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The plastome of a brown alga, *Dictyota dichotoma**

II. Location of structural genes coding for ribosomal RNAs, the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and for polypeptides of photosystems I and II

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Summary. The plastid DNA of *Dictyota dichotoma* contains an inverted repeat of about 4.7 kb as determined with cloned *Bam*HI – *Sal*I subfragments. The repeat separates two single copy regions of 72 and 42 kb, respectively, and contains the genes for the 16 S and 23 S rRNAs. The *rbcL* gene was located 27 kb from the *rrnA* operon in the large single copy region, whereas no Southern hybridization signals could be obtained using two *rbcS* probes from higher plants. The genes coding for the polypeptides of photosystems I and II appear to be highly rearranged in comparison with plastomes from higher plants. The *psaA* and *psaB* genes map closely together inside the large single copy region, less than 8 kb from the *rrnA* operon. About 60 kb separates these genes from a second locus hybridizing with the *psaA* probe. Genes for photosystem II associated polypeptides (*psbA-E*) are spread over both single copy regions. The close association of the *psbC* and *psbD* genes within a 4.1 kb *Bam*HI – *Bgl*II subfragment suggests that they may form a single transcriptional unit as in higher plants.

Key words: *Dictyota* – Plastome – Inverted repeat – Southern hybridization – Gene mapping

Introduction

We have recently reported on the restriction map of the plastome of *Dictyota dichotoma* as the first example from the chlorophyll *a*/*c* line of the algal kingdom (Kuhsel and Kowallik 1985). As in all other land plants and algae, with the possible exception of *Sphacelaria* and *Pylaiella* (Dalmon et al. 1983), the *Dictyota* plastome is represented by circular molecules belonging to a single size class. Its size of 123 kb is slightly smaller than that of most land plants but is the same as that of certain bryophytes (Herrmann et al. 1980; Ohyama et al. 1983) and that of *Cyanophora paradoxa* (Bohnert and Löffelhardt 1982).

Since little information about plastomes from algae belonging to the brown algal line is available, we have further characterized the plastome of *Dictyota* for the following reasons: (i) it is expected that members of the chlorophyll *a*/*c* line, as the second major group of photoautotrophic plants, may exhibit specific differences in both gene arrangement and gene structure. This expectation is based on a comparison of the plastomes of members of the Chlorophyta: whereas the overall gene arrangement is rather conserved among higher plants, the plastome in *Chlamydomonas* differs in several respects including the arrangement, duplication, and structure of genes (Rochaix 1981; Erickson et al. 1984; Lemieux and Lemieux 1985; Palmer 1985). Peculiarities in gene arrangement and gene structure also exist in the plastome of the Euglenophyte *Euglena gracilis* (Wurtz and Buetow 1981; Keller and Stutz 1984). (ii) The considerable taxonomic heterogeneity in the chlorophyll *a*/*c* line provides a suitable basis for investigating plastome evolution at different taxonomic and phylogenetic levels. This is of special interest since both freshwater and marine organisms of probably different phylogenetic age belong to several phyla of this algal branch.

In this contribution we report the location of genes on the plastome of *D. dichotoma*, including those coding for the 16 S and 23 S rRNAs, the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase and polypeptides associated with photosystems I and II.

Materials and methods

Restriction enzymes were purchased from Boehringer, Mannheim, and used according to the supplier's advice. High melting agarose (SeaKem) was from FMC Corporation, Rockland, the nitrocellulose membrane filters from Schleicher and Schüll, Dassel, and the [^{32}P]ATP from Amersham, UK.

The ptDNA of *Dictyota* was isolated and purified as described (Kuhsel and Kowallik 1985). DNA fragments produced by single and double digestions using the restriction endonucleases *Sal*I, *Bgl*II and *Bam*HI were transferred from agarose gels onto nitrocellulose filters according to Southern (1975). Fragments carrying structural genes coding for rRNAs and for soluble and structural polypeptides were detected by heterologous hybridization experiments using nick-translated probes (Rigby et al. 1977) from *Sinapis alba* and *Spinacia oleracea* ptDNA (Table 1).

If not otherwise stated, all hybridization experiments were carried out under reduced stringency conditions. These include overnight hybridization at room temperature in 50% formamide and 5 × SSC (Maniatis et al. 1982), followed by several washings in decreasing salt (2 × 30 min, 5 × SSC; 2 × 5 min, 2 × SSC; 1 min, 1 × SSC). The nitrocel-

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Abbreviations: ptDNA, plastid DNA; B, *Bam*HI; Bg, *Bgl*II; BBg, *Bam*HI – *Bgl*II; S, *Sal*I; BS, *Bam*HI – *Sal*I; IR, inverted repeat; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate

* Dedicated to Prof. W. Nultsch on occasion of his 60th birthday

Table 1. Probes used for Southern hybridization

Gene	Clone	Redigestion of insert	Probe	Reference
16 S rDNA	pSA 103a	–	Insert (1.45 kb noncoding region at 5′ end)	Przybyl et al. (1984)
23 S rDNA	pSA 107	–	Insert (3.4 kb noncoding region at 3′ end)	Przybyl et al. (1984)
rbcL	pWHsp 403/E_2	–	Insert (0.4 kb noncoding region at 5′ end)	Zurawski et al. (1981)
rbcS	pBT 76-2	*Bam*HI + *Pst*I	Subfragment 2	Nelson et al. (1984)
psaA	pWHsp 415b	*Kpn*I + *Bam*HI	Subfragment 2	Kirsch et al. (1986)
psaB	pWHsp 420	–	Insert	Kirsch et al. (1986)
psbA	pWHsp 206	*Pst*I + *Xba*I	Subfragment 2	Zurawski et al. (1982)
psbB	pWHsp 207/E_3	–	Insert (0.3 kb noncoding region at 3′ end)	Morris and Herrmann (1984)
psbC	pWHsp 408b	*Sac*I	Subfragment 3	Alt et al. (1984)
psbD	pWHsp 408b	*Sac*I	Subfragment 2 (0.2 kb noncoding region at 5′ end, 160 bp of *psbC* at 3′ end)	Alt et al. (1984)
psbE	pW 205/E_5	*Hpa*II	Subfragment 2 (0.2 kb noncoding region at 5′ end)	Herrmann et al. (1984)

lulose filters were exposed to Fuji RX or Kodak X-Omat AR film for 1–7 days.

Cloning of BamHI–SalI subfragments. Highly purified ptDNA at a concentration of 0.5 µg/µl was digested by *Bam*HI followed by *Sal*I. This combination produced a total of 18 fragments of moderate size, ranging from 0.5 to 19.2 kb (Kuhsel and Kowallik 1985). The fragments were cloned into the vector pUC18 (Norrander et al. 1983). Ligation was performed in a volume of 20 µl, containing 1 µg vector DNA, 1 µg *Dictyota* ptDNA, 10 mM Tris, pH 7.4, 7 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT, 1 mM ATP, 1 unit T4 DNA ligase, for 12 h at 4° C followed by 2 h at room temperature. Transformation of *Escherichia coli* RR1 ΔM15 by the calcium chloride procedure and amplification were as described in Maniatis et al. (1982). Transformants were selected using the β-galactosidase reaction according to Horwitz et al. (1964). Inserts were cut out with the same enzyme combination and separated from the vector DNA by electrophoresis in preparative gels. The identity of each fragment was determined both by its size and by hybridization against single and double digests of whole ptDNA. This method was used for cloning all fragments, with the exception of BS 1, 11, and 15.

Results

Identification of fragments carrying the rRNA genes

Southern hybridization experiments using rDNA probes from *S. alba* ptDNA identified fragments from both single and double digests which are situated almost exactly opposite each other on the circular plastome of *Dictyota*. Since this molecule does not have large regions of repeated sequences (Kuhsel and Kowallik 1985), we examined whether duplications of small fragments might be present that had previously been below the level of detection. Following reciprocal *Bam*HI + *Bgl*II redigestions, it was possible to identify subfragments with a minimum size of about 500 bp. In this way a total of 22 primary *Bgl*II fragments and 34 *Bam*HI – *Bgl*II subfragments were identified (Fig. 1). This resulted in the rearrangement of certain primary *Bgl*II fragments on the physical map. It turned out that Bg19/20 as well as BBg24/25 and BBg33/34 form two groups of fragments in inverted orientation (Fig. 7).

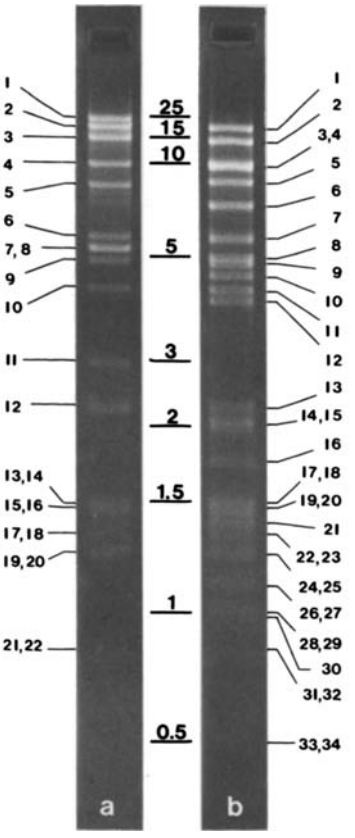

Fig. 1 a, b. Restriction patterns of ptDNA from *Dictyota dichotoma* after *Bgl*II digestion (**a**) and *Bam*HI + *Bgl*II double digestion (**b**) showing designation of individual fragments according to decreasing size. A molecular weight scale in kb is shown between the two lanes

Figure 2 shows the fragments from *Sal*I, *Bam*HI, and *Bgl*II digests, and from *Bam*HI + *Bgl*II double digests that hybridize to probes carrying either the 16 S rRNA or the 23 S rRNA genes from *S. alba* ptDNA. Strong signals resulting from the 16 S rDNA probe are observed with fragments S5, B7, B10, BBg24/25, and Bg19/20. This indicates that the greater part of the gene is located on these fragments. In particular, using the *Bam*HI restriction pattern, probe pSA 103a exclusively hybridizes to two fragments

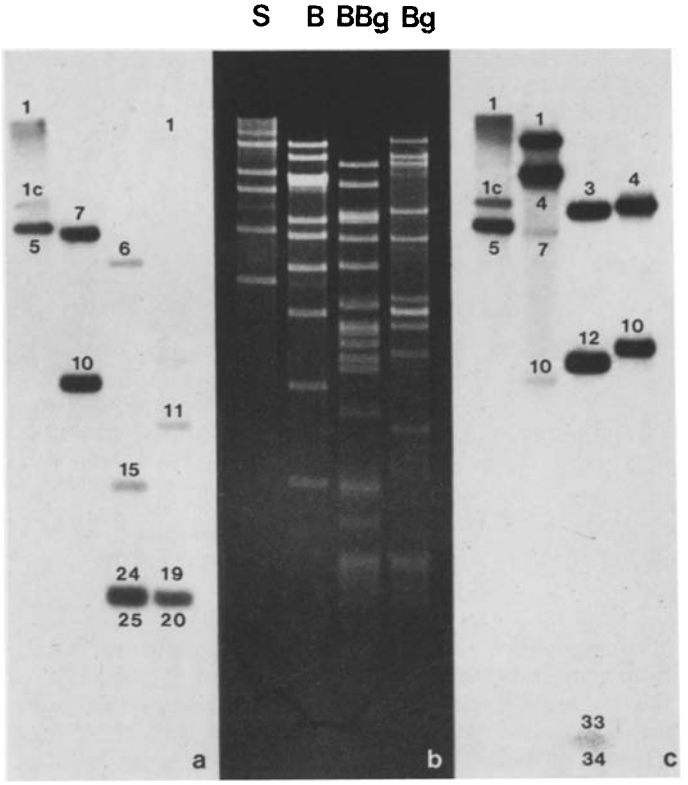

Fig. 2a–c. Restriction patterns of *Dictyota* ptDNA resulting from *Sal*I (S), *Bam*HI (B) and *Bgl*II (Bg) single digests and from a *Bam*HI + *Bgl*II (BBg) double digest (**b**). The corresponding hybridization signals with probes from *Sinapis alba* ptDNA are shown for the 16 S rDNA (**a**) and 23 S rDNA (**c**)

whereas in both *Bgl*II and *Bam*HI + *Bgl*II digests additional weak signals are observed with fragments joining the double fragments Bg19/20 and BBg24/25 at their distal ends. The position of the *Sal*I fragment 1c which we considered to be a part of S1 (Kuhsel and Kowallik 1985) is confirmed since it is also labelled with the 16 S rDNA probe.

The 23 S rRNA gene is also located on the *Sal*I fragments 1c and 5. As with the 16 S rDNA the hybridization signal with S1 appears somewhat blurred which can be explained by slight degradation of this exceptionally large fragment. There are strong signals from two fragments of the *Bam*HI pattern (B1, B4) that do not hybridize with the 16 S rDNA probe. In addition, the 5′ terminal region of the probe extends slightly into B7 and B10. Hybridization with the *Bgl*II fragments shows that the 23 S rRNA gene maps exclusively within the fragments Bg4 and Bg10. Hybridization with fragments from *Bam*HI + *Bgl*II double digests results in two strong signals (BBg3, BBg12) but also shows a weak signal at BBg33/34.

Determination of the inverted repeat (IR) region

In order to determine the extent of the inverted repeat region, the border of which lies within the fragments BS5 and BS8 on one side and within BS14 and BS10 on the other, each of the four cloned fragments was digested with *Sau*3A to yield a moderate number of subfragments ranging from 0.1 to 2.2 kb (Table 2). Cross hybridization experiments ("reciprocal hybridizations") using the *Sau*3A subfragments from BS8 and BS10 and from BS5 and BS14

Table 2. Restriction analysis of *Sau*3A subfragments resulting from primary *Bam*HI – *Sal*I fragments containing the inverted repeat sequence of the *Dictyota* plastome

*Bam*HI – *Sal*I fragment							
BS 5		BS 14		BS 8		BS 10	
1	1.55			1	2.18		
2	1.40			2	1.42		
3	1.05[a]	1	1.15	3	1.37[a]	1	1.37[a]
4	0.74[a]	2	0.74[a]			2	1.14[a]
5	0.61			4	0.72[a]	3	0.72[a]
6	0.55			5	0.44	4	0.54 (2×)
7	0.52			6	0.42[a]	5	0.42[a]
8	0.44[a]	3	0.44[a]	7	0.36[a]	6	0.38
9	0.40			8	0.19	7	0.18
10	0.39[a]	4	0.39[a]	9	0.12	8	0.11
11	0.32 (2×)	5	0.30[a]				
12	0.24	6	0.18				
13	0.20	7	0.12				
14	0.18						
15	0.13						

Sizes are given in kb
[a] Subfragments containing identical base sequences

revealed that in each combination three subfragments were identical (Fig. 3). In addition, subfragment 3 from BS5 and subfragment 5 from BS14 showed faint hybridization signals, indicating that these subfragments contain the border of the repeat at their distal terminus. Thus, the maximum extension of the repeat into fragments BS5 and BS14 is 1.87 kb.

The border of the repeat inside fragments BS8 and BS10 is clearly defined by *Sau*3A subfragment 7 generated from BS8 and by subfragment 2 derived from BS10 (Fig. 3). It is thus obvious that fragments BS8 and BS10 contain at most 2.87 kb of the inverted repeat each. Therefore, the total size of the repeat cannot exceed 4.74 kb.

Search for the rbcL *and* rbcS *genes*

Probe pWHsp403/E2 containing about 90% of the *rbcL* gene from spinach hybridizes exclusively to one fragment from *Bam*HI (B11) and *Bgl*II (Bg8) digests as well as from BamHI + *Bgl*II (BBg14) double digests (Fig. 4). Hybridization at $T_m - 25°$ C resulted in strong signals, whereas no hybridization signals were detectable at 18° C below T_m. According to Marmur and Doty (1962) and Bonner et al. (1973) this indicates that the homology between the hybridizing framents of the *Dictyota* ptDNA and the probe used is in the range of 80%.

The fragments B11 and BBg14 which map 27.3 kb from the left-hand inverted repeat (*rrnA*) within the large single copy region allow the location of the *rbcL* gene within 2.1 kb (Fig. 7).

Since there is some evidence that in the Chromophyte *Olisthodiscus luteus* the *rbcS* gene is plastome encoded, it was of interest to extend the search for this gene to the plastome of *Dictyota*, thus providing a possible common feature within the chlorophyll *a/c* line of the algal kingdom. However, even under low stringency conditions, no signals were obtained using two-thirds of the entire coding region of the nuclear-encoded gene from *Zea mays*. From the hy-

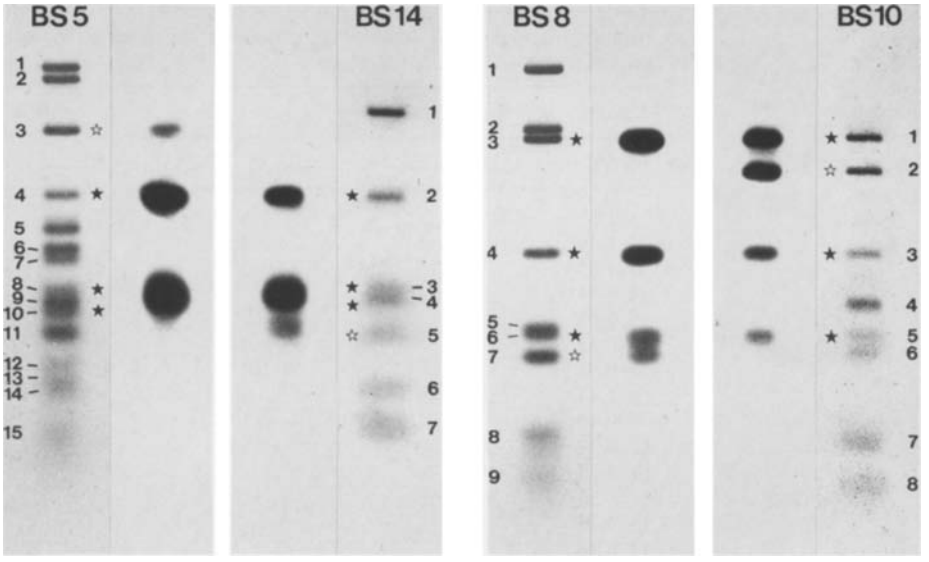

Fig. 3. *Sau*3A restriction patterns of two pairs of cloned *Bam*HI–*Sal*I fragments (BS5/BS14 and BS8/BS10). The corresponding hybridization signals result from cross hybridization experiments (see text). Identical subfragments of the IR are marked with *filled stars*, border subfragments are indicated by *open stars*. Fragments are numbered according to decreasing size

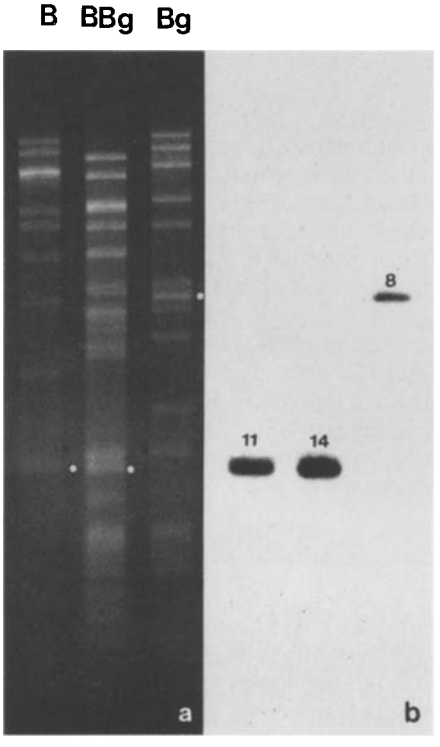

Fig. 4a, b. Restriction patterns of *Dictyota* ptDNA generated by *Bam*HI (B) and *Bgl*II (Bg) single digests, and from a *Bam*HI + *Bgl*II (BBg) double digest (**a**) to which the *rbcL* gene from spinach ptDNA was hybridized (**b**). The hybridizing fragments are marked with *dots*

bridization conditions used the base sequence homology, if any, between the *rbcS* gene of *Z. mays* and that of the *Dictyota* plastome must be less than 76%, if a similar average G + C-content of both ptDNAs is assumed.

Location of structural genes coding for photosystem I polypeptides

The position of genes coding for the two chlorophyll *a* apoproteins of photosystem I (*psaA, psaB*) was determined by heterologous Southern hybridization using probes prepared from spinach ptDNA (Table 1). We identified two loci inside the large single copy region, which gave signals with the *psaA* probe. The loci are about 7 kb from the distal terminus of both IR regions. Whereas fragments B7 and BBg6 resulted in strong hybridization signals, the labelling of fragments B9 and BBg10 was less intense (Fig. 5a). Even under increased stringency conditions the fragments at both loci hybridized with the probe, although the intensity of all signals gradually decreased with increasing hybridization temperature and formamide concentration.

The *psaB* gene was found to hybridize with fragments B2, B12, BBg1, BBg27, and Bg1 (Fig. 5b), indicating that this gene maps closely to one of the *psaA* genes about 8 kb from the *rrnA* operon.

Arrangement of genes coding for photosystem II polypeptides

Restriction fragments of *Dictyota* ptDNA labelled with probes for *psbA-E* are shown in Fig. 6. Whereas the genes for the 32 kDa herbicide-binding protein and the gene for cytochrome *b*-559 (larger chain), *psbA* and *psbE*, map within the large single copy region, *psbB* (the gene for the 51 kDa chlorophyll *a* apoprotein), *psbC*, and *psbD* are located inside the small single copy region. As Fig. 6c, d demonstrates, the Bg3 and BBg11 fragments harbour the genes for both the 44 kDa chlorophyll *a* apoprotein (*psbC*) and the "D-2" protein (*psbD*). The genes have also both been located on fragments B4 and BS7 (not shown). Since the bordering fragments remain unlabelled, it is concluded that both genes map entirely within 4.1 kb (fragment BBg11).

Figure 6b shows the Bg3, Bg12 and S6 fragments to which *psbB* from spinach ptDNA hybridizes. The corresponding base sequences of the *Dictyota* ptDNA can thus be located almost midway between both *rrn* operons, inside the small single copy region. According to additional Southern hybridizations (not illustrated) this gene can be restricted to 3.3 kb (fragments BBg13 plus BBg28).

In contrast, the position of *psaA* is less accurately determined, since it maps within the large fragments Bg1, BBg1, and B2 (Fig. 6a). Therefore, the distance of this gene from

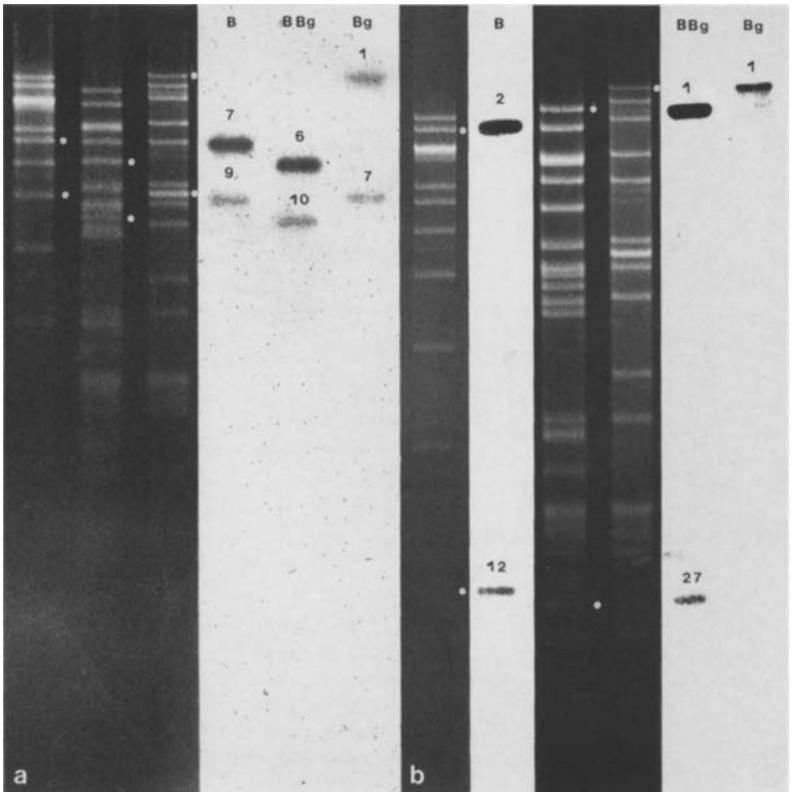

Fig. 5a, b. Restriction patterns of *Dictyota* ptDNA resulting from *Bam*HI (B), *Bgl*II (Bg), and *Bam*HI + *Bgl*II (BBg) digestion, together with the hybridization signals obtained with probes from spinach ptDNA coding for *psaA* (**a**) and *psaB* (**b**). The hybridizing fragments are marked with *dots*

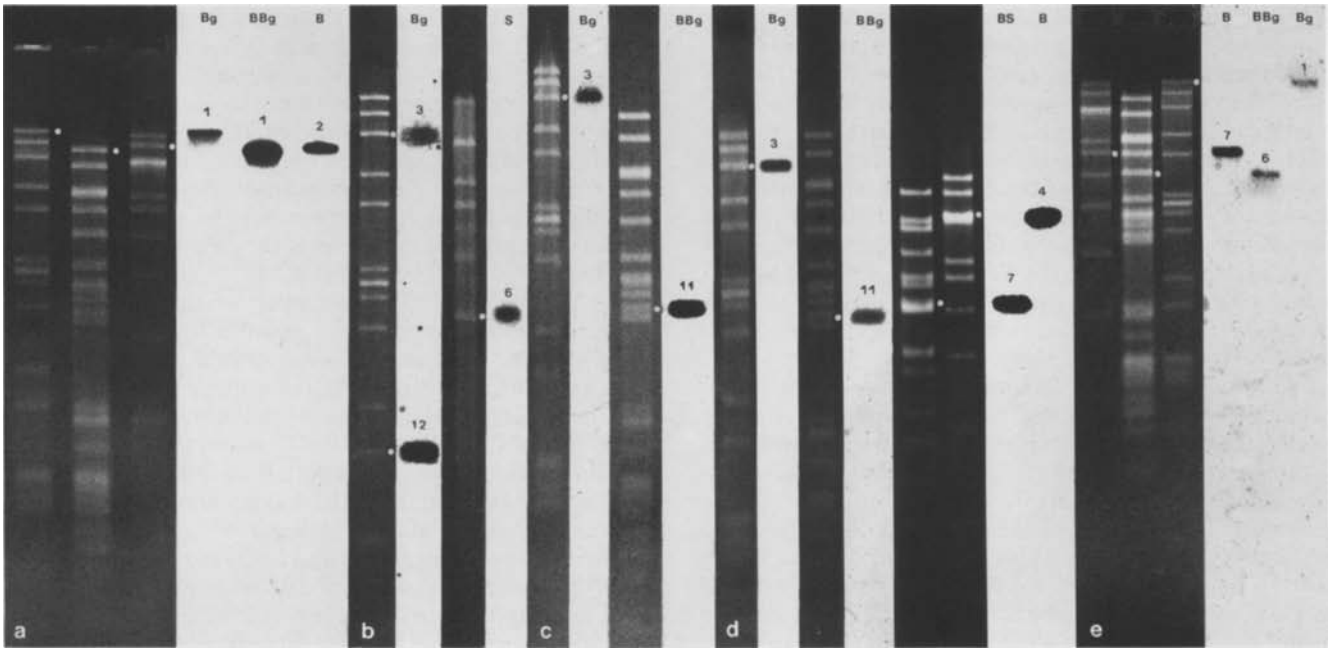

Fig. 6a–e. Restriction patterns of *Dictyota* ptDNA resulting from digestion with *Bam*HI (B), *Bgl*II (Bg), *Sal*I (S), *Bam*HI + *Bgl*II (BBg), and *Bam*HI + *Sal*I (BS), together with the hybridization signals obtained with probes from spinach ptDNA coding for *psbA* (**a**), *psbB* (**b**), *psb*C (**c**), *psb*D (**d**) and *psb*E (**e**). The hybridizing fragments are marked with *dots*

the border of the IR containing the *rrnA* operon is at least 8 kb and does not exceed 25.4 kb.

The gene coding for cytochrome *b*-559 (*psbE*) can be located on the fragments Bg1, BBg6, and B7 within a distance of less than 6.9 kb from the 5′ terminus of the *rrnA* operon (Fig. 6e).

Discussion

The determinaton of duplicated sequences of the *Dictyota* plastome which carry the *rrn* operon, by cross hybridization using redigested cloned fragments, revealed the smallest size so far known for any photoautotrophic organism (Fig. 7).

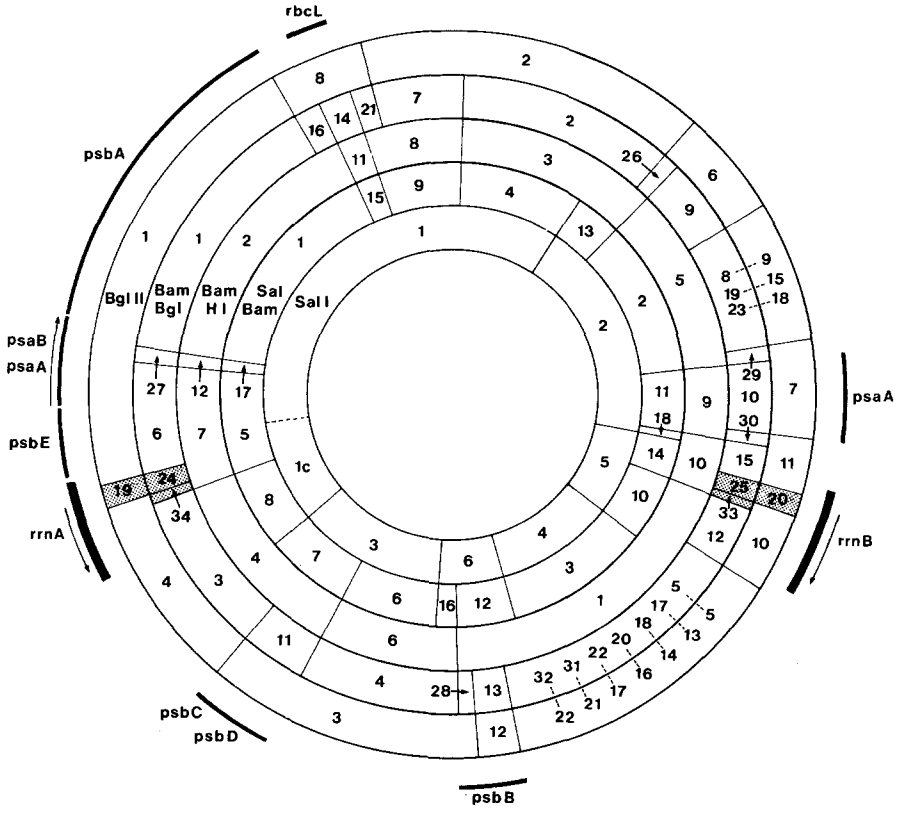

Fig. 7. Restriction map of *Dictyota* ptDNA. Identical fragments are *stippled*, whereas the whole IR region containing the rDNA is indicated by *heavy lines*. The position of genes is marked on the outer circle according to the smallest hybridizing restriction fragment

Whereas the IR region in most higher plants ranges from about 20 to 25 kb, with the *rrn* operon at its proximal terminus (Bohnert et al. 1982), a considerably smaller rDNA repeat of 5.6 kb, though not in inverted orientation, is found among certain strains of *E. gracilis* (Wurtz and Buetow 1981). Repeated rRNA cistrons of about the same size as in *Euglena* are also known from *E. coli* and blue-green algae such as *Anacystis nidulans* (Tomioka et al. 1981). In all these cases the repeated regions contain, in addition to the rRNA genes, sequences at both the 5′ and 3′ termini, as well as spacer sequences ranging from 250 bp for *Euglena* to more than 2 kb for higher plants which separate the 16 S and the 23 S rDNA.

In *Dictyota*, however, the maximum size of the repeat is about 4.7 kb indicating that it comprises essentially only the rRNA genes. In this respect it contrasts also with that of the putative Chloromonadophyte *Olisthodiscus luteus* (Cattolico, in Palmer 1985).

Since both the extent of the IR region of *Olisthodiscus* and its total plastome size are equivalent to those of higher plants, it might be argued that the size differences between the plastomes of the members of the chlorophyll *a*/*c* line are the result of the dissimilar extent of their repeats (cf. Palmer 1985). However, due to the lack of information on plastome organization within members of the brown algal line, this interpretation must remain speculative.

The presence of duplicated rRNA genes, now also demonstrated for members of the chlorophyll *a*/*c* line, does not necessarily imply a monophyletic origin for plastids. It might also be the result of convergent evolution among different lineages of algae leading to identical structures which prove to be the most stable elements for replication and gene arrangement, as has already been postulated by Palmer and Thompson (1982).

Of particular interest is the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase which in the *Olisthodiscus* plastome appears to be duplicated and maps within the repeat in close proximity to the *rbcS* gene. The genes are probably co-transcribed (Cattolico, in Palmer 1985). Although the question remains open as to whether the *rbcS* gene in *Dictyota* is plastid or nuclear encoded, the position of the *rbcL* gene appears to be conserved within the large single cope region as is the case in higher plants (Bowman et al. 1981; Seyer et al. 1981). Since in *Olisthodiscus* the direction of transcription of the *rrn* operon is as yet undertermined, it is not possible to decide whether the *rbcL* gene occupies the same position relative to the rDNA in both members of the chlorophyll *a*/*c* line.

The possibly duplicated *psaA* gene seems to be an intrinsic feature of the *Dictyota* ptDNA, since in the marine centric diatom *Odontella sinensis* both the *psaA* and *psaB* genes map at one position only (Kowallik, unpublished), as is the case for higher plants (Palmer 1985) and the cyanelle DNA of *Cyanophora* (Bohnert et al. 1985). However, despite the peculiar situation in *Dictyota*, in each case one *psaA* gene appears to be closely associated with the *psaB* gene; these genes have been shown to be transcribed together into a single messenger RNA in higher plants (Fish et al. 1985; Kirsch et al. 1986; Lehmbeck et al. 1986).

The presence of duplicated genes as determined by heterologous Southern hybridization raises the question as to whether the hybridization signals are unequivocal. Although in spinach ptDNA both *psa* genes share about 50% common base pairs (Kirsch et al. 1986), the absence of any cross reactions with fragments of the *Dictyota* plastome strongly indicates that the experimental conditions used are reliable. Even under increased stringency conditions, requiring at least 82% base sequence homology, the hybridization

patterns for both *psaA* genes, although gradually decreasing in intensity, did not change.

If the solitary *psaA* gene in *Dictyota* ptDNA is also transcribed and translated into protein, the question arises as to how these genes are regulated, since both chlorophyll *a* apoproteins constitute the photosystem I reaction centre in an equimolar ratio. Imbalances of both gene of protein ratios have also been reported for the constituents of the functional ATP synthase complex of spinach plastids (Hennig and Herrmann 1986). However, the regulatory mechanism is still unknown.

In addition, the distribution of the genes *psbA-E,* which have now been demonstrated as constituents of the *Dictyota* plastome, also shows several remarkable features. (i) The location of these genes, in both single copy regions, contrasts with the situation in all plastomes so far described. (ii) The *psbA* gene which is usually located in close proximity to the IR, within the large single copy region in higher plants, or which is part of the IR in *Chlamydomonas* (Rochaix 1981) and *Olisthodiscus* (Cattolico, in Palmer 1985) ptDNA, maps at least 8 kb from the distal terminus of the IR in *Dictyota.* (iii) As in higher plants (Alt et al. 1984), *psbC* and *psbD* appear to be closely associated in the plastome of *Dictyota.* It may thus be suggested that both genes also form a transcriptional unit. However, in *Odontella* these genes are distributed over one single copy region, indicating major gene rearrangements within the chlorophyll *a*/*c* line (Kowallik, unpublished).

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