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Physical Mapping of Differences in Chloroplast DNA of the Five Wild-type Plastomes in *Oenothera* Subsection *Euoenothera*

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Summary. 1) DNA has been isolated from the five genetically distinguishable plastid types of *Oenothera*, subsection *Euoenothera*. DNA of plastomes I to IV was obtained from plants with identical nuclear backgrounds containing the genotype AA of *Oenothera hookeri* whereas the DNA of plastome V came from *Oenothera argillicola* (genotype CC).
2) The DNAs of the five basic *Euoenothera* wild-type plastomes can be distinguished by restriction endonuclease analysis with Sal I, Pst I, Kpn I, Eco RI and Bam HI. The fragment patterns exhibit distinct common features as well as some degree of variability.
3) Physical maps for the circular DNAs of plastome I, II, III and V could be constructed using the previously detailed map of plastome IV DNA (Gordon et al. 1981). This has been achieved by comparing the cleavage products generated by restriction endonucleases Sal I, Pst I and Kpn I which collectively result in 36 sites in each of the five plastome DNAs, and by hybridization of radioactively labelled chloroplast rRNA or chloroplast cRNA probes of spinach to Southern blots of appropriate restriction digests. The data show that the overall fragment order is the same for all five plastome DNAs. Each DNA molecule is segmentally organized into four regions represented by a large duplicated sequence in inverted orientation whose copies are separated by two single-copy segments.
4) The alterations in position of restriction sites among the *Euoenothera* plastome DNAs result primarily from insertions/deletions. Eleven size differences of individual fragments in the Sal I, Pst I and Kpn I patterns measuring 0.1–0.8 Md (150–1,200 bp) relative to plastome IV DNA have been located. Most changes were found in the larger of the two single-copy regions of the five plastomes. Changes in the duplication are always found in both copies. This suggests the existence of an editing mechanism that, in natural populations, equalizes or transposes any change in one copy of the repeat to the equivalent site of the other copy.
5) Detailed mapping of the two rDNA regions of the five plastomes, using the restriction endonucleases Eco RI and Bam HI which each recognize more than 60 cleavage sites per DNA molecule, disclosed a 0.3 Md deletion in plastome III DNA and a 0.1 Md insertion in plastome V DNA relative to DNA of plastome IV, I and II. These changes are most probably located in the spacer between the genes for 16S and 23S rRNA and are found in both rDNA units.

Key words: *Oenothera* – Evolution of plastid DNA – Comparative restriction site mapping – rDNA spacer differences – Insertions and deletions.

Abbreviations

bp	base pairs
kbp	kilobase pairs
Md	Megadalton
rDNA	ribosomal DNA
rRNA	ribosomal RNA
cRNA	complementary RNA

Introduction

The subsection *Euoenothera* is one of five in the section *Oenothera*. It consists of three morphologically distinct genomes, A, B, C, that occur in homozygous (AA, BB, CC) and complex heterozygous (AB, AC, BC) combinations, and five basic plastid genomes (plastomes designated I to V) (Stubbe 1959). The five plastomes are recognizable by their compatibility or incompatibility with specific nuclear genomes (reviewed in Kutzelnigg and Stubbe 1974; Herrmann and Possingham 1980).

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For the analysis of differences in moderately complex DNA, such as chloroplast DNA, restriction endonucleases represent a sensitive and convenient experimental approach (see Botchan et al. 1973; Upholt 1977), which may yield information on base sequence changes or on rearrangements within DNA molecules. Earlier comparisons of plastid DNA from different genera and families showed considerable differences in restriction endonuclease cleavage patterns (Atchison et al. 1976; Vedel et al. 1976). In the genus *Nicotiana* and in the genus *Pelargonium,* several species have been analysed and specific changes in cleavage patterns found (Atchison et al. 1976; Vedel et al. 1976; Frankel et al. 1979; Metzlaff et al. 1981). In general, closely related species display more similar DNA fragment patterns than do more distant ones. Nucleotide polymorphism at an Eco RI cleavage site has been discovered in several different wild populations of *N. debneyi* (Scowcroft 1979). In F_1-hybrids, obtained by reciprocal crossing, Scowcroft demonstrated strict maternal inheritance of plastid DNA by comparing restriction patterns. A similar observation was made for four of six male sterile *Nicotiana* lines and their parents (Frankel et al. 1979). Analysis of the plastid DNA of *Pelargonium* F_1-hybrids, using the restriction endonuclease Eco RI, provided evidence for a biparental mode of inheritance (Metzlaff et al. 1981).

Since restriction endonuclease cleavage site maps are essential, we have recently detailed the physical map for the 100 Md (160 kbp) chromosome of the *Euoenothera* plastome IV using the restriction endonucleases Sal I, Pst I, Kpn I, Bam HI and Eco RI (Gordon et al. 1981). The plastid type IV DNA is believed to represent the ancestral plastome in the *Euoenothera* plastome pedigree (Stubbe 1959). The present study was designed (1) to map and compare the relative positions of the alterations in fragment patterns on the five basic *Euoenothera* plastid chromosomes and (2) to gain information on the type of such alterations (e.g., rearrangements, insertions, deletions, inversions). A progress report of this work has been presented (Herrmann et al. 1980c). Recently, Palmer and Thompson (1981) mapped a number of rearrangements of homologous sequences common to the mung bean and pea plastomes.

Materials and Methods

The *Euoenothera* plastomes I–V occur in the following natural species: AA (genotype) – I: *Oe. elata, Oe. hookeri, Oe. jamesii, Oe. longissima, Oe. strigosa, Oe. wolfii;* AB–II: *Oe. biennis* (part), *Oe. suaveolens;* AB–III: *Oe. biennis* (part), *Oe. erythrosepala* (syn. *lamarckiana*); BB–III: *Oe. grandiflora, Oe. austromontana;* BC–IV: *Oe. parviflora, Oe. atrovirens;* AC–IV: *Oe. oakesiana;* CC–V: *Oe. argillicola.* (For details about the modern *Oenothera* nomenclature, see Raven et al. 1979.) In the current investigation the DNAs of plastome I, II, III and IV were obtained from plants with the nuclear genotype AA (^{h}*hookeri* strain Johansen · ^{h}*hookeri* strain Johansen) containing the corresponding plastid type. The combination AA–I represents the wild species *Oe. hookeri.* Plastomes II, III and IV are not found in natural species homozygous for the genotype AA (Stubbe 1960, 1964). These genome/plastome hybrids were produced by combining the genotype of *Oenothera hookeri* (^{h}*hookeri* Johansen · ^{h}*hookeri* Johansen) with the plastids of *Oe. suaveolens* strains Standard, Friedrichshagen, Fünfkirchen and Grado (plastome II; genome AB, *albicans · flavens*); of *Oe. lamarckiana* line Schweden (plastome III; genome AB, *velans · gaudens*) and of *Oe. atrovirens* (plastome IV; genome BC, *pingens · flectens*) as described by Stubbe (1960) and Drillisch (1975). Plastome V is incompatible with the A genome (Stubbe 1960, 1964) and its DNA was thus obtained directly from the wild species *Oe. argillicola* (genome CC). All plants were grown as described in Gordon et al. (1980).

For methods used, the reader is referred to Driesel et al. (1979), Herrmann et al. (1975, 1980a, b) and Gordon et al. (1980, 1981).

Results

Comparison of Restriction Endonuclease Cleavage Patterns

The DNA fragment patterns obtained upon gel electrophoresis of restriction endonuclease digests, using Sal I, Pst I and Kpn I, are strikingly similar for DNA from each of the five *Euoenothera* plastomes. For each DNA, 14, 11 and 12 fragments, respectively, were resolvable in agarose gels. However, analysis of the patterns shows

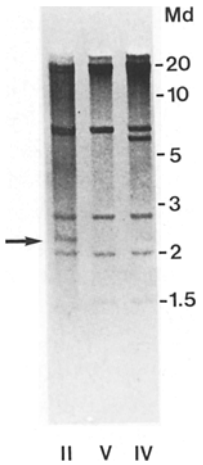

Fig. 1. Agarose slab gel electrophoresis (0.6% SeaKem agarose) of *Euoenothera* chloroplast DNAs after digestion with restriction endonuclease Kpn I. A molecular weight scale in Md is given and the roman numerals indicate the plastome from which the DNA was isolated. Arrow indicates a partial digestion band at 2.2 Md in plastome II DNA

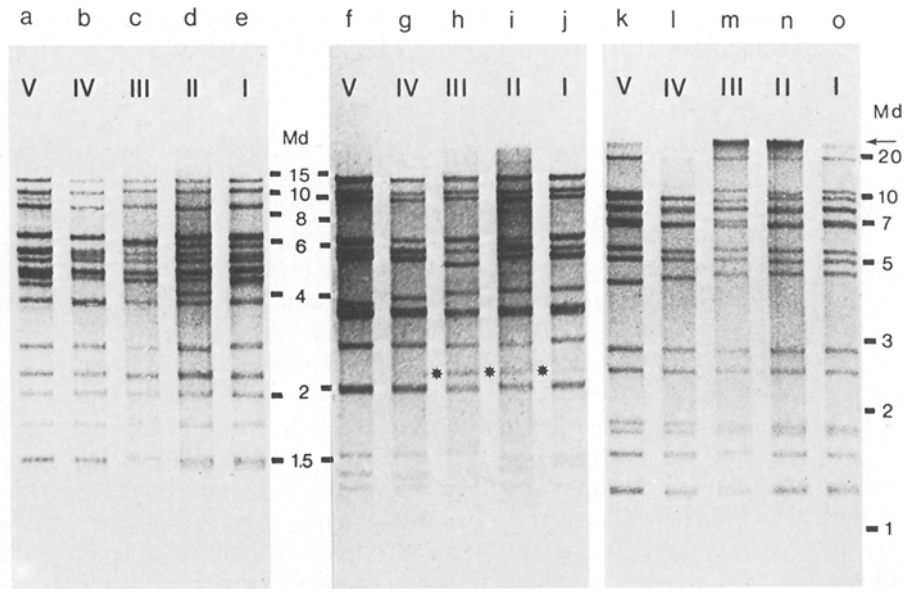

Fig. 2. Agarose slab gel electrophoresis of restriction endonuclease double digests of DNA from the *Euoenothera* plastomes I – V. The restriction fragments were separated by electrophoresis on 0.6% agarose gels. Molecular weight scales are given in Md for each gel. Tracks a – e: double digestions with Kpn I and Sal I; tracks f – j: double digestions with Kpn I and Pst I; tracks k – o: double digestions with Pst I and Sal I. The respective plastome DNA is designated with the roman numerals I to V. The star indicates fragments resulting from incomplete digestion. The high molecular weight zone in tracks m and n (arrow) represents contaminating nuclear DNA as evidenced by buoyant density analysis of electroeluted material (Herrmann et al. 1975; Gordon et al. 1981)

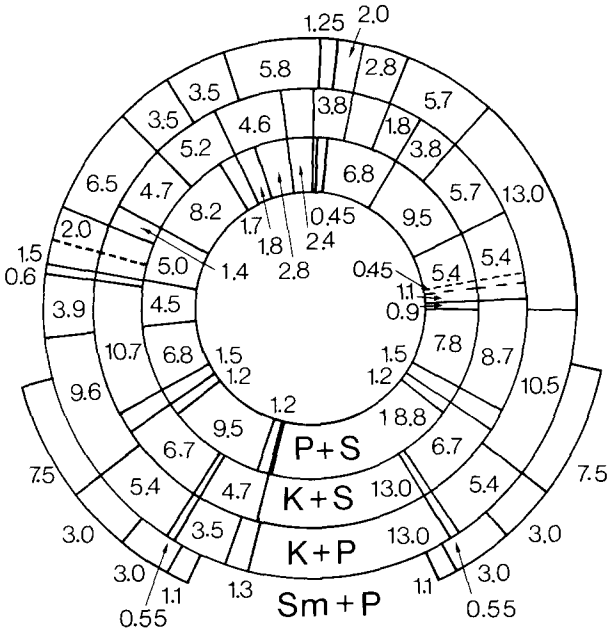

Fig. 3. Restriction endonuclease cleavage site map of DNA from *Oenothera* plastome IV, showing the relative locations of each of the secondary fragments produced by double digestions, as detailed in the preceding paper (Gordon et al. 1981). The symbols P+S, K+S and K+P refer to double digestions with Pst I+Sal I, Kpn I+Sal I and Kpn I+Pst I, respectively. Sm+P indicates a partial map obtained by double digestion with Sma I+Pst I covering most of the inverted repeat region. The size of each secondary fragment is given in Md. The relative order of the fragments separated by the dashed lines remains to be determined

distinct size differences between individual fragments, e.g. for the 5.7 Md Kpn I fragment (Fig. 1) and, to a lesser extent, the 4.5 Md Pst I fragment, the 9.5 Md or the 5.4 Md Sal I fragments of plastome IV DNA.

Many fragments found in single digests are too large for accurate analysis of small changes in size. The likelihood of observing differences among fragment patterns increases with greater frequency of restriction sites. Double digestions using these three enzymes result in secondary fragments with a size of less than 8 MD from about 85% of the circular DNA molecule. This allows a more complete comparison of the differences in the restriction patterns of the five plastome DNAs (Fig. 2). A complete listing of fragment sizes for DNA of plastomes I, II, III and V, relative to those of plastome IV, is presented in Tables 1 to 3.

Molecular weight determinations of DNA fragments by agarose gel electrophoresis allow differences of 0.15 Md and larger to be detected for the range of fragment sizes from 6 to 8 Md. In the size range of 1.5 to 6 Md, changes smaller than 0.1 Md may be measured and for fragments smaller than 1.5 Md, changes can be measured to an accuracy of about 0.03 Md (equivalent to about 50 base pairs). The reliability of size determination was ensured by co-electrophoresis of the sample to be compared or by including commercial length standards in the same gel slot (Gordon et al. 1981). Clearly, with larger DNA fragments, i.e. greater than 10 Md, small size differences will escape detection.

The differences in the fragment sizes among DNAs of the five plastome types after double digestion with Sal I, Pst I and Kpn I were small enough to permit their relative location on the physical map established for plastome IV DNA (Fig. 3). It was concluded that the serial order of restriction sites in DNA from all plastomes was preserved. Evidence for this rests in the following observations:

a) The same number of secondary fragments was observed for each of the three pairs of double digests of all five plastomes (Fig. 4).

b) Redigestion of individual fragments of plastome I DNA (data not given) corroborated that the serial order of the fragments was the same as for plastome IV DNA.

c) Hybridization of labelled ribosomal RNAs occurred to fragments of the same or nearly same size (Fig. 6).

d) Hybridization of labelled cRNA from a spinach chloroplast DNA fragment, occurred to fragments of the same or nearly the same size (experiment not shown; cf. Gordon et al. 1981; Herrmann et al. 1980c).

e) Increasing the number of restriction fragments by frequently cutting enzymes (Eco RI or Bam HI) still resulted in very similar patterns (Fig. 5).

Mapping of the Differences in DNA from the Five Plastomes

Using the restriction fragment map obtained with endonucleases Sal I, Pst I and Kpn I for plastome IV DNA (Fig. 3, Gordon et al. 1981), the secondary fragments produced by the same double digestions of the other four plastome DNAs can be directly mapped. The location of observed differences in the secondary fragments is narrowed down by finding the smallest secondary fragment which shows the difference. The overlapping secondary and, where possible, primary fragments from the other two double digestions are then checked for verification of the size difference. In some cases the large size of the overlapping fragments, and thus the limited accuracy of molecular weight determinations, precludes this verification.

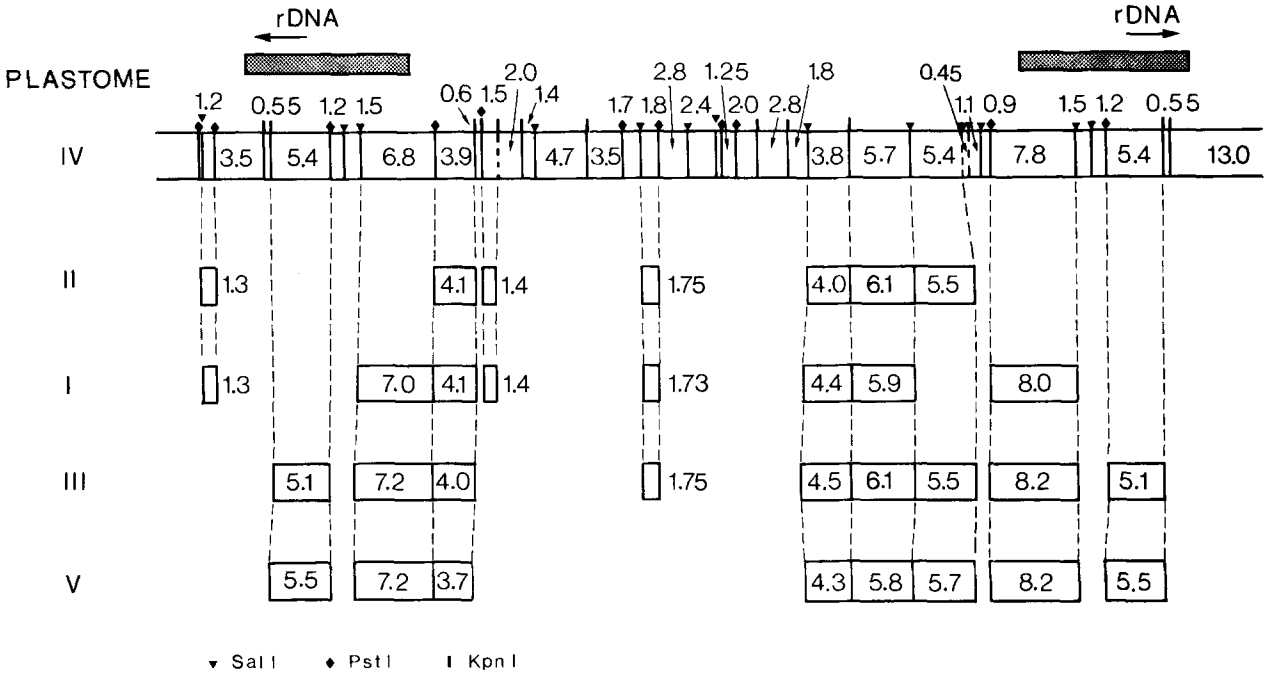

Fig. 4. Comparative restriction endonuclease cleavage site maps of DNA from the five related *Euoenothera* plastomes. The complete map of the DNA from plastome IV (see preceding paper: Gordon et al. 1981) is presented in linearized form by cutting the small single-copy region. The cleavage sites of the restriction endonucleases Sal I, Pst I and Kpn I are indicated by symbols. Arabic numbers represent the sizes in Md of the primary or secondary fragments between each pair of nearest adjacent restriction sites. Broken lines indicate that the order of two neighbouring fragments is ambiguous. The shaded bars indicate the approximate extent of the two copies of the inverted repeat region, and the location and orientation (from 16S to 23S) of the rDNA units within these are indicated by arrows. For plastomes I – III and V, only segments which differ in size from the corresponding one of plastome IV are shown in their correct locations together with the size of that particular segment in each plastome. The dashed lines indicate which cleavages on the plastome IV chromosome correspond to the ends of the segments differing in other plastomes. The sizes and location of the segments which are shown as differing from those of plastome IV are based on data in Fig. 2, Tables 1, 2 and 3 and the map in Fig. 3 (see text)

Figure 4 shows the difference among the DNAs from plastomes I, II, III or V in relation to the map of plastome IV DNA (Fig. 3) which has been linearized by cutting within the 13 MD Kpn I + Pst I secondary fragment (see map Fig. 3). This DNA fragment lacks a Sal I site and is too large for an exact size comparison between the different plastomes.

Starting from the left in Fig. 4, the first Sal I + Pst I secondary fragment in plastome IV DNA is 1.2 Md in size. This fragment is somewhat larger in DNA of plastomes I and II (Table 2). The difference was not detected in the Kpn I + Sal I digestion (Table 1) but is clearly apparent in the corresponding primary Pst I fragment (1.3 Md in plastome IV DNA) (see Kpn I/Pst I double digestion Fig. 2 f–j and Table 3).

The next fragment exhibiting differences is the 5.4 Md Kpn I + Pst I secondary fragment in the inverted repeat. Both this (Table 3) and the overlapping 6.7 Md Kpn I + Sal I secondary fragment (Table 1) are about 0.1 Md larger in the DNA from plastome V and 0.3 Md smaller in the plastome III DNA. This change in plastome III DNA is also reflected by the smaller size of the corresponding 9.2 Md Pst I primary fragment (9.5 Md in plastome IV DNA, Table 2), and the 11.5 Md Sal I primary fragment (11.8 Md in plastome IV DNA). The 5.4 Md Kpn I + Pst I secondary fragment of plastome IV DNA carries sequences for ribosomal RNAs and the fine mapping of this region of the inverted repeat is discussed below.

Difference in fragment size among the plastome DNAs have also been observed in the plastome IV 6.8 Md Pst I + Sal I secondary fragment (Table 2) which covers the other end of the inverted repeat region and part of the large single-copy region. This fragment is larger in DNA of plastomes I, III and V. The changes can be localized to this 6.8 Md fragment because the other Pst I + Sal I secondary fragment equivalent to the 6.8 Md fragment of plastome IV DNA which is located within the large single-copy region (Fig. 3) contains two cleavage sites for Kpn I. The 2.8 Md primary Kpn I fragment and the two

Table 1. Number and molecular weights (Md) of fragments of DNA from each of the five plastomes of *Oenothera* produced by double digestion with restriction endonucleases Kpn I and Sal I. Fragments marked '=' could not be distinguished in size from those of plastome IV. Observed differences are rounded to the nearest 0.1 Md for fragments between 10.5 and 1.5 Md and below this to the nearest 0.05 Md. The number of fragments in each instance is 26

Plastome				
IV	I	II	III	V
13.0	=	=	=	=
10.7	=	=	=	=
8.7	8.9	=	9.1	9.1
6.7 (2×)	=	=	6.4 (2×)	6.8 (2×)
5.7	5.9	6.1	6.1	5.8
5.4	=	5.5	5.5	5.7
5.2	=	=	=	=
4.7 (2×)	=	=	=	=
4.6	4.5	4.5	4.5 (2×)	=
	4.4	4.0		4.3
3.8 (2×)	3.8	3.8	3.8	3.8
2.8	=	=	=	=
2.4	=	=	=	=
2.0	=	=	=	=
1.8	=	=	=	=
1.5 (3×)	1.5 (2×)	1.5 (2×)	=	=
1.4	1.4 (2×)	1.4 (2×)	=	=
1.1	=	=	=	=
0.6	=	=	=	=
0.55 (2×)	=	=	=	=
0.45	=	=	=	=
Σ 101.85	102.65	102.35	102.75	103.35

Table 2. Number and molecular weights (Md) of fragments of DNA from each of the five plastomes of *Oenothera* produced by double digestion with restriction endonucleases Pst I and Sal I. Fragments marked '=' could not be distinguished in size from those of plastome IV. Observed differences are rounded to the nearest 0.1 Md for fragments between 10.5 and 1.8 Md and below this to the nearest 0.03 Md. The smallest, 0.1 Md secondary fragment is anticipated but has not been demonstrated in DNA of plastomes I–V. The number of fragments in each instance is 25

Plastome				
IV	I	II	III	V
18.8	=	=	=	=
	10.3	9.9	10.4	9.9
9.5 (2×)	9.5	9.5	9.2	9.5
8.2	=	=	8.2 (2×)	8.2 (2×)
7.8	8.0	=		
	7.0		7.2	7.2
6.8 (2×)	6.8	=	6.8	6.8
5.4	=	5.5	5.5	5.7
5.0	4.9	4.9	=	=
4.5	4.7	4.7	4.6	4.3
2.8	=	=	=	=
2.4	=	=	=	=
1.8	1.73	1.75	1.75	1.83
1.7	=	=	=	=
1.5 (2×)	=	=	=	=
	1.3	1.3		
1.25	=	=	=	=
1.2 (3×)	1.2 (2×)	1.2 (2×)	=	=
1.1	=	=	=	=
0.9	=	=	=	=
0.45 (2×)	=	=	=	=
(0.1)	(=)	(=)	(=)	(=)
Σ 101.85	103.18	102.5	103.4	103.18

Table 3. Number and molecular weights (Md) of fragments of DNA from each of the five plastomes of *Oenothera* produced by double digestion with restriction endonuclease Kpn I and Pst I. Fragments marked '=' could not be distinguished in size from those of plastome IV. Observed differences are rounded to the nearest 0.1 Md for fragments between 10.5 and 1.5 Md and below this to the nearest 0.03 Md. An expected 23rd secondary fragment in the Kpn I/Pst I series has not been directly demonstrated because the Kpn and Pst restriction site are very close together (Gordon et al. 1981). The number of fragments in each instance is 22

Plastome				
IV	I	II	III	V
13.0 (2×)	=	=	=	=
10.5	10.7	=	10.9	10.9
9.6	9.8	=	10.0	10.0
6.5	=	=	=	=
	6.3	5.9	6.4	6.2
5.8	=	=	=	=
5.7				
5.4 (2×)	=	=	5.1 (2×)	5.5 (2×)
3.9	4.1	4.1	4.0	3.7
3.5 (3×)	=	=	=	=
2.8	=	=	=	=
2.0 (2×)	=	=	=	=
1.5	1.4	1.4	1.55	=
1.3	1.35	1.38	=	=
1.25	=	=	=	=
0.6	=	=	=	=
0.55 (2×)	=	=	=	=
Σ 101.85	103.0	102.23	102.9	103.15

secondary fragments 2.0 and 1.8 Md obtainable with Kpn I from this latter fragment did not reveal significant differences among the plastomes.

The 3.9 Md Kpn I + Pst I secondary fragment of plastome IV DNA adjacent to the 6.8 Md Pst I + Sal I secondary fragment is enlarged in plastome I, II and III DNA and diminished in plastome V DNA (Table 3). It is contained within the 4.5 Md Pst I fragment, which is present as a single fragment in the Pst I/Sal I double digestion and there shows corresponding changes in size (Table 2).

While in plastome I and III DNA both the 6.8 Md Sal + Pst fragment and the 3.9 Md Pst + Kpn fragment of plastome IV DNA are enlarged, an increase of 0.4 Md in the former fragment and a decrease of 0.2 Md in the latter is found for plastome V DNA. Such a change might be due to adjacent insertion/deletion or to the inversion of a short DNA segment, which contains the Pst I recognition site, and a small insertion.

Next in order on the map of plastome IV DNA is the 1.5 Md Kpn I fragment which is smaller by 0.1 Md in plastome I and II DNA (Tables 1 and 3). Then follows a long stretch of DNA which contains only one

Table 4. Summary of overall differences in Md[a] in the DNA of plastomes I, II, III and V relative to plastome IV DNA.

Plastome	I	II	III	V
Total gains	1.5	1.0	2.1	1.8
Total losses	0.2	0.2	0.7	0.2
Total change	+1.3	+0.8	+1.4	+1.6

[a] These values are based on the data in Tables 1, 2 and 3 and Fig. 4.

fragment showing measurable variation, namely the 1.8 MD Pst I + Sal I secondary fragment with a size less than 0.1 Md smaller in the DNA from plastomes I, II and III (Table 2).

The 9.5 Md primary Sal I fragment of plastome IV DNA had an increased size in the other plastomes as is most apparent for the 5.7 Md primary Kpn I fragment which overlaps. Also, the adjacent 5.4 Md primary Sal I fragment of plastome IV DNA was increased in three plastome DNAs (II, III and V). Analysis of the secondary fragments in this region reveals the 1.8 Md Kpn I + Sal I secondary fragment to be constant, whereas both the 3.8 Md Kpn I + Sal I secondary fragment (overlapping the 9.5 Md Sal I and 5.7 Md Kpn I primary fragments) and the 5.7 Md Kpn I + Sal I secondary fragment (overlapping the 9.5 Md Sal I and 29 Md Kpn I primary fragments) are larger in DNA of the plastomes I, II, III and V than in plastome IV DNA (Tables 1, 2). The considerable size changes in the 5.7 Md Kpn I fragment are obvious in Figs. 1, 2.

As shown in Table 2, the 7.8 Md Pst I + Sal I secondary fragment (plastome IV DNA) is slightly larger in the DNA of plastomes I, III and V. Due to the large size of this secondary fragment, it is the least accurately determined size difference. Secondary fragments, in other double digests, which overlap here (Fig. 3, Tables 1, 3) do show some variation, but are too large for accurate measurement.

The remaining fragments (Fig. 4) belong to the inverted repeat and therefore reveal the same differences as have been discussed above.

The sizes of the smallest fragments (i.e. the 0.6 Md and 0.55 Md Kpn I and the 0.45 Md Sal I fragments) from DNA of plastomes I, II, IV and V were compared by electrophoresis on a 2% agarose slab gel and found not to differ in size (experiment not shown). In Table 4, the overall differences between DNA from the five *Euoenothera* plastomes are summarized.

Comparison Using Endonucleases Eco RI and Bam HI

For further characterization of the differences of the DNA from the five plastomes, digestions using Eco RI

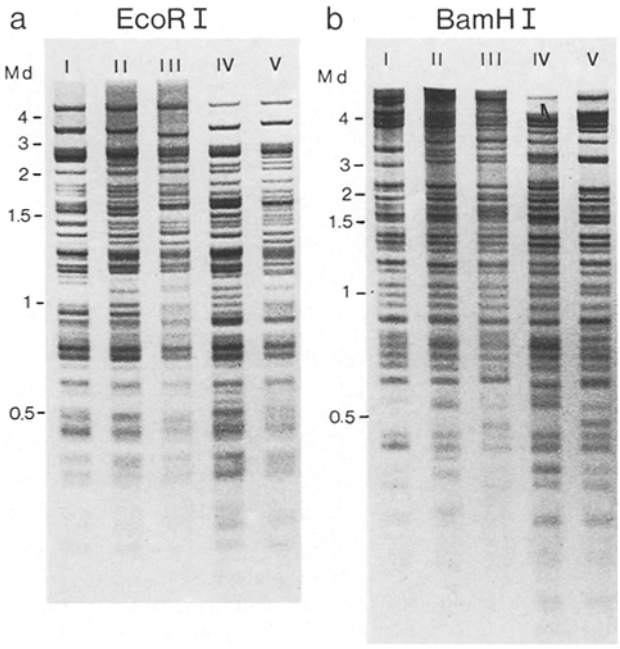

Fig. 5 a and b. Digestion of DNA from the five *Euoenothera* plastomes with restriction endonuclease Eco RI (**a**) and Bam HI (**b**). Electrophoresis was on 1.4% SeaKem agarose slab gels. Molecular weights are indicated in Md. The tracks contain DNA from plastid types I to V from right to left

and Bam HI (Fig. 5) were compared. These show that the DNAs are similar to a great extent, but many differences in the mobilities of individual fragments are observed. The Eco RI cleavage pattern (Fig. 5 a) can be resolved into about 52 fragments down to 0.6 Md in size, with resolution below this size being poor on the agarose gel used. These fragments account for over 80% of the total molecule, suggesting that there are more than 30 fragments smaller than 0.6 Md in size. Except for the rDNA region (see next section) the many differences observed have not yet been located on specific fragments.

The Bam HI cleavage patterns (Fig. 5 b) show that there are about 65 fragments down to 0.3 Md in size. Because these account for approximately 95% of the total molecule for each plastome, it is unlikely that there are many fragments smaller than 0.3 Md in size. Many fragments show size differences among the five DNA types. Again it was possible to analyse specific fragments using hybridization of labelled rRNAs (see below).

Mapping of Differences in Fragments Containing Ribosomal RNA Genes

As shown in Fig. 4, DNA from plastome V contains a small insertion and that of plastome III a deletion in the 5.4 Md Pst I + Kpn I secondary fragment which in plastome IV DNA includes part of the rDNA unit (Gordon et al. 1981). Hybridization of labelled chloroplast rRNAs from spinach was used to confirm the identity of the fragments containing the ribosomal RNA genes in the DNA from the other plastomes, and to locate changes within this DNA region.

In Figure 6 a, 16 S rRNA has been hybridized to Kpn I + Sal I double digestions of the five plastomes (i). This ribosomal RNA hybridizes, as expected, to the 6.7 Md fragment in plastome IV, II and I DNA, to a slightly smaller fragment in plastome III DNA and to a slightly larger fragment in plastome V DNA (Table 1; Fig. 2). Hybridization of 16 S rRNA to the Eco RI fragments of the five plastomes [Fig. 6 a (ii)] reveals complementarity with the 2.5 Md fragments in DNA from all plastomes and the 3.4 Md fragment of DNA from the plastomes IV, II and I, a fragment with slightly smaller size of plastome III DNA and a fragment of slightly larger size of plastome V DNA. The location of these fragments in plastome IV DNA has been previously identified (Gordon et al. 1981) and is given in Fig. 7.

The result of hybridization of the 23S rRNA to the Eco RI digests of the DNA from the five plastomes is presented in Fig. 6 b (i). This rRNA hybridizes to the 0.45 Md fragments from all plastomes. Hybridization is also observed to a 3.4 Md band of DNA from the plastomes IV, II and I, to the corresponding slightly larger fragment of plastome V DNA and the slightly smaller band in plastome III DNA (cf. Fig. 5). The weaker bands present in some tracks may have resulted from incomplete transfer of DNA fragments to the filter. The original autoradiograph shows clear bands, however, and it is, for example, the same 3.4 Md double band as can be observed for plastome III DNA in Fig. 6 a (ii).

Figure 7 shows a map of the rRNA genes, within the inverted repeat region of plastome IV DNA. It is taken from the preceeding paper (Gordon et al. 1981). The two DNA fragments containing rRNA genes which have been found to differ in size in some plastome DNAs, i.e. the 5.4 Md Kpn I + Pst I secondary fragment (plastome IV) and the 3.4 Md Eco RI fragment (plastome IV), overlap. The length of the overlapping segment, the DNA stretch common to both fragments and bounded by an Eco RI and a Kpn I cleavage site, is 2.4 Md in plastome IV DNA. This overlap is represented by a double-headed arrow in Fig. 7. In plastome III DNA the same differences (losses of 0.3 Md) have been measured both in the Kpn I/Pst I and Eco RI fragments. It is therefore concluded that this change has occurred in the overlapping segment; it is 2.1 Md long in plastome III DNA, as shown in Fig. 7. Similarly, a 0.1 Md increase is concluded to have occurred in

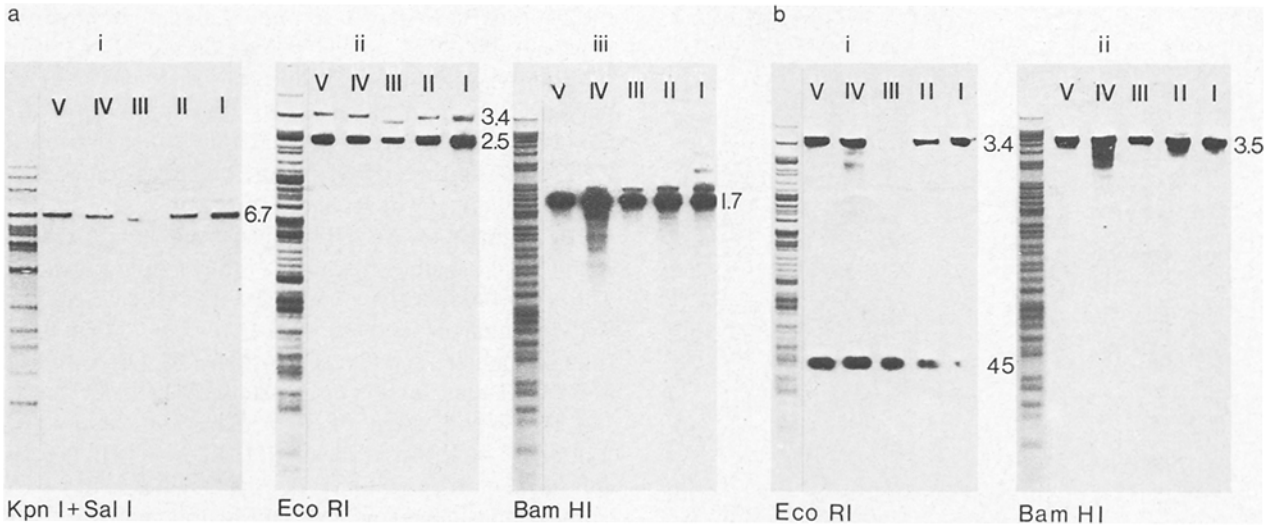

Fig. 6 a and b. Hybridization of spinach chloroplast 16S rRNA (**a**) and 23S rRNA (**b**) to DNA from the five *Euoenothera* plastomes. One to 4 μg of DNA were used per gel slot. Electrophoresis was on a 0.6% agarose gel for **a**, (i); and on 1.4% agarose gels for **a**, (ii), (iii) and **b**, (i), (ii). Only the cleavage pattern of DNA from plastome IV is shown (left), together with the autoradiograph of hybridizations of rRNA to DNA from the five plastomes (right). The individual tracks in the autoradiographs contain, from right to left, DNA from plastid types I to V, as indicated. The molecular weights in Md of the DNA fragments from plastome IV to which hybridization is observed are given to the right of the autoradiographs. Note that faint hybridization bands in **a**, (iii) are due to partial digestion products which indicate the presence of several Bam HI cleavage sites close to those at one or both ends of the 1.7 Md fragment. **a**: (i) double digestion with Kpn I + Sal I; (ii) digestion with Eco RI; (iii) digestion with Bam HI. **b**: (i) digestion with Eco RI; (ii) digestion with Bam HI

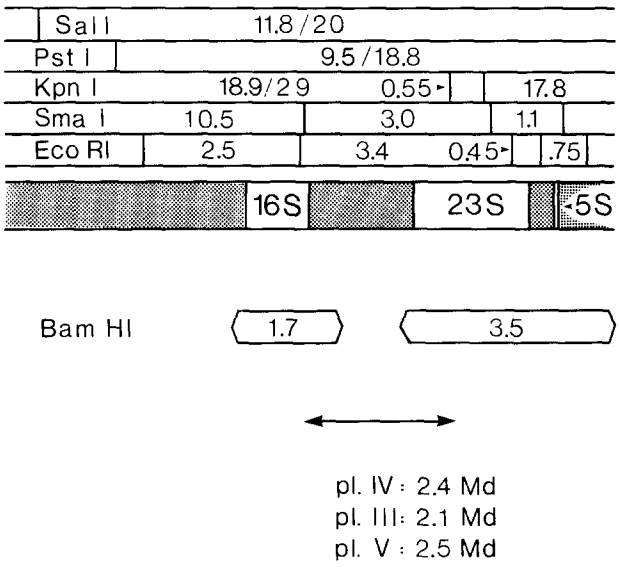

Fig. 7. Detail map of the restriction endonuclease cleavage sites within the part of the inverted repeat region which contains the rRNA genes (from Gordon et al. 1981, cf. Figs. 3, 4). The DNA segment, covering the spacer between the 16S and 23S rRNA genes, and bounded by an Eco RI and Kpn I cleavage site, is represented by the double-headed arrow. This segment represents the minimum overlap for the DNA fragments, from plastomes III and V which have been found to differ in size relative to that from plastome IV DNA (cf. Fig. 6). The sizes of this segment are given in Md for DNA from these three plastomes. In DNA from plastomes I and II, no size difference was noted compared to plastome IV DNA

the size of the corresponding segment in plastome V DNA which for this DNA is thus 2.5 Md long (Fig. 7). This is based on the observed small increases in the size of the 5.4 Md Kpn I + Pst I fragment (cf. Fig. 2) and of the 3.4 Md Eco RI fragments (cf. Fig. 5). No changes have been found in these fragments in DNA from plastomes II or I, relative to plastome IV.

The 2.4 Md overlapping segment in DNA from plastomes I, II and IV includes about one-third of the 23S rRNA gene, the spacer between the 16S and 23S rRNA genes and a minor part of the 16S rRNA genes.

As shown in Fig. 7, each of the two large rRNA genes is located within a specific Bam HI fragment. Hybridization of the 16S rRNA to the Bam HI digests [Fig. 6a (iii)] shows that a fragment of 1.7 Md from all five plastomes hybridizes with this rRNA. Likewise, a fragment of 3.5 Md from all five plastomes hybridizes with the 23S rRNA [Fig. 6b (ii)]. No loss of 0.3 Md in size is apparent for plastome III DNA, nor can a change be discerned in DNA from plastome V. Therefore, both changes have almost certainly occurred in the spacer between the 16S and 23S rRNA genes, i.e. in the Bam HI fragment(s) which must separate the 1.7 Md and 3.5 Md fragments. This implies both that the hybridization data for all plastomes are consistent with the map for plastome IV DNA and that the observed size differences in this fragment in DNA from plastome III and V are caused by the deletion/insertion of nucleotide sequences, respectively, since no changes

have occurred in the sizes of the 2.5 and 0.45 Md Eco RI fragments flanking the 3.4 Md fragment in plastome IV.

Other Differences Within the Inverted Repeat

The increases of 0.2 Md (plastome I DNA) or 0.4 Md (DNA from plastome III and V) observed in the Sal I + Pst I secondary fragments 6.8 and 7.8 Md respectively, overlapping the ends of the inverted repeat towards the larger single-copy region (Fig. 4), could be located within the inverted repeat or outside. To verify this, cRNA was made from the 6.6 Md Sal I fragment of spinach plastid DNA, which is located within the repeat just at the border with the large single-copy region (Crouse et al. 1978; Herrmann et al. 1980b; Gordon et al. 1981). Hybridization of this cRNA to DNA from plastomes I, III and IV, digested with Sal I + Pst I, showed identity of these secondary fragments and confirmed the size changes approximately. Hybridization of this cRNA to Sma I/Pst I double digests (cf. Figs. 3, 4 in Gordon et al. 1981) and Eco RI digests of DNA from plastomes I to IV, suggests that fragments located entirely within the inverted repeat region, e.g. the plastome IV DNA 7.5 Md Sma I + Pst I fragment (Fig. 3) differ significantly in size and can account for much of the total changes observed. It is important to note that the changes among the five plastome DNAs both in the rDNA and elsewhere within the inverted duplication are consistently found in both copies.

Discussion

In the foregoing paper, the procedure adopted for mapping *Oenothera* plastid DNA has allowed the localization of 54 restriction endonuclease cleavage sites on the complete 100 Md molecule of plastome IV DNA (Gordon et al. 1981). Comparison of this DNA with those from the other four basic plastomes of *Euoenothera* (Stubbe 1960, 1964) is presented in this paper. The DNAs from each of these five plastomes are distinguishable by small differences in the mobilities of individual restriction fragments. The study was performed using the restriction endonucleases Sal I, Pst I and Kpn I, for which a total of 36 cleavage sites have been located and the serial order of fragments established.

Location and Nature of the Differences in DNA from the Five Plastomes

In the present analysis of DNA from the five closely related plastomes of *Euoenothera,* it is concluded that the relative positions of restriction sites in their physical maps are conserved. The similar cleavage patterns for all five plastomes provide immediate evidence for the order of cleavage sites and, thus, the segmental organization of the molecule, being conserved. Hybridization of spinach chloroplast rRNAs and a specific cRNA confirm the identity of fragments of equal or similar sizes as does the redigestion of selected restriction fragments from various plastomes.

There are many small differences in the sizes of specific fragments generated from the five plastomes with the restriction endonucleases Sal I, Pst I and Kpn I. These differences in size have been mapped at eleven locations around the circular molecule. The patterns produced with the restriction endonucleases Eco RI and Bam HI have revealed additional small differences among the DNA fragments from these plastomes. These latter differences have not yet been mapped, except for those occurring in the rDNA unit.

Many of the differences found in this study are located in the large single-copy region of the molecule. Large segments of this region, however, show no changes in any of the plastomes. Some differences have also been located among fragments mapping in the inverted repeat region and one location of differences is in the small single-copy region. Finer analysis of most of this region is not, however, possible, due to the lack of sufficient restriction sites with the selected enzymes. The observed differences are concluded to be largely insertions (of 0.1–0.8 Md) with respect to DNA from plastome IV, which is, according to genetic data, presumed to be the most primitive of these plastomes (Stubbe 1964). Some deletions with respect to plastome IV DNA have also been observed. No cleavage sites of these enzymes have been gained or lost. The limited number of 36 studied does not, however, allow generalization on the conservation of base sequences, as described, for example, by Upholt (1977).

Redigestion of the plastome I DNA fragments equivalent to the 5.7 Md Kpn I and 9.5 Md Sal I primary fragments from plastome IV DNA with Hind III, an endonuclease which recognizes more than 30 cleavage sites on the total molecule, suggests that the large insertion (0.6 Md for plastome I) found in the overlapping segment of these primary fragments consists of several smaller ones (unpublished observations).

A specific example of insertion or deletion is observed in fragments containing the rDNA unit, and these rearrangements most probably are located in the spacer between the 16S and 23S rRNA genes. The rDNA spacer is known to be transcribed in spinach (Bohnert et al. 1977) and to contain a gene for an isoleucine transfer RNA (Driesel et al. 1979; Bohnert et al. 1979). Although the effect of changes in this spacer is unknown, such changes may represent a common polymorphism in *Oenothera* plastid DNA (cf. Gordon et al. 1980; Herrmann et al. 1980c).

There is probably no strong evolutionary pressure to conserve spacer DNA sequences (Federoff 1979).

A significant observation in regard to possible recombination events in plastid DNA is that rearrangements (e.g. deletions) of DNA within the inverted repeat occur in both copies. This strongly supports the suggestion that a molecular mechanism exists to ensure sequence homology for both copies of the repeat (Bedbrook et al. 1977, Herrmann et al. 1980c). No evidence has been found for heterogeneity of the chloroplast DNA within specific plants, e.g. as a result of the inversion of DNA stretches. This might result in several isomeric forms, as have been found for the herpes simplex virus (Roizman 1979) and is suggested to occur in plant mitochondrial DNA (Levings and Pring 1979). Nonetheless, the 0.2 Md deletion from the 3.9 Md Kpn I + Pst I secondary fragment in plastome V DNA, and part of the adjacent 0.4 Md deletion (see Fig. 4) may be due to the inversion of a short DNA segment, including a Pst I recognition site. If such is the case, this sort of change must occur at a low rate, being segregated thereafter into all plastid DNA molecules within a population.

The large rearrangements present within a short segment (the 3.8 Md Kpn I + Sal I secondary fragment from the 9.5 Md Sal I primary fragment of plastome IV DNA) raise the question of whether it has a base composition or sequence organization which is especially susceptible to changes. Comparison of mitochondrial DNA from various strains of yeast (Prunell et al. 1977; Sanders and Borst 1977; Sanders et al. 1977) or *Drosophila* (Shah and Langley 1979) has shown that major insertions are located in A + T-rich stretches. Physico-chemical analysis of spinach plastid DNA has shown considerable intramolecular base-compositional heterogeneity (Crouse et al. 1978; Schmitt et al. 1981), but, especially as far as A + T-rich stretches are concerned, not to the extent found for yeast mitochondrial DNA (Borst et al. 1977; Prunell et al. 1977). The large single-copy region is the location of most of the extra approximately 5 Md size of *Oenothera* plastid DNAs compared to spinach plastid DNA. This extra DNA has not appreciably altered the average base composition of *Oenothera* plastid DNA (Herrmann et al. 1975) compared to that of spinach and it thus appears unlikely to be extremely A + T-rich, although this remains to be examined.

The Plastid DNA Differences and Oenothera Plastid Genetics

The genetic material of the five *Euoenothera* plastomes has been found to differ at the molecular level. The plastomes are known to be inherited independently of the nuclear genotype, subject to the constraint that genome and plastome be physiologically compatible for the production of viable progeny. The interchange of plastomes and genomes can result in plants of varying viability. Although the DNA physical maps of the five plastomes differ slightly, it is not known if and how these differences establish the genetic identity of a plastome. Since the genetic function of about 90% of the plastid DNA molecule is not yet elucidated (Bedbrook and Kolodner 1979; Herrmann and Possingham 1980), most of the differences found in the present study cannot be related to specific genes or their products.

The one exception is that the spacer in the rDNA unit differs among the five plastomes. Furthermore it is of interest that the central part of the large single-copy region appears virtually free of observable DNA differences among the five plastomes. In spinach chloroplast DNA, the corresponding stretch has been shown to contain many tRNA genes arranged in two large "clusters" (Driesel et al. 1979), and the structural genes for the large subunit of the ribulose-bisphosphate carboxylase as well as of the beta and epsilon subunits of the thylakoid-located ATP synthetase (Westhoff et al. 1981).

Polymorphism at the molecular level has, in fact, been found to occur within a single genetically identified plastome, when comparison is made using plastids derived from different species. Such polymorphisms are found even within genes, as shown by peptide mapping of the large subunit of ribulose-bisphosphate carboxylase (EC 4.1.1.39), a major plastome product (Coen et al. 1977) and genetic marker (v. Wettstein et al. 1978; Wildman 1979) from the five *Euoenothera* plastomes (Holder 1978). Furthermore, gel electrophoretic analysis of thylakoid membrane proteins from different *Euoenothera* species with the same plastome (but in combination with different nuclear genotypes) has revealed polymorphisms (Herrmann et al. 1980c). These observations suggest that genome-plastome incompatibility may arise through alterations in the structure of plastid DNA-encoded subunits of certain proteins which also contain nucleus-encoded subunits. A possible example of a plastid DNA polymorphism, which is unlikely to have a genetic effect, is given by the plastid DNA of the mutant sigma in plastome I (Gordon et al. 1980).

On the basis of genome-plastome incompatibility, (as determined by studies of hybrid variegation) and the multiplication rates of plastids, Stubbe (1964) has deduced a phylogeny for the five plastomes. Comparison of this pedigree to the results of the present study shows that the three plastomes, whose DNAs show the largest differences (Table 4) to plastome IV DNA (which is regarded as the oldest of the presently existing plastomes) are those which are regarded by

Stubbe (1964) as being the newest, i.e. plastomes I, III and V. In addition, the DNA from plastome II is between that from plastomes I and IV in size, as well as sharing several of the observed differences (to plastome IV DNA) with DNA from plastome I (Fig. 4). This suggests that it might be, as proposed by Stubbe (1964), an intermediary between plastome IV and I. DNA from plastome II is clearly distinguishable from plastome III DNA, supporting the distinction made between these plastomes, in contrast to the view, based on genetic studies, of Cleland (1962).

Plastome V is incompatible with the homozygous A genome (Stubbe 1959, 1960, 1964) and also with the genomes (AB and BB) from which plastomes II and III, respectively, were derived (Stubbe 1960) but is specifically adapted to the homozygous C genotype (and, less successfully, the BC genotype). This phenomenon raises the question of whether one of the observed differences between this plastid DNA and that from other plastomes is responsible for such a specific adaptation of this plastome. The 0.2 Md deletion from the 3.9 Md (plastome IV) Kpn I + Pst I secondary fragment is the only unique observed change and may be responsible for this, although there is no evidence to exclude any other changes.

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