RESEARCH ARTICLE

A duplicated *coxI* gene is associated with cytoplasmic male sterility in an alloplasmic *Brassica juncea* line derived from somatic hybridization with *Diplotaxis catholica*

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Abstract

A cytoplasmic male sterile (CMS) line of *Brassica juncea* was derived by repeated backcrossing of the somatic hybrid (*Diplotaxis catholica* + *B. juncea*) to *B. juncea*. The new CMS line is comparable to euplasmic lines for almost all characters, except for flowers which bear slender, needle-like anthers with aborted pollen. Detailed Southern analysis revealed two copies of *coxI* gene in the CMS line. One copy, *coxI-1* is similar to the *coxI* gene of *B. juncea*, whereas the second copy, *coxI-2* is present in a novel rearranged region. Northern analysis with eight mitochondrial gene probes showed altered transcript pattern only for the *coxI* gene. Two transcripts of 2.0 and 2.4 kb, respectively, were detected in the CMS line. The novel 2.4 kb transcript was present in floral bud tissue but absent in the leaf tissue. In plants where male sterility broke down under high temperature during the later part of the growing season, the 2.4 kb *coxI* transcript was absent, which suggested its association with the CMS. The two *coxI* genes from the CMS line showed two amino acid changes in the coding region. The novel *coxI* gene showed unique repeats in the 5′ region suggesting recombination of mitochondrial genomes of the two species. The possible role of the duplicated *coxI* gene in causing male sterility is discussed.

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Introduction

Cytoplasmic male sterility (CMS), a maternally inherited trait wherein the plants fail to produce functional pollen, results from either mitochondrial mutations or nuclear–mitochondrial incompatibilities (Schnable and Wise 1998; Hanson and Bentolila 2004). Besides its use for hybrid seed production, CMS has been widely used to study mitochondrial gene function and to understand the influence of nuclear background on mitochondrial gene expression. A CMS plant may arise spontaneously in a population or in breeding programmes involving wide hybridization.

Belliard *et al*. (1979) first demonstrated the use of protoplast fusion as a powerful tool for genetic analysis of organelle genomes, to dissociate chloroplast and mitochondrial components, to transfer CMS from one parental species to another, to induce recombination between parental mitochondril genomes, and established correlation between floral morphology, male fertility or sterility and mtDNA restriction pattern. Subsequently, protoplast fusion technique has been employed to transfer CMS from one species to another (Pelletier *et al*. 1988).

Investigations of several CMS systems have revealed that male sterility is associated with reorganization of the mitochondrial genome, which, in many cases, results in the formation of chimeric genes that are responsible for the CMS character. In maize, *Petunia*, sunflower, bean, *Brassica*, radish, rice, sorghum etc., CMS-associated mtDNA rearrangements have generated novel open reading frames (orfs) which have been implicated in male sterility (reviewed in

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Schnable and Wise 1998; Kempken and Pring 1999). In a number of cases, transcripts originating from these altered mitochondrial regions are translated into proteins that appear to interfere with mitochondrial function and pollen development (Forde and Leaver 1980; Krishnasamy and Makaroff 1994; Song and Hedgecoth 1994; Abad *et al*. 1995; Shinada *et al*. 2006). Nuclear genes that suppress the CMS trait and thereby restore male fertility have also been identified for several CMS systems. These restorer genes affect CMS-associated mitochondrial genes at transcriptional, posttranscriptional, translational or posttranslational level.

A number of CMS systems have been identified in the Brassicaceae. One of the most extensively studied system is the Ogura cytoplasm of radish in which the *orf138* region has been implicated in male sterility (Bonhomme *et al*. 1992). A decrease in the amount of ORF138 protein in the floral buds accompanies fertility restoration (Krishnasamy and Makaroff 1994). Similarly, *orf125* showing sequence homology with *orf138* has been identified as the CMS-associated gene in Kosena radish and fertility restoration was accompanied by decrease in the amount of ORF125 protein (Iwabuchi *et al*. 1999). In pol CMS of *B. napus*, a chimaeric region *orf224* has been created upstream of *atp6* that gives rise to CMS-specific transcript pattern that is absent in fertile *B. napus* (Singh and Brown 1991; L'Homme and Brown 1993). Similarly, in nap CMS of *B. napus*, *orf222* with homology to *orf224*, gives rise to unique CMS-associated transcript pattern (L'Homme *et al*. 1997). In both these systems, restoration occurs by processing of these transcripts by the novel mechanism of RNA processing (Singh *et al*. 1996). In alloplasmic CMS *B. juncea* or *B. napus* carrying *B. tournefortii* mitochondria, an *orf263* is cotranscribed with *atp6* gene giving rise to a 29-kDa protein (Landgren *et al*. 1996). Fertility restorer genes of Ogura and Kosena radish CMS systems have been cloned and shown to belong to pentatricopeptide repeat family of genes which are involved in processing of transcripts of mitochondrial and plastid genomes (Brown *et al*. 2003; Desloire *et al*. 2003; Koizuka *et al*. 2003).

An alloplasmic CMS system of *B. juncea* carrying *Diplotaxis catholica* cytoplasm obtained by sexual hybridization was found to be associated with floral abnormalities and poor female fertility (Pathania *et al*. 2003). Therefore, attempts were made to derive better CMS lines through somatic hybridization between *B. juncea* and *D. catholica* (Kirti *et al*. 1995a). Here we report the molecular characterization of the improved CMS line and show that mitochondrial recombination has led to duplication of *coxI* gene which appears to be associated with the CMS.

Materials and methods

Plant material

B. juncea cv. Pusa Bold, the CMS line, and the wild species *Diplotaxis catholica* were used in the present study. The CMS line was derived by repeated backcrossing of the somatic hybrid (*D. catholica* + *B. juncea*) (Kirti *et al*. 1995a) with *B. juncea*. Stable CMS line was obtained in the BC_4 generation. However, male fertile flowers were observed on some CMS plants during the end of the season when day temperature was >30°C. These male fertile flower buds were used for transcript analysis.

DNA isolation and Southern hybridization

The mtDNA was isolated from flower buds and total DNA was isolated from the leaf tissue of *B. juncea*, *D. catholica* and the CMS line. Details of the protocols used for DNA isolation and Southern hybridization are as described earlier (Kirti *et al*. 1995a). Mitochondrial gene probes used for analysis were *atpA, atp6, atp9, coxI, coxII, coxIII, nad3/rps12, cob* and 18S rRNA (Pathania *et al*. 2003).

RNA isolation and northern hybridization

Mitochondria were isolated from young flower buds of the CMS line, fertile plants, euplasmic *B. juncea* and *D. catholica*. Mitochondria were lysed in Trizol reagent and RNA was isolated according to manufacturer's protocol (Invitrogen). For Northern blot, about 5 μg of RNA was mixed with formamide, formaldehyde and MOPS buffer, and heated to 65°C for 15 min. The samples were electrophoresed in 1.2% agarose–formaldehyde gel at a constant voltage (4 V/cm). After electrophoresis, the RNA was transferred to Hybond N^+ nylon membrane (Amersham-Pharmacia) and immobilized by UV crosslinking. Blots were hybridized with eight mitochontrial gene probes as described earlier (Pathania *et al*. 2003).

RAPD analysis

RAPD amplifications were performed in a 25-μl reaction mixture containing 50 ng of mtDNA, 20 ng of random primers (Operon Technologies), 2 mM each of dNTPs, 1X *Taq* polymerase buffer and 1U of *Taq* polymerase (MBI Fermentas). The amplification was carried out in an MJ Research Thermal Cycler (PTC-100-60) programmed as follows: initial step of 92°C for 2 min, 45 cycles of 92°C for 1 min, 37°C for 1 min and 72°C for 2 min, followed by a final step of 72°C for 7 min. Amplified products were electropheresed in 0.8% agarose gel and visualized by ethidium bromide staining.

Polymerase chain reaction

PCR was performed in 25-μl aliquots essentially as above, except for the primers (forward + reverse). The thermal cycler was programmed as follows: an initial step of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 90 s, followed by a final amplification step of 72°C for 10 min. Primer sequences were as follows:
Primer P1: 5′ ATGACAAATCCGGTCCGATGGCTGT 3′ (forward, *coxI*)
Primer P2: 5′ CTTCACATAGCTTTTCGTCTCCTT 3′ (reverse, *coxI*)

Primer P3: 5′ GCATTGAGATTCCGTAAGTAA 3′ (3′ BJ)
Primer P4: 5′ CGAGAGGTGCTTTAGCAACT 3′ (5′ *coxI-2*)

Reverse transcriptase (RT) PCR and cloning of coxI transcript

One μg of DNAaseI-treated mtRNA from flower buds was used in a 50-μl reaction. RT-PCR was performed following single-step RT-PCR system kit according to the manufacturer's instruction (Promega). The amplification product was electrophoresed in 0.8% agarose gel, and the eluted product was cloned in a pGemT cloning vector (Promega).

Mitochondrial DNA library construction and cloning of coxI gene

The mtDNA libraries of *B. juncea* and the CMS line were constructed in BlueScript KS$^-$ phagemid vector (Stratagene). The ligation mixture contained 1 μg *Hin*dIII (Boehringer Mannheim) digested mtDNA, 100 ng of *Hin*dIII-digested and dephosphorylated (calf intestinal alkaline phosphatase, Promega) vector DNA and 1 U of T4 DNA ligase (Promega). The reaction was incubated at 4°C overnight or 15°C for 4–18 h and subsequently used to transform *E. coli* strain DH5α (Sambrook *et al*. 1989). Blue white selection was done on X-Gal/IPTG-containing LA-Amp plates. The resulting library was screened with PCR-amplified *coxI* as probe to identify clones carrying *coxI* insertion. The insert size of positive clones was determined using *Hin*dIII and *Hin*dIII + *Eco*RI digestion.

DNA sequencing and analysis

For DNA sequencing, amplifications were performed using dye terminator premixes (ABI Prism Bigdye Terminator cycle sequencing kit). Reaction products were resolved and analysed using ABI PRISM 373 DNA sequencer. The sequences were assembled and analysed using Vector NTI program (Infomax, North Bethesda, MD) and Internet-based tools (http://www.ncbi.nlm.nih.gov/; http://tandem.bu.edu/trf/trf.html).

Results

Plants of the CMS line are green and vigorous. However, flowering is delayed by about 10 days. Flowers are slightly larger than normal *B. juncea* and show well-developed petals and nectaries. Anthers are slender, needle-like with short filament, and do not produce pollen (figure 1). Ovaries are well developed with an elongated style. Female fertility is around 97%. CMS trait is stable over generations, but some plants developed male fertile flowers late in the season when ambient temperature rose to >30°C.

Comparative analysis of mitochondial genome of the CMS line with the parents RAPD analysis: mtDNA isolated from *B. juncea, D. catholica* and the CMS line was used for RAPD analysis using 30 random primers (OPB and OPC series, Operon Technologies). Majority of the primers (18) were uninformative as they showed either no DNA amplification or displayed monomorphic amplification pattern. However, 12 primers detected polymorphism between the two parents. With nine of these primers, the amplicon pattern of the CMS line was similar to *B. juncea*. Only three primers showed unique RAPD profile for the CMS line compared with the parents. For example, with OPC11 primer, the CMS line showed a distinct RAPD profile compared with *B. juncea* and *D. catholica*, whereas with OPC7 primer, RAPD profile of the CMS line was identical to that of *B. juncea* (figure 2). Thus, the RAPD results suggested that the mitochondrial genome of the CMS line is, to a large extent, similar to that of *B. juncea*.

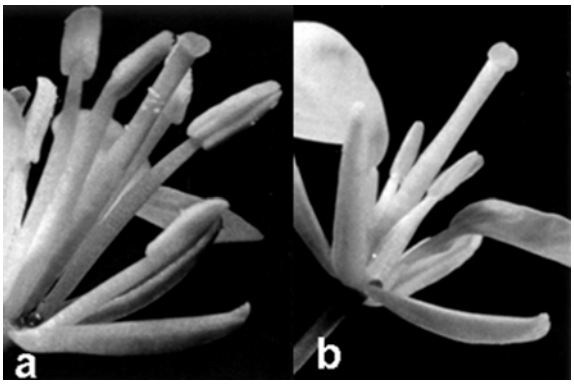

Figure 1. Flowers of (a) euplasmic *B. juncea*, and (b) the improved CMS line with well developed nectaries.

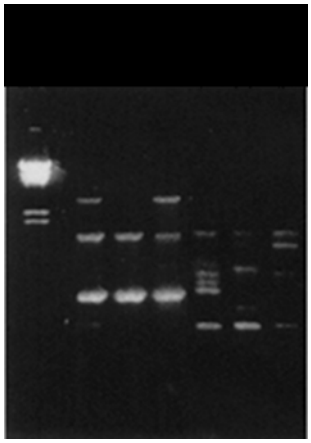

Figure 2. RAPD profile obtained with mitochondrial DNA of *B. juncea* (J), *D. catholica* (C) and the CMS line (S) with OPC7 and OPC11 primers.

Southern analysis: Nine mitochondrial gene-specific probes were used to detect structural changes in and around these genes. Parental species *D. catholica* and *B. juncea* show distinct RFLP patterns for most of the probe–enzyme combinations (Pathania *et al*. 2003). Southern hybridization patterns of the CMS line were identical to those of *B. juncea*

for majority of the probe–enzyme combinations examined. However, three probes, namely *coxI, coxII* and *nad3/rps12*, revealed differences between the CMS and *B. juncea*. For example, with *coxII* probe, the *Xho*I digest of *B. juncea* showed two bands (4.5 kb and 2.4 kb) while *D. catholica* showed a 3.7-kb band. The CMS line displayed a novel pattern with four bands of sizes 0.5, 2.0, 2.3 and 4.3 kb (figure 3a). Similarly, in *Xba*I digest, *nad3/rps12* hybridized to 7.1-kb and 6.3-kb fragments in *B. juncea*, and to a single 6.3-kb fragment in *D. catholica* (data not shown). In the CMS line, besides *B. juncea*-specific fragments, a new fragment of 10.1 kb was observed.

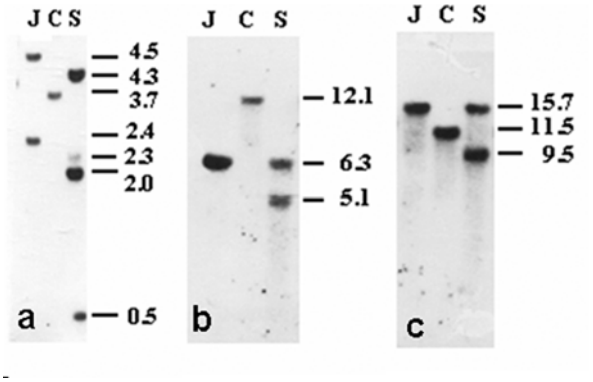

Figure 3. RFLP patterns of *B. juncea* (J), *D. catholica* (C) and the CMS line (S) obtained with various restriction enzyme and mitochondrial gene probe combinations: (a) *Xho*I/*coxII*, (b) *Eco*RV/*coxI*, (c) *Bam*HI/*coxI* (Fragment sizes in kb are indicated on the right).

When Southern blot prepared with *Eco*RV-digested DNA was probed with *coxI*, a 6.3-kb fragment of *B. juncea* and a 12.1-kb fragment of *D. catholica* were visualized. The CMS showed the *B. juncea*-specific band of 6.3 kb, and also a novel band of 5.1 kb (figure 3b). With *Bam*HI-digested DNA, fragments of 15.7 and 11.5 kb were detected in *B. juncea* and *D. catholica*, respectively. The CMS line showed two fragments of 15.7 and 9.5 kb hybridizing to the probe (figure 3c). Similarly, with *Bst*XI, *Eco*RI, *Hpa*II, *Hin*dIII, *Pvu*II *Sac*I, and *Xho*I the CMS line showed presence of novel bands, besides the *B. juncea*-specific bands. These data from Southern hybridization clearly established that there are two copies of *coxI* gene in the CMS line whereas the parents carry only one copy.

Analysis of mitochondrial gene expression

The Northern analysis was undertaken with RNA from flower bud tissue to find association between male sterility and transcript variation. Out of eight probes used, including *coxII* and *nad3/rps12*, altered transcript pattern was observed for only the *coxI* gene. A 2000-nt-long transcript was present in *B. juncea*, and *D. catholica*, as well as the CMS line. However, in the CMS line an additional transcript of 2400 nt was detected (figure 4a). The novel transcript was, moreover, more abundant than the normal transcript. In the male fertile flowers, this novel 2400-nt transcript was absent. When four separate plants were studied for *coxI* transcript pattern in the male-sterile and the male-fertile flower buds, the longer *coxI* transcript was found only in the male-sterile flower buds (figure 4b). Interestingly, RNA from leaf tissue of the CMS and parents showed only a 2000-nt transcript hybridizing to the *coxI* probe (figure 4c). Thus the CMS-associated 2400-nt transcript appears to be tissue specific. Since this is the only gene for which major rearrangement and transcript variation were detected in the CMS line, its involvement in causing CMS was suspected.

PCR amplification of coxI gene

In an attempt to detect the two *coxI* genes in the CMS by PCR, *coxI* gene was amplified using mtDNA of *B. juncea, D. catholica* and the CMS line. The amplification was carried out between 55°C and 60°C. A single band of 1.5 kb was obtained at all temperatures in all the three lines (figure 5). Only *Hpa*II and *Bst*XI could digest the amplicon. However, no polymorphism was detected. Interestingly, *Hpa*II had detected presence of a novel fragment in the CMS in Southern hybridization, but digestion of the amplicon with *Hpa*II did not detect any polymorphism. Similarly, RT-PCR amplification of *coxI* gene from RNA of flower buds of *B. juncea, D. catholica* and the CMS line also amplified a single fragment of 1.5 kb in the CMS and the parents. These results suggested two possibilities: either the two *coxI* coding regions are nearly identical, or the primers employed could amplify only one copy of *coxI*.

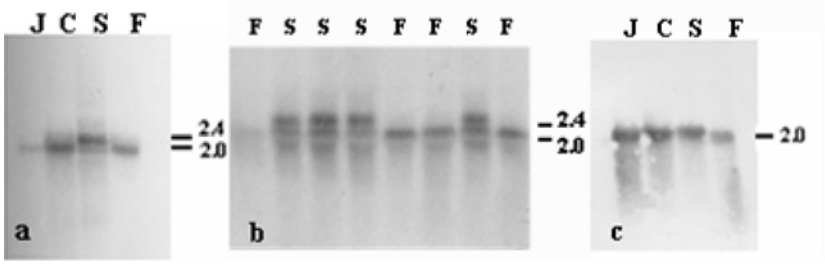

Figure 4. Northern blots prepared with RNA of flower bud (a, b) and leaf (c) probed with *coxI* (J — *B. juncea*, C — *D. catholica*, S — CMS line, F — fertility reverted plants). Transcript sizes in kb are indicated on the right.

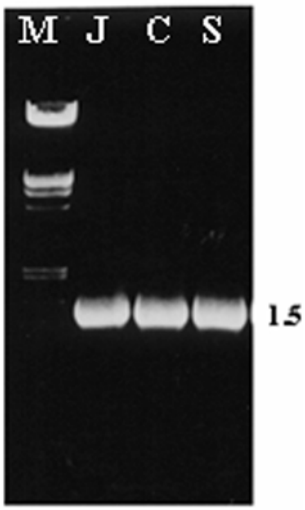

Figure 5. PCR amplification of *coxI* from mitochondrial DNA of *B. juncea* (J), *D. catholica* (C) and the CMS line (S). Amplicon size in kb is shown on the right.

Screening, identification and sequence analysis of the two coxI coding loci

For further characterization of the CMS line, the *Hin*dIII library of mtDNA from the CMS line was screened for *coxI* clones. Two clones of insert sizes 6.3 and 5.8 kb, designated as MS1 and MS2, respectively, were identified. For comparison, the 5.8-kb *Hin*dIII clone (BJ1) was chosen from mtDNA library of *B. juncea*. Restriction endonuclease analysis showed that the 5.8-kb *Hin*dIII fragment (BJ1) was similar to MS1 clone of the CMS line. However, the 6.3-kb clone (MS2) was found to have a different organization. This confirmed our observation from Southern hybridization that a normal copy of *coxI* gene similar to *B. juncea* is present in the CMS line. The BJ1 and MS1 clones do not carry the *Eco*RI site, whereas it is present in the 6.3-kb fragment of MS2 clone. The partial restriction maps of the 5.8-kb and 6.3-kb clones are given in figure 6.

The *coxI* coding and flanking regions of BJ1 clone spanning 2876 bp and the entire 6.3 kb of MS2 clone were sequenced. The coding region of *coxI* gene of BJ1 shares high sequence homology with *coxI* genes of Ogura radish, pea, soybean, potato and *Arabidopsis thaliana* mitochondrial genomes. Near-identical, uninterrupted ORFs of 1584 nucleotides with ATG start codon and TAA stop codon, corresponding to the coding region of *coxI* gene, were present in both the sequences. Only two amino acid changes were detected in the coding region between the two *coxI* genes (NCBI accession numbers AY300014 and AY300015). The presence of C at the 13th codon in *coxI-2* (from MS2 clone) instead of T as in *coxI-1* (BJ1 clone) leads to substitution of histidine residue with tyrosine. Similarly, a single base pair change at the 24th codon of *coxI-2* (A in place of G) results in replacement of glycine with serine residue. These codon changes in the MS2 clone were not found in any other *coxI* gene sequences in the database.

In the 5′ region, a stretch of about 100 bases from the start codon ATG was nearly identical between the two *coxI* genes. Beyond this, the 5′ region of *coxI-2* showed duplication of 31-nt-long sequence present in the *coxI-1*. A closer examination of the *coxI-2* sequences up to −324 bases revealed duplication and rearrangement of sequences separated by a homologous region of about 37 bases (figure 7). No significant homology was detected beyond this point. In the 3′-flanking region, the two *coxI* genes shared a continuous stretch of homologous region of 61 bp downstream of the stop codon, after which no significant homology was seen. Interestingly, 3′-flanking region of *coxI-2* gene showed sequences homologous to chloroplast sequence *ycf3* from *A. thaliana,* and *psaA* gene from *Sinapis alba*. ORF analysis of the two sequences did not reveal presence of any chimaeric orf that could be transcribed or expressed along with *cox1* transcript. Thus, sequences at the 5′ and 3′ regions of *coxI* strongly pointed to the involvement of these regions in mitochondrial recombination leading to the creation of *coxI* gene duplication.

Northern analysis of floral bud mtRNA

To know whether the novel transcript is derived from the additional *coxI* gene, Northern blot was sequentially probed with sequences from 5′-flanking region of *coxI-2* clone and 3′-flanking region of *coxI-1* clone. Primers were designed corresponding to the region outside the homology of the two

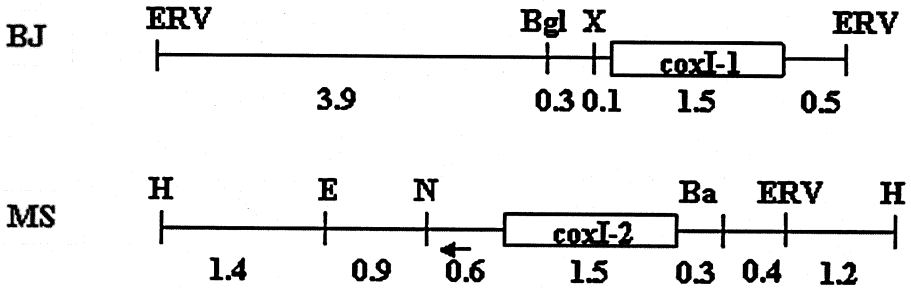

Figure 6. Partial restriction maps of BJ1 and MS2 clones containing the *coxI* gene. (B — *Bam*HI, E — *Eco*RI, H — *Hin*dIII, N — *Nco*I) The location of the P4 primer is indicated by an arrow below the main line. The numbers below the line indicate fragment size in kb.

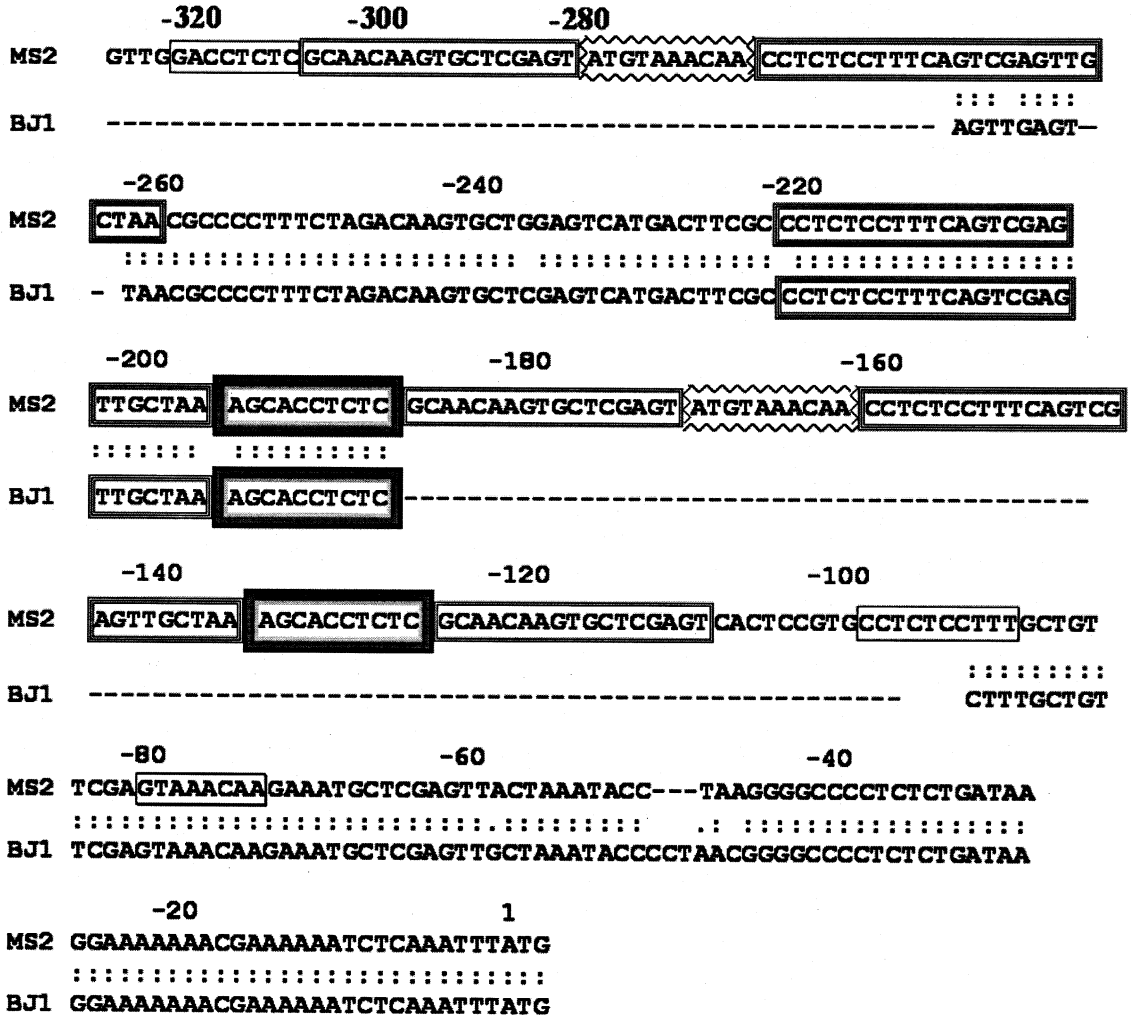

Figure 7. Comparison of the sequences upstream to the coding region of the two *coxI* genes. BJ1- clone with normal *coxI* gene of *B. juncea*, MS2 — clone with duplicated *coxI* from the CMS line. Various duplicated sequences are shown in different types of boxes.

cox1 genes and PCR amplification was done using P3+T7 and P4+T3 corresponding to the 3′-flanking region of *coxI-1* and 5′-flanking region of *coxI-2*, respectively. When the probe corresponding to the 3′-flanking region of *coxI-1* was used, only a 2000-nt-long transcript was detected (figure 8a). However, when 5′-flanking region of the *cox1-2* was used as a probe, only a 2400-nt-long transcript was detected in the CMS line (figure 8b). This confirmed that both the *coxI* genes are transcribed in the CMS line, and the longer transcript indeed originates from the recombined *coxI-2* gene.

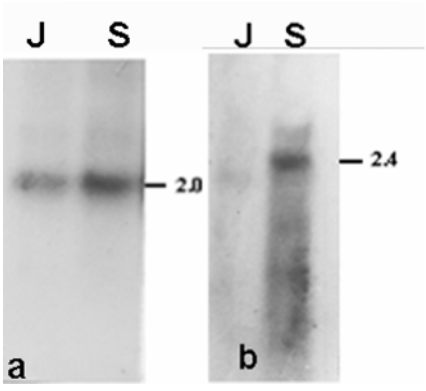

Figure 8. Northern blots of flower bud RNA probed with (a) 3′ sequences of *coxI* from BJ1 clone, and (b) 5′ sequences of *coxI* of MS2 clone (S — CMS line, J — *B. juncea*). The transcript sizes in kb are indicated at the right.

Discussion

CMS has been of great interest for its usefulness in the production of hybrid seeds thereby permitting exploitation of heterosis. Although several alloplasmic CMS lines have been developed in *B. juncea* (Prakash *et al*. 1998, 2001; Banga *et al*. 2003), they have not been well characterized at the molecular level. A CMS system based on wild-type cytoplasm of *D. catholica* was found to be associated with altered *atpA* transcription in the flower buds. Further, this

system was found to suffer from gynoecium abnormalities and poor female fertility (Pathania *et al*. 2003). Somatic hybridization was used to develop an improved CMS line involving *D. catholica* and *B. juncea* cytoplasms. The new CMS line bears normal flowers, instead of flowers with petaloid anthers, as seen in the sexual CMS, and shows very high female fertility. Similar improvement of CMS through somatic hybridization has been achieved earlier, where in undesirable characteristics of the ogura CMS (chlorotic leaves, low nectar secretions, feminization of the androecium) were eliminated through chloroplast substitution and mitochondrial recombination (Pelletier *et al*. 1988; Kirti *et al*. 1995b).

Molecular comparison between mitochondrial genomes of the CMS line and parents based on RAPD and RFLP analyses revealed that the mitochondiral genome of the CMS line is largely derived from *B. juncea*. However, a recombination of the mitochondrial genome was apparent in the CMS line, which resulted in the duplication of the *coxI* gene. Further, a strong association between duplicated *coxI* gene and the CMS trait was recorded based on Northern hybridization. The origin of duplicate *coxI* gene is, however, unclear. In Southern hybridization with *coxI* probe, and involving various restriction enzymes, one of the bands always corresponded with *B. juncea* whereas the other (duplicated, *coxI-2*) did not match with either of the parents. The mitochondrial genome in plants exists as a master circle and in multiple circular subgenomic forms which result from recombination between repeat elements spread over the genome (Palmer 1988; Hanson and Folkerts 1992). The 5′ region of *coxI-2* gene showed sequences that were duplicated and rearranged. Similar repeated sequences present at the *orf138* locus of Ogura and *orf125* of Kosena CMS radish are thought to be involved in recombination and origin of these CMS lines (Makaroff *et al*. 1989; Iwabuchi *et al*. 1999). In S-CMS of maize a repeat element present on the episome is known to recombine with a similar element on the master mitochondrial genome to give rise to a linear mitochondrial molecule leading to CMS (Zabala *et al*. 1997). The occurrence of long repeats at the 5′ region of *coxI-2* may thus represent the recombination event that has taken place at this region.

It is interesting to note that in the CMS line carrying unaltered mitochondrial genome of *D. catholica*, transcription pattern of *coxI* in the CMS line is similar to *B. juncea* and an altered transcription pattern of *atpA* gene is associated with male sterility (Pathania *et al*. 2003). Therefore, mere transfer of *D. catholica coxI* to *B. juncea* mitochondrial genome could not have led to CMS. Instead, the recombination event seems to be involved in causing CMS. In particular, the 5′ region extension was found in the novel transcript, suggesting either a change in the transcription-initiation site or transcript processing in a tissue specific manner. Further, the two amino acid differences between *coxI-1* and *coxI-2* may also be significant because histidine and glycine are highly conserved at 13th and 24th positions, respectively, of *coxI*. In 9E cytoplasm of male-sterile sorghum, mtDNA rearrangement has been found at the *coxI* locus. This rearrangement led to an extension of *coxI*-coding region, resulting in the synthesis of a modified form of protein (Bailey-Serres *et al*. 1986).

Our results are similar to CMS rice carrying bo cytoplasm where two copies of *atp6* were detected. The coding region of duplicated *atp6* (*B-atp6*) was identical to that of normal *atp6* but its 3′-flanking sequence was different, starting at 49 bases downstream of the stop codon. The *B-atp6* gives rise to CMS-associated 2000-nt transcript that is processed to normal 1500 nt and 450-nt transcripts in the presence of the restorer gene (Iwabuchi *et al*. 1993). Molecular analysis revealed that the RNA processing in presence of *Rf1* influences sequential posttranscriptional processing and editing of the *B-atp6* gene. The unprocessed RNA is not efficiently edited, presumably generating altered polypeptides, if they are translated. Similar posttranscriptional regulation of mitochondrial gene expression in which RNA processing and editing play a role in controlling CMS expression and restoration of fertility is also seen in A3 cytoplasm of sorghum (Tang *et al*. 1998; Pring *et al*. 1999). In *B. oleracea* CMS line carrying *D. muralis* cytoplasm, a partially duplicated *atp9* gene was found to give rise to a novel orf 72 that was associated with male sterility (Shinada *et al*. 2006).

However, the present results differ from those for rice in that the transcription of the normal *coxI* gene is drastically reduced in the CMS line. Further, male-sterile cybrids of rice derived from recombination between CMS-inducing and normal cytoplasms always contained a recombination involving the CMS-associated *B-atp6* gene region (Akagi *et al*. 1994). Similarly, recombination with *orf138* region was found in the *B. napus* male-sterile cybrids obtained from somatic hybridization with Ogura cytoplasm (Bonhomme *et al*. 1992; Grelon *et al*. 1994). In *B. juncea* with wild-type *D. catholica* cytoplasm, *atpA* gene is associated with CMS, but in the recombined mitochondrial genome of the somatic CMS line, this region is not at all involved. Thus, somatic hybridization in this case has created a new CMS-inducing locus involving the *coxI* gene.

It is intriguing that the normal copy of *coxI* is unable to function effectively to confer male fertility. Thus, in this instance CMS appears to be dominant. Since there is no major difference between the two *coxI* genes in the coding region and no novel ORFs could be identified in the duplicated *coxI* gene region, the male sterility may be due to defects in posttranscriptional processing or translation of the novel transcript. We have found that the *coxI* gene is not edited in the floral tissue of *B. juncea* (data not shown), which is also the case in *Arabidopsis* leaf tissue (Giege and Brennicke 1999). Therefore, editing problems may not be the cause of CMS. Transcript cloning and protein expression studies will be needed to clarify these possibilities.

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