Theor Appl Genet (1995) 90: 1087–1093

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Organization of the mitochondrial *Cob* 2 pseudogene in different lines of rice

Received: 16 November 1994 / Accepted: 8 December 1994

Abstract In the fertile rice line IR 36 there are two copies of the apocytochrome b (*cob*) gene: a functional copy, *cob* 1, and a pseudogene, *cob* 2 (Kaleikau et al. 1992). In a survey of diverse rice lines, we found that *cob* 2 was absent in the wild abortive(WA)-type cytoplasmic male-sterile cytoplasm, but was present in the fertile lines. While *cob* 1 was conserved among all the lines, fertile and sterile, the *cob* 2 region was different in the fertile lines tested. The 5′ regions of most *cob* 2 loci were similar to *cob* 1 (about 4 kb of the flanking region and most of the coding region), but the 3′ region varied among different fertile lines. The point of divergence, the break-point, from the *cob* 1 sequence was conserved in all the *cob* 2 regions tested. In all the *cob* 2 regions, this break-point seems to be linked to the variable region of *cob* 2 through a conserved 192-bp segment, which is not a part of *cob* 1. It is proposed that the *cob* 2 regions could have been produced by recombination or insertion events involving *cob* 1 and the 192-bp segment which is present at different locations in the mitochondrial genomes of the various rice lines.

Key words Apocytochrome b · Mitochondria · Pseudogene · Rice

Introduction

Rearranged genetic regions are a common feature of higher-plant mitochondrial (mt) genomes (Lonsdale 1989). In many instances, sequences of expressed genes are involved in such rearrangements (Bailey-Serres et al. 1986; Schuster and Brennicke 1986; Morikami and Nakamura 1987; Fragoso et al. 1989). Most of the rearranged, or chimeric, genetic regions are transcriptionally silent; however, the chimeric *T-urf 13* region in maize (Dewey et al. 1986) and the *S-pcf* region in petunia (Young and Hanson 1987) are expressed and seem to play a role in cytoplasmic male-sterility (CMS). Homologous recombination between sets of repetitive sequences is proposed to be the primary cause of the rearrangements that create chimeric genes (Lonsdale 1989). All plant mt genomes that have been mapped, except that of *Brassica hirta* (Palmer and Herbon 1987), have been found to have recombinogenic repeat sequences (Newton 1988). The size of the repeat regions involved in recombination varies considerably; even a region as small as a 7-bp repeat has been implicated in the formation of a chimeric *atp* 6 gene in rice mitochondria (Kadowaki et al. 1990). Although most of the variation in plant mt genomes can be explained by the homologous-recombination model, there is only descriptive evidence for this mechanism (Fauron et al. 1992). Thus, other mechanisms may contribute to the complexity of plant mt genomes.

The fertile rice mt genome contains two copies of the *cob* gene, an intact copy, *cob 1*, and a pseudogene, *cob* 2 (Kaleikau et al. 1992). *Cob 2* is a chimeric gene which is identical to *cob 1* through most of the coding region as well as in the 5′ flanking region. The point of divergence is in the C-terminal region, 63 codons upstream of the *cob 1* translation termination codon. We have examined the organization of *cob 1* and *cob 2* regions in different lines of rice, and found that *cob 1* organization is highly conserved whereas that of *cob 2* varied between rice lines. The N-terminal regions of *cob 2* were conserved, but the sequence beyond the break-point was characteristic of individual lines.

Materials and methods

Plant material

The male-sterile rice line, IR 62829A, its maintainer, IR 62829B, two restorer lines, IR 9761–19-IR (R) and IR 30864, the fertility re-

Communicated by R. Hagemann

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stored line, IR 62829A × IR 9761–19-IR (H line), and cultivars IR 36 and IR 64, were used in this study. The male-sterile line has the wild abortive (WA)-type of cytoplasm while all the other lines have the normal or fertile cytoplasm.

Isolation of mtDNA and restriction digestion

The crude mt fraction was isolated from a tissue homogenate of 7-day-old etiolated shoots in HB buffer [400 mM mannitol, 1 mM EGTA, 0.5% (w/v) cysteine and 10 mM TES, pH 7.2; to which 0.1% (w/v) bovine serum albumin and 50 mM β-mercaptoethanol were added just prior to use] through differential centrifugation as described by Kemble and Bedbrook (1980). The crude mt suspension was treated with DNaseI to remove contaminating nuclear DNA and intact mitochondria were purified on a discontinuous sucrose gradient (Narayanan et al. 1993). The mitochondria were lysed and the mtDNA was isolated according to Mulligan et al. (1991).

For restriction digestion, approximately 3 g of the mtDNA was suspended in the appropriate buffer and incubated with different restriction enzymes, as recommended by the suppliers.

Gel electrophoresis and Southern hybridization

Horizontal agarose-gel electrophoresis was carried out using 0.8% agarose gels in 1×TBE (89 mM Tris-Borate, 1 mM EDTA, pH 8.3) at a constant field strength of 2 V/cm until the bromophenol blue dye-marker reached the anodic end. The gels were stained with ethidium bromide (0.1 g/ml) and destained in the electrophoresis buffer for about 30 min prior to visualizing the bands on a UV transilluminator.

The DNA resolved by gel-electrophoresis was blotted onto an uncharged nylon membrane (Flash^{TM}, Stratagene) by capillary transfer (Southern, 1975) and fixed either by baking the filter at 80 °C for 2 h or by UV cross-linking. The different *cob* regions indicated in Fig. 1 were used as probes for hybridization.

The probes were labelled by incorporation of biotinylated UTP using the Flash^{TM}/Prime-It, Random Primer Labelling Kit (Stratagene); hybridization and chemiluminescent detection of the hybridization pattern was carried out using the Flash^{TM} Detection System (Stratagene). For labelling and detection the protocol outlined by the manufacturer was used.

Polymerase chain reaction (PCR) analysis

The three synthetic primers used for PCR amplification reactions were:

a) 5′-G C G G G G G G T G T A G C C-3′
b) 5′-G T A T G T G C G T A G T T C A A G-3′
c) 5′-C G A A G T A G C A G G G G C-3′

The relative positions of these primers are indicated in the map of the *cob* pseudogene region (Fig. 1B).

The PCR amplification was performed in a 50-µl reaction mixture containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 M each of dATP, dGTP, dTTP and dCTP, 0.1 mg/ml gelatin, 20 p-mole of each primer, 2.5 units of *T. aquaticus* DNA polymerase enzyme and 1–200 ng of template DNA. The template DNA was either mtDNA or DNA cloned in the plasmid vector pBluescript SK$^+$ (Stratagene). The samples, overlaid with mineral oil, were subjected to 40 amplification cycles in a programmable thermal cycler (COY Corporation). Each amplification cycle consisted of a denaturing step at 94 °C for 1 min, except for the first cycle when it was extended to 3 min, an annealing step at 60 °C for 90 s, and an extension step at 72 °C for 2 min for the first cycle and progressively adding 2 s for every subsequent cycle.

The amplified PCR products were resolved on a 1.5% agarose gel and visualized on a UV transilluminator after staining with ethidium bromide.

Sequence analysis

Nucleotide sequencing of the single-stranded plasmid DNA generated by the helper phage VCM S13 (Stratagene) (Vieira and Messing 1987) was performed by the dideoxy method (Sanger et al. 1977) using the Sequenase V.2 kit (United States Biochemicals). The primers used in these sequencing reactions were a and b (Fig. 1). Sequence analysis was performed on an IBM compatible PC AT computer using DNASIS (Hitachi Software Engineering Co., Ltd.).

Results

The pseudogene *cob 2* has a different organization in fertile lines

Our initial results indicated that while the organization of the functional *cob 1* gene was conserved in different rice lines, the organization of the *cob 2* pseudogene varied. This observation was investigated in detail by Southern analysis using IR 36 probes specific to *cob 1* and/or *cob 2*. Probe 1 was a *SalI*/*Eco*RI fragment that consisted of regions common to the normal and pseudogene copies of *cob*, ie. the 5′ flanking region and most of the ORF (Fig. 1A). This probe hybridized to at least two fragments in the fertile lines B and R but detected only one band in the male-sterile cytoplasm (Fig. 2A). This indicates the presence of two *cob* analogs in fertile lines (the additional band in the *Hin*dIII-digested DNA was caused by an internal *Hin*dIII site in the probe). Similar results were obtained with different *cob* regions as probes (Table 1). The restriction fragments carrying the normal *cob* copy, the 4.8-kb *Eco*RI fragment, the 3.4- and 2.0-kb *Hin*dIII fragments, and the 13.8-kb *Bam*HI fragment, were all conserved in the different lines (Fig. 2A, Table 1: probes 1–4). The *cob 1* organization was also the same in the other lines of rice, IR 36, IR 64 and IR 30864, that were analysed (data not shown).

Probe 4 derived from IR 36 encompasses the region covered by probe 1 (region common to *cob 1* and *cob 2*) as well as the 3′ region specific to *cob 2*. Thus, this probe was expected to detect all the bands corresponding to *cob 1* and *cob 2* (i.e. the same fragments detected by probe 1 in Fig. 1A); in addition, it should also detect *cob 2*-specific sequences if present elsewhere in the genome. Probe 4, as expected, detected all the bands that were identified by probe 1 plus a 1.7-kb *Eco*RI fragment, a 4.8-kb *Hin*dIII fragment, and a 6.5-kb *Bam*HI fragment in the B- and H-line mt genomes (Fig. 2B). Because these additional bands represent another region of the mt genome with *cob 2*-specific sequences, it is possible that *cob 2* might have arisen by recombination of this region with *cob 1*. No such additional band was detected in the R-line mtDNA indicating that the segment that recombined with *cob 1* in the R line is different from that in IR 36 from which probe 4 is derived. Significantly, the pseudogene copy was not found in the WA cytoplasm but the region of IR 36 that recombined with *cob 1* to form *cob 2* in that line is indeed present. Thus it seems that the recombinogenic region is present in the male-sterile line, but that the recombination event that results in the formation of a chimeric gene has not taken place.

Table 1 Size of the rice mitochondrial DNA restriction fragments hybridizing to different *cob* probes. See Fig. 1 for details of probes

Probe number	Rice line (s)	Size of hybridizing fragment (kb)											
		*Eco*RI			*Hin*dIII				*Bam*HI				
1	A and H		4.8			3.4		2.0	13.8				
	B	8.0	4.8			3.4	2.2	2.0	13.8	10.0			
	R	6.5	4.8			3.4	2.4	2.0	13.8	11.2			
2	A, B R and H			2.3				2.0	13.8				
3	A and H		4.8	2.3				2.0	13.8				
	B	8.0	4.8	2.3			2.2	2.0	13.8	10.0			
	R	6.5	4.8	2.3			2.4	2.0	13.8	11.2			
4	A and H		4.8	1.7	4.8	3.4		2.0	13.8		6.5		
	B	8.0	4.8	1.7	4.8	3.4	2.2	2.0	13.8	10.0	6.5		
	R	6.5	4.8			3.4	2.4	2.0	13.8	11.2			
5	A and H			1.7	4.8						6.5		
	B	8.0		1.7	4.8		2.2			10.0	6.5		
	R	ND			ND				ND				

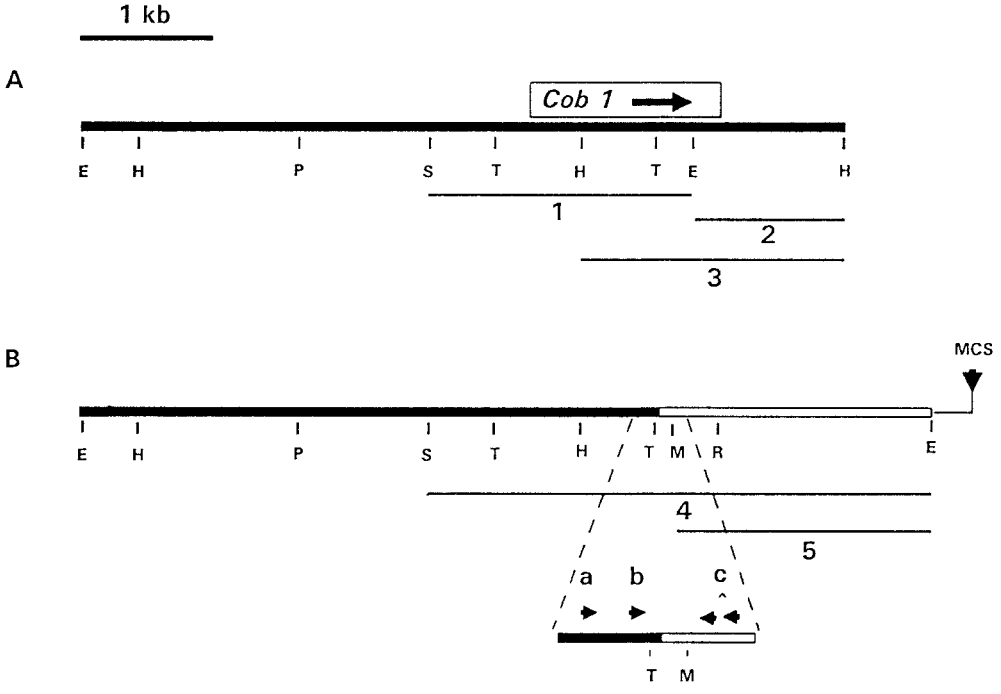

Fig. 1A, B Maps of the *cob* regions in the mitochondrial genomes of rice line IR 36 showing the location of different probes (*1–5*) used for Southern analysis and the positions of the primers (*a*, *b* and *c*) used for PCR analysis of the break-point region. The *vertical lines* indicate restriction sites for the enzymes *Eco*RI (*E*), *Hin*dIII (*H*), *Pst*I (*P*), *Sal*I (*S*), *Taq*I (*T*), *Sma*I (*M*) and *Eco*RV (*R*). **A** apocytochrome b gene, *cob 1*; **B** pseudogene, *cob 2*. The *solid line* in **B** represents the region that is colinear with *cob 1*

Physical maps of the different *cob* regions

The physical maps of the *cob 2* regions in the native B- and R-line mtDNA was deduced from the sizes of fragments hybridizing to different *cob* probes (Table 1). The maps of three *cob 2* regions are given in Fig. 3. A segment of about 4 kb, between the *Hin*dIII and the *Taq*I sites, appears to be conserved among the different *cob* regions. The break-point at which the *cob 2* sequence diverges from the *cob 1* sequence (Kaleikau et al. 1992), immediately downstream from the *Taq*I site, is also conserved in the different rice lines. The 3′ *cob 2* sequence after the break-point is the same in IR 36 and the B line but it is different in the R line. In the B line there also appears to be a divergence in the *cob 2* region from that of *cob 1* in the 5′ upstream region. Whereas probe 1 hybridized to a 8.0-kb *Eco*RI fragment from the *cob 2* region in the B line (Fig. 2, Table 1), it hybridized to a 6.0-kb fragment from the *cob 2* region in IR 36 (Narayanan et al. 1993). Probes 3, 4 and 5 also gave a similar result (Table 1) indicating that the *cob 2* region from the B line diverged from *cob 1* in the 5′ region. Divergent flanking regions suggest that the chimeric *cob 2* could have resulted from the insertion of a segment carrying part of *cob 1* into different locations in the rice mt genome of individual lines.

PCR amplification across the break-point

To determine if the *cob 2* break-point in the different rice lines is conserved, a PCR experiment was designed (Fig. 1B). Primer sequences were based on the *cob 2* sequence

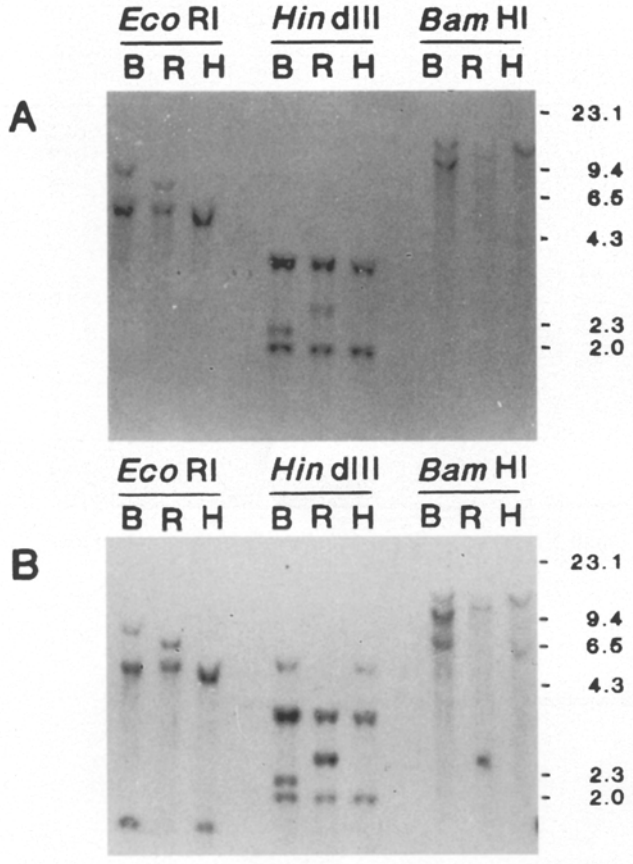

Fig. 2A, B Southern analysis of the mt genomes using *cob*-region probes. *Lane B* maintainer line, IR 62829B; *lane R* restorer line, IR 9761–19-IR; *lane H* restored line, IR 62829A×IR 9761–19-IR. The hybridization pattern for the male-sterile line IR 62829A was exactly similar to that of *H*. **A** hybridization pattern using probe 1 of Fig. 1A. **B** hybridization pattern using probe 4 of Fig. 1B

from IR 36 (Kaleikau et al. 1992) and included primers a and b upstream of the break-point and c from the downstream region. The PCR products using primers b and c are shown in Fig. 4. Primer c anneals in a region repeated three times and, therefore, three amplified products are expected – a 491-bp, a 453-bp and a 261-bp fragment. The major amplification products from IR 36 and the B line were the 491- and 453-bp fragments; the 261-bp fragment was also amplified (Fig. 4, lanes 1 and 4). A similar result was obtained with primers a and c. This confirms that *cob 2* of IR 36 and the B line starts diverging from the *cob 1* sequence at the same position. These fragments were also amplified from the R-line mtDNA along with two additional fragments of about 340 and 530 bp. This suggests that the primer sequence is repeated in additional regions of the R-line mt genomes. There was also weak amplification of the expected three bands in the A line (Fig. 4, lane 3). But in the H line, which has the same cytoplasm, there was no amplification of any band. Therefore, we suspect that there could have been a low-level contamination of the A-line mtDNA with that of some fertile line, most probably due to field admixture of A-line seeds with that of its maintainer, the B line. However, this contamination is not detected in the Southern analysis. Quite unexpectedly, only two amplication products, of about 261- and 300-bp, were obtained from the cloned *cob 2* region of IR 36 (Fig. 4, lane 2). This suggests that the clone in *E. coli* might have undergone a deletion.

A 192-bp sequence, immediately 3′ of the *cob 2* break-point is conserved

Sequencing of the break-point region in the IR 36 *cob 2* clone confirmed that it had indeed undergone a deletion;

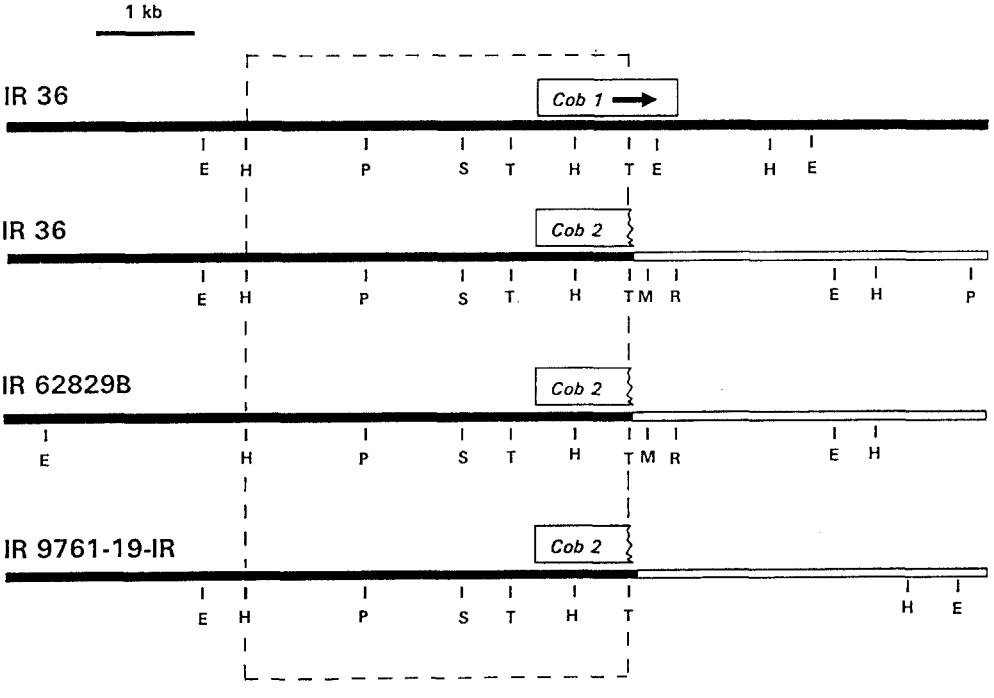

Fig. 3 Physical maps of *cob* regions from the different lines of rice. The conserved portion between the different *cob* regions is *boxed by broken lines*. The *vertical lines* denote restriction sites for different enzymes; *E Eco*RI; *H Hin*dIII; *P Pst*I; *S Sal* I; *T Taq* I; *M Sma*I, and *R Eco*RV

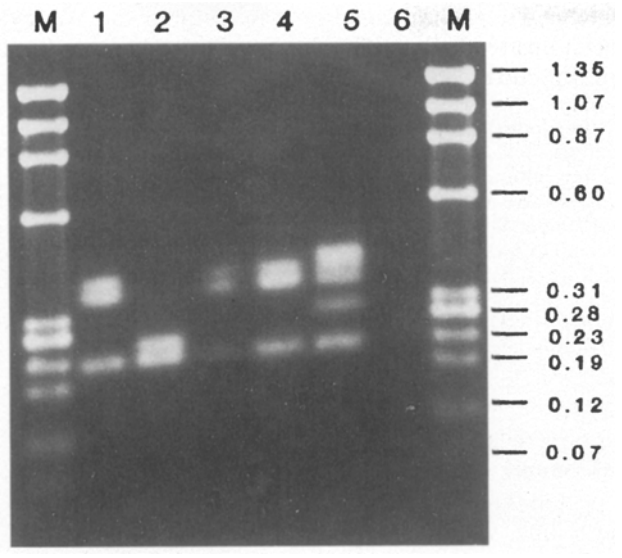

Fig. 4 PCR amplification of the break-point region of the *cob 2* pseudogenes from the mt genomes of different lines of rice. *Lane M* marker (X174 DNA-*Hae*III digest); *lane 1* IR 36; *lane 2* IR 36, *cob 2* clone; *lane 3* IR 62829A; *lane 4* IR 62829B; *lane 5* IR 9761–19-IR; *lane 6* IR 62829A×IR 9761–19-IR. The molecular sizes are marked in kilobases

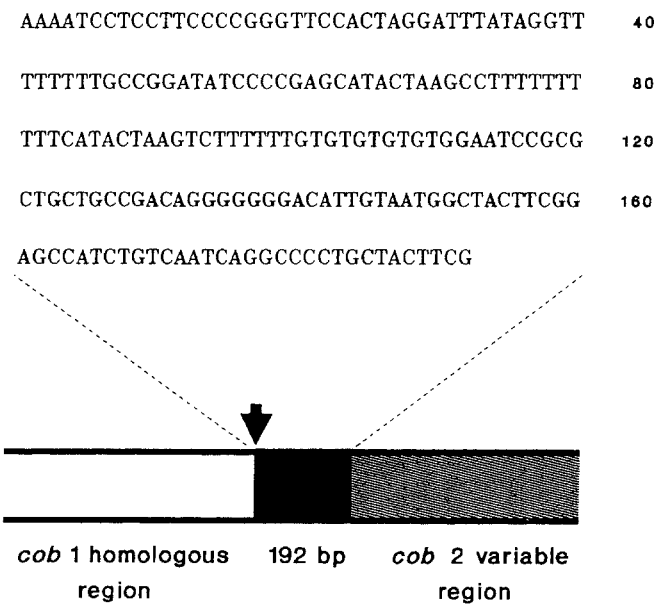

Fig. 5 The position and sequence of the 192-bp conserved region in relation to the break-point (indicated by *arrow*)

one copy of a 192-bp segment, which is present in two copies immediately downstream from the break-point in the native mtDNA (Kaleikau et al. 1992), has been precisely removed. A data-base search for the different rice *cob 2* sequences showed that this region has been sequenced only in one other rice line, Chinsurah Boro II (EMBL Acc. No. X53711). In this line too one copy of the 192-bp segment is conserved in both sequence and position. Interestingly, the conserved 192-bp segment of the *cob 2* pseudogene (Fig. 5) had no homology with the *cob 1* sequence. The *cob 2* sequence of Chinsurah Boro II differed from that in IR 36 further downstream from the 192-bp conserved sequence. Southern hybridization using the 192-bp region as a probe confirmed that this sequence was present in the *cob 2* regions of both B and R lines (data not shown). Its position, immediately downstream from the break-point, in at least two different rice lines suggests its involvement in the formation of the chimeric region. PCR amplification of this 192-bp segment using end primers indicated that this sequence was present in all the fertile rice lines tested, namely IR 36, B line, R line, IR 30864 and IR 64 (data not shown).

Discussion

Many chimeric genetic regions have been discovered in plant mt genomes (Lonsdale 1989). At least two such chimeric regions, the *T-urf 13* locus in maize (Dewey et al. 1986) and the *S-pcf* locus in petunia (Young and Hanson 1987), are expressed and are associated with CMS. The chimeric *cob* region, or *cob 2*, was first discovered and characterized in rice by Kaleikau et al (1992) in the variety IR 36. Rearranged genetic regions involving *cob* sequences have also been reported in other species including *Triticum aestivum* (Saalaoui et al. 1990), *Phaseolus vulgaris* (Chase and Ortega 1992), and the liverwort *Marchantia polymorpha* (Oda et al. 1992).

We analyzed the physical organization of the chimeric *cob 2* region in different lines of rice with a view to gaining some insight into the mechanism that generates such regions in plant mt genomes. The pseudogene *cob 2* was found in different forms in the various lines of rice. All *cob 2* copies appear to diverge from the normal *cob 1* region at the same break-point, which was the 1003rd base from the translation start site of *cob 1* (Kaleikau et al. 1992). The region downstream from this position was different in the several *cob 2* sequences analyzed (Fig. 3). There was some indication for instability at the 5′ flanking region also, because we found an *Eco*RI polymorphism between the B line and the other lines at the 5′ flanking region. The *cob 2* copy was not detected by Southern hybridization in the WA-CMS cytoplasm.

Chimeric regions in plant mitochondria are believed to originate through homologous recombination across sets of repeats (Lonodale 1989). Repetitive DNA is ubiquitous in plant mt genomes, and there is considerable evidence for recombination involving these sequences (Newton 1988; Lonsdale 1989; Fauron et al. 1992). If homologous recombination between repeats is responsible for *cob 2* organization in the different lines, then a *cob 1*-homologous sequence had to be present in more than one location in the genome, these locations being different in the different rice

lines. In addition, each *cob 2* region has to be a product of double recombination to have both flanking regions different from the *cob 1* flanking regions. Southern hybridization with probes having the *cob 1* sequence immediately downstream from the break-point did not detect any other region of homology in the IR 36 mt genome. It is notable that *cob 2* organization is different even between rice lines of quite recent origin. This suggests that the creation of *cob 2* regions is not an early event that occurred during the evolution of present-day rice. On the other hand, the rearrangements that produce the different *cob 2* regions appear to be independent and continuing events. Further, the presence of the precursor sequences does not automatically lead to the creation of the chimeric region; this is clearly demonstrated in the case of the A line where *cob 2* was not detected by Southern hybridization even though the normal *cob* region and the region that could have recombined with *cob 1* to produce *cob 2* in IR 36 were both present (Fig. 2B). Therefore, in addition to homologous recombination there may be other mechanisms that can produce chimeric genetic regions like *cob 2* in different rice lines.

There are many instances where direct evidence for homologous recombination leading to the creation of gene rearrangements in plant mitochondria is wanting. For example, the chimeric *S-pcf* locus has a portion of the *atp 9* sequence, but there is as yet no evidence that the *atp 9*-homologous sequence serves as a recombination repeat (Hanson 1991). Incontrovertible evidence for homologous recombination is the recovery of different genomic environments in repeat-containing clones. For instance, recombination across a two-copy repeat region should give rise to four genomic environments. The CMS-associated chimeric copy of *atp 6* in rice may have arisen by recombination across a 7-bp repeat region (Kadowaki et al. 1990). However, all four genomic environments could not be recovered in clones in spite of extensive screening of the mt genomic library. This could have been because of a problem in cloning but, again, the existence of alternative mechanisms for the generation of rearranged genetic regions in plant mt genomes cannot be ruled out.

It is note-worthy that a 192-bp sequence (Fig. 5) is conserved in the different *cob 2* regions, not only in sequence but also in location. Since this region is not part of the *cob 1* sequence it is unlikely that it is a recombination repeat. Although the mechanism of formation of the different *cob 2* regions is still unclear, the 192-bp sequence seems to play a definite role in the process.

One of the lines used in this study was a CMS line with WA cytoplasm. The pseudogene *cob 2* is present in all fertile indica lines that we examined, but was not detected in the male-sterile WA cytoplasm. A fertile japonica cultivar, Nipponbare, also does not seem to have the pseudogene copy (Iwahashi et al. 1992). The *cob 2* copy is reported to be present in another CMS cytoplasm, the BT cytoplasm from Chinsurah Boro II (Yamato et al. 1992). On continuous tissue culture, the *cob 2* copy disappeared in the sterile line with the BT cytoplasm, A-58CMS, but not in the fertile Chinsurah Boro II. It remains to be investigated whether the physiological insufficiency of the WA cytoplasm that leads to pollen abortion is also responsible for limiting the creation of the chimeric *cob 2* region.

Acknowledgements We thank M. Mahadevappa, University of Agricultural Sciences, Bangalore, and S.R. Sree Rangasamy, Tamil Nadu Agricultural University, Coimbatore, for seeds of the different rice lines. We are grateful to Virginia Walbot and Ed Kaleikau, Stanford University, for gene clones and comments on the manuscript. The mtDNA work in our laboratory is supported by grants from the Rockefeller Foundation, USA (grant no. RF 89026 # 34), and The Department of Biotechnology, Govt. of India (grant no. BT/AB/01/002/89). V.V.S. is a recipient of a Govt. of India CSIR fellowship.

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