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Conservation of sequence arrangement among higher plant chloroplast DNAs: molecular cross hybridization among the Solanaceae and between Nicotiana and Spinacia

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ABSTRACT

Isolated, nick-translated Pvu II fragments of Nicotiana tabacum chloroplast DNA produce specific intra- and intergeneric hybridization signals with chloroplast DNA digests from several representatives of the Solanaceae. These data, along with similarities in restriction enzyme patterns, permit construction of physical maps for Nicotiana line 92 (a cytoplasmic substitution line), Atropa belladonna and Petunia parodii. Plastid-DNA map differences among the Solanaceae are shown to result from single base-pair substitutions as well as local deletions or insertions. Several of these differences occur in the inverted, repeated region in a reciprocal manner. Hybridization of Nicotiana tabacum chloroplast DNA fragments to a chloroplast DNA digest of Spinacia oleracea defines a sequential arrangement of fragments for spinach DNA which is very similar to its published physical map. This is achieved although chloroplast-DNA restriction enzyme patterns from the two organisms are grossly dissimilar. Alignment differences which have been revealed involve the edges of the inverted repeat region where certain single copy stretches in tobacco have been duplicated in spinach.

INTRODUCTION

Various approaches have been used to compare chloroplast DNA from different plants (1), including intrageneric similarities in restriction endonuclease patterns (2,3). However, base pair drift can alter patterns significantly (4). DNA mapping by partial denaturation has been used to show conservation of the large, inverted, repeated region which appears in the stem structure of self-annealed, single-stranded chloroplast DNA from spinach, lettuce and maize (5). Similarly, the chloroplast rDNA region found in broad bean has been shown by partial denaturation mapping to have parallel counterparts in spinach (6). Indeed, the ribosomal cistron in all chloroplast DNA molecules investigated exhibits the same linear order of gene arrangement (1). The chloroplast tRNA gene maps from spinach and bean show many similarities (7) including the location of genes for tRNA Ile and tRNA Ala in the spacer region between the 16S and 23S rRNA genes (8,9). However, some differences in location of tRNAs in the small single copy region between corn and spinach

may exist (Weil, private communication). Positional conservation of abundantly transcribed genes, such as those of the large subunit of ribulose bisphosphate carboxylase and the 32 kd thylakoid protein, has also been noted (10).

In this work we examine chloroplast DNA homologies among five higher plants by systematically hybridizing individual restriction fragments of a characterized species to total digests of the other chloroplast DNAs. The probing species was Nicotiana tabacum while species analyzed were: Nicotiana line 92 (which has a non-tabacum-type cytoplasm but N. tabacum nucleus [11]), Petunia parodii, Atropa belladonna and Spinacia oleracea. In the cases investigated, a considerable degree of conservation of sequence arrangement along the entire chloroplast genome was revealed, even for more distantly related species.

MATERIALS AND METHODS

Sources and isolation of chloroplast DNA

Nicotiana tabacum var. Samsun, Nicotiana line 92 (11), Atropa belladonna and Petunia parodii were grown from seed in the greenhouse. Leaves of about 12 cm length were harvested after a 24 h period in the dark. Spinacia oleracea was obtained from the field.

Chloroplast DNA from all species was isolated by the procedure of Frankel et al. (3) with the modification reported in (12).

Isolation of restriction fragments

Two to three µg of tobacco DNA, digested with Pvu II, were applied to tube gels (40 x 0.7cm) containing 0.6% low-melting agarose (Sea Plaque, Marine Colloids) and a 2% agarose plug (2 cm). Gels were electrophoresed in 40 mM Tris acetate, 20 mM sodium acetate, 1 mM EDTA, 0.5 µg/ml ethidium bromide, pH 7.8 for 24-48 h at 1-2 V/cm. DNA bands were excised under UV illumination and transferred to silicone treated tubes. Bands from 3 to 4 gels were pooled and the DNA extracted essentially according to Langridge et al. (13). The fragments were soaked in 10 vol of distilled water for 20 min, the water removed, gel vol estimated, and an equal vol of water, equilibrated with butanol containing 1% hexadecyltrimethylammonium bromide (HTB), was added. The gels were melted at 70°C for 2 to 3 min and brought to 37°C. The aqueous phase was extracted with 0.5 vol of butanol containing 1% HTB by inverting fifty times and then allowing for phase separation. The extraction was repeated three times. The combined butanol phases were then extracted twice with 0.25 vol of 0.2M NaCl. The resulting aqueous phase was

extracted with chloroform, after which 10 µg of calf liver tRNA (Boehringer) were added to the aqueous phase as carrier. Nucleic acids were then precipitated with 2.5 vol of ethanol at -70°C for 2 h and resuspended in 100 µl of Tris-HCl, 10 mM EDTA, pH 8 (TE buffer). This solution was applied to a 0.5 ml Dowex AG 50W-X4 200-400 mesh resin column (Bio Rad Laboratories) in a 3 ml syringe, eluted by centrifugation, reprecipitated with ethanol and resuspended in 100 µl of TE buffer. Fragments isolated in this manner yielded high specific activities following nick translation.

Nick translation and hybridization

0.1-0.2 µg of isolated fragments were nick translated as described (14). Conditions for transfer of DNA fragments to nitrocellulose and hybridization of the nitrocellulose strips to labelled fragments were as described (12).

RESULTS

Chloroplast DNA of Solanacea species

Restriction endonuclease patterns - Chloroplast DNA patterns of several Solanaceae species digested with Bgl I and Pvu II are shown in Fig. 1 and the apparent molecular weights of the bands summarized in Table I. Clearly, there are striking similarities among restriction patterns of even more distantly related Solanaceae species. On the other hand, some restriction sites have been added, or deleted, and on close inspection, some of the similar bands show small differences in migration rates. Examination of the patterns obtained with the enzymes Bgl I, Pvu II and Xho I (∿ 50 bands in all) indicates restriction site conservation of 88, 71 and 62% for Nicotiana line 92, P. parodii and A. belladonna, respectively, relative to N. tabacum. This corresponds to < 3% random base substitution per nucleotide (4) and is in line with known phylogenetic relationships.

Interspecific hybridization - The meaning of these similarities was further tested by the following question: Do bands of similar mobility contain the same genetic information; or, possibly, has a shuffling of blocks of DNA sequences occurred so that despite the conservation (or near-conservation) of fragment size, the linear arrangement of genes has undergone change? Taking a direct approach, individual, nick-translated fragments of N. tabacum chloroplast DNA were hybridized to Southern blots of Bgl I- and Pvu II-digested chloroplast DNA from the other Solanaceae species. The results are summarized in Table II. A clear conservation of the sequential arrangement of the restriction fragments vis a vis N. tabacum DNA is apparent. At the level of sensitivity of these hybridization reactions (estimated to be ∿ 0.1 kbp)

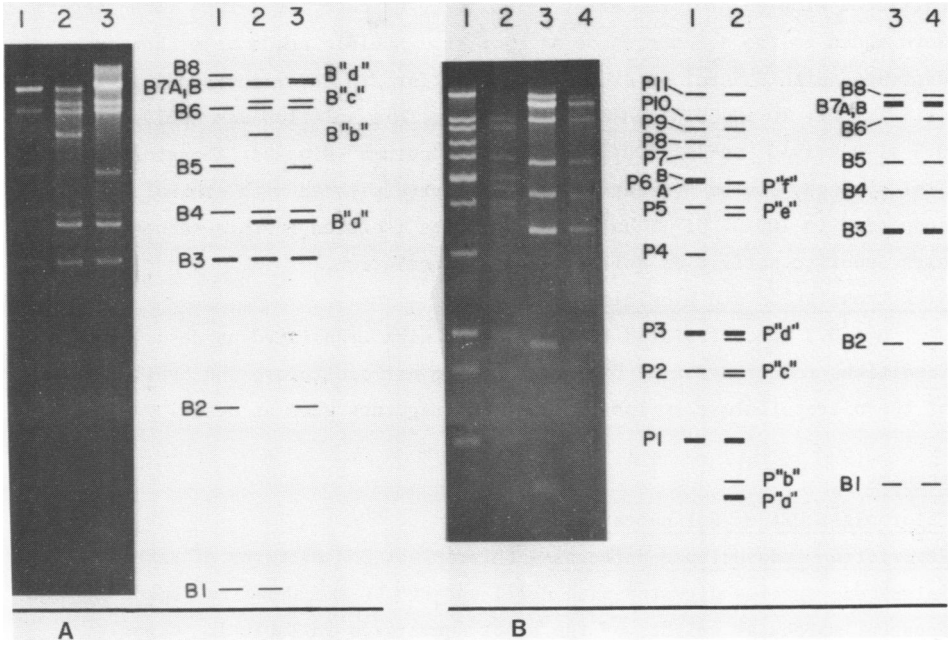

Fig. 1. Comparison of chloroplast DNA restriction patterns from several Solanaceae species. A) Bgl I digests of: 1, N. tabacum; 2, Nicotiana line 92; 3, P. parodii. B) Pvu II digests of: 1, N. tabacum; 2, A. belladonna; Bgl I digests of: 3, N. tabacum; 4, A. belladonna. Designation of N. tabacum restriction bands follows ref. 12 . Additional Bgl I bands present in other species are labeled B"a" to B"d", additional Pvu II bands are labeled P"a" to P"f". Electrophoresis was performed in 0.8% agarose gels.

no evidence was found for movement of sequences among fragments. Thus, the small differences in migration rate of some of the homologous bands among the four sample DNAs (Table I) suggests the existence of local (i.e., intra-fragment) deletions or duplications.

Physical mapping - From the data summarized in Tables I & II and the linear arrangement of chloroplast DNA restriction fragments determined for Nicotiana tabacum (12), physical maps were generated for Nicotiana line 92, Petunia parodii and Atropa belladonna (Fig. 2). A recently published restriction map for Petunia hybrida chloroplast DNA (15) shows the same order, number, and approximate size of Bgl I fragments as found by us for P. parodii.

One of the differences indicated in Fig. 2 between N. tabacum and both, P. parodii and Nicotiana line 92 upon restriction with Bgl I is an additional

Table I. Molecular weights, in kilobase pairs (kbp), of chloroplast DNA fragments from various Solanaceae species produced by restriction enzymes Bgl I and Pvu II.

Bgl I					Pvu II				
Fragment designation	Nicotiana tabacum	Nicotiana line 92	Petunia parodii	Atropa belladonna	Fragment designation	Nicotiana tabacum	Nicotiana line 92	Petunia parodii	Atropa belladonna
	Kbp					Kbp			
B8	41	X	X	-	P11	39.5	-	-	-
B"d"	X	X	31	X	P10	22.5	-	-	-
B7B	29	-	X	-	P9	17.3	-	-	-
B7A	27	-	-	-	P8	15.0	-	-	X
B"c"	X	25	25	X	P7	13.5	13.4	13.4	13.4
B6	23	22.9	-*	-*	P6B	10.5	-	-	-
B"b"	X	16.3	X	X	P6A	10.0	-	-	X
B5	12.5	X	12.4	-	P"f"	X	X	X	9.3
B4	8.3	-	-	8.2	P5	8.3	-	-	-
B"a"(x2)	X	8.0	8.0	X	P"e"	X	X	X	8.0
B3 (x2)	7.2	-	-	-	P4	6.1	-	6.2	X
B2	3.8	X	-	-	P3 (x2)	4.0	-	3.95	3.95
B1	2.0	-	X	-	P"d"	X	X	X	3.8
					P2	3.4	-	-	-
					P"c"	X	X	X	3.3
					P1 (x2)	2.4	-	-	-
					P"b"	X	X	X	2.1
					P"a"(x2)	X	X	X	2.0
Total	161	161	161	161		159	159	159	158

* No size changes were noted in large fragment B6; however, in smaller fragments P7 and X10(Xho I fragment, data not shown) which overlap a portion of B6 (12), size reductions were found.

X Band not present.

\- No change in migration of bands relative to Nicotiana tabacum was detected.

( ) Numbers in parentheses refer to molar concentration of fragments greater than one.

Table II Hybridization of individual, radioactive Pvu II fragments of N. tabacum chloroplast DNA to PVU II and Bgl I digests of chloroplast DNA from other Solanaceae species.

Radioactive fragments	Nicotiana tabacum	Nicotiana line 92	Petunia parodii	Atropa belladonna
	Pvu II digests			
P1	P1	P1	P1	P1
P2	P2	P2	P2	P2
P3	P3	P3	P3	P3
P4	P4	P4	P4	P"d", P"b"
P5	P5	P5	P5	P5
P6A	P6A, P8	P6A, P8	P6A, P8	P"e", P"a"
P6B	P6B	P6B	P6B	P6B
P7	P7	P7	P7	P7
	Bgl I digests			
P1	B8	B"a"	B"a"	B8
P2	B7B	B7B	B"d"	B7B
P3	B8	B"a"	B"a"	B8
P4	B7A	B7A	B7A	B7A
P5	B1, B4, B7B	B1,B4,B7A	B4,B"d"	B1, B4, B7B
P6A	B8	B"a", B"c"	B"a", B"c"	-
P6B	B4,B7A	-	B4, B7A	B4,B7A
P7	B6	B6	B6	B6
P8	B8	B"a", B"c"	B"a",B"c"	B8
P9	B3, B5, B6	B3,B"b", B6,B"a"	B3,B5,B6,B"a"	-
P10	B7B	-	-	-
P11	B2,B3,B5,B6,B7A	-	B2,B3,B"a",B5, B6,B7A	-

Dashes indicate data not available. Further corroborating hybridizations are: fragment B1 of Nicotiana tabacum hybridizes to B"d" of Petunia parodii: fragment B2 of Nicotiana tabacum hybridizes to B"b" of Nicotiana line 92. Refer to Fig. 1 for fragment designations.

site within the inverted, repeated region which cuts fragment B8 into fragment B"c" and bimolar fragment B"a". From our knowledge of the physical map of N. tabacum chloroplast DNA we estimated the position of this bimolar restriction site to be about 200 bp distal to the 3' end of the 5 S ribosomal gene. Since the nucleotide sequence in this region of the N. tabacum plastid genome has been published (16), we were able to pose a specific question: Could a single base-pair substitution in the chloroplast DNA of N. tabacum generate the Bgl I site present in P. parodii and Nicotiana line 92? Investigation reveals the existence of a sequence CCCGAAATGGC,184 Base-pairs from the

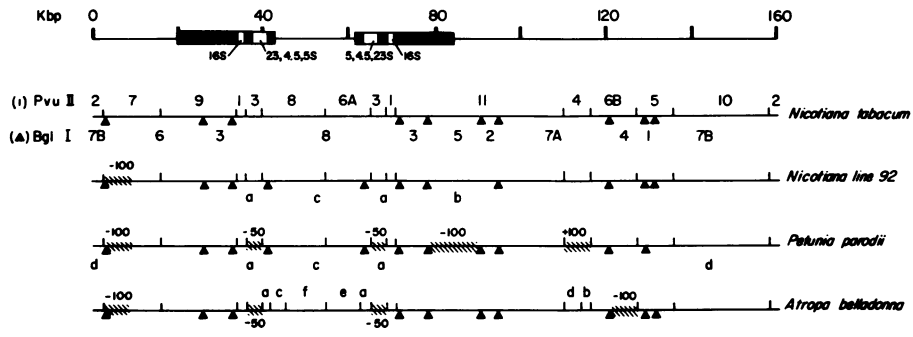

Fig. 2. Differences in restriction endonuclease maps of representatives of the Solanaceae. ı, Pvu II sites; ▲, Bgl I sites; \\\\, deletion or addition (estimated numbers of base pairs as indicated). All restriction fragments for Nicotiana tabacum are designated while only those differing from the above are designated for the other species. The positions of the inverted repeats and their accompanying ribosomal RNA genes are indicated on the kbp scale. The region of the deletion in P7 and B6 is deliniated by Xho I fragment X10 (see footnote, Table I).

3' end of the 5 S gene of N. tabacum chloroplast DNA (base pairs 674-684 in Fig. 3 of Takaiwa and Sugiura[16]). As the recognition sequence for Bgl I is GCCNNNNNGGC (17), a single base substitution of C to G at the first nucleotide would create the necessary conditions for the appearance of the new fragments B"a" and B"c". Thus, it appears likely that this restriction-site change in the Solanaceae arose via a single base-pair substitution.

Other site changes which dramatically alter the restriction patterns of the chloroplast DNAs investigated can be seen in Fig. 1. One of them, affecting the Pvu II pattern of A. belladonna and generating the bimolar fragment P"a", is within 200 bp of the site-change in fragment B8 discussed above. In this case, as well as the one cited above, changes within the inverted repeated region have occurred in a reciprocal manner, i.e. the repeat units have remained identical. Similar observations, using restriction endonucleases, have been made for diverged subtypes of the Euoenothera plastomes (18). Thus, some type of mechanism appears to exist where one of the inverted sequences is corrected by the other, perhaps through heteroduplex repair events (19) or intermolecular recombination within the repeated region coupled with sorting-out of molecules (5).

Restriction fragment hybridization of Nicotiana tabacum and Spinacea oleracea chloroplast DNAs

We attempted to apply the techniques used in studying sequential re-

latedness among chloroplast DNAs of the Solanaceae to comparisons between plants from different families. Fig. 3 shows the patterns of tobacco and spinach chloroplast DNAs digested with various restriction enzymes . From these patterns a maximum estimate of fragments-in-common is 20%; hence, base substitutions between the two chloroplast genomes are predicted to have occurred in at least 10% of the nucleotides (4). Probably, not all regions of these chloroplast genomes diverged at the same average rate. For example, Pvu II fragments P1 and P3 of tobacco, which contain the 16 S and 23 S rRNA genes and spacer regions (12), have similar counterparts in spinach. Similarly, conservation of certain small, plastid-DNA restriction fragments among widely different species has been reported by Herrmann et al (20). Be this as it may, at the level of pattern dissimilarity exhibited in Fig. 3, restriction enzyme analysis is clearly a poor guide for comparative fragment allignment.

For this purpose DNA fragment hybridization was performed. Isolated, radio-

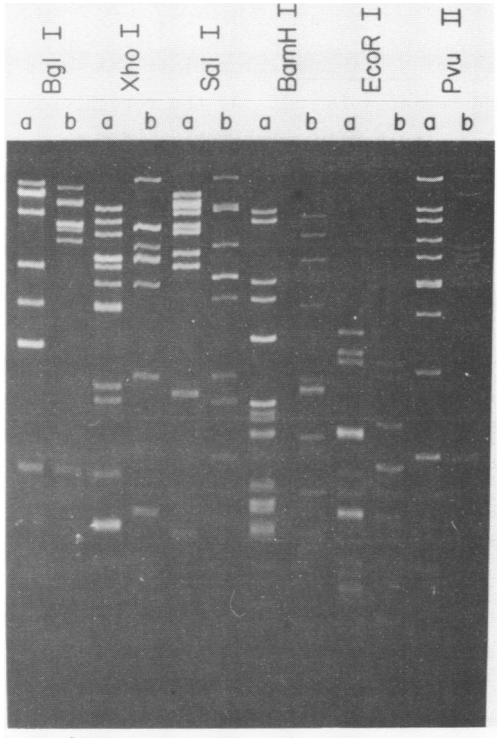

Fig. 3. Restriction endonuclease digests of a) tobacco and b) spinach chloroplast DNAs electrophoresed in a 0.8% agarose slab gel.

active, Pvu II fragments from tobacco chloroplast DNA were hybridized to a Sal I digest of spinach chloroplast DNA which had been transferred to nitrocellulose paper (Fig. 4). The results are summarized in Table III as either major or weak hybridization signals. For example, in addition to specific major hybridizations, bimolar fragment S5 of spinach hybridizes weakly with many sections of the tobacco genome. Analogous phenomena have been noted in other fragment-hybridization studies (21). The order of tobacco chloroplast DNA fragments digested with Pvu II is given in Table III. Based on major hybridization signals, the data in Fig. 4 and Table III suggest a Sal I fragment sequence of : S4-S2-S3-(S7-S8-S11)-S9-S5-S1-S5-S6-S10 for spinach chloroplast DNA, with ambiguity among S7, S11 and S8. This order corresponds to that previously established for spinach by conventional means (22). Thus, not withstanding the considerable differences in restriction-site patterns

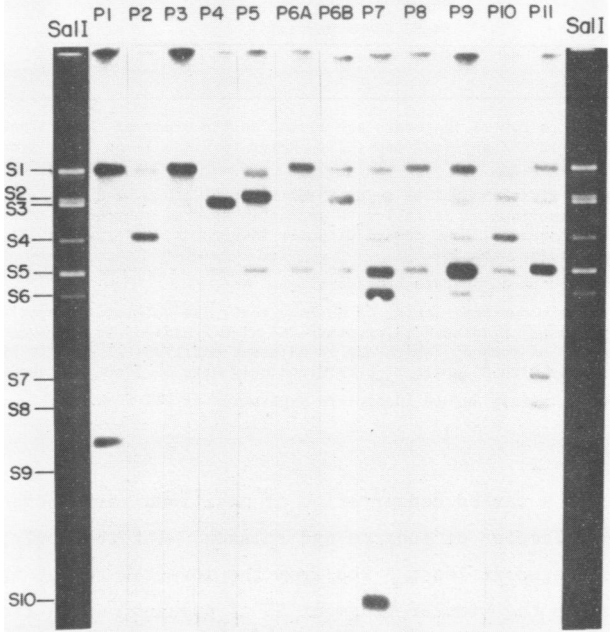

Fig. 4. Hybridization of nick-translated Pvu II fragments from tobacco chloroplast DNA to a Sal I digest of spinach chloroplast DNA. Outer lanes are restriction enzyme digests of spinach DNA; inner lanes are the corresponding nitrocellulose transfers hybridized to the individual bands of tobacco DNA as indicated. The very faint band below S1 is the result of incomplete digest of the Sal I site between S2 and S4. The band between S8 and S9 obtained with tobacco fragment P1 as probe was not repeated in other experiments (see footnote b Table III).

Table III. Hybridization of isolated Pvu II bands of tobacco chloroplast DNA to Sal I digests of spinach chloroplast DNA

Pvu II bands, (tobacco)[a]	Sal I digest (spinach)	
	Major signals	Weak signals
P7	S6,S5,S10	(S1,S4,S3)
P9	S5,S1,S6	(S4,S3)
P1	S1,S8/9[b]	
P3	S1	
P8	S1	(S5)
P6A	S1	(S5)
P3	S1	
P1	S1,S8/9[b]	
P11	S5,S1,S7,S8,S3,S11[c],S9[d]	
P4	S3	(S5)
P6B	S3	(S5,S1[e])
P5	S2,S2+S4 partial	(S5)
P10	S4,S2,S2+S4 partial	(S5)
P2	S4	(S1, S5)

a. The tobacco Pvu II fragments are listed in the order of their linear alignment in the physical map (12). Refer to Figs. 1 and 4 for fragment designations.

b. Although first thought to be an S9+S11 partial, this band is now considered spurious as it failed to appear in several subsequent experiments. Moreover, when tested directly in separate experiments, P1 did not hybridize with S11.

c. Determined in a separate hybridization.

d. The unambiguous positioning of S9 (see text) was obtained by hybridizing digests of spinach chloroplast DNA with isolated Xho I fragments X4 and X2 of tobacco (which map in adjacent positions [12]). X4 hybridized only with S5 while X2 hybridized only with S9 (data not shown).

e. Probably, mainly due to incomplete separation of P6B from P6A.

(Fig. 3), there is a marked conservation of positional arrangement along the chloroplast DNA molecules of tobacco and spinach. Interestingly, a unique fragment, P7, situated at least 3 kbp from the inverted repeat in tobacco (12), hybridizes to the bimolar fragment S5 of spinach, which lies totally within the inverted repeat in this organism (22). Thus, sequences present once in tobacco have been duplicated in spinach. Moreover, tobacco Xho I fragment X4, which spans the edge of the inverted repeat and maps within Pvu II fragment P11 (Fig. 2 and ref. 12), hybridizes only to bimolar fragment S5 of spinach (see footnote c, Table III). Perhaps a local inversion or a non reciprocal crossing-over event has occurred in spinach relative to tobacco chloroplast DNA, in an area adjacent to the inverted repeat.

DISCUSSION

Comparative arrangements of chloroplast DNA genomes can be studied at several levels, including restriction enzyme patterns, fragment hybridization and nucleotide sequencing. The last method is ultimately the most informative; however, due to its present tediousness, it is applicable only after detailed knowledge of a specific, and relatively limited, region has been gathered (for example, the tRNA-containing spacer region between the 16 S amd 23 S chloroplast rRNA genes [9]). Restriction enzyme patterns, on the other hand, are easily and rapidly obtained. However, because of pattern sensitivity to site deletions or additions, this method is valuable for comparing genome arrangements mainly among closely related organisms (4). Fragment hybridization is far less sensitive to random base changes and can yield information on positioning of larger blocks of DNA in both closely and distantly related organisms.

Several chloroplast genomes have been physically mapped using fragment hybridization (12, 21, 23, 24). In all of these studies, weak hybridization signals were found alongside the major ones. For the most part, the weak signals involved bimolar bands mapping within the inverted repeat region (e.g. bands P1 and P3 of tobacco [12] or Ba1 , Ba4 and E23 of Chlamydomonas [21]). The physical basis for these weak hybridizations has not been defined. The presence of small repeated sequences (25), possibly related to common chloroplast gene-recognition-signals, may be responsible. We note that the mapping data for tobacco chloroplast DNAs based on major hybridization signals has been confirmed by other physical techniques (10).

Concerning the chloroplast DNAs analyzed in this study, site changes have been sufficiently infrequent so that comparisons at the level of restriction enzyme patterns appear useful as a guide for mapping differences among genera of the Solanaceae. This was not the case in the interfamilial comparison between tobacco and spinach chloroplast DNAs, where recourse to fragment hybridization was necessary to expose significant patterns of similarity in linear arrangement. The results of Seyer et al (10), who carried out detailed hybridizations within an 8 kbp fragment encompassing the gene for the large subunit of ribulosebisphosphate carboxylase, and our results, on a coarser level over the entire genome, both indicate a remarkably high level of positional conservation between these two chloroplast DNAs. Gross rearrangements due to transpositions were not detected, however a change analogous to an inversion may have occurred. As pointed out by Palmer and Thompson (26), small scale rearrangement events occurring within a large fragment

would probably go undetected by the procedures used. There are points of similarity between the results of this study and those with animal mitochondria. In intergeneric comparisons between Drosophila (27), and Xenopus (28) mitochondrial DNAs, independent evolution produced large variations in primary sequence but not general changes in sequence alignment.

The hybridization results with chloroplast DNA of tobacco and spinach, which both have inverted repeat regions, are in marked contrast to the extensive rearrangements found between the chloroplast genomes of mung bean, which contains an inverted repeat region, and pea, which does not (26). This sharp difference in results lends some credence to the hypothesis that inverted repeats may contribute to the stability (or in our case, phylogenetic conservation) of circular DNA genomes (5, 26). However, other factors and other rules may well apply. Further phylogenetic comparisons of chloroplast genome arrangement among higher plants should help clarify this point.

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