Localization and Phylogenetic Analysis of Enzymes Related to Organellar Genome Replication in the Unicellular Rhodophyte Cyanidioschyzon merolae

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Accepted: January 1, 2014

Abstract
Plants and algae possess plastids and mitochondria harboring their own genomes, which are replicated by the apparatus consisting of DNA polymerase, DNA primase, DNA helicase, DNA topoisomerase, single-stranded DNA maintenance protein, DNA ligase, and primer removal enzyme. In the higher plant Arabidopsis thaliana, organellar replication-related enzymes (OREs) are similar in plastids and mitochondria because many of them are dually targeted to plastids and mitochondria. In the red algae, there is a report about a DNA replicase, plant/protist organellar DNA polymerase, which is localized to both plastids and mitochondria. However, other OREs remain unclear in algae. Here, we identified OREs possibly localized to organelles in the unicellular rhodophyte Cyanidioschyzon merolae. We then examined intracellular localization of green fluorescent protein-fusion proteins of these enzymes in C. merolae, whose cell has a single plastid and a single mitochondrion and is suitable for localization analysis, demonstrating that the plastid and the mitochondrion contain markedly different components of replication machinery. Phylogenetic analyses revealed that the organelle replication apparatus was composed of enzymes of various different origins, such as proteobacterial, cyanobacterial, and eukaryotic, in both red algae and green plants. Especially in the red alga, many enzymes of cyanobacterial origin remained. Finally, on the basis of the results of localization and phylogenetic analyses, we propose a model on the succession of OREs in eukaryotes.

Key words: evolution of replication apparatus, mitochondria and plastids, subcellular localization, GFP-fusion proteins, red algae.

Introduction
Plants and algae possess plastids and mitochondria. Both organelles contain their own genome to perform their metabolic functions. The organelar genomes are thought to be replicated by the machinery including DNA polymerase, DNA primase, DNA helicase, DNA topoisomerase, single-stranded DNA binding protein (SSB), DNA ligase, and primer removal enzyme (Langston et al. 2009). As plant organelar genomes do not encode these enzymes, the organelar genomes must be replicated by the enzymes that are encoded by the nuclear genome and transported to the organelles after their synthesis (Wang et al. 1997).

In Escherichia coli, DNA polymerase III (Pol III) holoenzyme consisting of ten subunits functions in DNA elongation in replication. The catalytic α subunit (encoded by the dnaE gene) of Pol III belongs to Family-C DNA polymerase, and it is conserved in all bacteria (Langston et al. 2009; Sanyal and Doig 2012).

DnaB helicase unwinds double-stranded DNA and then SSB maintains the DNA unwound. DNA gyrase, which consists of two subunits, gyrase A and B, is a type II DNA topoisomerase, and alleviates the strain resulting from DNA unwinding. DnaG synthesizes an RNA primer at the origin of replication in the leading strand and synthesizes primers in about every 1 kb in the lagging strand. Ribonucleotides within the RNA primer are removed by nick translation with 5’-3’ exonuclease and polymerase activity of DNA polymerase I (Pol I), and the nicked DNA is sealed by NAD+-dependent DNA ligase LigA.

In humans, the mitochondrial genome is replicated by a replisome consisting of DNA polymerase γ (Poly), human mitochondrial RNA polymerase (POLRMT), TWINKLE helicase (T7 gp4-like protein with intramitochondrial nucleoid localization), topoisomerases 1 and 3a, SSB, ligase 3, and RNase H1 (Arnold et al. 2012; Kasiviswanathan et al. 2012). Poly is responsible for the replication and repair in animal
mitochondrial genomes, and belongs to Family-A DNA polymerase, which shares distant sequence similarity to bacterial Pol I. Animal Poly comprises two subunits: a large subunit with DNA polymerase and 3’-5’ exonuclease activities, and a small subunit that enhances processivity and primer recognition (Kaguni 2004). POLRMT is a homolog of the RNA polymerase of T3/T7 phage (RPOT), which is a single polypeptide enzyme. POLRMT had been thought to function in transcription, but recently this RNA polymerase was demonstrated to function also as a primase (Wanrooij et al. 2008). In plants, the homolog of POLRMT is called RPOT type, and RPOT was localized to plastids and/or mitochondria for transcription (Kühn et al. 2007). TWINKLE is a homolog of the gp4 protein of T7 phage, which has helicase and primase activities. TWINKLE is widely conserved in eukaryotes including animals, bikonts (plants and protists), and amoebozoa (Shutt and Gray 2006). Animal TWINKLE shows only helicase activity and retains a nonfunctional domain of primase. Mitochondrial replisome was reconstituted with Poly, TWINKLE, and SSB, and the resulting replisome showed rolling-circle replication with high processivity (Korhonen et al. 2004). Processivity is defined as the number of nucleotides added by a DNA polymerase per one binding to the template DNA, and in general, replicative DNA polymerase has a high processivity value, and repair polymerase has a low processivity value.

Recently, in higher plant Arabidopsis thaliana, enzymes related to organellar genome replication have been identified. The genome of Arabidopsis encodes two genes, each encoding a DNA polymerase belonging to Family-A DNA polymerase, which are called Pol I-like or Poly (Christensen et al. 2005; Mori et al. 2005; Parent et al. 2011; Cupp and Nielsen 2013). These polymerases function in replication in both plastids and mitochondria. Subsequently, we reported that these polymerases are also conserved in many protists, and the enzymes are phylogenetically distinct from bacterial Pol I and Poly. Because it was thought that a new name is needed, we proposed to call them POP (plant/protist organellar DNA polymerase, Moriyama et al. 2011; Moriyama and Sato 2013). Arabidopsis thaliana has a single gyrase A that is localized to both plastids and mitochondria, and has two gyrase B1 and B2 that are localized to chloroplasts and mitochondria, respectively. Inhibitor assay and complement test in E. coli revealed that A. thaliana gyrase A is involved in organellar genome replication (Wall et al. 2004). Arabidopsis thaliana has an A-type topoisomerase I (ATOP1), which is a homolog of bacterial topoisomerase I, TopA. Localization analysis using green fluorescent protein (GFP) fusion protein showed dual localization of ATOP1 to chloroplasts and mitochondria (Carrie et al. 2009). In addition to this report, by proteome analysis, ATOP1 was detected in the chloroplast nucleoids in A. thaliana (Olinares et al. 2010). Arabidopsis thaliana TWINKLE is localized to both chloroplasts and mitochondria (Carrie et al. 2009), and has both helicase and primase activities unlike animal TWINKLE retaining only helicase activity (Diray-Arce et al. 2013). Recombinant Arabidopsis SSB (AtSSB) binds to single-stranded DNA (ssDNA), but not to double-stranded DNA, and AtSSB-GFP protein is localized to mitochondria (Edmondson et al. 2005). Organellar SSB (OSB), which has a SSB-like domain, is a plant-specific SSB. Arabidopsis thaliana has four OSBs: AtOSB1 and 2 are localized to mitochondria, chloroplasts, respectively, and AtOSB3 is localized to both chloroplasts and mitochondria (Zaegel et al. 2006). Replication protein A (RPA), a nucleus-localized SSB in eukaryotes, comprises three subunits, RPA70, RPA32, and RPA14. Rice has three RPA70s, three RPA32s, and one RPA14. These subunits make three types of complexes, namely, type A, B, and C complexes with different combinations of the three types of subunits. Among them, type A RPA complex is localized to chloroplasts in rice (Ishibashi et al. 2006). DNA ligase 1 (LIG1) is localized to both mitochondria and nucleus, but not to plastids, and plastidial ligase remains unclear (Sunderland et al. 2006). In bacteria, Pol I acts in primer removal with its 5’-3’ exonuclease activity. The Arabidopsis genome encodes two 5’-3’ exonuclease genes (5’-3’EXO1 and 2) having sequence homology to 5’-3’ exonuclease domain of bacterial Pol I, and these enzymes were predicted to be localized to chloroplasts or mitochondria (Sato et al. 2003).

Previously, we demonstrated that a rhodophyte Cyanidioschyzon merolae has two organellar DNA polymerases, POP and Pol I (Moriyama et al. 2008). By immunoblot analysis using isolated organelles and observation of GFP-fusion proteins in onion epidermal cells, we showed that CmPOP was localized to both plastids and mitochondria, while CmPol I was localized to plastids. We also determined enzymatic activity of these polymerases. CmPOP showed the high processivity value (>1,300 nt) and had 3’-5’ exonuclease activity, while CmPol I showed middle level processivity (<70 nt), and had no 3’-5’ exonuclease activity (Moriyama et al. 2008). Other organellar replication-related enzymes (OREs) have not been identified in red and green algae.

The unicellular rhodophyte C. merolae has a remarkably simple cell structure consisting of a single mitochondrion and a single plastid per cell. Its normal habitat is hot springs, which is warm (up to 50 °C) and acidic (pH 1.5–2.5) with sulfuric acid. The size of its completely sequenced genome is 16,546,747 bp, with 4,775 predicted protein-coding genes (Matsuzaki et al. 2004; Nozaki et al. 2007). The method of transformation by polyethylene glycol (PEG) was recently established in C. merolae (Ohnuma et al. 2008). Subcellular localization of GFP-fusion protein in C. merolae was first demonstrated by Watanabe et al. (2011). They constructed the pCG1 vector, in which the target protein-GFP fusion gene is overexpressed by C. merolae apC promoter.

In the present study, we identified the components of OREs, and analyzed their organellar localization by observation of GFP-fusion protein. We also estimated the origin of OREs
by phylogenetic analysis. Finally, we discuss a model on the succession of organellar replisome in eukaryotes.

Materials and Methods

Culture Conditions

Cells of *C. merolae* strain 10D (Toda et al. 1998) were inoculated in the 2 × Allen’s medium (Minoda et al. 2004) at pH 2.5. Flasks were shaken under continuous light provided by two fluorescent tubes (30 μmol/m²/s) at 40 °C.

Construction of GFP-Fusion Genes

Genes of putative OREs were amplified by PCR with specific primer sets (supplementary table S1, Supplementary Material online). Using In-Fusion HD cloning kit (Clontech Laboratories, Mountain View, CA), the amplified DNA fragments were inserted into XbaI-cut pCG1 vector (Watanabe et al. 2011) that contains the apcC gene promoter of *C. merolae*, sGFP, and NOS terminator.

Transformation in *C. merolae* Cells

Cells of *C. merolae* were transformed by the PEG-method according to Ohnuma et al. (2008) except for the preparation of recipient cells. Subcultured cells were grown to OD750 of recipient cells. Subcultured cells were grown to OD750 according to Ohnuma et al. (2008) except for the preparation of recipient cells. Subcultured cells were grown to OD750 according to Ohnuma et al. (2008) except for the preparation of recipient cells. Subcultured cells were grown to OD750 according to Ohnuma et al. (2008) except for the preparation of recipient cells. Subcultured cells were grown to OD750 according to Ohnuma et al. (2008) except for the preparation of recipient cells. Subcultured cells were grown to OD750 according to Ohnuma et al. (2008) except for the preparation of recipient cells. Subcultured cells were grown to OD750 according to Ohnuma et al. (2008) except for the preparation of recipient cells. Subcultured cells were grown to OD750 according to Ohnuma et al. 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January 22, 2014) were used for the prediction of subcellular localization of putative OREs. The results were considerably different among the three programs (table 1). We also made alignments of putative OREs and determined whether putative OREs have an N-terminal extension for organellar targeting (supplementary fig. S2-1 to S2-9, Supplementary Material online). On the basis of these results, we selected proteins for analysis of subcellular targeting.

### Intracellular Localization of Putative OREs in C. merolae

The C. merolae cell has a simple cell structure with a single plastid and a single mitochondrion, and reproduces itself by binary fission (fig. 1A). The N-terminal extension sequence of each putative ORE was cloned into pCG1 vector for overexpression of its GFP-fusion protein driven by the apcC promoter (fig. 1B). The amino acid sequences of putative OREs fused with GFP are given in supplementary figure S2, Supplementary Material online. The plasmids were introduced into C. merolae cells by the PEG method, and GFP fluorescence was observed (fig. 1C–E and supplementary fig. S3, Supplementary Material online). When GFP fluorescence in the nucleus-like structure was faint, the protein targeting was confirmed by immunostaining using anti-GFP antibody with 4',6-diamidino-2-phenylindole (DAPI) staining (supplementary fig. S3, Supplementary Material online).

| Annotation | Gene Identifier | Phylogenetic Origin | Prediction Results | GFP |
|------------|-----------------|---------------------|--------------------|-----|
| DNA polymerase | | | | |
| POP | CMO270C | Unknown | mt pt ER pt mt |
| Priming | | | | |
| DnaG | CMQ286C | Cyanobacterial | mt mt None pt |
| DNA helicase | | | | |
| DnaB (pt-encoded) | CMV098C | Cyanobacterial | (pt*) |
| Primase/helicase | | | | |
| TWINKLE | CMT452C | Eukaryotic | mt pt mt mt |
| Topoisomerases | | | | |
| Gyrase A | CMS243C | Cyanobacterial | None pt ER pt |
| Gyrase B | CMH166C | Cyanobacterial | pt pt None pt |
| Topoisomerase I (type IA) | CMQ252C | Cyanobacterial | pt pt None pt |
| Topoisomerase I (type IB) | CMV263C | Eukaryotic | None nuc None nuc |
| Topoisomerase II a | CM013C | Eukaryotic | pt pt None cyto |
| Topoisomerase II b | CML330C | Eukaryotic | mt cyto None mt |
| Topoisomerase III alpha | CML066C | — | mt pt mt |
| Topoisomerase VI A-1 | CME071C | — | None pt None |
| Topoisomerase VI A-2 | CML010C | — | mt pt None |
| Topoisomerase VI A-3 | CML111C | — | mt pt None cyto |
| Topoisomerase VI A-4 | CMR274C | — | mt pt None |
| Topoisomerase VI B | CMT273C | — | None nuc None |
| ssDNA maintenance | | | | |
| SSB | CMI135C | a-Proteobacterial | mt mt mt mt |
| RPA 70 kDa | CMC123C | — | mt pt mt nuc |
| RPA 30 kDa | CMI291C | — | None nuc None nuc |
| Ligation | | | | |
| DNA ligase | CMK235C | Eukaryotic | mt pt None pt mt |
| Primer removal | | | | |
| DNA Pol I | CMT462C | a-Proteobacterial | mt pt mt pt |
| RNase HI large subunit | CMK297C | — | None nuc None — |
| RNase HI | CMT626C | Bacterial (unknown origin) | mt pt mt mt |
| DNA2 | CMK133C | — | mt pt (nuc) None nuc |
| FEN1 | CMG106C | — | None pt mt nuc |

Notes: Predicted results of localization consistent with the GFP results are marked by bold italic. Abbreviations: pt, plastid; mt, mitochondrion; nuc, nucleus; cyto, cytosol; ER, endoplasmic reticulum.

**a**No GFP analysis was done because dnaB gene is encoded by the plastid genome.

**b**In this study, N-terminal peptides were fused with GFP for targeting analysis, and cytosol localization was observed in these enzymes. However, these enzymes have a nuclear localization signal within the protein sequence after the N-terminus.
The DNA replicase, POP, was dually targeted to plastid and mitochondrion (fig. 1E), which is consistent with our previous results by immunoblotting (Moriyama et al. 2008). There are two methionine residues in the N-terminus region of LIG1 (supplementary fig. S2-1, Supplementary Material online). A construct starting from the first methionine residue in LIG1 showed strong GFP-fluorescence in the mitochondrion and weak fluorescence in the plastid, whereas another construct starting from the second methionine residue showed GFP-fluorescence in the plastid (fig. 1E). LIG1 might be translated from the two different methionine residues.

TWINKLE, which had helicase/primase activity and was localized to both plastids and mitochondria in *A. thaliana*, was observed only in mitochondria in *C. merolae* (fig. 1D). DnaG primase, which was conserved in red algae and not in green plants, was localized to plastid (fig. 1C). In *C. merolae*, DnaB helicase is encoded by the plastid genome, and the enzyme is supposed to be localized to plastid. Among DNA topoisomerases, two subunits (A and B) of gyrase and type-IA topoisomerase I (TOP1) were localized to the plastid, while topoisomerase II-b (TOP2b) was localized to the mitochondrion (fig. 1C and D). Other tested topoisomerases, namely, TOP1 (type IB), TOP2A, and TOP6, were not observed in either plastid or mitochondrion (supplementary fig. S3, Supplementary Material online). The *C. merolae* genome encodes five enzymes related to primer removal, RNase HII and HII, DNA2, FEN1, and Pol I that has 5′-3′ exonuclease and polymerase domains. The GFP-fusion results suggested that Pol I and RNase HII are localized to plastid and mitochondrion, respectively (fig. 1C and D). DNA2 and FEN1 were observed
in the nucleus in C. merolae (supplementary fig. S3, Supplementary Material online).

The fluorescence of SSB-GFP was observed only in the mitochondrion (fig. 1D). We also tested a construct starting from the second methionine residue in SSB, and the construct showed nonorganellar localization (data not shown). In rice, an RPA was detected in isolated plastids, by immunoblotting, as well as in nuclei (Ishibashi et al. 2006). However, C. merolae RPAs have no extension sequence at its N-terminus, and these were localized to the nucleus (Supplementary fig. S3, Supplementary Material online). No other gene for ssDNA binding protein is found in the C. merolae genome. RNA binding protein may function also as a ssDNA binding protein. It is known that some RNA binding proteins, such as TIA-1 and TIAR in humans, bind to ssDNA (Suswam et al. 2005).

To examine localization of enzymes translated from native start codon(s) including non-AUG start codon, we first observed using constructs, authentic promoter:full-length protein:GFP. However, no fluorescent signal of these constructs was observed even if immunofluorescence using anti-GFP antibody was performed. Next, we prepared constructs overexpressed by apcC promoter, namely, apcC promoter:full-length protein:GFP. Also in these constructs except SSB, GFP-fluorescence could not be observed. Overexpressed SSB-GFP was observed as a single granule in mitochondrion, not in nucleus in C. merolae cell (data not shown). This granular structure might occur by artifact with overexpression of full-length SSB. Subcellular localization of other full-length OREs could not be determined yet, and we do not discard the possibility that some of the proteins we found in this study that went to only mitochondria, plastids, or the nucleus could still be dual-localized.

Phylogenetic Analysis of OREs

Excepting some clearly eukaryotic enzymes, such as POP, type IB and IIB topoisomerases, and DNA ligase I, we analyzed the phylogenetic origin of OREs. With respect to the POP, we previously reported that the origin of POP was unclear because POP did not originate from Pol I of cyanobacteria nor \(\alpha\)-proteobacteria (Moriyama et al. 2008).

Four types of simplified trees of OREs are shown in figure 2, and original trees are also shown in supplementary figure S4-1 to S4-6, Supplementary Material online. DnaG and DnaB, which are not conserved in green plants, originated from cyanobacteria (fig. 2A and supplementary fig. S4-1, Supplementary Material online). Gyrases A and B originated from cyanobacteria in red algae and green plants (fig. 2B and supplementary fig. S4-2, Supplementary Material online). Type IA topoisomerase originated from cyanobacteria in red algae, but the enzyme was obviously related to \(\alpha\)-proteobacterial homologs in green plants (fig. 2C and supplementary fig. S4-3, Supplementary Material online). Red algal and green plant SSB originated from the SSB in \(\alpha\)-proteobacteria (fig. 2D). Animal SSB is also \(\alpha\)-proteobacterial origin (supplementary fig. S4-4, Supplementary Material online). Because green plants have 5'-3' exonuclease but not a full-domain of Pol I consisting of 5'-3' exonuclease, 3'-5' exonuclease, and DNA polymerase domain, we examined phylogenetic relationship of the 5'-3' exonuclease domain in bacteria and photosynthetic eukaryotes. The results showed that exonucleases in the red and green lineages are monophyletic, and their origin is \(\alpha\)-proteobacterial exonuclease domain (fig. 2D and supplementary fig. S4-4, Supplementary Material online). Red
algae and green plants have bacterial RNase HII, but their RNase HII was not related to RNase HII in $\alpha$-proteobacteria nor cyanobacteria. The enzyme might have been acquired by lateral gene transfer from bacteria such as Chlamydiae, Firmicutes, or Chlorobi (supplementary fig. S4-S, Supplementary Material online). We also performed phylogenetic analysis of TWINKLE. The TWINKLE of C. merolae and diatoms are closely related to that of animals and ciliates rather than to that of plants. It was reported that animal TWINKLE does not have primase activity because some key amino acid residues for primase activity are not conserved in animal TWINKLE. In C. merolae, the key residues are conserved, and C. merolae TWINKLE is assumed to possess primase activity (Shutt and Gray 2006).

**Discussion**

Components of Replication Enzymes in Organelles

In the present study, we identified the intracellular localization of OREs in C. merolae, namely, six plastid-localized OREs, four mitochondrion-localized OREs, and two dually localized OREs. In C. merolae, ORE components of plastid differed significantly with those of mitochondrion except dually localized ones, POP and LG1. This is in contrast with the situation in A. thaliana, in which components of OREs in plastids and mitochondria are nearly identical.

Dual localization of POP was found in both the red alga C. merolae (Moriyama et al. 2008) and the green plants, A. thaliana (Christensen et al. 2005) and tobacco (Ono et al. 2007). In contrast, localization of other OREs is variable in various species of red algae and green plants. POP consists of a single polypeptide and showed high processivity, although POP belongs to Family A-type DNA polymerase that has sequence similarity to E. coli Pol I having a middle-level processivity (Moriyama et al. 2008). POP also showed a high fidelity with its 3'-5' exonuclease activity (Takeuchi et al. 2007). These properties of POP suggest that POPs are widely conserved organelar replicase in eukaryotes including plants, amoebzoa, alveolates, heterokonts, and discristates (Moriyama and Sato 2013).

TWINKLE is identified as a DNA helicase in human mitochondria (Spelbrink et al. 2001). In A. thaliana, AtTWINKLE showed dual activity, primase and helicase, and this TWINKLE was thought to be involved in the replication of genomes in plastids and mitochondria in green plants (Diray-Arce et al. 2013). In C. merolae, however, TWINKLE is targeted to only mitochondria. Instead, single domain enzymes originating from cyanobacteria, DnaB helicase and DnaG primase, are localized to the plastid. A rhodophyte Porphyridium purpureum, and diatoms Thalassiosira pseudonana and Phaeodactylum tricornutum also have a plastid-encoded DnaB and a nucleus-encoded DnaG (supplementary fig. S4-1, Supplementary Material online). We confirmed that Porphyridium DnaG was localized to plastid in transformed C. merolae cells (data not shown). These results suggest that rhodophytes and diatoms (NB: the latter is thought to originate from the secondary endosymbiosis involving a rhodophyte) retain DnaB and DnaG of cyanobacterial origin, and these enzymes function in plastids, whereas the TWINKLE consisting of helicase and primase domains functions in mitochondria.

Cyanidioschyzon merolae has 12 genes encoding various subunits of topoisomerases, among which gyrase and bacterial TOP1 are localized to plastids, while eukaryotic TOP2b is localized to mitochondria. Organellar localization of eukaryotic TOP2 has not been reported in plants. In C. merolae, the effects of a gyrase-specific inhibitor, nalidixic acid, were reported by two groups (Itoh et al. 1997; Kobayashi et al. 2009). In these reports, nalidixic acid arrests not only the replication of plastid genome but also that of mitochondrial and nuclear genomes. Considering the plastid localization of gyrase in C. merolae, these results may suggest that the arrest of plastid replication influences mitochondrial and nuclear replication by an unknown mechanism. In another possible explanation, nalidixic acid might inhibit mitochondrial and/or nuclear topoisomerases in C. merolae.

Evolution of Enzymes Involved in Organellar Genome Replication in Photosynthetic Organisms

Previously, we proposed a model for the succession of organelar DNA polymerase in eukaryotes (Moriyama et al. 2011; Moriyama and Sato 2013). In the present report, addition of other OREs completes the entire view of the model (fig. 3). First, when the ancestor of eukaryotes acquired mitochondria, the genome of the protomitochondrion had been replicated with the $\alpha$-proteobacterial replication machinery, but then the host cell started to use a distinct set of replicase, helicase, and primase. Namely, Pol III was replaced by POP, and DnaB and DnaG were replaced by TWINKLE. The origins of POP and TWINKLE are still unknown. In animals, POP was replaced by Poly whose origin is estimated as a T-odd phage DNA polymerase (File`e et al. 2002). In addition, a recent finding that some cyanophages have a homolog of Poly suggests that Poly might originate from a cyanophage enzyme (Chan et al. 2011). Moreover, in animals, TWINKLE lost primase activity and animal mitochondria started to use POLRMT (T3/T7 phage type RNA polymerase) for priming on replication in addition to transcription. SSB is a sole ORE of the $\alpha$-proteobacterial origin in animals. After the second endosymbiosis that engendered the plastids in the common ancestor of Viridiplantae and Rhodophyta, cyanobacterial Pol III was replaced by POP, originally functioning in mitochondria. Plastids of rhodophytes continued to use DnaB and DnaG of cyanobacterial origin, while plastids of green plants started to use TWINKLE in place of DnaB and DnaG. Pol I of the $\alpha$-proteobacterial origin was retained in photosynthetic
eukaryotes; however, its 3’-5’ exonuclease and DNA polymerase domains were lost in land plants, and in rhodophytes, 3’-5’ exonuclease activity was lost.

Accordingly, in land plants, components of replication enzymes had been exchanged between plastids and mitochondria, and are now homogeneous in these organelles. In red algae, however, components of OREs are essentially different between plastid and mitochondrion. Plastid in red algae retains many OREs originating from cyanobacteria. Among these cyanobacteria-derived OREs, DnaB is encoded by the plastid genome, while others are encoded by the nuclear genome in red algae. The same is true in diatoms. The mechanism of replication in the plastid genome in the red lineages might need to retain plastid-encoded dnaB gene for helicase activity or its nucleotide sequence. Cooperative function of DnaB and DnaG in replication and protein–protein interaction of these enzymes have been reported in E. coli (Tougu and Marians 1996), and this relationship may be conserved in red algae.
lineages. In the next step of research, functional analysis of replication apparatus in each organelle will be important. Reconstitution experiments in vitro of the organelar DNA replication by a combination of various ORE proteins will provide clues for the problem.

Supplementary Material

Supplementary tables S1, S2, and figures S1–S4 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

Acknowledgments

The authors thank Dr H. Yoshikawa and Dr S. Watanabe from Tokyo University of Agriculture for providing pCG1 plasmid, overexpression vector in C. merolae. This work was supported by Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Agency (JST); and Grants-in-Aid for Young Scientists (B) from JSPS (no. 25870155).

Literature Cited

Arnold J, Smidansky E, Moustafa I, Cameron C. 2012. Human mitochondrial RNA polymerase: structure‐function, mechanism and inhibition. Biochim Biophys Acta. 1819:948–960.
Carrie C, et al. 2009. Approaches to defining dual‐targeted proteins in overexpression vector in C. merolae. This work was supported by Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Agency (JST); and Grants‐in‐Aid for Young Scientists (B) from JSPS (no. 25870155).
Chan Y, et al. 2011. Discovery of cyanophage genomes which contain presequences in mitochondrial DNA polymerase. Mol Biol Evol. 28:2269–2274.
Christensen A, et al. 2005. Dual‐domain, dual‐targeting organellar protein prerequisites in Arabidopsis can use non‐AUG start codons. Plant Cell 17:2805–2816.
Cupp JD, Nielsen BL. 2013. Arabidopsis thaliana organellar DNA polymerase Ib mutants exhibit reduced mtDNA levels with a decrease in mitochondrial area density. Physiol Plant. 149:91–103.
Diray‐Arce J, Liu B, Cupp J, Hunt T, Nielsen B. 2013. The Arabidopsis At1g30680 gene encodes a homologue to the phage T7 gp4 protein that has both DNA primase and DNA helicase activities. BMC Plant Biol. 13:36.
Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–1797.
Edmondson AC, et al. 2005. Characterization of a mitochondrially targeted single‐stranded DNA‐binding protein in Arabidopsis thaliana. Mol Genet Genomics. 273:115–122.
Fiée J, Forterre P, Sen‐Lin T, Laurent J. 2002. Evolution of DNA polymerase families: evidences for multiple gene exchange between cellular and viral proteins. J Mol Evol. 54:763–773.
Ishibashi T, Kimura S, Sakaguchi K. 2006. A higher plant has three different types of RPA heterotrimeric complex. J Biochem. 139:99–104.
Itoh R, Takahashi H, Toda K, Kuroiwa H, Kuroiwa T. 1997. DNA gyrase involvement in chloroplast‐nucleoid division in Cyanidioschyzon merolae. Eur J Cell Biol. 73:252–258.
Jobb G, von Haeseler A, Strimmer K. 2004. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol Biol. 4:18.
Kaguni L. 2004. DNA polymerase gamma, the mitochondrial replicase. Annu Rev Biochem. 73:293–320.
Kakisuvanathan R, Collins T, Copeland W. 2012. The interface of transcription and DNA replication in the mitochondria. Biochim Biophys Acta. 1819:970–978.
Kobayashi Y, et al. 2009. Tetrapyrole signal as a cell‐cycle coordinator from organelle to nuclear DNA replication in plant cells. Proc Natl Acad Sci U S A. 106:803–807.
Korhonen J, Pham X, Pellegrini M, Falkenberg M. 2004. Reconstitution of a minimal mtDNA replisome in vitro. EMBO J. 23:2423–2429.
Kühn K, Bohne A, Liere K, Weihe A, Börner T. 2007. Arabidopsis phage‐type RNA polymerases: accurate in vitro transcription of organellar genes. Plant Cell 19:959–971.
Langston LD, Indiani C, O'Donnell M. 2009. Whither the replisome: emerging perspectives on the dynamic nature of the DNA replication machinery. Cell Cycle 8:2686–2691.
Matsuzaki M, et al. 2004. Genome sequence of the ultrasmall unicellular red alga Cyanidioschyzon merolae 10D. Nature 428:653–657.
Minoda A, Sakagami R, Yagisawa F, Kurotawa T, Tanaka K. 2004. Improvement of culture conditions and evidence for nuclear transformation by homologous recombination in a red alga, Cyanidioschyzon merolae 10D. Plant Cell Physiol. 45:667–671.
Mori Y, et al. 2005. Plastid DNA polymerases from higher plants, Arabidopsis thaliana. Biochem Biophys Res Commun. 334:43–50.
Moriyama T, Sato N. 2013. The plant and protist organellar DNA replication enzyme POP showing up in place of DNA polymerase gamma may be a suitable antiprotozoal drug target. In: Stuart D, editor. The mechanisms of DNA replication. Rijeka: InTech. p. 287–311.
Moriyama T, Terasawa K, Fujisawa M, Sato N. 2008. Purification and characterization of organellar DNA polymerases in the red alga Cyanidioschyzon merolae. FEBS J. 275:2899–2918.
Moriyama T, Terasawa K, Sato N. 2011. Conservation of POPs, the plant and protist organellar DNA polymerases, in eukaryotes. Protist 162:177–187.
Nishida K, et al. 2004. Triple immunofluorescent labeling of FtsZ, dynamin, and EF‐Tu reveals a loose association between the inner and outer membrane mitochondrial division machinery in the red alga Cyanidioschyzon merolae. J Histochem Cytochem. 52:843–849.
Nozaki H, et al. 2007. A 100%‐complete sequence reveals unusually simple genomic features in the hot‐spring red alga Cyanidioschyzon merolae. BMC Biol. 5:28.
Ono Y, et al. 2007. NtPol‐like1 and NtPol‐like2, bacterial DNA polymerase I homologs isolated from BY‐2 cultured tobacco cells, encode DNA polymerases engaged in DNA replication in both plastids and mitochondria. Plant Cell Physiol. 48:1679–1692.
Parent J, Lepage E, Brisson N. 2011. Divergent roles for the two PolI‐like DNA polymerases of Arabidopsis. Plant Physiol. 156:254–262.
Sanyal G, Doig P. 2012. Bacterial DNA replication enzymes as targets for antibacterial drug discovery. Expert Opin Drug Discov. 7:327–339.
Sato N. 2000. SISEQ: manipulation of multiple sequence and large database files for common platforms. Bioinformatics 16:180–181.
Sato N. 2009. Gclust: trans‐kingdom classification of proteins using automatic individual threshold setting. Bioinformatics 25:599–605.
Sato N, Terasawa K, Miyajima K, Kabeya Y. 2003. Organization, development, and mechanisms of DNA replication. Rijeka: InTech. p. 287–311.
Sato N. 2009. Gclust: trans‐kingdom classification of proteins using automatic individual threshold setting. Bioinformatics 25:599–605.
Sato N. 2009. Gclust: trans‐kingdom classification of proteins using automatic individual threshold setting. Bioinformatics 25:599–605.
Spelbrink JN, et al. 2001. Human mitochondrial DNA deletions may be a suitable antiprotozoal drug target. In: Stuart D, editor. The mechanisms of DNA replication. Rijeka: InTech. p. 287–311.
Spelbrink JN, et al. 2001. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage
T7 gene 4-like protein localized in mitochondria. Nat Genet. 28: 223–231.
Sunderland PA, West CE, Waterworth WM, Bray CM. 2006. An evolutionarily conserved translation initiation mechanism regulates nuclear or mitochondrial targeting of DNA ligase 1 in Arabidopsis thaliana. Plant J. 47:356–367.
Suswam EA, Li YY, Mahtani H, King PH. 2005. Novel DNA-binding properties of the RNA-binding protein TIAR. Nucleic Acids Res. 33: 4507–4518.
Takeuchi R, Kimura S, Saotome A, Sakaguchi K. 2007. Biochemical properties of a plastidial DNA polymerase of rice. Plant Mol Biol. 64:601–611.
Toda K, Takano H, Miyagishima S, Kuroiwa H, Kuroiwa T. 1998. Characterization of a chloroplast isoform of serine acetyltransferase from the thermo-acidophilic red alga Cyanidioschyzon merolae. Biochim Biophys Acta. 1403:72–84.
Tougu K, Marians KJ. 1996. The extreme C terminus of primase is required for interaction with DnaB at the replication fork. J Biol Chem. 271: 21391–21397.
Wall M, Mitchellall L, Maxwell A. 2004. Arabidopsis thaliana DNA gyrase is targeted to chloroplasts and mitochondria. Proc Natl Acad Sci U S A. 101:7821–7826.
Wang Y, Farr CL, Kaguni LS. 1997. Accessory subunit of mitochondrial DNA polymerase from Drosophila embryos. Cloning, molecular analysis, and association in the native enzyme. J Biol Chem. 272: 13640–13646.
Wanrooij S, et al. 2008. Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro. Proc Natl Acad Sci U S A. 105: 11122–11127.
Watanabe S, Ohnuma M, Sato J, Yoshikawa H, Tanaka K. 2011. Utility of a GFP reporter system in the red alga Cyanidioschyzon merolae. J Gen Appl Microbiol. 57:69–72.
Zaegel V, et al. 2006. The plant-specific ssDNA binding protein OSB1 is involved in the stoichiometric transmission of mitochondrial DNA in Arabidopsis. Plant Cell 18:3548–3563.

Associate editor: Geoff McFadden