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Genetic characterisation of a novel male sterile two-type line system 19F08AB in *Brassica napus* L.

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Abstract: Rapeseed (*Brassica napus* L.) is a major global oilseed crop and exhibits significant heterosis. The discovery and characterisation of novel male-sterile accessions remain fundamental for harnessing heterosis in rapeseed breeding. Previously, we developed a male sterile two-type line system 19F08AB in *B. napus*. In this study, anther abortion in 19F08A was characterised using the squash method. The inheritance of male sterility in 19F08A and its genetic relationship to reported male sterile accessions in rapeseed was investigated using classical genetic analysis and male-sterility-gene-specific molecular markers. Results indicated that male sterile flowers of 19F08A exhibit flat petals, reduced floral organs, short filaments, and completely degenerated stamens devoid of pollen. Pollen mother cells in 19F08A degenerated at the pre-meiotic stage and aborted completely at the tetrad stage, with no dyad or tetrad formation observed. This suggested that 19F08A represents a meiosis abnormality-type male sterility. Classical genetic and molecular marker analysis revealed that male-sterile plants 19F08A carry the genotype of *pol* (*RfpRfpMsms*), whereas fertile plants 19F08B possess *pol* (*RfpRfpmsms*). The effect of the *pol* cytoplasm was masked by the *Rfp* gene. Therefore, fertility in 19F08AB is controlled by a pair of nuclear genes (*Ms*/*ms*), with male sterility exhibiting dominance over fertility. The application prospects of this male-sterile accession are also discussed. These findings expand the pool of male-sterile resources available for *B. napus* hybrid breeding and contribute to plant male sterility theory.

Keywords: anther abortion; cytoplasmic male sterility; genic male sterility; inheritance; rapeseed

Rapeseed (*Brassica napus* L.) is a globally important oilseed and vegetable crop, which has quite obvious heterosis (Fu 2019). Cytoplasmic male sterility (CMS) and genic male sterility (GMS) are two main pollination management systems for heterosis utilisation in rapeseed and other *Brassica* crops (Fu 2019; Singh et al. 2019).

CMS is a maternally inherited trait that produces offspring with no viable pollen while it maintains female fertility in higher plants. Up to now, several types of CMS have been reported in the Brassicaceae family (Fu 2019; Singh et al. 2019; Gautam et al. 2023), such as *ogu*, *nap*, *pol*/*Shaan 2A*, *hau*, *Nca*, *Nsa*, *Moricandia arvensis* CMS and so on, among them,

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only *ogu* and *pol*/*Shaan 2A* CMS are widely used in rapeseed hybrid breeding programmes (Fu 2019). Molecular analysis reveals that these CMS systems are associated with the co-transcription of chimeric open reading frames (ORFs) besides mitochondrial genes (Gautam et al. 2023; Kitazaki et al. 2023; Bhattacharya et al. 2024). For examples, *ogu* CMS, *nap* CMS, *pol* CMS is closely related to mitochondrial genes *orf138*, *orf222/nad5c/orf139*, *orf224/atp6*, respectively. The detrimental effect of CMS proteins on tapetal and pollen development can be masked by the interaction between mitochondria-associated CMS genes and *Rf* (restorer-of-fertility) genes via various mechanisms (Kim & Zhang 2018; Singh et al. 2019; Kitazaki et al. 2023; Bhattacharya et al. 2024). Until now, *Rfo* for *ogu* CMS, *Rfp* for *pol* CMS, and *Rfn* for *nap* CMS have been characterised; they encoded pentatricopeptide repeat (PPR)-containing proteins that are localised to mitochondria (Liu et al. 2016, 2017).

GMS accessions identified in rapeseed fall into two categories: recessive and dominant. The recessive GMS types comprise three classic genetic models: the first involves monogenic control by a single recessive locus, exemplified by the 1205A line (Xiao et al. 2025); the second features duplicate recessive gene control (*BnMs1* and *BnMs2*), as observed in lines S45A and 117A (Pan et al. 1988; Hou et al. 1990); and the third demonstrates multi-allelic control at two loci (*BnMs3* and *BnMs4*), where *BnMs3* has two alleles (*BnMs3* > *Bnms3*) and *BnMs4* exhibits a tri-allelic dominance hierarchy ($BnMs4^a > BnMs4^b > BnMs4^c$) (Chen et al. 1998; Dong et al. 2010; Zu et al. 2010). The corresponding male-fertility genes *BnMs1* and *BnMs2* (Yi et al. 2010), *BnMs3* (Dun et al. 2011), and *BnMs4* (Deng et al. 2016) have been isolated and characterised (Yi et al. 2010; Xia et al. 2016).

The dominant GMS types include two classic examples: one controlled by a single dominant gene (Mathias 1985; Wang et al. 2006); the other governed by either a digenic interacting model or a multiple allele model (Li et al. 1985; Song et al. 2005; Liu et al. 2008). So far, several cases of rapeseed digenic interacting model have been reported (Li et al. 1985; Dong & Du 1993; Hu et al. 1999; Wang et al. 1999); of them, Yi3A and its derivatives have been regarded as representative of the digenic interacting model (Li et al. 1985). However, latter results of allelism test and molecular marker investigation suggest that 609AB and Rs1046AB derived from Yi-3A are inherited as a multiple allele model (Song et al. 2005, 2006; Liu et al. 2008), with three alleles in the one locus *BnMs5*, with the dominance relationship of $BnMs5^a > BnMs5^b > BnMs5^c$. The underlying gene (*BnMs5*) was map-based cloned (Xin et al. 2016). *Ms5* (*BnaA08g25920D*) encodes a *Brassica*-specific protein carrying conserved coiled-coil and DUF626 domains with unknown function. The protein Ms5 is essential for pairing of homologs in meiosis, but not necessary for the initiation of DNA double-strand breaks. In addition, another allele at *BnMs5* locus, $Ms5^d$, was map-based cloned in a *B. napus* thermo-sensitive dominant GMS line TE5A (Zeng et al. 2017), which had the same restoring-maintaining relationship with RS1046AB (Xin et al. 2016; Zeng et al. 2021). A C-to-T transition in $Ms5^d$ resulted in a Leu to Phe (L281F) substitution and caused abnormal male meiosis in TE5A. Shaan-GMS is another spontaneous mutant of the dominant GMS line in *B. napus* (Hu et al. 1999, 2004); its male sterility is controlled by a monogenically multi-allelic locus with three different alleles (*Ms*, *ms,* and *Mf*), with a relationship expressed as *Mf* > *Ms* and *Ms* > *ms* (Zhang et al. 2020, 2025).

The predominant use of a limited number of CMS or GMS sources in rapeseed heterosis breeding poses significant risks to production, including increased genetic vulnerability, reduced stress resistance, and unstable fertility (Fu 2019). Thus, discovery and characterisation of new male sterile resources is always the most important basic work for heterosis utilisation in *B. napus*. Previously, we developed a male sterile two-type line system 19F08AB by sib-mating male sterile plants and fertile plants within an F_2 population derived from the cross between two *pol* CMS restorer lines (Yu 2022). The objectives of the present study were to (1) characterise microspore development of 19F08AB by squash method; (2) investigate the inheritance model of 19F08AB and its genetic relationship with reported male sterile accessions in rapeseed by classical genetic method and male sterile gene-specific molecular markers.

MATERIAL AND METHODS

Plant materials. Rapeseed (*B. napus*) 19F08AB, a male sterile two-type line system, was developed through sib-crossing male-sterile plants with fertile siblings within an F_2 population generated by crossing two *pol* CMS restorer lines, CZL-20A and S11R (Yu 2022). A panel of representative rapeseed accessions was used to investigate the inheritance of 19F08AB and its relationship with previously reported CMS and

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GMS resources (Table 1). They included *pol* CMS restorers and maintainers, several different types of GMS lines, such as YY10AB derived from 117AB (Hou et al. 1990), 9012AB (Chen et al. 1998), Shaan-GMS heterozygous two-type line ZS02AB and homozygous two-type line 803AB (Hu et al. 1999, 2004), restorers for the dominant GMS line Shaan-GMS, temporary maintainer for 9012A, and two thermo-sensitive lines 373S (Yu et al. 2007; Sun et al. 2020) and H50S (Sun et al. 2009) (Table 1). Additionally, five lines, 3A333 (*ogu* CMS), 3A327 (*IP-ogu* CMS), 3C01 (*pol* CMS/*Shaan* 2A CMS), 3C10 (*nap* type), and 3C03 (*cam* type), were used as reference materials to characterise cytoplasmic variation (Zhao et al. 2010). All plant materials were provided by the College of Agronomy, Northwest A&F University, Yangling, China. Field experiments were conducted during the 2019–2024 crop seasons at the experimental station of Northwest A&F University in Yangling, Shaanxi, China (34°16'N, 108°4'E; altitude 530 m). Crops were sown in mid-September and harvested in late May of the following year. Experimental plots consisted of 2-m-long rows with 0.40 m between-row spacing and 0.10 m within-rows spacing. Standard agronomic practices-including soil preparation, fertiliser, and irrigations-followed local rapeseed production protocols.

Morphology and fertility observation. The experiment was conducted in the crop season of 2022–2023. In the flowering period of April 2023, 20 flowers each of five male sterile and fertile plants of 19F08AB, and 20 flowers each of four different types (fertile, GMS, CMS, and double MS) plants of 2A65, one F_2 populations derived from the cross between 19F08A and ZS05R, were selected to record the following traits: diameter of corolla, length of pedicel, length of petal, width of petal, length of four stronger stamens, length of two weaker stamens, length of four stronger anthers, and length of two weaker anthers. The plants and their flowers were also photographed to record the inflorescences, flower buds, and flowers. At maturity, ten open-pollinated male sterile and ten fertile plants of line 19F08AB were selected for evaluation. The following agronomical traits were recorded: plant height, branch height, branch number, length of the main inflorescence, total siliques per plant, seed numbers per silique, and silique length.

Cytological observation of pollen development. All specimens were collected from field-grown plants of 19F08AB in April 2023. When the male fertility of the first opened flowers of 19F08AB plants was visually detectable, the main inflorescences of the plants were collected and fixed at room temperature in a mixture of ethanol and acetic acid (3 : 1) for 24 h, transferred to 70% ethanol and stored at 4 °C until use. The cytological observation of microspore development was made by squashing, and bright-

Table 1. Plant materials used in the present study

No.	Name	Background	Genotype	References
1	ZS02AB	Shaan-GMS heterozygous two-type line	*Msms* × *msms*	Zhang et al. (2020)
2	803AB	Shaan-GMS homozygous two-type line	*MsMs* × *MfMs*	
3	YY10AB	two duplicate recessive GMS line	*ms1ms1ms2ms2* × *Ms1ms1ms2ms2*	Hou et al. (1990)
4	9012AB	Recessive epistatic interacting GMS line	$ms3ms3ms4^bms4^b$ × $Ms3ms3ms4^bms4^b$	Chen et al. (1998)
5	H50S	thermo-sensitive GMS line		Sun et al. (2009)
6	373S	thermo-sensitive GMS line	$Bnms^{t1}ms^{t1}$	Sun et al. (2020)
7	TAM	temporary maintainer for 9012A	$ms3ms3ms4^cms4^c$	Li et al. (2012)
8	ZS05R	restorer for Shaan-GMS	*MfMf*	Zhang et al. (2020)
9	2A16	restorer for Shaan-GMS	*MfMf*	
10	2011B2	maintainer for *pol* CMS	*rfrf*	Zhao et al. (2010)
11	S11R	restorer for *pol* CMS	*RfRf*	
12	T16	restorer for *pol* CMS	*RfRf*	
13	Q10C	restorer for *pol* CMS	*RfRf*	
14	CZL-20A	restorer for *pol* CMS	*RfRf*	
15	ZS09	maintainer for *pol* CMS	*rfrf*	
16	SH11	restorer for *pol* CMS	*RfRf*	
17	3C243	*pol* CMS F_1	*Rfrf*	

GMS – genic male sterile plants; CMS – cytoplasmic male sterile plants

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field photographs were taken by an OLYMPUS BX51 microscope (Olympus Corporation, Tokyo, Japan).

Genetic research. Male sterile plants 19F08A in the two-type line system 19F08AB, was crossed with a panel of representative rapeseed lines SH11, ZS09, CZL-20A, Q10C, T16, S11R, 2011B2, ZS05R, and TAM (Table 1). 19F08AB was also testcrossed with several different types of GMS lines, including YY10AB, 9012AB, ZS02AB, 803AB, 373S and H50S to reveal their inter-relationship (Table 1). All the resultant F_1, F_2, BC_1 populations and their corresponding parents were planted in the experimental field in Yangling, Shaanxi, China, during the crop seasons of 2019–2024. Male fertility was recorded for these populations at the flowering period.

Identification of 19F08AB cytoplasm type. Total genomic DNA was isolated from three-leaf stage plantlets of all the tested accessions using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle 1990). The following materials, including 19F08A and 19F08B, ZS02A and ZS02B, 803A and 803B, CZL-20A, and S11R were used to investigate their cytoplasmic type by the multiplex PCR analysis (Table S1 in Electronic Supplementary Material (ESM), Zhao et al. 2010).

Genotyping 19F08AB at the *Rfp* and *Ms* loci by molecular markers. According to the DNA sequence of *pol* CMS restorer allele (*Rfp*) and maintainer allele (*rfp*) reported by Liu et al. (2016), primers RFP-F/RFP-R1, rfp-9F/rfp-8R were designed for identifying the *Rfp/rfp* alleles (Table S1). The total volume of PCR reaction was 10 µL, including 1.5 µL DNA (100 ng/µL) template, 5 µL 2 × Rapid *Taq* Master Mix (Vazyme, China), 1 µL (10 µM) of each forward and reverse primers, and 1.5 µL ddH_2O. The PCR procedure was performed in a C1000 thermal cycler (Bio-Rad Co., Ltd., USA) following these steps: 95 °C for 3 min; 33 cycles of 95 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min. The PCR products were separated on 1% agarose gel, stained with ethidium bromide, and visualised by a gel imaging system (Alpha Innotech, Shanghai, China).

To verify the male sterile gene in 19F08AB, we designed three pairs of primers (Ms5a-1F/Ms5a-1R, 5c-12F/ac-2R, and Ms5a-1F/Ms5b-1R, Table S1) for detecting *Mf*, *ms*, and *Ms* alleles of Shaan-GMS, respectively. PCR reaction was performed same as that described above. The amplification program was performed in C1000 thermal cycler following these steps: 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min. The PCR products were detected as described above.

All the experiments for molecular markers tests were carried out for three replications.

Data analysis. Analysis of variance of the flower and agronomical traits was conducted by SPSS software (Ver. 19, 2010).

RESULTS

Morphological and agronomical characteristics of male sterile two-type line system 19F08AB. The two-type line system 19F08AB was morphologically characterised. Before flowering, no remarkable difference was found between male sterile plants 19F08A and male fertile plants 19F08B. After flowering, compared with 19F08B, 19F08A showed male sterility (Figure 1), exhibiting more small floral organs

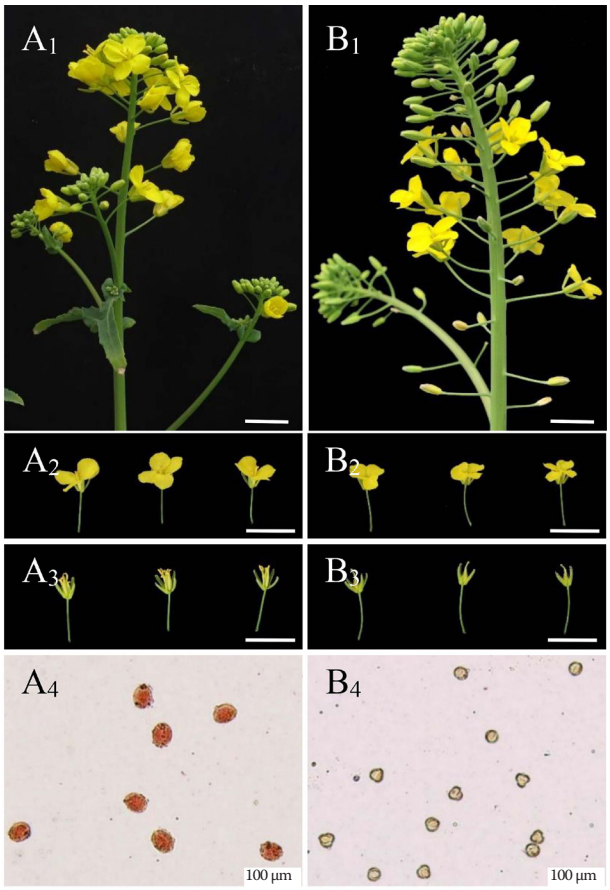

Figure 1. The fertility performance of 19F08AB in *Brassica napus* L.: inflorescence (A_1, B_1), flowers (A_2, B_2), petals-removed flowers (A_3, B_3), and pollens (A_4, B_4) of male fertile plants 19F08B (A_1–A_4) and male sterile plants 19F08A (B_1–B_4)

A_1–A_3, B_1–B_3 – scale bar = 2 cm; A_4, B_4 – scale bar = 100 µm

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(Table S2 in ESM). At maturity, most agronomic traits showed no significant differences between 19F08A and 19F08B open-pollinated plants, except for branch height and total siliques per plant, which were significantly increased or decreased, respectively, in 19F08A compared to 19F08B (Table S3 in ESM).

Cytological observations of microspore development in 19F08AB. We characterised the stamens of fertile (19F08B) and sterile (19F08A) plants by light microscopy to identify microscopic differences in cellular morphology. Based on the result of cytological observation, the meiotic process in 19F08B plants was normal, and finally, mature pollen could be formed (Figures 2A–H). However, the meiotic process in 19F08A plants exhibited significant abnormalities (Figures 2a–h). In sterile anthers, most pollen mother cells (PMCs) arrested at the pachytene stage; only a small number of PMCs entered diakinesis (Figure 2d), metaphase I (Figure 2e), and anaphase I (Figure 2g). Nuclei in sterile PMCs was condensed, and the named "microspore's analogue" (Yu & Fu 1990) was formed directly from the PMCs without the meiosis process. Ultimately, the cytoplasm of the 'microspore analogue' underwent nearly complete degradation, leaving only empty shells or degraded cell walls (Figures 2g, h). Notably, among all bud sizes examined in sterile plants, neither dyads nor tetrads were observed.

Inheritance. Within the 19F08AB population, sib-mating between male-sterile (19F08A) and male-fertile (19F08B) plants produced progeny with a 1 : 1 fertile-to-GMS segregation ratio. Conversely, self-pollination of 19F08B plants resulted in fully fertile offspring (Table 2). The results suggested that male sterility of 19F08A was controlled by a pair of nuclear gene, with male sterility to be dominant.

To investigate 19F08AB inheritance and its relationship with the reported CMS and GMS lines, 19F08AB was crossed with a panel of representative rapeseed lines and several different types of TGMS

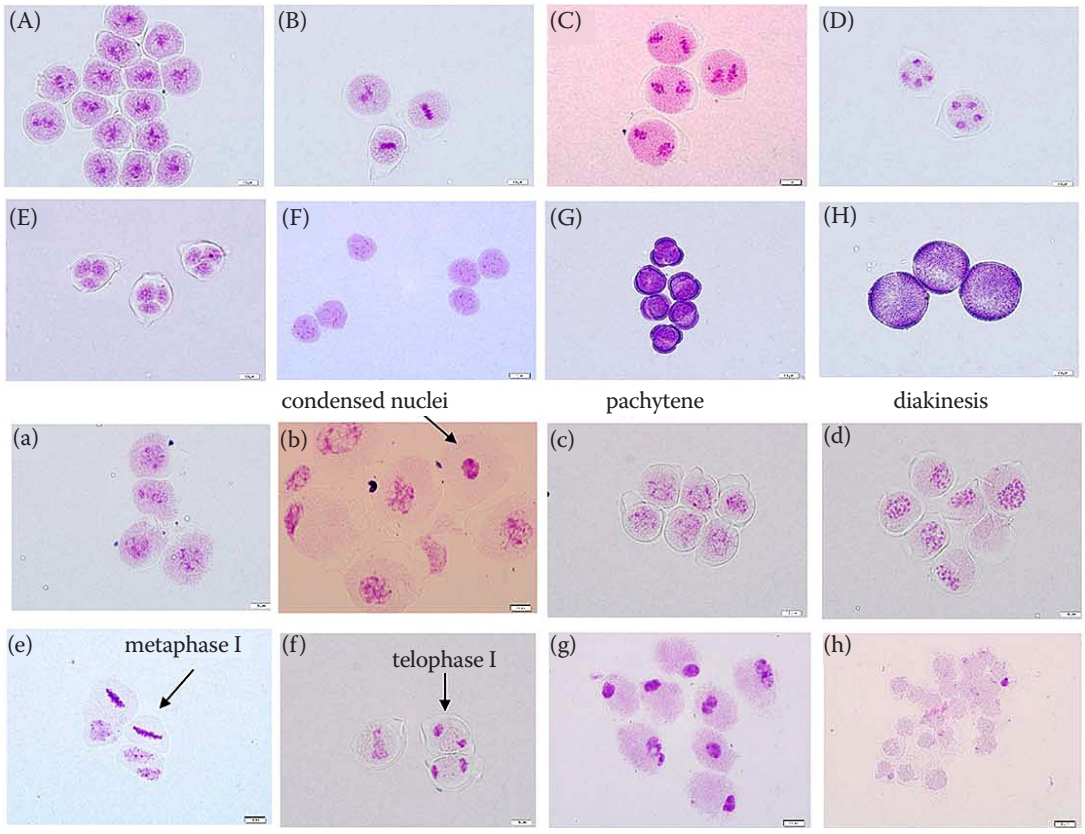

Figure 2. Cytological observation of microspore development in anthers of 19F08AB in *Brassica napus* L.: (A) to (H) 19F08B; prophase I (A), metaphase I (B), metaphase II (C), telophase II (D), tetrad stage (E), early microspore stage (F), trilateral thickening stage (G), mature pollen stage (H); (a) to (h) 19F08A; prophase I (a–d), metaphase I (e), anaphase I (f), microspore analogue (g), aborted microspores (h)
scale bar = 10 μm

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Table 2. Fertility performance of the male sterile two-type line system 19F08AB in *Brassica napus* L.

Code	Code in the last year	Fertile plants	GMS plants	Expected ratio (F : GMS)	χ_c^2 value
0A181	9F08-2S × 9A08F-6	10	13	1: 1	0.17
1A80	0A181-1S × 0A181-1F	24	19	1: 1	0.37
1A81	0A181-2S × 0A181-2F	27	31	1: 1	0.16
1A86	0A181-1F⊗	47	0	1: 0	–
1A87	0A181-2F⊗	41	0	1: 0	–

F – fertile plants; GMS – genic male sterile plants; $\chi^2_{0.05,1} = 3.84$, $\chi^2_{0.01,1} = 6.63$; – not applied

and GMS lines (Table 1). The tested F_1 progenies between 19F08A and two TGMS lines, H50S and 373S, segregated in a ratio of 1F : 1GMS (Table S4 in ESM), indicating that male sterility of 19F08A was controlled by different genes from both TGMS lines. The F_1 progeny between 9012A and 19F08B showed fertility, and the F_1 progeny between 19F08A and TAM, a temporary maintainer line for 9012A, segregated in a ratio of 1F : 1GMS (Table S4 in ESM), indicating that male sterility of 19F08A was controlled by different genes from 9012A. The tested F_1 progenies between 19F08A and five restorer lines (SH11, CZL-20A, Q10C, T16, and S11R) and two maintainer lines (2011B2 and ZS09) of *pol* CMS segregated in a ratio of 1F : GMS (Table S4 in ESM), indicating that 19F08A had a different maintaining and restoring relationship with *pol* CMS, or possessed a distinct genetic identity.

Interesting, two restorer lines ZS05R and WW1291 for Shaan-GMS could restore the male fertility in 19F08A (Table S4 in ESM).

The relationship between 19F08AB and the dominant GMS line Shaan-GMS. To further investigate the relationship between 19F08AB and Shaan-GMS, we crossed 19F08A with ZS05R (one restorer line for Shaan-GMS), and reciprocal crossed 19F08AB with ZS02AB (heterozygous two-type line of Shaan-GMS) and 803AB (homozygous two-type line of Shaan-GMS). The F_1 progenies between 19F08A and ZS05R were fertile (Table 3). Among the five tested crosses between the F_1 and SH11 (one restorer line for *pol* CMS), four crosses showed fertility, and one cross showed fertility segregation with 1F : 1GMS. Among three F_2 populations derived from the cross between 19F08A and ZS05R, one showed fertility segregation of 3F : 1CMS. In two other F_2 populations, we observed four different types of plants with different fertility performances: fertility plants, GMS plants, CMS plants, and double MS (CMS + GMS) plants (Figure S1 in ESM), and they showed fertility segregation of 9F : 3GMS : 3CMS : 1double MS (Table 3). From these results, it was inferred that 19F08AB has *pol* CMS cytoplasm and a pair of *Rfp* genes (Figure S2 in ESM). In these two F_2 population, compared with fertile plants (Figure S1 in ESM, A_1–A_3), the GMS plants flowers possessed fully-degenerated stamens with no pollen and their petals were flat, round, and overlapping (Figure S1 in ESM, B_1–B_3); the anthers of the CMS plants were not fully degenerated, but contained traces of pollen in early flowering, the petals were typically separated, wrinkled, ellipsoidal (Figure S1 in ESM, C_1–C_3); the anthers of the double MS plants were completely degenerated, the petals were separated and ellipsoidal, but flat (Figure S1 in ESM, D_1–D_3). The floral organ of the double MS plants was the smallest of the four types of plants, with the fertile plants the largest ones, then GMS and CMS plants (Figure S1, Table S5 in ESM). Phenotypic observations revealed that the floral organs of double MS plants exhibited interactions between the GMS and *pol* CMS gene systems. The double MS plants developed smaller, flatter petals and completely degenerated stamens that produced no pollen. Fertility analysis of the crosses between ZS02A × 19F08B, 803A × 19F08B, 803B × 19F08B, and 19F08A × 803B indicated that 19F08A has the same maintaining and restoring relationship with Shaan-GMS (Table 3, Zhang et al. 2020). The results confirmed that 19F08A carries the *Msms* genotype, while 19F08B is homozygous recessive (*msms*). The inferred genotypes of all parental lines are summarised in Table 3.

The relationship between 19F08AB and *pol* CMS. To investigate the cytoplasmic type and its relationship with *pol* CMS, we crossed 19F08A with ZS09 (one maintainer line for *pol* CMS) and SH11 (one restorer line for *pol* CMS). Fertility observation of the obtained F_1, F_2, and BC_1 progenies further indicated 19F08AB has *pol* CMS cytoplasm and a pair of *Rfp*

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Table 3. The relationship between 19F08AB and the dominant GMS line Shaan-GMS

Code	Generation	Fertile plants	GMS plants	CMS plants	MS plants	Expected ratio	χ_c^2 value	Deduced genotypes of parents
1A80-2 × 1A23-2	F_1 (19F08A × ZS05R)	33				F		*S* (*RfpRfpMsms*) × *S* (*rfprfpMfMf*)
1A81-1 × 1A23-2		33				F		*S* (*RfpRfpMsms*) × *S* (*rfprfpMfMf*)
1A82-2 × 1A23-2		31				F		*S* (*RfpRfpMsms*) × *S* (*rfprfpMfMf*)
2A64-1 × 2C378-1	BC_1 ((19F08A × ZS05R) × SH11)	99				F		*S* (*RfprfpMfms*) × *S* (*RfpRfpmsms*)
2A64-2 × 2C378-2	-	68				F		*S* (*RfprfpMfms*) × *S* (*RfpRfpmsms*)
2A64-3 × 2C378-3	-	82				F		*S* (*RfprfpMfms*) × *S* (*RfpRfpmsms*)
2A64-5 × 2C378-7	-	48				F		*S* (*RfprfpMfms*) × *S* (*RfpRfpmsms*)
2A64-4 × 2C378-6	-	30	14			1F:1GMS	2.43	*S* (*RfprfpMfMs*) × *S* (*RfpRfpmsms*)
2A64-21⊗	F_2 (19F08A × ZS05R)	49		18		3F:1CMS	2.72	*S* (*RfprfpMfms*)⊗
2A65-1⊗	-	57	8	15	6	9F:3GMS:3CMS:1MS	1.65	*S* (*RfprfpMfMs*)⊗
2A65-2⊗	-	56	7	10	4	9F:3GMS:3CMS:1MS	3.07	*S* (*RfprfpMfMs*)⊗
2A05-2S × 2A95-1F	F_1 (ZS02A × 19F08B)	40	33			1F:1GMS	0.49	*N* (*Msms*) × *S* (*msms*)
2A05-3S × 2A95-2F	F_1 (ZS02A × 19F08B)	23	37			1F:1GMS	2.82	*N* (*Msms*) × *S* (*msms*)
2A07-2F × 2A95-1F	F_1 (803B × 19F08B)	52	33			1F:1GMS	3.81	*N* (*MsMf*) × *S* (*msms*)
2A07-2F × 2A95-2F	F_1 (803B × 19F08B)	45	35			1F:1GMS	1.01	*N* (*MsMf*) × *S* (*msms*)
2A07-1S × 2A95-2F	F_1 (803A × 19F08B)	0	12			GMS		*N* (*MsMs*) × *S* (*msms*)
2A95-8S × 2A07-1F	F_1 (19F08A × 803B)	12	9			1F:1GMS	0.19	*S* (*Msms*) × *N* (*MsMf*)

F – fertile plants; GMS – genic male sterile plants; CMS – cytoplasmic male sterile plants; MS – cytoplasmic and genic male sterility (CMS + GMS) plants; *S* – *pol* CMS (*Shaan 2A* CMS); *N* – normal cytoplasm; *Rfp* – restorer gene for *pol* CMS; *rfp* – maintainer gene for *pol* CMS; *Ms* – dominant male sterile gene for Shaan-GMS; *ms* – male fertile gene for Shaan-GMS; *Mf* – male restorer gene for Shaan-GMS; $\chi^2_{0.05,1} = 3.84$, $\chi^2_{0.01,1} = 6.63$. $\chi^2_{0.05,3} = 7.81$, $\chi^2_{0.01,3} = 11.34$

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Table 4. The relationship between 19F08AB and *pol* CMS

Generation[1]	Fertile plants	GMS plants	CMS plants	MS plants	Expected ratio	χ_c^2 value	Deduced genotypes
19F08A × ZS09							*S* (*RfpRfpMsms*) × *N* (*rfprfpmsms*)
F_1	68	47			1F : 1GMS	3.48	1 *S* (*Rfprfpmsms*) : 1 *S*(*RfprfpMsms*)
F_2	116		26		3F : 1CMS	3.04	3 *S* (*Rfp_msms*) : 1 *S* (*rfprfpmsms*)
BC_1 (2A69)	23	10	5	12	1F : 1GMS : 1CMS : 1MS	3.46	1 *S* (*Rfprfpmsms*) : 1 *S* (*RfprfpMsms*) : 1 *S*(*rfprfpmsms*) : 1 *S* (*rfprfpMsms*)
19F08A × SH11							*S* (*RfpRfpMsms*) × *S* (*RfpRfpmsms*)
F_1	83	64			1F : 1GMS	2.2	1 *S*(*RfpRfpmsms*) : 1 *S* (*RfpRfpMsms*)
F_2	45				F		*S* (*RfpRfpmsms*)
BC_1	38	28			1F : 1GMS	1.23	1 *S* (*RfpRfpmsms*) : 1 *S* (*RfpRfpMsms*)

[1]F_2 is obtained by selfing fertile plants in the F_1 generation; F – fertile plants; GMS – genic male sterile plants; CMS – cytoplasmic male sterile plants; MS – cytoplasmic and genic male sterility (CMS + GMS) plants; *S* – *pol* CMS (*Shaan 2A* CMS); *Rfp* – restorer gene for *pol* CMS; *rfp* – maintainer gene for *pol* CMS; *Ms* – dominant male sterile gene for Shaan-GMS; *ms* – male fertile gene for Shaan-GMS; $\chi^2_{0.05,1} = 3.84$; $\chi^2_{0.01,1} = 6.63$; $\chi^2_{0.05,3} = 7.81$; $\chi^2_{0.01,3} = 11.34$

genes (Table 4). The deduced genotypes of the involved accessions are indicated in Table 4.

Collectively, genetic analysis revealed that the genotype of 19F08A is *pol* (*RfpRfpMsms*), and that of 19F08B is *pol* (*RfpRfpmsms*). The effect of *pol* cytoplasm was masked by the *Rfp* gene. Therefore, male sterility in 19F08A is controlled by a pair of nuclear genes (*Ms*/*ms*), with male sterility being dominant over fertility.

Investigation of cytoplasmic type and genotype at the *Ms*/*Mf/ms* and *Rf/rf* loci of 19F08AB using molecular markers. The multiplex PCR analysis was used to identify the cytoplasmic type of 19F08AB (Table S1 in ESM, Zhao et al. 2010). The results indicated that ZS02AB and 803AB had *nap* cytoplasm. However, 19F08AB and, lines CZL-20A and S11R, has *pol* CMS cytoplasm (Figure 3).

Based on the DNA sequence of *pol* CMS restorer (*Rfp*) and maintainer allele (*rfp*) (Liu et al. 2016), we designed two pairs of primers for identifying the *Rfp*/*rfp* locus of 19F08AB (Table S1 in ESM). The results indicated that these primers could successfully distinguish the *Rfp* from *rfp* alleles for *pol* CMS, with SH11 (*RfpRfp*), ZS11 (*rfprfp*), and 3C243 (*Rfprfp*) as reference, and 19F08AB along with lines CZL-20A and S11R each contain a pair of *Rfp* genes (Figure 4).

To investigate the genotype of 19F08AB at the *Ms/Mf/ms* locus, we designed three primers (Ms5a-1F/Ms5a-1R, 5c-12F/ac-2R, and Ms5a-1F/Ms5b-1R, Table S1 in ESM) to detect the *Mf/ms*/*Ms* alleles of Shaan-GMS. The results showed that 19F08A had *Msms* genotype, 19F08B had *msms* genotype.

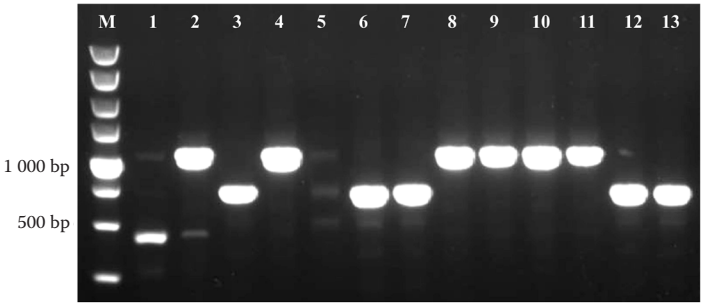

Figure 3. Identification of cytoplasm type of 19F08AB by the multiplex PCR analysis
M – marker; lane 1 – 3A333 (*Ogu* CMS); lane 2 – 3A327 (*IP-Ogu* CMS); lane 3 – 3C01 (*pol* CMS); lane 4 – 3C10 (*nap* type); lane 5 – 3C03 (*cam* type); lane 6 – 19F08A; lane 7 – 19F08B; lane 8 – ZS02B; lane 9 – ZS02A; lane 10 – 803B; lane 11 – 803A; lane 12 – CZL-20A; lane 13 – S11R

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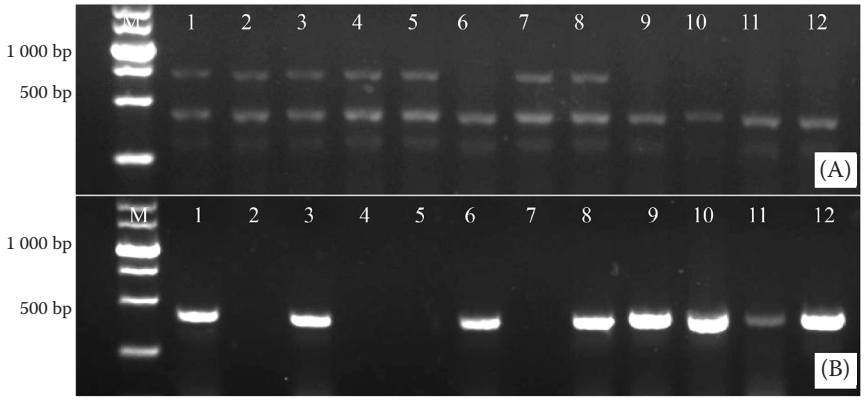

Figure 4. Development of molecular markers for the restorer (*Rfp*) and maintainer (*rfp*) alleles for *pol* CMS: PCR amplification results by primers rfp-9F/(rfp-8R+RFP-R1) for detecting the *rfp* allele (A) and by primers RFP-F/RFP-R1 for detecting the *Rfp* allele (B)

M – DS5000 Marker; lane 1 – 2A07F (*Rfprfp*); lane 2 – 2A07S (*rfprfp*); lane 3 – 3A03F (*Rfprfp*); lane 4 – 3A03S (*rfprfp*); lane 5 – 2A14 (*rfprfp*); lane 6 – SH11 (*RfpRfp*); lane 7 – ZS11 (*rfprfp*); lane 8 – 3C243 (*Rfprfp*); lane 9 – 19F08B (*RfpRfp*); lane 10 – 19F08A (*RfpRfp*); lane 11 – CZL-20A (*RfpRfp*); lane 12 – S11R (*RfpRfp*)

In contrast, both lines, CZL-20A and S11R, were found to have the *msms* genotype (Figure 5).

Collectively, the gene-specific molecular marker analysis confirmed that 19F08A carries the genotype *pol* (*RfpRfpMsms*), while 19F08B is *pol* (*RfpRfpmsms*). These genotypic assignments align with the genetic findings presented earlier.

Genotyping of four different types of plants in progenies of 19F08AB by the gene-specific markers. The above genetic and molecular analysis indi-

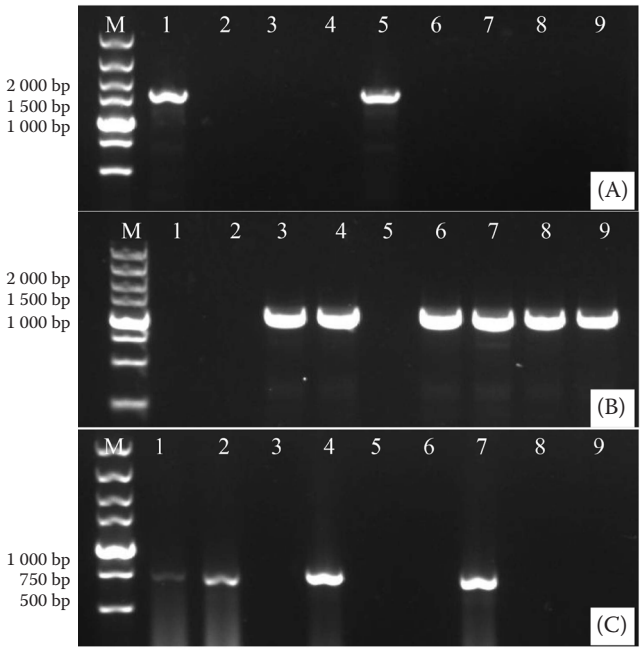

Figure 5. Development of molecular markers for identification of three different alleles (*Ms/Mf/ms*) at the *Ms5* locus of Shaan-GMS: PCR amplification results by primers Ms5a-1F and Ms5a-1R for detecting the *Mf* allele (A), primers 5c-12F and ac-2R for detecting the *ms* allele (B) and primers Ms5a-1F and Ms5b-1R for detecting the *Ms* allele (C)

M – DS5000 marker; lane 1 – 2A07F (*MfMs*); lane 2 – 2A07S (*MsMs*); lane 3 – 3A03F (*msms*); lane 4 – 3A03S (*Msms*); lane 5 – 2A14 (*MfMf*); lane 6 – 19F08B (*msms*); lane 7 – 19F08A (*Msms*); lane 8 – CZL-20A (*msms*); lane 9 – S11R (*msms*)

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cated that genotype of 19F08A was *pol* (*RfpRfpMsms*), and that of 19F08B was *pol* (*RfpRfpmsms*). In the F_2 population and BC_1 population (Tables 3 and 4) derived from 19F08A, we observed four different types of plants: fertile plants, GMS plants, CMS plants, and double MS plants (Figure S1 in ESM). To further support the above genetic hypothesis, we genotyped one BC_1 population (2A69) derived from the cross between 19F08A and ZS09 (19F08A/ZS09//ZS09) (Table 4), by the molecular markers designed for the *Rfp/rfp* alleles of *pol* CMS and the *Mf/ms*/*Ms* alleles of Shaan-GMS (Table S1 in ESM). The results-revealed distinct genotype-phenotype correlations in the 2A69 population: six fertile plants showed the *msmsRfprfp* genotype, four GMS plants carried *MsmsRfprfp*, five CMS plants exhibited *msmsrfprfp*, and 12 double MS plants possessed *Msmsrfprfp* (Figures S3, S4, Tables S6, S7 in ESM). These molecular genotyping results were fully consistent with the classical genetic analysis.

DISCUSSION

The identification and characterisation of novel male sterile resources remain essential for advancing heterosis breeding in *B. napus*. In the present study, a new male sterile two-type line system 19F08AB in *B. napus* was characterised at levels of floral organ morphology, cytological, genetic and molecular levels. The classical genetic and molecular analysis revealed that the male-sterile plants (19F08A) had genotype *pol*(*RfpRfpMsms*), while the male-fertile plants (19F08B) had the genotype *pol*(*RfpRfpmsms*). Because the *Rfp* gene masked the effect of *pol* cytoplasm (Fu 2019), male fertility in 19FAB is controlled by a pair of nuclear genes (*Ms*/*ms*), with male sterility being dominant over fertility. The findings will broaden male-sterile germplasm resources available for *B. napus* hybrid breeding and enrich the theory of plant male sterility.

Previous studies demonstrate that male sterility in the dominant GMS Shaan-GMS results from defective tapetal development, middle-layer abnormalities, and meiotic impairment (Xiao et al. 2013; Zhang et al. 2020). PMCs in Shaan-GMS initiated degeneration during early meiosis and failed to progress beyond anaphase I. Consequently, neither dyads nor tetrads formed during microsporogenesis. Cytological analyses of other dominant GMS lines, including Yi-3A, its derivatives Rs1046A and FM195A, and TE5A, revealed that meiosis ceased at the leptotene or pachytene stages. PMCs failed to undergo meiosis, resulting in no dyad or tetrad formation and culminating anther abortion (Yu & Fu 1990; Xin et al. 2016; Yan et al. 2016). These lines are genetically controlled by a multiple-allele system at *Ms5* locus, harbouring three distinct alleles (Song et al. 2006; Hong et al. 2006; Liu et al. 2008; Yan et al. 2016; Zhang et al. 2020). In this study, we observed that most PMCs in sterile anthers of 19F08A arrested at the pachytene stage, only a small number of PMCs entered diakinesis, metaphase I, and anaphase I, but could not pass the anaphase I stage, with no dyads or tetrads formed (Figure 2). This pattern mirrors that of the dominant GMS lines described above and suggests that 19F08A belongs to the male sterile type of meiosis abnormality (Yu & Fu 1990).

Previously, in experiments with improving fertility restorers for *pol* CMS, male-sterile plants were unexpectedly observed within an F_2 population generated by crossing two *pol* CMS restorer lines (CZL-20A and S11R), the male sterile two-type line system 19F08AB was developed by sib-crossing (Yu 2022). In the present study, classical genetic (Tables 3, 4) and molecular marker analysis (Figures 3, 5) demonstrated that the male-sterile plants (19F08A) carry the genotype *pol*(*RfpRfpMsms*), whereas the male-fertile plants (19F08B) possess *pol* (*RfpRfpmsms*). The *Rfp* gene was found to mask the effect of *pol* cytoplasm (Fu 2019), indicating that fertility in 19FAB is governed by a pair of nuclear genes (*Ms*/*ms*), with male sterility dominant over fertility. The *pol* CMS cytoplasm and *RfpRfp* genes of 19F08AB are traced back to its two parents, both of which had *pol* CMS cytoplasm and *RfpRfp* genes (Figures 3, 4). However, the origin of the male sterile gene *Ms* in 19F08A remains unclear, since this gene is absent in both parents (Figure 5). There was most likely an unwanted crossing of *pol* CMS restorer with the donor, fertile plants (genotype *MsMf*) of homozygous two-type GMS line, which may introduce the *Ms* gene into 19F08AB. Further, genetic (Table 3) and molecular (Figure 5) analysis suggest that the male sterility in 19F08A is likely governed by the same *Ms/ms* locus as that characterised in Shaan-GMS (Zhang et al. 2020). However, unlike Shaan-GMS, which possesses *nap* cytoplasm, the 19F08AB carries *pol* cytoplasm (Figure 3).

The 19F08AB two-type line system developed in this study offers two distinct applications in rapeseed breeding, differing from those of Shaan-GMS. Both enable the development rapeseed hybrid via either two-line or three-line system (Li et al. 1985).

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Furthermore, 19F08AB provides a foundation for developing Genic and Cytoplasmic Male Sterility (GCMS) lines in rapeseed – a promising heterosis utilisation strategy proposed previously (Yang & Fu 1993; Li et al. 1995; Yang et al. 1996). The male-sterile material also holds value for recurrent selection of restorer lines for *pol* CMS (*Shaan* 2A CMS), a dominant male sterile system in rapeseed hybrid breeding. Because 19F08A carries both the *pol* CMS sterile cytoplasm and the *Rfp* gene, the *pol* cytoplasm serves as an indicator for the fertility restorer allele (*Rf*) retention. This eliminates the need for test-crossing to track *Rf* allele across generations (Fu 2019).

In addition, gene-specific markers for *pol* CMS and dominant GMS systems developed here and in previous studies (Liu et al. 2016, 2017; Havlickova et al. 2012; Xin et al. 2016; Zhao et al. 2010; Zhang et al. 2023) will facilitate basic research and Shaan-GMS/*pol* CMS hybrid breeding.

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