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Development and Application of KASP Markers for Cytoplasmic Male Sterility Genes in Polima CMS and Ogura CMS Systems of Brassica napus

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Abstract

The cytoplasmic male sterility (CMS) is the main strategy for exploiting heterosis in rapeseed, with Polima CMS (Pol CMS) and Ogura CMS (Ogu CMS) being the most widely utilized systems. To enable high-throughput identification of cytoplasm types, we developed four Kompetitive Allele-Specific PCR (KASP) markers targeting or flanking the mitochondrial sterility genes *orf224/atp6* (Pol CMS) and *orf138* (Ogu CMS). Genotyping of 101 rapeseed accessions with diverse genetic backgrounds demonstrated accurate discrimination of cytoplasm types by these markers. By combining the above cytoplasmic markers with markers for the nuclear fertility restorer gene (*Rf*), we established a dual-marker system capable of simultaneously determining cytoplasmic type (S/N) and nuclear genotype (*RfRf*/*Rfrf*/*rfrf*). This system enables systematic tracing of the specific causes of purity decline in male-sterile lines, restorer lines, and hybrids, with results fully consistent with field phenotyping. Furthermore, using this dual-marker-assisted selection strategy, we efficiently identified 24 isoplasmic restorer lines from 187 germplasm accessions. In conclusion, the KASP markers developed in this study provide an efficient and precise tool for identifying mitochondrial sterility genes in the Pol CMS and Ogu CMS systems, thereby substantially improving the efficiency of parental line identification and purity assessment.

Introduction

Rapeseed (*Brassica napus*) is one of the world's most important oilseed crops, contributing substantially to global edible oil supply and agricultural economy (Zandberg et al. 2022). Exploiting heterosis represents a major strategy for improving both yield and stress tolerance in this crop. The CMS-based three-line system serves as the cornerstone of commercial hybrid seed production (Zandberg et al. 2022; Farooq et al. 2025). This system operates through the coordinated use of three genetically distinct lines: a male-sterile line (A line), its isogenic maintainer line (B line), and a fertility restorer line (R line) (Eckardt 2006). To date, more than ten CMS types have been identified or artificially developed in rapeseed worldwide. Among these, six-namely, Ogu CMS, Nap CMS, Pol CMS, Shan2A CMS, Nsa CMS, and Songyou CMS-have been successfully established as three-line systems with identified fertility restorers (Ogura 1968; Thompson 1972; Rawat 1979; Fu 1981; Prakash et al. 1998; Hu et al. 2002). Among these, Pol CMS and Ogu CMS are the most extensively utilized in rapeseed three-line hybrid breeding.

In Pol CMS, the CMS-associated gene is *orf224*, which is located upstream of *atp6* on the mitochondrial genome and is co-transcribed with it (Witt et al. 1991; Handa and Nakajima 1992; L'Homme and Brown 1993; Wang et al. 1995; Singh et al. 1996; Yang et al. 1996; Chen et al. 2013; Wang et al. 2025). Ogura CMS, originating from radish (*Raphanus sativus* L.), was introduced into rapeseed through distant hybridization, and its sterility-associated gene is *orf138* (or its homolog *orf125*) (Ogura 1968; Bannerot et al. 1974; Heyn 1976; Heath et al. 1994; Kirti et al. 1995; Primard-Brisset et al. 2005; Chen et al. 2013; Yamagishi and Bhat 2014; Yu et al. 2016; Wei et al. 2019). Both CMS systems cause pollen abortion through the expression of chimeric mitochondrial open reading frames, which impair mitochondrial function and disrupt pollen development (Wang et al. 1995; Ge et al. 2024). Regarding fertility restoration, the major restorer genes *Rfp* (for Pol CMS) and *Rfo* (for Ogu CMS) have been identified. Both genes encode pentatricopeptide repeat (PPR) proteins that are targeted to mitochondria, where they restore pollen fertility by modulating the processing of mitochondrial sterility gene(Desloire et al. 2003; Brown et al. 2003).

KASP markers have gained widespread adoption in crop genetics and breeding, owing to their high-throughput capability, cost-effectiveness, and gel-free detection (Gudi et al. 2020; Semagn et al. 2014). For the nuclear restorer genes of these CMS systems, numerous KASP markers have been developed and successfully applied for restorer line selection and hybrid seed purity testing (Semagn et al. 2014; Devran et al. 2016; Priya et al. 2019; Gudi et al. 2020). These markers have substantially improved the efficiency of restorer breeding and enabled high-throughput purity assessment of hybrid seeds. In other crops where CMS is widely employed, advances have also been made in developing KASP markers targeting mitochondrial sterility genes. For example, in tobacco, KASP markers based on SNP differences in the mitochondrial *atp6* gene, combined with background selection using a 50K SNP array, enabled the successful introgression of CMS cytoplasm into nine germplasm accessions (Priya et al. 2019). In onion, KASP markers have facilitated the rapid identification of S- or N-type cytoplasms; when combined with restorer gene markers, they greatly enhance the propagation efficiency of male-sterile lines (Anand et al. 2025). However, the development of KASP markers specifically targeting the mitochondrial sterility genes of Pol CMS and Ogu CMS in rapeseed has been limited. In current breeding practice, cytoplasm type identification still relies mainly on field fertility observations at the flowering stage, which is time-consuming and labor-intensive, thereby representing a technical bottleneck constraining efficient breeding in rapeseed.

To address this gap, we developed KASP markers targeting *orf224*/*atp6* and *orf138* based on publicly available mitochondrial sterility gene sequences and validated their accuracy across a diverse panel of germplasm resources, including sterile and maintainer lines. By integrating these cytoplasm-specific markers with KASP markers for the nuclear fertility restorer genes (*Rfp* for Pol CMS and *Rfo* for Ogu CMS), we established a dual-marker system capable of simultaneously determining cytoplasmic type and nuclear genotype in a single KASP reaction. This system enables systematic diagnosis of purity decline in male-sterile lines, restorer lines, and F_1 hybrids, and facilitates the rapid identification of isoplasmic restorer lines through marker-assisted selection. Collectively, the markers and the dual-marker strategy developed herein provide a robust technical platform for parental line selection, purity assessment, and restorer line development, thereby enhancing the efficiency and precision of three-line breeding and hybrid seed production in rapeseed.

Materials and Methods

Plant Materials

All plant materials were provided by the Hunan Crop Research Institute, Changsha, China, and were classified into four categories:

a) KASP marker validation materials: The following 8 control materials were used as KASP marker validation materials: 2 Pol CMS sterile lines (20A and 318A); 1 Pol CMS isoplasmic restorer line (SC4); 2 Ogu CMS sterile lines (20QA and 293QA); 1 Ogu CMS isoplasmic restorer line (679R); and 2 maintainer lines (20B, 318B).
b) Validation panel: The validation panel consisted of 101 rapeseed accessions with known cytoplasm types, including 20 Pol CMS sterile lines, 20 Pol CMS isoplasmic restorer lines, 9 Ogu CMS sterile lines, 12 Ogu CMS isoplasmic restorer lines, 30 maintainer lines, and 10 accessions carrying other CMS types.

c) Materials for purity testing of male sterile lines, restorer lines, and F_1 hybrids: Seeds from different batches of field production were sampled for KASP marker detection and field purity identification. The specific materials were as follows: Pol-A male sterile lines: 318A, 2018A, and 20A; Ogu-A male sterile lines: 293QA and 76024QA; Pol-R restorer lines: 09-5R and Rong R; Ogu-R restorer lines: 703R, 927R, and 679R; Pol F_1 hybrid: FY789; Ogu F_1 hybrid: FY9379.

d) Germplasm for restorer line identification: A total of 187 rapeseed breeding lines with diverse genetic backgrounds were collected for simultaneous screening of cytoplasmic and nuclear backgrounds.

DNA Extraction

DNA Extraction
Genomic DNA was extracted from young leaf tissue using the standard cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990). DNA concentration and purity were assessed using a NanoDrop spectrophotometer. All DNA samples were adjusted to a working concentration of 40–100 ng/μL and stored at 4°C until required.

KASP Marker Development and Genotyping

Reference mitochondrial genome sequences for Pol CMS (NCBI accession NC_008285.1, containing *orf224/atp6*) and Ogu CMS (NCBI accession AP013066.1, containing *orf138*) were retrieved from public databases (Zhang et al. 2022). Multiple sequence alignment was performed to identify cytoplasm-specific polymorphisms. Two stable, CMS-specific single-nucleotide polymorphism (SNP) sites were identified within the *orf224/atp6* region of Pol CMS and the flanking *orf138* region of Ogu CMS, respectively. KASP assay primers targeting these SNPs were designed using the LGC KASP Assay Design Service (version 4.0). Initial screening of candidate primer sets was conducted using genomic DNA from the control materials. KASP genotyping reactions were performed in a total volume of 5 μL per reaction, containing 2.5 μL of 2× KASP Master Mix, 0.07 μL of KASP primer assay mix, and approximately 20 ng of template genomic DNA. PCR amplification was carried out according to the standard KASP cycling protocol. Endpoint fluorescence signals (FAM and HEX) were detected using a LightCycler® 480 II instrument. Genotype clustering and allele calling were performed using the instrument's integrated analysis software. Markers exhibiting clear allele discrimination, well-separated genotype clusters, and high call rates were selected for subsequent validation and application.

Using the same design strategy, we developed and validated two additional KASP markers for the nuclear fertility restorer genes Rfp (Pol CMS) and Rfo (Ogu CMS): BN9000004 (validated for Pol CMS, Wang et al. 2020) and BN9000018 (validated for Ogu CMS, Liu et al. 2025).

Field experiments were conducted in Changsha, Hunan Province, China (28.12° N, 112.59° E). Plants were grown under standard agronomic practices, with a row spacing of 33 cm and an intra-row spacing of 7 cm. For each material subjected to purity assessment, 186 individual plants were randomly selected. Young leaf tissues were collected at the five- to six-leaf stage for genomic DNA extraction and subsequent KASP marker genotyping. At the flowering stage, fertility and morphological characteristics of each individual plant were investigated to validate the accuracy of the KASP marker assay.

Field Evaluation of Isoplasmic Restorer Lines

Field-based fertility restoration tests were performed to assess the presence of nuclear restorer genes and to confirm the cytoplasm types of candidate materials:

(i) Identification of fertility restorer alleles. Candidate lines were used as male parents and crossed with Pol CMS sterile line 20A (for Pol CMS) or Ogu CMS sterile line 20QA (for Ogu CMS) as the female parents. The resulting F_1 plants were grown in the field, and male fertility was assessed during the flowering stage based on anther dehiscence and pollen viability.

(ii) Confirmation of cytoplasm type in candidate restorer lines. To verify the cytoplasmic background of candidate restorer lines, each candidate line was used as the female parent and crossed with the corresponding maintainer line 20B. The resulting F_1 plants were selfed to produce F_2 populations. Fertility segregation in the F_2 generation was evaluated at the flowering stage to infer the cytoplasm type.

Results

Identification of Cytoplasm-Specific SNP Variants

Using BLASTn sequence comparisons, four high-quality SNPs were identified from publicly available mitochondrial genome sequences of rapeseed. These SNPs clearly differentiated between cytoplasm types while remaining highly conserved within each cytoplasmic background. Two SNPs were specific to the Ogu CMS cytoplasm, enabling its discrimination from both the maintainer and Pol CMS cytoplasms (Fig. 1A, B), while the other two SNPs were specific to the Pol CMS cytoplasm, clearly distinguishing it from the maintainer and Ogu CMS cytoplasms (Fig. 1C, D).

Development and Validation of KASP Markers

We developed four KASP markers: two Pol CMS-specific markers (BN9000344_K01 and BN9000345_K01) and two Ogu CMS-specific markers (BN9000568_K01 and BN9000570_K01). Primer sequences and primer information for each marker are provided in Supplementary Table S1 and S2.

The four mitochondrial KASP markers were validated using a 96-well plate format containing 94 control DNA samples—comprising 16 Pol CMS sterile lines, 12 isoplasmic Pol CMS restorer lines (sharing the Pol CMS cytoplasm), 18 Ogu CMS sterile lines, 12 isoplasmic Ogu CMS restorer lines (sharing the Ogu CMS cytoplasm), and 36 maintainer and conventional fertile lines with normal (N) cytoplasm—plus two no-template controls (NTC). All markers generated distinct allelic discrimination plots with complete separation of homozygous clusters and zero ambiguous calls. For the Pol CMS-specific markers, the 28 accessions carrying Pol CMS cytoplasm (sterile and restorer lines) uniformly displayed the cytoplasm-specific allele (FAM-homozygous cluster) and clustered distinctly from the 66 accessions with normal cytoplasm (maintainer, Ogu CMS, and conventional fertile lines) that carried the wild-type allele (HEX-homozygous cluster). Similarly, for the Ogu CMS-specific markers, the 30 accessions carrying Ogu CMS cytoplasm (sterile and restorer lines) formed a separate cluster from the 64 normal-cytoplasm accessions. Because these markers target mitochondrial SNPs, the sterile lines and their respective isoplasmic restorer lines—which share identical cytoplasms but differ in nuclear restorer genotypes—exhibited the same cytoplasmic allele and co-segregated within the same cluster. All four markers achieved 100% concordance (94/94) in cytoplasmic status assignment(Fig. 2).

Validation of Marker Universality Applicability across Diverse Germplasm

Using a validation panel comprising 101 rapeseed accessions with known cytoplasm types (Table 1), we further evaluated the broad applicability and robustness of the four KASP markers. Across this diverse germplasm set, all four markers consistently and accurately distinguished CMS sterile cytoplasms. For the Pol CMS-specific marker BN9000344_K01, all Pol CMS sterile lines and isoplasmic restorer lines exhibited TT or TC genotypes, while maintainer lines and other accessions displayed the homozygous CC genotype. Similarly, for marker BN9000345_K01, all Pol CMS sterile lines and isoplasmic restorer lines were homozygous for the T allele (TT), while maintainer lines and other materials were heterozygous (TA) or homozygous (AA) for the A allele. For the Ogu CMS-specific marker BN9000568_K01, all Ogu CMS sterile lines and isoplasmic restorer lines displayed a single-base deletion (−/−), while maintainer lines and other materials were homozygous for the C allele (CC). Consistently, marker BN9000570_K01 identified all Ogu CMS sterile lines and isoplasmic restorer lines as CC, while maintainer lines and other materials were homozygous for the A allele (AA).

Table 1
Validation results of KASP marker universal applicability in rapeseed resources

Resource Type	Number of Samples	KASP Markers/Genotypes			
		BN9000344	**BN9000345**	**BN9000568**	**BN9000570**
Pol CMS Male Sterile Lines	20	T: T / T: C	T: T	C: C	A: A
Pol CMS isoplasmic restorer lines	20	T: T / T: C	T: T	C: C	A: A
Ogu CMS Male Sterile Lines	9	C: C	A: A / T: A	-:-	C: C
Ogu CMS isoplasmic restorer lines	12	C: C	A: A / T: A	-:-	C: C
Maintaining Lines	30	C: C	A: A / T: A	C: C	A: A
accessions carrying other CMS types	10	C: C	A: A / T: A	C: C	A: A

Combined KASP markers for purity assessment of CMS lines, restorer lines, and their F_1 hybrids

As shown in Supplementary Table S3, the factors contributing to purity decline in rapeseed CMS lines, restorer lines, and their F1 hybrids are complex and diverse in commercial seed production. A single molecular marker is insufficient to comprehensively trace the underlying causes. Therefore, in this study, newly developed CMS cytoplasm-specific KASP markers were combined with nuclear fertility restorer gene (*Rf*) KASP markers and applied for purity assessment of the aforementioned materials.

The detection results (Table 2) revealed that S(*Rfrf*) and N(*rfrf*) genotypes were identified in CMS line samples, indicating that purity decline was primarily attributable to volunteer seedling contamination and maintainer line admixture. In restorer line samples, S(*Rfrf*), S(*rfrf*), and N(*rfrf*) genotypes were detected, suggesting that purity reduction was associated with foreign pollen contamination, volunteer seedlings, or mechanical seed mixture. In Pol-F_1 hybrids, S(*RfRf*) and S(*rfrf*) genotypes were observed, indicating paternal line contamination and maternal self-fertilization, respectively; additionally, a low proportion of N(*rfrf*) genotype was detected, which may have resulted from volunteer seedling contamination. In Ogu-F_1 hybrids, the presence of S(*RfRf*) genotype was mainly attributed to paternal line contamination, whereas the high proportion of N(*rfrf*) genotype may reflect adulteration with counterfeit seeds by farmers.

Compared with conventional field-based fertility and morphological evaluations at the flowering stage, the purity assessment results obtained by KASP markers were completely consistent (Table 2). These results demonstrate that the developed combined KASP marker system is efficient, accurate, and practical for rapid purity detection of CMS lines, restorer lines, and F_1 hybrids in rapeseed, enabling precise identification of the specific causes underlying purity decline and thereby providing targeted guidance for quality control measures in seed production.

Table 2
Comparison of purity assessment results between combined KASP marker assay and field phenotyping, and analysis of purity decline causes in male-sterile lines, restorer lines, and F_1 hybrids in rapeseed

No.	Material Type	Variety Name	Number of Tested Samples	Genotype					Field Phenotype			Concordance between the two identification methods (%)	Analysis of Purity Decline Causes
				Nuclear Genotype			Cytoplasmic Type		Fertile plants	Sterile plants	Off-type plants		
				rfrf	*RfRf*	*Rfrf*	N	S					
1	Pol-A	318A	186	181	0	5	0	186	5	181	0	100	Volunteer Seedling Contamination: S(*Rfrf*)
2	Pol-A	318A	186	182	0	4	0	186	4	182	0	100	Volunteer Seedling Contamination: S(*Rfrf*)
3	Pol-A	2018A	186	186	0	0	2	184	2	184	2	100	Maintainer Line Contamination: N(*rfrf*)
4	Pol-A	2018A	186	186	0	0	2	184	2	184	2	100	Maintainer Line Contamination: N(*rfrf*)
5	Pol-A	20A	186	186	0	0	0	186	0	186	0	100	
6	Ogu-A	293KQA	186	186	0	0	0	186	0	186	0	100	
7	Ogu-A	293KQA	186	186	0	0	1	185	1	185	1	100	Maintainer Line Contamination: N(*rfrf*)
8	Ogu-A	76024QA	186	186	0	0	2	184	2	184	2	100	Maintainer Line Contamination: N(*rfrf*)
9	Ogu-A	76024QA	186	186	0	0	0	186	0	186	0	100	
10	Ogu-A	76024QA	186	186	0	0	0	186	0	186	0	100	
11	Pol-R	09-5R	186	2	181	3	2	184	186	0	5	100	Foreign pollen contamination: S(*Rfrf*); Volunteer seedlings: N(*rfrf*)
12	Pol-R	09-5R	186	0	186	0	0	186	186	0	0	100	
13	Pol-R	09-5R	186	0	185	1	0	186	186	0	1	100	Foreign pollen contamination: S(*Rfrf*)
14	Pol-R	Rong R	186	0	186	0	0	186	186	0	0	100	
15	Pol-R	Rong R	186	0	186	0	0	186	186	0	0	100	
16	Ogu-R	703R	186	2	184	0	0	186	184	2	0	100	Mechanically mixed seeds: S(*rfrf*)
17	Ogu-R	703R	186	2	184	0	2	184	186	0	2	100	Volunteer seedlings: N(*rfrf*)
18	Ogu-R	927R	186	0	186	0	2	184	186	0	2	100	Volunteer seedlings: N(*rfrf*)
19	Ogu-R	927R	186	0	186	0	0	186	186	0	0	100	

20	Ogu-R	679R	186	0	180	6	0	186	186	0	6	100	Foreign pollen contamination: S(*Rfrf*)
21	Pol-F_1	FY789	186	10	10	166	0	186	176	10	0	100	Paternal contamination: S(*RfRf*); Maternal selfing: S(*rfrf*)
22	Pol-F_1	FY789	186	6	12	168	0	186	180	6	0	100	Paternal contamination: S(*RfRf*); Maternal selfing: S(*rfrf*)
23	Pol-F_1	FY789	186	18	2	166	4	182	172	18	4	100	Paternal contamination: S(*RfRf*); Maternal selfing: S(*rfrf*); Volunteer seedlings: N(*rfrf*)
24	Pol-F_1	FY789	186	16	7	163	5	181	170	16	5	100	Paternal contamination: S(*RfRf*); Maternal selfing: S(*rfrf*); Volunteer seedlings: N(*rfrf*)
25	Pol-F_1	FY789	186	18	5	163	0	186	168	18	0	100	Paternal contamination: S(*RfRf*); Maternal selfing: S(*rfrf*)
26	Ogu-F_1	FY9379	186	26	48	112	26	160	186	0	26	100	Paternal contamination: S(*RfRf*); Adulterated seeds: N(*rfrf*)
27	Ogu-F_1	FY9379	186	64	0	122	64	122	186	0	64	100	Adulterated seeds: N(*rfrf*)
28	Ogu-F_1	FY9379	186	15	0	171	15	171	186	0	15	100	Adulterated seeds: N(*rfrf*)
29	Ogu-F_1	FY9379	186	0	5	181	0	186	186	0	0	100	Paternal contamination: S(*RfRf*)
30	Ogu-F_1	FY9379	186	0	3	183	0	186	186	0	0	100	Paternal contamination: S(*RfRf*)

Accelerated Identification of Isoplasmic Restorer Lines via Combined MAS

To enhance the efficiency of developing isoplasmic restorer lines, the newly developed CMS cytoplasm-specific KASP markers were integrated with established KASP markers for the corresponding nuclear fertility restorer (*Rf*) genes, thus establishing a dual-marker-assisted selection strategy (Fig. 3B).

Using this dual-marker MAS strategy, we screened 187 diverse rapeseed germplasm accessions. We identified 14 accessions carrying Pol CMS cytoplasm and homozygous restorer alleles, and 10 accessions carrying Ogu CMS cytoplasm with homozygous restorer alleles. Field validation demonstrated that all 24 selected accessions exhibited full fertility. When crossed with their respective CMS sterile lines (A lines), the resulting F_1 hybrids showed complete fertility restoration, confirming the presence of homozygous restorer alleles. When test-crossed with their respective CMS maintainer lines (B), the resulting F_2 populations exhibited fertility segregation, confirming that these accessions carried sterile cytoplasm. These results confirmed the reliability of the combined marker system. Collectively, this combined MAS strategy effectively bypasses the multiple generations of field-based phenotypic evaluation required in conventional breeding (Fig. 3A), thereby substantially accelerating the identification of isoplasmic restorer lines.

Discussion

MAS is a cornerstone in modern crop breeding. By enabling precise selection at the DNA level, MAS substantially shortens breeding cycles and improves both selection efficiency and accuracy (Guo et al. 2022; Zhang et al. 2025). In major crops such as sorghum (Klein et al. 2008), rice (Kumar et al. 2023), and maize (Karim et al. 2020), molecular markers linked to fertility restorer genes or key agronomic traits have been successfully developed and widely deployed, greatly facilitating breeding processes, including backcrossing and gene pyramiding (Klein et al. 2008; Karim et al. 2020). Among currently available genotyping platforms, KASP has emerged as a leading technology for MAS due to its high throughput, accuracy, cost-effectiveness, and robustness to variable DNA quality. These advantages have established KASP as a core platform for applied molecular breeding (Dipta et al. 2024). The effectiveness of KASP-based MAS has been demonstrated across a wide range of crops, including pepper (Du et al. 2019), maize (Shen et al. 2025), and rice (Ishwarya Lakshmi et al. 2022).

The purity of male-sterile lines, restorer lines, and their F_1 hybrids in CMS hybrid seed production systems is a critical determinant of yield and quality in hybrid rapeseed (Waza 2015). Conventional purity testing relies primarily on field-based fertility observation at flowering and morphological trait evaluation. Although intuitive and reliable, these methods are time-consuming, labor-intensive, and susceptible to environmental interference, and they provide limited capability for tracing the specific causes of purity decline (Dadlani et al. 2023). The development of molecular marker technologies has provided new avenues for rapid seed purity testing. However, single markers can only detect variation at either the cytoplasmic or nuclear level, and cannot simultaneously resolve the combinatorial relationship between cytoplasmic background and nuclear restorer gene status, thereby limiting their utility in diagnosing complex purity problems.

In this study, we combined a CMS cytoplasm-specific KASP marker with a KASP marker for the nuclear fertility restorer gene (*Rf*) to establish a multiplex marker system capable of simultaneously determining cytoplasmic type (S/N) and nuclear genotype (*RfRf*/*Rfrf*/*rfrf*). The key advantage of this system is that it enables the acquisition of complete genotypic information from a single DNA extraction and a single KASP reaction, allowing systematic tracing of the underlying causes of purity decline. For example, detection of the S(*Rfrf*) genotype in a male-sterile line indicates that, although the cytoplasm is of the sterile type, the nuclear genome carries a restorer allele-an aberrant genotype that can only arise from contamination by volunteer seedlings carrying the restorer gene. By contrast, the presence of the N(*rfrf*) genotype unambiguously points to maintainer line contamination. This genotype-to-phenotype reasoning ensures that each aberrant genotype can be assigned a unique biological explanation, thereby providing a precise basis for decision-making in field quality control.

In conventional three-line breeding systems, the identification of isoplasmic restorer lines relies on multigenerational phenotypic evaluations, often requiring at least three generations, which severely limits breeding efficiency (Fig. 3A). In contrast, MAS with tightly linked markers can effectively overcome this bottleneck. By simultaneously applying the CMS cytoplasm-specific KASP markers developed in this study in conjunction with existing KASP markers for nuclear *Rf* genes, we efficiently identified 24 isoplasmic restorer lines from a panel of 187 diverse germplasm accessions. Compared with conventional breeding approaches, this combined MAS strategy offers several clear advantages, including high precision through direct genotype identification, substantially reduced breeding cycles (shortened by more than one year), high-throughput capability, and the feasibility of early-generation selection. Collectively, these advantages greatly enhance both the efficiency and success rate of homozygous restorer line development in rapeseed breeding programs.

In conclusion, we developed four KASP markers targeting the mitochondrial sterility genes *orf224*/*atp6* (Pol CMS) and *orf138* (Ogu CMS) in rapeseed. These markers accurately discriminated sterile from fertile cytoplasms across 101 diverse germplasm accessions. By integrating these cytoplasmic markers with KASP markers for the nuclear fertility restorer genes (*Rfp* for Pol CMS and *Rfo* for Ogu CMS), we established a dual-marker system that simultaneously determines cytoplasmic type (S/N) and nuclear genotype (*RfRf*/*Rfrf*/*rfrf*). This system enables systematic tracing of purity decline causes-including maintainer line contamination, volunteer seedlings, foreign pollen contamination, paternal/maternal contamination, mechanical mixing, and seed adulteration-in a single KASP reaction, providing precise guidance for quality control in hybrid seed production. Furthermore, using this combined marker-assisted selection strategy, we efficiently identified 24 isoplasmic restorer lines from 187 germplasm accessions, substantially shortening the breeding cycle compared with conventional multigenerational phenotyping. The markers and the dual-marker strategy developed herein provide an efficient, accurate, and practical tool for parental line identification, purity assessment, and restorer line development in rapeseed CMS hybrid breeding programs.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have consented to the publication of this manuscript.

Competing interests

The authors declare no competing interests.

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Author Contribution

Tonghua Wang and Bao Li conceived and designed the study and supervised the entire research process. Xinhong Liu conducted the experiments and drafted the manuscript. Qian Yang, Yiming Guo, and Liang Qu participated in field cultivation of materials, sample collection, and purity identification. Xing Zhou and Yuqing Li performed data analysis and prepared figures. All authors read and approved the final manuscript.All authors reviewed the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Figures

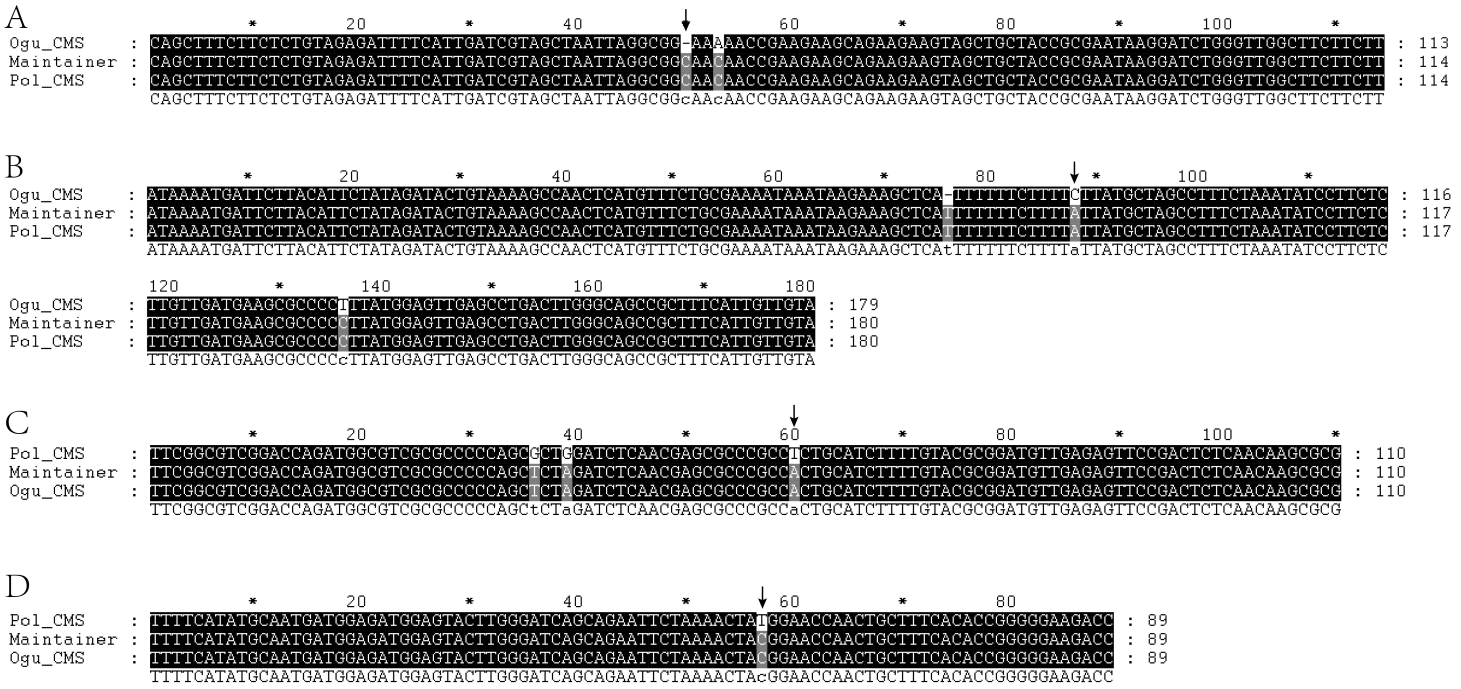


Figure 1

Diagnostic SNP markers for molecular identification of Ogu CMS and Pol CMS.

Arrows indicate diagnostic SNP alleles. Panels A-B represent Ogu CMS-specific markers: Ogu CMS carries - and C, which are distinct from Pol CMS and the maintainer (both carrying C and A). Panels C-D represent Pol CMS-specific markers: Pol CMS carries T and T, which are distinct from Ogu CMS and the maintainer (A and C).

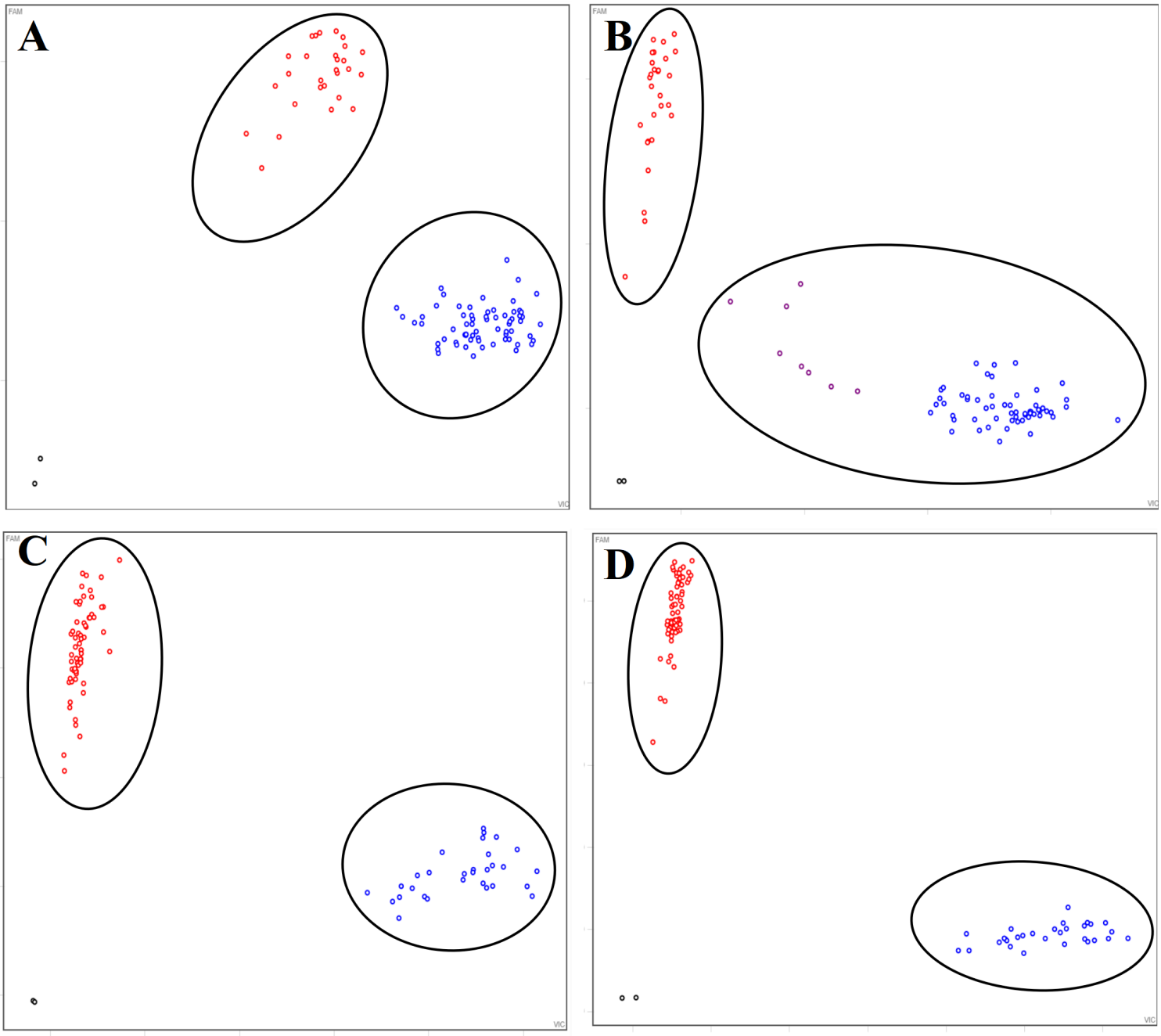


Figure 2

KASP genotyping of rapeseed materials using CMS-specific functional markers.

(A-B) Genotyping with markers BN9000344_K01 (A) and BN9000345_K01 (B) distinguished Polima sterile (T/T or T allele) from fertile (C/C or A allele) cytoplasms. (C-D) Genotyping with markers BN9000568_K01 (C) and BN9000570_K01 (D) distinguished Ogura sterile (lacking C or C/C) from fertile (C/C or A allele) cytoplasms, with undetected samples shown in gray for all markers.

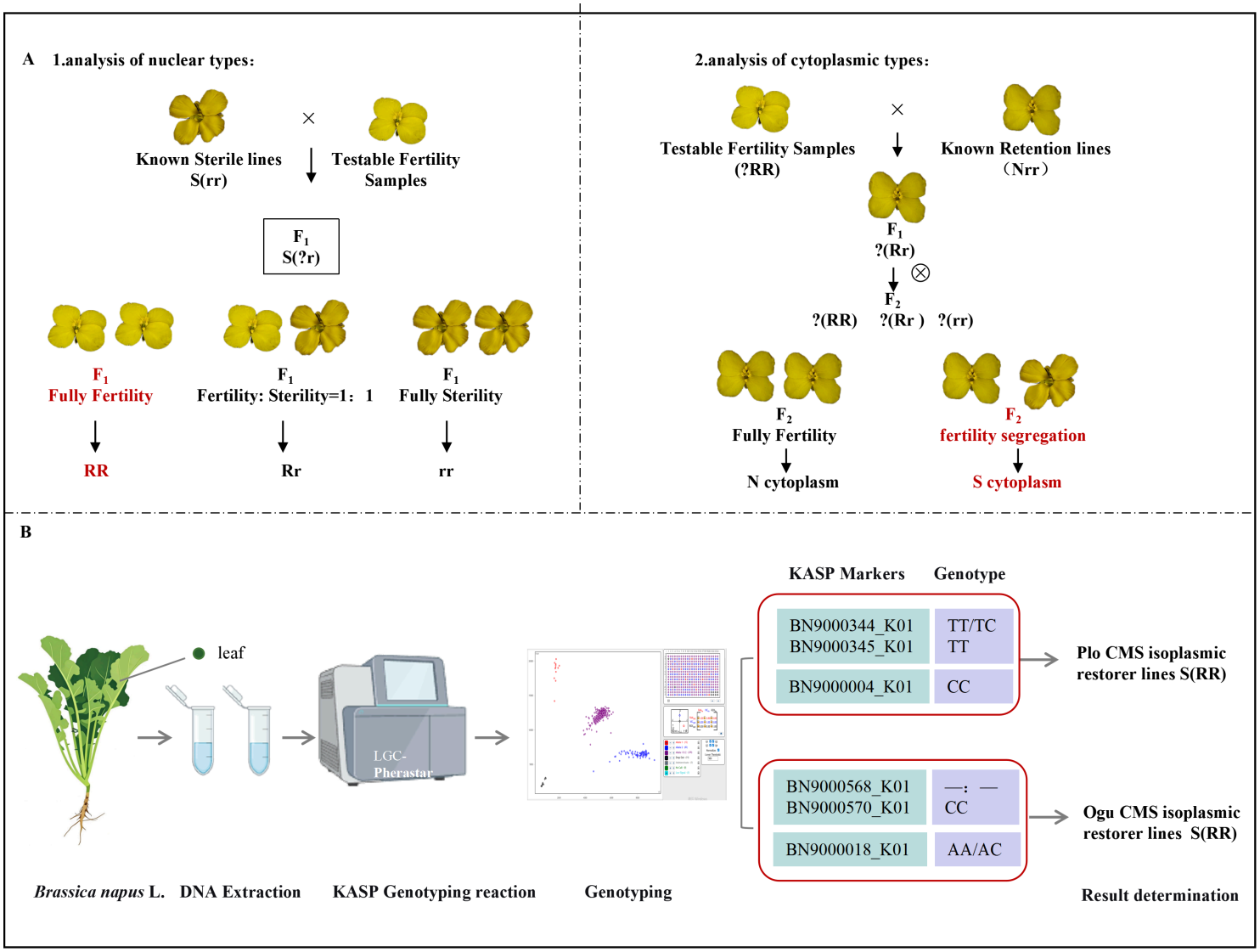


Figure 3

Schematic comparison of conventional and marker-assisted methods for identifying isoplasmic restorer lines in rapeseed

(A) Conventional phenotyping relies on multigenerational testcrosses and F_2 segregation analysis to evaluate cytoplasm type and *Rf* alleles, but is labor-intensive and time-consuming. (B) High-throughput MAS employs KASP markers for early-stage genotyping of cytoplasmic sterility and nuclear restorers. Pol CMS isoplasmic restorers are defined by BN9000344 (TT/TC), BN9000345 (TT), and BN9000004 (CC); Ogu CMS restorers by BN9000568 (−/−), BN9000570 (CC), and BN9000018 (AA/AC). This strategy enables rapid and precise early-generation selection.