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Recombination events across the *atpA*-associated repeated sequences in the mitochondrial genomes of beets

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Abstract The mitochondrial *atpA* gene sequence of the normal fertile sugarbeet (cv 'TK81-0') exists in one full-length version and one truncated version, both of which are present in normal stoichiometry and have a 406-bp segment in common. The PCR approach as well as prolonged exposure of Southern blots indicates that the products of the recombination across the 406-bp repeat are present in substoichiometric amounts in the 'TK81-0' genome. Intriguingly, one of these substoichiometric sequence arrangements was revealed to be preferentially amplified in an evolutionary lineage that led to a cytoplasmic male-sterile variant [I-12CMS(2)] in wild beets. We also found the 406-bp repeat to be part of a 6.5-kb repeat in the mitochondrial genome of I-12CMS(2). This 6.5-kb duplication is likely to involve recombination between two sets of repeats (the above-mentioned 406-bp repeat and a 7-bp repeat) in an ancestral beet mitochondria.

Key words Beets · Mitochondrial genome · Recombination · Repeat · Substoichiometric sequences

Introduction

Recombination is a major cause of gross DNA alterations involving deletion, inversion and duplication, and repeated DNA sequences are a feature common to many recombination systems (Conklin and Hanson 1994; Palmer 1992). With the exception of *Brassica hirta* (Palmer and Herbon 1987), plant mitochondrial genomes contain one or more small (two to three copies) families of long (1–14 kb) repeats, many of which appear to be implicated in the high-frequency recombination process that generates the multipartite organization of mitochondrial DNAs (mtDNAs) (André et al. 1992; Conklin and Hanson 1994). Plant mtDNAs are also characterized by an abundance of short dispersed repeats which allow infrequent recombinations leading to sequence rearrangements (André et al. 1992; Conklin and Hanson 1994). Some of the short repeats include the transcribed regions of the mitochondrial genes. This raises the possibility that some short repeats could have arisen via reverse transcription and random integration of active genes (André et al. 1992).

The mitochondrial *atpA* reading frame of the normal fertile sugarbeet (*Beta vulgaris* L.) exists in one full-length version (intact copy) and one truncated version (pseudocopy), both of which have a 406-bp segment in common (Senda et al. 1993). We also found that the male-sterile cytoplasms derived from wild beets contain the *atpA* arrangement, which is likely to have resulted from homologous recombination mediated by the 406-bp repeat. In this paper, we present the further analysis of the rearrangement involving the *atpA* locus. The expectation was that the data will aid in our understanding of evolutionary mechanisms for the generation of mitochondrial genome diversity in *Beta* species.

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Materials and methods

The sugarbeet line 'TK81-0', which carries the normal, male-fertile cytoplasm, is a maintainer of sterility of the Owen cytoplasm (Mikami et al. 1984). The line I-12CMS(2) contains the male-sterile cytoplasm derived from wild beets collected in Turkey and belonging to section *Beta* of the genus *Beta* (Mikami et al. 1985). TK81-0

and I-12CMS(2) were provided by the Hokkaido National Agricultural Experiment Station, Japan, and Dr. R. K. Oldemeyer, Mono-Hy Sugar Beet, USA, respectively.

MtDNA was prepared from tap roots of field-grown plants by the DNaseI procedure (Mikami et al. 1984). *Hin*dIII digests of mtDNAs from 'TK81-0' and I-12CMS(2) were 'shotgun' cloned into the Bluescript plasmid (Stratagene) and transformed into *E. coli* host strain JM109 (Senda et al. 1993). A mtDNA library of I-12CMS(2) was also constructed in the phage vector lambda DASH (Stratagene) as described in Kubo et al. (1995). Procedures for restriction enzyme digestion, agarose gel electrophoresis, Southern blot analysis and nucleotide sequencing have been described previously (Senda et al. 1991, 1993). *Taq* DNA polymerase-based polymerase chain reaction (PCR) amplification of mtDNA was conducted according to the manufacturer's recommendation (Promega Corporation, USA). The sequence of the primers used for the PCR experiments were: primer A, 5′-CACAATATGCACCACTTCCAAT-3′; primer B, 5′-AGATCAAGACATTACAAGGTTACG-3′; primer C, 5′-ACTTC-CTCTTCTCAACCCGTC-3′; and primer D, 5′-AGGATTCAAG-CACGACCCCAA-3′.

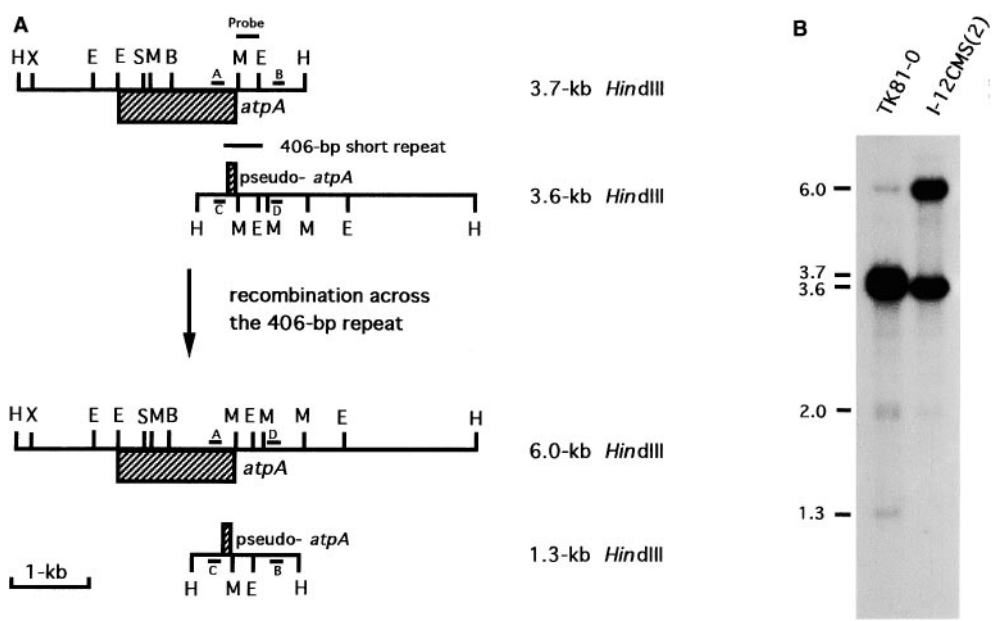
Fig. 1A,B **A** Restriction maps of the four *Hin*dIII fragments encompassing the 406-bp recombinogenic repeated sequence. The 3.7-kb and 3.6-kb *Hin*dIII clones were retrieved from the TK81-0 mtDNA library, and the 6.0-kb and 3.6-kb *Hin*dIII clones from the I-12CMS(2) mtDNA library. Their maps are taken from Senda et al. (1993). The 1.3-kb *Hin*dIII segment is present in substoichiometric amounts in the mitochondrial genome of TK81-0 and was identified by Southern blot analysis and the PCR approach (see Fig. 1B and Fig. 2). Primers A, B, C and D used for PCR experiments are indicated by *bold bars*. The location and extent of a 250-bp *Sma*I-*Eco*RI subfragment which was used as a probe for Southern blot analysis is also shown. Restriction sites are: *B Bam*HI, *E Eco*RI, *H Hin*dIII, *M*, *Sma*I, *S Sal*I, *X Xho*I. **B** Detection of *Hin*dIII fragments corresponding to the predicted products of recombination across the 406-bp repeat. *Hin*dIII-digested mtDNAs from TK81-0 and I-12CMS(2) were hybridized with the 250-bp *Sma*I-*Eco*RI subfragment of the 3.7-kb *Hin*dIII clone (see Fig. 1A). The Southern filter was exposed to Fuji medical X-ray film (Fuji Photo Film Co) for 2 days at −80°C. Sizes of the hybridizing fragments are indicated in kilobases

Results and discussion

Role of the 406-bp repeat in the rearrangement involving *atpA* locus

The restriction maps in Fig. 1A demonstrate the sequence arrangements of an intact copy (3.7-kb *Hin*dIII clone) and a pseudocopy (3.6-kb *Hin*dIII clone) of the *atpA* gene found in mitochondria from sugarbeet line 'TK81-0'. The 'TK81-0' *atpA* and the pseudocopy share a common segment of 406 bp which contains 11 bp of the C-terminal coding region and 395 bp past the *atpA* stop codon. If the 406-bp repeat could act as a region of recombination, we would expect to find it present in four genomic environments: as diagrammed in Fig. 1A, the two parental *Hin*dIII fragments of 3.7 kb and 3.6 kb could give rise to the 6.0-kb and 1.3-kb *Hin*dIII fragments through a homologous recombination event.

Interestingly, the 6.0-kb recombinant fragment [I-12CMS(2) *atpA* arrangement] was found in male-sterile line I-12CMS(2) that had a cytoplasmic origin in wild *Beta* beets (Senda et al. 1993, see Fig. 1A), while no trace of the reciprocal 1.3-kb recombinant fragment could be detected on 'normally' exposed autoradiograms (data not shown). In order to ascertain whether such recombinant configurations are actually present in the 'TK81-0' genome, we used a repeat internal probe (250-bp *Sma*I-*Eco*RI fragment; see Fig. 1A) to hybridize Southern blots carrying *Hin*dIII digests of 'TK81-0' mtDNA. As shown in Fig. 1B, prolonged exposure of the autoradiograms enabled us to detect the presence of two fragments (6.0 kb and 1.3 kb) representing the predicted recombination products in 'TK81-0'. In contrast, no hybridization signals corresponding to



the 3.7-kb and 1.3-kb fragments could be obtained for I-12CMS(2) mtDNA (Fig. 1B). The repeat internal probe revealed an additional weak signal (2.0 kb) in mtDNA digests of 'TK81-0' and I-12CMS(2) (Fig. 1B), but its source could not be determined.

The sequence data of the intact *atpA* and the pseudo-*atpA* from 'TK81-0' (Senda et al. 1993) were used to synthesize oligonucleotides that were subsequently employed for PCR amplification of the recombination products. Four primers (primers A–D; see Materials and methods) were designed to anneal to the unique sequences which lay outside the 406-bp repeat (Fig. 1A). When the two primers A and D were used with 'TK81-0' mtDNA, an 800-bp region specific to the 6.0-kb *Hin*dIII recombinant fragment was amplified (Fig. 2). Moreover, PCR experiments using primers C and B allowed the detection of an 850-bp signal specific to the reciprocal 1.3-kb recombinant fragment (Fig. 2). The nature of the PCR products was checked by sequencing the 800-bp and 850-bp amplified fragments (data not shown). We can conclude that the recombinant configurations encompassing the 6.0-kb *Hin*dIII fragment [I-12CMS(2) *atpA* arrangement] and the 1.3-kb *Hin*dIII fragment are present in substoichiometric amounts in 'TK81-0' mtDNA. On the other hand, no amplification products were obtained when two pairs of primers (A/B and C/B) were used with I-12CMS(2) mtDNA (Fig. 2). The results thus led us to suppose that the I-12CMS(2) *atpA* arrangement originated in a homologous recombination event mediated by the 406-bp repeat in the common ancestral genome of 'TK81-0' and I-12CMS(2) and was preferentially amplified in the lineage that gave rise to the I-12CMS(2) genome.

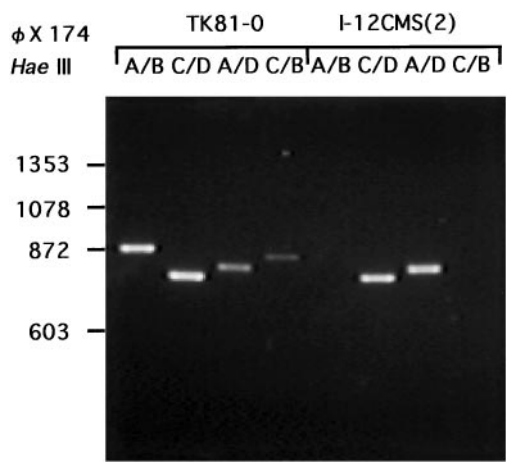


Fig. 2 PCR amplification of the predicted products of recombination across the 406-bp repeat. The experiments were performed using mtDNAs from TK81-0 and I-12CMS(2) as templates, and primers A–D (see Fig. 1A). *Numbers* on the *left* indicate the sizes of the øX174 DNA *Hae*III fragments used as molecular weight markers in basepairs

The 406-bp repeat is part of a 6.5-kb repeat

An examination of the sequences upstream of the *atpA* pseudogene revealed significant homology with the mitochondrial gene encoding a transmembrane subunit of an ABC transporter (Bonnard and Grienenberger 1995; Jekabsons and Schuster 1995). The detailed characterization of this sugarbeet gene will be presented in a separate paper. On the other hand, fine mapping downstream of the *atpA* reading frame indicates that I-12CMS(2) *atpA* and the pseudo-*atpA* copy [from both 'TK81-0' and I-12CMS(2)] share identical restriction maps at least up to 6.5 kb downstream of the 406-bp repeat. A 1.7-kb *Eco*RI-*Hin*dIII fragment within the 6.5-kb repeat (Fig. 1A) was used to screen the phage library of I-12CMS(2) mtDNA. We could isolate phage clones representing four configurations that corresponded to the reciprocal exchange of the 5′ and 3′ sequences flanking the 6.5-kb repeat (the ends of the repeat are described as 5′ or 3′ relative to the orientation of the *atpA* gene), evidence that the 6.5-kb repeat is recombinationally active (Fig. 3).

Recombination across the 7-bp repeat

Small et al. (1989) proposed a three-step model whereby two different sets of short repeats could give rise to new long repeats in plant mitochondrial genomes. This model has been invoked to explain the duplication of the 12-kb segment bounded by the two original short repeats in the normal fertile maize mtDNA. An another example illustrating the same mechanism is the mitochondrial genome reorganization in a fertile revertant line (V3) derived from the maize cytoplasmic male-sterile type T (Fauron et al. 1990). With this in mind, we

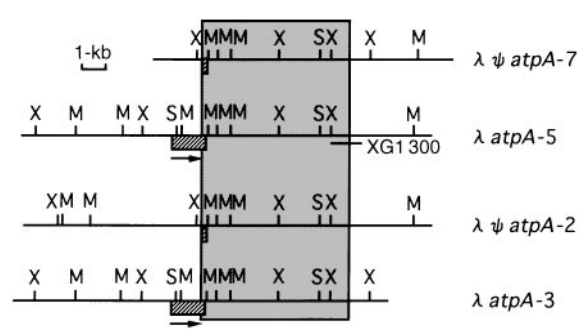


Fig. 3 Restriction maps of the four different genomic environments of the 6.5-kb repeat in I-12CMS(2) mtDNA. The *hatched box* represents the *atpA* coding sequence, while the *stippled box* shows the 6.5-kb repeat. The polarity of transcription is indicated by the *horizontal arrow*. The location and extent of a 1.3-kb *Xho*I-*Bgl*II subfragment which was used as a probe for the colony hybridization (XG1300, see Fig. 4A) is also shown. Restriction sites are: *M Sma*I, *S Sal*I, *X Xho*I

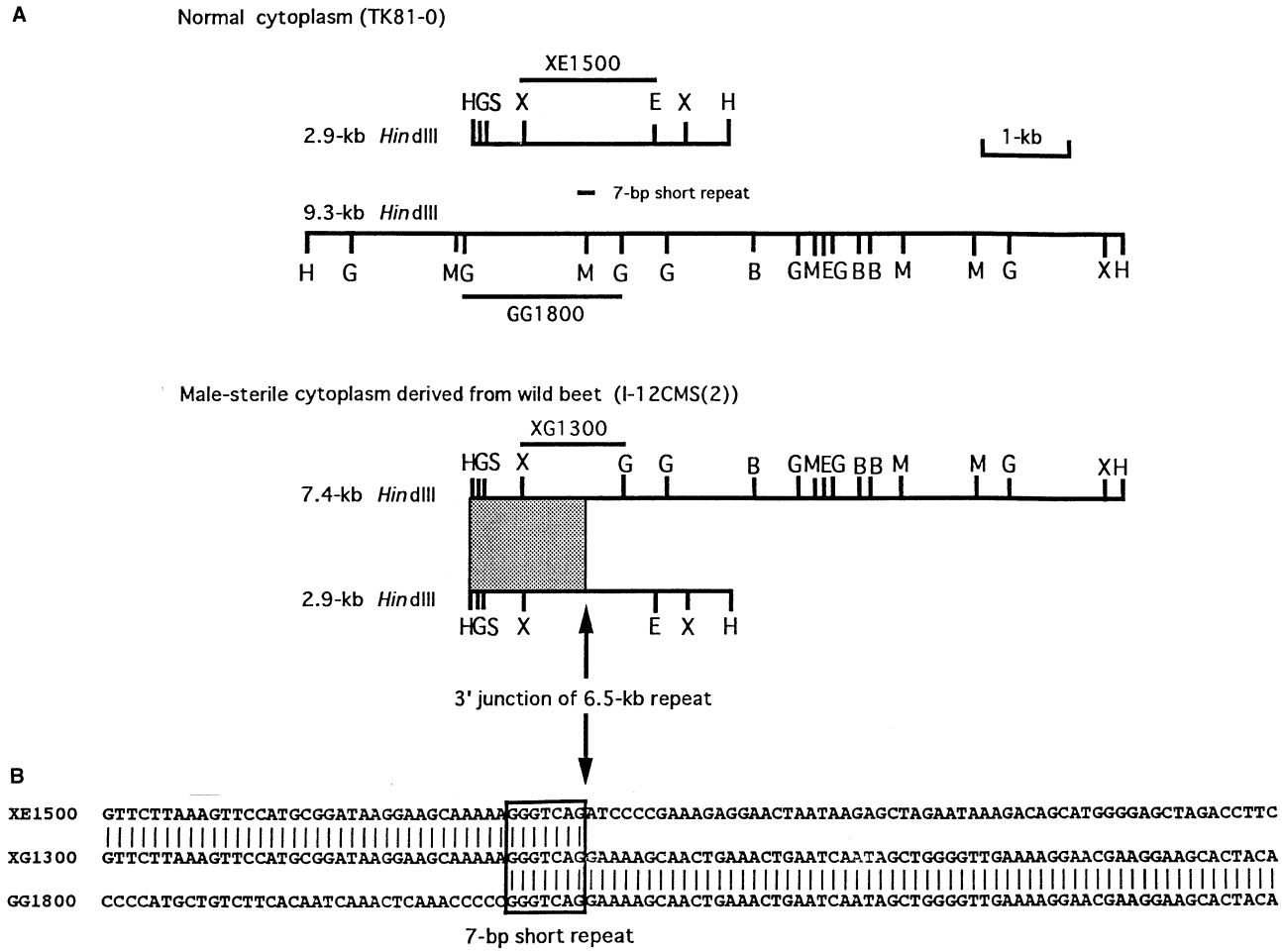


Fig. 4A,B **A** Restriction maps of the four *Hin*dIII fragments hybridized with a 1.3-kb *Xho*I-*Bgl*II subfragment (XG1300) spanning the 3′ junction of the 6.5-kb repeat. Using this probe, we retrieved the 2.9-kb and 9.3-kb *Hin*dIII clones from the TK81-0 mtDNA library, and the 7.4-kb and 2.9-kb *Hin*dIII clones from the I-12CMS(2) mtDNA library. Restriction sites are: *B Bam*HI, *E Eco*RI, *G Bgl*II, *H Hin*dIII, *M*, *Sma*I, *S Sal*I, *X Xho*I. **B** Sequence alignments of the homologous portions of 1.5-kb *Xho*I-*Eco*RI subclone (XE1500), 1.3-kb *Xho*I-*Bgl*II subclone (XG1300) and 1.8-kb *Bgl*II subclone (GG1800). The 7-bp sequence common to all three sequences is *boxed*. The point of the 3′ terminus of the 6.5-kb repeat is denoted by the *vertical arrow*. The sequence data reported here have been submitted to the EMBL/GeneBank/DDBJ Data Libraries under the accession numbers AB007030 (XE1500), AB007032 (XG1300) and AB007031 (GG1800)

determined the nucleotide sequences spanning the 3′ end of the 6.5-kb repeat. A 1.3-kb *Xho*I-*Bgl*II subfragment of the phage *λatpA*-5 (Figs. 3 and 4) was used as a probe to identify two clones (7.4-kb and 2.9-kb *Hin*dIII clones) containing the 3′ borders of the 6.5-kb repeat copies from a plasmid library of I-12CMS(2) mtDNA (Fig. 4). DNA sequence analysis located the 3′ end of the 6.5-kb repeat (Figs. 3 and 4).

The same probe was also used to identify the 9.3-kb and 2.9-kb *Hin*dIII clones from the 'TK81-0' mtDNA library (Fig. 4). Comparative restriction mapping revealed a 9.3-kb 'TK81-0' fragment to be identical to a 7.4-kb I-12CMS(2) fragment beyond the 3′ end of the 6.5-kb repeat. Moreover, sequencing studies showed that the terminal heptanucleotides, GGGTCAG, of the 6.5-kb repeat are also shared by the two TK81-0 clones in question (Fig. 4). A crossover in these 7-bp regions in 'TK81-0' mtDNA could give rise to the configuration seen in the 7.4-kb I-12CMS(2) clone. The sequences at the extreme termini of the 6.5-kb repeat of I-12CMS(2) proved to exist as short repeats (406-bp and 7-bp repeats) in 'TK81-0'.

Provided that the progenitor is the mitochondrial genome of 'TK81-0', one could hypothesize that (1) two copies of a 406-bp repeat and a 7-bp repeat in the progenitor master chromosome would recombine independently to produce four subgenomes, (2) two of the resultant subgenomes would subsequently recombine to create a new genome [I-12CMS(2)-like genome] containing the 6.5-kb repeat which is surrounded by the two original short repeats and (3) the sequence (the 3′ flanking region of 'TK81-0' *atpA*) between the other two copies of the short repeats might be lost (see Small et al. 1989). Failure of the PCR amplification from

I-12CMS(2) mtDNA using two sets of primers, A/B and C/B, suggests that the sequence downstream from the 'TK81-0' *atpA* gene is missing in the I-12CMS(2) genome (see Fig. 2). This was further confirmed by the Southern hybridization using a 600-bp *Eco*RI-*Hin*dIII end-subfragment ('TK81-0' *atpA* 3′ flank) of the 3.7-kb *Hin*dIII clone from 'TK81-0' (see Fig. 1A), which failed to detect homologous sequences in I-12CMS(2) mtDNA (data not shown). It thus appears that the sequential recombination events across the *atpA*-associated repeated sequences, followed by selective amplifications and/or losses of genomic recombinant forms, provide the basis for the mitochondrial genome diversity in beets.

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