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A small portion of plastid transcripts is polyadenylated in the flagellate *Euglena gracilis*

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ABSTRACT

Euglena gracilis **possesses secondary plastids of green algal origin. In this study, *E. gracilis* expressed sequence tags (ESTs) derived from polyA-selected mRNA were searched and several ESTs corresponding to plastid genes were found. PCR experiments failed to detect SL sequence at the 5′-end of any of these transcripts, suggesting plastid origin of these polyadenylated molecules. Quantitative PCR experiments confirmed that polyadenylation of transcripts occurs in the *Euglena* plastids. Such transcripts have been previously observed in primary plastids of plants and algae as low-abundance intermediates of transcript degradation. Our results suggest that a similar mechanism exists in secondary plastids.**

1. Introduction

Euglena gracilis is a fresh-water photosynthetic flagellate belonging to the order Euglenida and to the protist phylum Euglenozoa [1,2]. This phylum includes also the orders Kinetoplastida, Diplonemida and Symbiontida [3,4], which comprise exclusively heterotrophic species. Most euglenid species are free-living heterotrophic flagellates, but some of them possess secondary plastids that arose via secondary endosymbiosis of a green alga [5]. Phylogenetic analysis of plastid genome sequences revealed that euglenid plastids are derived from a *Pyramimonas*-related prasinophyte alga [6].

A common euglenozoan feature is the processing of primary nuclear transcripts by spliced leader (SL) RNA-mediated *trans*-splicing [7]. This process includes replacement of the 5′-end of pre-mRNA by the 5′-end of SL-RNA, donating identical 5′-termini to the mRNA molecules. Similar to nuclear *cis*-splicing, *trans*-splicing process is also mediated by spliceosomes, but a Y-branch intron structure is formed instead of a lariat [8]. The only currently known nuclear mRNA lacking the SL sequence in *E. gracilis* is that of the fibrillarin gene [9]. Since SL-*trans*-splicing does not occur in organelles (mitochondria, plastids), the presence (or absence) of an SL sequence at the 5′-end of a euglenozoan mRNA is diagnostic for its synthesis in the nucleus (or organelles, respectively).

E. gracilis cell possesses approximately eight secondary plastids bounded by three membranes [10,11]. The plastid genome of this species is circular and comprises 143.17 kb. It contains 96 protein and RNA gene loci [12], group II and III introns, and twintrons (i.e. introns within introns) [13].

The evolutionary transition from an endosymbiont to the plastid organelle was accompanied by a loss of many genes and gene transfer from the endosymbiont genome(s) to the host genome [14]. Gene transfer from plastids and mitochondria is an ongoing process, as it has been demonstrated in animals, plants, fungi as well as protists [15,16].

Nuclear copies of plastid DNA (NUPT) can be found in a variety of species [17]. However, some species, e.g. the pelagophyte alga *Aureococcus anophagefferens* or the apicomplexan parasite *Babesia bovis*, have been found to contain no nuclear organellar DNA (norgDNA) [16]. Species containing only one plastid per cell have fewer NUPTs than those with more plastids per cell. The total length of NUPTs is higher in polyplastidic species [16,18]. There is no obvious correlation between the norgDNA content and the organellar

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genome size. However, there is a strong positive correlation between the nuclear genome size and the NUPT content [16].

3′-terminal polyadenylation of nucleus-encoded transcripts is a very well-studied process that is essential for stability and proper function of most mRNA molecules in the cytosol [19]. Less is known about polyadenylation in organelles. It has been documented in metazoan mitochondria, where it is particularly important for some transcripts by restoring their termination codons [19]. On the other hand, polyadenylation of transcripts in primary plastids has been shown to serve as a signal for degradation [20–22]. As a result, polyadenylated transcripts are much more abundant in the cytosol and mitochondria than in plastids [23].

Polyadenylation-facilitated RNA degradation in plastids in those species, where it was studied in detail, proceeds in three steps: (i) endonucleolytic cleavage, (ii) polyadenylation of fragments and (iii) exonucleolytic degradation of polyadenylated fragments [20–22,24]. The polyA tails can be homopolymeric, composed solely of adenosines, or heteropolymeric composed of all four nucleotides, but with adenosines being the most abundant ones. The enzymes performing polyadenylation are polynucleotide phosphorylase (PNPase) and several polyA polymerases (PAPs) [21,22].

We anticipated that expressed sequence tag (EST) data from *E. gracilis*, which were obtained by sequencing of cDNAs synthesized by reverse transcription using oligo(dT) priming [25–27], should primarily include sequences from nuclear mRNA. However, our analysis of the EST data revealed that they include transcripts apparently derived from genes known to reside on the plastid genome. These sequences could represent transcripts of functional nucleus-localized copies of plastid genes or polyadenylated transcripts of plastid genes. The aim of the current study was to distinguish between these two possibilities.

2. Materials and methods

2.1. Nucleic acid isolation and purification, cDNA synthesis

E. gracilis wild type strains *Z* and *bacillaris* (hereafter abbreviated as EgZ and EgB, respectively) were cultivated under constant illumination or in the dark in Hutner medium [28]. Genomic DNA was isolated as in the protocol described earlier using phenol–chloroform method [29]. RNA extraction and mRNA isolation were carried out as described previously [29,30]. cDNA synthesis was performed with oligo(dT) and random hexanucleotide primers using ImProm-II Reverse Transcription System (Promega, Madison, WI, USA).

2.2. Polymerase chain reaction

PCR was performed as described earlier [29,30] except the annealing temperature of 60 °C instead of 58 °C. In the first set of PCR reactions, the 26 bp-long SL sequence present at the 5′-end of *E. gracilis* nuclear mRNAs was used as a forward primer (SL). Reverse primers (R) were designed based on the sequence of six *E. gracilis* EST sequences with 100% identity with plastid genes. In the second set of PCR reactions, the same reverse primers were used, but forward primers (F) were derived from the 5′-end sequence of the respective ESTs. Sequences of primers are listed in Supplementary Table 1. cDNAs synthesized using both oligo(dT) and random hexanucleotide primers were used as templates in quantitative PCR reactions. Total DNAs and cDNAs were used as templates in control reactions. Control PCRs were performed using primers for the nuclear genes *Nop1* (data not shown) and *PsbO*, and for the plastid gene *rpl16* (Supplementary Table 1). The identity of the products obtained by PCR was verified by sequencing.

2.3. Quantitative PCR

Quantitative PCRs were performed using LightCycler® 480 SYBRGreen Master mix (Roche, Basel, Switzerland) and the protocol according to the manufacturer's instructions, using the same primers as in the second set of PCRs (see above). All measurements were taken in triplicates. The ratios of (i) total mRNA or rRNA (full set of RNAs for individual genes) to polyadenylated mRNA or rRNA (polyadenylated fraction of total RNAs for individual genes), and (ii) RNAs from light- compared to dark-grown cultures were calculated using standard curve method for relative quantification [31]. Hereafter, total RNA means the full complement of mRNAs for individual genes and polyadenylated RNA means the polyadenylated fraction thereof. We used the expression of nuclear gene *PsbO* to normalize the calculations, because our previous studies have revealed that its mRNA level is the same in the light- and dark-grown *E. gracilis* [30,32].

2.4. Bioinformatics analyses

The *E. gracilis* plastid genome sequence RefSeq accession number NC_001603.2 [12] was used as a query in a BLASTn search of currently available *E. gracilis* EST data [25–27].

To search for enzymes potentially involved in polyadenylation-facilitated RNA degradation pathway in euglenid plastids, PNPase and PAP sequences from *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* were used as queries in tBLASTn search in *E. gracilis* EST data [25–27] and in the EST data of the marine euglenid *Eutreptiella gymnastica* (Marine Microbial Eukaryote Transcriptome Sequencing Project; http://camera.calit2.net/mmetsp/). Homologs found were screened for the presence of targeting presequences using the program TargetP (http://www.cbs.dtu.dk/services/TargetP/).

3. Results

Transcripts with 100% sequence identity with parts of six plastid genes – *atpA, ccsA, psbD, ycf13, rrn23S and rrn16S* – were found in *E. gracilis* EST data. Therefore, we tested whether these ESTs were derived from functional copies of plastid genes transferred to the nucleus, or whether representation of these sequences in the EST dataset reflected the fact that some plastid transcripts are polyadenylated. Our approach was based on the assumption that mRNAs originating by transcription of nuclear loci possess the SL sequence at their 5′-ends.

No PCR products were detected (lanes SL in Fig. 1) in PCRs using a forward primer (SL) corresponding to the conserved SL sequence and a reverse primer (R) designed based on particular EST sequences (listed in Supplementary Table 1). This suggests that primary transcripts of all studied genes are not *trans*-spliced. For control PCR reactions we used total DNA, as well as cDNA synthesized using oligo(dT) and random hexanucleotide primers as templates. All reactions yielded products of the expected sizes (Fig. 1). This confirms presence of the intact templates. The *rpl16* gene was detected in both *E. gracilis* strains using gene-specific F and R primers and cDNA synthesized using random hexanucleotide primers as the template. No PCR product was detected when oligo(dT) primed cDNA was used (lanes oligoT cDNA in Fig. 1). In all control reactions using primers for the nuclear gene *PsbO*, a product of the expected length was obtained (Fig. 1).

The most plausible interpretation of these results is that the studied genes presented in the *E. gracilis* EST database are not localized in the nucleus, but are exclusively present in the plastid and a part of their transcripts can be polyadenylated, at least to some extent. Therefore, quantitative PCR experiments were

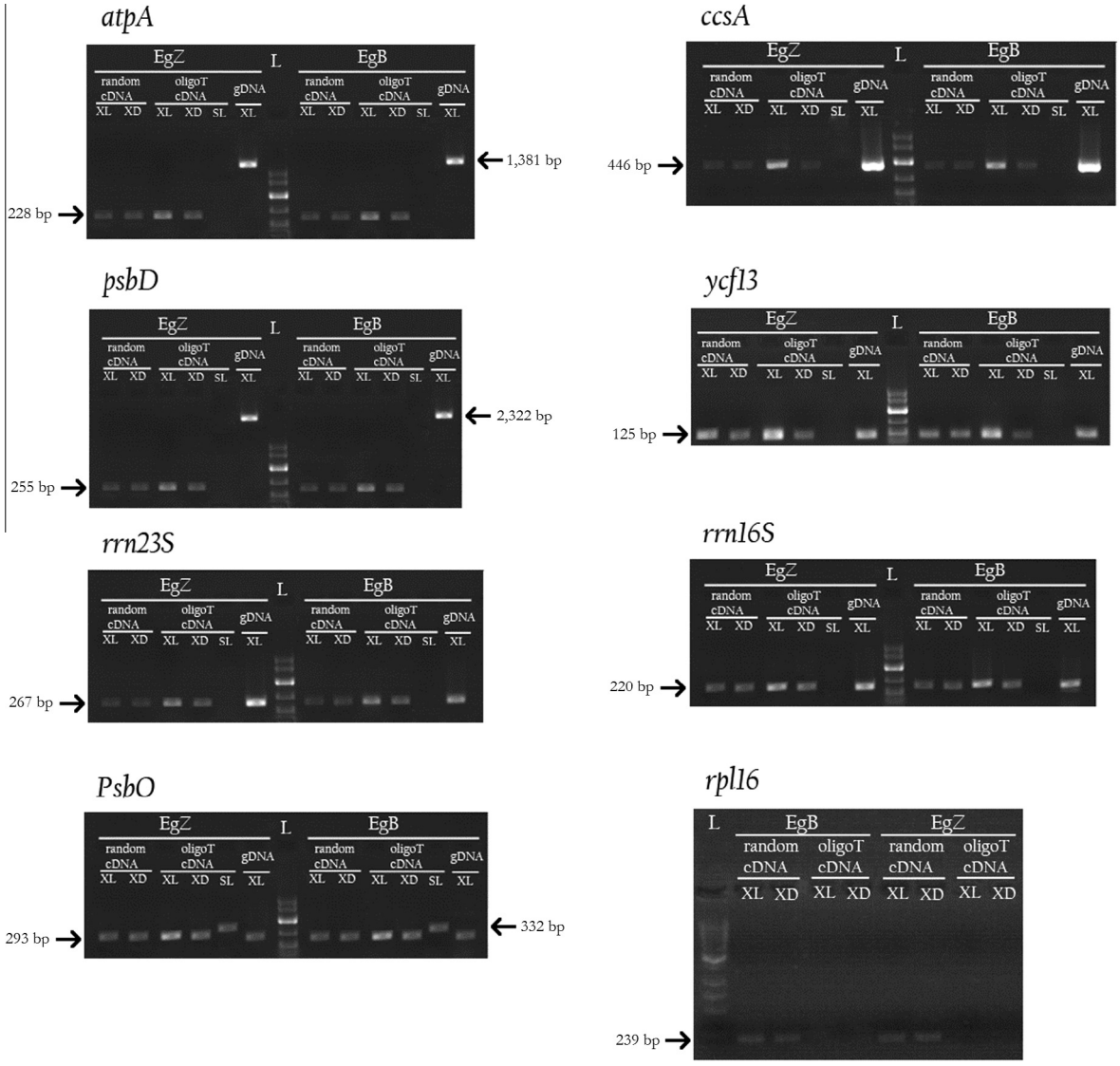

Fig. 1. Detection of polyadenylated transcripts in *Euglena gracilis* plastids. Polymerase chain reactions were performed with primers for *atpA*, *ccsA*, *psbD*, *psbO*, *ycf13*, *rpl16*, *rrn23S* and *rrn16S* genes. Total DNA (labeled gDNA) and cDNAs from light- and dark-grown *Euglena gracilis* strains *Z* and *bacillaris* (marked EgZ and EgB, respectively) were used as templates. cDNA was synthesized using random hexanucleotides (labeled random cDNA) and oligo(dT) (labeled oligoT cDNA) primers as described in Materials and Methods. The lengths of the PCR products are indicated. SL, forward primer corresponding to the SL sequence present at the 5′-end of *E. gracilis* mRNAs (SL) and reverse (R) primer, cDNA from light-grown culture; XD, forward (F) and R primers designed based on the respective EST sequence, cDNA from dark-grown culture; XL, F and R primers as above, cDNA or gDNA from light-grown culture. For the description of genes see Supplementary Table 1.

performed to compare the ratios of polyadenylated and non-polyadenylated transcripts of the plastid genes. The ratios of total versus polyadenylated RNAs for individual genes are indicated in Fig. 2. These experiments revealed that the amounts of total RNAs are ~350–1000-fold higher compared to the amounts of polyadenylated RNAs in the case of *ccsA* and *ycf13* genes; ~1000–10 000-fold higher in the case of *atpA*, *psbD* and *rrn16S* genes, and ~20 000–100 000-fold higher in the case of *rrn23S* (Fig. 2).

In addition, we tested possible differences in the rate of polyadenylation in plastids depending on the light conditions by using RNA isolated from light- and dark-grown cultures. The amounts of total mRNAs of all studied genes were slightly higher (1–6-fold) in the light than in the dark, except for *ycf13* in EgB (Fig. 3). The amounts of polyadenylated RNAs were in most cases higher in the light in EgZ, but they were in most cases higher in the dark in EgB (Fig. 3). The highest differences in the amounts of polyadenylated RNA in light- in comparison to dark-grown cultures were observed in the strain EgZ for the *rrn16S* gene (3.71-fold). The lowest ratio (0.18-fold) was observed in the strain EgB for the *ccsA* gene (Fig. 3).

4. Discussion

Our PCR experiments suggested that mRNAs of all examined protein-coding plastid genes (*atpA*, *ccsA*, *psbD* and *ycf13*) are not *trans*-spliced, since no PCR products were obtained when the SL primer was used. Although the SL region may be occasionally lacking in nuclear-encoded mRNAs in *E. gracilis* [9], its absence from the transcripts investigated here indicates that they likely originated exclusively in the plastid. Our experiments also confirmed that a small portion of these mRNAs is polyadenylated, since they could be amplified with gene-specific primers and cDNA prepared using oligo(dT) primer. In contrast, in the case of the *rpl16* gene (a negative control), no PCR product was obtained when cDNA synthesized with oligo(dT) primer was used as the template, suggesting that *rpl16* transcripts are not polyadenylated in *E. gracilis*

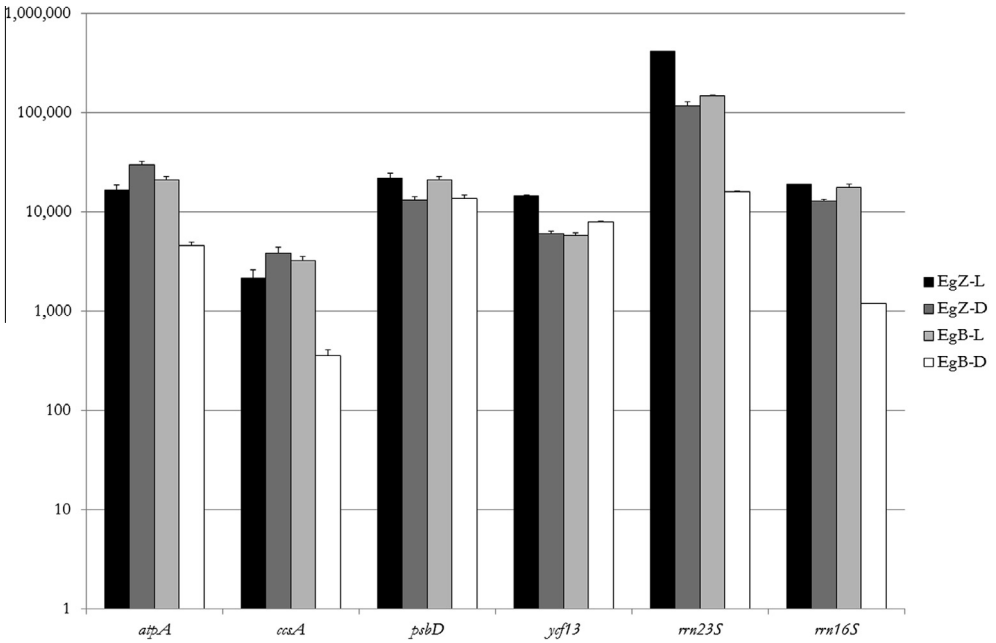

Fig. 2. Average ratios of total to polyadenylated RNAs in light-grown (L) and dark-grown (D) cultures of *Euglena gracilis*. The ratios were calculated using standard curve method for relative quantification and are presented in logarithmic scale. The expression of the nuclear *PsbO* gene was used for data normalization. EgB-D, cDNA from dark-grown *E. gracilis* strain *bacillaris*; EgB-L, cDNA from light-grown *E. gracilis* strain *bacillaris*; EgZ-D, cDNA from dark-grown *E. gracilis* strain *Z*; EgZ-L, cDNA from light-grown *E. gracilis* strain *Z*.

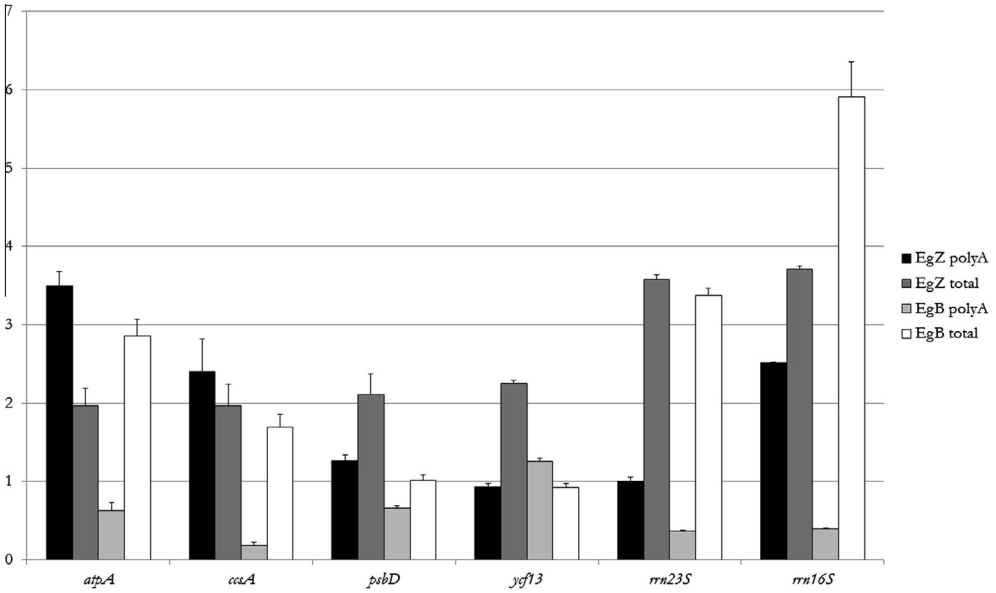

Fig. 3. Average ratios of the amounts of total and polyadenylated (polyA) RNAs in light- over dark-grown cultures of *Euglena gracilis*. The ratios were calculated using standard curve method for relative quantification. The expression of the nuclear *PsbO* gene was used for data normalization. EgB polyA, cDNA from *E. gracilis* strain *bacillaris* synthesized using the oligo(dT) primer; EgB total, cDNA from *E. gracilis* strain *bacillaris* synthesized using random hexanucleotide primers; EgZ polyA, cDNA from *E. gracilis* strain *Z* synthesized using the oligo(dT) primer; EgZ total, cDNA from *E. gracilis* strain *Z* synthesized using random hexanucleotide primers.

plastids (Fig. 1). However, the nuclear localization of the rRNA genes *rrn23S* and *rrn16S* cannot be directly ruled out by the absence of the SL sequence at the 5′-end, because nuclear (e.g. 18S and 28S) rRNAs have not been reported to possess SL-leaders in *Euglena*. However, a polyA tail is usually missing in nucleus-encoded rRNAs and SL-RNA-mediated processing of nuclear pre-18S rRNA has been recently described in trypanosomatids which are close relatives to euglenids [33]. Moreover, no pathway for rRNA import to plastids is known in euglenids thus far. Therefore, functional 23S and 16S rRNAs are most likely encoded solely by the plastid genome of *E. gracilis*. Supporting this conclusion, most of the genes studied here could not be detected in at least one of the stably bleached *E. gracilis* mutant strains possessing only remnants of the plastid genome (data not shown).

Quantitative PCRs revealed that the amounts of total RNAs in *E. gracilis* plastid are about 350–100000 higher compared to the amounts of polyadenylated RNAs (Fig. 2). These ratios may reflect a low rate of RNA polyadenylation or a low stability of polyadenylated transcripts in *E. gracilis* plastids. The latter cause would be consistent with the fact that transcript polyadenylation serves as

a signal for exonucleolytic degradation in primary plastids of the green lineage [20–22]. Indeed, only incomplete sequences of *E. gracilis* plastid genes are found in the EST database, suggesting that they may correspond to degradation intermediates.

PAPs and/or PNPase are the enzymes responsible for polyadenylation in plastids of plants and green algae [21,22]. PNPase function was demonstrated in polyadenylation and degradation not only of mRNAs, but also tRNAs and rRNAs [20,23,34]. We failed to identify homologs of the enzymes involved in polyA-degradation pathway in the currently available *E. gracilis* EST data, but this may be due to an inherent incompleteness of any EST survey based on limited Sanger sequencing of cDNA libraries. Recently, a plastid genome sequence [35] and extensive transcriptomic data (see Materials and Methods) have become available for a distantly-related plastid-bearing euglenid, *E. gymnastica*. We searched the transcriptome obtained by the Illumina sequencing of polyA RNA, and identified sequences corresponding to plastid genes *rrn23S*, *rrn16S*, *psbA*, *psbC*, *psbH*, and *tufA*, suggesting that polyadenylation of plastid transcripts may take place also in *Eutreptiella*. However, we also failed to identify candidates for plastid-targeted PNPases or PAPs, although the transcriptome of *E. gymnastica* has been sequenced much more deeply than that of *E. gracilis*. Thus the identity of the putative enzymatic machinery responsible for transcript polyadenylation in euglenid plastids remains unknown.

The amounts of gene-specific total RNAs were 1–3-fold and 3–6-fold higher for plastid mRNAs and rRNAs, respectively, in light-compared to dark-grown cultures (Fig. 3). The *E. gracilis* plastid transcriptome exhibits significant quantitative changes under illumination [36]. The latter case might be detected because of the fewer copies of plastid DNA molecules in the dark in comparison to the light conditions. It was shown that after transition from dark to light, the number of plastid DNA molecules and plastid rRNA genes in *E. gracilis* increases from 200 to 1400–2900 and from 1900 to 5200 per cell, respectively [10,37].

The amounts of polyadenylated RNAs were generally higher (0.9–3.5-fold) in the light in EgZ, but they were generally lower (1.1–5-fold) in the light in EgB (Fig. 3). This may reflect molecular pecularities of strains *Z* and *bacillaris*.

The polyadenylation of plastid transcripts in *E. gracilis* was previously studied by hybridization experiments of labeled plastid RNA to plastid DNA, and it was concluded that plastid rRNAs were polyadenylated to some extent, while plastid mRNAs were not [38]. Using more precise PCR and quantitative PCR methods, in this study we have demonstrated that a small portion of plastid mRNAs is polyadenylated as well.

In conclusion, this is the first study revealing that a small portion of mRNAs is polyadenylated in *E. gracilis* plastids. Future studies should shed more light on the functional significance and mechanisms of polyadenylation of plastid transcripts in euglenids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.01.034.

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