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Insertion polymorphism in pea chloroplast DNA

T. H. Teeri, A. Saura and J. Lokki

Department of Genetics, University of Helsinki, P. Rautatiekatu 13, SF-00100 Helsinki 10, Finland

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Summary. The chloroplast DNA of higher plants is suitable for restriction endonuclease analysis due to its size and homogeneity. We have analysed 48 different cultivars of pea (*Pisum sativum*) with EcoRI and HindIII. Of these, only 24 show the standard genotype, the remaining 24 comprise four different classes of short insertions, three of which are found at the same site. Even though this kind of insertion polymorphism has not been detected elsewhere in the plant kingdom, it is consistent with the discovery that the chloroplast DNA of pea is destabilised through the loss of an inverted repeat.

Key words: *Pisum sativum* – Chloroplast DNA – Restriction endonuclease analysis

Introduction

The molecular weight of the circular higher plant chloroplast DNAs (cpDNAs) ranges from 120 to 180 kilobases (kb). The mean size is 140–150 kb (Wallace 1982). These molecules show a remarkable structural homogeneity which is found even in the cpDNA of unicellular algae. An inverted repeat of about 22 kb divides the molecule into a larger (about 80 kb) and a smaller (about 19 kb) unique sequence. This repeated region contains the genes for chloroplast ribosomal RNAs. Accordingly, these genes are present at double dosage. A small number of protein coding genes have already been mapped in the larger unique sequence. One of these is the gene for the large subunit of ribulose-1,5-bisphosphatecarboxylase, one of the most extensively transcribed and translated sequences in the biosphere. In addition, 27 different transfer RNA species, capable of translating 16 of the required 20 amino-acids, have been mapped in different regions of the genome (Steinmetz et al. 1978). The above findings finally make plausible the hypothesis according to which the autonomously acting transcription-translation machinery of the chloroplast does not require nucleic acid transport through the double chloroplast envelope. Even though isolated chloroplasts synthesize some 90 separable polypeptides (Herrmann and Possingham 1980) (a number which roughly corresponds to the coding capacity of cpDNA), these organelles are completely dependent on nuclear coded, cytoplasmically synthesized proteins. Accordingly, a protein transport system of considerable capacity must exist between the cytoplasm and organelles.

Each chloroplast contains some 20 copies of its circular DNA molecule. As the number of chloroplasts per cell may rise to as high as 50, the degree of ploidy of this extrachromosomal genome is quite large (Walbot 1977). Yet the cpDNA pool of one plant individual (or strain) appears to be homogenous for all practical purposes. This has led to the application of restriction endonuclease analysis of these molecules, in which the fragment patterns produced by digestion with various restriction enzymes are compared (with or without mapping them).

Materials and methods

The cpDNAs were prepared by first isolating intact chloroplasts from 10–15 g of pea seedlings following a modification of the method of Mills and Joy (1980). The plastids (transferred in 150 mM NaCl, 5 mM EDTA–40 mM Tris-Cl, pH 7.9) were lysed by addition of SDS to 0.5% and the DNA was purified by extracting twice with redistilled phenol-chloroform (1:1). Following removal of the phenol by ether extraction, the RNA was digested with 10 μg of pancreatic RNase (15 min, 37 °C), and the cpDNA was precipitated with two

volumes of cold ethanol. After resuspending the pellet in 1 mM EDTA–6 mM Tris-Cl, pH 7.9, the DNA was digested essentially according to the instructions of the enzyme manufacturer. The DNA fragments were separated in 0.5% and 1.5% agarose slab gels (resolution ranges 2–20 kb and 0.2–2.5 kb, respectively) and the gels were photographed under ultraviolet illumination. The electrode and gel buffer used was 50 mM Tris–50 mM H_3BO_3–2 mM EDTA–0.2 μg/ml ethidium bromide, pH 8.3.

Results

We have analysed the cpDNAs of 48 different cultivars of pea (*Pisum sativum*) by comparing the band patterns produced by these DNA molecules in agarose gels after digestion with EcoRI and HindIII. A peculiarity of pea cpDNA is that it does not possess any larger repeated regions (Chu et al. 1981). Accordingly, the band patterns obtained lack true double bands, which usually arise in restriction analysis of cpDNA. The double bands that appear due to chance comigration can be detected most reliably by studying the densitograms of the photographed gels. The HindIII pattern contains two double bands and the appreciably denser EcoRI pattern 13 (a few of which may be triple). The molecular weight of pea cpDNA, calculated from the sizes of the individual fragments, (obtained by comparison with phage lambda DNA standard fragments) is 120.5 kb based on the HindIII digestion and it is similar to that obtained by Palmer and Thompson (1981). The lower value given by EcoRI fragments (115.4 kb) can be accounted for by the triple bands (which remained undetected) and lack of resolution below the limit of 0.2 kb.

Five different variations in the band patterns were repeatedly detected in the analysed cultivars. These variant patterns appeared in the gels for the larger fragments (Figs. 1 and 2). In all but one case the different variations obtained by the two enzymes coincided perfectly. This means that each HindIII variant has a counterpart in the EcoRI pattern. By designating the most abundant (24/48) variant as the basic chloroplast genotype, each of the other variations can be derived from this by an insertion of a few hundred base pairs. Remarkably, three of these insertions take place in a short region represented by a 2.2 kb EcoRI fragment (and a 6.8 kb HindIII fragment); this fragment is missing from the mutated types and a new band appears at three different locations in the three different variants. The fourth insertion is located in a different restriction fragment. The single case, which is not in accord with the coincidence pattern, can be explained through the limited relative resolution in the 7 kb region of HindIII digestion pattern. Any further variation in the 6.8 kb HindIII band (H8 in Fig. 2) might, therefore, be obscured. Accordingly, chloroplast genotypes B and D have a similar HindIII pattern, even though they differ clearly when analysed with EcoRI.

Table 1. EcoRI and HindIII restriction enzyme fragments obtained from pea chloroplast DNA. The list of fragments which are summed represent the genotype *O*. The representation of the multiple band H8 as fragments of three distinct molecular sizes only serves the purpose of obtaining the most probable structure of the complex in the calculations (i.e. that the genotypes B and D arise from *O* by the disappearance of H8c and appearance of H8a) and it is not an indication of gel resolution. Also, the representation of the estimates of the sizes by accuracy of one base pair is only illustrative

EcoRI				HindIII	
E 2	7.555 kb	E29	1.130	H 1	13.101 kb
E 3	6.798	E30	1.035	H 2	10.813
E 4	5.042	E31	0.996	H 3	8.645
E 5	5.042	E32	0.965	H 4	8.398
E 6	4.943	E33	0.861	H 5	7.857
E 7a	4.007	E34	0.820	H 6a	7.362
E 7b	4.007	E35	0.784	H 6b	7.362
E 8	3.446	E36	0.766	H 8b	6.834
E 9	3.346	E37	0.731	H 8c	6.745
E10	3.290	E38	0.722	H 9	6.589
E11a	2.949	E39	0.685	H10	5.584
E11b	2.949	E40a	0.657	H11	4.737
E12	2.829	E40b	0.657	H12	3.865
E13	2.764	E41a	0.638	H14	3.519
E14	2.587	E41b	0.638	H15	3.033
E15a	2.431	E42	0.602	H16	2.609
E15b	2.431	E43	0.589	H17	2.352
E16a	2.350	E44	0.545	H18	2.271
E16b	2.350	E45a	0.466	H19	1.522
E18a	2.176	E45b	0.466	H20	1.376
E18b	2.176	E46a	0.408	H21	1.300
E19	2.088	E46b	0.408	H22	1.245
E20a	1.978	E47	0.385	H23	1.150
E20b	1.978	E48a	0.375	H24	1.093
E21	1.904	E48b	0.375	H25	0.734
E22	1.783	E49	0.356	H26	0.545
E23	1.665	E50	0.342	H27	0.334
E24	1.637	E51a	0.320		
E25	1.525	E51b	0.320	Sum	120.468 kb
E26a	1.473	E52	0.301		
E26b	1.473	E53	0.288		
E27	1.408	E54	0.252		
E28	1.366			Others:	
		Sum	115.403 kb	H 7	6.965
				H 8a	6.923
				H13	3.718
		Others:			
		E 1	7.718		
		E17	2.307		

Discussion

The large degree of overall structural homology of cpDNAs of different species suggests that the molecule is highly conserved even at the level of restriction sites. Therefore, the band patterns within a single plant

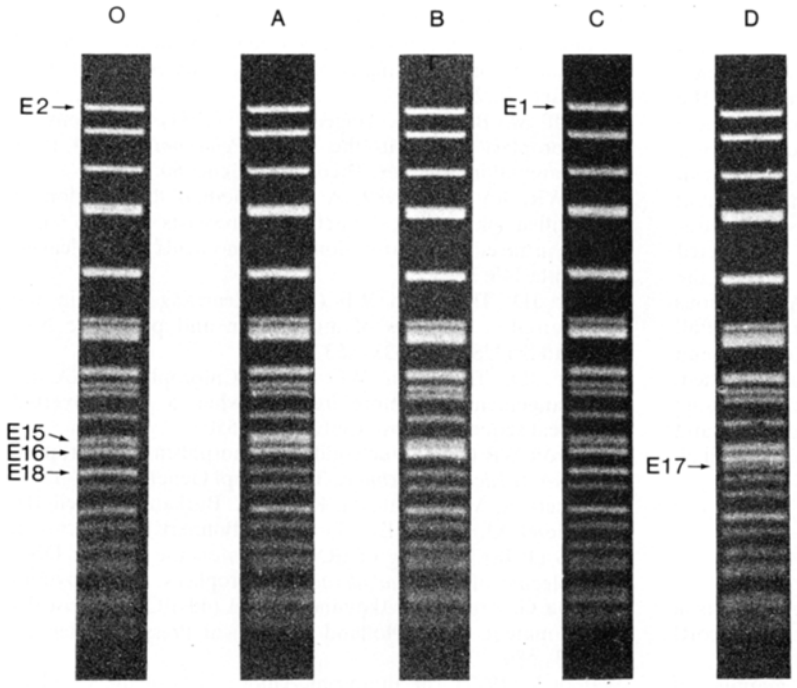

Fig. 1. The EcoRI patterns of the five different pea cpDNA variations. Genotype *O* is the basic genotype (24 cultivars out of 48) and type *A* (14/48) is characterized by a disappearance of the other fragment in the double band *E18* and its reappearance to comigrate with the double band *E15*, type *B* (3/48) by the reappearance of the same band to comigrate with the double band *E16* and type *D* (1/48) by its reappearance between bands *E16* and *E18* (forming band *E17*). Type *C* (6/48) is characterised by a shift in the largest fragment

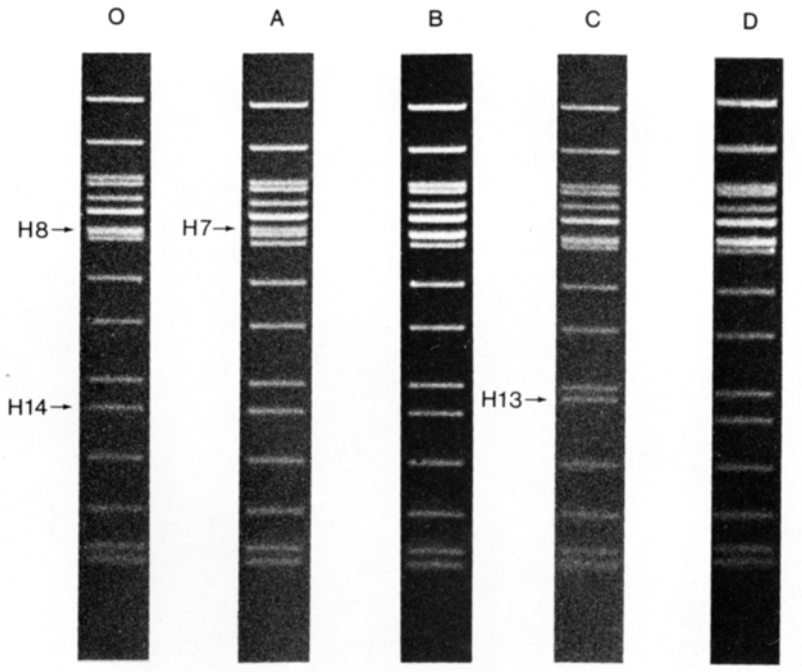

Fig. 2. The HindIII patterns of the same pea cpDNA variants as in Fig. 1. In type *A* the other fragment of the double band *H8* shifts upwards forming *H7*, and both types *B* and *D* seem to be characterized by a rise in the whole double band *H8*. In type *C*, band *H14* has disappeared and band *H13* has appeared. The band patterns with the two enzymes coincide so that each cultivar can be labelled *O, A, B, C* or *D* for its cpDNA

population or cultivar show homogeneity and often even related species are indistinguishable by this kind of analysis (Atchison et al. 1976). There are only two previous reports on within species variation in cpDNA restriction patterns. Scowcroft (1979) found in two of his nine *Nicotiana debneyi* populations a difference in EcoRI pattern, which can be accounted for by a single base pair substitution. Metzlaff et al. (1981), on the other hand, noticed a polymorphism in cpDNA in three out of 16 cultivars of *Pelargonium zonale*. One of their variants could be accounted for by a base substitution, while the other remained obscure.

Restriction enzyme analysis is sensitive enough to detect small insertions and deletions (down to 50 base pairs), but it is rather insensitive in detecting point mutations (because only the recognition sequences of the enzymes are screened). The insertion polymorphism in pea is the first one to be described. Chloroplast DNAs within a particular species could harbor some point mutation polymorphism. Such a phenomenon can not be effectively revealed without DNA sequencing, but it could occasionally appear in different experiments. The intraspecific polymorphism in pea is, however, not an unexpected result. Palmer and Thompson (1981, 1982) have studied the sequence organisation differences between cpDNAs of various angiosperms. The differences are consistent with the overall phylogeny of these species in all cases except for the pea and the broad bean, which have diverged further than expected. This may be connected with the (hypothetical) stabilising effect of the inverted repeat of the molecule. Both pea and broad bean have been shown to lack this feature (Chu et al. 1981; Koller and Delius 1980).

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