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# Expression of Nucleus-Encoded Genes for Chloroplast Proteins in the Flagellate *Euglena gracilis*

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ABSTRACT. Reverse transcription PCRs (RT-PCRs), real-time RT-PCRs and microarrays containing 50-mer oligonucleotides representing nucleus-encoded genes for chloroplast proteins from *Euglena gracilis* were used to compare light- and dark-grown wild-type mRNA levels to those of light- and dark-grown *E. gracilis* stable white mutant strains $W_{gm}ZOflL$, $W_3BUL$ and $W_{10}BSmL$. The analyses revealed no light-dependent regulation of mRNA levels. Moreover, the mRNA levels of most genes were unchanged in all white mutants in comparison with wild-type. These results suggest that mRNA levels of nucleus-encoded genes for chloroplast proteins in *E. gracilis* do not depend on either light or plastid function.

**Key Words.** Bleached mutants, chloroplast-to-nucleus signaling, Euglenozoa, light-dependent regulation, microarrays, plastids, real-time RT-PCR, transcription.

*EUGLENA gracilis* belongs to the order Euglenida that forms, together with the orders Kinetoplastida and Diplonemida, the protist phylum Euglenozoa. These organisms share various morphological features, such as discoidal mitochondrial cristae, a characteristic feeding apparatus (Simpson 1997; Triemer and Farmer 1991), and various molecular traits, such as the presence of modified base ''J'' in nuclear DNA (Dooijes et al. 2000) and the addition of non-coding capped spliced-leaders to cytoplasmic mRNAs (Bonen 1993; Liang et al. 2003). Molecular phylogenies recover monophyly of Euglenozoa (Simpson and Roger 2004), although it seems that diplonemids and kinetoplastids are more closely related to each other than to euglenids (Breglia, Slamovits, and Leander 2007; Simpson and Roger 2004).

Within the Euglenozoa, some euglenids contain plastids. These are surrounded by three membranes (Lefort-Tran et al. 1980) and arose by a secondary endosymbiotic event in which the ancestor of euglenids engulfed a eukaryotic green alga (Ahmadinejad, Dagan, and Martin 2007; Gibbs 1978; Morden et al. 1992). Chlorarachniophytes, which contain plastids with four envelope membranes harboring a nucleomorph, and euglenids obtained their plastids via two independent events of secondary symbiosis involving green algal endosymbionts (Rogers et al. 2007).

The treatment of *E. gracilis* with xenobiotics affecting prokaryotic translation, transcription and DNA replication can result in the loss of chloroplast DNA reflecting the cyanobacterial ancestry of *E. gracilis* chloroplast genome (see review by Krajčovič, Ebringer, and Schwartzbach 2002). This process is accompanied by irreversible elimination of functional chloroplasts (bleaching phenomenon). Various stable bleached mutant strains incapable of photosynthesis exist (Polónyi et al. 1998; Schiff, Lyman, and Russel 1971).

Many euglenoid species are heterotrophic and a few are even parasitic (Leedale 1967). At least five branches within the Euglenophyceae have lost photosynthesis (Marin 2004; Marin et al. 2003). Among the heterotrophic euglenids, *Astasia longa*—*Euglena longa* according to Marin et al. (2003) and Triemer et al. (2006)—is a colorless relative of *E. gracilis* that possesses a circular 73-kb plastid DNA (Gockel and Hachtel 2000), which is about half the size of *E. gracilis* chloroplast DNA (143 kb) (Hallick et al. 1993). Chloroplast genes for photosynthesis-related proteins are absent from *E. longa* plastid DNA except for *rbc*L, the gene encoding ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Gockel and Hachtel 2000).

In higher plants, plastid-derived signals can affect the expression of chloroplast-encoded genes (Puthiyaveetil et al. 2008) and the expression of nuclear genes that encode chloroplast proteins (Nott et al. 2006). A functional plastid transcription apparatus was shown to be necessary for the transcription of nucleus-encoded genes *Lhcb* (formerly *cab* gene, encoding light-harvesting chlorophyll *a/b*-binding protein of photosystem II—LHCPII) and *Rbc*S (encoding ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit) in wheat and barley (Rapp and Mullet 1991). Similarly, ribosome-deficient plastids repress transcription of light-induced nuclear genes in higher plants (Hess et al. 1994). Recent experiments with the gun mutants of *Arabidopsis thaliana* suggest that accumulation of Mg-protoporphyrin, the first committed precursor of chlorophyll, is involved in the regulation of transcription of nuclear genes by chloroplasts (Larkin et al. 2003), in line with earlier findings on *Chlamydomonas reinhardtii* (Johanningmeier 1988; Johanningmeier and Howell 1984). Experiments in plants and algae have shown that the transcription of several light-inducible nuclear genes, including *Lhcb*, *Rbc*S, and *Apx*—ascorbate peroxidase, is dependent on photosynthetic electron transport (Escoubas et al. 1995; Pfannschmidt et al. 2001; see reviews by Nott et al. 2006; Rodermel 2001). Photosynthetic electron transport also affects chloroplast gene transcription in *Arabidopsis* via a redox-dependent sensor kinase (Puthiyaveetil et al. 2008).

In *Euglena*, however, the situation appears to differ somewhat with respect to the influence of plastids on nuclear gene expression. The Northern hybridization experiments showed that mRNA levels of nuclear photosynthetic genes *Lhcb*, *Rbc*S and *Pbgd* (porphobilinogen deaminase) are similar in wild-type cells and non-photosynthetic *E. gracilis* white mutants (Vacula et al. 2001). In addition, constant levels of *Apx* transcripts were reported in *E. gracilis* during light adaptation while *Apx* mRNAs in white mutants and in the wild-type were comparable (Madhusudhan et al. 2003).

In this study, we explored the influence of plastid functionality and light/dark growth conditions on the expression of 76 nuclear genes from *E. gracilis*, of which 64 encode chloroplast proteins, to determine the influence of chloroplasts on the expression of nucleus-encoded genes. Furthermore, we specifically compared

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mRNA levels of *Lhcb* (*Lhcbm1* according to Koziol and Durnford 2008), *Rbc*S, *Pet*J (cytochrome $c_6$), *Psb*O (OEC30) and *Psb*W (13 kDa OEC protein W) in light- vs. dark-grown *E. gracilis* wild-type cells, as well as mRNA levels of these genes in wild-type *E. gracilis* vs. those of three different stable white mutants.

## MATERIALS AND METHODS

**Strains and nucleic acid extraction.** *Euglena gracilis* Pringsheim var. *Z* (wild-type, strain *Z*) was kindly provided by Prof. E. Stutz (University of Neuchâtel, Neuchâtel, Schwitzerland). Three *E. gracilis* stable bleached mutant strains with non-functional chloroplasts were used in this study: $W_3BUL$, $W_{10}BSmL$ and $W_{gm}ZOflL$. The mutants $W_3BUL$ and $W_{10}SmL$ were kindly provided by Prof. S.D. Schwartzbach (University of Memphis, Memphis, TN). The stable mutant $W_3BUL$ was induced by UV exposure of *E. gracilis* Klebs var. *bacillaris* Cori (Schiff et al. 1971). It contains an eyespot with carotenoids and plastid remnant with thylakoid membrane-specific sulfolipid (Schiff et al. 1971). The treatment of *E. gracilis* strain *Z* with *N*-succinimide derivative of ofloxacin produced the stable bleached mutant $W_{gm}ZOflL$, which possesses a stigma with carotenoids (Polónyi et al. 1998). $W_{gm}ZOflL$ also possesses permanently altered mitochondria, some about 5–8 µm long and wide having an unusual shape, others giant disfigured with unusual shape, giant swollen oval-shaped megamitochondria, and some very long (50–60 µm) mitochondria are also seen in addition to normal mitochondria (Polónyi et al. 1998). The stable mutant $W_{10}BSmL$ was induced by streptomycin treatment of *E. gracilis* Klebs var. *bacillaris* Cori and it lacks detectable carotenoids, eyespot and sulfolipids (Schiff et al. 1971). It is currently unknown whether mutant $W_{10}BSmL$ has a plastid remnant or not. Its plastid cannot be identified by light microscopy as it can be identified in $W_3BUL$ or $W_{gm}ZOflL$, and our unpublished PCR results suggest that it has no plastid DNA. Cultures were grown statically in modified liquid Cramer–Mayers medium pH 6.9 (Buetow and Padilla 1963) at 26 °C under constant light conditions (using cool white light, 30 µmol photons/m$^2$/s) and in the dark. All four strains were grown under both permanent dark and permanent light conditions. Total RNAs were isolated from 50 ml of both light- and dark-grown cultures in the exponential growth phase with densities of $5 \times 10^5$ cells/ml. Total RNA and DNA were isolated using TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA). Total RNAs were isolated twice independently from each strain (twice from light- and twice from dark-grown cells). Total RNA from the first isolation was used for mRNA isolation. The mRNA fraction was isolated from 100 µg of total RNA using PolyATtract mRNA Isolation Systems III (Promega Corp., Madison, WI). mRNAs were used for RT-PCRs and real-time RT-PCRs. Total RNAs from different independent RNA isolations (the second RNA isolation from each strain grown in the light as well as in the dark) were used for the microarray experiments.

**cDNA synthesis and RT-PCRs.** cDNA synthesis was performed using ImProm-II Reverse Transcription System (Promega) for 1 h. Sixty nanograms of mRNA were used as a template for cDNA synthesis in each reaction of 20 µl final vol. The final concentration of $Mg^{2+}$ in cDNA synthesis reactions was 6 mM. Five microliters of cDNA reaction were used as template in RT-PCRs and 400 ng of total DNA from *E. gracilis* were used as a template in PCRs. The PCRs and RT-PCRs were performed in 100 µl reaction vol. with the final concentration of $Mg^{2+}$, primers and dNTPs as 2 mM, 0.2 µM and 0.5 mM, respectively. Five units of Taq DNA polymerase (Invitrogen) were used per reaction. Samples were denatured by heating for 5 min at 94 °C, subjected to 34 cycles of 1-min denaturation at 94 °C, 1-min annealing at 58 °C and 2-min extension at 72 °C, and a final cycle of 8 min at 72 °C. Sequences of primers used were: 5′-AACCACCTTCAACTC CATCAA-3′ (Eg*act*for), 5′-TAGAAGCACTTGCGATGCAC-3′ (Eg*act*rev), 5′-AACATCTTCCAGCTGACC-3′ (*EgLhcb*for), 5′-TATAAAGCACCATTTTCATGGTCC-3′(*EgLhcb*rev), 5′-CAT GTGAACGGAGGAAAC-3′ (Eg*Pet*Jfor), 5′-AATAGGGAAGG GATCGAC-3′ (Eg*Pet*Jrev), 5′-TACGACGAACTTCAGTCC-3′ (Eg*Psb*Ofor), 5′-GTCACCACCAACATTCAG-3′ (Eg*Psb*Orev), 5′-CGAGGGGTTTGGTTATCT-3′ (Eg*Psb*Wfor), 5′-AGTACATC CGTGCTGGTCT-3′ (Eg*Psb*Wrev), 5′-TAAGGCGGCTTGTGT GTCTGT-3′ (Eg*Rbc*Sfor), 5′-CACAAACCACAGTTGTAGCTG-3′ (Eg*Rbc*Srev).

**Real-time RT-PCR.** Real-time RT-PCRs were performed using a ABI Prism 7900 HT Fast thermocycler (Applied Biosystems, Foster City, CA) with the use of a Power Sybr Green Master Mix (Applied Biosystems). The identical primers as for RT-PCR were exploited. The concentrations of primers were optimized to obtain similar reaction efficiency (90–110%); the final concentrations were as follows: 0.125 µM for genes *Pet*J and *Psb*W; 0.25 µM for genes *Act* and *Rbc*S; and 0.5 µM for genes *Lhcb* and *Psb*O. Six microliters of 50 times as well as 100 times diluted cDNA were used in 20 µl total vol. In real-time RT-PCR, samples were denatured by heating for 10 min at 94 °C, subjected to 40 cycles of 30-s denaturation at 94 °C, 1-min annealing at 58 °C and 2-min extension at 72 °C. All measurements (using 50 times diluted as well as 100 times diluted cDNAs as templates) were performed in duplicate or triplicate with standard deviations between parallels below $0.3C_t$ (cycle threshold). The level of expression of particular genes in different cDNA samples was calculated based on the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001; Pfaffl 2001). The expression of the *Act* gene (actin) was used to normalize the calculations, as in previous real-time PCR experiments monitoring the *E. gracilis* expression levels in response to stress treatments (Deloménie et al. 2007). Light-grown *E. gracilis* wild-type samples were used as the reference samples for dark-grown wild-type samples as well as for all light-grown mutant samples. Dark-grown wild-type samples were used as the reference samples for all dark-grown mutant samples. Expression ratios were obtained from the total number of four to six independent measurements (using 50 times diluted cDNAs as templates in duplicate or triplicate, and using 100 times diluted cDNAs as templates in duplicate or triplicate). Calculated average values together with standard deviations were used for final evaluation. Only those genes with expression ratios $\leq 0.5$ or $\geq 2$ were scored as being differentially expressed.

**Microarrays.** The DNA oligoarrays (MWG Biotech, Ebersberg, Germany) contained 50-mers of *E. gracilis* cDNA sequences derived from an EST library (Ahmadinejad et al. 2007). Sixty-four sequences from the total of 76 *E. gracilis* sequences on the arrays represented nucleus-encoded genes for chloroplast proteins (Table 1, supporting Table S1). Negative controls included $H^+/K^+$-ATPase α2b subunit (Accession no. AAB93902) and vacuolar ATPase subunit F (Accession no. NP_446336), both from *Rattus norvegicus*. Positive controls included ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Accession no. NP_198659) from *A. thaliana* and three ESTs found to be expressed in *E. gracilis* under aerobic conditions (Ahmadinejad et al. 2007) and represented in other euglenid EST projects searchable at GenBank. All sequences were printed in duplicate, yielding a total of 160 spots on each slide. The comparisons of expression profiles were performed as listed on the Table 2.

*Labeling.* Direct labeling of 25 mg total RNA was performed using Cy3- or Cy5-dCTP according to the protocol from the Microarray Center at the Ontario Cancer Institute, University Health Network, Toronto, ON, Canada, with subsequent isopropanol precipitation for probe cleanup.

*Microarray hybridization.* Hybridization was performed at 45 °C for 18 h with DIG Easy Hyb solution (Roche, Basel,

Table **1.** The list of BLASTX hits of 76 ESTs from *Euglena gracilis* (Ahmadinejad et al. 2007) whose expression was studied using microarrays.

| Putative gene product | Organism with the top BLASTX hit | e-value |
|---|---|---|
| Asparagine synthetase | *Helianthus annuus* | 1e − 49 |
| Glutamine synthetase | *Prevotella melaninogenica* | 5e − 21 |
| Glutamine synthetase | *Chaetoceros compressum* | 2e − 26 |
| Hypothetical protein b3830 | *Escherichia coli* | 2e − 11 |
| Pyrroline-5-carboxylate reductase | *Actinidia chinensis* | 1e − 19 |
| Tryptophan synthase β subunit | *Thermotoga maritima* | 6e − 57 |
| ATP synthase γ chain | *Phaeodactylum tricornutum* | 1e − 20 |
| Carbonic anhydrase | *Deinococcus radiodurans* | 2e − 18 |
| Hypothetical protein | *Nicotiana tabacum* | 2e − 05 |
| Putative thylakoid lumen rotamase | *Arabidopsis thaliana* | 1e − 52 |
| FKBP-type peptidyl-prolyl *cis–trans* isomerase | *Nostoc* sp. | 2e − 17 |
| 5-aminolevulinate synthase | *Euglena gracilis* | 2e − 41 |
| Glutamate-1-semialdehyde 2,1-aminomutase | *Nicotiana tabacum* | 2e − 26 |
| A chain of Schiff-base complex of ALADH | *Saccharomyces cerevisiae* | 6e − 21 |
| Porphobilinogen deaminase | *Euglena gracilis* | 1e − 60 |
| Coproporphyrinogen III oxidase | *Chlamydomonas reinhardtii* | 9e − 33 |
| Mg-protoporphyrin IX methyltransferase | *Arabidopsis thaliana* | 4e − 65 |
| Mg-chelatase subunit ChlH | *Synechocystis* sp. PCC 6803 | 4e − 53 |
| Protochlorophtyllide reductase | *Chlamydomonas reinhardtii* | 2e − 27 |
| Ferrochelatase (Heme synthetase) | *Pseudomonas aeruginosa* | 1e − 23 |
| Cytochrome $c_{552}$ | *Euglena gracilis* | 2e − 75 |
| Cytochrome *f* | *Chlamydomonas reinhardtii* | 1e − 17 |
| Rieske FeS protein | *Arabidopsis thaliana* | 1e − 33 |
| Cytochrome $b_6f$ complex subunit IV | *Guillardia theta* | 1e − 09 |
| CcsA protein | *Euglena gracilis* | 4e − 17 |
| Acyl carrier protein II | *Spinacea oleracea* | 5e − 13 |
| Hypothetical protein APE0889 | *Aeropyrum pernix* | 2e − 15 |
| Hypothetical protein XP_040148 | *Homo sapiens* | 2e − 08 |
| Delta-8-fatty acid desaturase | *Euglena gracilis* | 1e − 87 |
| Ferredoxin [2Fe-2S] | *Euglena viridis* | 9e − 42 |
| Ferredoxin NADP reductase | *Chlamydomonas reinhardtii* | 7e − 60 |
| Enolase | *Euglena gracilis* | 2e − 55 |
| Cytosolic D-fructose-1.6-bisphosphatase | *Brasica natans* | 6e − 24 |
| Fructose-1,6-bisphosphate aldolase class I | *Euglena gracilis* | 4e − 60 |
| Fructose-1,6-bisphosphate aldolase class II | *Euglena gracilis* | 1e − 67 |
| Phosphofructokinase | *Leishmania donovani* | 1e − 42 |
| Cytosolic glyceraldehyde-3-P dehydrogenase | *Euglena gracilis* | 1e − 148 |
| Plastid glyceraldehyde-3-P dehydrogenase | *Euglena gracilis* | 9e − 49 |
| Rubisco small subunit | *Euglena gracilis* | 3e − 65 |
| Putative transketolase | *Cyanophora paradoxa* | 1e − 49 |
| Phosphoribulokinase | *Chlamydomonas reinhardtii* | 4e − 39 |
| CG7998 gene product | *Drosophila melanogaster* | 6e − 48 |
| Malate dehydrogenase | *Vitis vinifera* | 9e − 21 |
| Photosystem II 30 K protein OEC30 | *Euglena gracilis* | 5e − 57 |
| Photosys. II oxygen-evolving complex protein 2 | *Nicotiana tabacum* | 2e − 38 |
| Oxygen-evolving enhancer protein 3 | *Chlamydomonas reinhardtii* | 3e − 22 |

Table **1.** (Continued).

| Putative gene product | Organism with the top BLASTX hit | e-value |
|---|---|---|
| Chlorophyll *a*/*b*-binding protein of LHCPII | *Euglena gracilis* | 1e − 13 |
| Chlorophyll *a*/*b*-binding protein Lhcb4 | *Chlamydomonas reinhardtii* | 6e − 37 |
| Chlorophyll *a*/*b*-binding protein LH38 | *Euglena gracilis* | 1e − 87 |
| Photosystem II reaction center W protein | *Chlamydomonas reindhardtii* | 1e − 12 |
| Light-harvesting complex I protein LH35 | *Euglena gracilis* | 4e − 36 |
| Photosystem I reaction centre subunit III | *Chlamydomonas reinhardtii* | 2e − 39 |
| Photosystem I reaction center subunit IV | *Chlamydomonas reinhardtii* | 7e − 17 |
| DegP protease | *Arabidopsis thaliana* | 1e − 28 |
| Chloroplast FtsH protease | *Nicotiana tabacum* | 5e − 15 |
| Hypothetical protein | *Euglena longa* | 4e − 23 |
| Hypothetical protein | *Euglena longa* | 2e − 71 |
| Carboxy-terminal proteinase homolog | *Arabidopsis thaliana* | 1e − 07 |
| Chloroplast ribosomal protein S10 | *Mesembryanthemum crystallinum* | 1e − 31 |
| Ribosomal protein L19 | *Synechocystis* sp. PPC6803 | 6e − 26 |
| Hypothetical protein | *Euglena gracilis* | 7e − 14 |
| Ribosomal protein L27 | *Thermotoga maritima* | 2e − 11 |
| Chloroplast ribosomal protein L9 | *Arabidopsis thaliana* | 2e − 15 |
| Chloroplast ribosomal protein L3 | *Nicotiana tabacum* | 1e − 17 |
| Ribosomal protein L17 | *Aquifex aeolicus* | 1e − 12 |
| Ribosomal protein S16 | *Rickettsia prowazakii* | 5e − 21 |
| Elongation factor TS | *Synechocystis* sp. PCC 6803 | 7e − 26 |
| Putative H+-pyrophosphatase | *Oryza sativa* | 7e − 10 |
| Rubisco activase | *Chlamydomonas reinhardtii* | 2e − 15 |
| Rubredoxin-oxygen oxidoreductase | *Desulfovibrio gigas* | 3e − 30 |
| Probable superoxid dismutase Fe protein | *Ralstonia solanacearum* | 1e − 38 |
| Thioredoxin reductase | *Nostoc* sp. PCC 7120 | 2e − 44 |
| β-tubulin | *Entosiphon sulcatum* | 1e − 149 |
| Luminal-binding protein 1 | *Arabidopsis thaliana* | 5e − 05 |
| Cytochrome *c* | *Euglena gracilis* | 3e − 54 |
| Cytochrome $B_5$ | *Oryza sativa* | 2e − 18 |

Switzerland) and herring sperm DNA (10 mg/ml, 7.5 μl added to 100 μl Hyb solution). After removal of the cover slip in 2 × SSC, 0.1% SDS, slides were washed at 30 °C in pre-warmed 1 × SSC with gentle agitation and subsequently in 0.5 × SSC for 5 min each and then spun dry for 2 min. For each experiment a dye swap (Churchill 2002; Quackenbush 2002) was performed.

*Image processing and data analysis.* Scanning was performed on a Axon GenePix 4000B scanner with GenePix Pro 4.1 software (Molecular Devices Corp., Sunnyvale, CA). The photomultiplier gain was adjusted to avoid saturation of spots. The local background signal was subtracted automatically from the hybridization signal of each spot for both wavelengths. Poor-quality spots, either flagged manually or by the software as well as spots where background signals were higher than hybridization signals, were excluded from normalization and data analysis. Global normalization was performed using the corresponding Genepix default normalization factor. For individual transcripts to be considered differentially regulated, the mean of the median of ratios of all included spots needed to be $\leq 0.5$ or $\geq 2$. Deviant

Table **2.** List of microarray expression profile comparisons.

1. Light-grown *Euglena gracilis* wild-type (wt) *vs*. dark-grown *E. gracilis* wt
2. Light-grown *E. gracilis* wt vs. light-grown mutant $W_3BUL$
3. Light-grown *E. gracilis* wt vs. light-grown mutant $W_{10}BSmL$
4. Light-grown *E. gracilis* wt vs. light-grown mutant $W_{gm}ZOflL$
5. Dark-grown *E. gracilis* wt vs. dark-grown mutant $W_3BUL$
6. Dark-grown *E. gracilis* wt vs. dark-grown mutant $W_{10}BSmL$
7. Dark-grown *E. gracilis* wt vs. dark-grown mutant $W_{gm}ZOflL$

array elements were identified using replicate filtering method (Quackenbush 2002). Genes were scored as deviant if RoM1 $\times$ RoM2 was $\leq 0.66$ or $\geq 1.66$.

## RESULTS

**RT-PCRs.** The RT-PCRs using primers for *Lhcb*, *Pet*J, *Psb*O, *Psb*W and *Rbc*S with the *Act* gene as a control and template cDNAs from wild-type and all mutants grown in the light and in the dark yielded identical signals suggesting that the mRNA levels of these five genes in dark-grown cells and all mutant cells were comparable to those of light-grown wild-type cells (Fig. 1). These preliminary experiments led us to assume that there were hardly any differences in the mRNA levels between *E. gracilis* light-grown and dark-grown wild-type and mutant cells. To support or reject this we performed real-time RT-PCRs using the same set of primers and cDNAs.

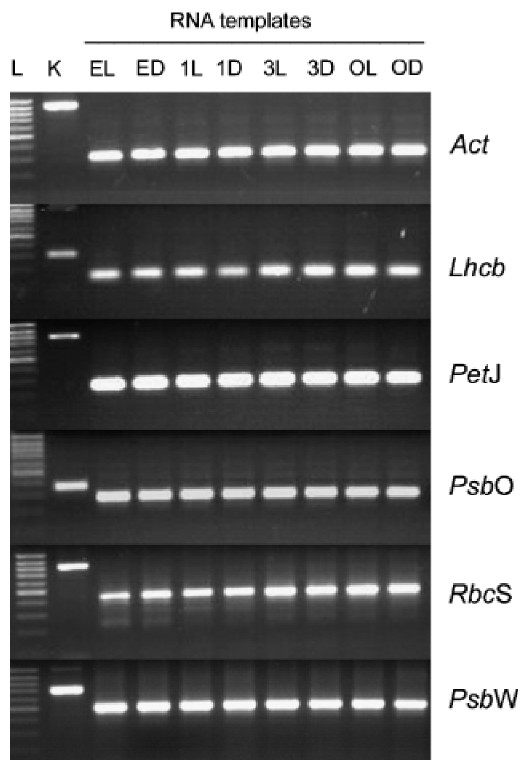


Fig. **1.** Reverse transcription PCRs. Rows: Primers were derived from (either DNA or cDNA) *Euglena gracilis* sequences of *Act* (actin), *Lhcb* (LHCPII), *Pet*J (cytochrome $c_6$), *Psb*O (OEC30), *Rbc*S (small subunit of Rubisco) and *Psb*W (13 kDa OEC protein W). Lanes: L, 100 bp ladder; K, control PCR using *E. gracilis* DNA as a template; EL, light-grown *E. gracilis* strain Z; ED, dark-grown *E. gracilis* strain Z; 1L, light-grown *E. gracilis* mutant strain $W_{10}BSmL$; 1D, dark-grown *E. gracilis* mutant strain $W_{10}BSmL$; 3L, light-grown *E. gracilis* mutant strain $W_3BUL$; 3D, dark-grown *E. gracilis* mutant strain $W_3BUL$; OL, light-grown *E. gracilis* mutant strain $W_{gm}ZOflL$; OD, dark-grown *E. gracilis* mutant strain $W_{gm}ZOflL$.

**Real-time RT-PCRs.** Real-time RT-PCRs revealed no differences in mRNA levels of *Lhcb*, *Pet*J, *Psb*O, *Psb*W and *Rbc*S between light-grown and dark-grown *E. gracilis* wild-type cells. Furthermore, in most cases there were no significant differences in mRNA levels of these genes between wild-type cells and mutant cells (Table 3). However, we found a 0.5-fold decrease of *Lhcb* mRNA levels in $W_{10}BSmL$ mutant in comparison with wild-type and a 0.45-fold decrease of *Lhcb* mRNA levels in dark-grown $W_{gm}ZOflL$ mutant in comparison with dark-grown wild-type (Table 3). A roughly 2-fold increase of *Rbc*S mRNA in $W_3BUL$ mutant in comparison with wild-type and a $\sim$2.5-fold increase of *Rbc*S mRNA in light-grown $W_{gm}ZOflL$ in comparison with light-grown wild-type were also observed (Table 3). In addition, we identified a 2.75-fold increase of *Pet*J mRNA levels in dark-grown $W_3BUL$ in comparison with dark-grown wild-type (Table 3). The remaining real-time RT-PCR comparisons did not reveal significant differences in the mRNA levels in mutants in comparison with wild-type cells (Table 3).

**The visual control of array experiments.** The visual inspection of the array experiments after hybridization and scanning suggested that the mRNA levels of the vast majority of nuclear genes for chloroplast proteins were apparently unchanged or were changed only slightly in all three *E. gracilis* white mutants in comparison with wild-type. However, the mRNA levels of three genes (i.e. *Psb*W, *Lhcb*4 and *Pet*D) in $W_{10}BSmL$ and $W_3BUL$ and one gene (*Psb*W) in $W_{gm}ZOflL$ were apparently lower than in wild-type as suggested by visual inspection of arrays. The dye-swap experiments confirmed these results. In experiments in which the mRNA profile of light-grown *E. gracilis* wild-type was compared with the mRNA profile of a certain light-grown mutant the visual inspection of arrays did not differ (or differed only slightly) from experiments in which the mRNA profiles of the dark-grown *E. gracilis* wild-type and the same dark-grown mutant were compared, suggesting that growing *E. gracilis* strains in light or dark had only a marginal effect on mRNA levels. This prediction was further confirmed in experiments comparing light-grown and dark-grown mRNA profiles of wild-type *E. gracilis* as there was no apparent difference in expression as suggested by

Table **3.** Real-time RT-PCR results, fold expressions with standard deviations.

| | *Lhcb* | *Pet*J | *Psb*O | *Psb*W | *Rbc*S |
|---|---|---|---|---|---|
| EL[a] | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ± 0.00 |
| ED[b] | 1.11 ± 0.05 | 0.77 ± 0.09 | 0.92 ± 0.05 | 0.98 ± 0.05 | 1.01 ± 0.01 |
| 1L[c] | 0.45 ± 0.04 | 1.12 ± 0.28 | 1.15 ± 0.01 | 0.90 ± 0.29 | 0.78 ± 0.03 |
| 1D[d] | 0.51 ± 0.01 | 1.52 ± 0.17 | 1.32 ± 0.20 | 1.15 ± 0.03 | 1.02 ± 0.05 |
| 3L[e] | 1.65 ± 0.19 | 1.13 ± 0.03 | 1.07 ± 0.01 | 1.19 ± 0.03 | 2.24 ± 0.25 |
| 3D[f] | 0.71 ± 0.04 | 2.75 ± 0.06 | 1.56 ± 0.11 | 1.21 ± 0.40 | 2.06 ± 0.15 |
| OL[g] | 0.84 ± 0.04 | 1.45 ± 0.03 | 1.69 ± 0.06 | 1.50 ± 0.50 | 2.54 ± 0.25 |
| OD[h] | 0.45 ± 0.03 | 1.64 ± 0.32 | 1.31 ± 0.08 | 1.75 ± 0.38 | 1.67 ± 0.06 |

*Act* gene (actin) was used to normalize the data. Light-grown *Euglena gracilis* wild-type samples were used as the reference samples for dark-grown wild-type samples as well as for all light-grown mutant samples, while dark-grown wild-type samples were used as the reference samples for all dark-grown mutant samples.

[a]Light-grown *E. gracilis* strain *Z*.
[b]Dark-grown *E. gracilis* strain *Z*.
[c]Light-grown *E. gracilis* mutant strain $W_{10}BSmL$.
[d]Dark-grown *E. gracilis* mutant strain $W_{10}BSmL$.
[e]Light-grown *E. gracilis* mutant strain $W_3BUL$.
[f]Dark-grown *E. gracilis* mutant strain $W_3BUL$.
[g]Light-grown *E. gracilis* mutant strain $W_{gm}ZOflL$.
[h]Dark-grown *E. gracilis* mutant strain $W_{gm}ZOflL$.

Table **4.** Differentially expressed genes (as revealed by microarray analysis) in the dark-grown $W_{10}BSmL$ mutant as compared with the dark-grown *Euglena gracilis* wild-type.

| Putative gene product | Fold exp[a] | St dev[b] |
|---|---|---|
| Lhcb4 protein | 0.225 | 0.057 |
| Sub IV of cytochrome $b_6f$ | 0.275 | 0.064 |
| Cytochrome *f* | 0.376 | 0.045 |
| Rieske FeS protein | 0.382 | 0.007 |
| Fru-1,6-bP-ase (cyt) | 0.423 | 0.042 |
| Phosphoribulokinase | 0.441 | 0.036 |
| PsaF protein | 0.471 | 0.116 |
| Chlorophyll *a/b*-binding protein LH38 | 2.053 | 0.079 |

[a]Fold exp represents the fold expression in the mutant as compared with the wild-type and it is the average value calculated from four values of ratio of medians (two from the original experiment and two from the dye-swap experiment).

[b]St dev is the standard deviation and was calculated in Excel from all four values of ratio of medians.

Table **5.** Differentially expressed genes (as revealed by microarray analysis) in the light-grown mutant $W_{10}BSmL$ as compared with the light-grown *Euglena gracilis* wild-type.

| Putative gene product | Fold exp[a] | St dev[b] |
|---|---|---|
| Lhcb4 protein | 0.191 | 0.035 |
| Sub IV of cytochrome $b_6f$ | 0.278 | 0.086 |
| Cytochrome *f* | 0.382 | 0.091 |
| Rieske FeS protein | 0.403 | 0.101 |
| Ribosomal protein L27 | 2.102 | 0.117 |
| GAPDHase (GapA) | 2.178 | 0.299 |
| Chlorophyll *a/b*-binding protein LH38 | 2.441 | 0.299 |

[a]Fold exp represents the fold expression in the mutant as compared with the wild-type and it is the average value calculated from four values of ratio of medians (two from the original experiment and two from the dye-swap experiment).

[b]St dev is the standard deviation and was calculated in Excel from all four values of ratio of medians.

visual inspection of both the original and the dye-swap experiment.

**Identifying questionable array elements.** After global normalization, several transcripts did not meet RoM1 × RoM2 criteria for reliability across experiments and were thus excluded from further consideration. Asparagine synthase, cytochrome *c* and cytochrome $b_5$ showed RoM1 × RoM2 < 0.66 in all experiments. Protochlorophyllide reductase had RoM1 × RoM2 > 0.66 only for dark-grown $W_{10}BSmL$ vs. dark-grown wild-type, and ATP synthase $\gamma$ chain had RoM1 × RoM2 > 0.66 only for dark-grown $W_{gm}ZOflL$ vs. dark-grown wild-type and dark-grown $W_{10}BSmL$ vs. dark-grown wild-type. *Psb*W did not meet RoM1 × RoM2 in most experiments. In total, 5–15% of transcripts did not meet the RoM1 × RoM2 criteria in individual experiments and were excluded, except in $W_3BUL$ vs. wild-type experiments, where 35% were excluded, although most of them had RoM1 × RoM2 close to 1.66. Thus, stringent application of RoM1 × RoM2 criteria yielded a smaller but more reliable set of gene expression comparisons.

**The expression ratios (mutants vs. wild-type) after normalization.** The expression comparison of dark-grown and light-grown *E. gracilis* wild-type revealed no significant differences. Moreover, the results from expression comparisons of dark-grown wild-type vs. dark-grown mutants were quite similar to results from experiments comparing light-grown wild-type vs. light-grown mutants (data not shown). This was consistent with the visual control of array experiments, RT-PCRs as well as real-time RT-PCRs. The $W_{gm}ZOflL$ mutant did not differ significantly from wild-type (deviant elements excluded) in the patterns of gene expression using global normalization. The expression profiles of $W_{10}BSmL$ and $W_3BUL$ were more different from the wild-type, although the expression of most genes was also not significantly different from the wild-type (data not shown). The analysis showed lower expression of *Lhcb*4 and *Pet*D in both $W_{10}BSmL$ and $W_3BUL$ in comparison with wild-type cells. The mRNA levels of cytochrome *f* (*Pet*A) and Rieske Fe-S protein were more than 2.5 times lower in $W_{10}BSmL$ in comparison with wild-type cells and the mRNA levels of nucleus-encoded plastid ribosomal protein L27 and chlorophyll *a/b*-binding protein LH38 of the light-harvesting complex were about 2 times higher in $W_{10}BSmL$ than in wild-type cells (Tables 4 and 5). The differences between $W_3BUL$ and wild-type cells were quite similar to differences observed for $W_{10}BSmL$ vs. wild-type cells (data not shown). A few moderate differences in mRNA levels of mutant vs. wild-type cells observed in real-time RT-PCR experiments (Table 3) were not confirmed using microarrays. Instead, we found no significant differences in all these cases in our microarray experiments.

## DISCUSSION

In most model eukaryotes including land plants, many signaling pathways are ultimately directed toward modulating gene expression by influencing transcription factor recruitment to promoter elements. In most cases, the final target is modulation of the position-specific transcriptional activity of RNA polymerase on the chromosomes. Barring a few exceptions, such a system is absent from trypanosomes (Elias et al. 2001; Field 2005; Koumandou et al. 2008). Most genes and even non-coding sequences are constitutively transcribed in trypanosomatids and their expression is regulated post-transcriptionally by modulating mRNA stabilization and translation (Furger et al. 1997; Hotz et al. 1997; Teixeira 1998). In trypanosomatids, the predominantly polycistronic transcription with subsequent resolution of nascent RNAs trough *trans*-splicing and polyadenylation (Benz et al. 2005; Campbell, Thomas, and Sturm 2003; Clayton 2002) reduces the influence of promoters as the main players in controlling gene expression, with major potential impact on signaling systems (Field 2005). Although the global changes in RNA amounts occur gradually throughout the life cycle of *Trypanosoma cruzi*, these correlate with changes in chromatin condensation, and do not seem to be due to activation/repression of stage-specific promoters and/or modulation of RNA stability (Elias et al. 2001).

As *Euglena* has chromosomes condensed throughout the whole cell cycle (Leedale 1967) and does not pass through morphologically different life stages, no stage-specific global changes in RNA amounts in *Euglena* can be proposed. Various lines of evidence suggest that *E. gracilis* regulates gene expression at the post-transcriptional rather than at transcriptional level (Hoffmeister et al. 2004; Keller et al. 1991; Kishore and Schwartzbach 1992; Madhusudhan et al. 2003; Levasseur, Meng, and Bouck 1994; Saint-Guily, Schantz, and Schantz 1994; Vacula et al. 2001). The levels of histone H2A and H3 mRNAs remain constant throughout the *E. gracilis* cell cycle, while in animals and plants the levels vary (Saint-Guily et al. 1994). The levels of $\alpha$- and $\beta$-tubulin mRNAs remain constant in *E. gracilis* after deflagellation, while in *Chlamydomonas* they increase (Levasseur et al. 1994). The Northern hybridization experiments revealed comparable mRNA levels of RbcS and LHCPII in light- and dark-grown *E. gracilis*, while the protein levels were higher in light-grown cells (Keller et al. 1991; Kishore and Schwartzbach 1992); Northern hybridization with probes for Rubisco SSU, LHCPII and PBGD (stromal protein porphobilinogen deaminase) yielded

identical signals in the case of *E. gracilis* wild-type and bleached mutants cultured under standard nutritional and light conditions (Vacula et al. 2001). The mRNA level of *apx* (light-inducible gene in plants) has been reported to show no response to light in *E. gracilis*, and its mRNA level in white mutants is comparable to wild-type, while APX protein level increases during illumination of wild-type cells (Madhusudhan et al. 2003). All these findings are consistent with the comparisons of mitochondrial proteomes from aerobically and anaerobically grown *E. gracilis* cells showing that this flagellate clearly does regulate gene expression at the level of protein accumulation (Hoffmeister et al. 2004).

Using microarrays of spotted cDNA amplification products, Ferreira et al. (2007) identified some changes in mRNA levels in wild-type *E. gracilis* strain UTEX 753 and strain MAT in response to streptomycin treatment, chromium stress or darkness. Out of 350 nucleus-encoded plant-related genes comprising 240 genes for chloroplast proteins, about 30 revealed moderate light/dark effects on their mRNA levels. For the 14 genes overlapping between our experiment and that of Ferreira et al. (2007) in the light–dark comparison (Rubisco, cytochrome $c_6$, GADPH, acyl carrier protein, superoxide dismutase, two photosystem II proteins, etc.), the changes in mRNA levels that we observed for the strain *Z* were not as prominent as those observed by Ferreira et al. (2007).

In the comparison of stable bleached mutants vs. wild-type some parallels to the comparison of streptomycin-treated vs. streptomycin-untreated *E. gracilis* become apparent, yet the results of Ferreira et al. (2007) differed from our results. For example, our real-time RT-PCR experiments revealed about 2 times higher *Rbc*S mRNA levels in $W_3BUL$ in comparison with wild-type, and about 2.5 times higher *Rbc*S mRNA levels in light-grown $W_{gm}ZOflL$ in comparison with light-grown wild-type. In contrast, Ferreira et al. (2007) identified a 2-fold decrease of *Rbc*S mRNA levels after Streptomycin treatment. However, our other real-time RT-PCR comparisons as well as all microarray comparisons revealed no differences in *Rbc*S mRNA levels in *E. gracilis* and its mutants whether grown in the dark or in the light. The minor differences between our results and those of Ferreira et al. (2007) likely reflect strain-specific or other biological variation of mRNA levels in *E. gracilis*. Keeping in mind that the differences in mRNA levels observed here and those observed by Ferreira et al. (2007) are typically on the order of 2-fold, whereas in higher plants, the differences in gene expression in response to light often span orders of magnitude (Jiao et al. 2005), neither the number of genes affected nor the extent of the effect observed here support the view that a chloroplast-specific signal affects nuclear transcription in *Euglena*.

Our study clearly reveals that light (or dark) has little influence on the mRNA levels of nuclear genes for chloroplast proteins in *E. gracilis*. Our real-time RT-PCRs and microarray experiments comparing mRNA levels of light- and dark-grown *E. gracilis* wild-type cells clearly indicate that genes such as *Lhcb* or *Rbc*S, whose mRNA levels are strongly light inducible in organisms with primary plastids, are not light inducible in this flagellate. Therefore promoters activated by light and/or regulation of mRNA stability by light might be missing or highly modified in this euglenozoan possessing chloroplasts of secondary symbiotic origin.

The disruption of plastid functions has little impact on mRNA levels in *E. gracilis* in most cases. Our microarray experiments identified somewhat lower mRNA levels of genes encoding Lhcb4, subunit IV of cytochrome $b_6f$ complex, cytochrome *f* and Rieske FeS protein (about 0.2, 0.3, 0.4 and 0.4 times, respectively; see Tables 4 and 5) in white *E. gracilis* mutant $W_{10}BSmL$ in comparison with wild-type. However, the mRNA levels of most nucleus-encoded genes, including highly expressed genes such as *Rbc*S and *Lhcb*, were the same or nearly the same in all three white *E. gracilis* mutants as in *E. gracilis* wild-type. In higher plants and *Chlamydomonas*, *Rbc*S and *Lhcb* represent typical light-inducible genes whose transcription is repressed also in the absence of functional plastid transcription, translation and/or redox potential created through photosynthetic electron transport. It is possible that a signaling pathway that would block the transcription of nuclear genes for chloroplast proteins when the photosynthetic activity is stopped and/or blocked does not exist in *Euglena*.

Most nuclear genes appear to be transcribed more or less constitutively and appear to have more or less constant mRNA levels in *Euglena*. In this respect *Euglena* is clearly similar to the trypanosomatids. Although about 6% of the transport-associated transcriptome seems to be developmentally regulated in *Trypanosoma brucei* (probably via post-transcriptional mechanisms), the vast majority of trypanosoma transcriptome is not, and trypanosome mRNA levels are insensitive to external stimuli within life stages (Koumandou et al. 2008). It has been suggested that the trypanosome transcriptional inflexibility might be due to operonal organization of genes (Clayton 2002) or arose through parasitic life-style (Koumandou et al. 2008). Although nearly nothing is known about the genome structure of euglenids, no nuclear genes for chloroplast proteins with operonal organization are known in euglenids so far. Nevertheless, the addition of SL-RNA leaders to cytosolic mRNAs via *trans*-splicing is one of the features common to Euglenozoa (Frantz et al. 2000). This unusual molecular characteristic shared by kinetoplastids, diplonemids and euglenids might suggest that the regulation of gene expression might have at least some common patterns in these closely related organisms. Thus, the absence of signaling pathways modulating the activity of RNA polymerase by influencing transcription factor recruitment to promoter elements might even be another feature common to Euglenozoa. Likely, the green algal endosymbiont originally had the capability for plastid-to-nucleus signaling. However, this feature was apparently not assimilated by the euglenozoan host cell, which appears to have retained its peculiar mode of (not) regulating nuclear gene expression.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table **S1.** List of sequences printed on the arrays.

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