RESEARCH PAPER

MutS HOMOLOG1 silencing mediates ORF220 substoichiometric shifting and causes male sterility in *Brassica juncea*

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Abstract

Cytoplasmic male sterility (CMS) has consistently been associated with the expression of mitochondrial open reading frames (ORFs) that arise from genomic rearrangements. Spontaneous fertility reversion in CMS has been observed in several cases, but a clear understanding of fertility reversion controlled by nuclear genetic influences has been lacking. Here, we identified spontaneous fertile revertant lines for *Brassica juncea* CMS cytoplasm in which the mitochondrial genome has undergone substoichiometric shifting (SSS) to suppress *ORF220* copy number. We placed *ORF220*, with or without a mitochondrial targeting presequence, under the control of the CaMV35S and AP3 promoters in Arabidopsis to confirm that *ORF220* causes male sterility when mitochondrially localized. We found that copy number of the *ORF220* gene was altered under conditions that suppress MSH1, a nuclear gene that controls illegitimate recombination in plant mitochondria. *MSH1-RNAi* lines with increased *ORF220* copy number were male sterile compared with wild type. We found that a wide range of genes involved in anther development were up- and down-regulated in revertant and *MSH1-RNAi* lines, respectively. The system that we have developed offers valuable future insight into the interplay of MSH1 and SSS in CMS induction and fertility reversion as a mediator of nuclear–mitochondrial crosstalk.

Key words: Cytoplasmic male sterility, DNA recombination, mitochondrial DNA, MSH1, pollen, substoichiometric shifting.

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that prevents the production of functional pollen. The phenomenon, observed in >150 plant species with several conserved features in common, comprises one of very few systems of nuclear–mitochondrial genetic interaction amenable to study in higher plants. CMS has been associated
with expression of novel mitochondrial open reading frames (ORFs) that arise by rearrangement of mitochondrial genomes (Hanson and Bentolila, 2004; Woodson and Chory, 2008). Fertility restorer (Rf) genes—pentatricopeptide repeat (PPR) proteins in most cases—are examples of nuclear genes that can alter mitochondrial CMS-associated gene expression. The PPR proteins usually operate at post-transcriptional levels, by RNA editing, processing, and polyadenylation, as well as post-translationally (Hanson and Bentolila, 2004; Schmitz-Linneweber and Small, 2008). In the system of Wild Abortive CMS in rice, for example, restorer genes Rf/4 and Rf/3 suppress the CMS-associated WA352 gene at transcriptional and translational levels, respectively (Luo et al., 2013).

Spontaneous fertility reversion in CMS has been seen in several plant species, and serves as an alternative means in nature to overcome mitochondrial encoded male sterility (Bellaoui et al., 1998; Arrieta-Montiel et al., 2001). Spontaneous fertility reversion in CMS is generally characterized by mitochondrial genomic substoichiometric shifting (SSS) (Arrieta-Montiel and Mackenzie, 2010), with the frequency of these genomic changes influenced by nuclear genetic background (Mackenzie et al., 1988; Small et al., 1988). An individual nuclear gene has been shown to reproducibly direct particular mitochondrial rearrangement events in common bean (Mackenzie and Chase, 1990), and natural or induced nuclear gene mutation can cause fertility reversion in carrot (Chahal et al., 1998) and rice (Shen et al., 1996). Tissue culture conditions can also give rise to fertility reversion in petunia and maize, again in association with mitochondrial genomic changes (Clark et al., 1988; Small et al., 1988; Fauron et al., 1990). However, the nuclear genes controlling mitochondrial genomic recombination to effect fertility reversion have not been identified in most cases.

The plant mitochondrial genome is known to undergo high frequency recombination and to comprise a multiparite organization (Arrieta-Montiel and Mackenzie, 2010; Marechal and Brisson, 2010). Asymmetric DNA exchange at small repeats appears to influence the stoichiometry of subgenomic mtDNA molecules—a phenomenon termed SSS (Small et al., 1989). This recombination is influenced by nuclear genes, including RecA3 and MSH1, which suppress ectopic mitochondrial recombination (Abdelnoor et al., 2003; Sledge et al., 2007). In Arabidopsis msh1, over 47 recombination repeat pairs become differentially active in the mitochondrial genome (Davila et al., 2011). Disruption of MSH1 has been shown to result not only in mitochondrial SSS, but also the appearance of CMS in several crops (Sandhu et al., 2007). Mitochondrial genome recombination plays an important role in plant mitochondrial genome evolution (Small et al., 1989; Chang et al., 2011b), generating novel mitotypes (Chen et al., 2011), and environmental adaptation (Sledge et al., 2010; Xu et al., 2011).

We previously developed a CMS line of *Brassica juncea* and identified the CMS-associated locus ORF220 in the mitochondrial genome (Yang et al., 2010). In this study, we identified fertile revertant lines in CMS *B. juncea*. We then established a link between MSH1 and mitochondrial genome rearrangements, effecting ORF220 SSS in association with fertility reversion. We suggest that the MSH1–mitochondrial interaction in plants may participate in the reversible male sterility–fertility transitions involved in gynodioecious reproductive systems.

**Materials and methods**

**Plant materials**

CMS and its fertile maintainer lines of *B. juncea* were used for identification of revertant lines and development of MSH1–RNAi lines. Wild type (WT) Arabidopsis (*Arabidopsis thaliana*) (Col-0) was used for transformation of ORF220 with and without a mitochondrial-targeting peptide under 35S (CaMV 35S) and AP3 (APETALA3) promoters. The mitochondrial targeting peptide was amplified from a previous construction plasmid (He et al., 1996). WT Arabidopsis (Col-0) was used for amplification of AP3 promoter sequences. A fertile isogenic maintainer line of *B. juncea* was used to generate the MSH1–RNAi line.

**Mitochondrial genome assembly**

Total DNA was isolated from fresh leaves of CMS and REV19 lines using a cetyl trimethylammonium bromide (CTAB) protocol. Total genomic DNA was prepared in paired-end libraries, tagged and sequenced on the Illumina Hisq2500 platform. High quality reads were mapped to the *B. juncea* mitochondrial genome sequence (Genbank: KF741445) using SAOP2, and paired mapping reads were extracted for mitochondrial genome assembly. These reads were assembled into scaffolds with the Velvet program (Zerbino and Birney, 2008).

**DNA gel blotting and SSS of ORF220**

Total genomic DNA samples were extracted from leaves for DNA gel blotting and SSS analysis of ORF220. For blotting, total genomic DNA samples were digested with HindIII endonuclease (Takara, Japan). Digested DNA samples were separated by electrophoresis for 24 h, and then transferred and fixed to nylon membrane (Amerham, Sweden) by capillary method. The ORF220 probe was prepared by PCR with the DIG probe synthesis kit (Roche, Switzerland). Hybridization was performed by standard pre-hybridization, probe denaturation, and hybridization in solution. The hybridization signal was detected using DIG High Prime DNA Labeling and Detection Starter II kit (Roche, Switzerland).

**SSS of ORF220**

ORF220 was monitored by varying PCR amplification cycles. The PCR reaction was performed in a total volume of 50 μl containing 5 μl 10× Ex Taq Buffer (Mg2+ plus), 4 μl 10mM dNTP, 10 pmol of forward and reverse primers, 200ng of template DNA and 0.25 μl Ex Taq™ DNA Polymerase. The amount of template DNA was adjusted to be equal in each sample. The PCR solutions were incubated for 5 min at 94 °C, and then run for 25, 30, and 35 cycles, respectively, at 94 °C for 30s, 50 °C for 30s, and 72 °C for 60 s, followed by final extension at 72 °C for 10 min. The amplifications were separated by electrophoresis on 1% agarose gels. The primers used for ORF220 SSS assays are listed in Supplementary Table S1 at *JXB* online.

**Expression analysis of ORF220**

RT-PCR and real-time RT-PCR were used for transcriptional expression of ORF220. Protein gel blotting was employed to investigate translational expression of ORF220. ORF220 polyclonal antibodies were prepared by BGI Protein (BGI, China). Plant total proteins were extracted from floral buds using Plant Protein Extraction kit (BestBio, China). Plant proteins were separated in 5% and 12% gradient acrylamide gels, and were transferred to PVDF.
of orthologous groups (COG) analysis was used as an online service (www.ncbi.nlm.nih.gov/COG/).

Differentially expressed genes by RNA-seq were annotated based on whole genome sequence information. Then we selected 15 annotated anther development-associated genes to represent candidate genes involved in Arabidopsis anther development (Chang et al., 2011a). Quantitative (q)PCR was used to study expression patterns of these selected anther development-associated genes. The primers for these anther-related genes are listed in Supplementary Table S1 at JXB online.

Results

Identification of fertility reversion in B. juncea

Thirty-three genes from self-pollination were collected from 39 CMS B. juncea (T84-66A) plants, of which two seeds gave rise to male fertile plants, designated revertants REV19 and REV21, and the remaining were male sterile plants (see Supplementary Table S2 and Supplementary Fig. S1 at JXB online). REV19 displayed earlier flowering than the CMS isolate (Fig. 1A), with full flower structure and normal stamens (Fig. 1B, C). Pollen from REV19 appeared normal based on Alexander staining (Fig. 1D), DAPI staining (Fig. 1E), and in situ germination on stigmas (Fig. 1F). Consequently, seed set was fully recovered in REV19 compared with the CMS line (Fig. 1G, H). REV19 progeny showed full fertility in three consecutive self-crossed generations, but could not restore fertility to the CMS line in crossing as a pollen parent, indicating that the reversion represents a cytoplasmic event.

Mitochondrial genome rearrangement and SSS of ORF220 in CMS and REV lines

We compared mitochondrial DNA in CMS and REV19 using assembled mt genome scaffolds, confirming that REV19 is not a fertile maintainer line contaminant (see Supplementary Fig. S2 at JXB online). We previously identified the CMS-associated ORF220 from CMS B. juncea (Yang et al., 2010). We compared mitochondrial DNA intervals encompassing CMS-associated ORF220 and flanking regions in the two lines, and observed two genome rearrangements around ORF220—a genomic insertion of atp4 and a reverse complement sequence composed of several mitochondrial genes (Fig. 2). Results indicated that ORF220 and its flanking regions undergo extensive genomic rearrangement between CMS and REV19 (Supplementary Fig. S3). We also observed several additional mitochondrial rearrangements in other regions of the mitochondrial genomes between CMS and REV19 (Supplementary data).

Different configurations of CMS-associated ORF220 were found in the CMS, REV19, REV21, and fertility maintainer (MF) lines, indicated by DNA gel blotting (Fig. 3A). We checked ORF220 copy number by PCR-based amplification, showing evidence of SSS in the various lines (Fig. 3B). Expression of ORF220 was significantly increased in the CMS line and decreased in REV19 based on RT-PCR and qRT-PCR (Fig. 3C, D), as well as protein gel blotting (Fig. 3E). The apparent correspondence of ORF220 copy number with
Zhao et al. gene expression levels in the male sterile and revertant lines suggests that SSS of ORF220 is associated with fertility reversion in CMS B. juncea. Mitochondrially targeted ORF220 causes male sterility in Arabidopsis

To further test the association of ORF220 with male sterility, we developed Arabidopsis lines containing ORF220 gene constructions with and without a mitochondrial targeting presequence and under control of the CaMV 35S (constitutive) and AP3 (flower-specific) promoters (see Supplementary Fig. S4 at JXB online). Plants containing the construct with the 35S promoter, with and without presequence, showed evidence of slightly reduced growth (Fig. 4A). In total, 17 plants that were transformed with the mitochondrially targeting construct were male sterile and plants transformed with the construct lacking presequence showed no evidence of sterility when expressed under control of the 35S promoter (Supplementary Table S3: Fig. 4B–E). Moreover, in constructs containing the AP3 promoter, 28 plants containing the construct with mitochondrial presequence were male sterile and one plant with the construct lacking presequence showed male sterility (Supplementary Table S3: Fig. 4G–J). These results are consistent with our hypothesis that mitochondrially localized ORF220 causes male sterility.

Phenotypes and ORF220 SSS in MSH1-RNAi lines

Two MSH1 genes were isolated from the B. juncea genome with high amino acid sequence similarity to their ortholog in Arabidopsis (see Supplementary Fig. S5 at JXB online). We developed four independent MSH1-RNAi lines of B. juncea with confirmed suppression of MSH1 expression, where two of the lines showed male sterility in the T1 generation. Varied leaf shape and normal flowering were also observed in the MSH1-RNAi lines (Fig. 5A). ORF220 copy number assays showed evidence of SSS following MSH1 suppression (Fig. 5B), and transcript levels of ORF220 were correspondingly increased in MSH1-RNAi lines (Fig. 5B). Comparison of three other mitochondrial genes in these lines indicated no evidence of gene alteration or copy number shifting (Supplementary Fig. S6).
B. juncea MSH1-RNAi lines produced small flowers (Fig. 5C), and stamen development was severely affected, such that anthers were not observed (Fig. 5C). The three MSH1-RNAi lines produced no seed by self-pollination (Fig. 5C), although seed set occurred with pollen from the WT. These results indicate that MSH1 suppression can lead to SSS of ORF220 and male sterility in B. juncea.

Anther development-associated gene expression

We employed RNA-seq to identify global transcriptional differences between CMS and REV19 lines of B. juncea, and to investigate the nature of mitochondrial retrograde regulation associated with fertility reversion. In total, we found 4880 differentially expressed genes between CMS and REV19 lines (see Supplementary Table S4; Supplementary Fig. S7). The identified genes were involved in metabolic processes, response to stimulus, biological regulation, developmental processes, reproduction, and reproductive processes by GO analysis (Supplementary Fig. S8). By COG analysis, the differentially expressed genes involved functions in replication, recombination and repair, energy production and conversion, carbohydrate transport and metabolism, cell cycle control, cell division, chromosome partitioning, and signal transduction (Supplementary Fig. S9).

We selected 15 anther development-associated genes (Table 1) to investigate transcription patterns among MF, CMS, REV19, and MSH1-RNAi lines of B. juncea. Expression of these anther development genes is up-regulated in REV19 compared with CMS lines, accompanied by reversion from male-sterile to fertile. Moreover, these genes are down-regulated in the MSH1-RNAi line compared with WT, with transition from male fertile to sterile (Fig. 6). For example, at early-stage initiation of anther development, the expression of WUS and several MADS-box genes, including AP3, AG, and PI, increased in REV19 relative to CMS, and showed decrease in the MSH1-RNAi line relative to WT (Fig. 6). During anther morphogenesis, the key regulatory gene for microsporogenesis SPOROCYTELESS (SPL) was restored to normal transcript levels in REV19, and decreased in the MSH1-RNAi line relative to WT (Fig. 6). We also found that expression of DYT1, AMS, MS1, MS2, MYB99, and MYB103 were correspondingly increased in REV19 compared with CMS, and decreased in the MSH1-RNAi line compared with WT at late-stage tapetum function and pollen development (Fig. 6). These results indicate that male-sterility induction by MSH1 suppression and fertility reversion—via SSS—are accompanied by corresponding changes in anther-associated gene expression, implying a relationship between mitochondrial genome behavior and anther development programs.

Discussion

CMS and fertility restoration are valuable components of hybrid breeding systems in crops, deriving from competitive interactions between mitochondrial and nuclear...
Zhao et al. (2013; Chen and Liu, 2014). Spontaneous fertility reversion sporadically occurs in some CMS systems, providing insight into the relationship of mitochondrial SSS and plant reproductive behavior (Escote et al., 1985; Rottmann et al., 1987; Janska et al., 1998; Feng et al., 2009). These spontaneous fertility reversion events are influenced in frequency by nuclear background (Mackenzie et al., 1988; Small et al., 1988), and can be problematic to

Table 1. Transcriptional analysis of anther development-associated genes by RNA-seq

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>CMS reads</th>
<th>REV19 reads</th>
<th>Log2 FC (CMS/REV19)</th>
<th>Ortholog in Arabidopsis</th>
<th>Annotation</th>
</tr>
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<tr>
<td>Bju009726</td>
<td>4</td>
<td>42</td>
<td>–3.325892244</td>
<td>WUS</td>
<td>WUSCHEL, homeobox gene controlling the stem cell</td>
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<td>Bju047574</td>
<td>108</td>
<td>321</td>
<td>–1.881915185</td>
<td>AP3</td>
<td>APETELA3, floral homeotic gene encoding a MADS</td>
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<tr>
<td>Bju012907</td>
<td>138</td>
<td>308</td>
<td>–1.472805418</td>
<td>PI</td>
<td>PISTILLATA, floral homeotic gene encoding a MADS</td>
</tr>
<tr>
<td>Bju010658</td>
<td>1</td>
<td>35</td>
<td>–4.246938286</td>
<td>SPL</td>
<td>SPOROCYTELESS, initiation of micro- and megagametogenesis</td>
</tr>
<tr>
<td>Bju083268</td>
<td>22</td>
<td>855</td>
<td>–5.518191891</td>
<td>DYT1</td>
<td>DYSFUNCTIONAL TAPETUM 1</td>
</tr>
<tr>
<td>Bju076135</td>
<td>12</td>
<td>634</td>
<td>–5.892604764</td>
<td>AMS</td>
<td>ABORTED MICROSPORES</td>
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<tr>
<td>Bju004296</td>
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<td>34</td>
<td>–4.992803589</td>
<td>MS1</td>
<td>MALE STERILITY 1</td>
</tr>
<tr>
<td>Bju014803</td>
<td>43</td>
<td>1911</td>
<td>–5.752855182</td>
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<td>MALE STERILITY 2</td>
</tr>
<tr>
<td>Bju003047</td>
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<td>MYB99</td>
<td>MYB transcription factor</td>
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<tr>
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<td>–4.325437835</td>
<td>MYB103</td>
<td>MYB transcription factor</td>
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<tr>
<td>Bju072885</td>
<td>2</td>
<td>47</td>
<td>–4.154850764</td>
<td>At1g02040</td>
<td>zinc finger (C2H2 type) family protein</td>
</tr>
<tr>
<td>Bju024267</td>
<td>56</td>
<td>169</td>
<td>–1.891531152</td>
<td>4CL3</td>
<td>pollen exine formation</td>
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<tr>
<td>Bju028126</td>
<td>32</td>
<td>2446</td>
<td>–4.325437835</td>
<td>AT5G13380</td>
<td>Auxin-responsive GB3 family protein, pollen exine formation</td>
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<td>54</td>
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<td>–2.080138807</td>
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<td>ERF/AP2 transcription factor</td>
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<td>1.702239773</td>
<td>SPL8</td>
<td>SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 8</td>
</tr>
</tbody>
</table>

Fig. 4. Mitochondrially targeted expression of ORF220 in Arabidopsis thaliana. (A) Transgenic plants with or without mitochondrial (mtT) presequence under control of the CaMV 35S promoter. (B) WT flower. (C) 35S::ORF220 flower. (D) 35S::mtT::ORF220 flower. (E) Silique from WT, 35S::ORF220, and 35S::mtT::ORF220 plant top-down. (F) Transgenic plants with or without mitochondrial presequence under the AP3 promoter. (G) WT flower. (H) AP3::ORF220 flower. (I) AP3::mtT::ORF220 flower. (J) Silique from WT, AP3::ORF220, and AP3::mtT::ORF220 plant top-down. (This figure is available in color at JXB online).
commercial interests for CMS implementation. In most fertility reversion cases previously reported, the nuclear genes involved in triggering mitochondrial genome rearrangement are largely unknown. In the case of \textit{MSH1}, previous evidence suggests that the loss of \textit{MSH1} function creates conditions conducive to mitochondrial asymmetric DNA exchange (Davila \textit{et al.}, 2011). We propose that the spontaneous SSS of \textit{ORF220} for fertility reversion in \textit{B. juncea} is associated with processes controlled, at least in part, by \textit{MSH1} (Fig. 7).

SSS of \textit{ORF220} is associated with spontaneous fertility reversion

Mitochondrial DNA rearrangements are often observed in some CMS systems upon reversion to fertility (Fauron \textit{et al.}, 1987; Smith \textit{et al.}, 1987; Escotecarlson \textit{et al.}, 1988; Mackenzie \textit{et al.}, 1988; Bellaoui \textit{et al.}, 1998; Janska \textit{et al.}, 1998), with dramatic reduction in relative copy number of the CMS sequence in each case. Here, we demonstrated that the SSS of CMS-associated \textit{ORF220} occurs in association with fertility reversion in CMS \textit{B. juncea}. To confirm that \textit{ORF220} is sufficient to condition the CMS phenotype, \textit{ORF220} was mitochondrially targeted, and the transgenic plants displayed male sterility in both Arabidopsis and \textit{B. juncea} (Yang \textit{et al.}, 2010). The amenability of this system to both transgenic induction and to fertility reversion provides a valuable opportunity for more detailed investigations of factors influencing nuclear–mitochondrial stability.

Depressed expression of \textit{MSH1} caused SSS of \textit{ORF220} and male sterility

It is not clear the extent to which \textit{MSH1} variation might have influenced spontaneous CMS reversion in natural systems. In the case of CMS common bean, SSS of the CMS-associated \textit{pvs-orf239} was associated with changes in a single nuclear gene that, at that time, was designated \textit{Fr} (Mackenzie and
Early anther development includes stamen identity determination, lobed anther structure morphogenesis, anther cell layer specification, and early microspore development processes. Molecular genetic studies have uncovered crucial molecules and transcription factors that function in determining anther cell types and in controlling gene expression regulatory networks for anther development (Chang et al., 2011a; Pearce et al., 2015). We observed that the reproductive dynamics created by manipulating mitochondrial genome behavior in B. juncea includes altered expression of several anther development genes in CMS, REV19, WT, and MSH1-RNAi lines. Increased expression of WUSCHEL (WUS), APETELA3 (AP3), and PISTILLATA (PI) occurred with recovery of floral structure development in the REV19 line. Correspondingly, reduced expression of these genes may cause the abnormal adhesive structure of petal and stamen observed in MSH1-RNAi lines. SPOROCYTELESS (SPL)—essential for the formation of reproductive cells and microsporogenesis—was altered in expression, suggesting its role in specifying the reproductive cell fate in these lines (Liu et al., 2009). We observed increased expression of SPL in REV19 and decreased expression in MSH1-RNAi lines, suggesting the action of SPL in fertility conversion. These results indicate that male sterility and fertility reversion, caused by the SSS of ORF220 and mediated by MSH1, involve differential regulation of anther development networks.

A distinctive characteristic of plant mitochondrial genomes is their recombinational versatility. The SSS activity of mitochondrial genomes likely serves as an important mechanism for maintaining appropriate function while retaining mitochondrial adaptive genetic diversity (Small et al., 1987). Our findings here suggest that it is feasible to directly manipulate the MSH1-mediated sterility–fertility reversion mechanism in crops, a promising first step toward enhancing breeding potential by creating CMS or controlling fertility reversion behavior.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Candidate revertant lines of *B. juncea*.

**Supplementary Fig. S2.** Comparison of mitochondrial DNA from CMS and revertant lines of *B. juncea*.

**Supplementary Fig. S3.** Mitochondrial genome rearrangement of the *atpA* gene.

**Supplementary Fig. S4.** Schematic diagram of ORF220 gene construction.

**Supplementary Fig. S5.** MSH1 from *B. juncea* and comparison with its ortholog in *Arabidopsis thaliana*.

**Supplementary Fig. S6.** SSS of mitochondrial genes in MSH1-RNAi lines relative to WT.

**Supplementary Fig. S7.** Genes differentially expressed between CMS and REV19 lines of *B. juncea* by RNA-seq.
**Supplementary Fig. S8.** Gene Ontology enrichment analysis of differentially expressed genes.

**Supplementary Fig. S9.** COG analysis of differentially expressed genes.

**Supplementary Table S1.** Candidate revertant events from CMS B. juncea.

**Supplementary Table S2.** Fertility of ORF220 expression in Arabidopsis.

**Supplementary Table S3.** Primers used in this study.

**Supplementary Table S4.** Genes differentially expressed between CMS and REV19 by RNA-seq.

**Supplementary data.** Assembled mitochondrial genomic scaffolds of CMS and REV19 lines.

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