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ORIGINAL PAPER

Substoichiometric shifting in the fertility reversion of cytoplasmic male sterile pearl millet

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Abstract Cytoplasmic male sterility (CMS) represents an important agricultural trait in pearl millet [*Pennisetum glaucum* (L.) R. Br.] with a value to the seed industry in facilitating economical hybrid seed production. Among the CMS systems available in millet, the A1 source is the most commonly used for hybrid production, but it can undergo low frequency reversion to fertility. Plant mitochondrial genomes are highly recombinogenic, becoming unstable and prone to ectopic recombination under conditions of tissue culture, somatic hybridization, or interspecific crossing. Similarly, CMS systems prone to spontaneous fertility reversion experience sporadic mitochondrial genome instability. We compared mitochondrial genome configurations between the male-sterile A1 line and fertile revertants of pearl millet to develop a model for millet mitochondrial genome reorganization upon reversion. Relative copy number of a subgenomic molecule containing the *CoxI-1-2* junction region, a component of the recombination process for reversion, is amplified tenfold following reversion, relative to the CMS A1 line. We propose that increased copy number of this molecule in a small number of cells or at low frequency triggers a recombination cascade, likely during reproductive development. The proposed recombination process initiates with ectopic recombination through a 7-bp repeat to produce a novel *CoxI-3-2* junction molecule and an unstable recombination intermediate. Subsequent intra-molecular recombination stabilizes the intermediate to form a new copy of *CoxI* accompanied by a deletion. This study furthers the argument that substoichiometric shifting within the plant mitochondrial genome plays an important role in the evolution of the mitochondrial genome and plant reproductive dynamics.

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Introduction

Pearl millet is one of the most important staple foods in the arid and semi-arid regions of Africa and India, and demonstrates marked heterosis in seed yield of hybrids. A favorable cytoplasmic male sterility (CMS) system for hybrid seed production requires a CMS line (A line), its fertility restorer line (R line) and a sterility maintainer line (B line). Among the CMS systems identified in pearl millet during the past 40 years, the A1 line is the most commonly used, with fertility restorers for this cytoplasm prevalent in pearl millet germplasm. Spontaneous fertility reversion in the A1 line of pearl millet occurs rarely (0.01% frequency), observed as a single pollen-shedding panicle surrounded by fully male-sterile panicles in a single CMS plant (Smith et al. 1987). Mitochondrial DNA (mtDNA) polymorphisms that distinguish spontaneous revertants from maintainer B and CMS A1 lines of millet have been reported to include

CoxI-related regions of the genome (Smith et al. 1987; Delorme et al. 1997). We capitalized on this observation to investigate the nature of the fertility reversion process in this line.

While higher plant mitochondrial genomes are known to undergo recombination and to comprise multipartite structures, the role of ectopic recombination in genome evolution is not clear (Mackenzie 2005). Recombination at repeats of a given size range (ca. 50–500 bp) is actively suppressed by at least three nuclear genes (Abdelnoor et al. 2003; Zaegel et al. 2006; Shedge et al. 2007). One of these nuclear genes, *Msh1*, appears to suppress recombination at nearly every repeat of this class within the Arabidopsis mitochondrial genome (Arrieta-Montiel and Mackenzie, unpublished). The recombination that occurs with disruption of *Msh1* is asymmetric, rare in frequency, and irreversible (Shedge et al. 2007). This type of recombination also appears to play a key role in plant mitochondrial genome evolution, generating novel mitotypes (Small et al. 1989) and serving as a possible mechanism for fertility reversion (Fauron et al. 1995) under conditions when fertility restorer genes or reproductively compatible partners are limiting in a population (Bellaoui et al. 1998; Janska et al. 1998; Budar et al. 2003).

Dramatic and rapid changes of mtDNA molecule stoichiometries, a phenomenon termed substoichiometric shifting, often accompanies ectopic recombination (Kanazawa et al. 1994; Janska et al. 1998; Kim et al. 2007). Involvement of substoichiometric DNA molecules in genomic rearrangements creates not only mitochondrial genome complexity, but associated alterations in gene expression that influence plant growth and cell metabolic processes (Sandhu et al. 2007), implying that this process is adaptive.

The degree of copy number variability in plant mtDNA molecules throughout plant development and in different tissue types (Suzuki et al. 1996) is still not well understood with regard to its role in mitochondrial function. It has been estimated, however, that some substoichiometric molecules are maintained at levels as low as one copy per every 100 cells (Arrieta-Montiel et al. 2001). This ratio appears to be maintained from generation to generation, except under conditions of CMS, when the substoichiometric form may be amplified (Janska et al. 1998). How substoichiometric molecules are retained through reproductive development is unclear, but their ability to reamplify, via recombination or replication, appears to represent a genomic feature distinct to plant mitochondrial evolution that may render these genomes more versatile or perhaps environmentally responsive.

Here, we demonstrate that at least one substoichiometric DNA molecule within the pearl millet mitochondrial genome regulates the process of fertility reversion.

Materials and methods

Plant materials

Tift 23DBE maintainer line (B line), Tift 23A1E male-sterile line (A1 line) and three independent revertant lines (Burton 1977) were grown in the greenhouse under semi-controlled conditions. Generally, the temperature was 30°C daytime and 22°C nighttime, with 16-h day length. Three spontaneous revertant lines, originating independently as single, pollen-shedding heads from Tift 23 A1E plants, are maintained by the UNL Millet Breeding Program and their progeny are 100% fertile.

DNA and RNA purification

Total genomic DNA was purified from 2-week-old seedlings or panicles, following a protocol described by Dellaporta et al. (1983). Mitochondria were isolated from panicles and mtDNA purified according to the procedure described by Mackenzie et al. (1988). Total mRNA, or mitochondrial mRNA (mtRNA) from panicles, was purified by using TRIzol (Sigma, St. Louis, MO), as recommended by the manufacturer, and then treated with DNase (Ambion, Austin, TX) to remove any contaminating DNA.

Sequencing of *CoxI*-related regions in CMS A1 line

Three *CoxI*-related mitochondrial genome regions in the CMS A1 line were sequenced by using Genome Walker Universal Kit (BD Biosciences Clontech, Palo Alto, CA). Experiments were designed and performed according to the manufacturer's instructions. MtDNA purified from the A1 line was digested with two restriction enzymes, *Hae*III and *Rsa*I, then purified and subsequently ligated to the adapter supplied in the kit. Sequence-specific forward primers *CoxIR1* and *CoxIR2* (Table 1) that anneal to the 3′ end of the highly conserved coding region of *CoxI* were used for the first round PCR reaction (Fig. 2a). PCR products were cloned to pDrive cloning vector (QIAGEN) for sequencing. The three *CoxI* regions were sequenced to the stop codon for each open reading frame (ORF).

Circularized RT-PCR (cRT-PCR)

The cRT-PCR experiments were performed essentially as described by Kuhn and Binder (2002), except that tobacco acid pyrophosphatase (TAP) treatment was included. Briefly, 5′ end triphosphates were converted to monophosphates by treating 50 µg of mtRNA from A1 line with 10 U TAP (Epicentre Technologies) at 37°C for 1 h with 40 U RNase inhibitor (Fermentas GmbH, Germany). The reaction was subjected to phenol/chloroform extraction, and

Table 1 Primers used in the study

Primer	Sequence 5′–3′	Location
CoxIR1-F-1699	GTCGCAATCACTTCAAGCAGTGGAA	CoxI 3′ end coding sequence
CoxIR2-F-1730	AAAGATGTGCGGAAAGTCCTTGGG	*CoxI* 3′ end coding sequence
*CoxI-1- 1845*F	CAGAAAACAAAGCAAAGTAAAGGAATTT	*CoxI-1* 3′ tail
CoxI-1-1869 F	TTTACAAGGCCCTCTAGCCCTTCA	*CoxI-1* 3′ tail
CoxI-2-1855 F	TCTCGAATCCTAACCGCGTGCT	*CoxI-2* 3′ tail
CoxI-2-1960 F	TGACTTCCAAATTCTGCCTTCTTC	*CoxI-2* 3′ tail
CoxI-3-1845 F	CTACAACAACTGGAGAGAAAGA	*CoxI-3* 3′ tail
HaeIIIf1-1991F	CCCTTFCCTTTGCTTTTTGC	*CoxI-3* 3′ tail
BE1- 309R	GAGAAGAGCCATCGGACCAGATTTGTC	*CoxI* 5′ UTR
BE2-236R	CACTAAGGCTGGATGTTGGCTGAAATC	*CoxI* 5′ UTR
CoxI-1R-2103	TTTGCCGTGGAGGATAGGCTGAAT	*CoxI-1* 3′ tail
CoxI-2-155R	GGCAGAATTTGGCCGTCATCCAAT	*CoxI-2* 3′ tail
CoxI-3-AluR	AGATCCAGAGGACAGACAAATTAG	*CoxI-3* 3′ tail
CoxIR-1350	TGGCAAATTCAGGGCTAGACATTGCTCT	*CoxI* 3′ end coding sequence
CoxIF-1820	GCAGGAGGACTTTGTACCATCCATTCCA	*CoxI* 3′ end coding sequence
Cox76F	CACTACGGTGAGACGTGAAAACA	*CoxI* 5′ UTR
*CoxI-1-2283*R	GGCGGAAGCTTTGCGTCATTTGT	*CoxI-1* 3′ tail
CoxI-2J1-1969R	CTGGTCCGTCATCCAATACAACGA	*CoxI-2* 3′ tail
CoxI-2J2-1878R	AGCACGCGGTTAGGATTCGAGAAA	*CoxI-2* 3′ tail
CoxI-3-AluR1	AGATCCAGAGGACAGAGAAATTAG	*CoxI-3* 3′ tail
CoxI-3-AluR2	TAGCCTCTTTGGTTCGAGAACTAATTC	*CoxI-3* 3′ tail
CoxI-1-1893R	TGAAGGGCTAGAGGGCCTTGTAAA	*CoxI-1-2* junction molecule
CoxI-2-56R	AGCACGCGGTTAGGATTCGAGAAA	*CoxI-1-2* junction molecule
Atp6F-286	GTGCCAAATGCATGGCAATCCTTG	*Atp6*
Atp6R-404	AAAGTGACCGAGATGCGAGGGAA	*Atp6*
AdhF-1820	TGGTGACCATGTCCTTCCTGTGTT	*Adh1*
AdhR-1954	TCGACTTGCCGTCACCAATCATCA	*Adh1*

5 µg of treated mtRNA was self-ligated using 10 U of T4 RNA ligase (New England Biolabs) in supplied buffer supplemented with 20 U RNase inhibitor in a total volume of 20 µl. For RT-PCR, 1 µg ligated mtRNA was mixed with 20 pmol of gene-specific primers *CoxI-1-1845*, *CoxI-2-1855* and *CoxI-3-1845* (Table 1), and incubated 5 min at 70°C. The cDNA first strand synthesis was performed 30 min at 42°C, using SuperScript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Aliquots were used as template for PCR. Regions outside of the highly conserved *CoxI* core sequence were amplified by inverse PCR followed by a nested PCR. Briefly, forward primers *CoxI-1-1845* and *-1869* from the *CoxI-1* 3′ tail, *CoxI-2-1855* and *-1960* from the *CoxI-2* 3′ tail, and *CoxI-3-1845* and *Haef1* from the *CoxI-3* 3′ tail, together with reverse primers *BE1* and *BE2* from the 5′ end of *CoxI* were used in cRT-PCR (Fig. 2a). PCR products were cloned with the pDrive cloning kit (QIAGEN) and sequenced. Primers used in these experiments are listed in Table 1.

Investigating mitochondrial DNA polymorphisms

Five microgram of total genomic DNA from B, A1 and revertant lines of pearl millet were digested with restriction enzymes *Hin*dIII, *Ssp*I, *Pvu*I and *Eco*RV, fractionated by agarose gel electrophoresis (1% agarose in 0.5× TBE buffer at 70 V/cm overnight) and transferred to Hybond-N (GE Healthcare, Little Chalfont, UK) for DNA gel blot hybridization. PCR-amplified mitochondrial fragments that complement the specific 3′ tail sequences of *CoxI-1* (P1; primers *CoxI-1-1845/CoxI-1R-2103*), *CoxI-2* (P2; primers *CoxI-2-1855/CoxI-2-155R*) and *CoxI-3* (P3; primers), the conserved *CoxI* coding region (P4; primers *CoxIR/CoxIF*), and the conserved *CoxI* 5′ UTR (P5; primers *Cox76F/BE1*) were used as probes. Primer sequences to amplify these probes are listed in Table 1. Probes were ^{32}P-labeled (Stratagene Prime-It II Random Primer Labeling Kit) for autoradiographic exposure.

Detecting the *CoxI-3-2* molecule and the substoichiometric *CoxI-1-2* molecule

Detecting presence of the *CoxI-3-2* junction in the revertant line involved primers *J1* and *J2* from the *CoxI-2* 3′ tail and primers, *AluR1* and *AluR2*, from the *CoxI-3* 3′ tail (Table 1) to amplify the region. PCR products were cloned using pDrive cloning kit for sequencing. Similarly, primers *CoxI-1-2103* and *CoxI-1-2283* from the *CoxI-1* 3′ tail and primers *J1* and *J2* from the *CoxI-2* 3′ tail (Table 1) were paired and PCR amplification was performed using mtDNA from the CMS A1 line to detect the presence of a substoichiometric molecule that contains the junction of *CoxI-1* and *CoxI-2 3′* tails. PCR products were cloned and sequenced. PCR reactions with primer pair *CoxI-1-2103* and *J1* were performed using total genomic DNA from maintainer (B line) and revertant as template to check the presence of this molecule in those lines. Primers *J1* and *AluR1* were used to check for presence of the *CoxI-3-2* junction in maintainer B and CMS A1 lines.

Quantifying relative levels of *CoxI-1-2* molecule by q-PCR and DNA gel blot analysis

Substoichiometric levels of the *CoxI-1-2* molecule were assessed in maintainer B, CMS A1 and revertant lines relative to the mitochondrial gene *Atp6* (present in equal copy number in B, A1 and revertant mitochondrial genomes) and a single copy nuclear gene represented by *alcohol dehydrogenase-1* (*Adh1*) using DNA based q-PCR. Total genomic DNA was prepared from seedlings and panicles to assess the stability of relative copy number ratios in the two tissues. q-PCR was performed using primers listed in Table 1. Primers *CoxI-1-1893* from the *CoxI-1* tail and *CoxI-2-56* from the *CoxI-2* tail amplified a 132-bp fragment from the substoichiometric *CoxI-1-2* molecule. Primer pair *Atp6F-286* and *Atp6R-404* amplified a 119-bp fragment from the highly conserved coding region of *Atp6*, and primers *1820F* and *1954R* amplified a 135-bp fragment from *Adh1* gene.

Primers to amplify the *Adh1* gene were designed from database accession M59082 for pearl millet *Adh1* genomic sequence. Amplification efficiencies of primers for *CoxI-1-2*, *Atp6* and *Adh1* were tested using different primer pairs from each locus, and PCR products were fractionated in agarose to compare the amplification efficiency. The pair of primers producing greatest consistency in amplification efficiency from each line was chosen for real time q-PCR experiments. q-PCR experiments were implemented using SYBR Green PCR master mix (PE Biosystems, Foster City, CA) according to manufacturer's instructions. The PCR reactions and quantifications were carried out with the real time PCR detection system iCycler iQ (Bio-Rad). In addition, DNA gel blot analysis was used to detect the presence of the molecule at substoichiometric levels. Five micrgram total genomic DNA from B, A1 and revertant lines was digested with *Eco*RV and hybridized with probes, P1 and P2, separately.

Results

Three *CoxI*-related regions exist in pearl millet

Total genomic DNA samples from maintainer B, CMS A1 and three revertant lines were probed with the 3′ end of the highly conserved *CoxI* coding region by DNA gel blot analysis. Results revealed three different *CoxI*-related regions in these lines (Fig. 1), with the 2.0-kb *Hin*dIII fragment representing *CoxI-1,* conserved in all three lines. The 2.3-kb fragment present in the B and CMS A1 lines was designated *CoxI-2*, and the 3.9-kb fragment, present only in the CMS A1 line, was designated *CoxI-3*. Two *CoxI*-related regions in the CMS A1 line disappeared in the three spontaneous revertants to fertility (Fig. 1, only one revertant shown).

PCR experiments initiated with primers *CoxIR1* and *CoxIR2*, specific for the 3′ end of the conserved *CoxI*

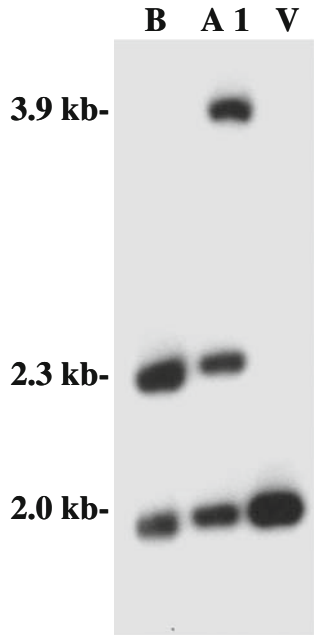

Fig. 1 Three mitochondrial *CoxI*-related regions detected by DNA gel blot hybridization. Total genomic DNAs from maintainer B (*B*), CMS A1 (*A*) and revertant (*V*) lines of millet were digested with *Hin*dIII and hybridized with a probe corresponding to the conserved *CoxI* coding region. The three *CoxI* regions identified were designated as *CoxI-1*, *CoxI-2* and *CoxI-3* as shown, and the size of each *Hin*dIII fragment is indicated to the *left*. Each gel lane was loaded with equal amounts (20 μg) of DNA; the doubling in hybridization intensity in the revertant lane is the consequence of recombination. Results for all three independent revertants were identical (other two not shown)

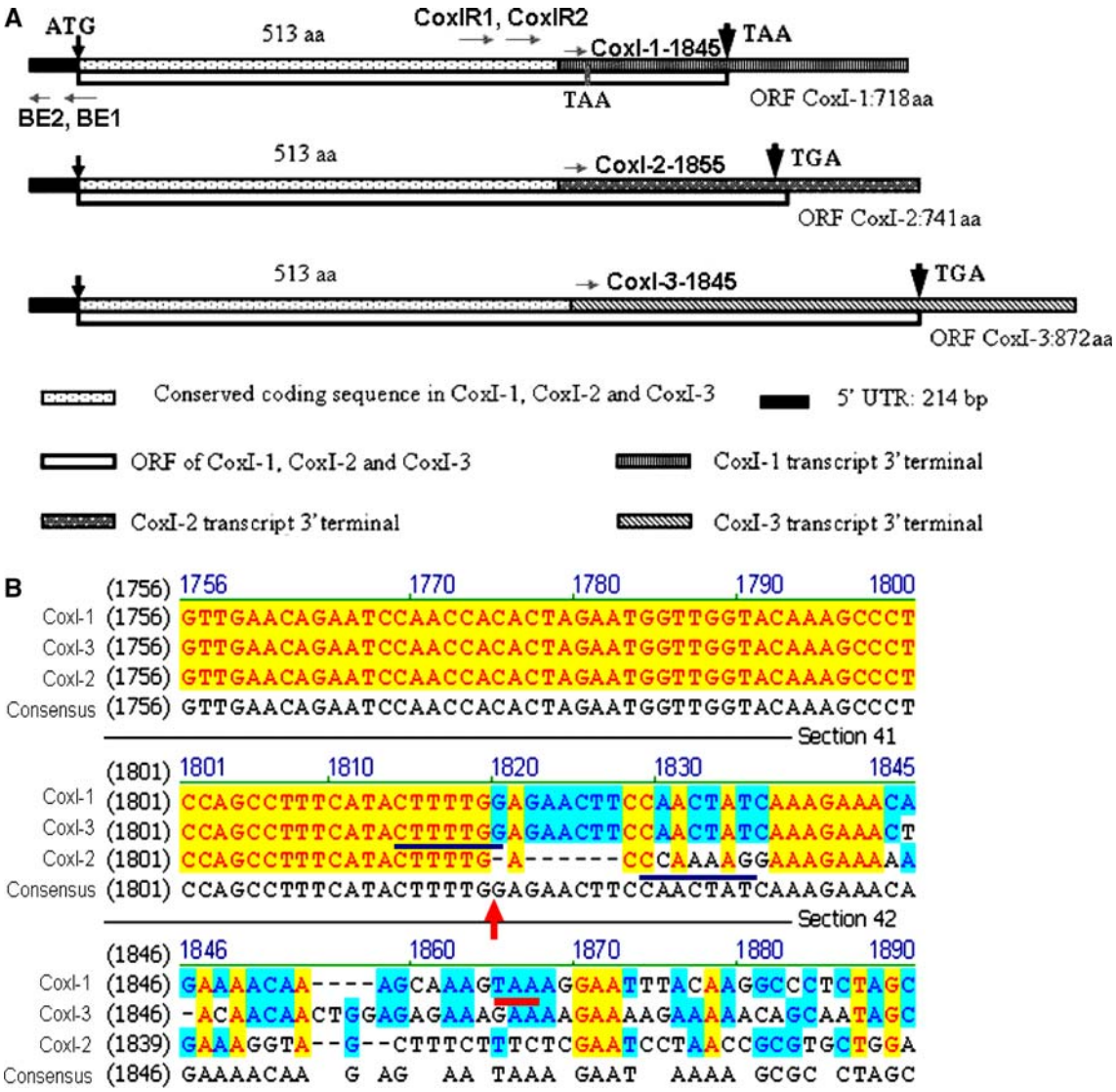

Fig. 2 The three *CoxI*-related open reading frames differ at their 3′ ends. **a** Diagram of the three *CoxI* -related open reading frames based on DNA sequence derived from primers *CoxIR1* and *CoxIR2*, located in conserved *CoxI* coding region, and a process of Genome walking. Primers *CoxI-1-1845*, *CoxI-2-1855* and *CoxI-3-1845*, derived from each unique 3′ tail, and primers *BE1* and *BE2* from the 5′ end of *CoxI*, were used in c-RT-PCR experiments. Sequencing data revealed an open reading frame within each *CoxI* region in the CMS A1 line, with the size of each indicated. The size of the *CoxI* fragment conserved in each (513 amino acids) is also shown. **b** Partial nucleotide sequence alignment of the three 3′ tails. A 7-bp repeat (*underlined*) connects the highly conserved *CoxI* coding region with the 3′ unique tail in each ORF. The breakpoint is indicated by a *red arrow*. Nucleotide sequence alignment of the 5′ UTR and the 513-amino acid coding area (not shown) indicates ca. 97% nucleotide identity

coding region (Fig. 2a), and mtDNA from CMS A1 produced three products of different sizes. These products were assumed to represent the three *CoxI*-related regions in the CMS A1 line. Sequencing results showed that the three products contained the same portion of *CoxI* sequence from the *CoxIR2* primer site until a breakpoint, followed by distinct sequence in each product (Fig. 2b). Therefore, each *CoxI* ORF terminated distinctly, encoding 718 amino acid for *orfCoxI-1*, 741 amino acid for *orfCoxI-2* and 872 amino acid for *orfCoxI-3* (Fig. 2a).

Circularized RT-PCR was used to test for mRNA editing within the three *CoxI* regions, to obtain 5′ and 3′ termini sequences, and to test for polyadenylation. The 5′ UTR and 97% of the *CoxI* coding region is shared by the three *CoxI* loci, with each containing a distinct 3′ extension. DNA–mRNA comparison showed evidence of transcript editing to create the *CoxI-1* stop codon, TAA, for a protein-coding region of 531 amino acid. No evidence of transcript editing was detected for the *CoxI-2* and *CoxI-3* transcripts. No evidence of polyadenylation was found in the three transcripts,

and RNA gel blot analysis detected transcripts for *CoxI-1* and *CoxI-3*, but not for *CoxI-2* (data not shown). Apparently, only two forms of *CoxI* are transcribed to detectable levels, and the *CoxI-3* transcripts were assumed to be associated with male sterility, because of their distinct association with the male-sterile line and disappearance upon reversion. *Cox1-2*, while also silent in the revertant line is present in identical form in the male fertile B line.

Mitochondrial genome reorganization upon reversion

To understand mitochondrial genome rearrangements that occur in the *CoxI* regions upon reversion, DNA gel blots were analyzed with mitochondrial site-specific probes. B, A1 and fertile revertant lines were analyzed with probes corresponding to the unique extended 3′ tails of *CoxI-1* (P1), *CoxI-2* (P2) and *CoxI-3* (P3), as well as the *CoxI* coding region (P4) (Fig. 3a). Figure 3b shows that probe, P1, hybridized to the 2.0-kb fragment contained in all three lines, recognized by probe P4, and representing the conserved *Cox1* coding region. This observation indicates that the *CoxI-1* region remains intact upon reversion. Probe, P2 recognized the 2.3-kb fragment contained in the B and A1 lines, to which probe, P4 also hybridized, and identified a novel fragment of ca. 2.6 kb in the revertant line. This observation indicates that the *CoxI-2* 3′ tail sequence was retained in the revertant, but the conserved *CoxI* coding sequence from *CoxI-2* disappeared during reversion. Probes, P2 and P3 recognized the same 2.6-kb fragment in the revertant, but P3 also hybridized to *CoxI-3* in the A1 line and not in the B line. These observations further lead us to postulate that *CoxI-3* is the male sterility-associated region, and the 2.6-kb P2/P3 hybridizing fragment represents a recombinationally-derived junction fragment linking the *CoxI-2* and *CoxI-3* 3′ tail sequences.

To test our assumption, primers annealing to the *CoxI-2* and *CoxI-3* 3′ tail sequences were designed and paired to amplify the predicted fragment in the revertant. A PCR product was amplified, cloned and sequenced to reveal *CoxI-2* and *CoxI-3* 3′ tail sequences joined in reverse orientation as shown in Fig. 4. A 7-bp repeat sequence, CTTTTGG, was identified close to the breakpoint in both *CoxI-2* (in reverse) and *CoxI-3*, so that the two tail sequences connect at this repeat. The identified junction was designated as *CoxI-3-2*, present in the revertant line, but absent in B and A1 lines are based on similar PCR-based analysis. From these observations, we assume that the 2.6-kb fragment recognized by probes, P2 and P3 in the

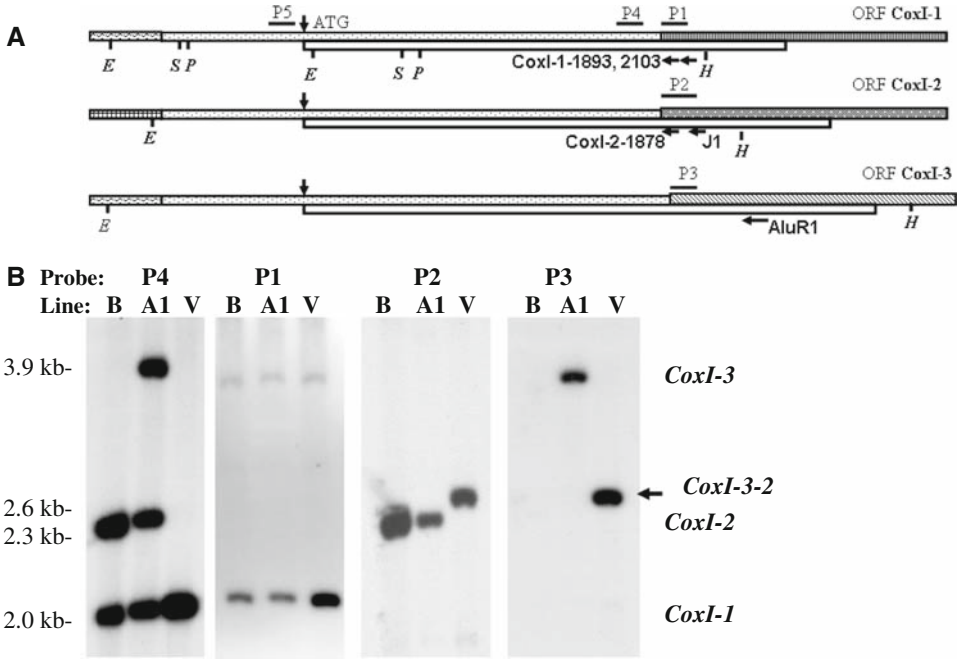

Fig. 3 Discovery of the *CoxI-2-3* configuration by diagnostic DNA polymorphisms. **a** Diagram of the *CoxI* regions indicating positions of relevant restriction sites, probes and primers. *Eco*RV (*E*), *Ssp*I (*S*), *Pvu*I (*P*) and *Hin*dIII (*H*) restriction endonucleases were used to digest total genomic DNA from maintainer (*B*), CMS (*A1*) and revertant (*V*) lines. Probes *P1*, *P2* and *P3* were derived from the unique 3′ tail regions of the *CoxI* loci; probes *P4* and *P5* represent the 3′ and 5′ ends of the *CoxI* conserved region, respectively. Primers *J1* and *AluR1* were used to identify the *CoxI-3-2* junction. Primers *J1* and *CoxI-1-2103* were used to identify the substoichiometric *CoxI-1-2* junction. Primers *CoxI-1-1893* and *CoxI-2-1878* were used to estimate the relative stoichiometric level of *CoxI-1-2* molecule in *B*, *A1* and revertant lines by q-PCR. **b** Total genomic DNAs from *B*, CMS (*A1*) and revertant lines were digested with *Hin*dIII and probed with *P1*, *P2*, *P3* and *P4*. *P1* recognized *CoxI-1* in all lines. *P2* recognized *CoxI-2* in *B* and *A1* lines, and a novel band in revertant, which was identified as junction *CoxI-3-2* (indicated by *arrow*). *P3* recognized *CoxI-3* in the *A1* line and junction *CoxI-3-2* in revertant. All three revertants showed identical results (other two not shown)

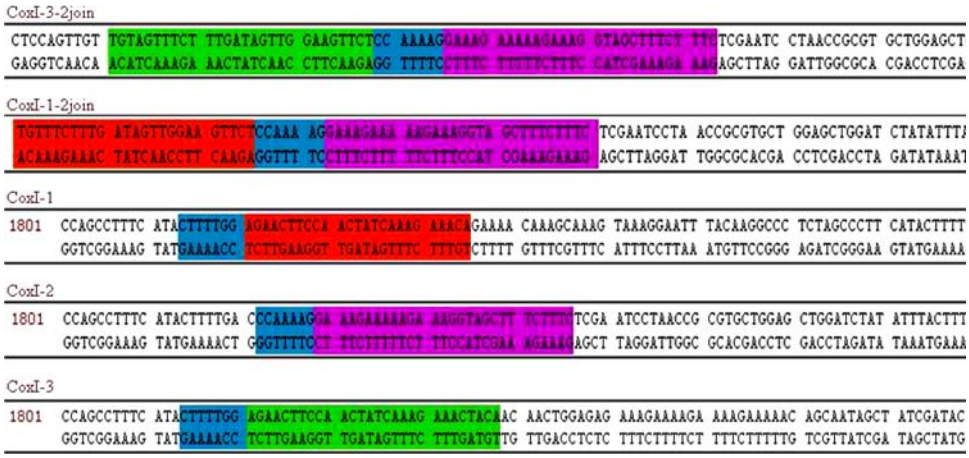

Fig. 4 Millet mitochondrial sequences containing the 7-bp CTTTTGG short repeat. The identified repeat connects each distinct 3′ tail sequence with the *CoxI* conserved coding sequence to form *CoxI-1*, *CoxI-2* and *CoxI-3*. The repeat is in reverse orientation in *CoxI-2* relative to *CoxI-1* and *CoxI-3*. This repeat also connects the *CoxI-1* and *CoxI-2* 3′ tail sequences to form the substoichiometric *CoxI-1-2* junction molecule, and the *CoxI-2* and *CoxI-3* 3′ tail sequences to form the *CoxI-3-2* junction molecule found in the revertant line. *Red* designates the partial *CoxI-1* 3′ tail sequence, *purple* designates *CoxI-2* and *green* *CoxI-3*. The 7-bp repeat is shown in *blue*

revertant line corresponds to a new mtDNA configuration created by the reversion process.

The results predict a rearrangement in which *CoxI-2* and *CoxI-3*, oriented inversely in the A1 line, recombine to produce deletion of the sequence between the two unique 3′ ends, forming the *CoxI-3-2* junction upon reversion. If the prediction above were correct, we would expect differences to be detectable in the regions upstream to the three *CoxI* regions in the revertant with the P5 probe that recognizes conserved 5′ termini for the three *CoxI* regions (Fig. 3a). Restriction enzymes *Ssp*I, *Pvu*I and *Eco*RV, each recognizing a single restriction site close to the 5′ termini, were selected to digest total genomic DNA from B, A1 and revertant lines for hybridization with the P5 probe. Figure 5 reveals that the two distinct *CoxI* upstream configurations in B and A1 lines were retained after reversion. The new *CoxI-1* configuration, created by reversion, differs from the original *CoxI-1* in its upstream sequence, but shares this sequence with *CoxI-2* and is, therefore, not visible as a novel fragment. The new *CoxI-1* configuration is, however, detectable in the doubled DNA band intensity observed upon reversion in Fig. 1.

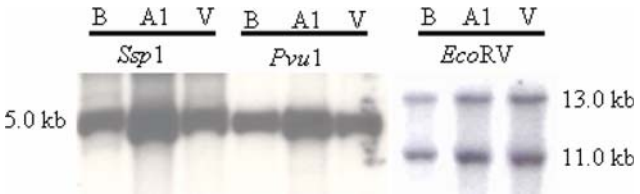

Fig. 5 Upstream configurations of the three millet *CoxI* regions. The upstream configurations of the three *CoxI* regions were examined by DNA gel blot analysis. Total genomic DNAs from *B*, CMS *A1* and revertant lines were digested with *Ssp*I, *Pvu*I and *Eco*RV, and probed with P5. The two different upstream configurations in the *CoxI*-related regions were both retained in the three revertants (other two not shown)

These results show that the reversion process alters *CoxI-2* and *CoxI-3* regions of the genome. The identified rearrangement apparently creates the observed *CoxI-3-2* junction as well as a second copy of *CoxI-1* in the revertant. The novel *CoxI-1* arises from recombination of an original *CoxI* upstream configuration and a *CoxI* coding sequence derived from either *CoxI-2* or *CoxI-3*. These observations raise two questions; namely, how the *CoxI-3-2* junction is formed, and from where the *CoxI-1* 3′ tail sequence in the new *CoxI-1* derives.

Substoichiometric forms may drive rare mitochondrial recombination

A model to account for observations upon reversion would require existence, within the A1 mitochondrial genome, of a repeat comprised of the *CoxI-1* 3′ tail linked to the *CoxI-2* 3′ tail by the 7-bp repeat identified previously. We suggest that this repeat exists substoichiometrically. The proposed model derives from the following four observations: (1) Probes P1 and P2, but not P3, recognized faint restriction fragments in the A1 line that could only be detected upon extended exposure times, (2) an additional *CoxI-1* 3′ tail sequence must be available within the genome to generate the observed new copy of *CoxI-1* in the revertant, (3) a *CoxI-3-2* tail junction is formed in the revertant, and (4) the 7-bp short repeat sequence of CTTTTGG connects *CoxI* conserved coding sequences with each unique 3′ tail in *CoxI-1*, *CoxI-2*, *Cox1-3* and the *CoxI-3-2* junction. Therefore, we tested for the presence of the requisite junction molecule predicted to link *CoxI-1* and *CoxI-2* 3′ tail sequences at the 7-bp repeat.

Primers from *CoxI-1* and *CoxI-2* 3′ tails (Table 1) were paired for PCR amplification in mtDNA samples from the A1 line. A product was amplified with primers *J1* and *CoxI-1-2103* (Fig. 3a), cloned and sequenced. As predicted, the sequence revealed a region joining *CoxI-1* and *CoxI-2* 3′ tails through the 7-bp short sequence CTTTTGG in reverse orientation (Fig. 4). This region was designated the *CoxI-1-2* junction, with organization identical to that of the *CoxI-3-2* junction. The *CoxI-1-2* junction was also detected in maintainer B and the three revertant lines. Substoichiometric levels of the *CoxI-1-2* junction molecule were estimated using, as interval standards, the mitochondrial *Atp6* and nuclear *Adh1* genes for real time q-PCR assays. Copy number of the *CoxI-1-2* junction molecule in B, A1 and revertant lines was estimated by q-PCR with results shown in Fig. 6. The junction molecule, relative to single copy nuclear gene, *Adh1*, was present at approximately one copy per every 128 cells in the A1 line, and one copy per every ten cells in the revertants, demonstrating more than tenfold increase in copy number of the junction molecule upon reversion. The copy number of *CoxI-1-2* in the B line was the same as in the CMS A1 line. Relative to *Adh1*, the mitochondrial *Atp6* gene in B, CMS A1 and revertants was present in 300-fold excess. No significant difference was detected in relative copy number estimates between leaf and panicle tissues. Presence of the junction molecule could also be detected in the three lines by DNA gel blot hybridizations with extended exposures (Fig. 7).

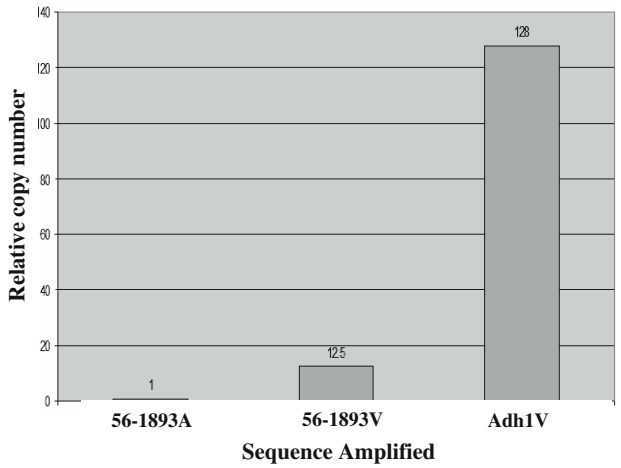

Fig. 6 Substoichiometric shift of the *CoxI-1-2* junction molecule in A1 and revertant lines. The *graph* shows the magnitude of copy number changes observed in the substoichiometric *CoxI-3-2* junction molecule in CMS A1 (56-1893A) and revertant (56-1893V) lines relative to the single copy nuclear gene *Adh1* [assayed in the revertant (V) line]. *CoxI-1-2* is estimated at one copy per 128 cells in the CMS A1 line, and about one copy per 12 cells in revertant, suggesting a tenfold increase in the junction molecule upon reversion. Analysis was conducted with DNA based real time q-PCR

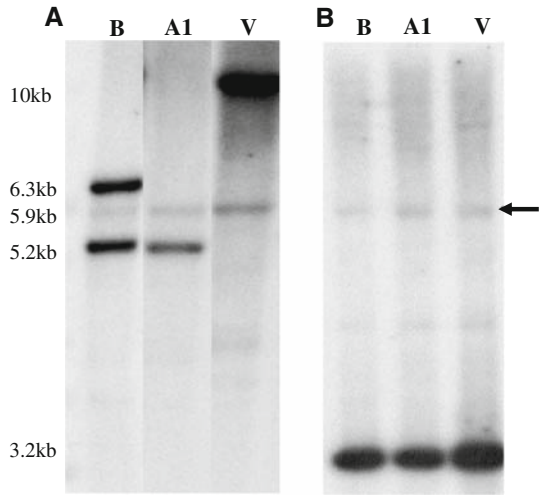

Fig. 7 The substoichiometric *Cox1-1–2* junction detected by DNA gel blot analysis. Total genomic DNAs from maintainer B, CMS A1 and revertant lines were digested with *Eco*RV, and probed with P2 (**a**) and P1 (**b**). The faint 5.9-kb fragment in both **a** and **b** corresponds to the substoichiometric *CoxI-3-2* junction (*arrow*). In **a**, the 5.2-kb band corresponds to *CoxI-2* in *B* and *A1* lines, and the 10-kb band corresponds to the *CoxI-3-2* junction molecule present in the revertant. In **b**, the 3.2-kb band corresponds to *CoxI-1* in the three lines

Discussion

In numerous previous studies, researchers noticed that changes in nuclear background could dramatically influence the reversion frequency and mitochondrial genome reorganization patterns in CMS lines (Small et al. 1988; Escote-Carlson et al. 1988; Mackenzie et al. 1988). Natural or induced nuclear gene mutations cause fertility reversion in carrot (Chahal et al. 1998) and rice (Shen et al. 1996), and tissue culture conditions also give rise to fertility reversion in petunia and maize (Clark et al. 1988; Small et al. 1988; Fauron et al. 1990). In most of these reversion events, the genomic conditions triggering the mitochondrial genome rearrangement are unknown. Spontaneous fertility reversion in the CMS A1 line of pearl millet occurs rarely (0.01% frequency), observed as a single pollen-shedding panicle surrounded by fully male-sterile panicles in a CMS plant (Smith et al. 1987). Our study suggests that this low frequency might be controlled by the substoichiometric nature of junction molecule *CoxI-3-2*, which appears to be essential to initiate the reversion phenomenon.

We propose a model of inter-molecular illegitimate recombination followed by intra-molecular homologous recombination to produce the novel revertant mitotype observed in millet A1 cytoplasm (Fig. 8). We assume that *CoxI-2* and *CoxI-3* reside in the same orientation on a molecule in the A1 line. Inter-molecular ectopic recombination at the 7-bp CTTTTGG repeat occurred between *CoxI-3* and

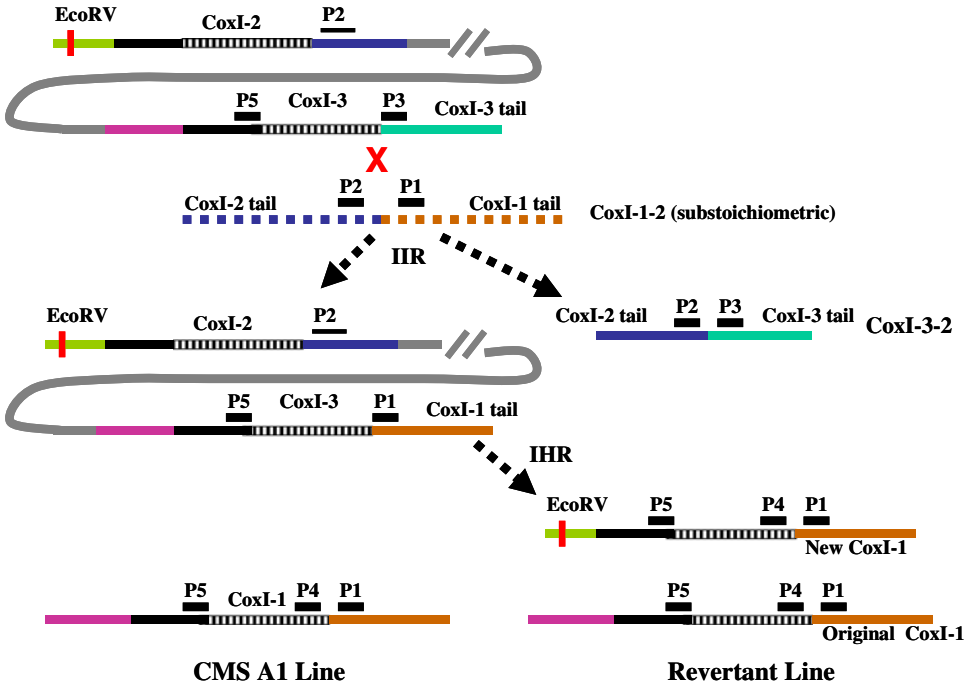

Fig. 8 Diagram of the proposed model involving two-step recombination. *CoxI-2* and *CoxI-3* reside in direct orientation on a single molecule, so that inter-molecular illegitimate recombination (*IIR*) is predicted between this molecule and the substoichiometric *CoxI-3-2* junction molecule via a 7-bp short repeat. This recombination produces a *CoxI-3-2* junction and the reciprocal recombinant, in which the original *CoxI-3* 3′ tail is replaced by the *CoxI-1* tail from the *CoxI-3-2* junction molecule. The reciprocal molecule is stabilized by a subsequent intramolecular homologous recombination (*IHR*) at the *CoxI* 7-bp repeat. This homologous recombination is predicted to cause a deletion, creating a new *CoxI-1* region. The original *CoxI-1* remains unaltered

the *CoxI-3-2* junction molecule, a rare event likely to be regulated by the infrequency of *CoxI-1-2* copy number amplification, but essential to trigger reversion. This recombination results in the *CoxI-3-2* junction molecule and a reciprocal molecule that replaces the original *CoxI-3* tail region with the *Cox- I-1* tail derived from *CoxI-3-2* junction molecule. This predicted reciprocal molecule could not be detected in either CMS A1 or revertant lines. We assume the molecule to be unstable, and intra-molecular homologous recombination within the two highly conserved *CoxI* regions would be predicted to result in deletion of the interval between the *CoxI-2* tail and the new *CoxI-1* tail. Remarkably, this recombination event would form a new *CoxI-1* region using the same CTTTTGG breakpoint (Figs. 2b, 8). The original *CoxI-1* would remain unaltered during the reversion process, but one would expect the hybridization intensity of *CoxI-1* to double upon reversion. This is, in fact, the case (Fig. 1).

Whether axillary meristems are produced de novo in leaf axils or they are derived from the apical meristem of the primary shoots is not clear (Grbic and Bleecker 2000), but the mitotype of revertants likely represents the consequence of mitochondrial genome reorganization at the cell differentiation stage of axillary bud formation. Presumably, this reorganization gives rise to the reversion mitotype that is maintained in subsequent cell divisions to produce the male fertile panicle. In rice roots, it was shown that a 2.5-fold increase in the stoichiometry of specific subgenomic mitochondrial molecules can occur during cell differentiation in the apical meristem compared to the differentiated elongation zone (Suzuki et al. 1996). Suzuki et al. postulate that the organization of the higher plant mitochondrial genome is variable during the very short period of replication and distribution of mtDNA in undifferentiated meristems, so that the variability in stoichiometry of mitochondrial subgenomic molecules could induce extensive variation in mitochondrial genome organization. We suggest that this reorganization might, in a small number of cells or in response to unknown environmental factors, result in differential amplification of the molecule containing junction *CoxI-3-2*. This suggestion would be consistent with the observed stoichiometric increase in this molecule in revertants relative to the male-sterile A1 line (Fig. 6). The two-step mitochondrial recombination process identified in our study alters the *CoxI-2* and *CoxI-3* regions in the CMS A1 line, effecting plant fertility. From these observations, we conclude that either *CoxI-3* or an adjacent ORF in the deleted region is associated with the CMS trait. Based on extensive gel blot hybridization analysis of the region of rearrangement, alteration of *CoxI-3* expression is the only consequence of the reversion event that has been detected to date.

Previous studies have shown that mitochondrial substoichiometric shifting in plants is controlled by at least three nuclear genes that function to suppress ectopic recombination activity (Abdelnoor et al. 2003; Zaegel et al. 2006; Shedge et al. 2007). Disruption of the nuclear gene, *Msh1* has been shown to result not only in mitochondrial substoichiometric shifting, but in the appearance of CMS (Sandhu et al. 2007). These observations imply that plants have evolved nuclear–mitochondrial interaction mechanisms that are adaptive in regulating reproductive strategies. Here,

we present evidence to further substantiate this view. While plants have been shown to maintain within their mitochondrial genomes, the capacity for CMS induction, demonstrated by Sandhu et al. (2007), we show in this study that they also appear to retain, substoichiometrically, the capacity for spontaneous reversion back to fertility.

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