Curr Genet (1990) 17:255–259

Current Genetics

The *psbA*-gene from a red alga resembles those from Cyanobacteria and Cyanelles

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Received November 8/November 27, 1989

Summary. Plastid DNA (ptDNA) from the unicellular red alga *Cyanidium caldarium* was isolated. A 5.8 kb *Eco*RI, fragment containing the entire *psbA*-gene was cloned and the nucleotide sequence of the *psbA*-gene determined. At the carboxyl terminus the encoded protein (D1) contains the seven amino acid-insertion which was found to be typical of the cyanobacteria and the cyanelles of *Cyanophora paradoxa*. However, the overall sequence homology does not support a direct relationship between the plastids of *Cyanidium*, cyanelles and the cyanobacteria. As in other photosynthetic organisms the *psbA*-gene is transcribed as a monocistronic mRNA. The ribosomal RNA operon was located 4 kb upstream of the *psbA*-gene.

Key words: Red algae – *psbA gene* – Plastid evolution

Introduction

The hypothesis of an endosymbiotic origin of plastids is now generally accepted (Margulis 1981). It still remains uncertain, however, whether all recent plastids have a common ancestor (monophyletic origin) or whether several endosymbioses have led to different types of plastids (chloroplasts, phaeoplasts, rhodoplasts; polyphyletic origin).

A large body of data is available on the ptDNA of chlorophyll a & b-containing plants (green algae and higher plants, see review by Palmer 1985). Similarities in gene arrangement, coding capacity and nucleotide sequences of these ptDNAs suggest a monophyletic origin for chloroplasts.

PtDNAs of a few chlorophyll a & c-containing algae (heterokontophyta) have been analyzed (Kuhsel and Kowallik 1985, 1987; Reith and Cattolico 1986; Linne v. Berg and Kowallik 1988). These data have been used as evidence for a monophyletic origin of chloroplasts and chlorophyll a & c-containing plastids (phaeoplasts; Kowallik 1989).

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Sequence analysis of the genes of Ribulose-1,5-bisphosphate-carboxylase (RubisCO) from the unicellular red alga *Porphyridium aerugineum* indicates a polyphyletic origin for the plastids of red algae (rhodoplasts) and other plastids. The organization of the RubisCO genes in *Porphyridium* is similar to that in cyanobacteria but sequence data show a large evolutionary distance between recent cyanobacteria and rhodoplasts (Valentin and Zetsche 1989).

In order to verify the findings obtained with the RubisCO genes we analyzed another well conserved plastid gene from a red alga. The *psbA*-gene encodes for the quinone-binding, Photosystem II associated D1 protein. As compared with chloroplasts, cyanobacterial D1 proteins contain a characteristic insertion of seven amino acids (Mulligan et al. 1984; Curtis and Haselkorn 1984). The same insertion was found in the D1 protein from *Cyanophora paradoxa* Janssen et al. 1989). The D1 protein from *Prochlorothrix hollandica*, a chlorophyll a & b-containing procaryote, lacks the seven amino acid-insertion (Morden and Golden 1989; cf. Turner et al. 1989). It seems very unlikely that exactly the same insertion/deletion has occurred twice during evolution. Therefore, Cyanelles and chloroplasts have probably originated polyphyletically from distinct cyanobacteria-like ancestors. The ancestor of chloroplasts may have been a *Prochlorothrix hollandica*-like organism. Hence it was of interest to ascertain whether a red algal D1 protein contains the insertion, and whether sequence comparisons would allow the evolutionary relationships between rhodoplasts, cyanelles, chloroplasts and cyanobacteria to be elucidated.

Cyanidium has been interpreted by some authors as an association between an uncolored eucyte and an endosymbiotic cyanobacterium (Trench 1982). We have recently obtained sequence data for the RubisCO-operon of *Cyanidium* which did not support this hypothesis. In fact we found a distinctly higher homology between the RubinsCO-operons of *Cyanidium* and the red alga *Porphyridium aerugineum* than between *Cyanidium* and *cyanobacteria* (unpublished results).

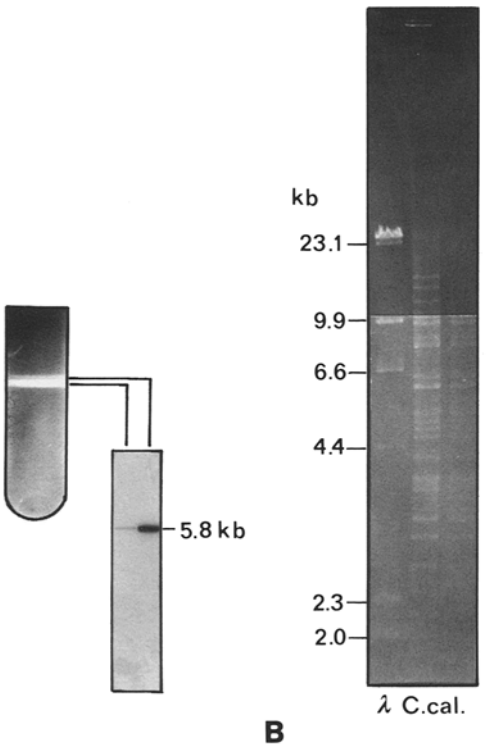

Fig. 1A. Ethidium bromide-stained CsCl-gradient of total cellular DNA from *Cyanidium caldarium*. Equal amounts of DNA from the upper and the lower band were digested with *Eco*RI, separated on agarose gels and hybridized with a *psbA*-gene probe from *Cyanophora paradoxa*. Upper-band DNA shows strong hybridization signal at 5.8 kb. Only a weak signal is detectable in the lower-band DNA lane, due to minor contamination of the upper-band DNA.
B DNA from the upper CsCl-gradient band, after *Eco*RI digestion, was electrophorezed on an agarose gel and stained with ethidium bromide. Separation of distinct bands indicates a DNA fraction with relatively small-sized molecules. Kb = kilobases

Materials and methods

Strain and culture conditions. Cyanidium caldarium Geitler, strain 14-1-1 was isolated by Allen (1959); it came as strain 107.79 from the collection of algae maintained by the Institute of Plant Physiology of the University of Göttingen. Cells were grown axenically under constant light (7,500 lux) in 5 litre flasks as described by Steinmüller et al. (1983). Cells were harvested by centrifugation for 10 min at 5,000 g.

Isolation of rhodoplast DNA. Cell pellets were resuspended in lysis buffer (50 mM Tris/HCl pH 7.5; 1% SDS; 50 mM EDTA; 500 µg proteinase K/ml) and incubated overnight at RT. Lysates were frozen, remelted, incubated again for 6 h at 60°C. Total cellular DNA was isolated by phenolization. PtDNA was separated from nuclear DNA by CsCl density gradient centrifugation in the presence of ethidiumbromide (Maniatis et al. 1982). DNA bands were characterized by heterologous hybridization and by restriction analysis (Fig. 1).

*Restriction endonuclease digestions. Eco*RI, *Bam*HI, *Hind*III, *Xba*I, *Alu*I and *Rsa*I (purchased from Boehringer Mannheim Biochemicals) digestions were worked out as described (Maniatis l.c.) and according to the manufacturer's recommendations. Fragments were separated in 0.5% to 1.5% ethidiumbromide stained agarose gels.

Mapping and hybridization procedures. DNA fragments were cloned in pUC18 vector. Collections of these fragments were screened by heterologous hybridization with a *psbA* probe from cyanelle DNA of *Cyanophora paradoxa*, which was also used to screen the two different DNA fractions from isopycnic centrifugation for nuclear or plastidal origin as described by Valentin & Zetsche (1989). As it was not possible to isolate more than several hundred ng ptDNA from *Cyanidium* we were not able to construct a physical map of ptDNA by restriction analysis. Therefore homologous hybridizations with isolated DNA fragments against filter-immobilized DNA fragments generated by other enzymes were carried out for further and more detailed mapping. A cloned 1.1 kb *Hind*III/*Xba*I fragment, representing about 95% of the *Cyanidium caldarium psbA*-gene, was used to screen for further *psbA* copies or pseudogenes. To elucidate the nearby plastome regions of the *psbA*-gene, a 16S + 23S rDNA probe from *Cyanophora paradoxa* cyanelle DNA was cloned (7.5 kb *Bam*HI/*Pst*I), using the mapping studies of Bohnert et al. (1985). Washing steps in the case of homologous probes were as described by Maniatis et al. (1987); in the case of heterologous probes, filters were washed three times with 1 × SSC; 0.1% SDS at 40°C for 30 min.

Labeling of DNA probes. Isolated DNA fragments were labeled either radioactively with ^{32}P or nonradioactively with digoxygenin-UTP, using nick-translation or nonradioactive labeling with hexanucleotide primers (Boehringer).

Northern analysis. Total cellular RNA preparations were kindly provided by R. Radetzky, Göttingen. Electrophoresis in 1.5% agarose gels containing formic aldehyde, blotting and hybridization were carried out as described (Maniatis et al. 1982; Davis et al. 1986).

Sequence analysis. Subclones of the *psbA* gene and its flanking regions were directly sequenced in pUC18, using the kilobase system from Bethesda Research Laboratories. Sequences of both DNA strands were determined and all restriction sites were crossed. Computer analysis was performed using the "Kroeger Menu" (Kröger & Kröger-Block 1984).

Results and discussion

Isolation of ptDNA and cloning of the psbA-gene and its flanking regions

After CsCl gradient centrifugation of total cellular DNA from *Cyanidium* one main DNA band and one satellite DNA band were visible (Fig. 1A). DNA from both bands was isolated, digested with *Eco*RI, electrophoresed and blotted. Blots were hybridized with a *psbA*-gene-probe from *Cyanophora paradoxa* (0.5 kb *Hind*II/*Hind*III fragment, unpublished). Only a 5.8 kb fragment from the upper satellite DNA band hybridized with this gene probe (Fig. 1A). The fact that the *psbA*-gene is located on the ptDNA of all plants analyzed so far (for recent review see Palmer 1985) strongly suggests that the upper satellite contains the ptDNA. In addition, digestion of this DNA with *Eco*RI yields a pattern of approximately 30 distinct bands (Fig. 1B) as is typical for the complexity of the DNA of mitochondria or plastids. (*Eco*RI digestion of the main band from the CsCl-gradient does not show any distinct bands, as was to be expected in the case of high molecular weight nuclear DNA).

The 5.8 kb fragment was cloned (pCCE 5.8) and physically mapped. An internal *Hind*III/*Xba*1 fragment (pCCpsbA), containing almost the whole *psbA*-gene [bases (–40)–1025, Fig. 2], was subcloned for further hybridization experiments.

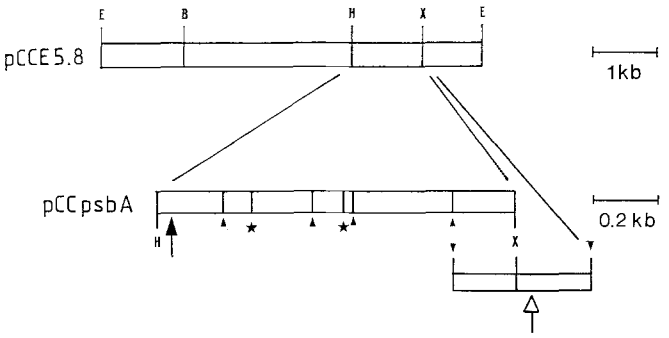

Fig. 2. Cloned DNA fragments containing the whole *psbA*-gene from *Cyanidium caldarium.* Restriction enzyme cleavage sites: *B* = *Bam*HI, *E* = *Eco*RI, *H* = *Hind*III, *X* = *Xba*I. *Asterisks* represent the *Rsa*I sites and *arrowheads* represent the *Alu*I sites which were chosen for subcloning and sequencing. The *solid* and *open arrow* indicate the position of the initiator and terminator codon, respectively, of the protein coding region

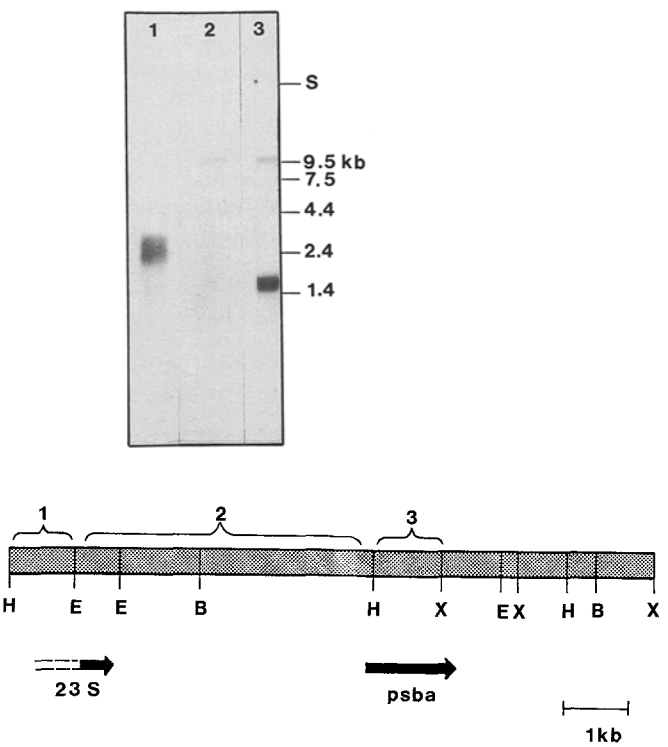

Fig. 3. Physical map of the entire cloned area containing the *psbA*-gene and parts of the ribosomal RNA operon from *Cyanidium caldarium.* (Abbreviations of enzyme cleavage sites as in Fig. 2). The corresponding ^{32}P-labeled Northern analysis is shown for the *psbA* locus and its upstream region. Exposure time for the left Northern blot lane (23S rRNA) was 1 h and for the middle and right lane 18

Whereas cyanobacteria and prochlorophyta contain several copies of slightly different *psbA*-genes (Curtis and Haselkorn 1984; Mulligan et al. 1984; Morden and Golden 1989) only one copy was found on the ptDNA from chloroplasts (Zurawski et al. 1982; Hirschberg and McIntosh 1983; Ohyama et al. 1986). On the chloroplast

Table 1. Percentage of amino-acid homology of *Cyanidium caldarium* D1 protein compared with counterparts in cyanobacteria, prochlorophyta, cyanelles and chloroplasts

Anabaena 7120 psbA-I (625)[1]	81.9
Cyanophora paradoxa[2]	84.4
Fremyella diplosiphon[3]	81.9
Prochlorothrix hollandica[4]	83.6*
Spinacia oleracea[5]	87.0*

* Calculation based on 353 amino acid residues
[1] Curtis and Haselkorn 1984
[2] Janssen et al. 1989
[3] Mulligan et al. 1984
[4] Morden and Golden 1989
[5] Zurawski et al. 1982

DNA of *Chlamydomonas reinhardtii* (Rochaix 1978), as well as on the phaeoplast DNA of *Olisthodiscus luteus* (Reith and Cattolico 1986), the *psbA*-genes are located within the inverted repeats and therefore exist in two identical copies. No further copy of the *psbA*-gene on the ptDNA of *Cyanidium* could be detected by hybridization experiments with the pCCpsbA fragment (data not shown). The loss of all but one copy of the *psbA*-gene during endosymbiotic evolution therefore seems to be a common feature of different lines of plastid evolution (rhodoplasts, chloroplasts). Several copies of the *psbA*-gene in cyanobacteria might be advantageous for complex regulation of D1 protein expression. In *Synechococcus* a differential response in expression of the two *psbA*-geneforms to varying light conditions was observed (Schaefer and Golden 1989).

Partially overlapping and neighbouring fragments of pCCE 5.8 were isolated and cloned (see Fig. 3).

Transcription analysis of the cloned area and localization of parts of the ribosomal RNA operon upstream the psbA-gene

Looking for further genes in the *psbA*-gene neighbourhood, in order to determine the length of noncoding regions on the rhodoplast DNA, we analyzed the transcription of the cloned areas.

The *psbA*-gene itself is transcribed as monocistronic RNA, approximately 1.5 kb in size as is shown in Fig. 3. For about 4.5 kb upstream of the *psbA*-gene, no transcript could be detected. At that distance, we found a region which is much more strongly transcribed than the *psbA*-gene itself (Fig. 3). As the *psbA*-gene is known for high transcript levels, which has been explained in terms of a comparatively stable mRNA (see review by Kyle 1984), it seemed likely for this gene to contain parts of the ribosomal operon. To verify this assumption we cloned the ribosomal RNA operon from *Cyanophora paradoxa* (7.5 kb *Bam*HI/*Pst*I fragment, Bohnert et al. 1985; Janssen et al. 1987) and used it to locate parts of the ribosomal RNA operon on the distal *Hind*III/*Eco*RI fragment (see Fig. 3, bracket No. 1) by heterologous hybridization (data not shown). Finally, sequence comparison with 23S rDNA data (e.g., *Euglena gracilis*,

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                                                                      57
ATG ACA GCT ACA TTA GAA AGA CGT CAA ACA GCA AGT TTA TGG GAA CGT TTT TGT TCT
Met Thr Ala Thr Leu Glu Arg Arg Gln Thr Ala Ser Leu Trp Glu Arg Phe Cys Ser
                                                                     114
TGG ATA ACT AGC ACA GAA AAT CGC CTA TAC ATA GGT TGG TTT GGT GTA TTG ATG ATA
Trp Ile Thr Ser Thr Glu Asn Arg Leu Tyr Ile Gly Trp Phe Gly Val Leu Met Ile
                                                                     171
CCT ACA TTA TTA ACA GCT ACA TCT GTA TTT ATA ATT GGT TTT ATA GCT GCT CCA CCT
Pro Thr Leu Leu Thr Ala Thr Ser Val Phe Ile Ile Gly Phe Ile Ala Ala Pro Pro
                                                                     228
GTT GAT ATA GAT GGT ATT AGA GAA CCA GGT TTC AGG TCC TTA TTA TAT GGA AAT AAT
Val Asp Ile Asp Gly Ile Arg Glu Pro Gly Phe Arg Ser Leu Leu Thr Gly Asn Asn
                                                                     285
ATT ATA ACC GGT GCT ATT GTA CCT ACA TCT AAT GCA ATA GGA ATA CAC TTT TAT CCT
Ile Ile Thr Gly Ala Ile Val Pro Thr Ser Asn Ala Ile Gly Ile His Phe Tyr Pro
                                                                     342
ATT TGG GAA GCA GCA TCA TTA GAT GAA TGG TTA TAT AAT GGT GGT CCT TAT GAA TTA
Ile Trp Glu Ala Ala Ser Leu Asp Glu Trp Leu Tyr Asn Gly Gly Pro Tyr Glu Leu
                                                                     399
ATA GTT TTG CAC TTC TTT ATT GGA ATT TGT GCA TAT ATG GGA CGT GAA TGG GAA TTA
Ile Val Leu His Phe Phe Ile Gly Ile Cys ALa Tyr Met Gly Arg Glu Trp Glu Leu
                                                                     456
AGT TAT CGT CTT GGA ATG CGT CCT TGG ATC GCT GTA GCT TTT TCA GCT CCT GTT GCT
Ser Tyr Arg Leu Gly Met Arg Pro Trp Ile Ala Val Ala Phe Ser Ala Pro Val Ala
                                                                     513
GCC GCC ACA GCT GTT TTT ATT ATA TAT CCT ATC GGA CAA GGT AGT TTT TCA GAT GGT
Ala Ala Thr Ala Val Phe Ile Ile Tyr Pro Ile Gly Gln Gly Ser Phe Ser Asp Gly
                                                                     570
ATG CCA TTA GGT ATT TCT GGT ACT TTT AAT TTT ATG TTA GTT TTC CAA GCT GAA CAT
Met Pro Leu Gly Ile Ser Gly Thr Phe Asn Phe Met Leu Val Phe Gln Ala Glu His
                                                                     627
AAT ATT TTA ATG CAT CCA TTC CAT ATG ATG GGT GTT GCT GGT GTA TTT GGT GGT TCA
Asn Ile Leu Met His Pro Phe His Met Met Gly Val Ala Gly Val Phe Gly Gly Ser
                                                                     684
CTA TTT AGT GCA ATG CAT GGT TCC TTG GTA ACT TCT AGT TTG ATC CGT GAA AGA ACA
Leu Phe Ser Ala Met His Gly Ser Leu Val Thr Ser Ser Leu Ile Arg Glu Arg Thr
                                                                     741
GAG AAT GAG TCT GCT AAC AAT GGT TAT AAA TTC GGT CAA GAA TAT GAA ACT TAT AAC
Glu Asn Glu Ser Ala Asn Asn Gly Tyr Lys Phe Gly Gln Glu Tyr Glu Thr Tyr Asn
                                                                     798
ATC GTT GCT GCT CAT GGT TAT TTT GGA AGA TTA ATT TTC CAA TAT GCA AGT TTT AAT
Ile Val Ala Ala His Gly Tyr Phe Gly Arg Leu Ile Phe Gln Tyr Ala Ser Phe Asn
                                                                     855
AAT TCA CGT TCA TTA CAT TTC TTC TTA GCT TTA TGG CCA GTA GTA TGT ATT TGC GTT
Asn Ser Arg Ser Leu His Phe Phe Leu Ala Leu Trp Pro Val Val Cys Ile Cys Val
                                                                     912
ACA GCT CTA GGT GTT AGC ACC ATG GCA TTT AAC TTA AAC GGA TTC AAT TTC AAC CAA
Thr Ala Leu Gly Val Ser Thr Met Ala Phe Asn Leu Asn Gly Phe Asn Phe Asn Gln
                                                                     969
TCT GTT GTT GAT TCT CAA GGA AGA GTA ATT AAT ACT TGG GCT GAT ATT TTA AAT CGT
Ser Val Val Asp Ser Gln Gly Arg Val Ile Asn Thr Trp Ala Asp Ile Leu Asn Arg
                                                                    1026
GCA AAT TTA GGA ATA GAA GTA ATG CAC GAA CGT AAT GCA CAT AAC TTC CCT CTA GAT
Ala Asn Leu Gly Ile Glu Val Met His Glu Arg Asn Ala His Asn Phe Pro Leu Asp
                                                                    1083
TTA GCA AGC GAG GTA TCT TTA CCA GTT GCT TTA AAT AAA GTA GAA ATA AAT GGT TAA
Leu Ala Ser Glu Val Ser Leu Pro Val Ala Leu Asn Lys Val Glu Ile Asn Gly  *

AAAAAAAACT AAACTCTTAA AAGAGTTTAG TTTTTTTTTT AACCATTTAT TTCTACTTTA TTTTTTTT
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Fig. 4. Nucleotide sequence and deduced amino acid sequence of the *Cyanidium caldarium psbA*-gene and the putative transcription-terminating region. This inverted repeat, consisting of a diad symmetry of 43 bases (*underlined*), extends into the protein-coding region (nucleotides in *italics*)

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LDLAAGEVAPVALTAPAING*      Anabaena 7120
LDLAAGEVAPVALTAPAING*      Fremyella diplosyphon
LDLASEVSLPVALNKVEING*      Cyanidium caldarium
LDLAAVK-------APSIIG*      Prochlorothrix hollandica
LDLAAVE-------APAVNG*      Marchantia polymorpha
LDLAAIE-------APSTNG*      Spinacia oleracea
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Fig. 5. The C terminus of the D1 protein. Comparison of amino-acid sequences from different genera starting from residue 341 to the termination codon (*)

Yepisz-Plaszencia et al. 1988) enabled us to determine the location and orientation of the 23S rDNA gene from *Cyanidium caldarium* as shown in Fig. 3.

Sequence analysis

Figure 4 shows the nucleotide sequence and the deduced amino acid sequence of the *psbA*-gene from *Cyanidium caldarium*. Sequence homologies between different D1 proteins are given in Table 1.

The *Cyanidium* D1 protein contains the cyanobacterial seven amino acid-insertion at the carboxyl terminus (see Fig. 4 + 5) which was also found in the D1 protein of *Cyanophora paradoxa* (Janssen et al. 1989), an association between an eucaryotic flagellat and an endosymbiotic cyanobacterium. D1 proteins from chloroplasts and from *Prochlorothrix hollandica* lack this insertion (Morden & Golden 1989). The absence of the seven amino acid-insertion has been interpreted as strong evidence of a close relationship between both (l.c.). It therefore seems legitimate to assume a phylogenetic relationship between the plastids of *Cyanidium*, Cyanobacteria and possibly also the endosymbionts of *Cyanophora paradoxa* based on the presence of the same insertion. Sequence comparison of different D1 proteins does not support these conclusions. In fact the D1 protein from *Cyanidium* is more similar to that from spinach than those from cyanobacteria (see Table 1). As a close relationship between *Cyanidium* and spinach seems very unlikely, the D1 protein function may not allow more variability than that found between cyanobacteria and *Cyanidium* or spinach (about 20% nucleotide divergence). The higher degree of *psbA*-sequence homology between *Cyanidium* and spinach may thus reflect a convergent development during evolution.

From this point of view, the occurence/nonoccurence of the seven amino acid-insertion is a better criterion than sequence homology for evaluating the phylogenetic relationship between the main lines of plastid evolution. Sequence comparisons of the *psbA*-gene should be used to distinguish between more closely related species, such as different members of red algae or green algae (cf. Meyer et al. 1986).

The structure of the *psbA*-gene, therefore, supports our previous findings with the RubisCO genes from the unicellular red algae *Porphyridium aerugineum* (l.c.). Both results support the idea of a polyphyletic origin of chloroplasts and rhodoplasts. The occurrence of the same insertion tn the D1-protein from Cyanidium and Cyanophora does not necessarily indicate a close relation between their organelles. Further sequence data are necessary to estimate the time scale of division of these different lines of plastid evolution. Sequence and transcript analyses of *psbA*-gene, RubisCO genes and ribosomal operons from other red algae are in progress in our laboratory.

Another interesting fact is that *C. caldarium* was originally isolated from hot springs (Allen 1959). The optimal growth temperature is about 45 °C but the algae is able to live up to 55 °C. This temperature is quite unusual for an eucaryotic photosynthetic organism (Brock 1985; Ford 1986) but is not reflected by a distinct change within the amino acid composition of the D1-protein compared with organisms growing in a lower temperature range.

Acknowledgements. We wish to thank I. Neul for documentation assistance, R. Radetzky for preparing the RNA and M. and A. Kröger for DNA software. This work was supported by the Deutsche Forschungsgemeinschaft (Schwerpunktprogramm Endocytosymbiose; Ze 71/21-1).

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Communicated by H. Kössel