

Development and characterisation of *Ogu* CMS root and leaf mustard

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Abstract: Mustard (*Brassica juncea*) is an important oilseed and vegetable crop in China with high genetic diversity. Among its various forms, root and leaf mustard are two important vegetable forms. Cytoplasmic male sterility (CMS) provides an important strategy for exploiting heterosis in mustard. *Ogu* CMS is a widely utilised sterility type in *Brassica* species; however, studies on this type of male-sterile material in root and leaf mustard remain limited. To address this gap, this study was designed to (i) introgress the *Ogu* CMS cytoplasm from *B. napus* into leaf and root mustard via backcrossing, (ii) determine the cytological stage and features of anther abortion in the newly developed CMS lines, and (iii) identify candidate genes and molecular pathways associated with male sterility through comparative transcriptome analysis. We successfully developed three leaf and one root mustard *Ogu* CMS lines and their maintainers. Both CMS and maintainer lines had $2n = 36$ chromosomes. Molecular typing confirmed the *Ogu* CMS type in mustard CMS lines and the *Cam* type in their maintainers. CMS lines showed shorter filaments, pale yellow-white non-dehiscent anthers, and no pollen. Cytological observation revealed that pollen abortion in the CMS line initiated at the tetrad stage, characterised by the tapetal enlargement and microspore compression. Transcriptome analysis identified 7 595 differentially expressed genes (DEGs) between the CMS line and its maintainer, including 3 952 upregulated and 3 643 downregulated genes. Genes involved in lipid, fatty acid, and carbohydrate metabolism were predominantly downregulated, whereas those associated with mitochondrial organisation and reactive oxygen species responses were upregulated. Among the DEGs, we identified 513 transcription factors and 107 kinase genes. Integrating cytological and transcriptome data, we propose a putative abortion mechanism for this CMS system. Our findings not only provide novel male-sterile resources, including the first *Ogu* CMS line in root mustard, but also establish a theoretical foundation for heterosis breeding in mustard.

Keywords: *Brassica juncea*; cytoplasmic male sterility; pollen abortion; tapetum development; transcriptome analysis

Heterosis refers to the phenomenon in which the F_1 progeny of two parents with a relatively distant genetic distance outperforms the parents in terms of yield, stress tolerance, quality, and other traits.

It has been widely exploited in crops such as maize, rice, rapeseed, and mustard (Sun et al. 2023). Mustard (*Brassica juncea*, AABB, $2n = 36$) originated from natural amphidiploidization following interspecific

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hybridisation between *B. rapa* (AA, $2n = 20$) and *B. nigra* (BB, $2n = 16$) (Sadhu et al. 2026), and is classified into oilseed and vegetable types. Vegetable mustard is further divided into four groups – croot, stem, leaf, and bolt types – comprising 16 varieties based on edible organ morphology (Yang et al. 1989). In China, the annual planting area of mustard is approximately 933 300 ha, accounting for about 3.4% of the total vegetable cultivation area, with a total production of 48 million tons, representing 4.7% of the national vegetable output. Mustard is rich in glucosinolates, vitamins, and trace elements such as calcium, phosphorus, potassium, sodium, and magnesium, and is used fresh, pickled, for oil extraction, and even for soil heavy metal remediation (Sha et al. 2025).

Mustard is hermaphroditic, selfcompatible, and exhibits strong heterosis. Cytoplasmic male sterility (CMS) is a key approach for harnessing heterosis in mustard (Wan et al. 2025). CMS source in mustard can be of intraspecific or interspecific origin. The former includes *Oxa* CMS, *hau* CMS, *Zhacai* CMS, and *WNJ01A* (Shi et al. 1991; Chen et al. 1993; Wan et al. 2006; Sun et al. 2022); the latter includes *Ogu* CMS, *Tour* CMS, *Oxyrrhina* CMS, *Siifolia* CMS, *Trachystoma* CMS, *Moricandia arvensis* CMS, *Erucooides* CMS, *Lyratus* CMS, among others (Ogura 1968; Deol et al. 2003; Singh & Srivastava 2006; Mukai et al. 2019).

Ogu CMS was originally discovered in radish (*Raphanus sativus*) (Ogura 1968) and is characterised by complete sterility. Bannerot et al. (1974) introduced it into *B. napus* via intergeneric distant hybridisation; however, the initial material suffered from chlorosis at low temperatures, poor nectary development, and low seed set. Pelletier et al. (1983) used protoplast fusion to substitute the chloroplasts of *R. sativus* with those of *B. napus* to overcome leaf chlorosis, and to obtain a recombinant mitochondrial genome involving both *B. napus* and *R. sativus* mtDNA, generating the improved *Ogu*-Institut National de la Recherche Agronomique (INRA) CMS line. Currently, *Ogu* CMS is the most widely used sterile cytoplasm type for heterosis utilisation in Brassicaceae crops (Ren et al. 2022). The male sterility gene has been identified as *orf138* (Bonhomme et al. 1991; Grelon et al. 1994), which contains three consecutive 39 bp repeats at its 3' end, and its function has been confirmed by transcription activator-like effector nucleases (TALENs) with mitochondria localisation signals (mitoTALENs) knockout (Ka-

zama et al. 2019). The fertility restorer genes *Rfo/Rfk* were cloned through mapbased cloning (Brown et al. 2003; Koizuka et al. 2003). Among the three highly similar pentatricopeptide repeat (*PPR*) copies (*PPR-A*, *PPR-B*, and *PPR-C*), only *PPR-B* encodes the ORF687 protein, which restores fertility by blocking the translational elongation of *orf138* mRNA (Uytewaal et al. 2009; Wang et al. 2021).

The transfer of *Ogu* CMS into mustard has been previously documented through successive backcrossing (Kirti et al. 1995) or protoplast fusion (Delourme et al. 1994). In China, *Ogu* CMS lines have been successfully developed in leaf mustard (Jin et al. 2006), heading mustard, and longpetiole mustard (Zhang et al. 2014), using improved *Ogu* CMS Chinese cabbage as the sterile cytoplasm source. Li et al. (2023) combined backcrossing with embryo rescue to generate *Ogu* CMS lines in leaf mustard. Despite these advances, the development of *Ogu* CMS in root mustard has not been reported. Considering the widespread application of *Ogu* CMS in rapeseed and its limited use in mustard – particularly in root mustard – its development would fill a critical gap for hybrid breeding.

Our laboratory previously developed the *Ogu*-NWSUAF CMS line in *B. napus* by using an *Ogu* CMS Chinese cabbage (Ke 2009) as the female parent, followed by six generations of backcrossing with *B. napus* (Chang et al. 2010). Although crossability between *B. napus* ($2n = 38$) and *B. juncea* ($2n = 36$) is low owing to chromosomal disparity, successful introgression would provide valuable germplasm for heterosis utilisation in mustard. To address these gaps, the present study was designed with three objectives: (i) to introgress the *Ogu*-NWSUAF CMS cytoplasm into both leaf and root mustard varieties via successive backcrossing; (ii) to characterise the cytological features of anther abortion and cytoplasmic sterility type; and (iii) to identify candidate genes and molecular pathways associated with male sterility via transcriptomic profiling. Our results are expected to provide both novel sterile resources and theoretical guidance for heterosis breeding in mustard.

MATERIAL AND METHODS

Breeding of *Ogu* CMS in root mustard and leaf mustard. In 2020, the *Brassica napus* *Ogu*-NWSUAF CMS line 19C137 was used as the female parent and pollinated with mixed pollen from mustard (*B. juncea*) accessions 19B26–19B31 to generate the F₁ genera-

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tion. During the flowering period in 2021, the resultant 30 F₁ plants were crossed with three leaf mustard inbreeding lines – Hengkoujie (23B36), Zhuyuanjie (23B39), and Mianxianjie (23B09) – as well as one root mustard line Bendatoujie (23B43), to obtain the BC₁ progeny. From 2022 to 2025, the resulting offspring were successively backcrossed with the four mustard inbreeding lines (recurrent parents) to generate the BC₅ generation. At each backcross round, sterile plants that displayed morphological traits closely resembling the recurrent parent were selected and backcrossed with the recurrent parent. Through this strategy, we developed four mustard CMS lines together with their corresponding maintainer lines. Subsequently, phenotypic observations and photographic records were carried out on the newly developed mustard *Ogu* CMS lines and their maintainer lines. Across the trial period 2019 to 2025, seeds of all plant materials were sown in late September at the experimental station of Northwest A&F University, Yangling, Shaanxi, China (N34.29°, E108.06°). The plants were arranged in 2.0 m-long rows, with a spacing of 0.4 m between rows and 0.10 m within rows. Field management practices – including soil preparation, fertilisation, and irrigation – were applied consistently across all experimental years.

Observation of chromosome number in root tips.

Root tips (1.5 cm) of the *Ogu* CMS line Bendatoujie (BC₅) and its maintainer line were treated with 0.002 mol/L 8-hydroxyquinoline at 4 °C in darkness for 4 h, fixed in Carnoy's solution (ethanol:acetic acid = 3:1, v/v) for 24 h, and stored in 70% ethanol. Prior to squashing, tips were macerated in 1 mol/L HCl for 15 min at room temperature, rinsed, and the meristem (2–3 mm) was stained with modified phenol-fuchsin for 1–2 min. After coverslipping, cells were dispersed by tapping. Chromosome numbers were observed and photographed under an OLYMPUS BX51 optical microscope (Olympus, Japan). For each accession, root tips from three seedlings were used for slide preparation and chromosome counts were taken from five randomly chosen microscopic fields per sample.

Morphological observation. At the full flowering stage in 2024, morphological observations were conducted on the *Ogu* CMS line Bendatoujie and its maintainer line. Whole plants, inflorescences, and flower organs (pistils and stamens) were photographed for documentation. Petals, sepals, stamens, and pistils were then carefully detached using forceps. The following traits were measured with a vernier

calliper: petal length, petal width, length of the four long (tetradynamous) stamens, length of the two short (didynamous) stamens, and pistil length. For each line, ten flowers from the main inflorescence of five individual plants were measured. All measurement data were statistically analysed using a two-tailed Student's *t*-test in SPSS (Ver. 26.0).

Cytological observation of stamen (anther) abortion. Flower buds of the *Ogu* CMS line Bendatoujie and its maintainer line were fixed in Carnoy's (12 to 24 h), dehydrated in graded ethanol (100% to 75%, 2 h each), and stored in 70% ethanol. Anthers were crushed on slides and stained with 1% acetic carmine (50–100 µL, 2–3 min) for developmental staging or with 4',6-diamidino-2-phenylindole (DAPI; 10 µg/mL, 5 min in the dark) for chromosome observation. Both bright-field and fluorescence imaging were performed on an Olympus BX51 microscope (Olympus, Japan) following Sun et al. (2023).

Molecular identification of cytoplasmic type. Young leaves of the tested materials were collected for genomic deoxyribonucleic acid (DNA) extraction using a modified cetyltrimethylammonium bromide (CTAB) method (Li et al. 2013). Cytoplasmic types were identified via a one-step multiplex polymerase chain reaction (PCR) assay (Zhao et al. 2010, 2025; Zhang et al. 2023). The PCR reaction was performed in a 10 µL volume containing 1.5 µL of template DNA (100 ng/µL), 5 µL of 2× RapidTaq MasterMix (Vazyme, China), 1 µL each of forward and reverse primers (10 µM), and ddH₂O to make up 10 µL (Table S1 in Electronic Supplementary Material (ESM)). The PCR program was: 95 °C for 3 min; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s, for 27 cycles; final extension at 72 °C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis, stained with SuperGelRed® 10 000X in water, and detected and photographed using a Qinxiang 1800 series gel imaging system (Qinxiang, China). All reactions were performed in triplicate.

Ribonucleic acid sequencing (RNA-seq) analysis. After flowering, flower buds ≤ 3 mm (before vacuolated microspore stage) were collected from the *Ogu* CMS line Bendatoujie and its maintainer line, with three biological replicates per line (each replicate pooling 10 plants), yielding a total of six samples. The samples were frozen in liquid nitrogen, and sent to Beijing Biomarker Technologies Co., Ltd. (Beijing, China) for RNA-seq. The raw sequence data were deposited in the National Centre for Biotechnology Information (NCBI) database

under accession number PRJNA1482477. Sequencing reads were aligned to the *Brassica juncea* reference genome (Braju_tum_V2.0 genome.fa) with HISAT2. Differential expression analysis was performed with EdgeR, using thresholds of $|\log_2 \text{fold change (FC)}| \geq 1$ and false discovery rate (FDR) < 0.001 . Additional analyses, including gene expression profiling, gene ontology (GO) enrichment, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation, were conducted on the Biomarker cloud platform.

Quantitative real-time PCR (qRT-PCR). Five CMS-related genes were validated by qRT-PCR. Total RNAs were extracted, and cDNA was synthesised using the GoScript™ Reverse Transcription System (A5001, Promega), using *BnGAPDH* as the internal reference gene (primers listed in Table S2 in ESM). Reactions were performed using GoTaq® qPCR master mix (A6001, Promega) on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). Three biological replicates and three technical replicates were analysed for each sample. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), and data are presented as means $\pm$ standard deviation (SD). Statistical analysis and graphing were conducted using GraphPad Prism (Ver. 9.5.1.).

RESULTS

Breeding of *Ogu* CMS in leaf and root mustard.

In 2020, the *Brassica napus Ogu*-NWSUAF CMS line 19C137 was pollinated with mixed pollen from mustard accessions 19B26–19B31 to produce the F_1 generation. During the following flowering season in 2021, 30 resultant F_1 plants were crossed with three leaf mustard inbred lines – Hengkoujie, Zhuyuanjie, and Mianxianjie – and one root mustard inbred line, Bendatoujie, to obtain the BC_1 progeny. These four mustard lines were subsequently used as recurrent parents and backcrossed for five successive generations, ultimately yielding four corresponding CMS lines and their respective maintainer lines (Figure S1 in ESM). Each CMS line was phenotypically identical to its maintainer line in all traits except for fertility. The four CMS/maintainer pairs were designated as: *Ogu* CMS Hengkoujie/Hengkoujie, *Ogu* CMS Zhuyuanjie/Zhuyuanjie, *Ogu* CMS Mianxianjie/Mianxianjie, and *Ogu* CMS Bendatoujie/Bendatoujie.

The three leaf mustard *Ogu* CMS lines – Hengkoujie, Zhuyuanjie, and Mianxianjie – exhibited no root enlargement and were characterised by predominat-

ing fibrous roots; their leaf shapes were differentiated into pinnate broad-lobed, pinnate fine-lobed, and pinnate deeply-lobed types, respectively (Figure S2 in ESM). In contrast, the root mustard *Ogu* CMS Bendatoujie displayed prominent root enlargement, with a short, cylindrical, fleshy root and shallow leaf sinuses (Figure S2 in ESM).

Observation of chromosome numbers in root tip meristems of root mustard. Chromosomes in root tip meristems of the root mustard *Ogu* CMS Bendatoujie (23B41) and its maintainer line Bendatoujie (23B43) were examined. The results revealed that both lines had a chromosome number of $2n = 36$ (Figure S3 in ESM), confirming that the nuclear genome of root mustard had been successfully introgressed into the *Ogu* CMS cytoplasmic background. This indicates that the mustard *Ogu* CMS lines and their maintainer lines had been successfully developed.

Morphological observation of mustard *Ogu* CMS. Morphological comparison between root mustard *Ogu* CMS Bendatoujie and its maintainer Bendatoujie showed no plant-level difference (Figure 1A1, 1B1). However, the CMS flowers had smaller petals, stamens, and pistils, with pale-yellow to whitish anthers and no dehiscent pollen, whereas maintainer anthers were bright yellow with abundant pollen (Figure 1; Table S3 in ESM).

Cytological observation of stamen (anther) abortion in mustard *Ogu* CMS. Cytological comparison of anther development between root mustard *Ogu* CMS Bendatoujie and its maintainer Bendatoujie showed no difference before the tetrad stage. In the CMS line, from tetrad onward, abnormal callose thickening hindered microspore release; released microspores showed vacuolation and failed exine formation. At the late uninucleate stage, irregular shape and cytoplasmic/nuclear disintegration occurred, leaving empty pollen shells and resulting in complete abortion (Figure 2).

DAPI staining showed no chromosome differences between the root mustard *Ogu* CMS Bendatoujie and its maintainer at the tetrad stage. At the early uninucleate stage, maintainer microspores had clear chromosomes, while abortive ones showed nuclear dispersion; at the late uninucleate stage, dispersion intensified, and some nuclei disappeared. At the binucleate stage, maintainer nuclei divided into generative and vegetative nuclei, whereas CMS nuclei nearly vanished (Figure S4 in ESM).

Anther paraffin sections revealed normal anther development up to the tetard stage in both lines.

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Figure 1. Plant and floral organ morphology of the *Ogu* CMS Bendatoujie line and its maintainer line Bendatoujie (both of root mustard): *Ogu* CMS Bendatoujie (A1–A4), maintainer line Bendatoujie (B1–B4)
Scale bar = 5 cm (A1 and B1); scale bar = 1 cm (A2–A4, B2–B4)

At the tetrad stage, CMS tapetal cells became slightly enlarged and tetrads appeared abnormal. At the uninucleate stage, the maintainer tapetum secreted

callase to release pollen, but the CMS tapetum became irregular and vacuolated. At the binucleate stage, the maintainer tapetum degenerated normally, while

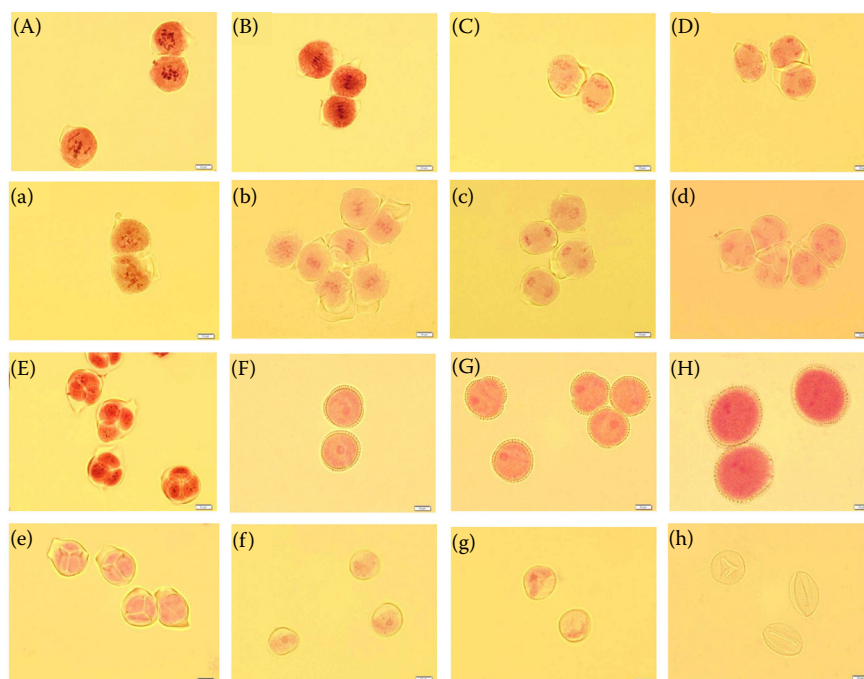


Figure 2. Cytological observation of anther development in *Ogu* CMS Bendatoujie and its maintainer line Bendatoujie (root mustard): Bendatoujie (A–H), *Ogu* CMS Bendatoujie (a–h), prephase-I (A, a), metaphase I (B, b), anaphase I (C, c), anaphase II (D, d), tetrad stage (E, e), early mononuclear stage (F, f), vacuolated microspore stage (G, g), mature stage (H), nuclear disappearance and cytoplasmic disintegration (h)

Scale bar = 10 μm

the CMS tapetum expanded radially, compressing microspores and depriving them of nutrients, leading to disintegration. Subsequently, CMS tapetum disintegrated, leaving empty locules and non-dehiscent anthers (Figure 3). Thus, the anther abortion in the root mustard *Ogu* CMS Bendatoujie occurs from the tetrad to the uninucleate stage, defining a uninucleate abortion type.

Molecular identification of cytoplasm types in mustard *Ogu* CMS lines. Using a previously reported one-step multiplex PCR method (Zhao et al. 2010; Zhang et al. 2023) with four primer pairs (P11/P12 for *Orf138*, P21/P22 for *Orf222*, P31/P32 for *Orf224*, and MSS26-L/MSS26-R for *Orf288*, Table S1 in ESM), we identify cytoplasm types of four mustard

Ogu CMS lines (23B35, 23B38, 23B45 and 23B41) and their maintainers, plus six controls (*Pol*, *Nap*, *Ogu*, *Ogu*-NWSUAF, *Cam*, *hau*). The method distinguished six cytoplasm types: *Pol A/Shaan 2A* (*orf224*), *Nap* (*orf222*), *Ogu* (*orf138*), *Ogu*-NWSUAF (*orf222* + *orf138*), *hau* (*orf288*), and *Cam* type (800/500 bp double bands). All the tested mustard CMS lines showed 465 and 1 102 bp bands, matching *Ogu*-NWSUAF CMS control, their maintainers showed 500/800 bp bands, matching *Cam* type (Figure 4). This confirmed the successful breeding of four mustard *Ogu* CMS lines and their corresponding maintainers.

RNA-seq analysis of mustard *Ogu* CMS. Based on the cytological observation, pollen abortion in the mustard *Ogu* CMS line was found to initiate

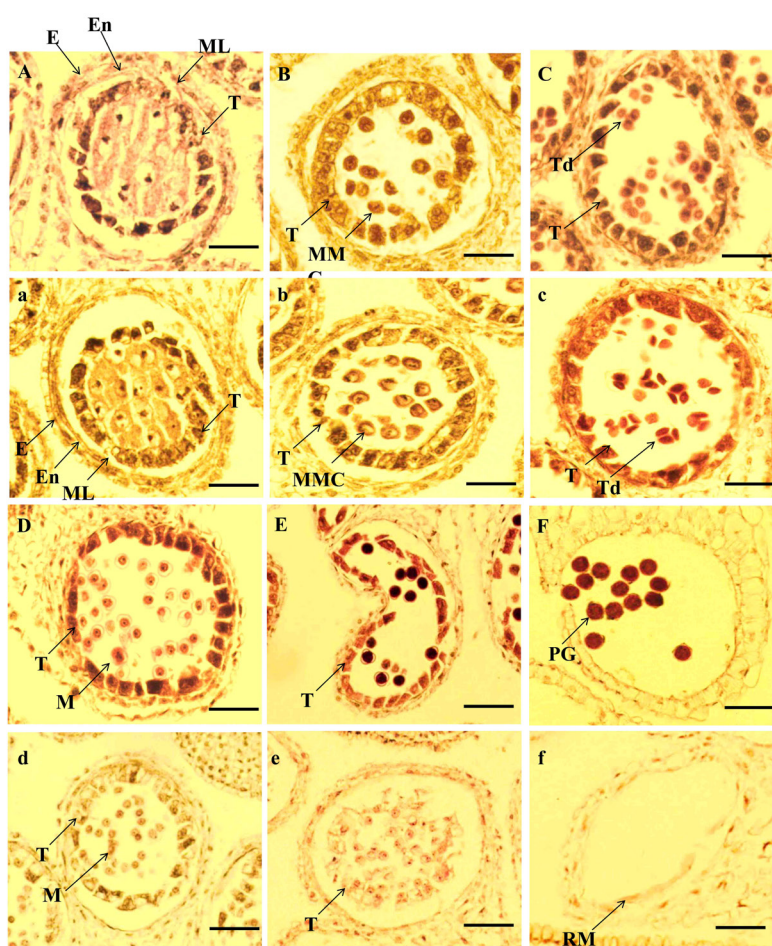


Figure 3. Observation of anther paraffin sections from the *Ogu* CMS Bendatoujie and its maintainer line Bendatoujie (root mustard): Bendatoujie (A–F), *Ogu* CMS Bendatoujie (a–f), sporogenous cell stage (A, a), meiosis stage (B, b), tetrad stage (C, c), mononuclear stage (D, d), binucleate pollen stage (E, e), mature pollen stage (F), microspore degradation and disappeared, and there was only residue in the drug room in sterile sample (f)

E – epidermis; En – endothecium; ML – middle layer; T – tapetum; MMC – microspore mother cell; Td – tetrads; M – microspore; PG – pollen grains; RM – residual microspores; scale bar = 20 μ m

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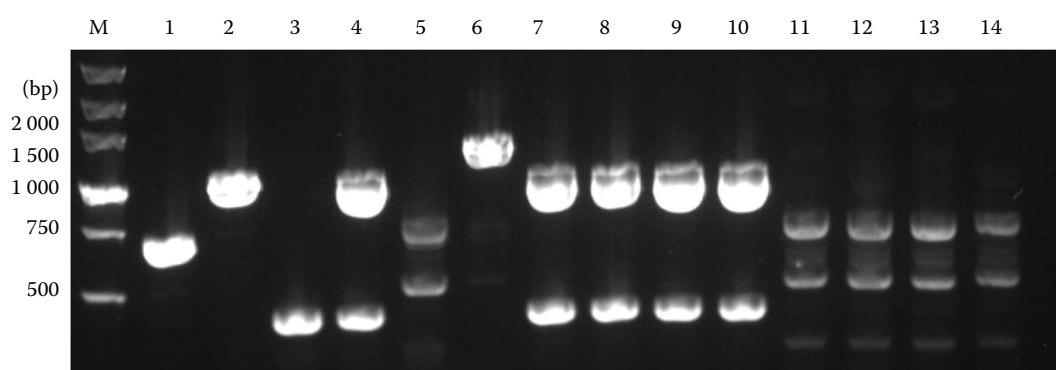


Figure 4. Identification of cytoplasmic types of *Ogu* CMS materials in mustard using one-step multiplex PCR

M – 5 000 marker; lane 1 – 23C01 (*pol* CMS-ZS2, *Brassica napus*); lane 2 – 23C10 (*Nap*-ZS9, *B. napus*); lane 3 – 23A333 (*Ogu* CMS-ZS2, *B. napus*); lane 4 – 23A327 (*Ogu*-NWSUAF CMS-S8R, *B. napus*); lane 5 – 23C03 (*Cam*-ZS2, *B. napus*); lane 6 – 23B19 (*hau* CMS, *B. juncea*); lane 7 – 23B35 (*Ogu* CMS Hengkoujie, *B. juncea*); lane 8 – 23B38 (*Ogu* CMS Zhuyuanjie, *B. juncea*); lane 9 – 23B45 (*Ogu* CMS Mianxianjie, *B. juncea*); lane 10 – 23B41 (*Ogu* CMS Bendatoujie, *B. juncea*); lane 11 – 23B36 (Hengkoujie, *B. juncea*); lane 12 – 23B39 (Zhuyuanjie, *B. juncea*); lane 13 – 23B09 (Mianxianjie, *B. juncea*); lane 14 – 23B43 (Bendatoujie, *B. juncea*)

at the tetrad stage. Accordingly, flower buds ≤ 3 mm in length – corresponding to pre-vacuolated microspore stages – were collected from the *Ogu* CMS line Bendatoujie and its maintainer line for RNA-seq. High-quality RNA-seq data were obtained for both lines, with an average of ~ 20.4 – 20.9 million clean reads per sample, $Q20 \geq 99.62\%$, $Q30 \geq 98.48\%$, mapping $> 90\%$, and GC content 45.8% (Table S4 in ESM). Principal component analysis (PCA) and correlation analysis confirmed reproducibility of the biological replicates. A total of 7 595 differentially expressed genes (DEGs) were identified (3 952 up-regulated, 3 643 down-regulated) under the criteria of $FDR < 0.001$ and $|\log_2FC| \geq 1$ (Figure S5 in ESM). These DEGs were clustered into ten groups (C1–C10); among which clusters C1–C6 were selected for GO enrichment analysis (Figure S6 in ESM).

Cluster C1 (1 103 upregulated genes) was enriched in protein folding and mitochondrial organisation (Figure S7A in ESM). Annotation genes in this cluster included an *ATAD3* homolog and a *TIM44* subunit (Figure 5). *ATAD3* spans both mitochondrial membranes and regulates nucleoid assembly; its dysfunction has been shown to affect complex I activity and heat tolerance (Kim et al. 2021). *TIM44* mediates protein translocation via ATP hydrolysis. The up-regulation of these genes in C1 may therefore disrupt nucleoid assembly, translocase complex function, and mitochondrial integrity.

Cluster C4 (398 upregulated genes) was enriched in responses to heat, H_2O_2 , and reactive oxygen spe-

cies (ROS) responses (Figures S6, S7D in ESM), with marked upregulation of small heat shock proteins (sHSP) (Figure 5).

The downregulated clusters C2 and C6 showed enrichment in lipid metabolism (Figures S6, S7B in ESM). Specifically, C2 was enriched in glycerolipid, glycerophospholipid, and α -linolenic acid metabolism pathways, while C6 was mainly enriched in glycerolipid metabolism. Both clusters exhibited significant downregulation of GDSL esterases (Figure 5). Cluster C3 (214 downregulated genes) was enriched in fatty acid biosynthesis (Figures S6, S7C in ESM), with *KCS7* and *KCS21* downregulated (Figure 5); these enzymes catalyse the elongation of very-long-chain-fatty acids (VLCFA) for cutin and wax precursors (Haslam & Kunst 2013).

Cluster C5 (18 downregulated genes) was enriched in carbohydrate metabolism, including genes encoding glycosyl hydrolases (e.g., *BjuVA02G43510*, *BjuVA03G62250*), pectate lyases (e.g., *BjuVB03G22540*, *BjuVA08G34400*), β -glucosidase functional genes (e.g., *BjuVB04G21510*, *BjuVB08G58930*), and peroxisomal NAD-malate dehydrogenase (*BjuVA04G17800*) (Figure 5).

Among all DEGs, 513 were transcription factors (TFs) belonging to 15 families, with NAC (96), AP2/ERF (86), MYB (57), C2H2 (45), WRKY (43), bHLH (37), HSF (25), LOB (21), C3H (19), and bZIP (16) being the top ten (Figure S8 in ESM). Eighteen TFs with known roles in plant male sterility were identified, including *MYB26* (*BjuVA01G40990*,

BjuVB07G06320) associated with endothecium thickening (Chen et al. 2024), *MYB21* (*BjuVB02G54520*) involved in jasmonate signaling (Cheng et al. 2009), *TDF1* (*BjuVB02G54850*) involved in the tapetum cas-

cade (Zhu et al. 2011), *AMS* (*BjuVA03G45600*) and its bHLH partners (*BjuVA10G04710*, *BjuVB06G37690*, *BjuVA04G24090*, *BjuVB01G11470*, *BjuVA05G14150*, *BjuVB01G11460*) related to tapetal programmed cell

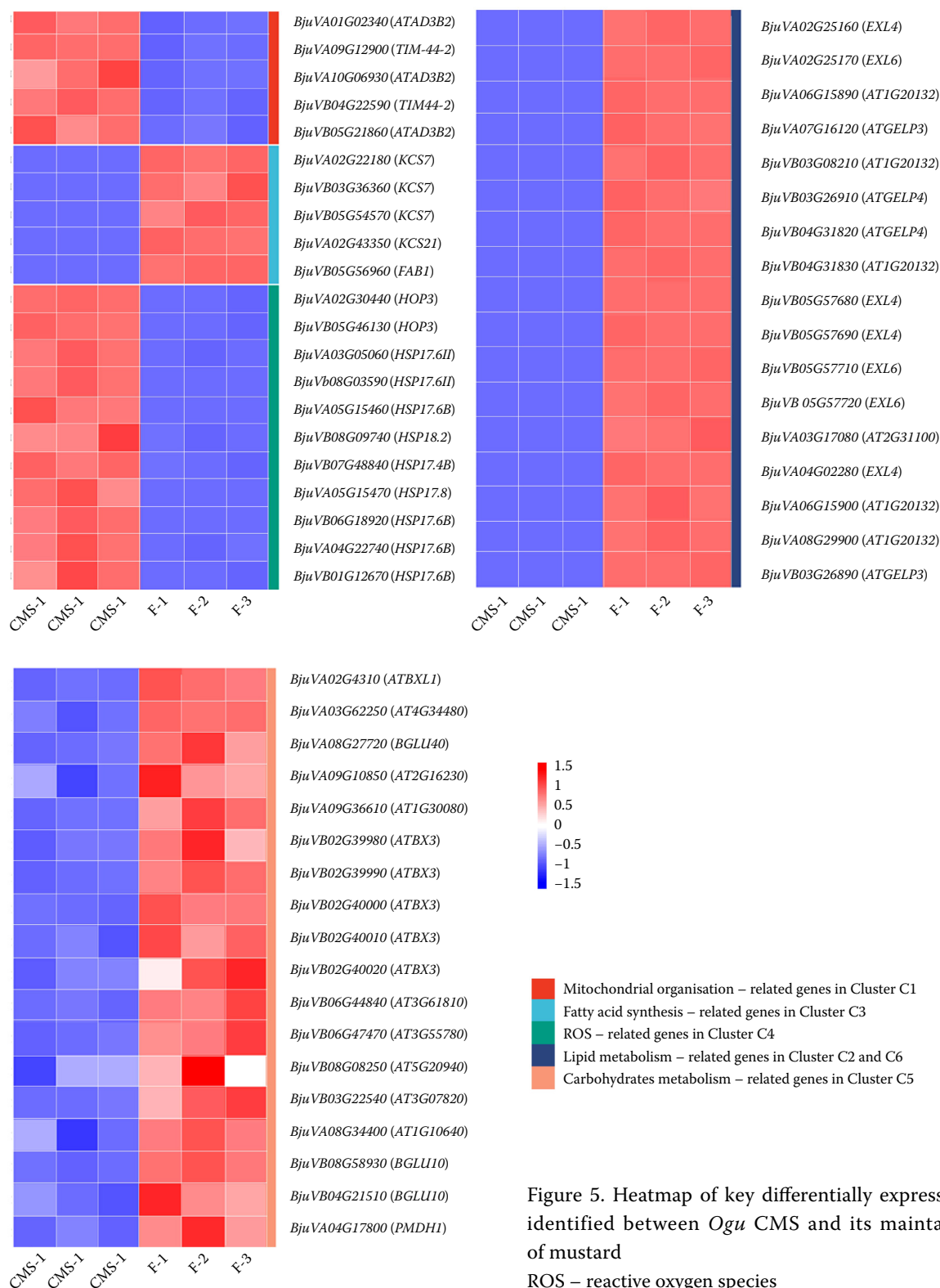


Figure 5. Heatmap of key differentially expressed genes identified between *Ogu* CMS and its maintainer line of mustard

ROS – reactive oxygen species

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death and pollen wall formation, *MYB80/MYB103/MS188* (*BjuVB05G32340*) involved in exine and callose regulation (Xu et al. 2025). *MYB101* (*BjuVA03G17790*, *BjuVA04G24960*, *BjuVA05G12920*, *BjuVB01G10670*) related to pollen tube reception; and *MYB43* (*BjuVB05G22070*, *BjuVA10G22850*) related to secondary cell wall biosynthesis (Pan et al. 2020). These transcription factors constitute regulatory networks governing anther maturation and fertility.

Additionally, 107 kinase genes were detected, belonging mainly to RLK/Pelle and CAMK families (Figure S8). Annotation identified *NDR5* homologs (*BjuVA09G58780*, *BjuVB02G12280*) (Figure S8 in ESM). The *ndr2 ndr4 ndr5* triple mutant showed abnormal callose deposition and precocious germination, suggesting involvement in Hippo-like signalling (Zhou et al. 2021).

qRT-PCR validation confirmed the expression trends observed in the RNA-seq data (Figure S9 in ESM), thereby ensuring the reliability of the transcriptomic analysis.

DISCUSSION

Development of *Ogu* CMS materials in root and leaf mustard. CMS is a cornerstone for heterosis exploitation in *Brassica* crops. The *Ogu* CMS system, originally identified in wild radish (*Raphanus sativus*; Ogura 1968), has been widely transferred into various *Brassica* species, including *B. napus*, *B. oleracea*, *B. rapa*, and *B. juncea*, making it the most extensively utilised sterile cytoplasm in Brassicaceae breeding (Ren et al. 2022). Previous efforts have successfully introduced *Ogu* CMS into oilseed, leaf, heading or long-petiole mustard (Delourme et al. 1994; Kirti 1995; Jin et al. 2006; Zhang et al. 2014; Li et al. 2023). However, to date, no report has documented the development of *Ogu* CMS in root mustard, nor have the molecular events underlying sterility in mustard *Ogu*-type CMS been systematically explored.

In our laboratory, Chang et al. (2010) developed a novel *Ogu* CMS line in *B. napus*, named *Ogu*-NWSUAF CMS, derived from an *Ogu* CMS Chinese cabbage accession provided by Prof. Guilan Ke (Northwest A&F University). This accession originated from a *B. napus* cybrid obtained from the Institut National de la Recherche Agronomique (INRA), France (Ke 2009). The *Ogu*-NWSUAF CMS is molecularly distinct from the original *Ogu* CMS, as it carries both *orf138* (*Ogu*-type) and *orf222*

(*Nap*-type) (Chang et al. 2010; Zhao et al. 2010). This finding was later confirmed by sequencing the mitochondrial genome of BVRC-CMS96, a *B. rapa* CMS line that shares the same INRA *B. napus* cybrid origin, revealing one *orf222* and two copies of *orf138* (Li et al. 2020).

In the present work, we employed a conventional backcrossing strategy rather than protoplast fusion, because it preserves the intact nuclear genome of the recurrent mustard parent while progressively replacing the cytoplasmic genome, thus minimising somaclonal variation and facilitating maintainer line development. Through five backcross generations, we successfully obtained four CMS/maintainer pairs – three from leaf mustard and, for the first time, one from root mustard. Molecular and cytological assays confirmed the complete replacement of the cytoplasm with *Ogu*-NWSUAF type and the stability of the nuclear background (Figures S3, S4 in ESM). Notably, all four sterile lines displayed no seedling chlorosis, floral malformation, or silique defects, and set seeds normally when crossed with their maintainers – features that are particularly advantageous for practical hybrid seed production.

The key innovations of this study are threefold. First, we have extended the applicability of *Ogu*-NWSUAF CMS to root mustard, a previously unexplored germplasm for this CMS system, thereby broadening the scope of heterosis utilisation in *Brassica* root crops. Second, our results demonstrate that conventional backcrossing, without embryo rescue or protoplast fusion, is sufficient to achieve a stable *Ogu*-type CMS in mustard, offering a simpler and more accessible route for breeders. Third, the newly developed lines retain the unique attributes of *Ogu*-NWSUAF CMS (dual *orf* genes) and exhibit excellent agronomic performance, making them valuable resources for both fundamental research on cytonuclear interactions and applied hybrid breeding in mustard. Collectively, these findings fill a notable gap in CMS germplasm and provide a solid foundation for subsequent mechanistic and breeding studies.

For vegetable-use mustard (both leaf mustard and root mustard), the development of a restorer line is not required, since commercial hybrids are grown for their vegetative organs or fleshy roots rather than for seed production. In contrast, for future oilseed-use mustard, transferring the restorer gene will be necessary.

Cytological characteristics of anther abortion in mustard *Ogu* CMS. Investigating the timing and

cytological features of anther abortion is fundamental to understanding CMS mechanisms. In *B. napus* *Ogu*-INRA CMS, González-Melendi et al. (2008) reported that abortion initiates at the tetrad stage, accompanied by the absence of typical mitochondria in tapetum cells and vacuolate microspores, and suggested that premature tapetal cell death (likely autolytic rather than normal programmed) lead to pollen failure at the vacuolated microspore stage. Subsequent studies in *B. napus* (Ge et al. 2025), *B. oleracea* (Han et al. 2018), and Chinese cabbage (Wei et al. 2025) have consistently implicated tapetal abnormalities from the tetrad stage through late uninucleate stages as the primary cause of *Ogu* CMS abortion (Liu et al. 2022). However, systematic characterisation of *Ogu*-type CMS in *B. juncea*, particularly in root mustard, has remained lacking.

In the present study, we performed a comprehensive cytological analysis of anther development in the root mustard *Ogu* CMS line and its maintainer, using paraffin sections and DAPI staining (Figures 2, 3, and Figure S4 in ESM). Our observations confirmed that abortion predominantly from the tetrad to the late uninucleate stage. Notably, while tapetal cells in the CMS line began to show slight enlargement at the tetrad stage, overt defects – including tapetal vacuolation, radial hypertrophy, and progressive compression of microspores – became pronounced only at the uninucleate and binucleate stages, ultimately resulting in complete microspore disintegration and empty locules. Concurrently, DAPI staining (Figure S4 in ESM) revealed that microspore nuclei exhibited dispersion and eventual disappearance. These findings for the first time delineate the full cytological progression of *Ogu* CMS abortion in a mustard background, with a clear temporal sequence in which tapetal abnormality precedes and likely drives microspore failure. Our results are broadly consistent with observations in Chinese cabbage and broccoli *Ogu* CMS, reinforcing the notion that tapetal hypertrophy and disrupted nutrient supply are conserved cytological hallmarks of *Ogu*-type male sterility across *Brassica* species.

Transcriptomic characterisation of mustard *Ogu* CMS. Previous transcriptomic studies across various *Ogu* CMS systems have consistently linked male sterility to dysregulated carbohydrate/lipid metabolism, oxidative stress, and altered transcriptional regulation (Dong et al. 2013; Wang et al. 2016; Shu et al. 2018; Wei et al. 2020; Hu et al. 2021; Ge et al. 2025). However, these studies have been largely confined

to *B. napus*, Chinese cabbage, and broccoli, with no transcriptomic characterisation of *Ogu*-type CMS in mustard – particularly in root mustard – reported to date. To fill this gap, we performed RNA-seq analysis on buds ≤ 3 mm (the critical transition from tetrad to vacuolate microspore stage, as defined by our cytological observations) from the root mustard *Ogu* CMS line and its maintainer.

Our transcriptomic data revealed a comprehensive molecular landscape associated with sterility. Upregulated clusters (C1 and C4) were prominently enriched in mitochondrial components and ROS/heat responses, highlighted by the elevated expression of an *ATAD3* homolog (involved in mitochondrial nucleoid organisation) and the TIM44 subunit (mediating protein translocation), along with small heat shock proteins. This suggests that mitochondrial dysfunction and oxidative stress are early and active responses in the sterile anthers. Conversely, downregulated clusters (C2, C3, C5, and C6) exhibited broad suppression of lipid biosynthesis (glycerolipid, glycerophospholipid, α -linolenic acid, and very-long-chain fatty acid pathways), carbohydrate metabolism, and energy production, indicating a global impairment of anther energy supply and cuticle/wax formation. Notably, the significant downregulation of GDSL esterases and fatty acid elongases (*KCS7* and *KCS21*) points to a specific defect in exine and sporopollenin precursor biosynthesis – a finding not previously highlighted in *Ogu* CMS transcriptomes.

A key innovation of this study is the integration of transcriptional changes with the core transcription factor (TF) network governing anther development. Among the 513 TFs identified, 18 sterility-related genes – including homologs of *MYB26*, *MYB21*, *TDF1*, *AMS*, *MYB80/MS188*, *MYB101*, *MYB43*, and several bHLH partners – were differentially expressed. Many of these belong to the conserved DYT1-TDF1-AMS-MS188-MS1 regulatory cascade (Zhu et al. 2011), which orchestrates tapetal programmed cell death (PCD) and pollen wall formation. Their coordinated downregulation in the CMS line suggests that the entire developmental program is repressed, likely as a consequence of upstream mitochondrial dysfunction. In addition, the detection of 107 differentially expressed kinases, including NDR5 homologs implicated in Hippo-like signalling and callose regulation (Zhou et al. 2021), points to potential novel signalling nodes involved in the abortion process.

Collectively, our results support a multi-pathway model in which *Ogu* CMS arises from the convergence

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of mitochondrial dysfunction and ROS imbalance, suppression of energy and lipid metabolism, and disruption of the core transcriptional cascade – ultimately leading to tapetal hypertrophy, impaired PCD, and microspore collapse.

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