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Organization and expression of a phycobiliprotein gene cluster from the unicellular red alga *Cyanidium caldarium*

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Abstract

We have sequenced a plastid gene cluster from the unicellular red alga *Cyanidium caldarium* which is located downstream from the *psbA* gene and contains, in the following order, genes for a β-allophycocyanin-like protein (*apcB'*), a putative 9.5 kDa allophycocyanin linker protein ($apcL^{9.5}$) and a putative 29 kDa phycocyanin linker protein ($cpcL^{29}$). The *apcB'* and $apcL^{9.5}$ genes are organized in the form of an operon. The $cpcL^{29}$ gene is transcribed monocistronically from the opposite strand of DNA. Both transcription units are probably terminated at a 25 bp inverted repeat 3 and 5 bp downstream of the stop codons of the $apcL^{9.5}$ and $cpcL^{29}$ genes, respectively. The levels of both transcripts are greatly reduced in the dark as is the *psbA* transcript. Downstream from the phycobiliprotein gene cluster two open reading frames (ORFs) were found which are homologous to ORFs from plastid DNAs and cyanelle DNA of *Cyanophora paradoxa*. Sequence homologies between genes analysed in this study and corresponding genes from cyanobacteria, chlorophytic plastids and cyanelles point to a large phylogenetic distance between the plastids of *Cyanidium* and cyanobacteria and other plastid types.

Introduction

Phycobilisomes (PB), the major light-harvesting complexes in red algae and cyanobacteria, contain three abundant phycobiliproteins (PBP): phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC), which account for about 85% of the PB mass. The phycobiliproteins are connected by linker proteins (LP [15, 16, 33]). It has been hypothesized that PBPs and LPs descend from a common biliprotein ancestor [40]. Additionally, a phylogenetic relationship may exist among light-harvesting proteins, phytochrome, chlorosome C-protein and phycobiliproteins [45]. The α- and β-subunits (SU) of a given PBP are more related to a similar SU from different species of cyanobacteria (about 75% homology) than to each other (about 30% homology) indicating that the divergence of different PBP genes from a common ancestor gene occurred earlier in evolution than the division of cyanobacteria into different lines (cf. DeLange *et al.* [8].

Phycobiliprotein genes may be clustered and cotranscription of genes for the α and β SU of a given PBP was found in several cases. Cotranscription of PBP genes with LP genes has also been described [2–6, 11, 20–22, 24, 25, 31, 36]. Inhibitor studies suggested that at least some of the linker proteins are nuclear-encoded in eucary-

otic algae [9]. Several PBPs are translated from poly(A)$^-$ mRNA in red algae and therefore appear to be plastid-encoded [1, 46].

As indicated by Rubisco sequence comparisons, the line of cyanobacteria that led to rhodoplasts of Rhodophyta and some species of cyanobacteria (e.g. *Anabaena*, *Anacystis nidulans*) may have separated at an early stage of the evolution of the majority of cyanobacteria [47]. This hypothesis indicates that there should be significant differences in organization and sequence of PBP genes from red algae and cyanobacteria. To date no DNA sequence of a red algal PBP gene or LP gene has been published and information about the organization of such genes is scanty [43]. In contrast, several PBP and LP genes from cyanobacteria have already been analysed (cited above).

Many efforts have been made to understand the regulation of gene expression in green chloroplasts (reviewed in [17]). However, gene expression of other plastid types (e.g. in the rhodoplasts of Rhodophyta and phaeoplasts of Chromophyta) is poorly understood. The putative large phylogenetic distance between chloroplasts on the one hand and rhodo- and phaeoplasts on the other hand [48] may not allow use of chloroplast models of plastid gene regulation to explain the situation in rhodo- and phaeoplasts.

Here we describe the organization of a plastid phycobilisome gene cluster and two additional plastid genes from the unicellular red alga *Cyanidium caldarium*. The investigation of transcript levels of these genes and the rhodoplast-encoded *psbA* gene under different growth conditions indicated that transcriptional regulation may play an important role in rhodoplast biogenesis.

Materials and methods

Growth of the algae and isolation of plastid DNA

Cyanidium caldarium Geitler (strain 14-1-1) was purchased from the Algae Culture Collection of the Institute for Plant Physiology of the University of Göttingen, Germany. (On the basis of cytological and biochemical criteria this species has been described as *Galderia sulphuraria* [32].) Cells were grown autotrophically as described [46], mixotrophically in the light (1% glucose added) or heterotrophically in the dark (1% glucose added), and were harvested by centrifugation. Cells were lysed by SDS/EDTA/Proteinase K treatment and total cellular DNA was isolated as described [47]. Plastid DNA (ptDNA) was separated from nuclear DNA by CsCl density gradient centrifugation in the presence of ethidium bromide [47].

Cloning and sequencing of ptDNA restriction fragments

In a previous study we described the location of the *psbA* gene on a 5.8 kb ptDNA *Eco* RI fragment from *C. caldarium* [28]. We generated partial plastid DNA libraries using several restriction enzymes (*Bam* HI, *Eco* RI, *Hin*d III, *Xba* I). Colonies were transferred to nylon membrane and hybridized with the 5.8 kb *Eco* RI fragment. Clones overlapping with this fragment were isolated and used for subsequent rounds of screening. By this 'gene walking' procedure we were able to clone a contiguous ptDNA fragment of about 14 kb (see Fig. 1). Those parts of the cloned areas which were found to be transcriptionally active (see below) were subcloned in pUC 18 and sequenced with a T7 sequencing kit from Pharmacia. Both strands of DNA were sequenced and all restriction sites were crossed.

Transcription analysis of cloned ptDNA fragments

Fragments of the cloned segment were labeled with ^{32}P with a random-primed labelling kit (Boehringer) and used as hybridization probes against total cellular RNA from *Cyanidium* fractionated on 1.5% formaldehyde/agarose gels using standard procedures [7]. Transcribed parts of the cloned area were chosen for sequence analysis (see above).

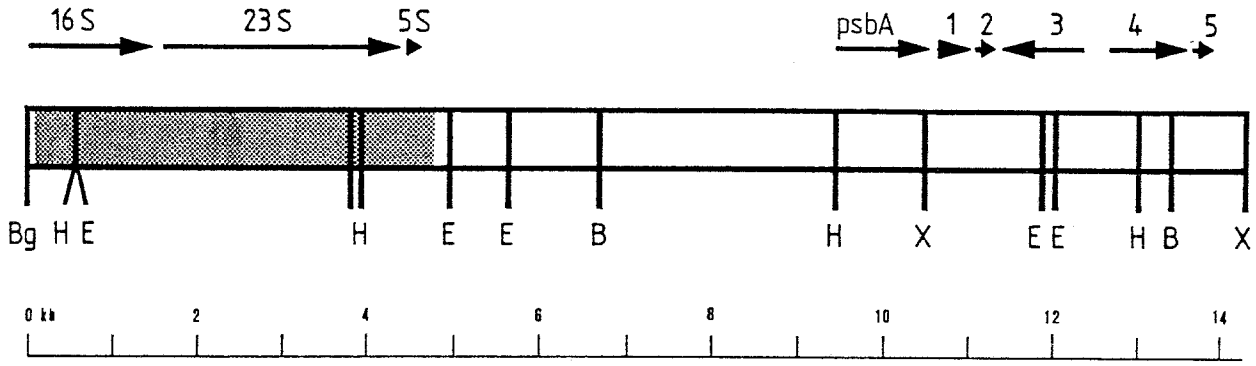

Fig. 1. Gene arrangement of a 14 kb region of the ptDNA from *Cyanidium caldarium*. The inverted repeat in the plastid DNA is indicated by hatching. Abbreviations are: 1, ORF 492 (*apcB'*); 2, ORF 252 ($apcL^{9.5}$); 3, ORF 732 ($cpcL^{29}$); 4, ORF 921; 5, ORF 210; B, *Bam* HI; Bg, *Bgl* II; E, *Eco* RI; H, *Hin* dIII; X, *Xba* I.

In order to analyse the transcription of sequenced regions in detail we used single-stranded probes. Recombinant plasmids containing parts of the sequenced areas were linearized with a restriction enzyme that cuts only at one side of the polylinker and subsequently denatured by boiling for 10 min and chilling on ice. Primers (17-mers) were annealed to the other side of the polylinker and a labelled complementary strand of the cloned fragment was synthesized using ^{32}P-labelled dATP and Klenow polymerase. This procedure enabled us to use full-length single-strand specific gene probes which do not contain labelled vector DNA and which were labelled to a very high specific activity. These probes were used in hybridization experiments with total cellular RNA isolated from auto-, mixo- and heterotrophically grown *Cyanidium* using standard procedures [7].

Construction of dendrograms

Dendrograms were constructed with the CLUSTAL program [18]. This program computes dendrograms based on 'similarity scores' between various sequences which indicate the number of matching residues between two sequences minus a fixed score for every gap introduced to maximize homology ('gap penalty' = 1 in this study).

Results

Sequencing of transcribed regions

Using a plastid *psbA* encoding 5.8 kb *Eco* RI fragment from *Cyanidium caldarium* [28] we cloned a contiguous 14 kb part of the ptDNA from this alga. By transcription and subsequent sequencing analysis we were able to localize the plastid inverted repeat containing the ribosomal RNA operon about 4 kb upstream of the *psbA* gene (Fig. 1; see [28, 29]). By northern hybridization no transcription activity of the region between *psbA* and the 5S rRNA gene could be detected. Preliminary sequencing data showed the presence of unidentified reading frames in this area (unpublished results). Downstream of the *psbA* gene, a 0.9 kb transcript was identified by northern analysis (data not shown). The sequence of the transcribed region and its flanking regions was determined (Fig. 2). Five open reading frames (ORFs) were identified in this area as indicated in Fig. 1. By sequence comparison to the EMBL sequence database these ORFs could be identified.

ORF 492 shows about 60% homology (on the amino acid level) to β-APC from various species and to a 'β-APC-like protein' from *Mastigocladus laminosus* ('$\beta^{16.2}$' [37]). The gene was therefore designated *apcB'*. ORF 252 encodes for a 9.5 kDa protein which shows the highest amino

Fig. 2. Nucleotide and deduced amino acid sequence of a gene cluster from *Cyanidium caldarium* containing genes for a β allophycocyanin-like protein ('ORF 492' = *apcB'*, 141–632), a 9.5 kDa APC linker protein ('ORF 252' = $apcL^{9.5}$, 647–898), a 29 kDa PC linker protein ('ORF 732' = $cpcL^{29}$, 1690–959, opposite strand of DNA), a hypothetical 35 kDa protein (ORF 921, 2007–2926) and a hypothetical 7.4 kDa protein (ORF 210, 2987–3196). Numbering starts at the stop codon of the *psbA* gene (base 1–3). Sequences that resemble procaryotic −35 and −10 promoter sequences and ribosome-binding sites (Shine-Dalgarno sequences, SD) are underlined. Arrows mark inverted repeats of 41 bp downstream of *psbA*, of 25 bp between $apcL^{9.5}$ and $cpcL^{29}$ and of 58 bp downstream of ORF 210. These may serve as transcription terminators and/or mRNA processing signals.

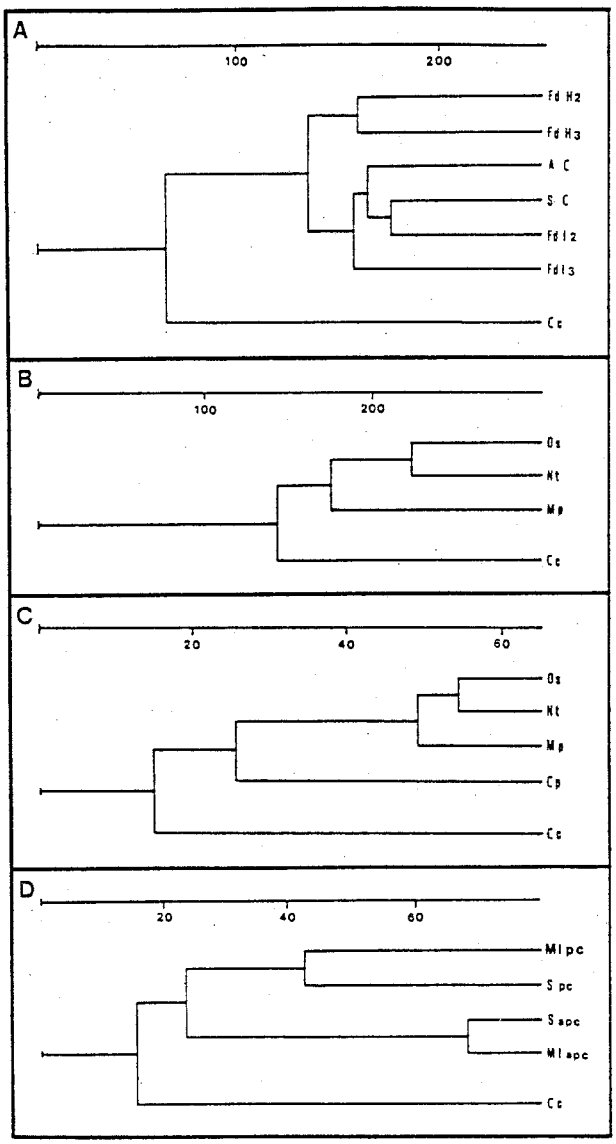

Fig. 3. Dendrograms derived by comparison of amino acid sequences of various PC 30 kDa linker proteins (A), ORFs 921 (B), ORFs 210 (C) and small linker proteins (D). Numbers are the similarity scores which indicate the number of matching amino acids minus a fixed score for each gap introduced to maximize homology. Abbreviations and references are: (A) Fd H2 = *Fremyella diplosiphon cpcH2* [24], Fd H3 = *F. diplosiphon cpcH3* [30], A C = *Anabaena cpcC* [2], S C = *Synechococcus* 7002 *cpcC* [4], Fd 12 = *F. diplosiphon cpcI2* [24], Fd I3 = *F. diplosiphon cpcI3* [30], Cc = *Cyanidium caldarium cpcL*[29]; (B & C) Os = *Oryza sativa* ORF321/ORF62 [19], Nt = *Nicotiana tabacum* ORF313/ORF62 [42], Mp = *Marchantia polymorpha* ORF321/ORF62 [35], Cp *Cyanophora paradoxa* ORF65 [10], Cc = *Cyanidium caldarium* ORF210; (D) Ml pc = *Mastigocladus laminosus* small phycocyanin linker (*cpcD* [13,14]), S pc = *Synechococcus* 7002 small phycocyanin linker (*cpcD* [4, 26]), S apc = *Synechococcus* 6301 small allophycocyanin linker (*apcC* [21]), Ml = *M.laminosus* small APC linker [12], Cc = *Cyanidium caldarium apcL*[9.5].

acid homology (about 23%) to small APC linker proteins from cyanobacteria (conservative amino acid exchanges about 63%) and amino acid homologies of 16–19% to other small linker proteins. We therefore propose to give it the gene name *apcL*[9.5]. ORF 732 was found to encode a 29 kDa putative phycocyanin linker protein (ca. 30% amino acid homology to other PC linker protein genes encoding proteins of about 30 kDa and was therefore designated *cpcL*[29].

The amino acid of ORF 921 is about 45% identical to hypothetical protein coding regions for proteins of about 37 kDa of the tobacco (ORF 313 [42]), *Marchantia polymorpha* (ORF 320 [35]) and rice ptDNA (ORF 321 [19]), respectively.

The amino acid sequence coded for by ORF 210 is about 25% identical and about 63% homologous to ORF 65 from cyanelles of *Cyanophora paradoxa* [10] and to ORF 62 of several chloroplasts [19, 35, 42], and is collinear with these ORFs (data not shown). Up to now the function of ORFs 921 and 210 is unclear. Hydropathy plots of gene products of both ORFs show the presence of several putative transmembrane domains (data not shown) indicating that the gene products are membrane-bound proteins. The presence of a terminator-like inverted repeat structure downstream of ORF 210 (Fig. 2) and the absence of such a structure between the ORFs indicates that they are cotranscribed. However we did not detect a transcription signal from either ORF.

Dendrograms for *cpcL*[29], *apcL*[9.5], ORF 921 and ORF 210 showing their relationships in different organisms have been constructed (Fig. 3).

Transcription analysis

Hybridization experiments with single-stranded probes revealed that the 0.9 kb transcription signal initially found (see above) was made up of two

signals of similar size from opposite strands, one specific for ORF 732 (*cpcL*^{29}) and the other for ORFs 492/252 (*apcB'*/*apcL*$^{9.5}$), respectively (Fig. 4). ORF 252 (*apcL*$^{9.5}$) appears to be cotranscribed with ORF 492 (*apcB'*). Between these transcription units a transcription terminator-like structure consisting of a 25 bp inverted repeat was found (Fig. 2). Upstream of the start codons of *apcB'* and *cpcL*^{29} sequences were identified that resemble procaryotic −35 and −10 promoter sequences (Fig. 2). Assuming that these sequences serve as transcription promoters and terminators transcripts of ca. 850 bp for both transcription units could be expected. This value is in good agreement with the size of both transcripts determined by northern hybridization (about 900 bp; see Fig. 4). We therefore suggest that the 25 bp inverted repeat between both transcription units may serve as a bidirectional transcription terminator.

Light-regulated expression of apcB', apcL$^{9.5}$, cpcL^{29} *and* psbA

In green algae the copy number of the ptDNA may depend on growth conditions [41]. Therefore we isolated total cellular DNA from auto-, mixo- and heterotrophically grown *Cyanidium* and hybridized these DNAs to a plastid specific *psbA* gene probe. Figure 5A shows that in this alga the proportion of plastid DNA in total cellular DNA is not influenced by these growth conditions. We then used *apcB'*/*apcL*$^{9.5}$- and *cpcL*^{29}-specific gene probes in hybridization experiments with total cellular RNA isolated from auto-, mixo- and heterotrophically grown *Cyanidium*. It was found that the amounts of both transcripts were not influenced when cells were grown in mixotrophic conditions but were strongly reduced in heterotrophically grown cells (Fig. 5B). Therefore we analysed the expression of another plastid-encoded photosynthesis-related gene. The *psbA* transcript level was strongly reduced in mixotrophically and heterotrophically grown *C. caldarium* (Fig. 5B).

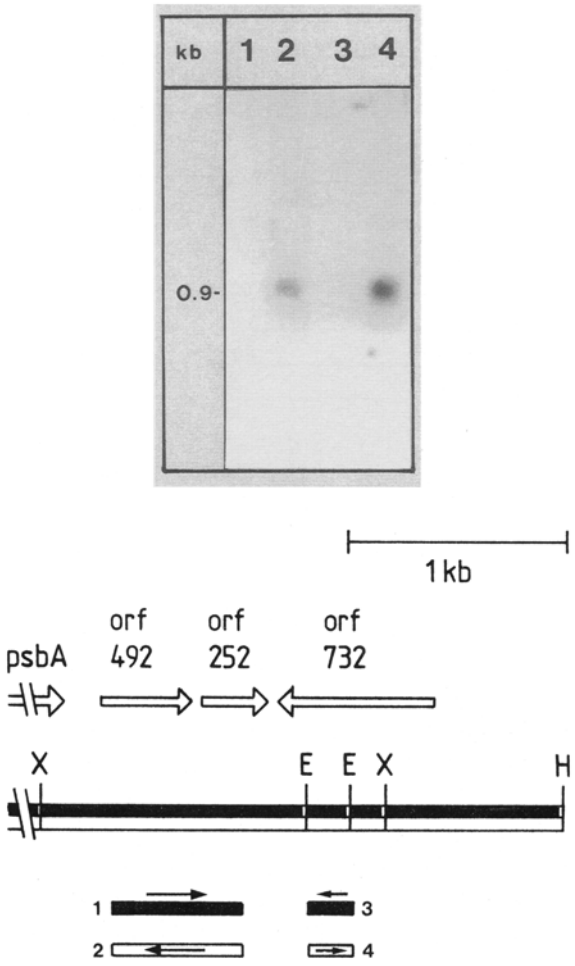

Fig. 4. Transcription of the region downstream *psbA* in *Cyanidium caldarium* plastid DNA. Bars give sizes and location of hybridization probes used in this experiment. The direction of the single-strand probes is indicated by arrows. Antisense probes of ORF 492/252 (*apcB'*/*apcL*$^{9.5}$) and ORF 732 (*cpcL*^{29}) hybridized to mRNAs of ca. 0.9 kb (lanes 2 and 4).

Discussion

We have sequenced a phycobilisome gene cluster from the unicellular red alga *Cyanidium caldarium* containing genes for a β-APC-like protein (*apcB'*), a putative 9.5 kDa APC linker protein (*apcL*$^{9.5}$) and a putative 29 kDa PC linker protein (*cpcL*^{29}). We believe that *apcB'* does not encode a 'regular' (major light-harvesting) β-APC protein. Firstly, it shows homology of only about 60% (on an amino acid level) to several β-APCs

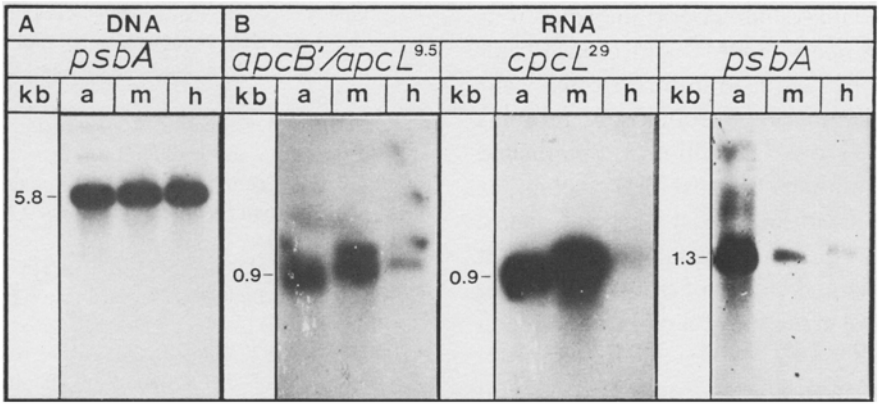

Fig. 5. A. Hybridization of a plastid DNA specific *psbA* gene probe to Southern blots of total cellular DNA isolated from auto- (a), mixo- (m) and heterotrophically (h) grown *Cyanidium caldarium*. B. Hybridization of *psbA*, *apcB'/apcL*$^{9.5}$ and *cpcL*^{29} specific gene probes to northern blots of total cellular RNA isolated from auto- (a), mixo- (m) and heterotrophically (h) grown *C. caldarium*.

from cyanobacteria and red algae (including a β-APC protein sequence from *C. caldarium* determined by protein sequencing [34]). Sequence homologies among 'regular' β-APCs from cyanobacteria and *Cyanophora paradoxa* are higher than 74% [3, 5, 8, 22, 23, 31, 44]. Secondly, the transcript level of the *apcB'/apcL*$^{9.5}$ operon is relatively low as compared to that of *cpcL*^{29} (Figs. 3 and 5). As β-APC is a prominent component of the PBS one would expect a much higher level of expression for the 'regular' *apcB*. We think *apcB'* might encode for a β-APC-like protein similar to the '$\beta^{16.2}$' protein from *Mastigocladus laminosus* [37, cf. 27]. Both the small APC linker protein and the β-APC-like protein are contained in small amounts in cyanobacterial phycobilisomes [27, 37–39]. This is in good agreement with the finding that *apcB'* and *apcL*$^{9.5}$ are probably cotranscribed.

The organization of *cpcL*^{29} and *apcL*$^{9.5}$ in *C. caldarium* differs significantly from that in cyanobacteria. In these organisms phycocyanin linker protein genes usually are part of a large gene cluster containing genes for α- and β-PC and PC linker proteins and are cotranscribed with the whole cluster or at least with other PC linker protein genes [2, 4, 6, 24, 30]. In *C. caldarium*, *cpcL*^{29} constitutes a single transcription unit. Cyanobacterial genes for small allophycocyanin linker proteins are parts of APC gene clusters and are cotranscribed with other APC and APC-linker genes [20, 21]. These differences might have been caused by genome rearrangements during the establishment of rhodoplast endosymbiosis. Alternatively, a large phylogenetic distance may exist between rhodoplasts of *Cyanidium* and some cyanobacterial species. Sequence comparisons indicate that the second possibility might be true (see Fig. 4). A deep branching was found between *Cyanidium* linker proteins and those from cyanobacteria (see Fig. 4). Recently we have found a similar gene cluster in the multicellular red alga *Antithamnion* sp. indicating that the different organization of these genes is possibly typical of red algae (unpublished results).

Sequence comparisons of plastid encoded Rubisco genes [47], *psbA* genes [28] and 16S rRNA genes [29] indicated a large phylogenetic distance between plastids of green plants and those of red algae and suggested a polyphyletic origin of these plastids. This hypothesis is supported by sequence comparisons of ORF 921 and ORF 210. In both cases the *C. caldarium* genes are widely divergent from the chloroplast cluster (Fig. 4).

We have found that transcript levels of genes analysed in this study are influenced by light and/or the presence of glucose in the growth medium (Fig. 5). Thus it is possible that in *C. caldarium* transcriptional regulation of photosynthe-

sis-related genes plays an important role. It is noteworthy that *apcB'/apcL*[9.5] and *cpcL*[29] transcript levels are not reduced in mixotrophic cells whereas the amount of phycobilisome proteins decrease at these growth conditions (unpublished results). These findings suggest that a complex regulation mechanism may exist for these genes: the level of transcripts might be influenced by light and the translation efficiency of transcripts might be reduced by the presence of a carbon source in the medium.

We are now analysing the organization and expression of phycobilisome genes from the multicellular red alga *Antithamnion* sp. in order to characterize red algae-specific gene arrangements and expression patterns of such genes.

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