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 RF4 and RF3 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 multipartite 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; Shedge 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 (Shedge 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 3SS (CaMV 3SS) 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 Hiseq2500 platform. High quality reads were mapped to the *B. juncea* mitochondrial genome sequence (Genbank: KJ461445) 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 were transferred and fixed to HyBond N nylon membrane (Amersham, 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 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 10 mM dNTP, 10 pmol of forward and reverse primers, 200 ng 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 30 s, 50 °C for 30 s, 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.
membrane and immunoblotted with anti-ORF220 polyclonal antibodies. The membrane was then combined with Enhanced Luminol Reagent and Oxidizing Reagent substrates. The signals were detected by FUJIFILM LAS-300 Luminescent Image Analyzer (FUJIFILM, Japan). The primers used for ORF220 assay are listed in Supplementary Table S1 at JXB online.

**ORF220 construction and transformation in Arabidopsis**

cDNA of ORF220 and the mitochondrial presequence were amplified according to our previous publication (Yang et al., 2010). The ORF220 and mt-ORF220 constructions were inserted into pMDC83 binary expression vector to generate ORF220-pMDC83 by gateway protocols (Curts and Grossniklaus, 2003). Four constructions of ORF220 plasmid were introduced into Agrobacterium tumefaciens strain (GV1301) and the floral dipping method was used to introduce the ORF220 construction to Arabidopsis (Clough and Bent, 1998). PCR and RT-PCR amplifications of ORF220 confirmed successful transformations. WT and transgenic Arabidopsis T2 lines were used in this study. The primers used for ORF220 constructions are listed in Supplementary Table S1 at JXB online.

**MSH1-RNAi line construction**

The MSH1-RNAi construction was prepared identically with that reported in tomato previously (Sandhu et al., 2007). The MSH1-RNAi construct was made by cloning part of MSH1 (AT3G24320) domain VI in the RNAi vector pFGC1008 (Kerschen et al., 2004) using primers MSH1RNAI-AscI-F: AGTCGCGCAGCCATTGAGGCTGAAGCAATAGATGTC; MSH1RNAI-SwaI-R: AGTCATTTAAAAGGACGCTTCC GAAATATCGGTC; MSH1RNAI-Spel-F: AGTCTAATGATT GAGCCTGAAGCAATAGATGTC; MSH1RNAI-BamHI-R: AG TCGATCCAGGACGTTCGATACTCGAGTC. Presence of MSH1 inserts and correct orientation was confirmed by PCR as well as sequencing using primers MSH1RNAI-AscI-F with Gus-5out: AAGGGTAAAGGCGGACGAGCA for the left fragment and MSH1RNAI-Spel-F with Gus-3out: AAGCAACGGTAAAACCTCGAC for the right fragment. The MSH1-RNAi plasmid was then introduced into Agrobacterium tumefaciens strain GV1301. The transformation procedure generating the MSH1-RNAi line of B. juncea was as described previously (Yang et al., 2010). Transgenic lines were identified by Hygromycin B selection and by PCR amplification of a junction fragment consisting of vector and MSH1 gene sequence. The primers used for MSH1-RNAi line confirmation are listed in Supplementary Table S1 at JXB online.

**Transcriptome analysis in CMS and revertant lines of B. juncea**

Total RNAs isolated from floral buds of CMS and REV19 lines of B. juncea were used for global transcript analysis by RNA-seq. The protocols for library construction and sequencing were the standard procedures provided by Illumina (NEBNextUltra™ RNA library Prep kit, Illumina, USA). The sequencing was performed using the Illumina HiSeq™ 2500 System according to the manufacturer’s protocol (50 bp single read module). An average of 8.5 G clean reads for each library was used for differential gene expression analysis. For each sequenced library, the read counts were adjusted using the edger program package through one scaling normalized factor. Differential expression analysis was performed using the DESeq R package. P values were adjusted using the Benjamini & Hochberg method. Corrected P-value of 0.005 and log2 (fold change) of 1 were set as the threshold for significantly differential expression. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by GOseq R package, in which gene length bias was corrected. GO terms with corrected P-value <0.05 were considered significantly enriched by differentially expressed genes. Clusters 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**

Fifty-three seeds 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 REV lines 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
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 targeted 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...
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

| Gene ID    | CMS reads | REV19 reads | Log₂ FC (CMS/REV19) | Ortholog in Arabidopsis | Annotation                                                                 |
|------------|-----------|-------------|----------------------|-------------------------|-----------------------------------------------------------------------------|
| Bju009726  | 4         | 42          | -3.325892244         | WUS                     | WUSCHEL, homeobox gene controlling the stem cell                           |
| Bju047574  | 108       | 321         | -1.881915185         | AP3                     | APETELA3, floral homeotic gene encoding a MADS domain transcription factor |
| Bju012907  | 138       | 308         | -1.472805418         | PI                      | PISTILLATA, floral homeotic gene encoding a MADS domain transcription factor |
| Bju010658  | 1         | 35          | 4.246938286          | SPL                     | SPOROCYTELESS, initiation of micro- and megamagametogenesis                |
| Bju083268  | 22        | 855         | -5.518191891         | DYT1                    | DYSFUNCTIONAL TAPETUM 1                                                     |
| Bju076135  | 12        | 634         | -5.892604764         | AMS                     | ABORTED MICROSPORES                                                        |
| Bju004296  | 0.01      | 34          | -4.992803589         | MS1                     | MALE STERILITY 1                                                           |
| Bju014803  | 43        | 1911        | -5.752855182         | MS2                     | MALE STERILITY 2                                                           |
| Bju003047  | 1         | 178         | -6.557555136         | MYB99                   | MYB transcription factor                                                    |
| Bju027475  | 0.01      | 21          | -4.325437835         | MYB103                  | MYB transcription factor                                                    |
| Bju072885  | 2         | 47          | -4.154850764         | At1g02040               | zinc finger (C2H2 type) family protein                                     |
| Bju024267  | 56        | 199         | -1.891531152         | 4CL3                    | pollen exine formation                                                     |
| Bju028126  | 32        | 2446        | -4.325437835         | AT5G13380                | Auxin-responsive GH3 family protein, pollen exine formation               |
| Bju002493  | 54        | 186         | -2.080138807         | SHN1/ WIN1              | ERF/AP2 transcription factor                                               |
| Bju038196  | 109       | 26          | 1.702239773          | SPL8                    | SQUAMOSA PROMOTER BINDING PROTEIN-LIKE                                      |

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 MSH1, previous evidence suggests that the loss of MSH1 function creates conditions conducive to mitochondrial asymmetric DNA exchange (Davila et al., 2011). We propose that the spontaneous SSS of ORF220 for fertility reversion in B. juncea is associated with processes controlled, at least in part, by MSH1 (Fig. 7).

**Fig. 5.** MSH1-RNAi construction in B. juncea and SSS of ORF220. (A) Seedling and inflorescence of WT and MSH1-RNAi lines. (B) SSS and transcript levels of ORF220 in WT and MSH1-RNAi lines. (C) Flowers, stamens, and silique of self-crossing from WT and MSH1-RNAi lines. (This figure is available in color at JXB online).

**Fig. 6.** Transcriptional analysis of anther development-associated genes in fertile maintainer, CMS, REV19, and MSH1-RNAi lines of B. juncea. (This figure is available in color at JXB online).

**SSS of ORF220 is associated with spontaneous fertility reversion**

Mitochondrial DNA rearrangements are often observed in some CMS systems upon reversion to fertility (Fauron et al., 1987; Smith et al., 1987; Escotecarlson et al., 1988; Mackenzie et al., 1988; Bellaoui et al., 1998; Janska 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 ORF220 occurs in association with fertility reversion in CMS B. juncea. To confirm that ORF220 is sufficient to condition the CMS phenotype, ORF220 was mitochondrially targeted, and the transgenic plants displayed male sterility in both Arabidopsis and B. juncea (Yang 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 MSH1 caused SSS of ORF220 and male sterility**

It is not clear the extent to which MSH1 variation might have influenced spontaneous CMS reversion in natural systems. In the case of CMS common bean, SSS of the CMS-associated pvs-orf239 was associated with changes in a single nuclear gene that, at that time, was designated Fr (Mackenzie and
Comparison of mitochondrial AP3). We observed increased expression of WUS-
PI-Schematic diagram of Chang-
Abdelnoor Liu-

three nuclear genes, recombination surveillance mechanisms that include at least
represent Chase, 1990 male sterility. (This figure is available in color at Fig. 7.

mitochondrial metabolism to confer biochemical activities associated gene expression has been modulated by nuclear restorer genes, which also sometimes affect fertility recovery by nuclear fertility restoration. CMS-associated ORF220 when MSH1 is suppressed. Pollen fertility was significantly reduced in MSH1-RNAi lines of B. juncea. However, the MSH1-associated SSS process observed in Arabidopsis Col-0 does not result in male sterility, indicating that not all SSS events necessarily give rise to a CMS phenotype (Abdelnoor et al., 2003). This response in Arabidopsis may be due to the lack of a CMS-associated mitochondrial sequence in the Col-0 ecotype (Gobron et al., 2013). Furthermore, only partial male sterility was observed in MSH1-RNAi lines of B. juncea in this study, and in MSH1-RNAi lines of tomato and tobacco plants previously (Sandhu et al., 2007). SSS events are bidirectional and dynamic, so that only a fraction of CMS-associated genes might achieve a threshold to cause male sterility.

This fertility reversion mechanism is distinguished from fertility recovery by nuclear fertility restoration. CMS-associated gene expression has been observed to be modulated by nuclear restorer genes, which also sometimes affect mitochondrial metabolism to confer biochemical activities that facilitate pollen development (Liu et al., 2001; Hanson and Bentolila, 2004; Chase, 2007; Ma, 2013; Chen and Liu, 2014). In nature, it is reasonable to assume that both restorer and reversion mechanisms are operational, with restorer systems providing a means of recovering self-fertility under conditions when CMS plants are located within cross-compatible populations, and reversion providing a means of self-pollination when CMS plants are in reproductive isolation.

Fertility reversion is associated with anther development-associated gene expression

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.

Fig. 7. A working model for MSH1 mediating ORF220 SSS and causing male sterility. (This figure is available in color at JXB online).
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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