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Intraspecific heterogeneity of the *Vicia faba* mitochondrial genome: evidence for multiregional rearrangements in the mitochondrial chromosome associated with *coxII-orf192* chimeric gene formation

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Summary

Previous RFLP-analysis of mtDNA isolated from different lines and cultivars of *Vicia faba* with respect to variability of the coxII gene revealed two types of mitochondrial genome: one with a normal coxII gene and the other with both normal *coxII* and chimeric *coxII-orf192* genes. In this study we analyzed other regions of these two types of mitochondrial genome and found significant differences in the arrangement of regions around the *coxII*, *coxIII*, *cob*, *rrn*26 and *atpA* genes. More detailed analysis of the *rrn*26 and *atpA* gene regions showed that these genes are associated with recombinationally active repeats. Restriction maps of the *rrn*26 and *atpA* gene regions in different recombinative variants are presented.

Key words: *Vicia faba* – Mitochondrial DNA – Mitochondrial DNA instability – Heterogeneity – Chimeric gene

Introduction

It is now obvious that mitochondrial genomes of different species of higher plants differ significantly not only by their size but additionally by their gene order and gene copy number (Lonsdale 1989). It has been shown that the mitochondrial genomes of fertile type and cytoplasmic male-sterile type T-lines of *Zea mays* also differ by both gene order and gene copy number (Fauron and Havlik 1989). These differences were probably caused by the recombination of short repeats which are different from those which maintain subgenomic circular mtDNA and genomic isomers (Brears and Lonsdale 1988; Palmer and Herborn 1988; Rottman et al. 1987; Joyce et al. 1988; Folkerts and Hanson 1989). The mechanisms of recombination which cause such genetic divergence are still unknown. Therefore, new model systems, where extensive rearrangement events leading to genetic divergence between closely related cytoplasms are demonstrated, would of interest for a more detailed analysis of such rearrangements. Previous RFLP-analysis of mitochondrial DNAs (mtDNA) from different lines and cultivars of *Vicia faba*, with respect to variability of the *coxII* gene, revealed two types of mitochondrial genome (Negruk and Kaushik 1988). More detailed analysis of these two types showed that type-I mtDNA contained only normal *coxII* and *orf192* genes, while type-II mtDNA, in addition to these two genes, contained a chimeric *coxII-orf192* gene which appeared as a result of non-homologous recombination (Negruk et al. 1991). In this paper, we present data showing that *coxII-orf192* chimeric gene formation is associated with significant rearrangements of the *coxII, coxIII, cob, rrn26, atpA* and *atp9* genes.

Materials and methods

We have used lines K and F from broad bean seeds of cv 'Black Russians' (a natural isolate cultivated in middle Russia, All-Union Institute of Selection and Seed Production of Vegetable Crops, Moscow District, USSR). These lines differ from the standard karyotype of *V. faba* by homozygous translocations between chromosomes I and VI (K) and II and III (F) respectively (Schubert et al. 1982). We have also used seeds of line cms447, kindly supplied by Prof. D. Bond via Prof. R. Rieger (Zentralinstitut für Genetik und Kulturpflanzenforschung, Gatersleben, Germany). Preparations of mtDNA from individual 6-day-old etiolated seedlings were made according to Synenki et al. (1978). Restriction enzyme digestion, electrophoresis, labelling of DNA

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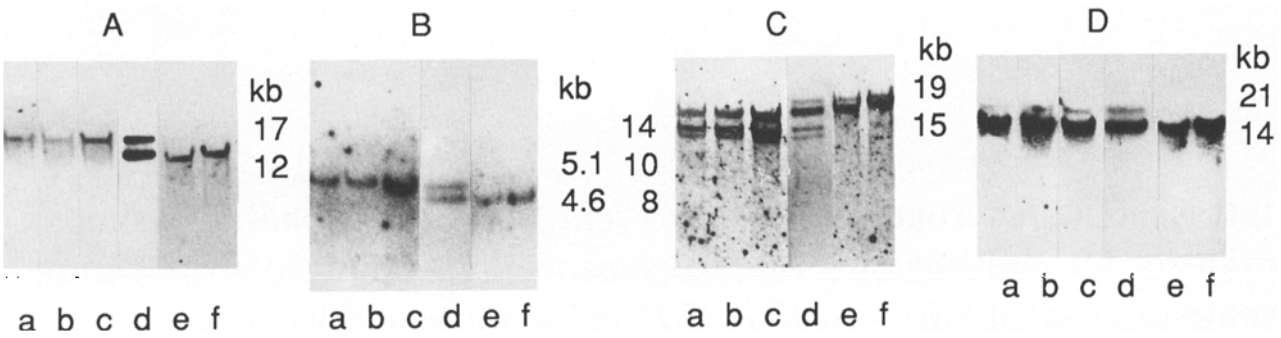

Fig. 1. Autoradiography of *V. faba* mtDNA digested with *Xba*I (*A, C, D*) or *Hin*dIII (*B*) and hybridized with the *cob* (*A*), *coxIII* (*B*), *atpA* (*C*), and *rrn26* (*D*) gene probes. mtDNA was prepared from individual 6-day-old etiolated seedlings of line K (**a**), line cms447 (**b**), cv Russian Black with a type-I mitochondrial genome (**c**), line F (**e**) cv Russian Black with a type-II mitochondrial genome. In lane (**d**), mtDNA was prepared from a pool of several dozen seedlings of cv Russian Black. After electrophoresis through 1% agarose gel in TAE buffer, DNA was blotted onto Nylon filters. The numbers at the sides of the electrophoregrams show the fragment sizes in kb

probes, and Southern hybridization were all done according to Maniatis et al. (1982). The cloned *Z. mays coxII* gene probe was kindly supplied by C. S. Levings III. The cloned *Oenothera coxIII, cob, rrn26, atpA* and *atp9* gene probes were kindly supplied by A. Brennicke.

Results

To analyze possible differences between the type-I and type-II mitochondrial genomes of *V. faba* we hybridized ^{32}P-labelled *coxIII, cob, rrn26* and *atpA* gene probes with mtDNA preparations isolated from individual 6-day-old etiolated seedlings of line K (mtDNA of type-I), line cms447 (mtDNA of type-I), individual seedling of cv Russian Black with mtDNA of type-I, line F (mtDNA of type-II), and individual seedlings of cv Russian Black with type-II mtDNA. We also tested mtDNA isolated from several dozen cv Russian Black seedlings representing a mixture of type-I and type-II mtDNAs. The results of the Southern hybridization experiments are shown in Fig. 1. The types of *V. faba* mitochondrial genomes contained different *Xbal* fragments hybridizing with the *cob* gene probe; mtDNA of type-I contained this gene in a 17 kb fragment and mtDNA of type-II in a 12 kb fragment (Fig. 1A). The *coxIII* gene region in these two types of mitochondrial genomes was also rearranged. We found different *Hin*dIII fragments hybridizing with the *coxIII* gene probe: a 4.6 kb fragment for type-II and a 5.1 kb fragment for type-I (Fig. 1B). *Xba*I fragments were also of different sizes: 12.3 kb for type-II and 11.3 kb for type-I (data not shown). All four fragments were found in a cosmid library, sublconed and mapped (Fig. 2). The comparison of these maps led us to conclude that a recombination event had occurred left of the *Bam*HI site located near *orf192*. As a consequence of this recombination the *atp9* gene sequence was transfered to a region several kb left of *orf192* in the type-II mitochondrial genome. The situation was even more complicated

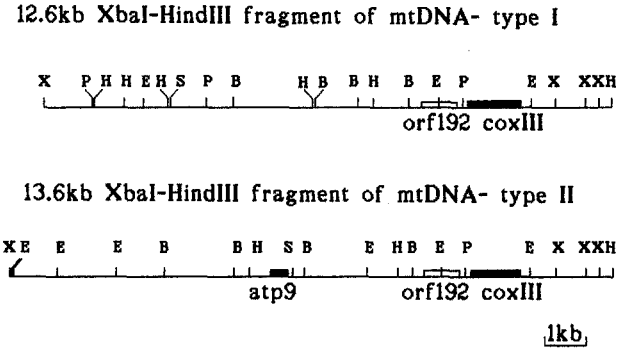

Fig. 2. Restriction maps of a 12.6 kb *Xba*I-*Hin*dIII fragment of *V. faba* mtDNA of type-I, and a 13.6 kb *Xba*I-*Hin*dIII fragment of *V. faba* mtDNA of type-II. The restriction endonucleases are indicated as: *H, Hin*dIII; *B, Bam*HI; *E, Eco*RI; *X, Xba*I; *P, Pst*I; *S, Sal*GI

when these two regions were compared at the cosmid level. In this case we found that mtDNA had also recombined at a distance of several kb from the right side of the *coxIII* gene (data not shown).

A *Xba*I cosmid library of the mitochondrial genome of *V. faba* cv Russian Black was constructed in the pHC79/*E. coli* DHI system. Parallel experiments revealed a heterogenous population of plants with mtDNA of type-I (about 70%) and type-II (about 25%) in this cultivar (Negruk et al. 1991). Southern hybridization of ^{32}P-labelled mitochondrial gene probes with type-I and type-II mtDNA allowed us to distribute a significant part of the cosmid library between two groups of cosmids corresponding to type-I and type-II mitochondrial genomes.

The *atpA* gene region had a complex organization in both mitochondrial genomes (Fig. 1C). Type-I mtDNA contained sequences homologous to the *atpA* gene in 15 kb, 14 kb, 10 kb, and 8 kb *Xba*I fragments. These fragments were present in non-equimolar amounts, and

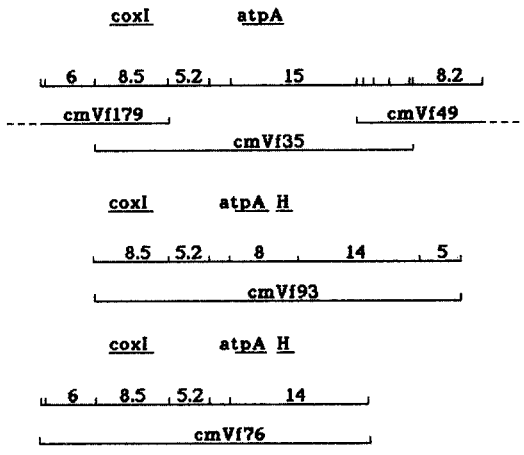

Fig. 3. *Xba*I restriction maps of cosmids with three different arrangements of the *atpA* region in a type-I mitochondrial genome. Similar data were obtained for *Sal*GI restriction maps of these cosmids. The tRNA-His gene is indicated as *H*. The numbers above restriction maps show the fragment sizes in kb

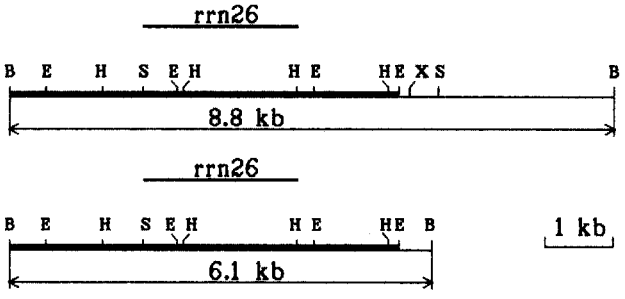

Fig. 4. Restriction maps of 8.8 kb and 6.1 kb *Bam*HI fragments of *V. faba* mtDNA containing the *rrn26* gene

some of them may be present on subgenomic molecules. Three of these fragments – 15 kb, 14 kb, and 8 kb – were found in the cosmid library and were mapped (Fig. 3). Mapping revealed that in all three cases recombination took place to the right of the *atpA* gene. In the 14 kb and 8 kb fragmens the *atpA* gene was flanked on the right side by the *trnH* gene (Kozhemyakin et al. 1991), but the *trnH* gene was absent from the 15 kb fragment. In all three fragments the region between the *coxI* and *atpA* genes was probably not reorganized (Fig. 3). Type-II mtDNA contained sequences hybridizing with the *atpA* gene probe in two or three of the largest *Xba*I fragments (Fig. 1 C). Thus, by analyzing the results shown in Figs. 1 C and 3 we could conclude that, in the mitochondrial genome of *V. faba*, the *atpA* gene is localized in a region of recombinationally active repeats and that type-I and type-II mitochondrial genomes had different sets of *Xba*I fragments homologous to the *atpA* gene probe.

Southern-hybridization analysis with a ^{32}P-labelled *rrn26* gene probe revealed that both types of *V. faba* mitochondrial genomes contained a 14 kb *Xba*I fragment hybridizing with this probe. A 21 kb *Xba*I fragment from type-I mtDNA gave only a weak signal (Fig. 1 D). Analysis of the *rrn26* gene in the *V. faba* mitochondrial genome, by hybridization of the corresponding gene probe with the cosmid library, revealed significant heterogeneity in this region too. To analyze the region in detail we hybridized a ^{32}P-labelled *rrn26* gene probe with *Bam*HI and *Sal*GI digests of type-I and type-II mtDNAs. Both types of mtDNA contained 8.8 kb *Bam*HI and 4.3 kb *Sal*GI fragments homologous to the *rrn26* gene, in agreement with the results published by Huh and Gray (1982). In addition, the type-I mtDNA contained a 6.1 kb *Bam*HI fragment and two *Sal*GI fragments – 4.5 kb and 21 kb in length. Both the 8.8 kb and the 6.1 kb *Bam*HI fragments were cloned into the vector Bluescript SK+/JM109 and mapped. Comparative analysis of restriction maps of these two fragments revealed that the *rrn26* gene was localised within a repeat of about 6 kb (Fig. 4). This repeat was found in four different arrangements in cosmids corresponding to the type-I mitochondrial genome (Fig. 5). These data lead us to suggest that this repeat was also recombinationally active. The mitochondrial genome of type-II contained only one copy of the *rrn26* gene corresponding to the arrangement shown in Fig. 5 C.

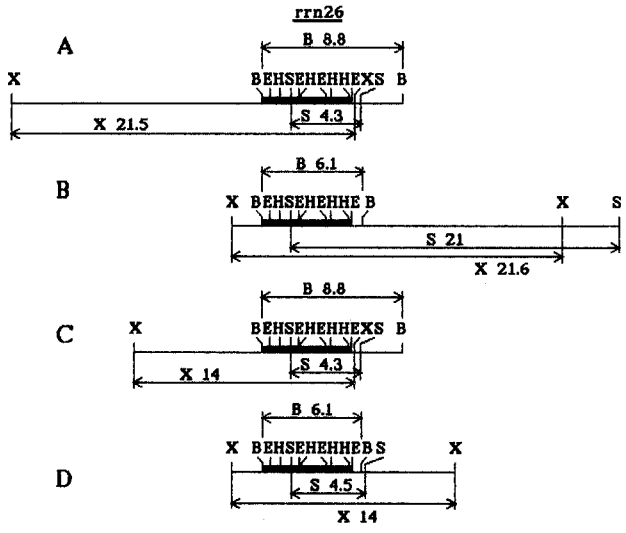

Fig. 5. Restriction maps of different arrangements of the *rrn26* gene in the *V. faba* type-I mtDNA. The restriction enzymes are indicated as in Fig. 2

As mentioned before, the type-II mitochondrial genome differed from type-I by the presence of a chimeric *coxII-orf192* gene in addition to normal *coxII* and *orf192* genes (Negruk et al. 1991). Comparative analysis of restriction maps of cosmid clones corresponding to the type-I mitochondrial genome, and containing *orf192* and *trnP* genes (Kozhemyakin et al. 1991), with cosmid clones corresponding to the type-II mitochondrial ge-

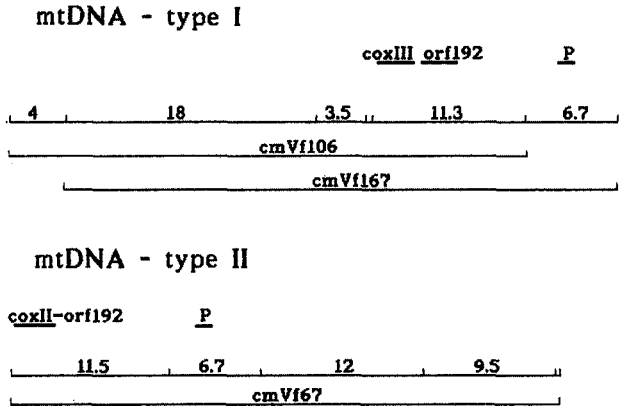

Fig. 6. *Xba*I restriction maps of cosmids with *coxIII* and *orf192* regions in a type-I mitochondrial genome and a *coxII-orf192* chimeric gene region in a type-II mitochondrial genome. Similar data were obtained for *Sal*GI restriction maps of these cosmids. The numbers above the restriction maps show the fragment sizes in kb. The tRNA Pro gene is indicated as *P*

nome, and containing a *coxII*-orf192 chimeric gene, confirmed the conclusion that the *coxII-orf192* chimeric gene appeared as a result of recombination between the *orf192* and *coxII* genes (Fig. 6). Comparative analysis of restriction maps of cosmid clones corresponding to mitochondrial genome of both types with each containing the *coxII* and *cob* genes lead us to suggest that chimeric gene formation was accompanied by rearrangements in these two regions also (Fig. 6). Analysis of *V. faba* cms447 mtDNA revealed a similarity at different loci between the mtDNA of this line and type-I mtDNA (Fig. 1).

Discussion

Detailed analysis of the differences between type-I and type-II mitochondrial genomes of *V. faba* has revealed significant rearrangements not only in the *coxII* and *orf192* genes but also in other regions. Sequence analysis of the *coxII*, *orf192* and chimeric *coxII-orf192* genes showed that the chimeric gene resulted from non-homologous recombination (Negruk et al. 1991). On the other hand, comparative restriction analysis of the *coxII*, *coxIII*, *cob*, *rrn26* and *atpA* genes also revealed significant differences in the physical maps of these regions. In this connection we suggest that chimeric gene formation can be associated with additional extensive rearrangements which lead to significant differences in the organization of type-I and type-II mitochondrial genomes. The mechanism of such recombination is still unknown, although site-specific recombination involving repeats is considered as the most probable cause (Rottman et al. 1987; Brears and Lonsdale 1988; Joyse et al. 1988; Palmer and Herborn 1988; Folkerts and Hanson 1989). There are also some data which suggest the possible participation of transposon-mediated insertions and deletions in mtDNA rearrangements (Pruit and Hanson 1989; Senda et al. 1991).

It is interesting that as yet there is no known case of two closely related mitochondrial genomes differing in only one rearrangement (Small et al. 1987; Young and Hanson 1987; Hanson et al. 1988; Leaver et al. 1988; Levings and Dewey 1988; Pring et al. 1988; Fauron and Havlik 1989). Of course, these data do not exclude the possibility of single recombination events within mitochondrial genomes, but the fomation of a new stable variant of the mitochondrial genome probably involves complex recombinative events. In this connection two facts deserve mention. Our data and those published, for example, by Small et al. (1987), Leaver et al. (1988), and Pring et al. (1988) demonstrate that mitochondrial genomes in whole plants from distinct lines were stable for long periods of time. However, after in-vitro cultivation of plant tissues, mitochondrial genomes of various species underwent complex recombinative rearrangements (Dale et al. 1981; Morgens et al. 1984; Negruk et al. 1986; Grayburn and Bendich 1987; Rode et al. 1987; Lonsdale 1989). To explain these data two principal steps in the evolution of higher plant mitochondrial genomes might be suggested. First, the hidden accumulation of single different recombinations and mutations at the level of "sublimons" (Leaver et al. 1988) and second, an explosion of recombinative processes resulting in a significant reorganization of the mitochondrial genome over a relatively short period of time. The mechanism inducing such recombination activity is not known. It is now obvious that the mitochondrial genome of a large number of higher plants consists of a multicircular, dynamically balanced, population of molecules resulting from frequent recombinations between relatively long repeats (Lonsdale 1989). This dynamic balance can be stably preserved for a long time in a pure line with a corresponding nuclear background. At the same time a mitochondrial genome may contain a number of variants involving different loci of the mitochondrial chromosome in very low copy number ("sublimons", see Leaver et al. 1988). For several reasons this dynamic balance might become disturbed; for example, by in-vitro cultivation or by exposure of whole plants to severe stress. Finally, it is possible that processes within the nuclear genome, similar to hybrid dysgenesis in *Drosophila* (Bregliano and Kidwell 1983), could affect the dynamic balance of the mitochondrial genome by increasing the copy number of some sublimons. In order to increase copy number in a given molecule it is necessary to either insert a more active replication origin (ori) or to activate an inactive one. We cannot exclude the existence of special mechanisms of activation or of recombinational ori transfer which become induced by stresses or some kind of functional imbalance between nucleus and mitochondria. Both the master chromosome and minicircular DNAs

could serve as ori donors. After one of the sublimons received a more active ori it could shift the balance within the population of circular mtDNA molecules. The appearance of the same or new repeats in different positions could then cause a recombinational burst eventually leading to a new dynamic balance. During this process some loci previously present in high copy number, could revert to the status of sublimons and significant parts of the mitochondrial chromosome could be recombinatively rearranged. In accordance with this speculation the type-I mitochondrial genome could be considered as an original dynamically balanced genome and the type-II mitochondrial genome as a new dynamically balanced genome which appeared after the formation of the *coxII-orf192* chimeric gene as a result of non-homologous recombination (Negruk et al. 1991). It is possible that such a recombination caused a functional imbalance between the nuclear and the mitochondrial genomes. To compensate for this imbalance 'an explosion' of several additional rearrangements took place culminating in a new exbalanced, type-II, mitochondrial genome.

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