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THEORETICAL AND APPLIED GENETICS

Analysis of the Plastid DNA in an *Oenothera* Plastome Mutant Deficient in Ribulose Bisphosphate Carboxylase

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Summary. Plastid DNA of the light green *Oenothera* plastome mutant sigma, from plastome I, which is deficient in ribulose bisphosphate carboxylase, has been compared with wild-type chloroplast DNA from plastome I and the related plastome IV. For this, double digestions with the restriction endonucleases Sal I, Pst I and Kpn I were used. Chloroplast DNA from plastomes I and IV differs in the sizes of several fragments, with the changes being from under 0.1 to about 0.6 Md in size. In the cleavage patterns of the mutant DNA compared to the wild-type DNA from plastome I, the only differences observed are two possible deletions of less than 0.1 Md from a fragment known to partly cover the genes for the ribosomal RNAs and from a fragment located in the small single-copy region of the molecule. It is concluded that the ribulose bisphosphate carboxylase deficiency in this mutant is not caused by a major deletion in the plastid DNA.

Key words: Plastid ribosomal RNAs – Inverted repeat region – Plastome mutant – Restriction endonucleases

Introduction

The analysis of the higher plant chloroplast genome, the plastome, has so far been limited by the unavailability of mutations with single biochemical defects. The plastome mutants studied in *Pelargonium* and barley (Hagemann and Börner 1978) have multiple biochemical defects. These white mutants lack plastid ribosomes and as a consequence thereof many plastid proteins, but also the levels of nuclear-coded cytosolically synthesized chloroplast enzymes are strongly reduced (Bradbeer and Börner 1978).

Species differences in the nucleotide sequence of the chloroplast gene coding for the large subunit of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) have been demonstrated by amino acid sequence determination (v. Wettstein et al. 1978; Poulsen et al. 1979). Likewise chymotryptic peptide mapping of this protein from different *Oenothera* plastomes indicates the occurrence of different alleles in this gene (Holder 1978).

A total of over 50 plastome mutants have been identified in the subgenus *Euoenothera* on the basis of pigment deficiencies (Kutzelnigg and Stubbe 1974; Kutzelnigg et al. 1975). A search among these has revealed the light-green lethal mutant sigma in plastome I to lack ribulose bisphosphate carboxylase (Hallier et al. 1978; Schmitt 1978). Both the large subunit and the small subunit of the enzyme are not detectable in the mutant. The activities of three chloroplast enzymes, considered to be translated in the cytosol, namely NADP-dependent glyceraldehyde phosphate dehydrogenase (EC 1.2.1.13), ribose-5-phosphate isomerase (EC 5.3.1.6) and phosphoribulokinase (EC 2.7.1.19) in the mutant are equal or greater than those in the wild-type (Hallier et al. 1978). It was further shown that the mutant was capable of photosynthetic electron transport. Since this mutant may represent a point mutation or a deletion of the gene for the large subunit, we have studied its plastid DNA by restriction endonuclease analysis. In addition we have analyzed the mutant for the presence of chloroplast ribosomal RNAs, which are known to be transcribed from chloroplast DNA (Bedbrook and Kolodner 1979; Herrmann and Possingham 1980).

Material and Methods

Material

The light-green mutant sigma of plastome I is a spontaneous lethal mutant which has been isolated and kindly supplied by Professor W. Stubbe (Düsseldorf). It is maintained as sectors on wild-type plants (Kutzelnigg and Stubbe 1974) or in pure form in heterotrophic tissue cultures (Mehra-Palta and Herrmann 1975) on the medium of Nagata and Takebe (1970). The wild-type material con-

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sisted of leaves from greenhouse grown plants of *Oenothera hookeri* line 'Johansen', with the genome AA and the plastome I or IV.

Isolation of Plastid DNA

Plastids were isolated in a Waring Blendor in 30 volumes of a medium containing 0.33 M sorbitol, 6 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM NaCl, 0.5 mM KH_2PO_4, 5 mM β-mercaptoethanol, 4 mM cysteine, 2 mM sodium isoascorbate, 0.3% polyvinylpyrrolidone 10 000, 50 mM 2-(N-morpholino)-ethanesulphonic acid-NaOH, pH 6.1 (Jensen and Bassham 1966; Heber 1973; Schneider and Hallier 1970). The chloroplasts were sedimented with 2,000 g for 1.5 min and then purified by centrifugation in 10-65% sucrose gradients at 90,000 g for 1.5 h in the isolation medium minus sorbitol (Bisanz et al., in preparation). The plastid DNA was isolated by a method modified from Gross-Bellard et al. (1973) as described in Driesel et al. (1979), but omitting the RNase treatment. The DNA solution was further purified in CsCl gradients, in a Beckman L5-75 ultracentrifuge (SW60Ti rotor) at 45,000 rpm for 35-40 h. After dialysis against 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, the DNA solution was concentrated by evaporation, first in air and then in vacuo, at room temperature to about 0.5-1 $\mu g/\mu l$ in about 5 mM Tris-HCl, 0.5 mM EDTA, pH 7.4.

Analysis with Restriction Endonucleases

Digestion with restriction endonucleases and agarose gel electrophoresis were performed as described in Herrmann et al. (1980). For determination of the molecular weights of the DNA fragments the digests of the total plastid DNA were co-electrophoresed with standard digests of lambda DNA (Eco RI, Hind III) from Boehringer, Mannheim, and ϕX 174 DNA (Hae III) from Biolabs, Beverly, Ma. The molecular weights of the larger fragments (over about 8 megadalton in size) are preferably computed as the sum of component fragments obtained by second digestion.

Isolation of Total Leaf Nucleic Acids

Between 0.2 g and 3 g (wet weight) of leaves were taken from plants of *Oenothera* (wild-type plastome I or the mutant I sigma), frozen in liquid nitrogen and crushed to a fine powder. This was dissolved in 150 mM NaCl, 150 mM Tris-HCl, pH 8.0, 0.5% SDS, 1% sarcosinate and, after 2 min, the debris remaining was pelleted by a short centrifugation. The lysate was deproteinized twice with buffered phenol and chloroform in the cold and the nucleic acids were precipitated, washed and dried. After dissolving the nucleic acids in 0.2 M potassium acetate they were passed through a Sephadex G 25 column. After reprecipitation the nucleic acids

Table 1. Number and molecular weights of restriction endonuclease fragments of chloroplast DNA from *Oenothera hookeri* with plastome IV or I produced by double digestion

	Double digestion with					
	Kpn I/Pst I		Kpn I/Sal I		Pst I/Sal I	
Plastomes	IV	I	IV	I	IV	I
	13.0 (2x)	13.0 (2x)	13.0	13.0	18.8	18.8
	10.5	10.7[a]	10.7	10.9[a]		10.3[a]
	9.6	9.8[a]	8.7	8.9[a]	9.5 (2x)	9.5
	6.5	6.5	6.7 (2x)	6.7 (2x)	8.2	8.2
	5.8	5.8	5.7	5.9[a]	7.8	8.0[a]
	5.7	6.3[a]	5.4	5.4		7.0[a]
	5.4 (2x)	5.4 (2x)	5.2	5.2	6.8 (2x)	6.8
	3.9	4.1[a]	4.7 (2x)	4.7 (2x)	5.4	5.4
	3.5 (3x)	3.5 (3x)	4.6	4.5	5.0	4.9[a]
	2.8	2.8		4.4[a]	4.5	4.7[a]
	2.0 (2x)	2.0 (2x)	3.8 (2x)	3.8	2.8	2.8
	1.5	1.4[a]	2.8	2.8	2.4	2.4
	1.3	1.35[a]	2.4	2.4	1.8	1.7 (2x)[a]
	1.25	1.25	2.0	2.0	1.7	1.5 (2x)
	0.6	0.6	1.8	1.8	1.5 (2x)	1.3[a]
	0.55 (2x)	0.55 (2x)	1.5 (3x)	1.5 (2x)	1.25	1.25
			1.4	1.4 (2x)[a]	1.2 (3x)	1.2 (2x)
			1.1	1.1	1.1	1.1
			0.6	0.6	0.9	0.9
			0.55 (2x)	0.55 (2x)	0.45 (2x)	0.45 (2x)
			0.45	0.45		
Sum	102	103	102	103	102	103
No of fragments	22	22	26	26	24	24

Molecular weights were determined as under Methods and are given in megadalton (Md)

[a] Fragments (under 11 Md) from Plastome I which differ in size from the corresponding fragment in plastome IV are marked

were dried again, dissolved in electrophoresis buffer containing 20% sucrose, 40 mM Tris-acetic acid, 20 mM sodium acetate, 1 mM EDTA, pH 7.2, and subjected to electrophoresis on 2.4% polyacrylamide gels.

Results

Both subunits of ribulose bisphosphate carboxylase were not detectable by polyacrylamide gel electrophoresis in extracts of tissue cultures from mutant I sigma as previously described for mutant sectors from green-house grown plants (Hallier et al. 1978). The total protein content per leaf from the cultures as judged by staining intensity on polyacrylamide gels was, however, somewhat lower than in the extracts from the green-house grown plants.

Under conditions of complete digestion the three endonucleases Sal I, Pst I and Kpn I cleave the plastid DNA molecule of *Euoenothera* into 37 fragments, which have been resolved by gel electrophoresis, occur in stoichiometric amounts and add up to about 100 Md. This value is in agreement with the contour length (about 95 Md) of circular DNA isolated from *Oenothera hookeri* chloroplast fractions (Herrmann et al. 1975). The number of fragments was the same for plastome I DNA, plastome IV DNA and mutant I sigma DNA. A few differences in migration of some fragments are found comparing plastome I and IV. Their significance in relation to mapping is discussed in separate papers (Gordon et al. 1980, Bisanz et al. in preparation).

By the three double digestions used 72 fragments were observed with plastome I and IV DNA as well as mutant I sigma (Figs. 1, 2 and Table 1). Two differences in migration between mutant I sigma and wild-type fragments are apparent. The 9.5 Md fragment after Pst I/Sal I digestion moves slightly faster when obtained from the mutant. The same is observed for the 6.7 Md fragment and most clearly for the 5.4 Md fragment obtained with the other two double digestions indicating possibly a deletion of some nucleotides. A further difference is observed for the 1.35 Md Pst I + Kpn I fragment, which moves slightly faster in the mutant.

Double digestions yield for 87% of the total DNA molecule fragments which are 8 Md in size or smaller.

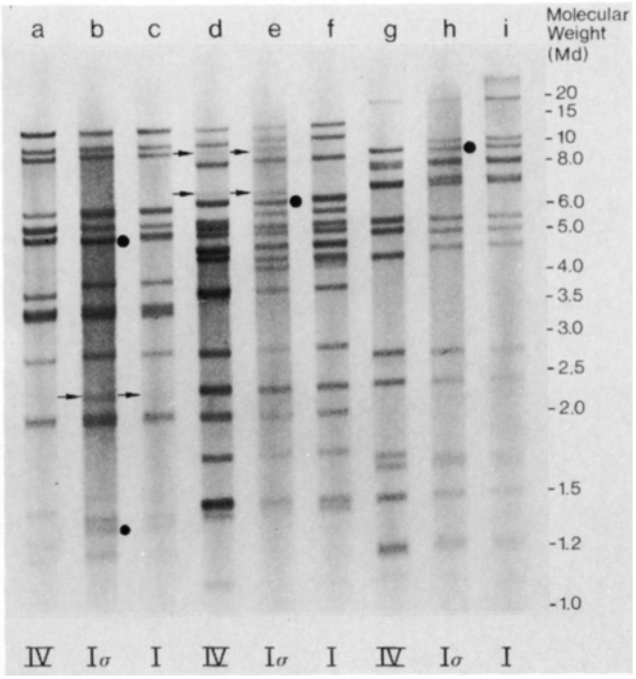

Fig. 1. Cleavage patterns on a 0.6% agarose slab gel obtained by double digestions of *Oenothera* plastid DNA with restriction endonucleases. Molecular weights are given in megadaltons (Md). The arrows indicate fragments resulting from incomplete digestion. The dots indicate fragments in the mutant I sigma DNA which migrate differently to those of the wild-type. Tracks a-c are digests with Kpn I and Pst I, tracks d-f with Kpn I and Sal I and tracks g-i with Pst I and Sal I. Tracks a, d, g: plastome IV DNA (wild-type). Tracks b, e, h: mutant sigma in plastome I DNA (Iσ). Tracks c, f, i: plastome I DNA (wild-type). (The uppermost band in lane i is undigestable high molecular weight DNA probably of nuclear origin).

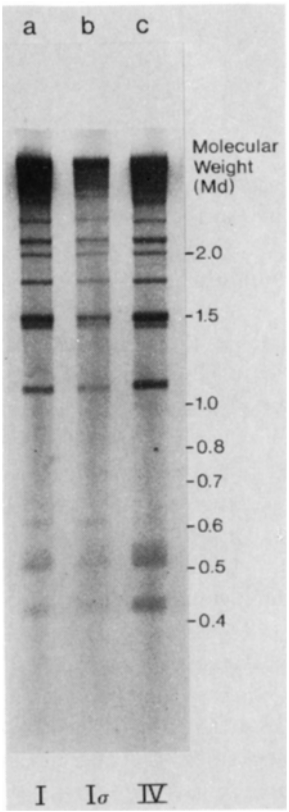

Fig. 2. Double digestions of *Oenothera* plastid DNA with Kpn I and Sal I. The fragments were separated by electrophoresis on a 1.8% agarose gel. Molecular weight are given in megadaltons (Md). Track a: plastome I DNA (wild-type). Track b: DNA of mutant sigma in plastome I (Iσ). Track c: plastome IV DNA (wild-type)

Since the gene for the large subunit of ribulose bisphosphate carboxylase (~500 amino acids) is about 1 Md in size, its complete deletion would be recognizable by a considerable change in migration of one or two fragments. The differences between some fragments in the cleavage patterns of the two wild-type chloroplast DNAs in Figure 1 are from under 0.1 Md to about 0.6 Md in size. The two differences observed between the DNA of plastome I and I sigma are smaller than 0.1 Md. The fragments (6.7 Md Pst + Sal I, 5.4 Md Pst I + Kpn I) giving rise to one of the differences have been mapped within the inverted repeat region and contain part of the 23S rRNA gene, the spacer between the 16S and 23S rRNA genes, the complete 16S rRNA gene and about 2.5 Md of DNA beyond the 16S rRNA gene (Gordon et al. 1980). The second difference is located within the small single-copy region.

The change in the size of fragments originating from the inverted repeat region did not cause the loss of ribosomal RNAs. The analysis presented in Fig. 3 reveals the mutant to contain both large chloroplast ribosomal RNAs. The 23S rRNA is present in less than expected amounts in both wild-type and mutant tissue. This is probably due to the generally observed lability of the 23S rRNA (Grierson 1974).

Discussion

The presence of functional ribosomes in plastids of the mutant is apparent from its ability to form cytochrome f (Hallier et al. 1978), a protein that has recently been shown to be synthesized in isolated pea chloroplasts (Doherty and Gray 1979). This indicates that the absence of ribulose bisphosphate carboxylase in the mutant is not caused by the organelle translation machinery itself. Moreover, the gene for the large subunit of this protein in *Oenothera* plastid DNA has been mapped by hybridizing the corresponding nick-translated nucleotide sequences of spinach to Southern blots of restricted *Oenothera* DNA (to be published). It is located in the conserved part (Gordon et al. 1980) of the large single-copy region of the circular molecule at a position analogous to that found in corn and spinach (Coen et al. 1977, Herrmann and Possingham 1980) and on fragments that are present in both the wild-type and the mutant DNA. Thus this refined analysis of I σ (Herrmann 1977) indicates that the difference in the nucleotide sequence within the inverted repeat between the mutant and the analysed plastome DNA, may not be a change connected with the sigma mutational event but reflect polymorphism for plastome I.

Evidence for polymorphisms has in fact been detected by chymotryptic peptide mapping of the large subunit of ribulose bisphosphate carboxylase among species with the

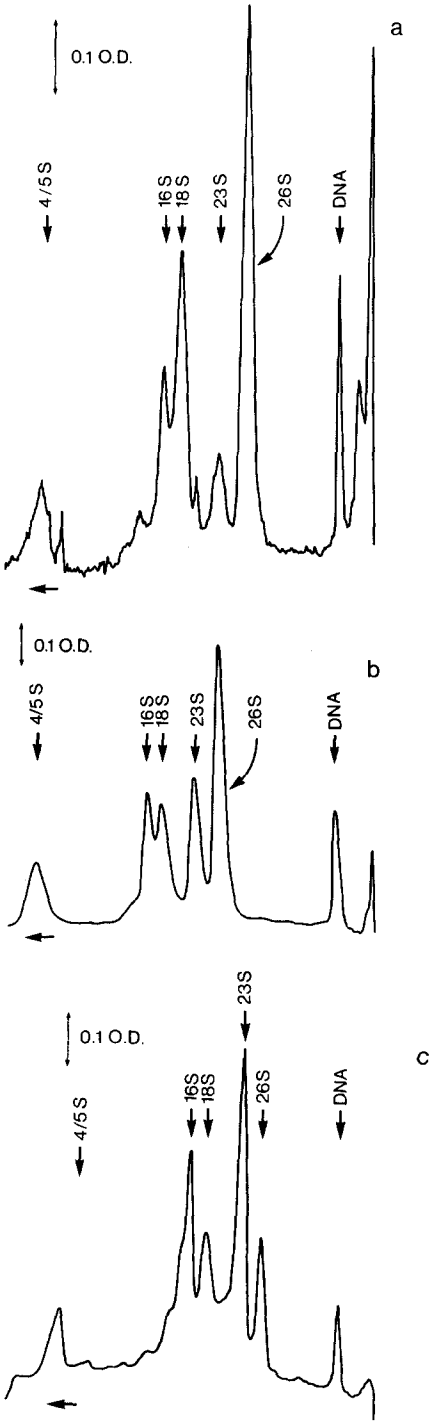

Fig. 3a-c. Total leaf nucleic acids of *Oenothera hookeri* with wild-type plastome I (b) and the mutant I sigma (a). The nucleic acids were separated by electrophoresis on 2.4% polyacrylamide gels. The identity of each peak is from right to left: DNA, 26S and 23S RNA from the large subunits of cytoplasmic and chloroplast ribosomes, 18 and 16S RNA from the small subunits of cytoplasmic and chloroplast ribosomes and 4 – 5S RNA. In (c) RNA from a 2000 × g plastid-enriched pellet from mutant I Tsigma has been separated and demonstrates the presence of both the 23S and 16S chloroplast ribosomal RNA. The molecular weights were determined on non-denaturing gels by comparison with *E. coli* rRNAs

same plastome type as defined by incompatibility relations (Holder 1978). It is also interesting that apparently the same nucleotide sequence change is observed in both members of the inverted repeat when the sigma mutant DNA is compared to the plastome I analysed. This gives another example to the previously observed phenomenon that homology of the repeated regions is strongly conserved (Bedbrook et al. 1976).

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