**

The changes in the chloroplast DNA level in the heterotrophic culture under the dark condition. *C. reinhardtii* 137c mt⁺ cells were cultured in the medium containing acetate (TAP medium) under complete darkness for 2 days. The nuclear (nuDNA) and chloroplast (cpDNA) DNA levels were determined by qPCR analyses. The error bars represent the standard error of 3 technical repeats of qPCR. Two independent experiments showed similar results.
**Figure 4**

The effects of redox reagents on chloroplast DNA replication *in vivo*. *C. reinhardtii* 137c mt+ cells were cultured in the photoautotrophic medium. **A**, The effect of methylviologen (MV; a H₂O₂ producer dependent on photosystem II) on chloroplast DNA and nuclear DNA replication. Methylviologen was added to a nonsynchronous log-phase culture that was grown under continuous light. DNA levels in the culture 4 h after the treatment with (MV) or without (-) methylviologen were examined by qPCR analyses. The error bars represent the standard error of 3 technical repeats of qPCR. Two independent experiments showed similar results. **B**, The effect of dimethylthiourea (DMTU; a H₂O₂ scavenger) on chloroplast DNA and nuclear DNA replication. DMTU added to synchronous culture at the end of a 12-h dark period. Nuclear and chloroplast DNA levels in the cultures that were treated with or without DMTU for 4 h under dark (D) or light (L) were examined by qPCR analyses. The error bars represent the standard error of 3 technical repeats of qPCR. Three independent experiments showed similar results. **C**, DAPI-staining images of cells treated with or without DMTU for 4 h under darkness. The intensity of the fluorescent DAPI staining that overlapped with the chloroplast red autofluorescence was measured using ImageJ. The error bars represent the standard deviation (n=50 cells). Scale bar = 10 µm. The levels just before the addition of the reagents (at 0 h for MV; at 12 h for DMTU) are defined as 1.0.

**Figure 5**
The chloroplast DNA polymerase level in the light and dark cycles. Immunoblot analyses were performed using anti-chloroplast DNA polymerase (CrPOP) antibodies. Twenty micrograms of proteins were separated in each lane. **A**, Total proteins from asynchronous log-phase cells were detected by the CrPOP antibodies that were raised in two rabbits. **B**, Aliquots of the synchronous culture were collected at the indicated timepoints and the total proteins were separated. Level of CrPOP protein was analyzed with the anti-CrPOP antibodies (rabbit 1), and the Rubisco large subunit (Rubisco LSU) was detected by CBB staining as the quantitative control.

**Figure 6**

The effects of redox reagents on chloroplast DNA replication in isolated chloroplasts. *C. reinhardtii* *cw-15* cells were cultured in the photoautotrophic medium. Chloroplasts were isolated from synchronous culture either at the end of the second dark period or the end of the second subjective night under light (indicated by the arrowheads) and then osmotically permeabilized in hypertonic buffer. To investigate the effects of reducing reagents on chloroplast DNA replication activity, dithiothreitol (DTT), reduced thioredoxin (trx), NADPH, or reduced glutathione (GSH) was added to chloroplasts isolated from the dark culture. To investigate the involvement of disulfide bonds on the regulation of the chloroplast DNA replication activity, diamide was added to chloroplasts isolated from the light culture. Ethidium bromide (EtBr), which inhibits DNA replication, was added as a negative control. Each reagent was added to the permeabilized chloroplasts. After preincubation for 15 min, chloroplasts were further
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**Figure 7**

The effects of redox reagents on chloroplast DNA replication in isolated chloroplast nucleoids. Chloroplast nucleoids were isolated from synchronous culture (the isolated time points were the same as in Figure 6). Diamide or DTT was added to chloroplast nucleoids. After preincubation for 15 min, DNA synthesis was examined as the incorporation of digoxigenin-labeled dUTP, as described in Figure 6. The relative levels of the incorporated digoxigenin are plotted. The level of the culture grown under darkness at 60 min is defined as 1.0. The error bars represent standard deviation of 3 biological replicates. Asterisks indicate that the significant difference from the dark by t test ($P < 0.001$).
Table I. Change in level of reduced glutathione (GSH) and oxidized glutathione (GSSG).

Cells were cultured in conditions as described in Figs. 1, 4, and 6. GSH and GSSG were extracted from cells at the end of a 12-h subjective night (light), dark period (dark), after 4-h treatment with (MV+, DMTU+) or without reduct peroxides (MV-, DMTU-) or from isolated chloroplasts. The values are the mean ± standard error of 3 technical repeats. Two independent experiments showed similar results. Asterisks indicate that the significant difference by t test ($P < 0.005$).

<table>
<thead>
<tr>
<th>Culture</th>
<th>GSSG (nmol/mg protein)</th>
<th>GSH (nmol/mg protein)</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>light</td>
<td>0.114 ± 0.015</td>
<td>0.377 ± 0.006</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>dark</td>
<td>0.110 ± 0.016</td>
<td>0.218 ± 0.021</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>MV+ (light)</td>
<td>0.094 ± 0.038</td>
<td>0.057 ± 0.004</td>
<td>0.7 ± 0.2</td>
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<tr>
<td>MV- (light)</td>
<td>0.111 ± 0.017</td>
<td>0.324 ± 0.008</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>DMTU+ (dark)</td>
<td>0.120 ± 0.046</td>
<td>0.425 ± 0.009</td>
<td>7.8 ± 3.2</td>
</tr>
<tr>
<td>DMTU- (dark)</td>
<td>0.104 ± 0.029</td>
<td>0.229 ± 0.011</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>isolated chloroplasts (light)</td>
<td>0.250 ± 0.067</td>
<td>0.778 ± 0.058</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>isolated chloroplasts (dark)</td>
<td>0.267 ± 0.054</td>
<td>0.550 ± 0.060</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
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