Residual plastids of bleached mutants of *Euglena gracilis* and their effects on the expression of nucleus-encoded genes*

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Abstract Bleached mutants of *Euglena gracilis* were obtained by treatment with ofloxacin (Ofl) and streptomycin (Sm) respectively. As shown by electron microscopy, the residual plastids contain prothylakoids in an Ofl mutant, and the highly developed and tightly stacked membranous structure found in cells of two Sm mutants. Nine genes of the plastid genome were examined with PCR, showing that ribosomal protein genes and most other plastid genes were lost in all but one Sm mutant. Using differential display and RT-PCR, it was shown that chloroplast degeneration could cause changes in transcription of certain nucleus-encoded genes during heterotrophic growth in darkness.

Keywords: *Euglena gracilis*, bleached mutant, residual plastids, tightly stacked membranous structure, nucleus-encoded genes, regulation.

Euglenoids are a group of single-celled and flagellated algae. Their chloroplasts have 3 layers of envelope membranes, chlorophyll a and b, and are thought to have originated from secondary symbiosis of a single-celled green alga[1,2]. When treated by some mutagens, antibiotics or environmental factors, *Euglena gracilis* could permanently lose its chloroplasts and produce bleached mutants, even though most of them still retain residual plastids and partial plastid genome[2~4]. Such bleaching mutations led to the origin of some colorless saprophytic species, for example, *Astasia longa*, a close relative to *E. gracilis*, which retains merely half of the plastid DNA and loses all photosynthetic genes except *rbcL*[5].

Like higher plants, chloroplasts of euglenoids are unable to form thylakoids and photosynthetic systems and present as proplastids in complete darkness, regain thylakoid membrane when transferred back to the light[6]. Residual plastids, usually resembling proplastids of the wild type, produce only prothylakoids when induced by light. Some whorl-like membranous structure would appear at an early stage of the biogenesis process[7]. Most genes that determine photosynthetic systems and biogenesis of chloroplasts are located in nuclei, regulated by light and the plastid status. Loss of plastid function in euglenoids may affect the expression of some nucleus-encoded photosynthetic genes. For instance, genes *petJ* and *psbO* are not transcribed in an ofloxacin (Ofl)-induced mutant in the light[8]. However, there is no conclusive study on whether loss of plastid DNA affects the expression of nuclear genes in darkness. In this study, electron microscopic observations of an Ofl- and two streptomycin (Sm)-induced bleached mutants showed the presence of plastid remnants in cells, and especially, a type of tightly stacked membranous structure in Sm mutants. Using a method of mRNA differential display, we proved that deletions in plastid genome of *Euglena* could influence the transcription of some nuclear genes in darkness.

1 Materials and methods

1.1 Strains and culture condition

E. gracilis FACHB47 was obtained from the Freshwater Algae Collection of Institute of Hydrobiology and its axenic monoclones were derived by serial dilution and plating. *Euglena* cells were grown in Polytomella medium[9] under the condition of 14h/10 h light (1000 lux)/dark or complete darkness at 25 ℃. Solid plates were supplemented with 1.2% agar.

1.2 Mutant selection and electron microscopy

Ofloxacin or streptomycin treatment of *E. gracilis* and selection of mutants were performed as de-

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scribed previously[3, 10, 11], but Polytomella liquid medium was used instead. Briefly, ofloxacin- or streptomycin- treated cells were spread on agar plates with Polytomella medium (1.2% agar). After about 7 days, white colonies were picked and transferred to fresh liquid medium. Those clones shown to be stable by repeated transfer and plating were selected for further studies. The fixation and preparation of samples for transmission electron microscopy were performed as the procedure described in Ref. [12], observations were carried out on a Hitachi H-7000 FA electron microscope and micrographs were taken.

1.3 Examination of plastid genome with PCR

A previous procedure[13] was followed for total DNA exaction. Nine pairs of PCR primers for *Englena* chloroplast genome are listed in Table 1. The PCR amplification was started with denaturation at 94 ℃ for 5 min, followed by 30 cycles of 1 min at 94 ℃, 55 s at 58 ℃ and 55 s at 72 ℃, and a final extension of 7 min at 72 ℃. PCR products were detected with 1.6% agarose electrophoresis.

Table 1. Sequences of primers (5′→3′)

16srRNA-1	gtggtgcatggctgtcgtca
16srRNA-2	cacctccagtacggctac
rpoB-1	gaaatgcttaggcctatccc
rpoB-2	accctaaagcgacctacttc
psaA-1	ggagatcttgtggtattacatctg
psaA-2	accataaaccaccccgttac
psbD-1	tggttcgacgtagcagacga
psbD-2	tgaaactgctgctgtcaaag
rps9-1	ttgtccagttctttttgaggg
rps9-2	ggtattactcggtcataacgct
rpl16-1	gtcctaagcgaacgaagtttc
rpl16-2	ccaaccaggctctaatgattg
rbcL-1	caagtggtgtgcgtttgcag
rbcL-2	caaatccacacggcaggaac
atpE-1	tcgttttttggggatttattctc
atpE-2	ctttaagaaccccatctgacc
rpB2-1	ggcggttccaaaaaagaaaatgtcca
rpB2-2	ctattgtggtaatttgtctcc
actin-1	gtgcttctcacagaggca
actin-2	aactgcctgaatgctgaca
WD1-1	tctacgcgtggtgcctgg
WD1-2	gagaagcaggccattcac
WD10-1	ttggcgctgttgtgttgg
WD10-2	agagaaagatggtggtga
WD12-1	agaagaccgcaagcccag
WD12-2	gggacaagagtcaagttgaaagtgaacaagcga
linkerB-1	ttttgtagacattctagtatctcgtcaagtcggaagggacatg
linkerB-2	tcccttccgacttgacgagatactagaatgtctacaa
PrimerA	gtagacattctagtatctcgt
D1	ttttttttttttttcag

To be continued

Continued

D2	ttttttttttttttctg
D3	ttttttttttttttcac
D4	ttttttttttttttctc
D5	ttttttttttttttcca
D6	ttttttttttttttcct
D7	ttttttttttttttccg
D8	ttttttttttttttccc
D9	ttttttttttttttcga
D10	ttttttttttttttcgt
D11	ttttttttttttttcgg
D12	ttttttttttttttcgc

1.4 Selective differential display of 3′-end restriction fragments of cDNA

Total RNA was prepared from the wild type *E. gracilis* and its bleached mutants by a method of Guanidine/phenol-chloroform extraction (Liu, 2000, http://mutant.lse.okstate.edu/chunming/arabidopsisrna.html). mRNA was isolated with PolyA-Tract mRNA Isolation System III system Kit (Promega).

The first strand of cDNA was synthesized with biotinylated polyT and SuperScript II reverse transcriptase (Gibco BRL Life Technologies), the second strand of cDNA was synthesized using RNase H and DNA polymerase I. The double-stranded cDNA was completely digested with *Nla* III (New England Biolabs) and adsorbed with streptavidin-paramagnetic particles (SA-PMP) (Progema) to purify the biotin-labeled 3′-end cDNA fragments, which were then ligated with a linker, rinsed with 50 μL of 0.1× SSC and resuspended in 25 μL of ddH_2O. The linker was designed according to linker B described for serial analysis of gene expression (SAGE)[14]. One microliter of suspension of paramagnetic particles was used in PCR which consisted of 30 cycles of 30 s at 94 ℃, 45 s at 42 ℃ and 50 s at 72 ℃. The selective primers (dT15)CNN and primer A are listed in Table 1. PCR products generated with different pairs of primers for both wild type and the Ofl mutant were compared after separation on 2% agarose gel. Differentially expressed bands were purified, re-amplified, cloned into pMD18-T vector (Takara) and sequenced.

1.5 RT-PCR

cDNA from both wild type and mutants grown under conditions of light/dark or complete darkness were used for PCR amplification. The 3 pairs of PCR primers designed according to the sequencing results of differentially expressed cDNA are listed in Table 1.

Actin gene of *E. gracilis* was used as a control to adjust the initial concentration of template to nearly the same level. cDNA samples were then serially diluted in 2-fold and used for PCR. The profile of PCR consisted of 30 cycles of 55 s at 94 ℃, 55 s at 42 ℃ and 150 s at 72 ℃.

2 Results and discussions

2.1 Electron microscopic observations of mutant cells

In our previous study, the absence of chloroplast in an Ofl-induced mutant was noticed under light and fluorescence microscopes[11]. In this article, five stable bleached mutants of *Euglena gracilis* were obtained by treatments with Olf and Sm respectively. Neither chloroplast structure under the light microscope nor red chlorophyll auto-fluorescence under the fluorescence microscope was found in the ten mutants. However, using transmission electron microscopy, residual plastids and a plenty of paramylum were found in one Ofl and two Sm mutants grown under light/dark conditions (Fig. 1). Residual plastids of the Ofl mutant are usually close to mitochondria, inside which are peripherally arranged prothylakoids. Besides a large quantity of paramylum, there are also some homogeneous and dark stained particles in the cells. For the two Sm mutants, there are highly developed and tightly packed membranous structures that are single- or multi-centered and occupy most space within the region confined by the envelope of residual plastids, but there are very few homogeneous and dark stained particles in cells. Such extraordinary membranous structures have not been described in any previous literature, and are distinctive from the whorl-like membranes in both size and structure. Their identity and biogenesis process await further investigations. Under the same conditions, fully developed chloroplasts and fewer paramylon particles were found in the wild type cells (data not shown).

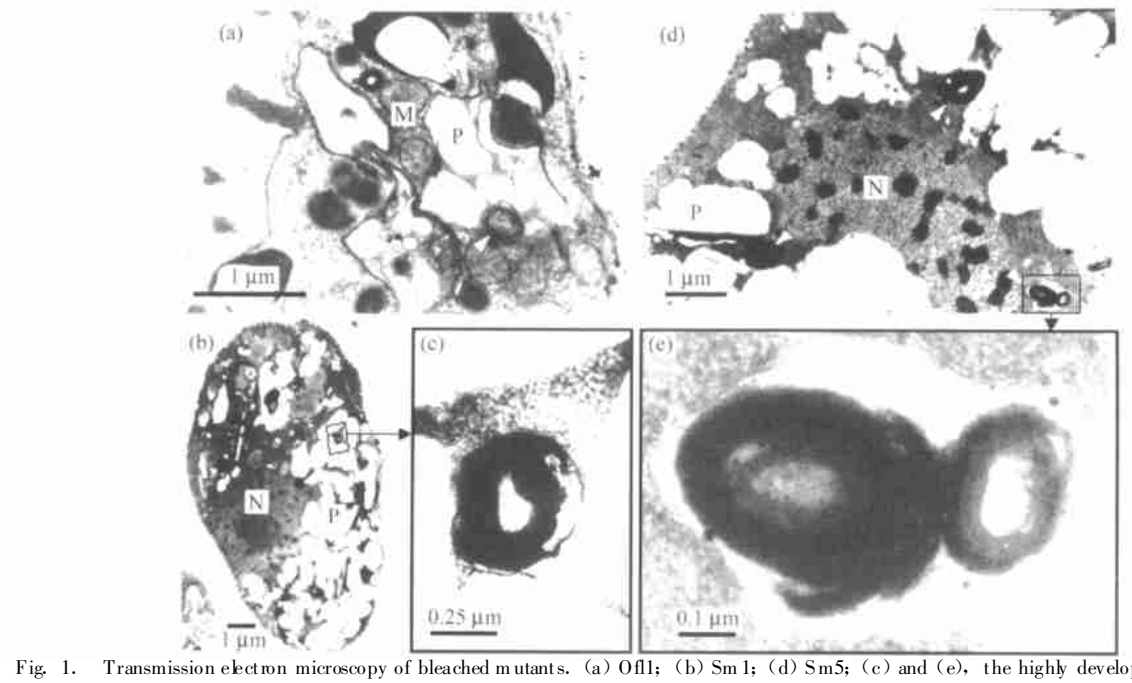

Fig. 1. Transmission electron microscopy of bleached mutants. (a) Ofl1; (b) Sm1; (d) Sm5; (c) and (e), the highly developed and stacked membranous structure. Arrows point to residual plastids. M, mitochondria; N, nucleus; P, paramylum.

2.2 Genomes of residual plastids

DAPI staining method could be employed to visualize plastid DNA of colorless saprophytic *Astasia longa*, but not that of Ofl mutants[11], which could be due to absence of or quite a low copy number of plastid DNA. Currently, the sequence of chloroplast genome[15] of *Euglena gracilis* has been available, and the extent of loss of plastid DNA could be well evaluated by PCR method. Nine genes in the circular chloroplast genome were chosen and PCR primers were designed. Using these primers and total DNA of wild type cells as the template, single bright PCR products of predicted sizes were produced for each of the genes. Detection of 5 Ofl and 5 Sm mutants with PCR showed that one mutant, Sm5, retained 16S rRNA gene, and *psbD*, *psaA*, *rpl16*, *rps9* and *rpoB* genes, while the rest mutants retained 16S

rRNA gene and the *rpoB* region but lost genes *rpl3*, *rbcL* and *atpE* (Fig. 2). In the plastid genome of *Euglena gracilis*, 16S rRNA gene and *rpoB* are next to the replication origin, therefore retained in most mutants. In a previous report, Southern blot hybridization result also showed the presence of ribosomal RNA gene region. An extra band was produced in PCR for 16S rRNA gene, which was shown to be an unspecific product by sequencing. Out of the 10 bleached mutants, residual plastids were found in Ofl1, Sm1 and Sm5 in the above electron microscopic observations. Comparison of Sm1 and Sm5 seemed to indicate that there is no direct relationship between the formation of tightly stacked membranous structure and the extent of deletions in plastid genome, while comparison of Sm1 and Ofl1 indicated that the arrangement of membrane could be different from each other given that both have a high extent of deletions in plastid genome. Genes *rpl16*, *rpl32* and *rps9* encode proteins of the large or small subunit of ribosome respectively, the absence of these genes provided indirect evidence that there is no functional ribosome and protein synthesis in the residual plastids, therefore the replication of plastid and its DNA should completely rely on nucleus-encoded genes.

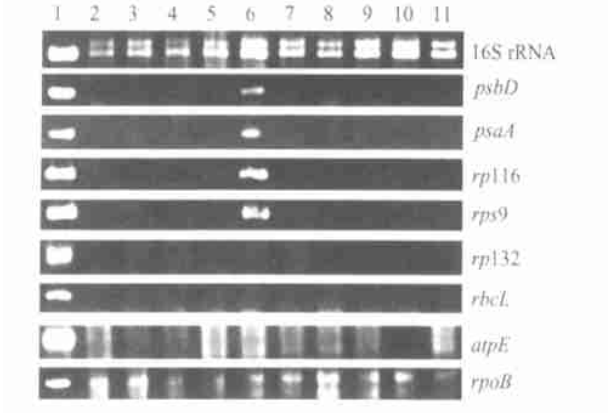

Fig. 2. Tests of plastid genome with PCR. 1, wild type; 2 ~ 6, streptomycin-bleached mutants Sm1 ~ 5 respectively; 7 ~ 11, ofloxacin-bleached mutants Ofl1 ~ 5 respectively.

2.3 Differential expression of nucleus-encoded genes

It has been documented that some photosynthesis genes are incapable of light-induced expression in bleached mutants of *E. gracilis*[6]. The residual plastids of mutants in this study lacked certain ribosomal protein genes; therefore, even under complete darkness they should be substantially different from the proplastids of wild type cells. Accordingly, certain nuclear genes could be altered in their expression. To look for evidence for differential expression of nuclear genes, we used purified poly-adenylated mRNA to conduct reverse transcriptions and compared the transcriptional level in wild type and mutant cells. With some modifications of a procedure for differential display of 3′-end cDNA fragments, some PCR fragments of presumptively differentially expressed genes were detected directly by electrophoresis on agarose gel and EB staining (Fig. 3). These DNA fragments were excised, purified and re-amplified, then followed by cloning and sequencing. According to their sequences, primers were designed for fragments WD1, WD10 and WD12 (Fig. 3) to evaluate their transcriptional levels with semi-quantitative RT-PCR, that is, to make comparisons by conducting PCR with serially diluted cDNA templates. The results showed that when both are grown in darkness, the transcriptional level of some genes were remarkably elevated in mutant Ofl1 compared to that in wild type (Fig. 4). Similar results were obtained when both were grown under light/dark conditions (data not shown), hence the effect of residual plastids on the transcriptional activity of these genes was independent of light induction. To exclude the possibility that mutations in nuclei or mitochondria that might have arisen during ofloxacin treatment changed the expression of some genes, the transcriptions of WD1, WD10 and WD12 were examined in the other two independent mutants Sm1 and Sm5 and showed comparable results. The transcription of WD1 in mutant Sm5 was seemingly lower than others. Whether it is due to certain difference in plastid DNA awaits further investigations. Because the transcription of genes underwent similar changes in 3 independent mutants, we conclude that the bleaching mutation of plastid was the direct cause for such changes. If more selective primers, e. g. (dT15)GNN and (dT15)ANN, were applied in our research, one can expect that more differentially expressed genes would have been identified. Because genes in *Euglena* are also regulated at translational and post-translational levels[8], change of transcription of certain genes might have reflected only one aspect of regulatory effects on nuclear genes exerted by plastids, hence the general differences of gene expression could be much more remarkable. The accumulation of large quantity of paramylum in mutants also reflects overexpression of genes involved in relevant synthesis process. Based on the results of this study, it is predictable that if strength of antibiotic treatments is further reduced, some mutants with deletion of a small portion of plas-

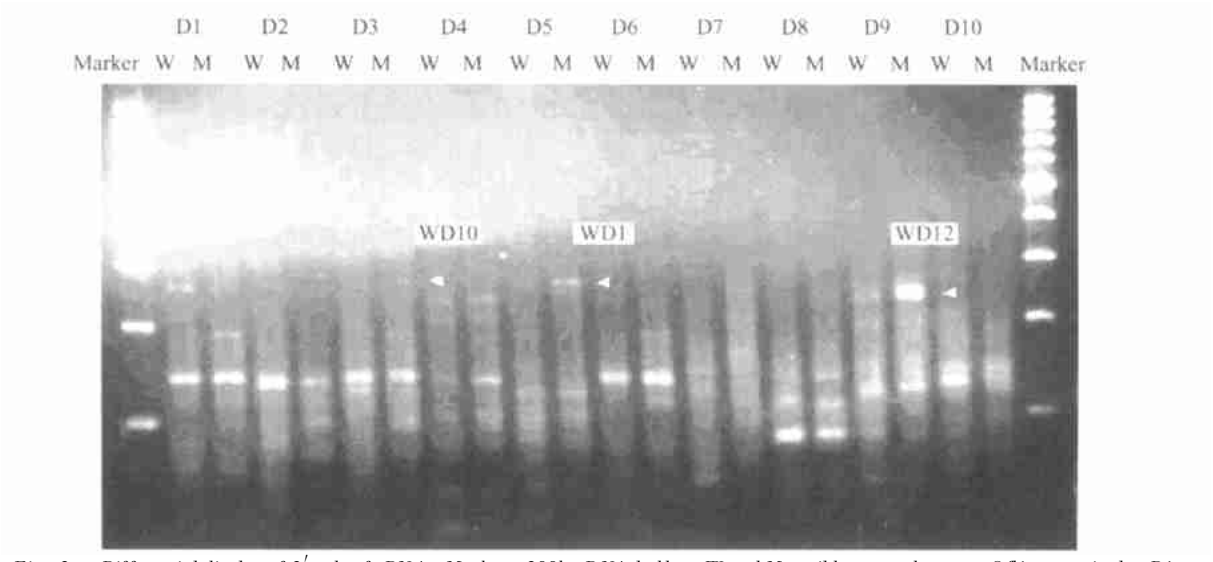

Fig. 3. Differential display of 3′ ends of cDNA. Marker, 200bp DNA ladder; W and M, wild type and mutant Ofl1 respectively; D1 ~ D10, selective primers, sequences are listed in Table 1. Arrows point to fragments of WD1, WD10 and WD12.

tid DNA or within a single gene could be obtained. By relating mutations in plastid genome to light-induced biogenesis of plastid and expression of nuclear genes, a research model might be developed from euglenoids for nucleus-plastid signal transductions and plastid biogenesis.

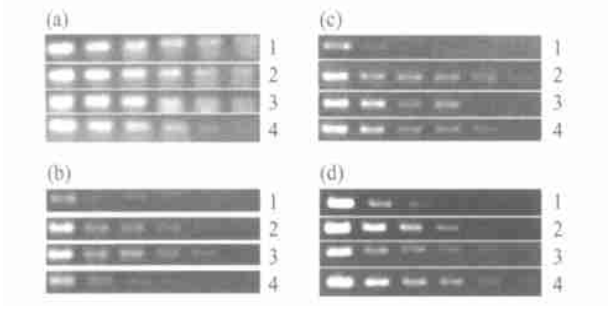

Fig. 4. The transcription of actin(a), WD1(b), WD10(c) and WD12(d) of *Euglena gracilis* in complete darkness as shown with semi-quantitative RT-PCR. cDNA from wild type and mutants were serially diluted by 2 fold for PCR amplification. 1, wild type; 2, Ofl1; 3, Sm1; 4, Sm5.

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