Current Genetics (1983) 7:1–6

Current Genetics

Chloroplast DNAs of *Spinacia, Petunia* and *Spirodela* Have a Similar Gene Organization

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Summary. We have located the positions of the genes coding for the α, β and ϵ subunits of the ATPase complex on *Spirodela oligorhiza* chloroplast DNA by means of heterologous hybridization with *Spinacia* cpDNA fragments.

The overall cpDNA sequence organization of *Petunia hybrida* and *Spirodela* was compared. We hybridized well-characterized, cloned *Spirodela* cpDNA fragments with size fractionated *Petunia* cpDNA digested by SalI. It appears that the monocotyledonous *Spirodela* and the dicotyledonous *Petunia* cpDNA share a common sequence organization around their entire circumference. These observations, together with data reported in the literature, indicate a strikingly similar genetic organization of the chloroplast genome in widely divergent plants.

Key words: Chloroplast DNA – Gene organization – Ribulose-1,5-bisphosphate carboxylase – ATPase

Introduction

Chloroplast DNA of higher plants consists of covalently closed circular molecules between 120 and 185 kbp in size. Most cpDNAs contain a large inverted repeat sequence, part of which is coding for the four ribosomal RNAs. These inverted repeats separate two unique regions: the so called large and small single copy region (Herrmann and Possingham 1980). *Pisum* and *Vicia* cpDNA are exceptions to this general pattern and lack one inverted repeat (Kolodner and Tewari 1979; Koller and Delius 1980; Palmer and Thompson 1981). In different plants the gene organization of ribosomal RNAs (Bedbrook et al. 1976; Crouse et al. 1978), tRNAs (Driesel et al. 1979; Groot and van Harten-Loosbroek 1981; Weil et al. 1981), the large subunit of ribulose-1,5-bisphosphate carboxylase (Bedbrook et al. 1979; Whitfeld and Bottomley 1980; Link 1981; Seyer et al. 1981) and the "32 kD" herbicide binding protein (Bedbrook et al. 1978; Driesel et al. 1980; Link 1981; van Ee et al. 1982) is very similar. Recently, Westhoff et al. (1981) have located the genes coding for the α, β and ϵ subunits of the ATPase complex on *Spinacia* cpDNA. We have determined the positions of these ATPase genes on *Spirodela oligorhiza* chloroplast DNA by means of hybridization with well-defined *Spinacia* cpDNA probes, containing structural parts of the genes only.

The sequence organization of *Spirodela oligorhiza* and *Petunia hybrida* chloroplast genomes was compared by hybridizing cloned *Spirodela* cpDNA fragments with a known map position, to *Petunia* cpDNA restriction fragments, which were separated on an agarose gel. Both sets of experiments show that cpDNA of *Spinacia, Petunia* and *Spirodela* share the same basic pattern of gene organization. Moreover, *Spirodela* and *Petunia* chloroplast genomes have a sequence homology around the entire DNA molecule, despite their distant phylogenetic relationship.

Abbreviations: ATPase = CF_1 = chloroplast coupling factor; cp = chloroplast; kbp = kilo base pairs; LSU = large subunit of ribulose-1,5-bisphosphate carboxylase; BSA = bovine serum albumine; SDS = sodium dodecylsulphate; kD = kilo dalton; PVP = polyvinylpyrrolidon; SSC = standard sodium saline citrate; PS II = photosystem II

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Materials and Methods

Spirodela oligorhiza was cultivated as described by van Ee et al. (1980a). cpDNA from *Spirodela* and *Petunia* was isolated as given by van Ee et al. (1980a) and Bovenberg et al. (1981), respective-

ly. Purification of *Spirodela* cpDNA fragments was carried out by centrifugation of 20 μg, with PstI digested cpDNA on a linear sucrose gradient at 125.000 g_{av} during 12 h at 4 °C. The sucrose gradient [10–30% (w/v)] was made in 0.1 M NaCl, 0.01 M Tris-HCl pH 7.6, 0.001 M EDTA and 0.1% Sarkosyl (Ciba-Geigy). The gradient was collected in 15 fractions (10 drops per fraction) from which the cpDNA was precipitated with ethanol; the precipitate was washed twice with 67% ethanol-water. Subsequently cpDNA (0.5-1 μg) was ligated to dephosphorylated (Worthington phosphatase) pBR325 (0.5-1 μg) with 2 units T_4-ligase from Boehringer. Ligation was carried out during 16 h at 13 °C in 0.1% BSA, 0.02 M Tris-HCl pH 7.6, 0.001 M $MgCl_2$, 0.01 M DTT and 0.5 mM ATP. This ligation mix was used directly for transformation.

E. coli K_{12}-C600, made transformation competent according to Dagart and Ehrlich (1979) was used for transformation with recombinant DNA. Tetracycline-resistant colonies were picked and screened for ampicillin sensitivity. Plasmid isolation was carried out as described by Birnboim and Doly (1979). Usually undigested plasmid was purified on CsCl gradient prior to ^{32}P-labeling by nick-translation (Jeffreys and Flavell 1977). The excess, unincorporated label was removed on Sephadex-G-50 (Penefski 1977).

Petunia hybrida cpDNA was digested with SalI and analyzed by agarose (Sigma) gelelectrophoresis and subsequently blotted to nitrocellulose (0.1 μm Sartorius) as described by Denhardt (1966) and Southern (1979).

Filter immobilized *Petunia* cpDNA was hybridized with ^{32}P-labeled, cloned *Spirodela* cpDNA fragments in 3 x SSC, 2‰ calf thymus DNA, 0.2% SDS, 0.2% Ficoll, 0.2% PVP and 0.2% BSA during 20 h at 60 °C. The filters were washed once at 60 °C during 1/2 h with a solution of the same composition and twice at room temperature with 2 x SSC and 0.2% SDS. After drying the blots (500 Watt lamp) autoradiography at -70 °C with Kodak Royal X-OMAT XR I was used to detect homologous *Petunia* cpDNA fragments.

We have used the nomenclature devized by van Ee et al. (1980b): fragments produced by BamHI are referred to as BA, BB, BC, etc. in the order of declining size. Similarly, SacI is abbreviated as S, XhoI as X, SmaI as Sm, PstI as P and BglI as Bg. Petunia cpDNA was digested with SalI and the fragments produced are referred to as Sal1, Sal2, Sal3, etc. in the order of declining size (Bovenberg et al. 1981).

Filter immobilized *Spirodela* cpDNA digests were hybridized with well-characterized, cloned *Spinacia* cpDNA fragments, which only contain structural parts of the ATPase genes and LSU gene. These fragments are described elsewhere (Bisanz et al. in preparation).

Results

Mapping of cpDNA-Coded CF_1 Subunit Genes

Westhoff et al. (1981) have located the genes coding for the α, β and ε subunits of the coupling factor on the *Spinacia* chloroplast genome. We have used cloned *Spinacia* cpDNA fragments, containing structural information for these genes only, to define the corresponding gene positions on the restriction map of *Spirodela oligorhiza* cpDNA. The clones were labeled in vitro by nick-translation and subsequently hybridized to different, nitrocellulose filter immobilized, *Spirodela* cpDNA digests. The results are shown in Fig. 1 and the gene locations are drawn on a part of the *Spirodela* chloroplast restriction map, which is given in Fig. 2. The LSU-probe (Fig. 1 – panel I) hybridizes with BgA, SG, SM and SmG. Apparently SM hybridizes more strongly with the LSU probe than SG. From these results we conclude that the LSU gene is located on the left side of a 8,500 bp fragment (see Fig. 2) and probably PM contains the entire gene.

Van Ee et al. (1982) have raised the possibility that *Spirodela* cpDNA contains two copies of the gene for LSU. We could not confirm this observation and suggest that the second copy they detected, in fact is the gene for the β-subunit of the ATPase complex, which hybridized to a contaminating DNA fragment of slightly lower molecular weight, present in the probe used. Recent experiments (van Ee, personal communication) have confirmed our interpretation.

The CF_1-β probe (Fig. 1 – panel III) hybridizes with several fragments, amongst which there are SmD, PG and XB. The CF_1-ε probe (Fig. 1 – panel IV) appears to hybridize much better than the CF_1-β probe and hybridizes clearly with BgA, SmD, SC, XB and PG.

The CF_1-α probe (Fig. 1 – panel II) hybridizes with BgB, SmF, XH/I and PC, therefore this gene can be localized within a 6 kb fragment (see Fig. 2).

From this we conclude that the CF_1-β and ε genes are located on a 5,000 bp fragment, with outer boundaries as drawn in Fig. 2. As can be seen from this figure, the positions of the α gene and the β-ε gene cluster are similar to that reported by Westhoff et al. (1981). Since the resolution of the *Spirodela* physical map is not extremely great, the β and ε gene could not be separated and they are drawn analogous to Westhoff et al. (1981).

Cloning of Spirodela cpDNA Restriction Fragments

In initial shotgun cloning experiments, to obtain *Spirodela* cpRNA restriction fragments, it appeared that larger fragments are severely underrepresented. To circumvent this we fractionated cpDNA, digested with PstI, on a linear sucrose gradient. In this manner we were able to enrich specifically certain restriction fragments. The large PA/PB fragments were cloned with the aid of a cosmid vector (Keus et al., to be published); the other fragments were cloned in pBR325. In this way we have cloned the entire *Spirodela* chloroplast genome, except the PF and PQ fragments. Despite several attempts we were not able to clone these distinct restriction fragments. A similar finding was reported by Palmer and Thompson (1981). The significance of this observation is not known.

Figure 3 shows the cloned PstI fragments. All fragments were characterized by nick-translation and hybridization to nitrocellulose filter immobilized, digested,

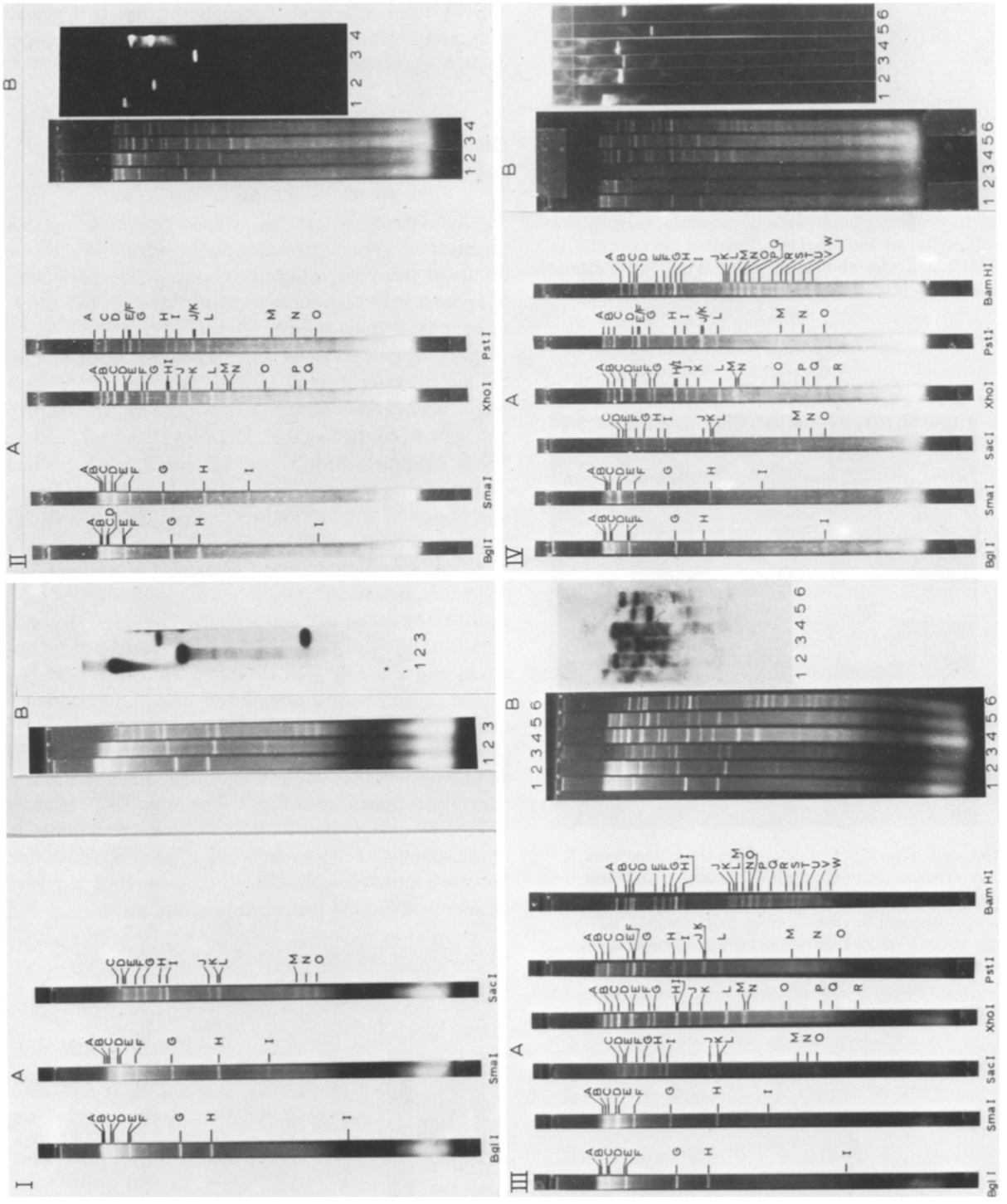

Fig. 1. Hybridization of various *Spinacia* cpDNA probes to restriction fragments of *Spirodela* cpDNA. Each panel is divided into two parts: A and B. In A an ethidiumbromide stain of an agarose gel analysis of different *Spirodela* cpDNA digests is shown. The fragments are indicated schematically beside each track. In B an ethidiumbromide stain of cpDNA digests, which have been transferred to nitrocellulose is presented. The numbers below those tracks refer to cpDNA digests in the same order as in part A. The right hand part of B shows an autoradiogram of the hybridization and the numbers below each track refer to the nitrocellulose transfers of *Spirodela* cpDNA. Panel I hybridization with *Spinacia* LSU probe; panel II hybridization with *Spinacia* CF_1-α probe; panel III hybridization with Spinacia CF_1-β probe; panel IV hybridization with Spinacia CF_1-ϵ probe

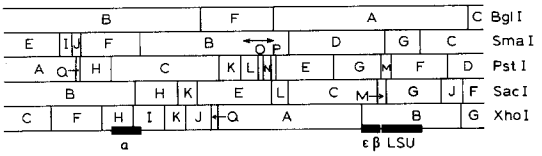

Fig. 2. Restriction fragment map of the *Spirodela* chloroplast DNA region containing the genes for the LSU of ribulose-1,5-bisphosphate carboxylase and the α, β and ε subunits of the ATPase complex. The fragment letters refer to digests of total cpDNA and gene positions are marked. The orientation from left to right corresponds to the orientation in the physical map of the chloroplast genome presented in Figs 5 and 6

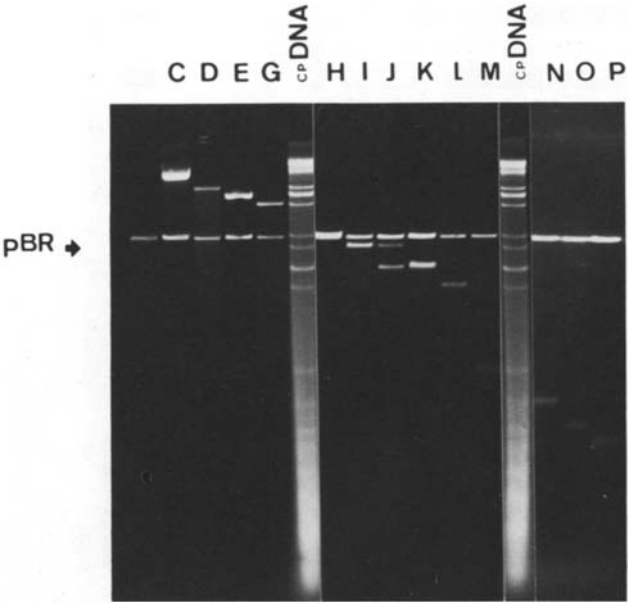

Fig. 3. *Spirodela oligorhiza* PstI chloroplast DNA clone bank. Recombinant plasmids containing pBR325 vector sequences (indicated by an arrow and the first track on the lefthand side) were digested with PstI and separated on a 1% agarose gel. The tracks marked by cpDNA are PstI restriction enzyme digest of *Spirodela* cpDNA and the letters above each track corresponds with a defined PstI fragment

cpDNA from *Spirodela* (data not shown). It appeared that each cloned fragment hybridized to the proper cpDNA restriction fragments.

Comparision of the Organization of Petunia and Spirodela cpDNA

We hybridized ^{32}P-labeled, cloned *Spirodela* cpDNA fragments to *Petunia hybrida* cpDNA. Figure 4 shows the results of these heterologous hybridization experiments. It appears that each Spirodela PstI fragment hybridizes to a similar *Petunia* fragment.

As could be expected PA and PB do hybridize exactly the same. Figure 4, track 2 and 3, shows that these large fragments hybridize with S1, S2, S3 and S7. The BamHI-H fragment, containing the 23S rRNA gene (van Ee et al. 1982) hybridizes with S1 and S3 (Fig. 4, track 17) and therefore we suggest that the entire ribosomal RNA gene unit is located within S1 and S3, respectively, at a similar position on both cpDNAs.

In order to obtain a good separation of the large SalI fragments, it was necessary to run the gel for a long time and therefore the small SalI fragments were lost. Hybridization of PI with S11, S12 and S13 could not be observed and since PI does hybridize with S7 and S8, we suggest that PI will be homologous to S11, S12 and S13 too.

We have observed that PN and PP are located near PO and therefore the map position of these fragments is corrected and drawn as in Fig. 2. The *Spirodela* fragments PN and PP do hybridize with S4, as can be seen in Fig. 4.

We summarize these results in Fig. 5 and conclude that *Spirodela* and *Petunia* cpDNA share a common sequence arrangement around their entire circumference.

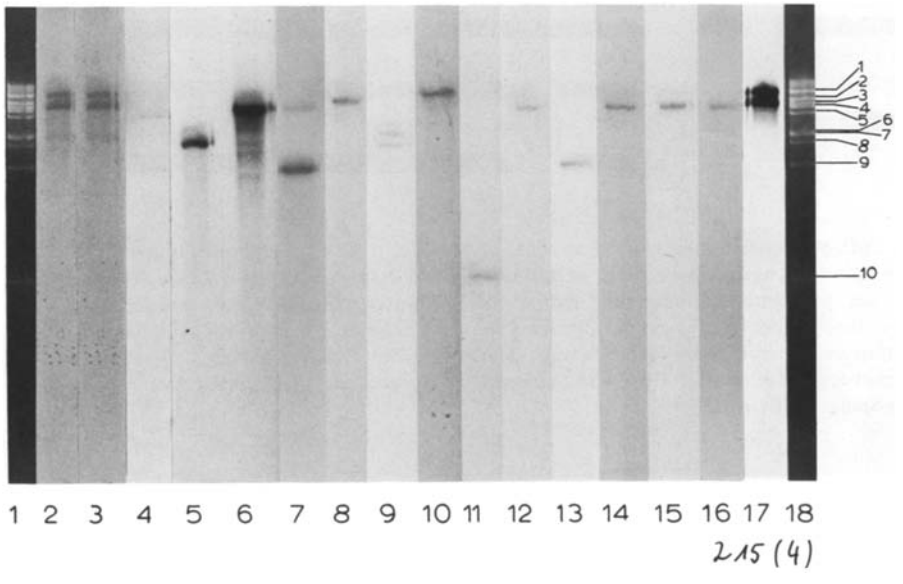

Fig. 4. Hybridization of nick-translated PstI fragments from *Spirodela* cpDNA to a SalI digest of *Petunia* cpDNA. Outer tracks 1 and 18 are ethidiumbromide stained SalI restriction enzyme digests of *Petunia* cpDNA; the numbers indicate the specific SalI fragments. Inner tracks 2 up to 16 are the corresponding nitrocellulose filter immobilized *Petunia* cpDNA fragments hybridized to *Spirodela* cpDNA PstI fragments: A (track 2), B (3), C (4), D (5), E (6), G (7), H (8), I (9), J (10), K (11), L (12), M (13), N (14), O (15) and P (16). Track 17 is a nitrocellulose transfer hybridized to 23S rDNA containing *Spirodela* BamHI-H fragment

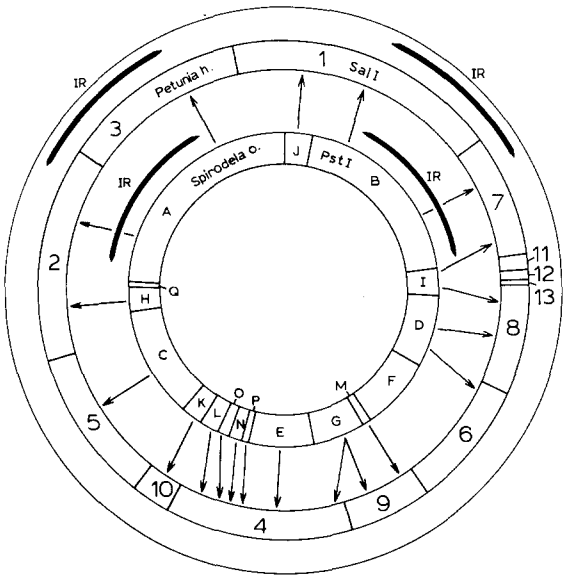

Fig. 5. Summary of hybridization experiments presented in Fig. 4. *Spirodela* cpDNA fragments which have been cloned and hybridize with a homologous *Petunia* cpDNA fragment, are marked with an arrow. The inverted repeat (IR) regions of both cpDNAs are marked by means of a thick line. *Spirodela* cpDNA contains 182 kbp and 1 kbp is equivalent to 2°. *Petunia* cpDNA contains 151 kbp and 1 kbp is equivalent to 2.4°

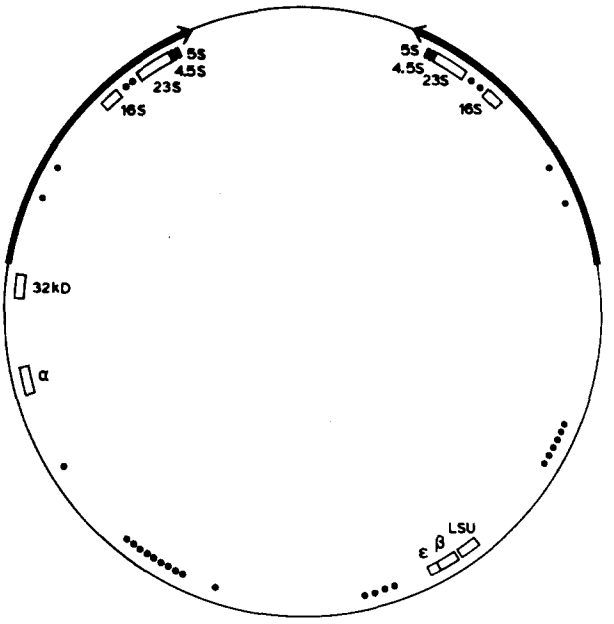

Fig. 6. Schematic general representation of the chloroplast genome and the relative positions of the genes located so far. The S-values correspond to the rRNA genes within the inverted repeats, which are marked by an arrow. The black dots represent tRNA genes and the protein genes are marked by 32 kD, α, β, ϵ and LSU, respectively. For details about the protein genes, see Discussion

Discussion

In recent years it has become evident that the general molecular anatomy of the cpDNAs of different higher plants, with the exception of *Vicia* and *Pisum*, is as shown in Fig. 6: two single copy regions separated by inverted repeats (Herrmann and Possingham 1980; Davies and Hopwood 1980). The size of these regions differ considerably, however. The inverted repeats vary from 14.4 to 26.9 kb in *Petunia* (Bovenberg et al. 1981) and *Spirodela* (van Ee et al. 1980); the small single copy region from 12.6 to 26.9 kb in *Zea* (Bedbrook et al. 1980) and *Spirodela* (van Ee et al. 1980b); the large single copy region from 82.7 kb (*Atriplex*, Palmer 1982) to 103 kb (*Cucumis*, Palmer 1982).

From the literature it is clear that in all cases studied, the rRNA genes are part of the inverted repeat. They are located in the small single copy proximal part in the order: 5′-16S-spacer-23S-spacer-4.5S-spacer-5S-3′ (Bedbrook et al. 1976; Crouse et al. 1978; Dyer and Bedbrook 1980; Schwarz and Kössel 1980; Edwards and Kössel 1981; Koch et al. 1981; Tohdoh and Sugiura 1982; Takaiwa and Sugiura 1982; van Ee et al. 1982). Moreover, the organization of the genes for tRNAs is similar in *Spinacia, Spirodela* and *Zea*, the three species studied (Driesel et al. 1979; Groot and van Harten-Loosbroek 1981; Weil et al. 1981).

A similar situation holds for the positions of the genes coding for LSU (Bedbrook et al. 1979; Whitfeld and Bottomley 1980; Link 1981; Seyer et al. 1981; van Ee 1982) and the "32 kD" herbicide binding protein, associated with photosystem II activity (Bedbrook et al. 1978; Driesel et al. 1980; Link 1981; Mattoo et al. 1981; van Ee et al. 1982). In *Spinacia, Sinapis, Spirodela, Nicotiana* and *Zea* the genes for these proteins are located at comparable coordinates.

In this paper we have extended the comparison of the genetic organization of *Spirodela* cpDNA with that of other chloroplast genomes. On the one hand we have shown that the genes coding for the α, β and ϵ subunits of the *Spirodela* chloroplast ATPase-complex and the LSU gene are located at map positions corresponding to those on *Spinacia* cpDNA. On the other hand we have shown that *Spirodela* and *Petunia* cpDNA, differing in size by more than 30 kbp, show complete positional conservation along the entire chloroplast DNA molecule.

Our findings also indicate that the large difference between the size of *Spirodela* cpDNA and that of other plants, can most likely not be explained by assuming a much larger coding capacity of the *Spirodela* chloroplast genome. It is much more plausible that *Spirodela* cpDNA contains an increased amount of non-coding (e.g. AT-rich) DNA. Indication for this is that PA and PG melt

out at a much lower temperature in comparison with whole cpDNA.

One might argue that the approach used by us, can lead to false conclusions because of the use of large fragments for the hybridization experiments. Indeed rearrangements occurring at short distance within a fragment would stay unnoticed in our approach. However, the striking homology of relatively small *Spirodela* PstI fragments with the corresponding *Petunia* fragments show that large-scale rearrangements do not occur.

Small-scale rearrangements do occur as has been shown by Fluhr and Edelman (1981) and by Kung et al. (1982) in regions bordering the inverted repeats. Herrmann and Possingham (1980) and Gordon et al. (1981) have also found that changes are allowed in two areas of the large single copy region, which lie near the inverted repeat in related *Euoenothera* cpDNAs.

Fluhr and Edelman (1981) have reported on the conservation of sequence arrangement for *Nicotiana* and *Spinacia* and Palmer (1982) has found that a part of *Spinacia* and *Atriplex* cpDNA is colinear in sequence. Moreover, Palmer (1982) announces that *Atriplex* and *Cucumis* cpDNA are extremely similar in all aspects examined. Taken together our findings and data from the literature suggest that a general anatomy exists for chloroplast genomes of a large number of higher plants, even for monocotyledons and dicotyledons, which have widely diverged in evolution.

When this manuscript was completed, Palmer and Thompson (1982) reported an extensive comparison of the sequence organization of the cpDNAs in different plants. Their results are very similar to ours. Moreover, they show that *Zea* and *Vigna* cpDNA differ from those of a large group which are essentially similar to *Spinacia* by inversions in the large single copy region amounting to 50 kb and less than 15 kb, respectively.

Acknowledgements. We are grateful to Prof. R. G. Herrmann for commenting the manuscript and for discussions throughout the course of this work.

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Communicated by R. J. Schweyen

Received September 20, 1982