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Inverted repeat of *Olisthodiscus luteus* chloroplast DNA contains genes for both subunits of ribulose-1,5-bisphosphate carboxylase and the 32,000-dalton Q_B protein: Phylogenetic implications

(chromophytic alga/restriction enzyme map/heterologous hybridization/chloroplast evolution)

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ABSTRACT The chloroplast DNA of the chromophytic alga *Olisthodiscus luteus* has been physically mapped with four restriction enzymes. An inverted repeat of 22 kilobase pairs is present in this 150-kilobase-pair plastid genome. The inverted repeat contains the genes for the large and small subunit polypeptides of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) and also codes for the 32,000-dalton Q_B protein. These observations demonstrate that significant differences exist in chloroplast genome structure and organization among major plant taxa.

In recent years, the concept of an endosymbiotic origin for chloroplasts has been generally accepted. Data that demonstrate potential relatedness between chloroplasts and photosynthetic prokaryotes are extensive and include similarities in protein-synthesizing systems, rRNA species, ribosomal composition, gene arrangement, mode of DNA transcription, and homology between structural and enzymatic proteins (see ref. 1 for review).

Extant plants are grouped into three distinct categories based on chloroplast morphology and biochemistry. Although chloroplast pigment composition is the primary basis for this separation (chlorophytes contain chlorophylls a and b; rhodophytes, chlorophyll a and phycobilins; and chromophytes, chlorophylls a and c and sometimes phycobilins), other plastid differences such as limiting membrane number, thylakoid arrangement, DNA localization, and storage product composition distinguish the chloroplasts found within these plant types (2).

Two hypotheses have been advanced to explain the origin of such extensive diversity among chloroplasts. The monophyletic scheme suggests (3, 4) that in early evolutionary time, a colorless host phagocytosed and maintained a photosynthetic prokaryote. Following this single symbiotic event the ancestral chloroplast genome diverged, resulting in plastid specialization. Alternatively, the observed variation seen among chloroplast types could have arisen in a polyphyletic manner (5–7)—that is, different plastid types may have had different photosynthetic prokaryotes as their progenitors. The polyphyletic hypothesis also suggests that the chloroplast of some plants (euglenoids, chromophytes) might have arisen through a second symbiosis in which a photosynthetic eukaryote served as the plastid source. Although chloroplast diversity may reflect ancestral genome source, observed variations among plastids may also have been influenced by different strategies of gene transfer from the symbiont DNA to the host nuclear DNA (8).

Data on genome size, structure, gene arrangement, and, ultimately, information on protein and DNA sequences for different chloroplast types and photosynthetic prokaryotes are needed to analyze the evolutionary development and relatedness of plant chloroplasts. To date, however, most studies of the chloroplast genome have focused on chlorophytic plant species. Restriction fragment size or contour length measurements of chloroplast DNA (ctDNA) for almost 250 algal, liverwort, fern, angiosperm, and gymnosperm representatives have been published (see ref. 9 for review) and, for some of these plants, there is extensive physical and genetic mapping information. Data obtained from these studies are remarkably consistent. The ctDNA of most chlorophytes exists as a homogeneous population of supertwisted, circular molecules that are ≈140 kilobase pairs (kb) in size. A feature prominent in most but not all chlorophytic ctDNAs is the presence of a large inverted repeat (IR) region that has been shown to contain the rRNA cistrons, some tRNAs, and, to date, one ribosomal protein (9). In all chlorophytes that do contain an IR, genes for photosynthetic functions are coded in the ctDNA single copy region except within the genus *Chlamydomonas* (10, 11) and in *Pelargonium hortorum* (12).

In contrast to the abundant data available for green plant systems, little work has been done on chromophyte or rhodophyte representatives. Recent restriction data show that the *Chattonella subsalsa* has a ctDNA of ≈150 kb in size (R.A.C., unpublished). An abstract by Linne von Berg and coworkers (13) provides genome sizes of 129, 120, 108–116, and 110 kb for *Tribonema verde, Botrydium granulatum, Vaucheria sp.*, and *Odentella sinensis*, respectively; *Dictyota dichotoma* ctDNA is 123 kb and size heterogeneity in ctDNAs has been reported for *Pylaiella littoralis, Spacelaria sp.* (14), *Monodus sp.* (M. Hedberg, personal communication), and *Ochromonas danica* (N. Li and R.A.C., unpublished). All these organisms are chromophytes. Recent data (N. Li and R.A.C., unpublished) demonstrate that the rhodophyte *Griffithsia pacifica* has a ctDNA genome that is 178 kb in size.

The only extensive analysis of any nonchlorophytic plant ctDNA has been done using the alga *Olisthodiscus luteus*. Restriction analysis (15), contour length measurement (16), and reassociation kinetic analysis (17) demonstrate that this chromophyte contains a ctDNA that is ≈150 kb in size. Like chlorophytic plants, the chloroplast genome is polyploid but homogeneous (17).

Recent *in vivo* chloroplast protein labeling studies (18) demonstrate that the ctDNA of plastids containing chlorophyll c codes for a spectrum of proteins that differs from that of the chlorophyll a, b plastid type. Ribulose-1,5-bisphosphate carboxylase (RbuP_2Case; EC 4.1.1.39) in chlorophytic

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Abbreviations: ctDNA, chloroplast DNA; kb, kilobase pair(s); RbuP_2Case, ribulose-1,5-bisphosphate carboxylase; IR, inverted repeat; LS, large subunit of RbuP_2Case; SS, small subunit of RbuP_2Case.

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plants is composed of eight large chloroplast-encoded and eight small nuclear-encoded polypeptides (19). When *O. luteus* chloroplast proteins were labeled in the presence of cycloheximide (an 80S ribosome inhibitor), the large and small subunits of RbuP_2Case (LS and SS) were synthesized (as detected by immunoprecipitation), demonstrating that both polypeptides are ctDNA encoded even though the enzyme has a polypeptide complement similar to that seen in chlorophytic plants (20).

In this communication, we demonstrate that the plastid of the chromophyte *O. luteus* contains a ctDNA that has a gene dosage, arrangement, and complement significantly different from that observed in any chlorophytic ctDNA analyzed to date.

METHODS

Cell Maintenance. *O. luteus* Carter was grown in O-3 medium on a 12-hr light:12-hr dark cycle as described (18).

Mapping Studies. *O. luteus* ctDNA was isolated as described (15) and digested with restriction enzymes *Bam*HI, *Bgl* II, *Pst* I, and *Sal* I according to the supplier's (Bethesda Research Laboratories) directions. Fragments were separated on 0.7% agarose submarine gels and visualized by ethidium bromide staining. For hybridization studies, restriction fragments were transferred to nitrocellulose filters by the method of Southern (21). A partial clone bank of ctDNA sequences was constructed by ligating *Pst* I-digested ctDNA into the *Pst* I site of pBR322 and transforming *Escherichia coli* HB101 (22). Additional *Bam*HI and *Bgl* II fragments were cloned in the *Bam*HI site of pUC9 (23). Clones were screened by the method of Holmes and Quigley (24) and plasmid DNA was isolated according to Godson and Vapnek (25). Cloned DNA was nick-translated (22), denatured, and hybridized overnight to nitrocellulose filters in hybridization buffer A [50% formamide, 0.75 M NaCl/75 mM sodium citrate, pH 7, 5× concentrated Denhardt's solution (26), 0.2% $NaDodSO_4$] at 37°C. Filters were washed twice in 0.3 M NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$ for 10 min at room temperature and twice in 30 mM NaCl/3 mM sodium citrate/0.1% $NaDodSO_4$ for 45 min at 68°C. Alternatively, labeled probes were generated by nick-translating total ctDNA in the absence of added DNase (27), restricting the labeled DNA with the appropriate restriction enzyme, and separating the fragments by agarose gel electrophoresis. The desired fragments were excised from the gel, melted at 100°C, and added directly to nitrocellulose filters in hybridization buffer A.

Hybridizations with mung bean *rbcL* (28) and maize *psbA* (a gift from L. McIntosh, Michigan State University) probes were carried out as described above except that hybridization buffer B (25% formamide, 0.75 M NaCl/75 mM sodium citrate, 5× concentrated Denhardt's solution, 0.2% $NaDodSO_4$) was used and the second series of washes was done with 0.3 M NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$ at 55°C.

Immunoprecipitation of Linked Transcription-Translation Products. An *E. coli* linked transcription-translation system (Amersham) was programed with 5 μg of plasmid DNA. Following incubation at 37°C for 60 min, aliquots were subjected to immunoprecipitation (29), using protein A-Sepharose. Antisera to *O. luteus* RbuP_2Case holoenzyme, LS or SS were prepared as described (18). Precipitated proteins were analyzed by electrophoresis on linear 12–18% polyacrylamide gradient gels followed by autoradiography (30, 31).

RESULTS

Physical Mapping. Restriction enzymes that produce a relatively simple fragment pattern after digestion of *O. luteus* ctDNA include *Pst* I, *Bam*HI, *Sal* I (15), and *Bgl* II. These enzymes were therefore used in the construction of a physical map of the ctDNA from this alga. A partial *O. luteus* ctDNA clone bank representing ≈70% of the *O. luteus* chloroplast genome was used to probe nitrocellulose filters that contained single or double digests of *O. luteus* ctDNA made with the four restriction enzymes listed above. Probes for additional, uncloned fragments were generated by isolating the labeled fragments from agarose gels as described in *Methods*. The results of these hybridizations are summarized in the restriction map shown in Fig. 1.

O. luteus ctDNA is a circular molecule ≈150 kb in length. This algal ctDNA contains two homologous regions that are present in an IR orientation. The boundaries of the IR are not well defined by the restriction enzymes chosen. The repeat length may range from approximately 15 to 22 kb. Preliminary evidence from further mapping studies suggests that the actual length is near the upper limit of this range. The single-copy regions are thus approximately 37 and 73 kb.

Chloroplast Gene Mapping. The synthesis of *O. luteus* ctDNA-encoded proteins has been studied by labeling cells in the presence of cycloheximide (18). Data indicated that the genes for three proteins, the 32,000-dalton Q_B protein and the LS and SS of RbuP_2Case, are among the products encoded on *O. luteus* ctDNA. The genes for these proteins are designated *psbA*, *rbcL*, and *rbcS*, respectively (32). The first two of these genes were mapped with heterologous DNA probes.

When a cloned mung bean 2.5-kb *Bgl* II fragment containing the *rbcL* gene (28) was used to probe *O. luteus* ctDNA cleaved with *Bgl* II, *Pst* I, *Bam*HI, or *Sal* I, the results shown in Fig. 2 were obtained. Hybridization to the *Bgl* II digest yielded a single band near the top of the gel where the two largest fragments (32.5 and 28.5 kb) comigrated. Using low-percentage agarose gels to resolve these fragments, it was observed that both fragments hybridized to the *rbcL* probe (results not shown). Mapping studies indicated that these fragments encompassed the entire IR portion of the genome. Further hybridization data (Fig. 2) verified this hypothesis and more precisely located the position of the *rbcL* gene. Two *O. luteus Pst* I restriction fragments (5.8 and 0.55 kb) that are located within the IR hybridized to the mung bean probe. Similarly, hybridization of the mung bean probe to three *Bam*HI fragments (19, 15.8, and 3.5 kb) was

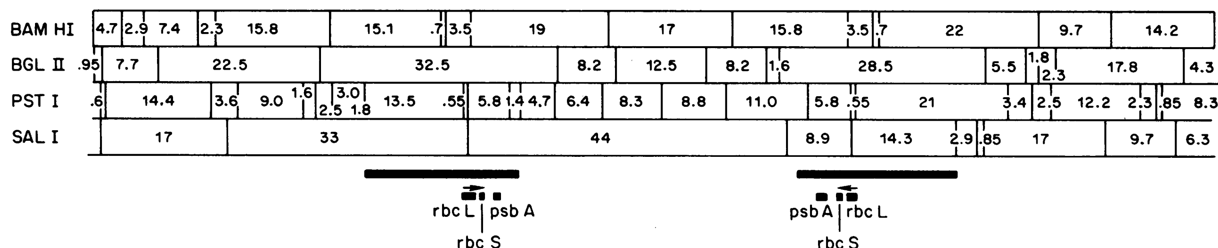

FIG. 1. Physical map of the *O. luteus* chloroplast genome constructed by Southern blot hybridization of cloned or isolated restriction fragments to restricted ctDNA. The circular map has been linearized at a *Bam*HI site in the large single-copy region of the genome.

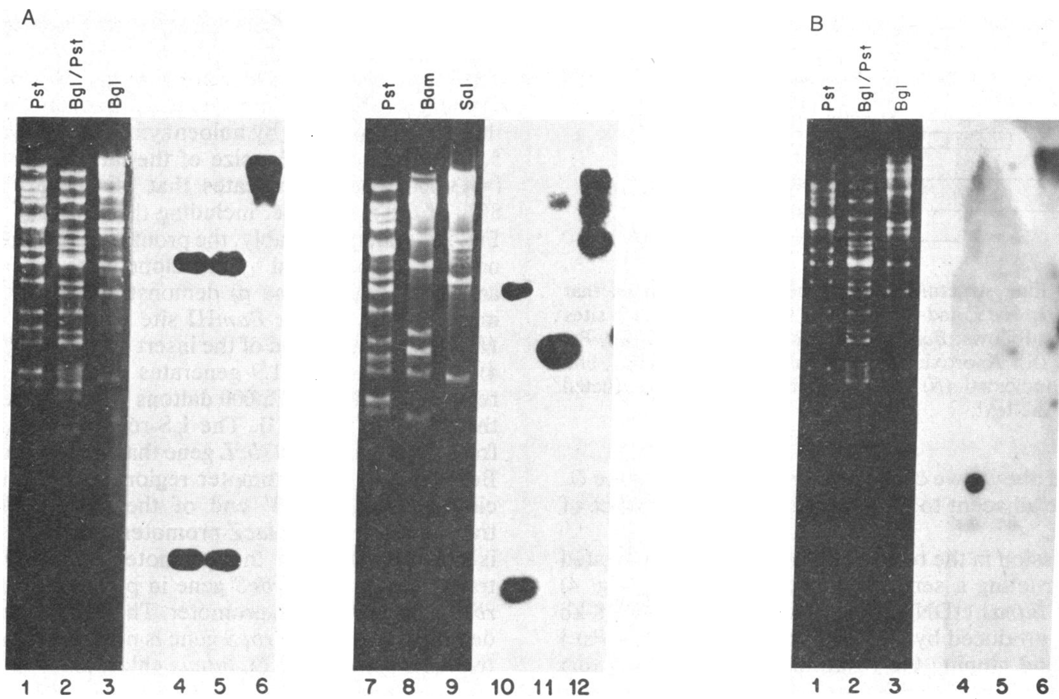

FIG. 2. (*A*) Hybridization of a mung bean *rbcL* probe to *O. luteus* ctDNA digested by *Pst* I (lanes 1, 4, 7, and 10), *Bgl* II/*Pst* I (lanes 2 and 5), *Bgl* II (lanes 3 and 6), *Bam*HI (lanes 8 and 11), and *Sal* I (lanes 9 and 12). Ethidium bromide-stained gels are shown in lanes 1–3 and 7–9, whereas hybridization results are shown in lanes 4–6 and 10–12. (*B*) Hybridization of a maize ctDNA probe containing only the 5′ end of the *rbcL* gene to *O. luteus* ctDNA digested by *Pst* I (lanes 1 and 4), *Pst* I/*Bgl* II (lanes 2 and 5), and *Bgl* II (lanes 3 and 6). Ethidium bromide-stained gels are shown in lanes 1–3, whereas hybridization results are shown in lanes 4–6.

observed. The 3.5-kb fragment is present in two copies per genome. Finally, four *Sal* I fragments (44, 33, 14.3, and 8.9 kb) hybridized with the *rbcL* mung bean probe. Identical data were obtained when an *rbcL* probe from *Anabaena cylindrica* was used. These results demonstrate that the *rbcL* gene of *O. luteus* is present in two copies per chloroplast genome and that the gene is located within the IR region of the ctDNA (Fig. 1) of this alga.

The polarity of the *O. luteus rbcL* gene was determined by hybridization with a maize *rbcL* probe containing 440 base pairs from the 5′ end of the gene (33). This probe hybridized to the *Pst* I 0.55-kb fragment but not the 5.8-kb fragment (Fig. 2*B*), indicating that transcription proceeds toward the small single-copy region of the genome (Fig. 1).

A maize *psbA* probe was used to map the location of *O. luteus* ctDNA 32,000-dalton Q_B protein gene. Both large *Bgl* II fragments (32.5 and 28.5 kb), the *Pst* I 5.8-kb fragment, two *Bam*HI fragments (19 and 15.8 kb), and two *Sal* I fragments (44 and 8.9 kb) hybridized (Fig. 3) to the maize probe. Thus, the *psbA* gene is also present in two copies in *O. luteus* ctDNA and is located within the IR. However, this gene is nearer the small single-copy region than the *rbcL* gene (Fig. 1). Further mapping studies (results not shown) placed this gene within a 2.3-kb *Hin*dIII/*Eco*RI fragment very close to the *rbcL* gene (Fig. 4).

Mapping the *rbcS* Gene. In all eukaryotic, chlorophytic plants studied to date, the *rbcS* gene has been shown to be a nuclear-encoded gene. However, inhibitor data coupled with immunological evidence (18) strongly indicate that, unlike chlorophytic plants, the chromophytic alga *O. luteus* codes for both subunits of this enzyme in the chloroplast. Because hybridization studies using several *rbcS* probes (pea, maize, cyanelle) were unsuccessful in localizing this gene on *O. luteus* ctDNA even when extremely low conditions of stringency were maintained, a second experimental approach was used in our attempt to map the *O. luteus rbcS*. It was reasoned that if *rbcS* is a ctDNA gene in *O. luteus*, it might be expected that the location and arrangement of *rbcS* with respect to *rbcL* would be similar to that seen in prokaryotic cells. For example, recent studies using the cyanobacteria *Anacystis nidulans* (34) and *A. cylindrica* (35) have shown that the *rbcS* gene is located just to the 3′ side of the *rbcL* gene. In *A. cylindrica* it has been demonstrated that both of these genes are transcribed as a single mRNA (35). Given the

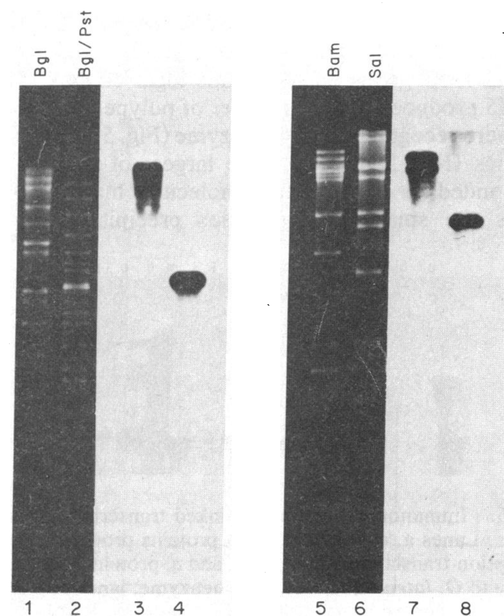

FIG. 3. Hybridization of a maize *psbA* probe to *O. luteus* ctDNA digested by *Bgl* II (lanes 1 and 3), *Bgl* II/*Pst* I (lanes 2 and 4), *Bam*HI (lanes 5 and 7), and *Sal* I (lanes 6 and 8). Ethidium bromide-stained gels are shown in lanes 1, 2, 5, and 6, whereas hybridization results are shown in lanes 3, 4, 7, and 8.

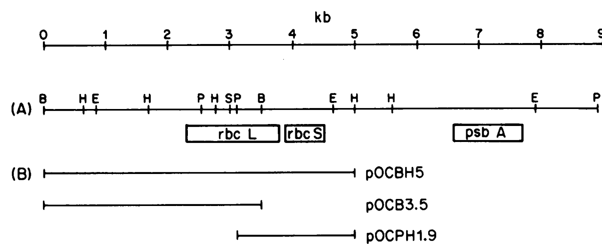

FIG. 4. (*A*) Fine structure map of the region of the IR that contains the *rbcL*, *rbcS*, and *psbA* genes. Restriction enzyme sites are designated as follows: *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst* I (P), and *Sal* I (S). Approximate positions of the *rbcL*, *rbcS*, and *psbA* genes are indicated. (*B*) Plasmid inserts that were constructed as described in the text.

premise stated above, we chose to search the region of the *O. luteus* genome adjacent to the *rbcL* gene for the presence of the *rbcS* gene.

Gene expression in the region of the *rbcL* was investigated by first constructing a series of chimeric plasmids (Fig. 4) containing *O. luteus* ctDNA. A subclone of the *Pst* I 5.8-kb fragment was produced by digesting this fragment with *Pst* I and *Hin*dIII and cloning the resulting 1.9-kb fragment that contains part of the *rbcL* gene into pUC8, generating pOCPH1.9. A second plasmid was constructed when the *Bam*HI 3.5-kb fragment was cloned into pUC9, producing pOCB3.5. The third plasmid containing the entire *rbcL* region was constructed by restricting pOCPH1.9 with *Bam*HI. This digestion removed a 0.4-kb fragment between the vector *Bam*HI site and the *O. luteus* ctDNA *Bam*HI site. Next the *O. luteus* *Bam*HI 3.5-kb fragment was inserted and the recovered clones were screened for the correct *O. luteus* ctDNA orientation by analyzing for the presence of the proper *Eco*RI restriction pattern. A plasmid reconstructing the *O. luteus* ctDNA sequence was isolated and designated pOCBH5.

These three plasmids were then expressed in an *E. coli* linked transcription-translation system and the resulting products were immunoprecipitated with antibody to the *O. luteus* RbuP_2Case holoenzyme, LS, or SS. The results demonstrate that the predicted contiguous arrangement of *rbcL* and *rbcS* genes exists in this algal ctDNA. Plasmid pOCBH5 produced a large number of polypeptides, most of which were recognized by holoenzyme (Fig. 5, lane f) and LS antibodies (Fig. 5, lane g). The largest of these proteins corresponded to the expected molecular mass of the LS, whereas the smaller polypeptides precipitated by holoenzyme or LS antibodies must represent premature termination products. Antibody to SS recognized only one polypeptide of a size appropriate for the SS (Fig. 5, lane h). Plasmid pOCB3.5 also produced a number of polypeptides that were recognized by holoenzyme and LS antibodies (Fig. 5, lanes n and o). The size of the largest of these peptides (≈43,000 daltons) indicates that pOCB3.5 contains about 80% of the *rbcL* gene, including the 5′ end of the gene (see Fig. 2*B*) and, presumably, the promoter. The observation that no peptides produced by this clone were precipitated by SS antibody (Fig. 5, lane p) demonstrates that the *rbcS* gene must lie between the *Bam*HI site in the *rbcL* gene and the *Hin*dIII site at the end of the insert of plasmid pOCBH5 (Fig. 4). Plasmid pOCPH1.9 generates two polypeptides: a LS-related peptide of ≈15,000 daltons (Fig. 5, lanes j and k) and the SS (Fig. 5, lane l). The LS-related peptide must come from the 3′ end of the *rbcL* gene that is included in this clone. Because the *rbcL* promoter region is not contained on the cloned insert, the 3′ end of the gene could have been transcribed from the *lacZ* promoter of pUC8. At this time, it is not clear whether this promoter is also involved in the transcription of the *rbcS* gene in pOCHP1.9 or whether the *rbcS* gene has its own promoter. The above data conclusively demonstrate that the *rbcS* gene is positioned on the 3′ side of the *rbcL* gene in the *O. luteus* chloroplast genome.

FIG. 5. Immunoprecipitation of linked transcription-translation products. Lanes a, e, i, and m: total proteins produced by *in vitro* transcription-translation; lanes b, f, j, and n: proteins precipitated by antibody to *O. luteus* RbuP_2Case holoenzyme; lanes c, g, k, and o: proteins precipitated by antibody to *O. luteus* RbuP_2Case LS; lanes d, h, l, and p: proteins precipitated by antibody to *O. luteus* RbuP_2Case SS. Linked transcription-translation was programed by pUC9 (lanes a–d), pOCBH5 (lanes e–h), pOCPH1.9 (lanes i–l), and pOCB3.5 (lanes m–p). Molecular mass markers (arrows) are bovine serum albumin (68,000 daltons), ovalbumin (45,000 daltons), carbonic anhydrase (29,000 daltons), and myoglobin (17,000 daltons).

DISCUSSION

A physical map of the chloroplast genome of the chromophytic alga *O. luteus* has been constructed. These mapping data and electron microscopic analysis (16) demonstrate that this alga contains a ctDNA that is circular and ≈150 kb in length. *O. luteus* ctDNA contains a large IR that is similar in size (≈22 kb) to that observed in most chlorophytes. Moreover, like the IR in green plants, the IR in *O. luteus* causes the genome to be arranged such that small (37 kb) and large (73 kb) single-copy regions occur.

The most striking feature that distinguishes the *O. luteus* chloroplast genome from chlorophyte ctDNAs is the presence of the gene for the SS of RbuP_2Case in the chloroplast genome. In all higher plants and green algae that have been investigated to date, *rbcS* is a nuclear DNA-encoded gene. However, there is evidence that the *rbcS* gene may also be ctDNA encoded in other nonchlorophytic plants. Several laboratories have demonstrated (ref. 36; C. Wasmann, personal communication) the presence of *rbcL* and *rbcS* on the cyanelle DNA of *Cyanophora paradoxa*, an organism often placed in the phylum Rhodophyta. In addition, Steinmuller *et al.* (37) found that the SS of RbuP_2Case is synthesized *in vitro* from non-poly(A) RNA in the true rhodophytes, *Cyanidium caldarum* and *Porphyridium cruentum*. These results indirectly suggest a plastid origin for the *rbcS* gene in these plants.

It is interesting to note that in cyanobacteria, cyanelles, and *O. luteus* (and possibly all rhodophytes and chromophytes), the *rbcL* and *rbcS* genes are closely linked, appear to be transcribed in the same relative direction, and are probably transcribed as a dicistronic mRNA. In addition, the order and spacing of the *rbcL*, *rbcS*, and *psbA* genes appear to be identical in *O. luteus* and cyanelles, although the cyanelle genes are found in the single-copy region of the genome. This conservation of gene order is consistent with models for the polyphyletic origin of chloroplasts, which suggest that cyanobacteria were the plastid precursors for rhodophytes and, through a second symbiosis, chromophytes. However, these observations do not eliminate the possibility of a monophyletic origin of plastids.

If more than one prokaryote was involved in the origin of the various chloroplast types, then similar mechanisms for the restructuring of the endosymbiont genome may have been

at work. The presence of an asymmetrically positioned IR containing rRNA cistrons is a feature of nearly all chloroplast genomes studied [the exceptions are *Euglena* and certain legumes (9)], including *O. luteus* (38). In addition, certain genes (e.g., *rbcL, psbA*) are present in the chloroplast genome of all plants investigated to date. Whether these similarities are due to a monophyletic origin of plastids or multiple, independent symbiosis events remains unclear. Further study of chromophyte and rhodophyte ctDNAs and the genomes of the putative chloroplast ancestors, cyanobacteria and *Prochloron*, should help resolve this controversy.

It is interesting that in the *O. luteus* chloroplast genome, the *rbcL, rbcS*, and *psbA* genes are all located within the IR. In chlorophytic plants, the presence of photosynthetic genes in the IR is the exception rather than the rule. Only in *P. hortorum* (*rbcL*) (12), *C. reinhardtii* (*psbA*) (10), and *C. eugametos* (*rbcL* and *psbA*) (11) have these genes been shown to be located in the IR. How (or if) genes are selected for maintenance within the IR is unknown at this time. It is possible that the presence of these genes in the IR confers a selective advantage because of either the increased gene dosage or the gene conversion/copy correction mechanism (12) that maintains sequence identity within the IR. Whether *psbA, rbcL*, and *rbcS* will be present within the IR of other chromophytic and rhodophytic plants (and perhaps be a useful evolutionary marker) remains an intriguing possibility.

Structural and kinetic analyses demonstrate (20) that though the coding location of *O. luteus* RbuP_2Case differs from the RbuP_2Case coding profile of land plants and green algae, the enzyme is similar in structure, having eight large and eight small polypeptide subunits, catalytic activation, pH requirements, and oxygenase activity, but has a K_m (CO_2) that is intermediate between cyanobacteria and chlorophytes. Amino acid composition studies (20) and high cross-reactivity with heterologous DNA probes (Fig. 2) demonstrate a high degree of sequence conservation for the LS. As expected, the SS polypeptide is significantly different in amino acid profile from that of chlorophytic plants. Preliminary data, however, show that the antibody to the SS of *O. luteus* precipitates the SS polypeptide of the rhodophytic alga, *G. pacifica* (20). Future comparison of DNA sequence data from chloroplast- and nuclear-encoded versions of this peptide should indicate conserved regions and possibly lead to a better understanding of SS evolution and information on the function of this polypeptide in the holoenzyme.

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