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Physical and gene mapping of chloroplast DNA from Atriplex triangularis and Cucumis sativa

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ABSTRACT

A rapid and simple method for constructing restriction maps of large DNAs (100-200 kb) is presented. The utility of this method is illustrated by mapping the Sal I, Sac I, and Hpa I sites of the 152 kb Atriplex triangularis chloroplast genome, and the Sal I and Pvu II sites of the 155 kb Cucumis sativa chloroplast genome. These two chloroplast DNAs are very similar in organization; both feature the near-universal chloroplast DNA inverted repeat sequence of 22-25 kb.

The positions of four different genes have been localized on these chloroplast DNAs. In both genomes the 16S and 23S ribosomal RNAs are encoded by duplicate genes situated at one end of the inverted repeat, while genes for the large subunit of ribulose-1,5-bisphosphate carboxylase and a 32 kilodalton photosystem II polypeptide are separated by 55 kb of DNA within the large single copy region. The physical and genetic organization of these DNAs is compared to that of spinach chloroplast DNA.

INTRODUCTION

The chloroplast genome of vascular plants consists of a single circular DNA molecule between 120 kb and 180 kb in size. The majority of chloroplast DNAs studied contain a large inverted repeat sequence of 22-25 kb, part of which codes for ribosomal RNA. Restriction maps which demonstrate the inverted repeat organization have so far been constructed for chloroplast DNAs from corn (1), spinach (2), wheat (3), tobacco (4), petunia (5), mustard (6), mung bean (7), Oenothera (8), and Spirodela (9). Two exceptions to this pattern are chloroplast DNAs from pea (7), and broad bean (10), both of which lack one entire segment of the inverted repeat

The relatively compact size, the absence of molecular heterogeneity, and the evolutionary conservatism of the chloroplast genome make it an ideal molecular tool for assessing evolutionary relationships among plants at practically all levels, from the intraspecific to the interdivisional. For many of these studies detailed physical maps of restriction endonuclease cleavage sites will be required. In this paper I present a strategy for

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constructing restriction maps of large DNAs of the size of the chloroplast genome. This method is quite rapid, can be adapted to mapping a number of DNAs at once, and requires only small amounts (<10μg) of chloroplast DNA. The utility of the method is illustrated by constructing restriction maps for the chloroplast genomes of Atriplex triangularis and Cucumis sativa. In addition, the positions of four genes have been localized on the physical map of these DNAs.

MATERIALS AND METHODS

DNA Isolation

Chloroplast DNA was prepared from one-week-old Cucumis sativa cotyledons (cv. Beit Alpha Mt.; seed obtained from FMC Corporation), 3-week-old corn leaves (Zea mays, cv. Trojan; seed obtained from Pfizer Genetics) and spinach leaves (Spinacia oleracea, obtained from a local grocery market), by treating chloroplasts with DNase I according to the method of Kolodner and Tewari (11).

Chloroplast DNA from Atriplex triangularis was prepared by a modification of the sucrose gradient technique described for the preparation of chloroplast DNA from tobacco leaves (12,13). 10-100 gm of leaves are placed in 100-500 ml of ice-cold isolation buffer (0.35 M sorbitol, 50 mM Tris-HCl, ph 8.0, 5 mM EDTA, 0.1% BSA (w/v), 15 mM β-mercaptoethanol, 1 mM spermine, 1 mM spermidine) and homogenized for 10-20 sec in a Waring blender or for 30-60 sec in a polytron homogenizer. The extract is filtered through cheesecloth and miracloth (Calbiochem) and centrifuged at 1000 g for 10-15 min at 4°C. The pellet is resuspended in 10 ml wash buffer (0.35 M sorbitol, 50 mM Tris-HCl, ph 8.0, 25 mM EDTA, 1 mM spermine, 1 mM spermidine) using a soft brush and loaded on a step gradient consisting of 18 ml of a 60% sucrose solution and 7 ml of a 30% sucrose solution, each containing Tris, EDTA, spermine and spermidine at the same concentrations as in the wash buffer. The gradient is placed in a SW-27 rotor and centrifuged at 25,000 RPM for 50 min at 4°C. The chloroplast band at the 30%-60% interphase is removed, diluted with 3-10 volumes wash buffer, and centrifuged at 1,500 g for 10-15' at 4°C. Depending on its size, the chloroplast pellet is resuspended in either 2 ml or 15 ml of wash buffer and one-tenth volume of a 10 mg/ml solution of Pronase (Calbiochem) is added. After 2 min at room temperature one-fifth volume of lysis buffer (5% sodium sarcosinate (w/v), 50 mM Tris-HCl, ph 8.0, 25 mM EDTA) is gently added. The tube is gently mixed by inverting several times and incubated at room temperature for 15' to several hours. 3.35 gm or 23.0

gm solid CsCl (Kawecki Berylco Industries Inc.), EtBr to a final concentration of 200 μg/ml, and 50 mm Tris, ph 8.0, 25 mM EDTA to a final volume of 4.45 ml or 32.0 ml are added to the small (2 ml) or large (15 ml) chloroplast lysates, respectively. The small gradient is centrifuged in the TV-865 rotor (Sorvall) for 4-16 hr at 58,000 RPM (319,000 g_{max}), while the large gradient is centrifuged in the TV-850 rotor (Sorvall) for 12-16 hr at 43,000 RPM (175,000 g_{max}). The DNA from either a small or large initial gradient is then banded a second time in the TV-865 rotor. Ethidium bromide is removed by three extractions with isopropanol saturated with NaCl and H_2O, and the DNA is dialyzed against at least three changes of 2 ℓ of 10 mM Tris, ph 8.0, 0.1 mM EDTA over a period of 1-2 days.

This method has proved extremely versatile in the purification of chloroplast DNA from well over 100 species of angiosperms, gynmosperms and ferns. The method gives higher yields (0.2 - 10 μg chloroplast DNA per gm F.W. of leaves) than the DNase I procedure (11) and is applicable to a much wider range of plants, including many for which it is not possible to isolate any DNA using the DNase I treatment. The purity of the chloroplast DNA is variable using the sucrose gradient method, but is generally high enough to enable visualization of all the chloroplast DNA restriction fragments on ethidium bromide-stained agarose gels. One final advantage is that nuclear DNA of high molecular weight can be obtained by resuspending the pellet from the sucrose gradient in wash buffer, and further treating this fraction in exactly the same manner as the chloroplast fraction.

Gels and Blots

Chloroplast DNA was digested with Sal I, Sac I, Hpa I or Pvu II (New England Biolabs) according to the supplier's instructions. Between 0.2-0.5 μg DNA was loaded per lane on a 0.7% neutral agarose (Sigma, Type I) horizontal slab gel 0.4 x 20 x 40 cm in size. Electrophoresis was for 12-20 hrs at 75 mA in 100 mM Tris-acetate (ph 8.1), 1 mM EDTA. The gel was prepared for transfer to nitrocellulose filters according to Wahl et al. (14). Two filter replicas of the same gel were prepared by blotting onto nitrocellulose filters placed on both sides of the gel exactly as described by Smith and Summers (15) except that the transfer buffer was 20 x SSC (3 mM NaCl, 0.3 M trisodium citrate).

Preparation of ^{32}P-Probes

2-5 μg intact chloroplast DNA was labeled with [α-^{32}P] dGTP (Amersham) by the nick-translation reaction according to Maniatis et al. (16) except that no DNase I was added. Chloroplast DNA prepared by the above methods

always has sufficient nicks to permit good incorporation of ^{32}P in such reactions. In this case the amount of ^{32}P in the reaction mixture was set at no more than 30 μCi/μg DNA in order to limit the specific activity, and hence prevent excessive degradation, of the labeled DNA. The nick-translation reaction was terminated by heating to 65° for 10' and restriction enzyme added after the buffer was adjusted to that prescribed for the enzyme. The ^{32}P-labeled restriction fragments were separated by agarose (Sigma, Type I) gel electrophoresis and gel slices containing each fragment were cut out and placed in a 5 ml polypropylene tube. One ml of 1x SSC was added and the tube boiled for 15' to melt the agarose and denature the DNA. The liquified agarose solution was then added to a bag containing the prehybridized filter (see below).

Tobacco chloroplast 16S and 23S ribosomal RNAs were alkali hydrolyzed to a few hundred nucleotides and labeled with [γ - ^{32}P] ATP according to Maizels (17).

Filter Hybridizations

Nitrocellulose filters were placed in heat-sealable plastic bags which contained 4 x SSC, 50 mM phosphate buffer, 0.1% SDS, 5 x Denhardt's solution (18), and 100 μg/ml sonicated calf thymus DNA, and the bags incubated for 14-16 hr in a shaking water bath at 65°C. ^{32}P-labeled RNA or DNA was added and hybridization at 65°C allowed to proceed for two days. The filters were washed in several changes of 2 x SSC, 0.1% SDS over a period of 4-6 hr at 65°C and exposed to preflashed (19) Kodak XAR-5 film, using a Dupont Lightning Plus intensifying screen, for 1-20 days at -70°C.

RESULTS

The restriction mapping strategy is to employ the complete set of fragments generated by a single restriction enzyme as hybridization probes against filters which contain single digests of chloroplast DNA produced by various other restriction enzymes and also double digests produced by each of the other enzymes plus the enzyme used to generate the probe fragments. Hybridization to single digests generates overlaps between probe and filter-bound fragments, while hybridization to double digests gives the precise localization of cleavage sites within probe fragments.

Atriplex and *Cucumis* chloroplast DNAs were screened with 15 different six-base restriction enzymes in order to find enzymes which produce simple patterns in which all the fragments are well resolved. Three enzymes were chosen for the *Atriplex* mapping and two for the *Cucumis* mapping (Fig. 1).

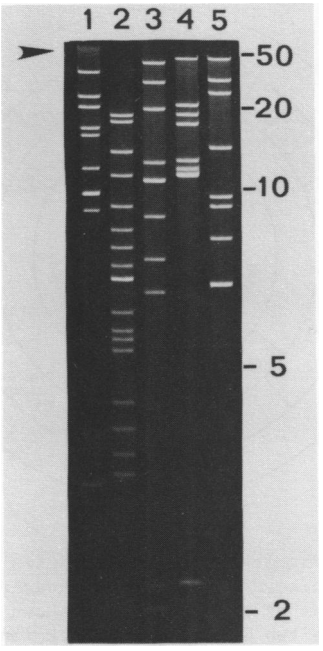

Figure 1. 0.7% agarose gel electrophoresis of Atriplex chloroplast DNA digested with (1) Sal I, (2) Sac I, and (3) Hpa I, and Cucumis chloroplast DNA digested with (4) Sal I and (5) Pvu II. Size scale at right is in kb. Arrow indicates a small amount of nuclear DNA present in the Atriplex chloroplast DNA preparation and resistant to digestion with Sal I as a consequence of its extensive methylation (24).

Sizes for the fragments shown in Fig. 1 are listed in Tables 1 and 2. When fragment stoichiometries are taken into account (Figs. 2 and 3) the size of the Atriplex chloroplast genome is estimated at 152 kb (Sal I: 152.7 kb; Sac I: 152.4 kb; Hpa I: 151.9 kb) and the Cucumis genome at 155 kb (Sal I: 155.7 kb; Pvu II: 154.5 kb).

Fig. 4 shows the hybridization pattern of each of the Hpa I fragments of Atriplex chloroplast DNA to filters which contained both Sac I and Sac I-Hpa I digests. In a second experiment, the Atriplex Sal I fragments were used as probes against Sac I and Sac I-Sal I digests. Data from these two sets of hybridizations are summarized in Table 1. From the autoradiograms (Fig.4) it is clear that some cross-contamination of smaller probe fragments occurred as a result of degradation of larger fragments. Cross-contamination with a given fragment is greatest in the fragment next smaller in size and decreases in

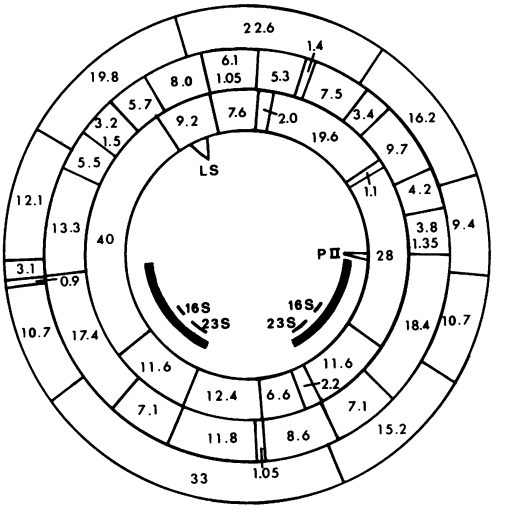

Figure 2. Physical and genetic map of the Atriplex chloroplast genome. Restriction endonuclease cleavage sites were deduced from the data presented in Table 2, while locations of the four genes shown are from the hybridization data of Table 4. The two long, heavy black lines represent the extent of the inverted repeat segments, which, as defined by these mapping data are bounded by the sites between the 7.1 and 11.8 (8.6) kb Sac I fragments and the 10.7 and 0.9 (9.4) kb Sal I fragments. Including the 6.3 kb Sac I-Sal I fragment internal to the repeat, the minimal length of the inverted repeat is 24.1 kb (7.1 + 10.7 + 6.3 kb). Sal I fragments are shown on the outer circle, Sac I fragments on the middle circle, and Hpa I fragments on the inner circle.

fragments further away. In some cases, when two probe fragments are very close in mobility, there may also be "upstream" contamination of the larger fragment by the smaller. When one assembles the filters in order of probe fragment size, true hybridization signals generally stand out and are easily recognized above the background of artefactual bands (Fig. 4).

The data of Table 1 yield the restriction map for the Atriplex chloroplast genome (Fig. 2). Note that the presence of the large inverted repeat introduces ambiguity in interpreting the single digest hybridizations but that this ambiguity is generally resolved by knowing the size of the double digest fragments for probe fragments which lie on the inverted repeat. While the Atriplex map was derived using probe fragments prepared with two different restriction enzymes, almost all the mapping information could have been obtained using just a single set of probe fragments, e.g., the Hpa I fragments, by including Sal I and Sal I-Hpa I lanes on the filter.

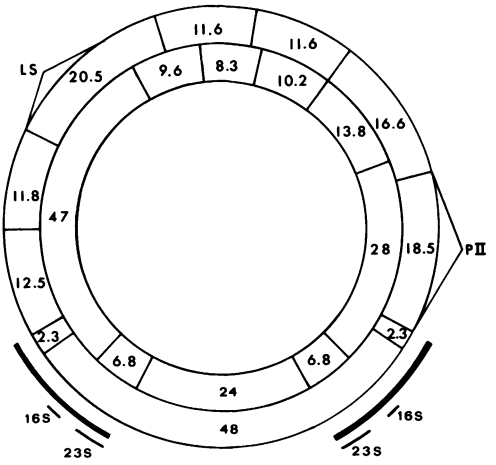

Figure 3. Physical and genetic map of the Cucumis chloroplast genome. Cleavage sites and gene locations are from Tables 3 and 4. The two long, heavy black lines represent the extent of the inverted repeat segments, which, as defined by these mapping data, are bounded by the sites between the 6.8 and 24 kb Pvu II fragments and the 2.3 and 12.5 (18.5) Sal I fragments. Including the 4.9 kb Pvu II-Sal I fragment internal to the repeat, the minimal length of the inverted repeat is 14.0 kb (6.8 + 2.3 + 4.9 kb). Sal I fragments are shown on the outer circle and Pvu II fragments on the inner circle.

Sal I and Pvu II sites for the Cucumis chloroplast genome were mapped by reciprocal sets of hybridizations of Sal I or Pvu II probe fragments to Sal I-Pvu II double digests plus either Pvu II or Sal I single digests, respectively. These experiments are summarized in Table 2 and the Cucumis restriction map is shown in Fig. 3. Note that the restriction mapping data do not allow unambiguous ordering of the three Pvu II fragments, two of 6.8 kb and one of 24 kb, which lie completely internal to the 48 kb Sal I fragment. The order of these three fragments was deduced on the basis of 16S ribosomal RNA hybridization (Table 3).

In light of their similarities in physical organization (Figs.2 and 3), it is of interest to determine whether the arrangement of specific genes is also similar in the Atriplex and Cucumis chloroplast genomes. To this end I have localized four different genes on the physical maps of Atriplex and Cucumis chloroplast DNA. Fig. 5 shows the hybridization of probes for the genes for the 16S and 23S chloroplast ribosomal RNAs, the large subunit (LS) of ribulose-1,5-bisphophate carboxylase and a 32 kilodalton photosystem II polypeptide (PII) to nitrocellulose filters containing restriction digests of Atri-

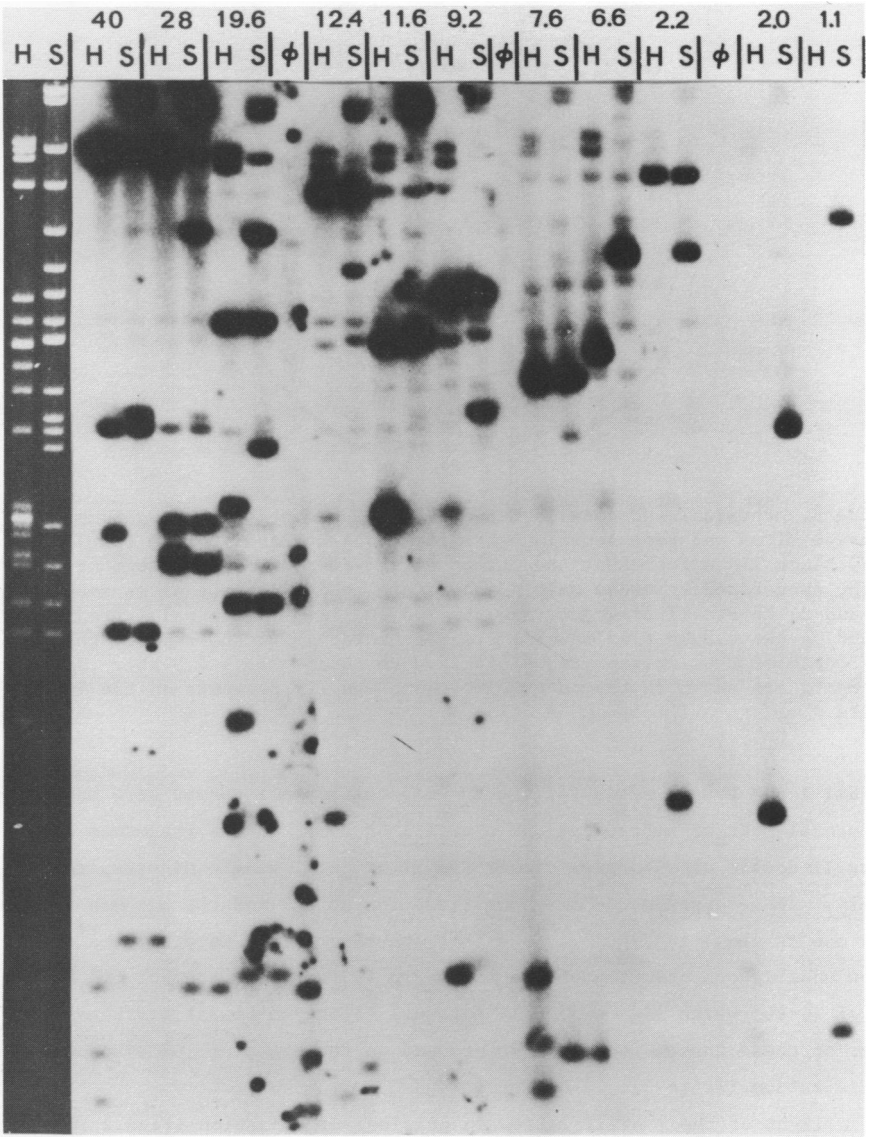

Figure 4. Hybridization of ^{32}P-labelled Atriplex Hpa I fragments to Atriplex (H) Hpa I-Sac I and (S) Sac I fragments separated on a 0.7% agarose gel and transferred to nitrocellulose filters. Numbers above the filters indicate the size in kb of the Hpa I fragments used as probes. Hae III restriction fragments of phage ØX 174 DNA are in lanes marked "Ø".

Table 1. Summary of *Atriplex* Restriction Mapping Hybridizations

Probe DNA	Filter-bound DNA	
Sal I	Sac I	Sac I-Sal I
33	18.4, 17.4, 11.8, 8.6, 7.1, 1.05	11.8, 7.1, 6.5, 6.3, 2.1, 1.05
22.6	8.0, 7.5, 6.1, 5.3, 1.4, 1.05	6.5, 6.1, 5.3, 2.2, 1.4, 1.05
19.8	8.0, 5.7, 5.5, 3.2, 1.5	5.8, 5.7,3.35, 3.2, 1.5
16.2	9.7, 7.5, 4.2, 3.4	9.7, 3.4, 2.0, 0.8
15.2	18.4, 17.4, 11.8, 8.6, 7.1	11.8, 7.1, 6.3, 2.1
12.1	13.3, 5.5	10.4, 1.85
10.7	18.4, 17.4	10.7
9.4	18.4, 17.4, 4.2, 3.8, 1.35	3.8, 2.4, 1.8, 1.35, 0.9
3.1	13.3	3.1
0.9	18.4, 17.4	0.9
Hpa I	Sac I	Sac I-Hpa I
40	18.4, 17.4, 13.3, 5.7, 5.5, 3.2, 1.5	14.0, 13.3, 12.8, 5.5, 4.1, 3.2, 1.5
28	18.4, 17.4, 9.7, 4.2, 3.8, 1.35	14.0, 12.8, 4.2, 3.9, 3.8, 1.35
19.6	9.7, 7.5, 5.3, 3.4, 1.4	7.5, 4.4, 3.4, 2.7, 1.4
12.4	11.8, 8.6, 1.05	11.8, 2.2, 0.95
11.6	18.4, 17.4, 7.1	7.0, 4.3
9.2	8.0, 5.7	7.9, 1.45
7.6	6.1, 5.3, 1.05	6.1, 1.05
6.6	8.6	6.5
2.2	11.8, 8.6	11.8, 2.2
2.0	5.3	2.0
1.1	9.7	1.1

Table 2. Summary of *Cucumis* Restriction Mapping Hybridizations

Probe DNA	Filter-bound DNA	
Pvu II	Sal I	Pvu II - Sal I
47	48, 20.5, 18.5, 12.5, 11.8, 2.3	18.5, 15.6, 12.5, 11.8, 4.9, 2.3
28	48, 18.5, 16.6, 12.5, 2.3	18.5, 12.5, 4.9, 2.9, 2.3
24	48	24
13.8	16.6	13.8
10.2	11.6	10.2
9.6	20.5, 11.6	4.9
8.3	11.6	6.8. 1.5
6.8	48	6.8
Sal I	Pvu II	Pvu II-Sal I
48	47, 28, 24, 6.8	24, 6.8, 4.9
20.5	47, 9.6	15.6, 4.9
18.5	47, 28	18.5, 12.5
16.6	28, 13.8	13.8, 2.9
12.5	47, 28	18.5, 12.5
11.8	47	11.8
11.6	10.2, 9.6, 8.3	10.2, 6.8, 4.9, 15
2.3	47, 28	2.3

Table 3. Summary of Gene Mapping Hybridizations

Probe	Filter-bound DNA					
	Atriplex triangularis			*Cucumis sativa*		
	Sac I	Sac I-Hpa I	Sac I-Sal I	Pvu II	Pvu II-Sal I	Sal I
16S rRNA	18.4, 17.4	4.3	6.3	47,28,6.8	6.8, 4.9	48
23S rRNA	7.1	7.0	7.1	6.8	6.8	48
LS	8.0	7.9	5.8	47	15.6	20.5
PII	18.4	14.0	1.8	28	18.5	18.5

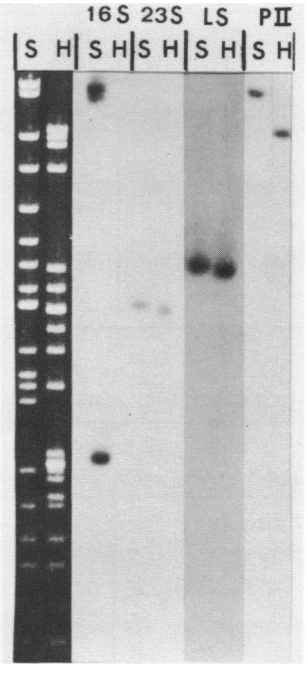

Figure 5. Mapping *Atriplex* chloroplast genes. Probes used are ^{32}P-labelled 16S and 23S tobacco chloroplast ribosomal RNA, the 0.58 kb Pst I fragment of corn chloroplast DNA, which is located entirely within the translated region of the gene for the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase (25), and the 1.1 kb Sal I-Pst I fragment of spinach chloroplast DNA (26) which contains most of the gene for a 32,000 dalton photosystem II polypeptide (PII) (27). Slices containing the corn and spinach fragments were cut out of polyacrylamide gels of total chloroplast DNA and the DNA eluted and nick-translated (16). *Atriplex* chloroplast DNA was digested with Sac I (S) and Hpa I-Sac I (H), electrophoresed on a 0.7% agarose gel and transferred to nitrocellulose filters.

plex chloroplast DNA. Data from these, as well as other Atriplex and Cucumis hybridizations, are summarized in Table 3 and the map locations of these genes are indicated on the restriction maps shown in Figs. 2 and 3.

DISCUSSION

The mapping strategy presented here is an amalgam of two approaches which have been commonly used to map large DNAs of the size of the chloroplast genome. The first is the determination of fragment overlaps by hybridization of labelled fragments produced by one enzyme to filter-bound fragments produced by a second enzyme (1,7,13). In the second method, fragment overlaps are recognized from common double digestion fragments produced by reciprocal digestions of individual fragments purified out of low-melting agarose gels (2,4,6,8,9). The approach taken in this study clearly incorporates the first of these approaches, by hybridizing purified fragments to single digests, and also the second, by hybridizing to double digests. This unified approach allows a complete map to be produced with reference to a single set of probe fragments.

By adding more lanes to each filter replica, one can easily scale up the experiment in order to map more enzymes for one DNA, or even to map several DNAs at once. This feature should permit comparative restriction mapping, as exemplified by the evolutionary studies of Brown and coworkers on primate mitochondrial DNA (20,21), to be performed on the much larger chloroplast genome.

An elegant approach which allows simultaneous determineation of all possible combinations of fragment homologies for two sets of restriction fragments is the cross-hybridization procedure described by Sato et al. (22). In principle, i.e., by hybridizing "hot" gels to both single and double-digest gels, this procedure could be adapted to provide each of the two data sets generated by the method described in this paper. However, this would require a minimum of four gels, to map fragments produced by two enzymes, and proportionately more gels to map additional enzyme digests.

The Atriplex and Cucumis chloroplast genomes are extremely similar in all aspects examined. They differ by only a few percent in size, both feature the inverted repeat which contains the ribosomal genes, and the genes for LS and PII are in the same approximate locations in the two genomes. One apparent difference between the two genomes is the size of the inverted repeat - 24 kb in Atriplex, but only 14 kb in Cucumis. Restriction mapping necessarily yields a minimal estimate for the size of the repeat. Since the 12.5

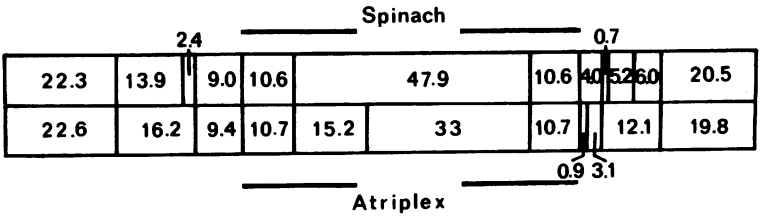

Figure 6. Comparison of Sal I cleavage sites in the Atriplex and spinach (2) chloroplast genomes. The two long lines represent the inverted repeat.

and 18.5 kb Sal I fragments adjacent to the apparent end of the inverted repeat cross-hybridize (Table 2), it can be concluded that the inverted repeat does extend into at least these two Cucumis fragments.

Comparison of the Sal I map of Atriplex with that of spinach (2), a member of the same family (Chenopodiaceae), suggests that these two chloroplast genomes are colinear in sequence (Fig. 6). The slight size differences between many of the fragments which map to the same locations in the two genomes probably reflect small deletion/insertions, known to occur quite frequently during chloroplast genome evolution (23), rather than restriction site changes near the ends of fragments. Accordingly, these small size differences have been neglected in aligning the two maps.

We have recently performed heterologous hybridizations using cloned mung bean restriction fragments and have found that the spinach and Cucumis genomes share a common sequence arrangement around their entire circumference (Palmer and Thompson, sub. for publ.). Thus it appears that all three of these chloroplast DNAs, from spinach, Atriplex and Cucumis, share the same basic pattern of sequence organization.

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