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Marker-assisted identification and selection of fertility restorers for wild abortive cytoplasmic male sterility-based three-line hybrid rice breeding

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Abstract

Reliable identification and selection of effective fertility restorers are essential for broadening the male-parent pool in three-line hybrid rice breeding. In the present study, 100 advanced rice breeding lines representing public and private breeding programmes were screened for the major fertility-restoration loci *Rf3* and *Rf4* associated with the wild-abortive cytoplasmic male sterility (WA-CMS) system. Genomic DNA isolated from 20-day-old seedlings was analysed using the candidate-gene-specific markers RMS-SF21-5 for *Rf3* and RMS-PRR9-1 for *Rf4*. The diagnostic restorer alleles were 172 bp and 185 bp, respectively, whereas the corresponding non-restorer alleles were 127 bp and 175 bp. Of the 100 lines, 44 (44 %) carried the diagnostic *Rf3* allele and 35 (35 %) carried the diagnostic *Rf4* allele. Eighteen lines (18 %) were homozygous for diagnostic alleles at both loci, one line exhibited a heterozygous marker profile, 25 carried *Rf3* alone, 16 carried *Rf4* alone and 40 lacked both diagnostic alleles. Based on the presence of both diagnostic loci and superior agronomic performance, 12 lines were prioritised as elite putative restorers for hybridisation with WA-CMS lines. The selected lines were crossed with CMS-59A, CMS-64A and RMS-2A, generating 36 experimental hybrids for subsequent fertility restoration evaluation. The results demonstrate that marker-assisted screening can efficiently enrich breeding populations for putative two-locus restorers and reduce the number of genotypes requiring phenotypic testcross evaluation. However, marker presence alone does not establish complete fertility restoration; therefore, pollen and spikelet fertility assessment across representative WA-CMS backgrounds is required for confirmation. These findings provide a practical molecular pre-screening strategy for accelerating restorer identification and utilisation in three-line hybrid rice breeding.

Keywords: fertility restoration; hybrid rice; marker-assisted selection; restorer breeding; Rf3; Rf4; wild-abortive cytoplasmic male sterility

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Introduction

Hybrid rice technology exploits heterosis to increase productivity and has become an important component of rice improvement programmes. The three-line system uses a cytoplasmic male-sterile (A) line, its isonuclear maintainer (B) line and a genetically diverse fertility restorer (R) line. Among the cytoplasmic male-sterility systems available in rice, the wild-abortive (WA) system is the most extensively deployed in indica hybrid breeding (1–3).

Fertility restoration of wild-abortive cytoplasmic male sterility (WA-CMS) is primarily governed by two dominant nuclear loci, fertility-restorer gene 3 (*Rf3*) on chromosome 1 and fertility-restorer gene 4 (*Rf4*) on chromosome 10, although additional modifiers and genotype-dependent interactions have been reported (4–6). The *Rf* genes largely encode or are associated with pentatricopeptide-repeat proteins that suppress or process aberrant mitochondrial transcripts responsible for cytoplasmic male sterility (7, 8). The

combined presence of *Rf3* and *Rf4* generally provides stronger and more stable restoration than either locus alone.

Conventional identification of restorers requires crossing each candidate male with one or more CMS lines, followed by pollen- and spikelet-fertility assessment. This procedure is reliable but expensive, season-dependent and time-consuming. Molecular markers linked to *Rf3* and *Rf4* permit early-generation screening independent of flowering and can therefore accelerate restorer-line development (6, 9, 10). Candidate-gene-specific markers have further improved diagnostic resolution by targeting polymorphisms within or close to functional regions (11, 12).

Despite the availability of molecular markers for major fertility-restoration loci, the identification of effective restorer lines remains dependent on the interaction between nuclear genotype and the cytoplasmic background of the female parent. Therefore, marker-assisted screening should be considered an early-generation selection tool rather than a substitute for phenotypic confirmation. Identification of lines carrying both *Rf3* and *Rf4* can nevertheless provide a rational basis for prioritising candidate male parents for subsequent testcrossing and evaluation of pollen and spikelet fertility across different WA-CMS backgrounds.

The present investigation screened a diverse set of 100 advanced rice breeding lines with markers diagnostic for *Rf3* and *Rf4*. The objectives were to (i) determine the frequency and distribution of the two restoration loci, (ii) identify lines possessing both genes and (iii) select agronomically useful putative restorers for subsequent hybridisation with WA-CMS lines.

Materials and Methods

Plant materials and experimental site

A set of 100 advanced breeding lines was assembled from the ICAR-Indian Institute of Rice Research, Hyderabad; the Rice Research Unit, Agricultural Research Institute, Professor Jayashankar Telangana Agricultural University, Rajendranagar; and selected private-sector breeding programmes. The material included advanced selections, breeding lines, maintainer lines and commercial restorer candidates. Plants were raised at the Rice Research Unit, Rajendranagar, Hyderabad (17°19′ N, 78°23′ E; 542 m above mean sea level) in vertisols. Twenty-day-old seedlings were used for molecular analysis. The complete germplasm list and source are provided in Supplementary Table 1.

Genomic DNA isolation and quantification

Young leaf tissue was collected from 20-day-old seedlings and stored at -20 °C. Genomic DNA was isolated using a cetyl trimethyl ammonium bromide (CTAB) method. Approximately 700 µL of extraction buffer was used per sample, followed by chloroform : isoamyl alcohol (24 : 1) purification, isopropanol precipitation, a 70 % ethanol wash and resuspension in TE buffer. DNA purity was evaluated spectrophotometrically from the A260/A280 ratio; samples with ratios of 1.8–2.0 were adjusted to 40 ng/µL. DNA integrity was additionally checked on 0.8 % agarose gels.

Marker assay and polymerase chain reaction amplification

The marker RMS-SF21-5 was used to assay *Rf3* and RMS-PRR9-1 to assay *Rf4*. KMR-3R was included as the positive restorer control and APMS-6B as the negative maintainer control. Primer sequences, chromosome locations and diagnostic allele sizes are presented in Table 1. Each 10 µL PCR contained 4.0 µL 4× amplicon master mix, 1.0 µL each of 10 µM forward and reverse primers, 1.0 µL template DNA (40 ng/µL) and 3.0 µL sterile water. Polymerase chain reaction (PCR) amplification was performed with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing for 30 s at 60.5 °C for RMS-SF21-5 (*Rf3*) and 55 °C for RMS-PRR9-1 (*Rf4*), extension at 72 °C for 1 min, a final extension at 72 °C for 5 min and a final hold at 4 °C.

Electrophoresis and allele scoring

The PCR products were resolved on 3.0 % agarose gels prepared in 1× TBE (Tris-borate-ethylenediaminetetraacetic acid) and stained with ethidium bromide. A 100-bp DNA ladder was used for fragment-size estimation. Electrophoresis was performed at 150 V for 10 min followed by 75 V for 2–3 hr and gels were documented using a gel-imaging system. A genotype was scored as carrying a target restorer locus when its amplicon co-migrated with the diagnostic restorer allele of KMR-3R. Lines were classified into four mutually exclusive groups: *Rf3* only, *Rf4* only, *Rf3* + *Rf4*, or neither locus.

Selection of elite putative restorers

Lines carrying both diagnostic loci were further considered together with their field performance for yield and key agronomic characters. Twelve lines with the two-locus genotype and superior *per se* performance were selected for use as testers in a line × tester crossing programme with the WA-CMS lines CMS-59A, CMS-64A and RMS-2A. The 36 resulting testcross hybrids were generated for subsequent pollen-fertility assessment using I2-KI staining and spikelet-fertility evaluation. Phenotypic restoration data were not included in the present marker-screening study; therefore, all selected lines are described as putative restorers until testcross confirmation is completed. The complete marker-assisted selection and utilisation pipeline is summarised in the workflow.

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Table 1. Molecular markers used for identification of *Rf3* and *Rf4* fertility-restoration loci

Marker	Target locus	Chromosome	Forward primer (5′-3′)	Reverse primer (5′-3′)	Restorer allele (bp)	Non-restorer allele (bp)
RMS-SF21-5	*Rf3*	1	GAGTTGGGGGTCGAGAAATC	CGTACGTGCGGCTAGGATCAA	172	127
RMS-PRR9-1	*Rf4*	10	GAGTTTTGAATAGATTTACGT GTGGA	AGTGTCCAGATTCGTAGTAATG C	185	175

Rf3: fertility-restorer gene 3; *Rf4*: fertility-restorer gene 4.

Results

Marker polymorphism and locus frequencies

Both marker systems differentiated the positive restorer control KMR-3R from the negative maintainer control APMS-6B. RMS-SF21-5 produced the 172-bp restorer-associated allele at the *Rf3* locus, whereas RMS-PRR9-1 produced the 185-bp restorer-associated allele at the *Rf4* locus. The corresponding non-restorer alleles were 127 and 175 bp, respectively (Fig. 1 and 2). Of the 100 advanced breeding lines screened, 44 (44 %) carried the diagnostic *Rf3* allele and 35 (35 %) carried the diagnostic *Rf4* allele. Eighteen lines were homozygous for the diagnostic alleles at both loci, while RNR-44242 exhibited a heterozygous marker profile. Twenty-five lines carried *Rf3* alone, 16 carried *Rf4* alone and 40 lacked both diagnostic alleles (Table 2). The classification and identity of the advanced breeding lines within each *Rf3* and *Rf4* marker class are presented in Table 3. Thus, 60 % of the evaluated material carried at least one major diagnostic restoration locus, while homozygous two-locus putative restorers occurred at a frequency of 18 %. The distribution of mutually exclusive marker classes and the overlap between the two restoration loci are illustrated in Fig. 3 and 4. The complete marker-assisted selection and utilisation pipeline is summarised in Fig. 5. For the five mutually exclusive marker classes, the corresponding 95 % Wilson confidence intervals for the observed frequencies were 17.5–34.3 % (*Rf3* only), 10.1–24.4 % (*Rf4* only), 11.7–26.7 % (*Rf3* + *Rf4*), 0.2–5.4 % (heterozygous) and 30.9–49.8 % (neither locus), respectively.

Elite two-locus putative restorers selected for hybridisation

Eighteen genotypes were homozygous for the diagnostic alleles at both restoration loci, while RNR-44242 exhibited a heterozygous marker profile. Twelve of the homozygous two-locus lines, namely SN-2071, SN-2183, SN-2696, SN-2705, SN-2717, SN-2743, SN-2744, SN-2754, SN-2756, SN-2769, SN-2775 and SN-2784, also showed superior agronomic performance and were selected as elite putative restorers (Table 4). All 12 originated from the Rice Research Unit, Rajendranagar and were subsequently crossed with CMS-59A, CMS-64A and RMS-2A to generate 36 experimental hybrids for subsequent phenotypic restoration testing.

Discussion

The frequency of marker-positive material observed here indicates that major WA-CMS restoration alleles were represented in the evaluated germplasm, although their distribution was uneven. The diagnostic *Rf3* allele was detected in 44 % of the lines, whereas the diagnostic *Rf4* allele was detected in 35 % of the lines and 18 % of the entries were homozygous for the diagnostic alleles at both loci.

Variation in the distribution of fertility-restoration alleles among rice breeding materials has also been reported in earlier studies. Previous studies demonstrated the utility of a two-gene marker-assisted selection system for identifying fertility-restoration genes in rice (3), while another researcher reported variation in the distribution and effects of *Rf3* and *Rf4* among diverse genetic materials (6). Differences among studies may arise from variation in germplasm composition, genetic background, breeding history, geographical origin and the relative contribution of individual restoration loci in different populations. Differences in marker systems and criteria used to classify marker-positive genotypes may also contribute to variation in the observed frequencies. Therefore, the marker frequencies obtained in the present study should be interpreted in relation to the specific breeding population evaluated rather than as estimates of the frequency of restoration genes in rice. In addition to this, earlier studies have also reported marker-based screening and genetic mapping of fertility-restoration loci in WA-CMS rice (13–15).

The two-locus class is particularly valuable for hybrid rice breeding. Fine-mapping studies have shown that the *Rf3* and *Rf4* regions jointly account for a large proportion of variation in fertility restoration and may interact epistatically. *Rf4* is frequently reported as a major restoration locus, whereas *Rf3* may enhance restoration or contribute strongly in genetic backgrounds. Consequently, simultaneous selection for both genes provides a rational strategy for prioritising candidate male parents, while recognising that nuclear background and cytoplasmic source can modify the final fertility-restoration phenotype.

Implications of marker-negative lines

The 40 lines that lacked both diagnostic *Rf3* and *Rf4* alleles should not automatically be designated as maintainers or absolute non-restorers. The absence of the diagnostic alleles indicates only that the marker-associated alleles detected by RMS-SF21-5

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and RMS-PRR9-1 were not present in these genotypes. Some of these lines may possess alternative fertility-restoration loci or allelic variants that are not detected by the two marker assays. Conversely, the absence of both marker alleles may indicate a reduced probability of restoring fertility through the two major loci assessed in the present study. Therefore, marker-negative lines possessing desirable agronomic or breeding characteristics should be evaluated through appropriate testcrosses rather than being excluded solely based on these two markers.

Table 2. Distribution of *Rf3* and *Rf4* marker classes among 100 advanced breeding lines

Marker class	Number of lines	Frequency (%)	Breeding interpretation
Rf3 only	25	25	Putative single-locus restorers
Rf4 only	16	16	Putative single-locus restorers
Rf3 + *Rf4*	18	18	Homozygous two-locus putative restorers
Heterozygous	1	1	Heterozygous marker profile (RNR-44242)
Neither locus	40	40	No diagnostic allele detected; restoration cannot be inferred from these markers
Marker-positive genotypes	60	60	At least one diagnostic restorer allele detected

Rf3: fertility-restorer gene 3; *Rf4*: fertility-restorer gene 4.

Table 3. Classification of advanced breeding lines based on diagnostic alleles at Rf3 and Rf4

Marker class	number	Breeding lines
Rf3 only	25	DUBRAJ Sel-1, TCDM-1, DHANGURI, HUR-917, HUR-105, JAWAPHOOL MUTANT, CR SUGANDHAN-907, SAMUND CHINI, RNR-44035, RNR-44045, RNR-44079, RNR-44089, RNR-44153, RNR-44163, RNR-44187, RNR-44194, RNR-31622, RNR-44229, RNR-44232, RNR-44233, RNR-44235, RNR-52253, RNR-52259, Yamuna and AMAR-88 (JSR-seg).
Rf4 only	16	NLR-40054, RNR-43965, RNR-44068, RNR-44175, RNR-52137, RNR-52258, RNR-52270, RNR-52274, CMS-52-B, CMS-59-B, RMS-1-B, RMS-7-B, SN-2160, SN-2281, Annapurna and Poornima.
Rf3 + *Rf4*	18	SN-2071, SN-2183, SN-2696, SN-2705, SN-2717, RNR-44039, SN-2743, SN-2744, SN-2754, SN-2756, RNR-52157, RNR-52240, SN-2769, SN-2775, SN-2784, Kanakabhushitha (HMT), Chandni and VAPL-678.
Heterozygous	1	RNR-44242
Neither	40	VISHNU BHOG Sel-1, BADHSH Bhog sel-1, BRRI DHAN-75, GAR-1, GR-101, GAR-14, TULASI MANJARI, HUR-1309, HUR 4-3, TARUN BHOG, RNR-44076, RNR-44082, RNR-44158, RNR-44189, RNR-44191, RNR-44222, RNR-44246, RNR-44294, RNR-44252, RNR-44257, RNR-44259, RNR-44265, RNR-52223, RNR-52226, RNR-52237, RNR-52246, RNR-52250, RNR-52140, RNR-52145, RNR-52197, RNR-52200, RNR-52241, RNR-52245, RNR-52254, RNR-52261, CMS-64-B, RMS-2-B, OM-1515 (Super Aman segment), VAPL-666 and Rudra P-205.

Rf3: fertility-restorer gene 3; *Rf4*: fertility-restorer gene 4.

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Table 4. Elite two-locus putative restorers selected for the hybridisation programme

Sl. No.	Selected line	*Rf3*	*Rf4*	Proposed use
1	SN-2071	Present	Present	Tester with three WA-CMS lines
2	SN-2183	Present	Present	Tester with three WA-CMS lines
3	SN-2696	Present	Present	Tester with three WA-CMS lines
4	SN-2705	Present	Present	Tester with three WA-CMS lines
5	SN-2717	Present	Present	Tester with three WA-CMS lines
6	SN-2743	Present	Present	Tester with three WA-CMS lines
7	SN-2744	Present	Present	Tester with three WA-CMS lines
8	SN-2754	Present	Present	Tester with three WA-CMS lines
9	SN-2756	Present	Present	Tester with three WA-CMS lines
10	SN-2769	Present	Present	Tester with three WA-CMS lines
11	SN-2775	Present	Present	Tester with three WA-CMS lines
12	SN-2784	Present	Present	Tester with three WA-CMS lines

Rf3: fertility-restorer gene 3; *Rf4*: fertility-restorer gene 4; WA-CMS: wild-abortive cytoplasmic male sterility.

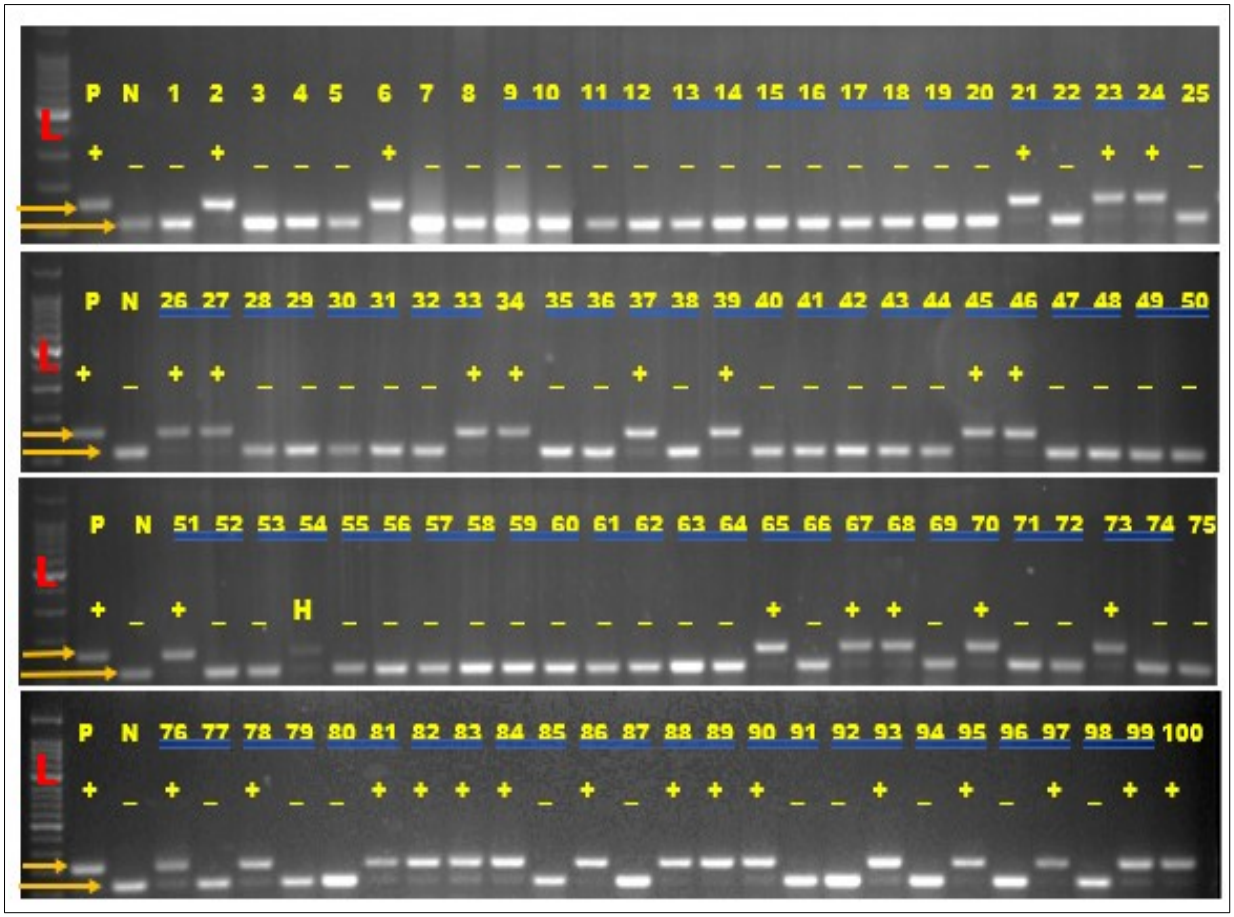


Fig. 1. Agarose gel electrophoresis profile generated with the *Rf4* candidate-gene-specific marker RMS-PRR9-1 among 100 advanced rice breeding lines. The four panels, from top to bottom, show breeding-line lanes 1–25, 26–50, 51–75 and 76–100, respectively; each panel begins with L, P and N. L, 100-bp DNA ladder; P, positive control KMR-3R (185-bp restorer allele); N, negative control APMS-6B (175-bp non-restorer allele); +, restorer-associated allele present; -, restorer-associated allele absent; H, heterozygous profile. Lane numbers correspond to the genotype list provided in Supplementary Table 1.

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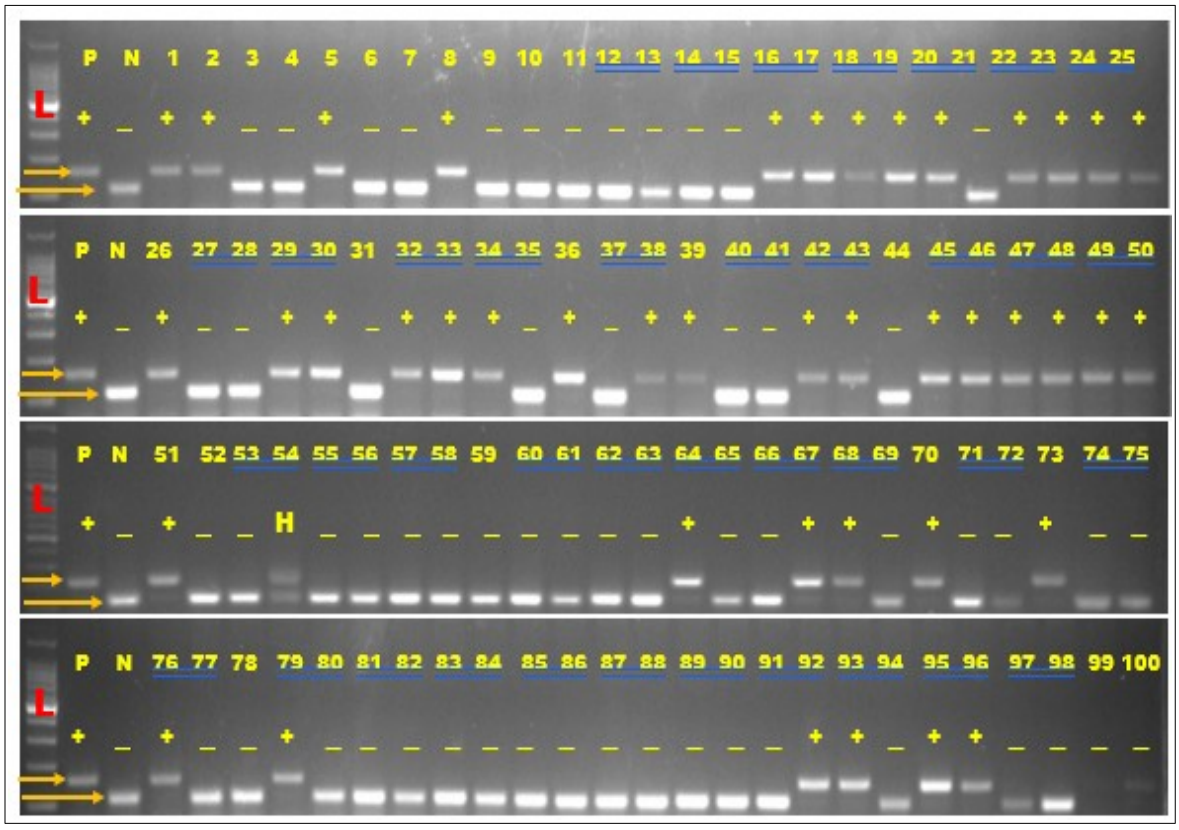


Fig. 2. Agarose gel electrophoresis profile generated with the *Rf3* candidate-gene-specific marker RMS-SF21-5 among 100 advanced rice breeding lines. The four panels, from top to bottom, show breeding-line lanes 1–25, 26–50, 51–75 and 76–100, respectively; each panel begins with L, P and N. L, 100-bp DNA ladder; P : positive control KMR-3R (172-bp restorer allele); N: negative control APMS-6B (127-bp non-restorer allele); +, restorer-associated allele present; -: restorer-associated allele absent; H: heterozygous profile. Lane numbers correspond to the genotype list provided in Supplementary Table 1.

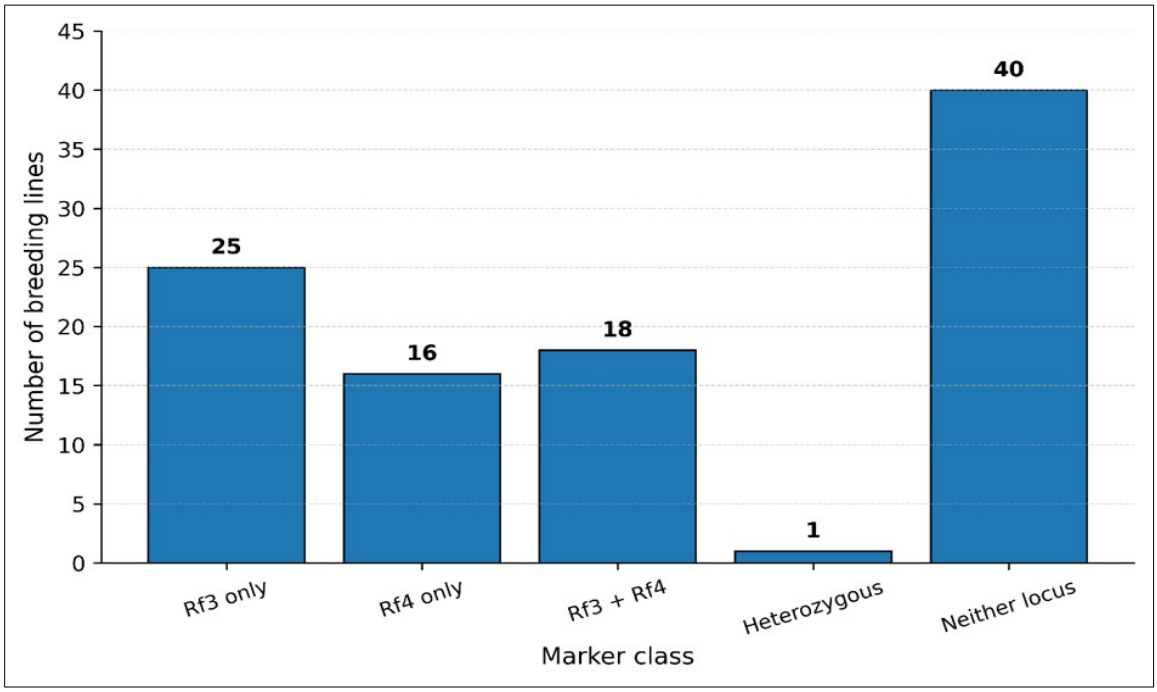


Fig. 3. Distribution of marker classes among 100 advanced rice breeding lines based on diagnostic markers for Rf3 and Rf4.

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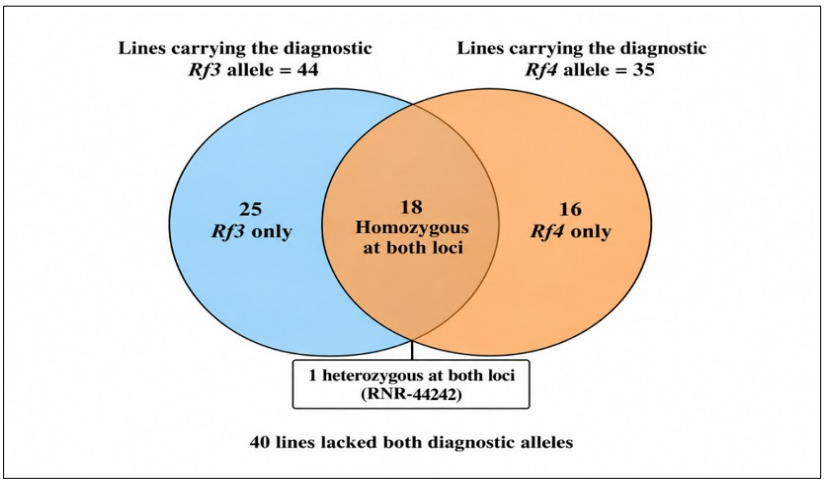


Fig. 4. Venn diagram showing the distribution and overlap of diagnostic *Rf3* and *Rf4* alleles among 100 advanced rice breeding lines. The non-overlapping sectors represent *Rf3*-only and *Rf4*-only lines, the intersection represents lines homozygous for diagnostic alleles at both loci and the boxed entry denotes the heterozygous profile of RNR-44242; 40 lines lacked both diagnostic alleles.

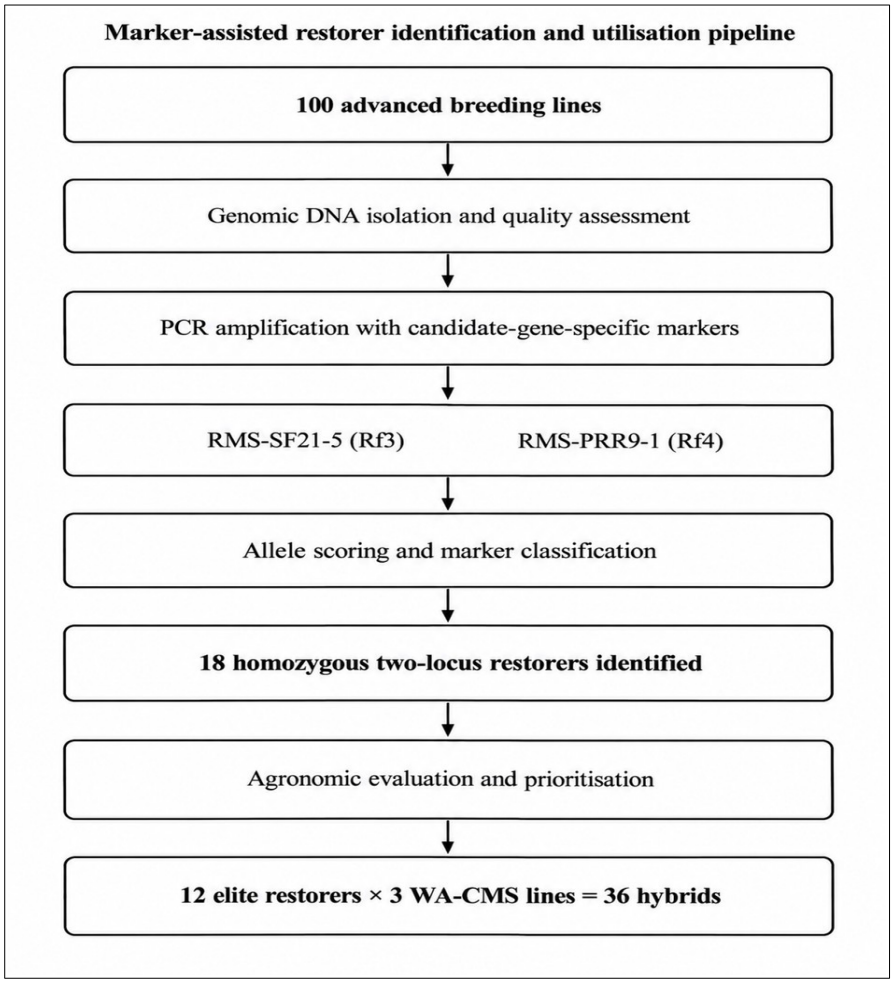


Fig. 5. Marker-assisted workflow used to identify and prioritise putative fertility restorers carrying *Rf3* and *Rf4* for development of experimental wild-abortive cytoplasmic male sterility (WA-CMS) rice hybrids.

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Limitations of the marker assays

RMS-SF21-5 and RMS-PRR9-1 generated clearly distinguishable allele-size differences and were suitable for rapid screening of the evaluated breeding material. Candidate-gene-specific and closely linked markers for *Rf3* and *Rf4* have been developed and validated for improving the efficiency of restorer identification. Nevertheless, diagnostic efficiency is not necessarily absolute across unrelated germplasm because recombination between a marker and the functional gene, allelic heterogeneity, additional restorer genes and cytoplasm nuclear interactions can cause disagreement between marker genotype and fertility phenotype.

The present study did not estimate the recombination frequency between RMS-SF21-5 or RMS-PRR9-1 and the corresponding functional genes. Therefore, the marker results should be interpreted as a molecular pre-screening criterion rather than definitive evidence of fertility restoration. Even lines carrying diagnostic alleles at both loci should be confirmed through appropriate testcrosses and pollen- and spikelet-fertility evaluation across representative WA-CMS backgrounds.

Breeding implications

The 12 selected two-locus lines provide a useful set of candidate male parents for subsequent WA-CMS hybrid breeding. Their selection combined the presence of diagnostic alleles at both *Rf3* and *Rf4* with superior agronomic performance. The selected lines were crossed with CMS-59A, CMS-64A and RMS-2A, generating 36 experimental hybrids for subsequent phenotypic evaluation.

The next step is to evaluate the resulting testcross hybrids for pollen fertility using I2-KI staining and for spikelet fertility under field conditions. Evaluation using multiple WA-CMS lines is important because fertility restoration may vary with the nuclear background of the male parent and the cytoplasmic background of the CMS line. Hybrids showing adequate and stable fertility restoration can subsequently be evaluated for combining ability, heterosis, agronomic performance, grain yield and grain quality across representative environments. Thus, the marker-assisted approach provides an initial molecular screening stage, followed by phenotypic confirmation and subsequent evaluation of promising hybrids.

Implications of the heterozygous line RNR-44242

RNR-44242 exhibited a heterozygous marker profile at the assayed restoration loci. Because the diagnostic alleles were not fixed, this genotype should not be considered a fixed two-locus putative restorer. However, RNR-44242 may have potential breeding value for further selection. Selfing followed by progeny genotyping could be used to identify homozygous progenies carrying the desired diagnostic alleles. Such progenies should subsequently be evaluated through testcrosses with appropriate WA-CMS lines to determine their actual fertility-restoration ability. Therefore, RNR-44242 may be considered a potential source for further allele fixation and selection rather than an immediately deployable restorer line.

Overall, the results demonstrate that molecular screening with RMS-SF21-5 and RMS-PRR9-1 can efficiently enrich breeding populations for candidate two-locus restorers. However, marker genotypes should be considered a pre-selection criterion and final designation as an effective fertility restorer should be based on phenotypic confirmation through appropriate testcrosses.

Conclusion

Marker-assisted screening of 100 advanced rice breeding lines identified 44 lines carrying the diagnostic fertility-restorer gene 3 (*Rf3*) allele and 35 lines carrying the diagnostic fertility-restorer gene 4 (*Rf4*) allele, while 18 lines were homozygous for the diagnostic alleles at both major wild abortive cytoplasmic male sterility (WA-CMS) restoration loci. RNR-44242 exhibited a heterozygous marker profile, whereas 40 lines lacked both diagnostic alleles. Based on the simultaneous presence of diagnostic *Rf3* and *Rf4* alleles and superior agronomic performance, 12 lines were prioritised as elite putative restorers for subsequent hybridisation with WA-CMS lines. The RMS-SF21-5 and RMS-PRR9-1 marker combination provides an efficient first-stage molecular screening approach for enriching breeding populations for candidate two-locus restorers and reducing the number of genotypes requiring intensive testcross evaluation. However, the presence of diagnostic marker alleles should not be interpreted as definitive evidence of complete fertility restoration because marker–phenotype associations may vary with genetic background and CMS cytoplasm. Future research should focus on confirming the fertility-restoration ability of the selected putative restorers through pollen and spikelet-fertility evaluation of testcrosses involving diverse WA-CMS backgrounds, followed by evaluation of promising hybrids for combining ability, heterosis, agronomic performance, grain yield, grain quality and stability across representative environments. Such integrated molecular and phenotypic validation will facilitate the reliable identification and utilisation of effective fertility restorers in three-line hybrid rice breeding. The pollen and spikelet-fertility data from the 36 testcross hybrids will be reported in a companion study to provide phenotypic validation of the marker-based predictions.

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Authors' contributions

BS conducted the field and laboratory experiments, performed the molecular marker analysis, analysed and interpreted the data and drafted the manuscript. CDR conceived and supervised the study and provided guidance throughout the research and manuscript preparation. BLP provided technical assistance and guidance in molecular marker analysis. CSR assisted with data analysis and interpretation and contributed to manuscript preparation. YCM provided guidance on experimental planning, data interpretation and manuscript preparation. AK provided guidance and inputs related to grain quality analysis and manuscript preparation. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

Authors do not have any conflict of interests to declare.

Ethical issues

None

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