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Chloroplast DNA differences between two species of *Oenothera* subsection *Munzia:* location in the chloroplast genome and relevance to possible interactions between nuclear and plastid genomes*

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Summary. The chloroplast DNAs (cpDNAs) of *Oenothera berteriana* and *Oe. odorata* (subsection *Munzia*) were examined by restriction endonuclease analysis with Sal I, Pvu II, Kpn I, Pst I, Hind III, and Bam HI. The fragment patterns show that these cpDNAs have all 133 restriction sites in common as well as a lot of individual bands. Nevertheless the cpDNAs of the two species can be distinguished by distinct differences in size between a small number of fragments. The 42 cleavage sites produced by Sal I, Pvu II and Kpn I were mapped on the circular cpDNAs. This was achieved by an approach which combined experimental and mathematical procedures. The overall serial order of the fragments was found to be the same for both cpDNAs. The size differences of individual fragments in the Sal I, Pvu II and Kpn I patterns between *Oe. berteriana* and *Oe. odorata* cpDNA are located within five regions scattered along the plastid chromosome. Two of these regions have been localized in the larger and one in the smaller of the two single-copy parts of the cpDNA molecule. The remaining two overlap the borders between the large single-copy and each of the duplicated parts of the molecule. The positions of distinct restriction sites are altered among the two *Oenothera* plastome DNAs by 0.02–0.4 MDa (30–600 base pairs). These alterations probably result from insertions/deletions.

Key words: *Oenothera* subsection *Munzia* – Chloroplast DNA – Cytoplasmic inheritance – Comparative restriction site mapping – Insertion and deletions

Abbreviations: cpDNA = chloroplast, plastid DNA; *Oe.* = *Oenothera;* MDa = Megadalton; rRNA, rDNA = ribosomal RNA, DNA

* Dedicated to Professor Berthold Schwemmle, Tübingen, on the occasion of his 60th birthday

Introduction

The interaction between nuclear genes and the genetic systems of plastids and mitochondria, respectively, is not only required for the biogenesis of the organelles but also has a fundamental influence on the growth and development of plants.

Several unique insights into the interactions of organellar and nuclear genetic systems are provided by interspecific reciprocal crossing in the genus *Oenothera* subsection *Munzia.* Starting with the complex-heterozygous species *Oenothera berteriana* and *Oe. odorata,* eleven different combinations of nuclear, plastid and mitochondrial genomes have been obtained (Schwemmle 1938). By comparing hybrids having different cytoplasms though identical nuclear chromosome-complexes it was possible to demonstrate that certain phenotypic effects are associated with specific organellar genomes (Schwemmle 1962; Hachtel 1972). In particular, the plastid genome is involved in the genetic control of chlorophyll biosynthesis (Hachtel 1981); lethality of embryos in some of the hybrids is caused by an incompatibility between specific plastid and nuclear genomes (Kistner 1955); and the character of leaf dentation is modified by different plastomes (Schwemmle 1941).

To understand more fully the part played in this interaction by the plastid genome, analyses using restriction endonucleases have been performed. The plastid DNAs from *Oe. berteriana* and *Oe. odorata* can be distinguished through their different fragment patterns generated by restriction enzymes. Moreover, the identity of the chloroplast genomes could be verified in hybrids with different nuclear genomes (Hachtel 1980; Alt et al. 1982). The present study was designed, first, to map the relative positions of the alterations in the fragment patterns on the plastid chromosomes of the two *Munzia* species and, second, to gain information on the type of such alterations (e.g. rearrangements, insertions, deletions, point mutations).

Materials and methods

Plant material

Oenothera berteriana and *Oe. odorata* (= *Oe. villaricae* and *Oe. picensis* subsp. *picensis,* respectively, of the South American subsection *Munzia* according to Dietrich 1977) are complex-heterozygous species which have been bred by self-pollination in the Botanical Garden of Erlangen (FRG) since 1925. Formation of homozygous plants is prevented by lethal factors. Plants were grown from seed in soil in a greenhouse for 8–12 weeks with supplementary light during the winter and then starved for two days in darkness prior to the harvest of the leaves.

Isolation of chloroplast DNA

Chloroplasts were isolated from leaves in a Sorvall omnimixer in 10 volumes of a medium containing 0.05 M Tris (Sigma), 0.33 M sorbitol, 0.02 M NaCl, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 8 mM EDTA, 5 mM mercaptoethanol, 1.5 g l^{-1} polyvinylpyrrolidone (average molecular weight 15,000), 2 mM Na ascorbate, adjusted to pH 7.6 at 4 °C; EDTA was raised up to 12 mM when needed to lower the viscosity of the homogenate. The slurry was strained through 100 µm and 20 µm Nylon meshes (Schweizer Seidengaze) and the chloroplasts sedimented at 2,500 g for 2 min. The plastids were resuspended in 0.05 M Tris-HCl, 0.3 M sucrose, 1–2 mM EDTA depending on the viscosity, pH 7.6, repelleted and further purified by centrifugation in a discontinuous sucrose gradient at 15,000 g in a Sorvall HB4 rotor for 75 min. The chloroplasts recovered at the interface of the 25% and 65% sucrose layers were lysed by 2.5% N-lauroylsarcosine (Sigma) in the presence of 37.5 mM EDTA. A DNAase treatment of the chloroplasts before lysis was omitted because it was detrimental to the cpDNA. DNA was purified from the lysate by ultracentrifugation at 20,000 rpm for 18 h in a Beckman SW 27.1 rotor employing the CsCl-cushion technique developed by Kolodner and Tewari (1975). The fractions of the cushion containing DNA were determined by a spotting technique and the DNA was recovered by ethanol precipitation as described by Herrmann (1982).

Restriction endonuclease analysis

Restriction endonucleases Sal I, Pvu II, Kpn I, Pst I, Sma I, Xba I, Xho I, Bam HI, and Hind III were purchased from Boehringer Mannheim. To achieve satisfactory restriction the incubation conditions as specified by the supplier were modified with regard to the concentrations of $MgCl_2$, Tris, bovine serum albumin, and Triton-X-100 in the storing and incubation buffers. Restriction fragments were separated by electrophoresis on horizontal agarose slab gels using a H4 submarine cell (Bethesda Research Laboratories). The concentrations varied from 0.3 to 1.75% for SeaKem-ME agarose and from 2 to 4% for NuSieve agarose (Marine Colloids). DNA fragment sizes were determined with the aid of a computer program (Duggleby et al. 1981) by coelectrophoresis of markers consisting of Lambda DNA digested with Eco RI, Hind III, Kpn I and Xho I. Fragments smaller than 0.37 MDa might have escaped from detection. Molecular sizes of the larger fragments were computed as the sum of secondary fragments obtained upon redigestion. Individual fragments were recovered from the gels by electrophoretic elution.

Cleavage site mapping was performed primarily by digestion of the cpDNA with Sal I, Pvu II and Kpn I which were applied either individually (single digestions) or in combination (double digestions). Because these endonucleases are different with regard to their NaCl requirements the means of the optimum concentrations were employed in the double digestions; the losses in activity were compensated by increasing the enzyme concentrations by 20–30%. From the experimental data obtained by single and double digestions, physical maps were constructed with the aid of a computer program developed by Durand and Bregegere (1984). This program originally was designed for mapping linear DNA but was easily adjusted to map circular DNA. To achieve this the data set derived from the circular cpDNA was reversibly converted into the data set of a linear molecule. Additional information was obtained by recovery of individual primary fragments and subsequent digestion with a second restriction endonuclease in a reciprocal manner. The computer program allows utilization of the attained relationships between some of the primary and secondary fragments in such a way that the number of possible solutions was drastically reduced.

Results

Cleavage of Oenothera cpDNA with restriction endonucleases

Restriction analyses using Eco RI (Hachtel 1980) and Bam HI/Bst I (Alt et al. 1982) have revealed that unique chloroplast DNAs are associated with the plastids of *Oenothera berteriana* and *Oe. odorata.* These enzymes, however, were not well suited to start mapping of the *Oenothera* cpDNAs because up to 39 Eco RI fragments and 46 Bam HI fragments could be resolved. In order to find endonucleases which recognize a lower number of cleavage sites and still allow detection of differences between the fragments patterns, cpDNA of both *Oe. berteriana* and *Oe. odorata* was digested with each of the following enzymes: Sal I, Pvu II, Kpn I, Pst I, and Hind III. For each cpDNA, 13 Sal I fragments, 16 Pvu II fragments and 13 Kpn I fragments were resolvable in agarose gels upon electrophoresis. The molecular sizes determined for these fragments are given in Fig. 2. Pst I generates 12 and Hind III produces 33 cpDNA fragments. Also performed were double digestions using Sal I + Pvu II, Sal I + Kpn I, Sal I + Pst I, and Pst I + Kpn I because the likelihood of observing differences among fragments increases with the greater frequency of restriction sites. Double restrictions with Sal I and Pvu II generated 28 fragments from both *Oe. berteriana* and *Oe. odorata* cpDNA. The 29th fragment, which is expected from single digestions, was observed only indirectly because a Sal I and a Pvu II restriction site are very close together; the distance is about 0.05 MDa (Fig. 2). Similarly, a Sal I and a Kpn I cleavage site were found to be only 0.08 MDa apart. In each of the other double digestions two expected fragments were not observed because of their small sizes (less than 0.05 MDa).

The DNA fragment patterns obtained show a striking similarity for the cpDNA of *Oe. berteriana* and

Table 1. Plastome sizes of *Oenothera berteriana* and *Oe. odorata* (MDa)

	Total of fragments	
	Primary[a]	Secondary[b]
Oe. berteriana	100.99	100.75
Oe. odorata	102.23	101.97

[a] Sal I, Pvu II, Kpn I, Pst I, Bam HI, and Hind III single digestions
[b] Sal I + Pvu II, Sal I + Kpn I, Sal I + Pst I, and Pst I + Kpn I double digestions

Oe. odorata and have many bands in common. However, distinct differences in the electrophoretic mobility between individual fragments are evident. The differences between the molecular sizes of such corresponding fragments range from 0.02 MDa to 0.4 MDa. Depending on the enzyme or enzyme combination used, 4 to 10 of the fragments obtained from *Oe. odorata* cpDNA were of a higher molecular size, and with all enzymes, one was of lower molecular size, than the corresponding fragments from *Oe. berteriana* cpDNA. The overall molecular size difference between the cpDNAs from *Oe. odorata* and *Oe. berteriana* is 1.22–1.24 MDa as indicated by the total of the fragments (Table 1).

Construction of a Sal I + Pvu II + Kpn I map of Oe. berteriana cpDNA

The two endonucleases, Sal I and Pvu II, each recognize a low number of sites on the complete molecule. Analysis of their double digestions showed that the secondary fragments can be reasonably resolved, enabling mapping of these restriction sites. Mapping proceeded in several steps. First, individual primary fragments obtained with one endonuclease were treated with the second enzyme, and vice versa. Part of this analysis is shown in Fig. 1. The resulting fragments were compared with and correlated to the fragments in the double and single digestion patterns of total cpDNA according to the principles detailed by Gordon et al. (1981). Now it was possible to determine those primary Sal I and Pvu II fragments which have a Sal I + Pvu II secondary fragment in common. The overlapping fragments determined in this way account for 11 of the 13 Sal I, and for all of the Pvu II, primary fragments. Next, using a published computer program (Durand and Bregegere 1984) the map of the Sal I and Pvu II cleavage sites was constructed on the basis of the data obtained from the Sal I and Pvu II single digestions, the Sal I + Pvu II double digestions and the reciprocal digestions of individual fragments (Fig. 2).

The aim of mapping the restriction sites recognized by Kpn I was to check the map obtained from Sal I and Pvu II and to achieve finer mapping of stretches of DNA where few cleavage sites for Sal I and Pvu II are located. Mapping of Kpn I cleavage sites was accomplished on the basis of the data from Sal I + Kpn I double digestions. An almost unambiguous correlation of the obtained secondary fragments to the Sal I primary fragments was possible. The extended map is also shown in Fig. 2.

To check the relative positions of the cleavage sites of Sal I, Pvu II and Kpn I on the map, a triple digestion with these three enzymes was performed. In the molecular size range above 3.0 MDa the tertiary fragments could be reasonably resolved and their sizes could be determined. These experimentally determined sizes agree exactly with the expected values derived from the map (not shown).

Occurrence of inverted repeats

Most of the two-molar fragments obtained with the three endonucleases Sal I, Pvu II and Kpn I are present in two clusters (Fig. 2). Within each cluster, these fragments are arranged in the same serial order: that is, 2.65 MDa, 2.0 MDa, 3.05 MDa and 1.53 MDa in the Sal I + Pvu II map and 0.5 MDa, 6.4 MDa and 1.53 MDa in the Sal I + Kpn I map. The identical cleavage maps within the clusters suggest that these DNA segments contain identical nucleotide sequences. Their relative locations on the circular DNA molecule show that they are present in inverted orientation. As indicated by the asymmetry of the adjacent cleavage sites, the copies of the duplication must be separated by two regions of single-copy DNA differing in size. A minimum length of 9.23 MDa for the inverted repeat can be calculated from the above data.

Physical mapping of differences between Oe. berteriana and Oe. odorata cpDNA

Independently, a Sal I + Pvu II + Kpn I restriction site map of *Oe. odorata* cpDNA was constructed in exactly the same manner as described above for *Oe. berteriana* cpDNA. It turned out that the serial order of restriction sites was identical to that determined for *Oe. berteriana* cpDNA. Redigestion of individual fragments of the *odorata* plastome corroborated this conclusion (data not shown). Moreover, those fragments of single and double digestions showing small differences between *Oe. berteriana* and *Oe. odorata* were in fact found to be localized at the same relative positions on the maps. The location of observed differences is narrowed down by finding the smallest secondary fragment which shows the difference. The overlapping secondary and, where possible, primary fragments from the other digestions are then checked for verification of the size difference.

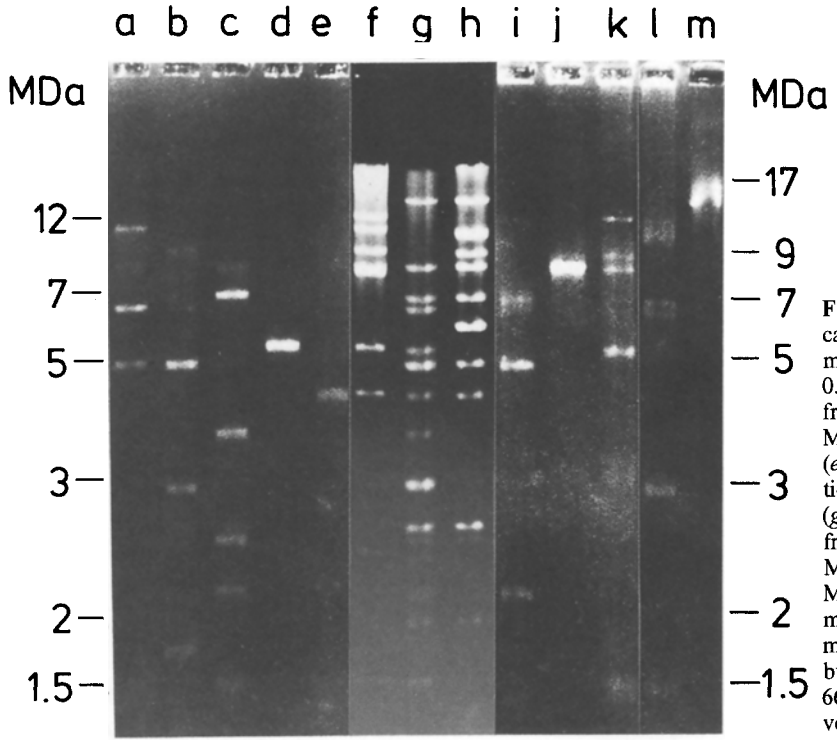

Fig. 1. Electrophoretic analysis of the reciprocal digestion of individual Sal I and Pvu II primary fragments of *Oe. berteriana* cpDNA on 0.5% agarose gels. *Tracks a–e:* Sal I primary fragments of 11.9 MDa (*a*), 9.8 MDa (*b*), 8.7 MDa + 8.6 MDa (*c*), 5.5 MDa (*d*) and 4.5 MDa (*e*) were digested by Pvu II; *tracks f–h:* digestion of total cpDNA by Sal I (*f*), Sal I + Pvu II (*g*) and Pvu II (*h*); *tracks i–m:* Pvu II primary fragments of 7.4 MDa (*i*), 8.7 MDa (*j*), 9.3 MDa (*k*), 11.5 MDa + 11.8 MDa (*l*) and 16.7 MDa (*m*) were digested by Sal I. Molecular mass scales (MDa) are provided in the lateral margins. All samples were run on the same gel, but four different exposure times (Polaroid film 665) were employed in order to visualize even very faint bands

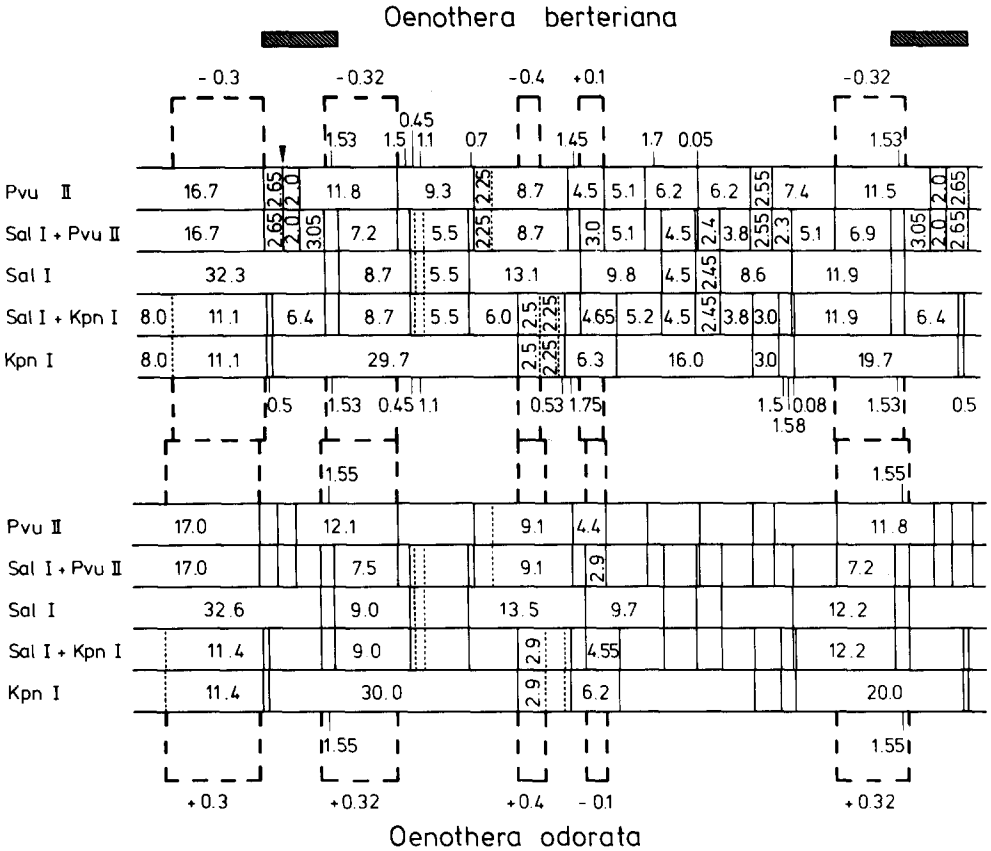

Fig. 2. Comparative maps of restriction endonuclease cleavage sites of chloroplast DNA from *Oenothera berteriana* and *Oe. odorata*. The serial orders of the fragments produced upon single and double digestion with the endonucleases Sal I, Pvu II and Kpn I are shown. For *Oe. berteriana*, the size of each primary and secondary fragment is given in MDa. For *Oe. odorata*, only those fragments which differ in size from the corresponding one of *Oe. berteriana* are identified by their size. The *dashed brackets* mark the maximum extent of regions where size differences (given in MDa) between *Oe. berteriana* and *Oe. odorata* cpDNA-fragments have been localized. *Broken lines* indicate that the order of the two adjacent fragments is ambiguous. The *shaded bars* illustrate the location and minimum extent of the two copies of the inverted repeat region. The maps are presented in a linearized form by cutting the small single-copy region

Starting from the left in Fig. 2, the first Sal I + Kpn I secondary fragment of the *Oe. berteriana* plastome is an 11.1 MDa Kpn I fragment which is somewhat larger (11.4 MDa) in the cpDNA of *Oe. odorata*. The maps indicate that these fragments are cut by Pvu II. Indeed, secondary fragments of 10.2 MDa and 10.5 MDa, respectively, have been found in the Pvu II + Kpn I double digestions. It is concluded that these are the smallest fragments in this region showing the observed size difference. They cover more than half of the small single-copy region and probably include the border of one of the copies of the inverted repeat (see "Discussion"). The difference is also clearly apparent in the 16.7/17.0 MDa Pvu II primary fragments. The next fragments exhibiting differences are the 1.53 MDa Sal I and the adjacent 7.2 MDa Sal I + Pvu II fragments. These and the overlapping 11.8 MDa Pvu II and 8.7 MDa Sal I fragments are 0.3 MDa larger in the cpDNA of the *Oe. odorata* plastome. These fragments cover the other end of the inverted repeat region and part of the large single-copy region.

Differences in fragment size among the two plastome DNAs have also been observed in fragments which are located within the large single-copy region. The smallest fragments showing the differences are the 2.5/2.9 MDa Kpn I fragments and the 3.0/2.9 MDa Sal I + Pvu II fragments, respectively. Finally, the 11.5 MDa primary Pvu II fragment and the overlapping 11.9 MDa primary Sal I fragments had an increased size in the *Oe. odorata* cpDNA (11.8 and 12.2 MDa). These fragments cover the border between the large single-copy region and the second copy of the inverted repeat. Analysis of the secondary fragments in this region reveals both the 5.1 and the 3.05 MDa Sal I + Pvu II fragments to be constant, whereas the 6.9 MDa Sal I + Pvu II secondary fragment (overlapping the 11.5 MDa Pvu II and the 11.9 MDa Sal I primary fragments) is larger (7.2 MDa) in the *Oe. odorata* cpDNA. The 1.53 MDa Sal I primary fragment belongs to the inverted repeat and reveals the same small difference as its double in the other repeat region (1.55 MDa in the *Oe. odorata* cpDNA).

Discussion

A circular restriction endonuclease cleavage site map of the plastome DNAs of two *Munzia-Oenothera* species has been constructed. We employed a combination of experimental techniques and mathematical methods first proposed by Durand and Bregegere (1984). This greatly facilitated the mapping procedure as compared to already published procedures to map cpDNAs (e.g. Gordon et al. 1981) because it was not necessary to digest the complete set of individual primary fragments with a second enzyme. However, two problems emerged from applying the computer program developed by Durand and Bregegere (1984). First, the program originally was deviced to construct restriction maps of linear DNA molecules. Second, the program is written for use with a small computer, and unreasonably long computing time is needed if a restriction enzyme produces more than ten fragments. Therefore, additional information was required to reduce the combinatory possibilities which have to be checked by the program. This information was obtained from reciprocal digestions of a small number of individual fragments.

The attained physical maps show an organization resembling that found with other higher plant chloroplast DNAs (Crouse et al. 1985; Palmer 1985). It is divided into four regions: two copies of an inverted repeat region are separated by a large and a small single-copy region. An especially high degree of similarity is observed between the cpDNAs of the *Munzia* species and of *Euoenothera* plastome IV DNA with regard to the location of Sal I and Kpn I cleavage sites in the duplications. This is shown in Fig. 3 using the data published by Gordon et al. (1981). The homology extends from one of the repeats into the adjacent part of the large single-copy region. Obviously the serial order of cleavage sites is preserved and the fragment sizes are largely unaltered in these parts of the molecules. This tentatively allows us to localize approximately the borders of the repeats as well as the rRNA gene clusters on the *Munzia* plastomes. In addition, the comparison reveals that none of the detected fragment differences between the *Munzia* cpDNAs is located in the spacer between the genes for 16S and 23S rRNA. This is in contrast to the situation in the subsection *Euoenothera* where the plastomes III and V are affected by deletions/insertions in this spacer relative to plastome IV (Gordon et al. 1982).

Polymorphism of restriction fragments can arise by several processes, including point mutations, base modification, insertions or deletions, and inversions and transpositions, all of which have been demonstrated to occur in cpDNA. The principle mechanism that accounts for most, if not all, observed differences between the plastomes of *Oe. berteriana* and *Oe. odorata* seems to be small deletions/insertions. This is readily demonstrated if polymorphic fragments are flanked by invariant secondary fragments. For example, the 13.1 MDa and 13.5 MDa Sal I primary fragments of *Oe. berteriana* and *Oe. odorata* are cleaved by Kpn I in terminal fragments of 6.0 and 1.75 MDa and in internal fragments of 2.25 and 0.53 MDa in both instances plus a 2.5 MDa fragment in *Oe. berteriana* which is 0.4 MDa shorter than the corresponding 2.9 MDa fragment in *Oe. odorata*. This size difference therefore is not due to

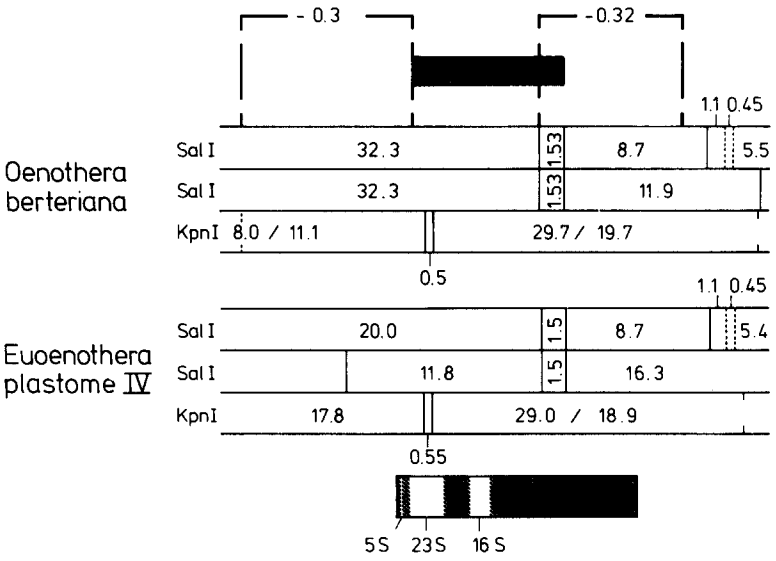

Fig. 3. Comparative maps of Sal I and Kpn I endonuclease restriction sites within the inverted repeat regions and adjacent parts of the single-copy regions for *Oenothera berteriana* cpDNA and for *Euoenothera* plastome IV DNA. *On the left,* the fragments overlapping the border between the repeat regions and the small single-copy region are shown. Fragments which partly or completely belong to the large single-copy region are shown the right hand side. The various possibilities in the order of these fragments are given. *Brackets* mark deletions in the cpDNA of *Oe. berteriana* relative to that of *Oe. odorata.* The *shaded bars* illustrate the location of the inverted repeat regions which contain the genes for 5S, 16S and 23S rRNA. The data for *Euoenothera* plastome IV are taken from Gordon et al. (1981)

a change in the cut frequency of either Sal I or Kpn I. The 0.1 MDa deletion in the 4.4 MDa Pvu II fragment and in the overlapping 9.7 MDa Sal I fragment of *Oe. odorata* compared to the 4.5 MDa Pvu II and 9.8 MDa Sal I fragment of *Oe. berteriana* is corroborated by an equivalent size change of the internal 2.9 versus 3.0 MDa Sal I + Pvu II secondary fragment. Similarly, one can exclude that gains or losses of closely spaced restriction sites cause the size alterations which have been localized in the small single-copy and in the border regions between the inverted repeats and the large single-copy. One large insertion/deletion, however, might consist of several smaller ones since an extensive sequence comparison between the transcribed rDNA spacers of tobacco and maize emphasizes that small length changes can occur in chloroplast evolution with a high frequency relative to larger changes (Takaiwa and Sugiura 1982).

It is striking that only length mutations and no restriction site mutations have been detcted in our study. A high incidence of length mutations appears to be a general aspect of chloroplast DNA evolution at least if related plastomes are considered. Comparisons of the five genetically distinct chloroplast genomes in *Euoenothera* revealed 24 length mutations without a single base substitution (Gordon et al. 1982). Only one base substitution and 14 length mutations were found among 14 species in *Triticum* and *Aegilops* (Bowman et al. 1983). Most of the cpDNA changes among 11 *Nicotiana* species obviously result from small insertions/deletions and, possibly, inversions (Salts et al. 1984). Lower ratios of chloroplast DNA length mutations to base substitutions were reported for *Pisum* (Palmer et al. 1985). There is, however, good reason to believe that the latter data considerably underestimate the frequency of length mutation events (Palmer et al. 1985).

The relative simple pattern of cpDNA divergence that is revealed by restriction endonuclease analysis is presumably a consequence of the close phylogenetic relationship between the two *Munzia* species. Such simplicity makes cpDNA analysis a useful tool for the identification and classification of extrachromosomal genomes. It is, however, impossible to attach any phenotypic significance to the cpDNA variation at this level. Even though the different cytoplasms chosen for analysis were already known to be associated with specific phenotypic effects in nuclear-cytoplasmic hybrid plants, and were then also found to contain distinctly different types of cpDNA, no correlation was observed between cpDNA organisation and the phenotypic effects of the cytoplasm. To understand the involvement of chloroplast genes in the production of the plant phenotype in these species, more detailed studies are needed. Appropriate analysis would reveal whether there are any specific DNA alterations which affect the transcription/translation of the chloroplast genome in vivo.

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