Plastid Genome of *Cyanidium caldarium* Strain RK-1 Encodes *trpA*

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Cyanidioschyzon merolae, *Cyanidium caldarium* strain RK-1 (*C. caldarium* RK-1, *C. caldarium* Forma A) and *C. caldarium* strain M-8 (*C. caldarium* M-8, *C. caldarium* Forma B, *Galdieria sulphuraria*) are very small unicellular eukaryotes which range from 1 to 5 μ m in diameter, have phycobilisomes containing pigments such as phycocyanin, phycoerythrin and chlorophyll a and thus show blue green color (Nagashima and Fukuda 1981, Seckbach 1991). In addition, they are often found living together in thermal acidic environments. Therefore, these three species have previously been classified into the same species. DeLuca *et al.* (1978) and Merola *et al.* (1981) classified these algae into three species on the basis of different morphologies, such as the number of endospore, mitochondria and chloroplasts, and the mode of cell divisions. Nagashima and Fukuda (1981) and Nagashima *et al.* (1984; 1989) supported DeLuca's classification by cytological and biochemical data.

The plastid nucleus (pt-nucleus, nucleoid) in *C. merolae* and *C. caldarium* RK-1 was limited to the center of the plastid and thus the plastids resemble cyanobacteria, while the pt-nucleus in *C. caldarium* M-8 was located at the periphery of plastids (Nagashima *et al.* 1984). In addition, the mean amounts of cell-nuclear DNA of *C. merolae*, *C. caldarium* RK-1 and *C. caldarium* M-8 were 8 Mbp, 13 Mbp, 31 Mbp, respectively (Ohta *et al.* 1992a, Suzuki *et al.* 1992). The results suggest that *C. merolae* has the smallest amount of DNA among eukaryotes. Therefore, *C. merolae* can be considered to be first photosynthetic eukaryotic organisms. The genome analyses of the border organisms between prokaryotes and primitive eukaryotes are essential to elucidate the origin of eukaryotes.

To detect the structural changes and differences of the genome systems in cell nuclei and organelle nuclei among the algae, Ohta *et al.* (1992a) and Suzuki *et al.* (1992) isolated chloroplast DNA, mitochondrial DNA and cell-nuclear DNA as three distinct CsCl bands from *C. caldarium* RK-1 and *C. merolae*, respectively, by an improved isolation method and sequenced some plastid genes such as *rbcL* (the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase) and *trnK*. In the process of the genome analyses of the alga, we found *trpA* gene (the gene for the α subunit of tryptophan synthase) by homology of the nucleotide sequence on the plastid genome of *C. caldarium* (Ohta *et al.* 1992b, 1993).

The aim of the present work is to examine whether or not the *trpA* is encoded on the plastid genomes of two algae, *C. caldarium* RK-1 and *C. caldarium* M-8.

Materials and methods

Strain and isolation of the plastid DNA

Cyanidium caldarium RK-1 was grown in Allen's medium as described elsewhere (Nagashima *et al.* 1989). After 14 days, cells were harvested by centrifugation at 5,000 $\times$ g for 10

minutes. The plastid DNA was isolated as described (Ohta *et al.* 1992a).

Cloning and sequence of the plastid DNA

The plastid DNA was digested with *Pst*I and cloned in pBluescriptII SK, M13+ (Stratagene, California USA), with *E. coli* XL1 Blue as host bacterium. Part of the 3.4-kbp *Pst*I fragment was sequenced by the chain-termination method (Sambrook *et al.* 1989) using a Sequenase Kit (United States Biochemical, Ohio, USA). The gene was identified by a homology search of EMBL/SWISSPROT data using a DNASIS gene/protein sequence database (Hitachi, Japan). Sequence was determined from both strands.

Southern hybridization analysis

Southern blotting was performed as follows. Cell-nuclear DNA (10 μ g) and plastid DNA (1 μ g) was digested with *Pst*I or *Hind*III and subjected to electrophoresis at 2V/cm. The DNA

Fig. 1. Phase-contrast (a, d) and fluorescent photomicrographs (b, c, e, f) showing whole cells (a, d), plastids (b, e), cell nuclei (small arrows in c, f) and plastid nuclei (large arrows in c, f) in cells of *Cyanidium caldarium* RK-1 (a-c) and M-8 (d-f) after DAPI staining. (a-c) and (d-f) are the same field in each strain. Note that the plastid nucleus is located in the central area of the plastid in *C. caldarium* RK-1, while the ring-shaped plastid nucleus is located at the periphery of the petal-like plastid in *C. caldarium* M-8. Bar represents 1 μ m.

was transferred to nylon membrane (Hybond-N⁺, Amersham, Buckinghamshire, England). The probe for *rbcL* was cloned by using *rbcL* from the pea *Pisum sativum* (Ohta *et al.* 1991). The probe for *trpA* was amplified from a cloned *Pst*I-*Eco*RI 1.8 kbp fragment by the PCR method using two primers that corresponded to the flanking region of *trpA* by using Gene Amp PCR Reagent Kit (Perkin Elmer Cetus, USA). The product was purified by agarose gel electrophoresis. Hybridization was performed at 37°C in the hybridization buffer (Sambrook *et al.* 1989) containing 50% formamide. Membrane was washed 3 times in 2×SSC. Then autoradiographed.

Fluorescence microscopy

Procedures for fluorescence microscopy were described in a previous paper (Nagashima *et al.* 1984).

Results

Fig. 1 shows representative phase-contrast (Figs. 1a and 1d), epi-fluorescent (Figs. 1b, 1c, 1e and 1f) photomicrographs of cell-nuclei, plastids and pt-nuclei in young cells of *C. caldarium* RK-1 and *C. caldarium* M-8. When the cells were excited with green light, the plastids, that emit red autofluorescence, appear in the cells (Figs. 1b and 1e). By contrast, when the cells were excited with UV light, the pt-nuclei, which emit blue fluorescence, appear in the plastids (Figs. 1b, 1c, 1e and 1f). Although the two algae are very small unicellular eukaryotes, have a blue green color, have phycobilisomes containing similar pigments and often are living together in thermal acidic environments, the distributions of the pt-nuclei are different each other.

ATGTTTCATAGCTTATCTTACAGCGGGGGCACCTGACTTAAACACTACCAAACAAGCGTTA	60
M F I A Y L T A G A P D L N T T K Q A L	20
TTAAACTTGGCGAATGATGGGGCAGATGTTATAGAAATAGGTGTCCTTATTCAGATCCT	120
L N L A N D G A D V I E I G V P Y S D P	40
TTAGCTGATGGAATGATTTTGC AAAAGCCTCACAACAGGCGTTAAAAAATGGATTTTCGT	180
L A D G M I L Q K A S Q Q A L K N G F R	60
TTAGAACATTTGTGGCATCTGCTAGCAGAAATAGAACTGCCTGTTCTGTAGTGATTTTA	240
L E Q L W H L L A E I E L P V P V V I L	80
GCGTATTACAACCAAAATTTTTCATTATGGTGTAGAAAAATGGGTGACCACCTTAGTGAAT	300
A Y Y N Q I F H Y G V E K W V T T L V N	100
TTAAAGGTAAAAGCCTTGATTGTGCCTGACTTACCTTATGAAGAAGCCGCCATTTTAAGA	360
L K V K A L I V P D L P Y E E A A I L R	120
GCAGCTTGTGCTCGACACCATTTACATATGATCTGGCTAATCTCTCCCACTACACCAAAA	420
A A C A R H H L H M I W L I S P T T P K	140
GTTAGAGCGAAACAATTAGCATTGGCATGTGATGATTGGATTATTTAGTAAGTCGTACC	480
V R A K Q L A L A C D D W I Y L V S R T	160
GGAGTCACAGGAGTAGATGCCCATTTTGATAACCAGATCCCTAATATGATAGCCGAATTA	540
G V T G V D A H F D N Q I P N M I A E L	180
AAACAAGTGACCACAACACCTATCGCATTAGGATTTGGGATTCACCAGAAACAACAATTA	600
K Q V T T T P I A L G F G I H Q K Q Q L	200
CAATTAGTAAAATCTTGGGGAGCGGATGGAGTCATTATAGGCACAGCATGCATGCAAATT	660
Q L V K S W G A D G V I I G T A C M Q I	220
TTATTAGAAAAAGGAGTAGATCAATTAACAGAATGGATAAGTGCGATGAAAAATCATCT	720
L L E K G V D Q L T E W I S A M K K S S	240
TACCCGTAG	729
Y P *	242

Fig. 2. Nucleotide sequence and deduced amino acid sequence of *trpA* on the plastid genome of *C. caldarium* RK-1. The number of nucleotide is shown on the right side with the start codon of *trpA* numbered 1. The numbering of amino acids is shown with the first amino acid residue of the tryptophan synthase numbered 1.

Then we examined the *rbcL* gene to show the difference between two algae at the molecular level. We cloned the DNA fragment which contained *rbcL* and *rbcS* from *C. caldarium* RK-1. In the fragment, we found an open reading frame that corresponds to *trpA* on the plastid genome. It encodes 242 amino acid residues, as indicated by its homology to *trpA* in other organisms. The gene is located 3.2 kbp upstream of *rbcL*. The nucleotide sequence and the deduced amino acid sequence of the *trpA* are shown in Fig. 2.

The deduced amino acid sequence encoded by *trpA* is compared to those encoded by analogous genes in *Escherichia coli* (Guest *et al.* 1967) and *Saccharomyces cerevisiae* (Zalkin

and Yanofski 1982). The three proteins have consensus sequences, which are boxed in the Fig. 3. As the tryptophan synthase in *E. coli* is functional, the results suggest the tryptophan synthase encoded on the plastid genome of *C. caldarium* RK-1 should also be functional.

To confirm that the *trpA* of *C. caldarium* RK-1 is encoded on the plastid genome, Southern hybridization analysis was performed. Cell-nuclear and chloroplast DNA was digested with *Pst*I or *Hind*III, subjected to electrophoresis and blotted to a nylon membrane. The probe hybridized to the plastid DNA of *C. caldarium* RK-1. The *trpA* of *C. caldarium* RK-1 was shown to be encoded on the chloroplast genome (Fig. 4). However, the *rbcL* and *rbcS* hybridized to both plastid DNA at 5.5 kbp of *C. caldarium* RK-1 and at 2.3 kbp of *C. caldarium* M-8, but *trpA* hybridized only to that of *C. caldarium* RK-1 (Fig. 5). The results show that the *rbcL* and *rbcS* are encoded on the chloroplast genomes of *C. caldarium* RK-1 and M-8, but *trpA* only on the chloroplast genome of *C. caldarium* RK-1.

Cc	MFIAYLTAGAPDLNTTKQALLNLANDGADVIEI
Ec	MERYESLFAQLKERKEGAFVFPFVTLGDPGIEQSLKIIDTLIEAGADALEL
Sc	MSEQLRQTFANAKKENRNALVTFMTAGYPTVKDTPVILKGFQDGGVDIIEI
Cc	GVPTSKPLADGMILQKASQQALKNGFRLEQLWHLLAEI--ELP-VPVVILA
Ec	GIPFSDPLADGPTIQNATLRAFAAGVTPAQCFEMLALIRQKHPTIPIGLLM
Sc	GMPFSDPIADGPTIQLSVTVALQNGVTLPQTLEMVSQARNEGVTVPILMG
Cc	YYNQIFHYGVEKVVTTLVNLKVKALIVPDLPTEEAAILRAACATHHLMIW
Ec	YANLVFNKGIDEFYAQCEKVGVDVSVLVADVPVEESAPFRQAALRHNVAPIF
Sc	YYNPILNYGEERFIQDAKAGANGFIIVDLPPEEALKVRNYIVDVGLSLIP
Cc	LISPTTPKVRAKQLALACKKWIYLVSRGTGVKAHFDNQIPNMIAE-LKQ
Ec	ICPPNADDLLRQIASYGRGYTYLLSRAGVTGAENRAALPLNLVAK-LKE
Sc	LVAPSTTDERLELLSHIADSFVYVVSMTGTGVQSSVASDLDELISVRKY
Cc	VTTPPIALGFGIHQKQQLQVKSAGDGVII GTACMQILLEKGVDTLQTEWI
Ec	YNAAPPLQGFGISAPDQVKAADAGAAGASGSAIVKII EQHINEPEKMLA
Sc	TKDTPPLAVGFGVSTREHFQSV-GSVADGVVIVSKIVTLCGDAPEGKRYDVA
Cc	SAMKKSSYP
Ec	ALKVFVQPMKAATRS
Sc	KEYVQGILNGAKHKVLSKDEFFAFQKESLKSANVKKEILDEFD

Fig. 3. Comparison of the α subunit of tryptophan synthase. The deduced amino acid sequence of the tryptophan synthase α subunit from *C. caldarium* RK-1 (Cc) was compared with those from *Escherichia coli* (Ec; Guest *et al.* 1967) and *Saccharomyces cerevisiae* (Sc; Zalkin and Yanofsky, 1982). The highly conserved regions are boxed. The percentages of amino acid residues in the proteins from *E. coli* and *S. cerevisiae* that are homologous to those from *C. caldarium* RK-1 (%) are shown after the sequences.

Discussion

In plants, tryptophan is synthesized via the shikimic acid pathway in plastids (Buchholz *et al.* 1979). Tryptophan is especially important in higher plants, because it is a precursor of

Fig. 4. Southern hybridization analysis of *trpA*. The cell-nuclear (nc) and plastid (pt) DNAs were digested with *Pst*I (P) or *Hind*III (H), and subjected to electrophoresis on a 1% agarose gel and blotted onto a nylon membrane. Left 4 lanes are etidium bromide stained restriction endonuclease digestion patterns and right 4 lanes Southern hybridization patterns, respectively. The probe of *trpA* hybridized to plastid DNA at 3.4 kbp by the digestion of both *Pst*I and *Hind*III.

Fig. 5. Southern hybridization analysis of *rbcL* (*rbcL*) and *trpA* (*trpA*) cloned from the plastid genome of *C. caldarium* RK-1 (R) and strain M-8 (M). Left 2 lanes are etidium bromide stained restriction endonuclease digestion patterns and right 4 lanes are Southern hybridization patterns, respectively. Their DNAs were digested with *Hind*III and hybridized to the gene probes of *rbcL* and *trpA* of *C. caldarium* RK-1. The *rbcL* hybridized to both plastid DNAs at 5.5 kbp of *C. caldarium* RK-1 and at 2.3 kbp of *C. caldarium* M-8, but *trpA* hybridized only to that of *C. caldarium* RK-1.

the plant auxin, indole acetic acid. The terminal reactions involving synthesis of tryptophan are identical in form and sequence in all procaryotes and eukaryotes examined (Gilchrist and Kosuge 1980). The gene for tryptophan synthase in plant is known in *Arabidopsis thaliana* and it has putative chloroplast transit peptides in N-terminal (Berlyn *et al.* 1989). Given that tryptophan synthase is used in plastids, it is not unreasonable that *trpA* should be encoded on the plastid genome of *C. caldarium* RK-1 and *C. merolae* (unpublished data).

The relocation of genes from the plastid genome to the cell nucleus is thought to have occurred as evolutionary gene transfer at very early stage of the appearance of primary eucaryotes, because the relocation of *trpA* appeared to occur during an evolution from *C. caldarium* RK-1 into *C. caldarium* M-8. The genes known so far to have been transferred from the plastid genome to the cell nucleus in the line of Chlorophyta are *tufA* (the gene for the chloroplast protein synthesis elongation factor Tu; Baldauf and Palmer 1990), *rp121* (the gene for ribosomal protein L21; Martin *et al.* 1990), *chlL* (the gene for chlorophyll synthase; Yamada *et al.* 1992) and *rp122* (the gene for ribosomal protein L22; Gantt *et al.* 1991).

There are some genes that are not encoded on the Chlorophyte plastid genomes but are encoded on the Rhodophyte and Chromophyte plastid genomes. Such genes known so far are *rbcS* (the gene for the small subunit of ribulose 1,5- biphosphate carboxylase/oxygenase; Palmer 1985), *hsp70* (the gene for heat-shock protein 70; Reith and Munholland 1991, Scaramuzzi *et al.* 1992) and the gene for acyl carrier protein (Hwang and Tabita 1991). The presence of these genes may be an indication that these genes have not yet been transferred to the cell nucleus in Rhodophyta and Chromophyte.

C. caldarium RK-1 is protoflorideophyceous alga and its plastid genome should encode several genes that have relocated to the cell nucleus in advanced members of plants.

Summary

We examined the organization of organelle DNA in the two algae, *Cyanidium caldarium* RK-1 and *C. caldarium* M-8 with epifluorescence microscopy and molecular-biological techniques. The plastid nucleus (pt-nucleus, a complex of DNA and proteins) is located in the central area of the plastid in *C. caldarium* RK-1, while the ring-shaped pt-nucleus is located at the periphery of the petal-like plastid in *C. caldarium* M-8. The results suggest that *C. caldarium* RK-1 markedly differs from *C. caldarium* M-8 in an evolutionary tree.

To confirm the result, we examined the fragment that contained *rbcL*. The *trpA* (the gene for the α subunit of the tryptophan synthase), which was located on the vicinity of the *rbcL*, was found on the plastid genome of the "primitive" unicellular red alga, *C. caldarium* RK-1, while the gene could not be found on the plastid genome of the *C. caldarium* M-8 (*Galdieria sulphuraria*). This is the first example of tryptophan synthase encoded on the plastid genome. The results suggest that the *trpA* gene may have relocated from the plastid genome to the cell-nuclear genome for longer ago than the genes that have been considered to date to have been lost from plastid genomes in the advanced members of plants.

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