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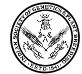

Molecular characterization reveals chlorosis-corrected CMS (*Brassica oxyrrhina*) *B. juncea* cybrid has recombinant mitochondrial genome involving male sterility inducing *orf108-atpA* gene

Vasupalli Naresh, Krothapalli Raja Surya Sambasiva Rao[1] and Shripad Ramachandra Bhat*

ICAR-National Research Centre on Plant Biotechnology, New Delhi 110 012; [1]Acharya Nagarjuna University, Guntur 522 510, Andhra Pradesh

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Abstract

Alloplasmic *Brassica juncea* lines with *B. oxyrrhina* cytoplasm show stable cytoplasmic male sterility (CMS) but display chlorosis due to incompatible interaction between the plastid and the nuclear genomes. Normal, green CMS cybrids were developed through somatic hybridization between chlorotic CMS line and *B. juncea*. This study was aimed at characterization of the mitochondrial genome constitution of two chlorosis-corrected *B. juncea* cybrids. Comparative RFLP analysis of parents and cybrids with mitochondrial gene specific probes revealed *B. oxyrrhina* specific pattern in cybrids for *atp4*, *ccmfn2*, *nad4L* and *cox3* but *atp1* and *orf108* gave novel pattern indicating recombinant mitochondria. RT-PCR showed co-transcription of *orf108* and *atp1* in the cybrids. PCR analysis with mitochondrial genome specific primers revealed that the cybrids had mostly *B. oxyrrhina* like mitochondrial genome. The two cybrids showed identical RFLP and PCR pattern suggesting that they are derived from the same event. Interestingly, RFLP pattern indicated two copies of *orf108-atp1* in the cybrid. One of the copies was derived from intermolecular recombination between *B. juncea* and *B. oxyrrhina* whereas the second copy resulted from intramolecular recombination between a pair of short repeat regions. The results show that *orf108-atp1* region is recombinationally active and could be involved in causing male sterility.

Key words: Cytoplasmic male sterility, mitochondrial recombination, Northern, RFLP, RT-PCR

Introduction

Plant mitochondrial genomes are relatively large and range in size from 200 to 2000 kb (Kubo et al. 2011). Despite this large difference, the coding capacity of mitochondrial genomes is limited and most of the size differences arise from the increase in the size of introns and number of short repeats (50-550 bp) (Kubo et al. 2011). In higher plants, mitochondria are uniparentally inherited from the maternal side. Further, each cell contains several hundred copies of the mitochondrial genome. Therefore, spontaneous or induced mutations in the mitochondrial genome often fail to get established. As a consequence, mitochondrial genome shows very limited sequence variation within a species. Further, transformation techniques have not been devised so far for plant mitochondria. Therefore, functional analysis of mitochondrial genes poses challenge.

Sequenced mitochondrial genomes assemble as a single circular molecule. However, mitochondrial genome *in vivo* is dynamic and exists as multipartite, subgenomic circles due to intramolecular recombination at short and large (>1 kb) repeats (Sun et al. 2012). Depending on cellular demands, stoichiometry of different subgenomic molecules varies in different tissues. The stoichiometric shift sometimes leads to generation of new mitotypes (Woloszynska 2010). Mitochondrial recombination appears to be under the control of nuclear genes. Mutations in the nuclear genes *MSH1* (Abdelnoor et al. 2003), *REC1* (Shedge et al. 2007) and *OSB1* (Zaegel et al. 2006) have been shown to generate mitochondrial genome variants due to increased mitochondrial recombination and substochiometric shift (Arrieta-Montiel et al. 2009).

Maternally inherited cytoplasmic male sterility

*Corresponding author's e-mail: srbhat22@gmail.com
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(CMS) is one of the few traits governed by the mitochondrial genes. Therefore, CMS is used as a model to understand mitochondrial genetics. Besides, CMS is also an important agronomic trait that facilitates hybrid seed production. CMS of spontaneous origin have been widely employed in commercial hybrid seed production in crops such as maize, sugar beet, sorghum, pearl millet, *Brassica*, sunflower etc. (Chen and Liu 2014). In addition, CMS is also frequently found in alloplasmic lines developed through interspecific hybridization (Yamagishi and Bhat 2014). Studies conducted in many different species have shown that novel mitochondrial open reading frames (orfs) usually co-transcribed with some essential genes of the oxidative phosphorylation pathway are associated CMS (Kubo et al. 2011).

To facilitate hybrid breeding of the Indian mustard (*Brassica juncea*), our laboratory has developed a number of CMS and fertility restorer lines by processing interspecific hybrids between cultivated *Brassica* species (*B. juncea or B. rapa*) and wild allies (Prakash and Chopra 1990; Pathania et al. 2003; Bhat et al. 2006, 2008). A *B. juncea* CMS line based on *B. oxyrrhina* cytoplasm was developed through sexual hybridization (Prakash and Chopra 1990). However, presence of co-inherited plastid genome of the wild species led to leaf chlorosis making it unfit for practical use. This defect was corrected through somatic hybridization involving recombination of plastid genomes of *B. juncea* and *B. oxyrrhina* (Kirti et al. 1993). A preliminary molecular analysis of these chlorosis-corrected cybrid CMS lines showed that these lines carry recombined plastid genome and *B. oxyrrhina* mitochondrial genome (Kirti et al. 1993). The *Rf* gene for *B. oxyrrhina* based CMS lines of *B. juncea* has not been identified so far. While *B. oxyrrhina* is known to carry CMS-inducing *orf108* (Kumar et al. 2012), the *M. arvensis Rf* gene fails to restore male fertility to *B. oxyrrhina* CMS lines (unpublished). Hence, the molecular basis of CMS in *B. oxyrrhina* based CMS lines of *B. juncea* remains to be determined. Mitochondrial recombination is frequently reported in somatic hybrids (Belliard et al. 1979; Vedel 1986). Further, tissue culture regeneration is known to bring about alteration in mitochondrial genome (Kanazawa et al. 1994). Sun et al. (2012) have shown that suppression of *MSH1* and *RECA3* gene function in *in vitro* cultures leads to mitochondrial genome changes. Therefore, we undertook a detailed study of mitochondrial genome organization in two chlorosis-corrected CMS (*B. oxyrrhina*) *B. juncea* cybrids with the hope that recombined mitochondrial genomes would help us in identifying candidate gene(s) associated CMS.

Materials and methods

Plant materials

Male sterile *B. juncea* carrying *B. oxyrrhina* cytoplasm (*oxy-cms*) (Prakash and Chopra 1990), CMS *B. juncea* cybrids (*oxy-cyb1, oxy-cyb3*) (Kirti et al. 1993), male fertile synthetic amphidiploid *B. oxyrrhina* × *B. rapa* (*oxy-camp*) (Prakash and Chopra 1988), and euplasmic *B. juncea* cv. RLM 198 were used in this study.

Molecular analysis

All the molecular analyses including nucleic acid isolation, Southern and Northern hybridizations (including details of probes), PCR and RT-PCR were done as described previously by Vasupalli et al. (2016). Southern hybridization was done as per Sambrook et al. (1989) using 5 μg of DNA.

Results

RFLP analysis shows oxy-cyb1 and oxy-cyb3 possess two copies of orf108 and atp1

Mitochondrial genome organization in the male sterile (*oxy-cms, oxy-cyb1* and *oxy-cyb3*) and the male fertile (*oxy-camp*, RLM198) lines was examined through RFLP analysis using five mitochondrial gene probes. No difference in the RFLP pattern was detected among the five lines when DNA blots prepared from *Hind*III or *EcoR*I digests were probed with *atp4*, *ccmfn2*, *nad4L* and *cox3* gene probes. However, major differences in RFLP profile were observed for both *atp1* and *orf108* genes. When total DNA was digested with *Eco*RI and probed with *atp1*, a 2.7 kb fragment was detected in *oxy-cms* and *oxy-camp*, whereas euplasmic *B. juncea* showed a 4.6 kb band. In contrast, *oxy-cyb1* and *oxy-cyb3* showed two bands of 4.6 and 7.0 kb (Fig. 1A). Similarly, with *Hind*III, *oxy-cyb1* and *oxy-cyb3* showed two fragments (5.0 and 6.0 kb) hybridizing to the probe while *oxy-cms* and *oxy-camp* showed a single band of ~5.5 kb. Likewise, with *BamH*I, two fragments of 1.7 and 1.45 kb were detected in *oxy-cms* and *oxy-camp*, whereas *oxy-cyb1* and *oxy-cyb3* gave three bands (8.0, 4.0 and 1.7 kb). With *EcoR*I and *BamH*I, a band corresponding to *B. juncea* was detected whereas it was not so with *Hind*III. This suggested that oxy-cyb1 and oxy-cyb3 contain recombinant mitochondrial genome. When the same blot was reprobed with *orf108*, *Eco*RI digests showed a single 2.6 kb fragment

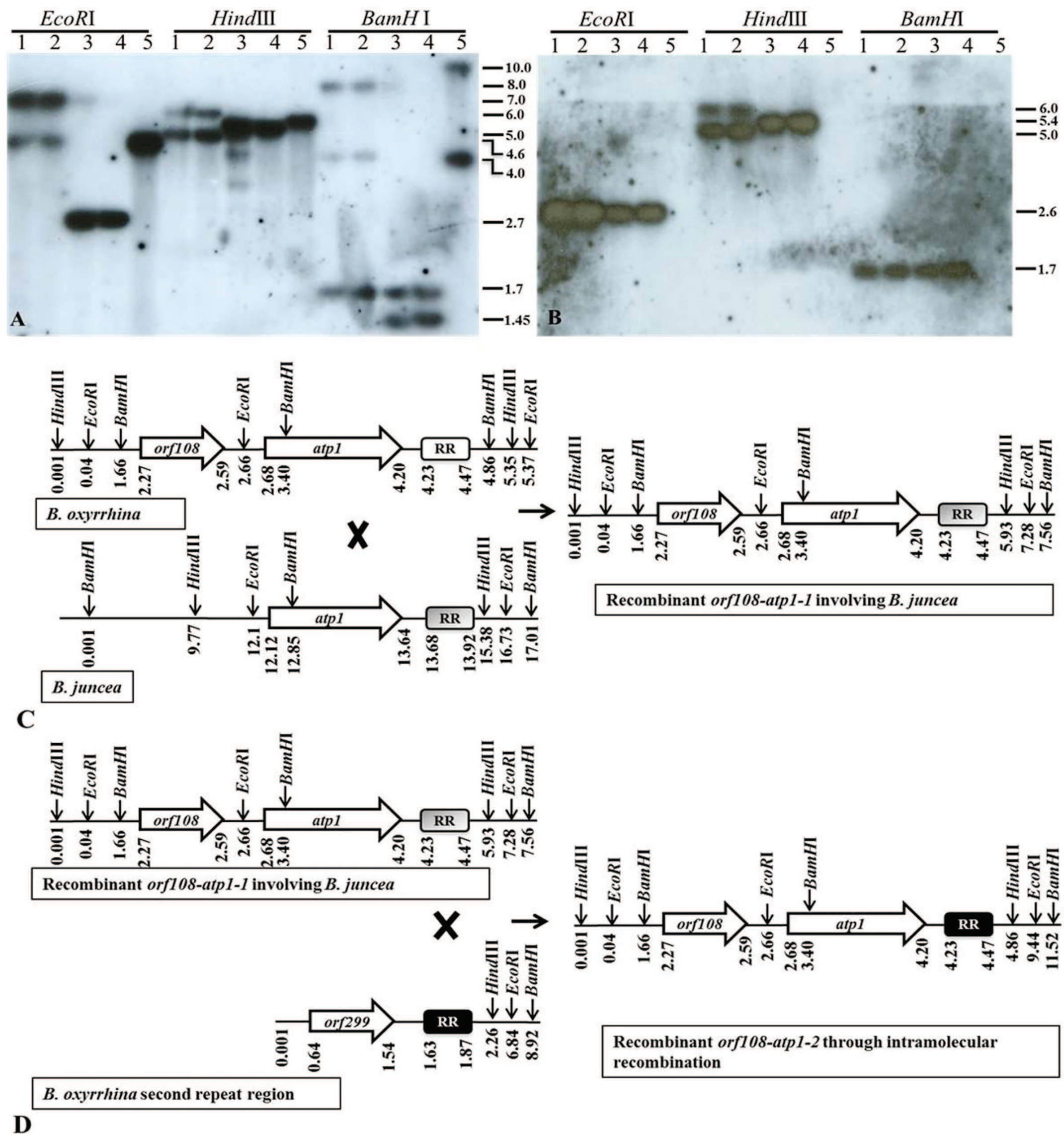

Fig. 1. Analysis of mitochondrial genome constitution of *B. oxyrrhina*-based cybrids of *B. juncea*. A and B = Southern blots hybridized with mitochondrial gene probes *atp1* (A) and *orf108* (B). 1. *oxy-cyb1*, 2. *oxy-cyb3*, 3. *oxy-camp*, 4. *oxy-cms*, and 5. *B. juncea.* Fragment sizes are indicated in kb. C and D = Schematic representation of origin of recombinant *atp1-1* (C) and *atp1-2*

in all lines except the euplasmic *B. juncea* whereas with *Hind*III digests two fragments of 5.0 and 6.0 kb were visualized in the cybrids. Also, with *BamH*I 1.7 kb fragment was detected in all lines except euplasmic *B. juncea* (Fig. 1B). However, the signal intensity in *oxy-cyb1* and *oxy-cyb3* was stronger than that recorded in *oxy-cms* and *oxy-camp* lines suggesting that *orf108* is duplicated in cybrids. We have sequenced the mitochondrial genome of *B. oxyrrhina* (unpublished data) and the sequence assembly of the *orf108-atp1* region is depicted in Fig. 1C. The RFLP patterns of *oxy-cms* and *oxy-camp* were found to match banding pattern expected for *B. oxyrrhina* based on its mitochondrial genome sequence data. In contrast, both the cybrids were found to be identical and contain recombined mitochondrial genome. In particular, the mitochondrial recombination appears to have occurred in the *atp1* gene region of *B. juncea* and *B. oxyrrhina*. For instance, *oxy-cyb1* and *oxy-cyb3* gave 4.6 kb *Eco*RI and 4.0 kb *BamH*I fragments, which corresponds to the RFLP pattern of *B. juncea atp1* gene.

We examined the mitochondrial genome sequence around *atp1* gene to identify the possible

recombination points that generated the new molecules. A pair of direct repeats of 240 bp and separated by ~100 kb intervening sequences is found in both *B. juncea* and *B. oxyrrhina*. RFLP pattern with three different restriction enzymes showed that one of the *atp1* copies in the cybrids matches the *B. juncea* copy (Fig. 1C). This copy, henceforth called *atp1-1*, appears to have originated from homologous recombination between *B. juncea* and *B. oxyrrhina* mitochondrial genomes in the region between *Eco*RI site upstream of *atp1* and the repeat region (Fig. 1C). The RFLP pattern of the second copy, designated as *atp1-2*, did not match either of the parents. Instead, an intramolecular recombination in *B. oxyrrhina* mitochondrial genome involving the two repeat regions appears to have led to the generation of *atp1-2* (Fig. 1C). In Southern blot, the band intensity of *atp1-2* was always more than the *atp1-1*. This strongly indicates that *atp1-2* is a substoichiometirc molecule originating from intramolecular recombination.

To further assess the mitochondrial genome constitution of cybrids, a total eight primer pairs (Table 1) yielding polymorphic amplification pattern between *B. juncea* and *B. oxyrrhina* were designed based on the mitochondrial genome sequence, and used in PCR. All primer combinations tested gave identical amplification patterns between the cybrids and *B. oxyrrhina* (Fig. 2). Thus based on RFLP and PCR

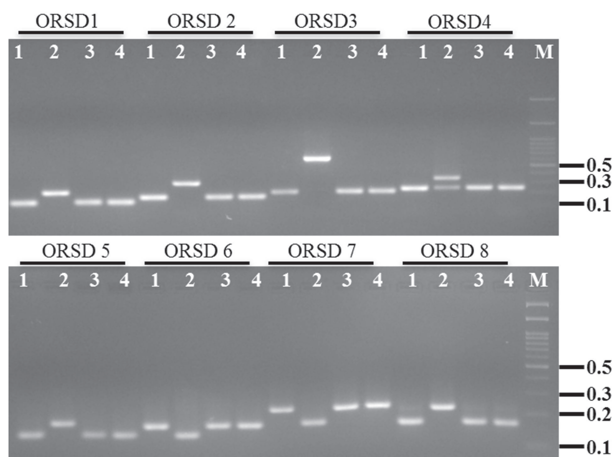

Fig. 2: Gel picture of PCR amplicons obtained in *B. oxyrrhina* derived *B. juncea* cybrids with primers targeted to amplify polymorphic mitochondrial regions of *B. oxyrrhina* and *B. juncea*. 1. *oxy-cms*, 2. *B. juncea*, 3. *oxy-cyb1*, 4. *oxy-cyb3*, M. 100 bp DNA marker

results we conclude that *oxy-cyb1* and *oxy-cyb3* are identical and carry recombined mitochondrial genome derived from *B. juncea* and *B. oxyrrhina* with the majority of sequences derived from the latter. Further, the mitochondrial recombination leads to generation of two different *atp1* and *orf108* molecules in the cybrids.

orf108 and atp1 are co-transcribed in the cybrid

Our previous study had shown that *orf108* is located upstream to *atp1* in *B. oxyrrhina* (Kumar et al. 2012). To verify whether these genes are co-transcribed as in other CMS systems, northern analysis was done using RNA isolated from flower buds of *oxy-cyb1*, *oxy-cyb3* and *B. juncea*. When the blot was hybridized with *orf108*, a 2.4 kb transcript was detected in both *oxy-cyb1* and *oxy-cyb3* while no transcript was found in *B. juncea* (Fig. 3A). When the blot was reprobed

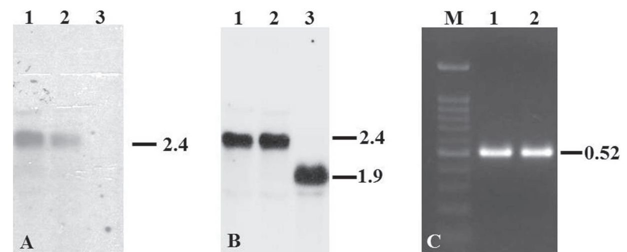

Fig. 3: Expression analysis of *orf108* and *atp1* genes in *B. oxyrrhina*-based CMS lines of *B. juncea*. A, B. Northern blots hybridized with mitochondrial gene probes *orf108* (A) and *atp1* (B). 1. *oxy-cyb1*, 2. *oxy-cyb3*, 3. *B. juncea*. C. RT-PCR analysis of co-transcription of *orf108-atp1*. 1. *oxy-cyb1* and 2. *oxy-cyb3*, M. 100 bp DNA marker. Fragments sizes are indicated in kb

with *atp1* a 2.4 kb fragment was detected in both *oxy-cyb1* and *oxy-cyb3* but *B. juncea* showed a 1.9 kb transcript (Fig. 3B). To further confirm co-transcription of *orf108* and *atpA*, RT-PCR was done using the forward primer from *orf108* and the reverse primer from *atp1* (Table 1). This gave a 520 bp amplicon in both *oxy-cyb1* and *oxy-cyb3* thereby confirming co-transcription of *orf108* and *atp1* genes (Fig. 3C).

Discussion

A previous study had shown that the chlorosis-corrected cybrids *oxy-cyb1* and *oxy-cyb3* carry wild type *B. oxyrrhina* mitochondrial genome and recombinant plastid genome (Kirti et al. 1993). However, our RFLP analysis showed that the cybrids are recombinant for the mitochondrial genome also and carry two copies of *orf108-atp1* genes. One of the *atp1* sequences was derived from *B. juncea*. However,

Table 1. Details of primers used in the study

Primer name	Forward primer (5′-3′)	Reverse primer (5′-3′)
ORSD 1	CGATAAAGTCCGTTCCTCAC	TACAGATTGCTCGCCTTAAC
ORSD 2	GAGGATGGAGAGCTTTCATTG	CAATTCCGTACTCAGGAGTTTC
ORSD 3	ATTGAACATATGACCGACCTAC	GGTTCGAATCCCTCTCTTTC
ORSD 4	GAGGTCTTCGGCTTGAATG	GAATCATTCTCGTCCTCCAAA
ORSD 5	GTATGGAAAGACGCCTACAC	GGAAGTTGCTTGGAAGGAT
ORSD 6	CTCGAGCATTTCTTGTTTACTC	ATAGCCCACGTCTTCCA
ORSD 7	CCAACAAACGGTTACATCAAAG	TTGGGAATGGAATGGGAATG
ORSD 8	TAGAAAGGGCGGTAGTAGAG	CTGACCACAGTCAATCAGTC
orf108 F/ atp1R	CCCGAAAATCAACTTCTACTTATGAAGAC	GACCGATCTCATCCACTTGAAA

for all other regions queried, the cybrids showed PCR pattern identical to *B. oxyrrhina* mitochondrial genome. Further, the two cybrids were indistinguishable at all the mitochondrial regions examined.

Mitochondrial recombination is frequently reported in somatic hybrids (Belliard et al. 1979; Kirti et al. 1995; Vedel et al. 1986). However, only a few studies have fine mapped the recombination points. Analysis of a cybrid derived from Kosena radish CMS and *B. napus* revealed that homologous recombination at the 63 bp repeat region shared by the two parental mitochondrial genome gave rise to a chimeric mtDNA molecule carrying CMS-inducing *orf125*/*orfB* region (Oshima et al. 2010). By sequencing mitochondrial genome of a cybrid between *Nicotiana tabacum* + *Hyosyamus niger*, Sanchez-Puerta et al. (2015) have shown high frequency mitochondrial recombination through homologous recombination. They found 35 intergenomic recombinations between the parental mitochondrial genomes leading to a mosaic of recombinant mitochondrial genome. In our study, we have found cybridization leading to mostly *B. oxyrrhina*-like mitochondrial genome. Interestingly, the *orf108*, which is known to cause CMS in *B. juncea* (Kumar et al. 2012), was found to occur in two different configurations in the cybrid. Sanchez-Puerta et al. (2015) have reported multiple copies of 15 mitochondrial genes. Of these, six genes were duplicated from one of the parental genomes. In a previous study involving somatic hybrids between *B. juncea* and *D. catholica*, *coxI* gene was found to be duplicated (Pathania et al. 2007). In the present case, *orf108*-*atp1* duplication event resulted from two homologous recombination events of which one was between the parental mitochondrial genomes whereas the other was through intramolecular recombination. Analyzing rapeseed cybrids with recombined mitochondrial genome between *B. napus* and *Ogura* radish CMS, Bellaoui et al. (1998) found different mitochondrial subgenomes, of which some were predominant. Further, a shift in the balance of the substoichiometric molecules led to reversion to male fertility. Their results suggested an active intramolecular recombination of the mitochondrial genome. In our study also, the *atp1-1* copy was less abundant than *atp1-2* copy, which strongly suggested that this is a subgenomic molecule generated from intramolecular recombination. Since independent mitochondrial recombinations are unlikely to result in identical recombinants, the two cybrids appear to have originated from the same event. Our results of mitochondrial genome sequence do not suggest any new orfs in *B. oxyrrhina*. Therefore, *orf108* appears to be the candidate male sterility gene in *B. oxyrrhina* based CMS lines of *B. juncea*.

Authors' contribution

Conceptualization of research (SRB, KRSSR); Designing of the experiments (VN, SRB, KRSSR); Contribution of experimental materials (SRB); Execution of field/lab experiments and data collection (VN); Analysis of data and interpretation (VN, SRB); Preparation of manuscript (VN, SRB).

Declaration

The authors declare no conflict of interest.

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