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Identification of stable restorer lines developed through inter-sub-specific hybridization in rice (*Oryza sativa*) using multi-trait stability index

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Abstract

Inter-sub-specific hybridization between *indica* and tropical *japonica* rice germplasm is the most efficient approach for broadening the genetic base of hybrid rice parental lines and enhancing the heterotic potential of hybrids in a tropical country like India. In the present study, 106 *indica*/tropical–*japonica* derived lines were developed through inter-sub-specific hybridization and screened using functional markers with respect to the major fertility restorer genes, *Rf3* and *Rf4*, and the wide compatibility gene, *S5n*. The fertility restoration ability of newly developed restorers was validated through test cross nursery performance. The functional markers were observed to accurately predict the trait of fertility restoration. From the present study, the decreasing order of efficiency of different gene combinations on fertility restoration ability are as follows: *Rf4/S5n* > *Rf4/Rf3/S5n* > *Rf4* > *Rf4/Rf3* > *Rf3/S5n* > *Rf3*. Based on multi-trait stability index (MTSI) analysis, the four newly identified restorers, that is, RP6388-90, RP6382-49, RP6375-81 and RP6368-38, were selected as the top best performing genotypes with high stability for multiple traits, and these genotypes will be useful for the development of superior rice hybrids in India.

KEYWORDS
fertility restoration, hybrid rice, *indicas*, parental lines, stability analysis, tropical *japonicas*

1 | INTRODUCTION

To achieve sustainability in hybrid rice technology, diversifying Cytoplasmic Male Sterility (CMS) sources and broadening the genetic base of hybrid rice parental lines is the key step. The major constraint to breeding heterotic hybrids in India is the availability of a limited number of parental lines, which have a narrow genetic base. To realize greater heterosis, future hybrid rice research should aim to develop a large number of genetically diverse parental lines as suggested in earlier studies (Brar & Khush, 2017; Rajendran et al., 2012; Sruthi

et al., 2020). The level of heterosis in rice has the following order: *Indica* × *Japonica*> *Indica* × *Javanica*> *Japonica* × *Javanica*> *Indica* × *Indica* > *Japonica* × *Japonica* (Yuan, 1994; Zeng et al., 1997; Zhang et al., 1997). Inter-sub-specific hybridization between *indica* and *japonica* subspecies is one of the best breeding strategies for achieving greater heterosis. Although their theoretical yield may be 30% more than *indica* /*indica* hybrids, but the practical exploitation of greater heterosis is limited by the constraints like hybrid sterility, poor adaptability and tall stature of derived hybrids, which leads to lodging susceptibility and also poor grain quality (Dan et al., 2014; Khush & Aquino, 1994; Yuan, 1994; Zhang, 2020). Instead of temperate *japonicas*, tropical *japonicas* suited for tropical ecology as restorers for wild abortive (WA)-based *indica* CMS lines were advocated for increased heterosis (Virmani et al., 1997). Tropical *japonicas*/*javanicas*/bulu rice are excellent sources for broadening the genetic base of hybrid rice parental lines suited for the tropics as they are quite diverse from the *indica* parental lines used in several countries (Garris et al., 2005; Glaszmann, 1987; Oka, 1958). Tropical *japonicas* cannot be used directly as pollen parents in the hybrid breeding programme as the restorer frequency among them is much less (Tai, 1995).

In the process of development of a stable heterotic hybrid, the primary role of a hybrid breeder is to breed and identify stable superior parental lines. Proper understanding of genotype by environment interaction provides an opportunity for planning breeding programmes and increases the efficiency of utilization of limited resources (Magari et al., 1996). Taking these points into consideration the present study was undertaken with major objectives as follows: (i) to identify potential restorer lines with new diversified genetic background from 106 *indica*/tropical *japonica* derived lines through marker-assisted selection and their validation and (ii) identify best performing stable lines for multiple traits through multi-trait stability index (MTSI) analysis.

2 | MATERIALS AND METHODS

2.1 | Plant materials

A total of 141 rice (*Oryza sativa* L.) lines consisting of 106 *indica*/tropical *japonica* derived lines, 16 female and 19 male parental lines were analysed for the presence of restorer genes, that is, *Rf3*, *Rf4*, and wide compatibility gene *S5n* using reported markers. These 106 *indica*/tropical *japonica* derived lines were developed by crossing potential *indica* hybrid parental lines with tropical *japonica* (TJ) germplasm. These 106 genotypes were developed from 32 different crosses. Out of 32 crosses, 25 crosses with 13 promising restorers (R lines) (13 R lines × TJ lines) and seven crosses with six potential maintainers (B lines) (6 B lines × TJ lines). From 25 crosses with restorers, 83 *indica*/tropical *japonica* derived lines were developed. From seven crosses with maintainers, 23 lines were developed. These 23 maintainer's derived lines developed from six potential maintainers, that is, APMS6B, IR79156B, DRR4B, DRR9B, IR58025B and CRMS32B. Segregating material was handled by the pedigree method of breeding. A set of 106 promising genotypes was developed by selecting important traits like *indica* plant type with medium plant height, *indica* grain type and strong culm (tropical *japonica* characteristic).

2.2 | Molecular screening of *indica*/tropical *japonica* derived lines for the presence of fertility restorer genes (*Rf3* and *Rf4*) and wide compatible gene (*S5n*)

Seeds of 141 lines were sown in Petri plates and leaf samples were collected from 14-day old seedlings for DNA isolation. The genomic DNA was isolated from these lines using the method followed by Rajendrakumar et al. (2007). For molecular screening, candidate gene-specific markers, namely, RMS-PRR9-1 (Pranathi et al., 2016) on chromosome 10 for *Rf4*, RMS-SF21-5 (Pranathi et al., 2016) on chromosome 1 for *Rf3* and *S5n* INDEL (Sundaram et al., 2010) on chromosome 6 for *S5n* were used. For polymerase chain reaction (PCR) analysis, master mix preparation and thermal profile followed as given in Sruthi et al. (2020).

2.3 | Validation of fertility restoration ability

Based on molecular screening, 54 genotypes with various allelic combinations of *Rf3*, *Rf4* and *S5n* were selected and crossed with four popularly used WA-CMS lines, namely, IR68897A, IR79156A, APMS6A and PUSA5A, during the wet season, 2018. For the four female lines (IR68897A, IR79156A, APMS6A and PUSA5A), pollen sterility studies were carried out before test crossing, and 100% sterile lines only were used for crossing purpose. The resulting F_1 hybrids were grown in a test cross nursery during the dry season of 2018–2019. F_1 hybrids were analysed for pollen and spikelet fertility per cent. Pollen and spikelet fertility study was carried out as given in Kumar et al. (2017).

2.4 | Stability analysis

The identified 31 new restorer lines were grown in three different seasons, that is, wet season 2018 (E1/*Kharif*), dry season 2018–2019 (E2/*Rabi*) and wet season 2019 (E3/*Kharif*) to identify stable restorers. In each row, 25 hills were transplanted with a spacing of 20 × 15 cm. Recommended agronomic practices were followed to grow a healthy crop. A randomized complete block design was followed with two replications. Two popular restorer lines RPHR1005 and KMR3R were used as checks. The data were collected on 11 yield attributing traits for five plants per genotype using the field book app on android (Rife & Poland, 2014), namely, days to 50% flowering (DFF), plant height (PH), panicle length (PL), productive tillers (PT), filled grains (FG), unfilled grains (UFG), spikelet fertility per cent (SF%), thousand-grain weight (TGW), single plant yield (SPY), biological yield (BY) and harvest index (HI) recorded as per prescribed protocols (SES;

IRRI, 2013) for three seasons at the research farm of ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad (17° 19′N and 78° 29′) at an altitude of 549 m above mean sea level. Each season is considered as one environment (E). MTSI has been calculated based on weighted average absolute scores (WAASB). WAASB index values for each trait are computed by the singular value decomposition of the best linear unbiased predictions matrix for the GE effects produced by the linear mixed-effect model (LMM) (Olivoto et al., 2019). WAASB scores are estimated as follows:

$$\mathrm{WAASBi} = \sum_{k=1}^{p} |\mathrm{IPCA}ik \times \mathrm{EP}k| / \sum_{K=1}^{P} \mathrm{EP}k$$

WAASB*i* is the weighted average of absolute scores of the *i*th genotype, IPCA*ik* is the score of the *i*th genotype in the *k*th interaction principal component axis (IPCA), and EP*k* is the amount of the variance explained by the *k*th IPCA. MTSI has been calculated using the WAASBY index through 'metan' package in R (R Core Team, 2020) by following Equation (2). WAASBY index considers weightage between mean performance (Y) and stability (WAASB). WAASBY index has been calculated as follows (Equation 1):

$$WAASBYi = \frac{(rYi \times \theta Y) + (rWi \times \theta s)}{\theta Y + \theta s} \quad (1)$$

where WAASYi is the simultaneous selection index for the *i*th genotype and rYi and rWi are the rescaled values (0–100) for the response variable (y) and the stability (WAAS or WAASB), respectively; θY and θW are the weights for mean performance and stability, respectively:

$$\mathrm{MTSI_i} = \left[\Sigma^{f}_{j=1} \left(F_{ij} - F_j\right)^2\right]^{0.5} \quad (2)$$

MTSI is the multi-trait stability index for the *i*th genotype, *Fij* is the *j*th score of the *i*th genotype, and *Fj* is the *j*th score of the ideotype. The genotype with the lowest MTSI is then closer to the ideotype and therefore presents a high mean performance and stability for all the traits.

3 | RESULTS

In the present study, 106 *indica*/tropical *japonica* derived lines were screened for the fertility restorer genes, *Rf3* and *Rf4*, and the major

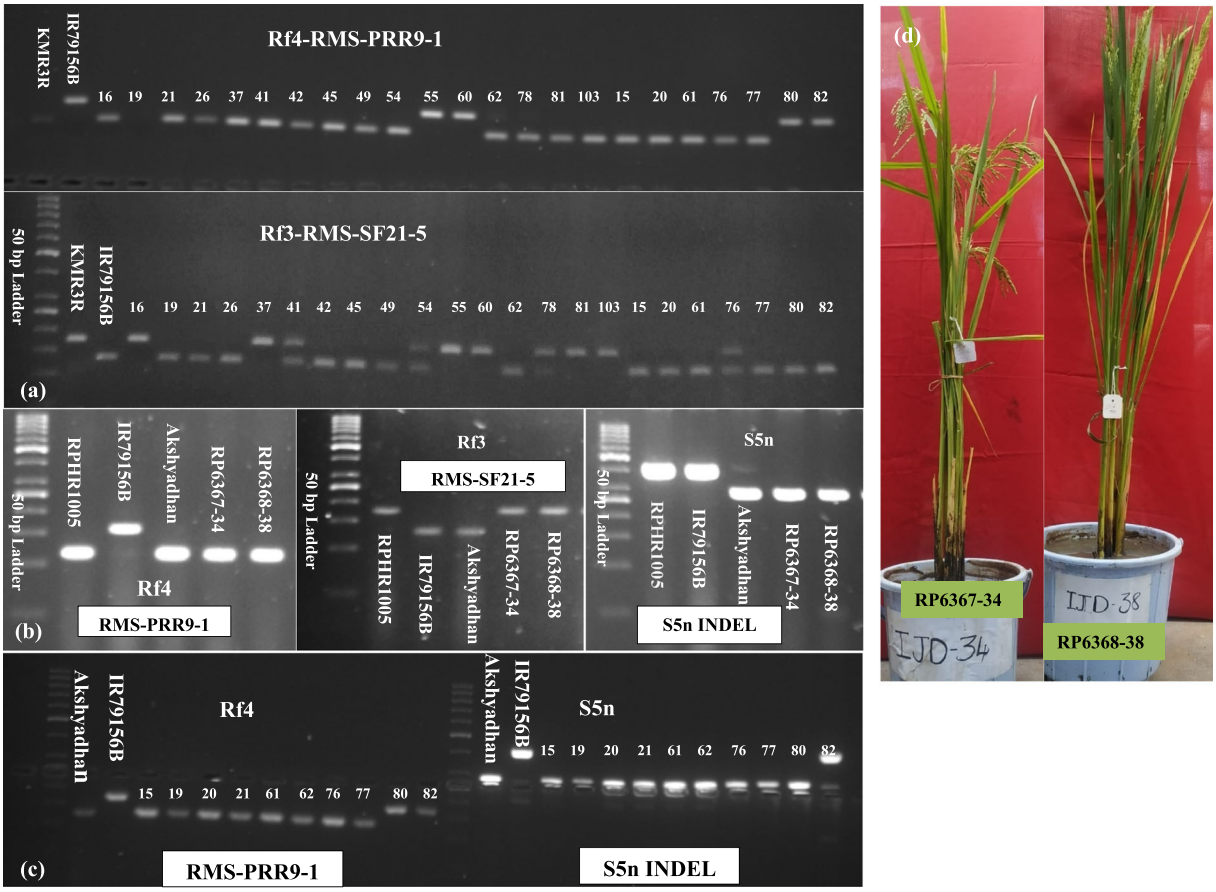

FIGURE 1 (a) The amplification profile of Rf4-RMS-PRR9-1 and Rf3-RMS-SF21-5 in a set of *indica* /tropical *japonica* derived lines for the presence of *Rf4* and *Rf3*. (b) Molecular screening of RP6367-34 and RP6368-38 for fertility restoration genes (*Rf4* and *Rf3*) and wide compatibility gene (*S5n*). (c) Gel picture showing the set of *indica*/tropical *japonica* lines both with *Rf4* and *S5n* genes. (d) Plant type of wide compatible restorer lines RP6367-34 and RP6368-38.

wide compatibility gene, *S5n*, with the help of gene-specific markers along with 16 *indica* female parents and 19 male parents. Among these 19 male parents, 17 are tropical *japonica*, and two are *indica* lines. In the molecular screening of *indica*/tropical *japonica* derived lines, RPHR 1005 (known restorer) was used as a positive control, and IR58025B (known maintainer) was used as a negative control for fertility restoration genes (*Rf4* and *Rf3*). Out of 106 *indica*/tropical *japonica* derived lines, two genotypes (RP6367-34 and RP6368-38) were having three gene combinations (*Rf4/Rf3/S5n*) (Figure 1b,d). For two other genotypes (RP6372-54 and RP6372-76) in three gene combinations, the *Rf3* gene was in heterozygous condition (*Rf4/H/S5n*) (Figure 1a,c). Sixteen genotypes had both *Rf4* and *Rf3* in homozygous conditions (*Rf4Rf4/Rf3Rf3*) (Figure 1a). Fifteen genotypes were identified with only *Rf4* (*Rf4Rf4*), 11 genotypes were having only *Rf3*

TABLE 1 Molecular screening of *indica*/tropical *japonica* derived lines for restorer and wide compatibility genes.

Identified gene combinations	Number of genotypes	Genotypes
Rf4/Rf3/S5n	2	RP6367-34 and RP6368-38
Rf4/H/*S5n*	2	RP6372-54 and RP6372-76
Rf4/Rf3	16	RP6370-4, RP6370-13, RP6370-16, RP6376-28, RP6376-29, RP6376-30, RP6375-31, RP6375-32, RP6375-33, RP6368-37, RP6380-46, RP6375-68, RP6372-75, RP6375-81, RP6388-90 and RP6395-103
Rf4	15	RP6372-18, RP6377-36, RP6380-42, RP6380-43, RP6380-44, RP6380-45, RP6380-47, RP6381-48, RP6382-49, RP6372-53, RP6373-63, RP6380-70, RP6396-104, RP6396-105 and RP6397-106
Rf4/S5n	11	RP6370-15, RP6372-19, RP6372-20, RP6372-21, RP6374-25, RP6374-26, RP6372-51, RP6372-52, RP6372-61, RP6372-62, RP6385-77
Rf3	11	RP6369-2, RP6370-6, RP6370-11, RP6373-22, RP6373-24, RP6378-39, RP6384-55, RP6369-58, RP6369-60, RP6373-64 and RP6373-65
Rf3/S5n	1	RP6394-99
Rf4/H	5	RP6370-14, RP6380-41, RP6380-69, RP6385-78 and RP6392-93
H/*Rf3*	11	RP6369-1, RP6370-3, RP6370-5, RP6370-7, RP6370-8, RP6370-9, RP6370-12, RP6385-57, RP6369-59, RP6373-66 and RP6373-102
H/H	4	RP6370-17, RP6385-6, RP6383-74 and RP6391-92
H/Absent/*S5n*	2	RP6393-97 and RP6393-98
H/Absent	5	RP6375-27, RP6377, RP6378-89, RP6389-96 and RP638372
Absent/H	4	RP6371-10, RP6379-40, RP6389-84 and RP6378-88
Absent/*S5n*	3	RP6373-23, RP6387-80 and RP6388-82
Absent	14	RP6383-50, RP6373-67, RP6382-71, RP6383-73, RP6386-79, RP6389-83, RP6389-85, RP6373-86, RP6373-87, RP6390-91, RP6392-94, RP6389-95, RP6389-100 and RP6389-101
Parents (*indica* female parents)		
Rf4/Rf3/S5n	1	DR-714-1-2-R
Rf4/Rf3	2	RPHR1005 and KMR3R
Rf4/S5n	4	Akshyadhan, RPHR1096, BCP123 and IBL-57
Rf4	4	RPHR-619-2, SG26-120, BK-49-80 and BCW56
Absent	5	DRR4B, IR58025B, APMS6B, CRMS-32B and DRR9B
Parents (tropical *japonica* lines)		
Rf4/Rf3	1	IRGC50865
Rf4/S5n	4	IRGC44076, IRGC69857, IRGC67614 and IRGC50836
Rf3/S5n	2	IRGC328 and IRGC8196
Rf4	1	IRGC67431
Rf3	2	IRGC1797 and IRGC25966
H/Rf3	1	IRGC15073
H/Rf3/S5n	1	IRGC16073
Absent/*S5n*	2	IRGC43372, IRGC47216
Absent	3	IRGC3388, IRGC5726 and IRGC34018

Abbreviation: H, heterozygote.

TABLE 2 Allelic combination of 54 selected genotypes and their test cross nursery details.

S. no.	Allelic combination	Number of genotypes selected × 4 WA-CMS lines	Crosses supposed to be successful	Successful crosses	Restorers	Partial restorers	Partial maintainers	Maintainers
1	*Rf4/Rf3/S5n*	2 × 4	8	4	3	0	1	0
2	*Rf4/H/S5n*	1 × 4	4	2	2	0	0	0
3	*Rf4/Rf3*	16 × 4	64	49	22	21	5	1
4	*Rf4*	6 × 4	24	16	10	4	2	0
5	*Rf3*	4 × 4	16	13	0	1	12	0
6	*Rf4/S5n*	8 × 4	32	19	15	4	0	0
7	*Rf3/S5n*	1 × 4	4	4	2	0	2	0
8	*Rf4*/H	4 × 4	16	14	7	6	1	0
9	H/*Rf3*	4 × 4	16	12	5	5	2	0
10	H/H	4 × 4	16	14	4	3	7	0
11	Absent/*S5n*	1 × 4	4	3	0	1	2	0
12	Absent	3 × 4	12	8	4	1	3	0
	Total	54 × 4	216	158	74	45	37	1

FIGURE 2 (a) Panicle type of hybrid combinations derived from *indica*/tropical *japonica* derived lines from various allelic combinations. (b) Panicle type of hybrid combinations derived from identified restorers based on molecular screening in comparison with varietal and hybrid checks.

(*Rf3Rf3*), 11 genotypes were observed with *Rf4* and *S5n* gene combination (*Rf4/S5n*) (Figure 1), and one genotype (RP6394-99) was observed with *Rf3/S5n* combination. Fourteen genotypes were observed with the complete absence of both fertility restoration genes (*Rf4* and *Rf3*) and wide compatibility allele (*S5n*) (Table 1).

Among the 16 *indica* female parents (Table 1 and Figure S1), one restorer (DR-714-1-2-R) was found with both fertility restorer genes (*Rf3* and *Rf4*) and wide compatibility gene (*S5n*); two restorers (RPHR1005 and KMR3R) were observed to have both *Rf3* and *Rf4* genes; Akshyadhan, RPHR1096, BCP123 and IBL-57 were having *Rf4* and *S5n* gene combination; RPHR-619-2, SG26-120, BK-49-80 and BCW56 were observed with only *Rf4*. All maintainers were observed with a complete absence of both the genes for fertility restoration and wide compatibility genes. Among 17 tropical *japonica* (TJ) lines, one line (IRGC50865) was observed with the presence of both the fertility restoration genes (*Rf3* and *Rf4*), and four lines (IRGC44076, IRGC69857, IRGC67614 and IRGC50836) were observed with both *Rf4* and *S5n* gene combination. Three TJ lines were observed with a

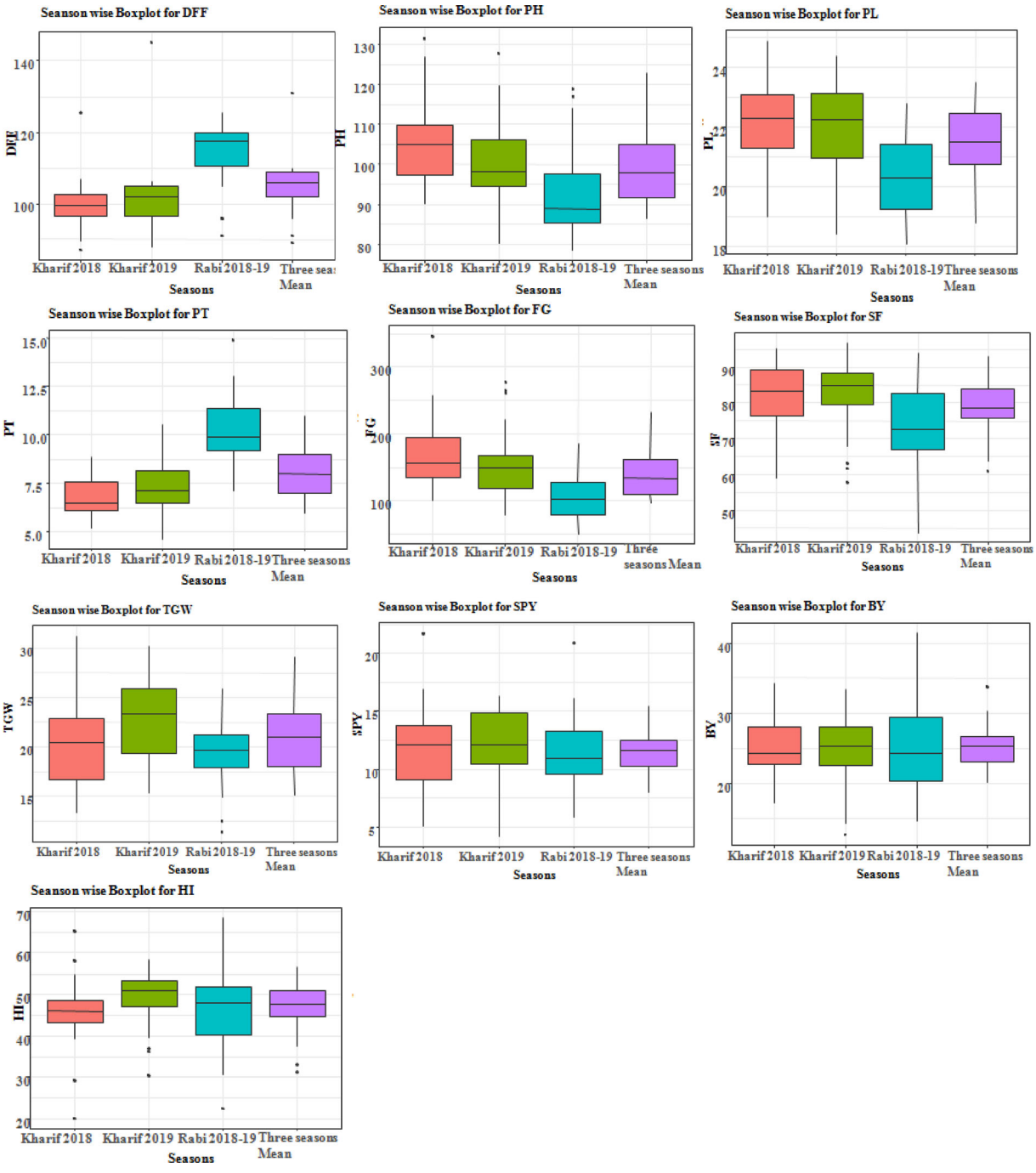

FIGURE 3 Box plot showing the season-wise phenotypic performance for different yield traits.

complete absence of genes, that is, both fertility restoration genes and wide compatibility gene. The allelic status of tropical *japonica* lines for *Rf3*, *Rf4* and *S5n* is given in Table 1.

3.1 | Validation of fertility restoration ability at field level by test crossing

A total of 216 test crosses were attempted; however, only 158 crosses were successful. Allelic combinations of the selected set of 54 genotypes are presented in Table 2, and pollen and spikelet fertility of 158 successful crosses with four CMS lines is presented in Table S1. Among 54 genotypes, two genotypes were having both the fertility restoration genes and wide compatibility gene. RP6367–34 (*Rf4/Rf3/S5n*) could restore the spikelet fertility above 80% in two out of three hybrid combinations (Figure 2b). With APMS6A, RP6367-34 (*Rf4/Rf3/S5n*) behaved as a partial maintainer. The other genotype with three gene combination RP6368-38 (*Rf4/Rf3/S5n*) could restore above 80% spikelet fertility with APMS6A as only one combination was successful (Figure 2a). Among 54 genotypes, 16 genotypes were having both *Rf3* and *Rf4* genes. In 64 combinations (4 × 16), 76% (49) of the crosses were successful. Out of 49 successful crosses having both *Rf3* and *Rf4*, 23 (47%) crosses could restore the spikelet fertility above 75%, 21 crosses could show 50%–75% spikelet fertility (partial restorers), four crosses showed <50% spikelet fertility (partial maintainers), and one cross was observed with 100% sterility (complete maintainer). Out of 16 genotypes with both *Rf3* and *Rf4*, 15 genotypes behaved as restorers with at least one of the CMS lines. Of these 16 genotypes, for seven genotypes, four combinations (4 × 7) were successful. Out of seven genotypes, none of the genotypes behaved like a complete restorer for the four WA-CMS lines. One particular genotype RP6372-75 (*Rf3/Rf4*) did not behave as a restorer with any of the CMS lines. Of 54 genotypes, six genotypes had only *Rf4*, and 16 (67%) crosses were successful. Out of 16 successful crosses, 10 (62.5%) hybrids could show >75% spikelet fertility, five crosses could show 50%–75% spikelet fertility (partial restorers), and one cross showed <50% spikelet fertility (partial maintainers). Four genotypes were selected with only *Rf3*, and the majority of the resulting F_1s (94%) could restore <50% spikelet fertility (partial maintainers). Eight genotypes were selected with *Rf4* and *S5n* gene combination; out of 32 (4 × 8) crosses, 19 crosses were successful; out of 19 crosses, 79% (15) could show >75% spikelet fertility (Restorers); and the remaining 22% (4) showed 50%–75% spikelet fertility (partial restorers). The genotype with *Rf3* and *S5n* combination was proved to be a restorer with two CMS lines (IR68897A and APMS6A) (Figure 2a), and with the remaining CMS lines, it behaved as a partial maintainer. With only *S5n*, it proved a partial restorer in one combination and partial maintainer in the other two combinations. Three genotypes were selected with the complete absence of any of the genes for fertility restoration and wide compatibility; two genotypes behaved as restorers (one genotype with three CMS lines and the other genotype with one CMS line) (Table S1).

Sixty-five per cent of *indica*/tropical *japonica* derived lines developed from restorer lines were identified as potential restorers. Among 23 *indica*/tropical *japonica* derived lines developed from maintainers, three lines (RP6369-60, RP6373-64 and RP6373-65) were validated for their complete sterility.

TABLE 3 Factorial loadings, eigenvalues, percentage of variance explained and cumulative variance under each factor based on factor analysis.

	Traits	FA1	FA2	FA3	FA4	FA5
1	DFF	.06	−.03	.11	.05	**.89**
2	PH	−.39	**.67**	.20	−.20	−.26
3	PL	−.41	**.54**	.32	−.27	−.12
4	PT	.09	−.05	.23	**.89**	.02
5	FG	**.79**	−.37	−.23	.16	−.07
6	UFG	**.83**	.06	−.02	−.02	.37
7	SF	−**.59**	−.09	−.09	.34	−.43
8	TGW	−**.71**	.30	−.02	−.22	.28
9	SPY	.02	−**.76**	.62	.03	−.01
10	BY	−.09	.09	**.88**	.25	.16
11	HI	.02	−**.92**	−.02	−.06	−.14
	Eigenvalues	3.61	1.89	1.65	1.06	.71
	Variance%	32.77	17.18	14.96	9.66	6.54
	Cumulative variance	32.77	49.96	64.92	74.58	81.12

Note: Bold values indicate traits grouped under each factor.

Abbreviations: BY, biological yield; DFF, days to 50% flowering; FG, field grains; HI, harvest index; PH, plant height; PL, panicle length; PT, productive tillers; SF, spikelet fertility; SPY, single plant yield; TGW, thousand grain weight.

3.2 | Identification of multi-trait stable restorers through MTSI

Based on molecular marker analysis and field performance for fertility restoration ability, 31 genotypes were selected and studied for stability analysis across three seasons along with popular restorer lines RPHR1005 and KMR3R as checks. The season-wise phenotypic performance of 33 genotypes for different yield attributing traits is given in Figure 3.

MTSI has been calculated among 33 genotypes for 11 traits to identify the best genotypes both in terms of mean performance and stability. As per the likelihood ratio test, the G × E interaction is not significant for DFF, PH, PL and SF. For all remaining traits such as PT, FG, UFG, SPY, TGW, BY and HI, significant G × E interaction was observed. The MTSI has been calculated based on factor analysis. The five principal components (PC) were selected having the eigenvalue of above 0.7 and explaining a cumulative variance of 81.12% (Table 3). The 11 yield attributing traits were

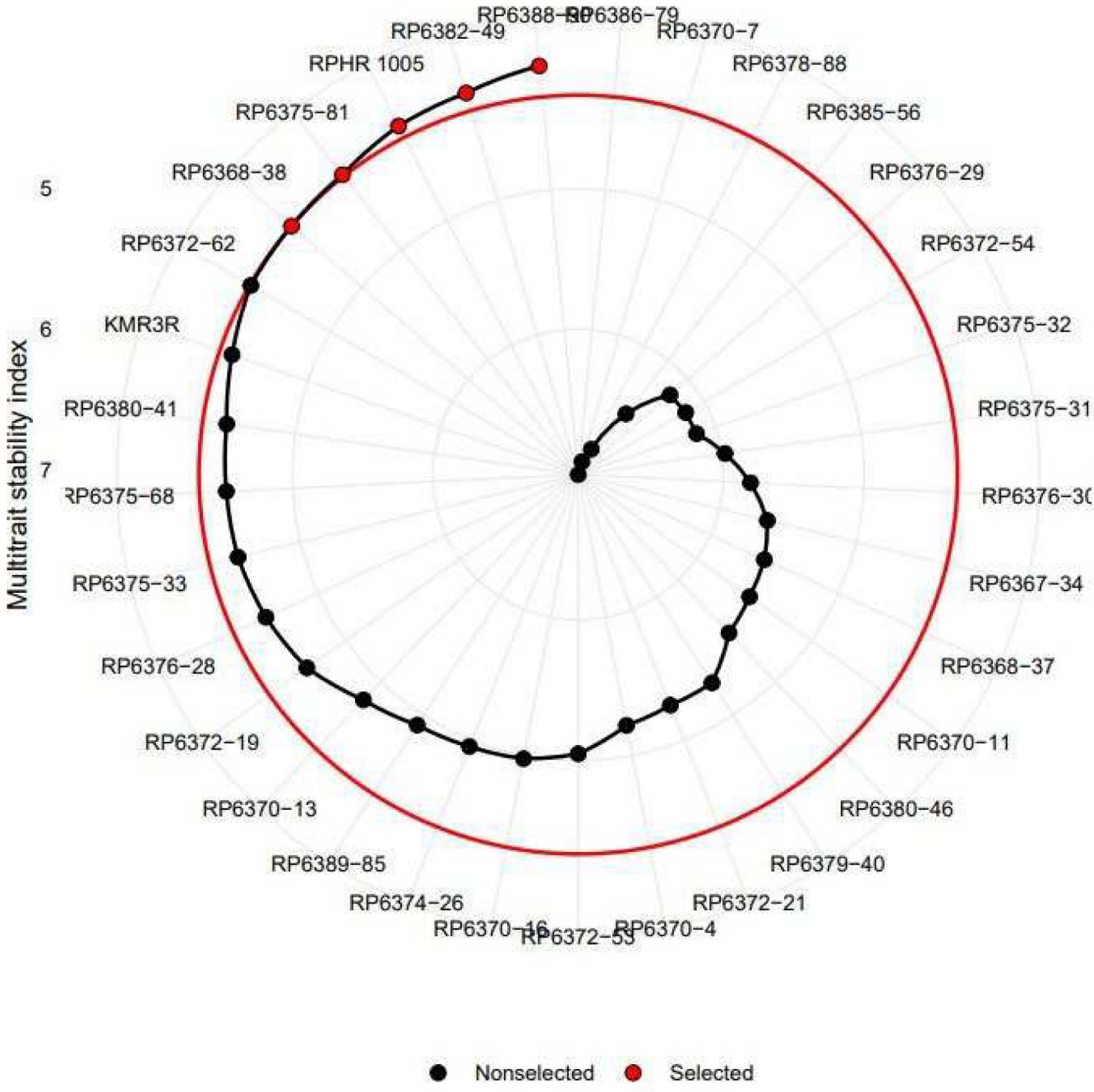

FIGURE 4 Multi-trait stability index.

grouped under five PCs. The traits FG, UFG, SF% and TGW were grouped under the first PC; PH, PL, SPY and HI under the second PC; BY under the third PC; PT under the fourth PC and DFF under the fifth PC (Table 3). The genotype ranking of 33 genotypes based on MTSI is given in Figure 4, and the MTSI value of 4.335 is the cut-off value represented by the red circle. Five genotypes, that is, RP6388-90 (4.115), RP6382-49 (4.206), RPHR1005 (4.245), RP6375-81 (4.322) and RP6368-38 (4.335), have been selected with selection intensity of 15% (Table 4). Among the five selected genotypes, RPHR1005, a check genotype, ranked third after RP6388-90 and RP6382-49. Among 11 yield attributing traits, the positive selection differential for the WAASBY index was observed for FG, SPY, BY and PT with a mean selection differential of 2.36% (Table 5).

4 | DISCUSSION

The conceptual heterosis levels in inter-sub-specific hybrids (*indica* × *japonica*) are 30%–50% higher than that of intra-sub-specific hybrids (Li et al., 2009). Unfortunately, it is harder to exploit the greater heterosis present between *indica japonica* hybrids, majorly because of semi-sterility problems in the hybrids (Dan et al., 2014; Zhang, 2020). After identifying the *japonica* germplasm adapted to tropical situations, the hybrid breeding strategy has been shifted from *indica japonica* hybrids to *indica* tropical *japonica* hybrids, and mostly, the focus is on breeding parental lines, particularly restorer lines from *indica* tropical *japonica* crosses. The present approach for inter-sub-specific hybrid breeding is to use tropical *japonicas* (TJs) in place of temperate *japonicas*. Once such tropical *japonica* restorer with *indica* grain quality becomes available, it can then be used as a TJ restorer line in crosses of hybrid rice breeding programmes with *indica* CMS lines to develop heterotic hybrids with *indica* type grain quality (Brar & Khush, 2017).

In a three-line hybrid rice breeding programme, identification of restorer lines from diverse *indica*, tropical *japonicas* and lines derived from *indica* × tropical *japonica* crosses are of great importance in breaking yield barrier with a broader genetic base. Previously, Shidenur et al. (2019) detected WA-based cytoplasmic restorer lines (WA-CMS) using 310 tropical *japonica*-based new plant type lines, which were developed from a common female parent background (Pusa 44), and evaluated a subset of 42 restorers at three diverse locations. In our study, we explored a different set of materials including 106 *indica*/tropical *japonica* derived lines, which were developed from 32 crosses involving 16 *indica* female hybrid rice parental lines and 19 male parents. This indicates the wide genetic variability of the material developed from this breeding programme. In addition to *Rf3* and *Rf4* screening, we have also screened the material for the presence of the *S5n* gene, which is highly essential in parental lines to repress the problem of semi-sterility in inter-sub-specific hybrids. In this direction, the restorers with *Rf3/Rf4/S5n* and *Rf4/S5n* combinations have been developed. Additionally, stable restorers were identified using MTSI in our study. Khush et al. (1998) based on their

TABLE 5 Estimates of grand mean of genotypes across three seasons (Xo), mean of selected genotypes (Xs) and selection differential (SD) for 11 yield attributing traits based on MTSI.

S.no.	Trait	Factor	Xo	Xs	SD
1	FG	FA 1	142.2	157.3	15.04
2	UFG	FA 1	39.91	59.29	19.38
3	SF	FA 1	79.21	74.32	−4.891
4	TGW	FA 1	22.64	20.99	−1.658
5	PH	FA 2	99.46	91.66	−7.803
6	PL	FA 2	21.5	21.27	−.2271
7	SPY	FA 2	11.74	12.64	.9064
8	HI	FA 2	47.06	45.23	−1.834
9	BY	FA 3	25.11	28.05	2.942
10	PT	FA 4	8.087	8.864	.7774
11	DFF	FA 5	105.2	108.4	3.29

Abbreviations: BY, biological yield, DFF, days to 50% flowering; FG, field grains; HI, harvest index; PH, plant height; PL, panicle length; PT, productive tillers; SF, spikelet fertility; SPY, single plant yield; TGW, thousand-grain weight.

TABLE 4 Mean performance of selected genotypes and genotypes near to the cut-off value for various yield attributing traits.

Selected genotypes	DFF	PH (cm)	PL (cm)	PT	FG	SF (%)	TGW (g)	SPY (g)	BY (g)	HI (%)
RP6388-90 (*Rf3/Rf4*)	106.00	91.60	22.68	9.00	167	83.24	23.22	14.23	27.52	51.52
RP6382-49 (*Rf4*)	110.00	104.32	21.92	9.00	108	60.91	24.71	10.06	30.35	33.15
RPHR 1005(*Rf3/Rf4*)	108.00	86.48	21.40	9.00	162	63.80	16.42	15.44	29.06	53.59
RP6375-81(*Rf3/Rf4*)	109.00	89.54	18.79	9.00	217	72.40	18.61	11.16	25.09	44.68
RP6368-38 (*Rf3/Rf4/S5n*)	109.00	86.35	21.57	9.00	132	91.24	21.97	12.32	28.26	43.20
Genotypes near to the cut-off value	**DFF**	**PH (cm)**	**PL (cm)**	**PT**	**FG**	**SF (%)**	**TGW (g)**	**SPY (g)**	**BY (g)**	**HI (%)**
RP6372-62 (*Rf4/S5n*)	105.00	101.24	21.72	10.00	108	87.47	21.50	13.10	27.17	49.59
KMR3R(*Rf3/Rf4*)	104.00	116.08	22.48	8.00	133	86.53	20.44	15.46	33.74	46.78
RP6380-41 (*Rf4*/H)	106.00	108.48	22.96	7.00	134	77.79	24.95	14.05	25.72	52.27

Abbreviations: BY, biological yield; DFF, days to 50% flowering; FG, field grains; HI, harvest index; PH, plant height; PL, panicle length; PT, productive tillers; SF, spikelet fertility; SPY, single plant yield; TGW, thousand-grain weight.

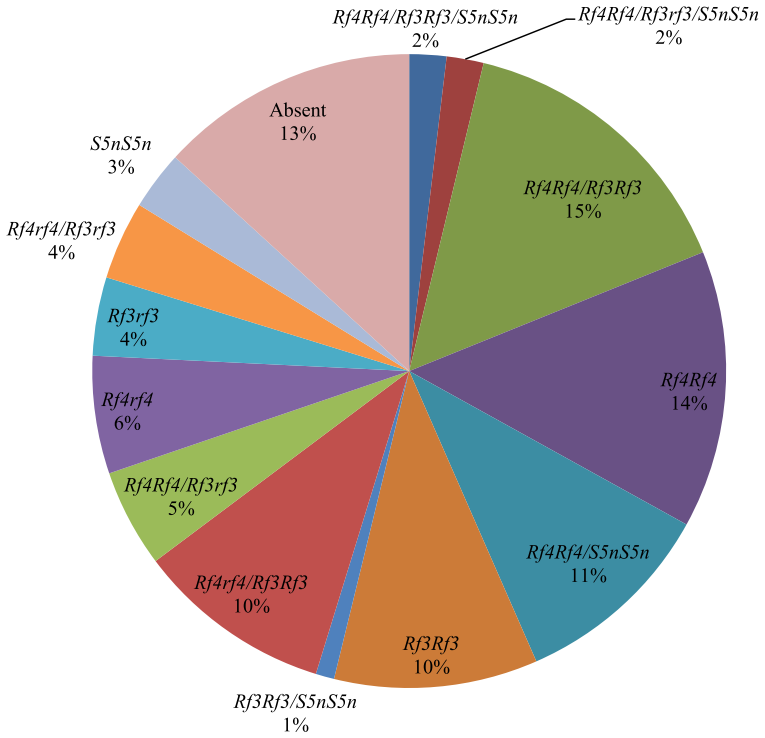

FIGURE 5 Pie chart showing the percent of *indica* /tropical *japonica* derived lines identified with different allelic combinations of *Rf3*, *Rf4* and *S5n*.

experimental evidence at IRRI indicated that further increase of heterosis levels in rice could be possible in the tropics by using crosses involving *indica*, *tropical japonica* or *indica/tropical japonica* derivative lines.

4.1 | Molecular screening of *indica*/tropical *japonica* derived lines for the presence of *Rf3*, *Rf4* and *S5n*

Out of 106 genotypes, through marker-assisted selection, 2% of the lines received three gene combination (*Rf3*/*Rf4*/*S5*n), 15.1% of genotypes had both *Rf3* and *Rf4*, 14% of genotypes possessed only *Rf4*, 10% of genotypes possessed *Rf4* and *S5n* gene combination, and 13% of genotypes were observed to be completely devoid of any of the genes tested in this study through marker analysis (Figure 5). The 31 newly developed restorers analysed in this study have been derived from six promising restorers RPHR1005, RPHR1096, Akshyadhan, IBL-57, RPHR 619-2 and BCP-123. Out of 83 restorer-derived genotypes, 28 genotypes were derived from RPHR1005 as one of the parents and 12 genotypes from RPHR1096, 12 genotypes from Akshyadhan as one of the parents, and the remaining 31 genotypes were developed from 10 different restorers. This indeed indicates the possibility of the existence of wide variability among the 106 genotypes. Of these 83 genotypes developed from the crosses between potential restorers and tropical *japonica* lines, 54 (65.06%) genotypes (after ignoring the lines with *Rf3* alone and heterozygous *Rf3* and *Rf4* loci, as it has a minor influence on fertility restoration) were identified as restorers and few of these lines confirmed through test crosses. Even though, no trait specific selection (for *Rf4* and *Rf3* genes) was carried out during the development procedure; around 50% of the lines carried the fertility restoration genes. This indicates the high heritability and easy transferability of the trait. Interesting to present the parent and progeny correlation for *Rf3*, *Rf4* and *S5n*, even though no trait-specific selection was carried out during the selection process, the gene status for *Rf3*, *Rf4* and *S5n* in derived lines was highly correlated with the gene status of parents (Table 6).

The two genotypes with three gene combinations (*Rf4*/*Rf3*/*S5n*) could restore fertility at least with any of the combinations out of four WA-CMS lines. The total 16 lines with both the fertility restoration genes behaved as complete restorers at least with one combination except RP6372-75. One particular line (RP6395-103) behaved like a complete maintainer with one CMS line and restorer (>80% SF) with the second combination and a partial restorer with the other/third combination. Seventy-five per cent of the crosses with *Rf4*/*Rf3*/*S5n* combination, 47% of the crosses with *Rf4*/*Rf3* combination, 62.5% of the crosses with only *Rf4* and 79% of the crosses with *Rf4*/*S5n* could restore the fertility to an extent of >75%. Ninety-four per cent of the crosses with *Rf3* alone behaved as partial maintainers. Three lines were selected with the complete absence of both the fertility restorer genes. Interestingly, RP6373-67 derived from a BXB cross; hence, it behaved as a partial maintainer with all successful combinations, RP6386-79 (derivative of RPHR1005) and RP6389-85 (derivative of RPHR619-2) derived from potential restorers; however, they did not

TABLE 6 List of 54 genotypes used for validation and their female parents along with their *Rf3*,*Rf4* and *S5n* status.

S.no.	Genotype/*Indica*/tropical *japonica* derived line	Allelic combination of the derived line	Female parent of the derived line
1	RP6367-34	*Rf4/Rf3/S5n*	RPHR-1096 (*Rf4/S5n*)
2	RP6368-38	*Rf4/Rf3/S5n*	IBL-57 (*Rf4/S5n*)
3	RP6372-54	*Rf4/H/S5n*	Akshyadhan (*Rf4/S5n*)
4	RP6370-4	*Rf4/Rf3*	RPHR1005 (*Rf4/Rf3*)
5	RP6370-13	*Rf4/Rf3*	RPHR1005 (*Rf4/Rf3*)
6	RP6370-16	*Rf4/Rf3*	RPHR1005(*Rf4/Rf3*)
7	RP6376-28	*Rf4/Rf3*	RPHR1005(*Rf4/Rf3*)
8	RP6376-29	*Rf4/Rf3*	RPHR1005(*Rf4/Rf3*)
9	RP6376-30	*Rf4/Rf3*	RPHR1005 (*Rf4/Rf3*)
10	RP6375-31	*Rf4/Rf3*	RPHR1005 (*Rf4/Rf3*)
11	RP6375-32	*Rf4/Rf3*	RPHR1005 (*Rf4/Rf3*)
12	RP6375-33	*Rf4/Rf3*	RPHR1005 (*Rf4/Rf3*)
13	RP6368-37	*Rf4/Rf3*	IBL-57 (*Rf4/S5n*)
14	RP6380-46	*Rf4/Rf3*	Uttrirajappan (NA)
15	RP6375-68	*Rf4/Rf3*	RPHR1005 (*Rf4/Rf3*)
16	RP6372-75	*Rf4/Rf3*	Akshyadhan (*Rf4/S5n*)
17	RP6375-81	*Rf4/Rf3*	RPHR1005 (*Rf4/Rf3*)
18	RP6388-90	*Rf4/Rf3*	DRR-9B (absent)
19	RP6395-103	*Rf4/Rf3*	L2-182 (NA)
20	RP6372-76	*Rf4*	Akshyadhan(*Rf4/S5n*)
21	RP6380-43	*Rf4*	Uttrirajappan (NA)
22	RP6380-45	*Rf4*	Uttrirajappan (NA)
23	RP6382-49	*Rf4*	RPHR-1096 ((*Rf4/S5n*)
24	RP6372-53	*Rf4*	Akshyadhan (*Rf4/S5n*)
25	RP6380-70	*Rf4*	Uttrirajappan (NA)
26	RP6369-60	*Rf3*	APMS6B (absent)
27	RP6373-64	*Rf3*	APMS6B (absent) IR79156B (absent)
28	RP6373-65	*Rf3*	APMS6B (absent) IR79156B (absent)
29	RP6370-11	*Rf3*	RPHR1005 (*Rf4/Rf3*)
30	RP6372-19	*Rf4/S5n*	Akshyadhan (*Rf4/S5n*)
31	RP6372-20	*Rf4/S5n*	Akshyadhan (*Rf4/S5n*)
32	RP6372-21	*Rf4/S5n*	RPHR1096 (*Rf4/S5n*)
33	RP6374-26	*Rf4/S5n*	Akshyadhan (*Rf4/S5n*)
34	RP6372-51	*Rf4/S5n*	Akshyadhan (*Rf4/S5n*)
35	RP6372-61	*Rf4/S5n*	Akshyadhan (*Rf4/S5n*)
36	RP6372-62	*Rf4/S5n*	RPHR-1096 (*Rf4/S5n*)
37	RP6374-25	*Rf4/S5n*	RPHR-1096 (*Rf4/S5n*)
38	RP6394-99	*Rf3/S5n*	BCW-56 (*Rf4*)
39	RP6380-41	*Rf4/H*	Uttrirajappan (NA)
40	RP6380-69	*Rf4/H*	Uttrirajappan (NA)
41	RP6385-78	*Rf4/H*	RPHR-1096 (*Rf4/S5n*)
42	RP6392-93	*Rf4/H*	BK 49-80 (*Rf4*)
43	RP6370-3	*H/Rf3*	APMS-6B (absent)
44	RP6370-5	*H/Rf3*	RPHR1005 (*Rf4/Rf3*)
45	RP6370-12	*H/Rf3*	RPHR1005 (*Rf4/Rf3*)

(Continues)

TABLE 6 (Continued)

S.no.	Genotype/*Indica*/tropical *japonica* derived line	Allelic combination of the derived line	Female parent of the derived line
46	RP6385-57	*H/Rf3*	RPHR1096 (*Rf4/S5n*)
47	RP6379-40	H	BCP-123 (*Rf4/S5n*)
48	RP6385-56	H/H	RPHR1096 (*Rf4/S5n*)
49	RP6378-88	H	IBL-57 (*Rf4/S5n*)
50	RP6391-92	H/H	SG 26-120 (*Rf4*)
51	RP6373-23	Absent/*S5n*	APMS6B (absent)
52	RP6373-67	Absent	APMS6B (absent) IR79156B (absent)
53	RP6386-79	Absent	RPHR1005 (*Rf4/Rf3*)
54	RP6389-85	Absent	RPHR 619-2 (*Rf4*)

Abbreviations: Hm heterozygote; NA, not available.

show the presence of *Rf4* and *Rf3* even they behaved as potential restorers both based on pollen and spikelet fertility percentage. This might be because two genotypes may harbour the novel recombination/gene responsible for fertility restoration ability (Balaji Suresh et al., 2012; Katara et al., 2017).

The differential fertility restoration behaviour of restorers with different CMS lines of the same cytoplasmic background may be because of the differential interaction of nuclear and cytoplasmic genes of CMS lines with the nuclear genes of restorers or could be due to the sterility-inducing genes in the nuclear background of CMS lines (Bobby & Nadarajan, 1994). The gene expression may be affected by the presence of minor or modifier genes in the restorer lines. According to Maan (1985), the penetrance and expressivity of restorer genes in wheat are known to be affected by the parental genotypes in a particular cross combination. The existence of modifier genes (Ganeshan et al., 1998; Pande et al., 1990) and G × E interactions may be the reason for variable expressivity of fertility restorer genes in different combinations. Similar observations of differential behaviour of restorers with different CMS lines were earlier observed by many rice researchers (Govinda Raj et al., 1984; Bijral et al., 1989; Virmani et al., 1997; Kumar et al., 2002; Hariprasanna et al., 2006; Rosamma & Vijayakumar, 2006; Upadhyay & Jaiswal, 2012). In this study, a significant correlation was found between pollen and spikelet fertility; however, in few combinations, the partial restorers based on pollen fertility analysis turned out to be potential restorers through spikelet fertility analysis; this may be because few fertile pollen is sufficient to effect fertilization (Govinda Raj & Virmani, 1988); inversely, the potential restorers identified through pollen fertility studies became partial restorers through spikelet fertility analysis; this could be due to the stage of pollen abortion (Chaudhury et al., 1981). Probably, the existence of other *Rf* loci may partly be responsible for the many partial restorers that we identified through test crosses in hybrid breeding source nurseries. Sheeba et al. (2009) reported that there are three major genes (*Rf3*, *Rf4* and *Rf(u1)*) and many minor QTLs and different combinations of the alleles of these genes and QTLs govern the degree of fertility restoration ability in a restorer line.

In the case of tropical *japonica* male parents, one line (IRGC50865) was observed with both the restorer genes (*Rf4* and *Rf3*), and four lines (IRGC44076, IRGC69857, IRGC67614 and IRGC50836) were observed with both *Rf4* and *S5n* gene combination. In the present study, we identified tropical *japonica* lines with restorer genes. This is also may be one of the reasons for getting a higher frequency of restorers in the derived population. The identified tropical *japonica* lines with restorer genes can be used directly in the hybrid breeding programme as pollen parents; later, these lines can be improved for grain quality and tolerance to pests and diseases.

Out of 19 male parents, nine (53%) tropical *japonica* lines were having *the S5n* gene. Concerning parents, similar kind of results was reported by Revathi et al. (2015, 2010). The present results were in congruence with many research studies stating that most of the tropical *japonica* lines contain wide compatibility gene (Bharaj et al., 1994; IRRI, 1996; Khush, 1996; Kumar & Virmani, 1992; Luo et al., 1990). In the present study, 16% of heterozygous loci for *the Rf4* gene (H/*Rf3* and H) and 9% for the *Rf3* gene (*Rf4*/H and H) and 4% for both the loci (H/H) were observed even after F_{11} generation. This might be because the crosses included tropical *japonica* subspecies genome, hence segregating for longer generations. The factors like crossing over and recombination also may result in the conservation of heterozygosity in the advanced generations.

4.2 | Confounding effect of *Rf3*, *Rf4* versus *S5n* in the restorer lines

The *indica*/*japonica* hybrids were considered impossible until the discovery of the wide compatibility gene (Ikehashi & Araki, 1986), which overcomes the problem of F_1 hybrid sterility in such parental combinations. The hybrid sterility consists of both male or pollen sterility and female or embryo sac sterility. Hybrid sterility for pollen and hybrid sterility for spikelets are independently controlled (Ikehashi & Araki, 1986). Perhaps a new gene may be responsible for the combined action of sterility in both pollen and spikelets. It has been reported that in the lines with the *S5n* gene, the pollen semi-sterility should not lower the spikelet fertility of *indica*/*japonica* or *indica*/tropical *japonica* hybrids. In the present study, there is no particular difference in spikelet and pollen

fertility percentages that have not been observed in many crosses with and without the presence of the *S5n* gene. However, increased efficiency in fertility restoration ability of *Rf4/S5n* gene combination compared to *Rf4* gene alone has been observed.

When we see the decreasing order of efficiency of different gene combinations on fertility restoration ability, *Rf4/S5n* > *Rf4/Rf3/S5n* > *Rf4* > *Rf4/Rf3* > *Rf3/S5n* > *Rf3*. The gene combination (*Rf4/S5n)* could show 79% (79% of the cross combinations with this gene combination showed potential fertility restoration, i.e. >75%) of efficiency, *Rf4/Rf3/S5n* 75%, *Rf4* alone showed 62.5% efficiency, and *Rf4/Rf3* showed 47% efficiency in fertility restoration. Interestingly, the genotypes with *Rf3* alone behaved as partial maintainers, and this was supported by the report of Katara et al. (2017) but, with *Rf3/S5n* combination, could restore fertility in two combinations out of four successful combinations. In the present study, it has been observed that the presence of the *S5n* gene with either the *Rf4* or *Rf3* increased the spikelet fertility percentage. However, the confounding effect of *Rf4* and *Rf3* with *S5n* needs to be studied in detail. To develop inter-sub-specific hybrids with increased heterosis, the parental lines should contain both fertility restoration genes and wide compatibility gene for overcoming the female or embryo sac sterility. In this direction, the newly developed wide-compatible restorer lines possessing *Rf4/Rf3/S5n* and *Rf4/S5n* combinations are a highly useful and valuable resource for future hybrid breeding programmes.

To identify multi-trait stable restorers, a multi-seasonal evaluation was carried out for three seasons: wet season 2018, dry season 2018–2019 and wet season 2019. The stability performance of 31 newly identified restorers was studied in comparison with two popular restorers as checks KMR3R and RPHR1005. To identify the best genotypes in terms of performance and stability for multiple traits and the advance multi-environment data analysis, a MTSI has been calculated. In the present study, five genotypes (RP6388-90, RP6382-49, RPHR1005, RP6375-81 and RP6368-38) were selected based on MTSI value. Selected five genotypes are of medium duration genotypes (100–110 days DFF). MTSI is based on a linear mixed effect model having combined features of AMMI (Additive Main Effect and Multiplicative Interaction) and BLUP (Best Linear Unbiased Prediction), which considers the weighted average of all IPCA scores with increased predictive accuracy unlike AMMI quantifies the stability only based on first IPCA scores (Olivoto et al., 2019). Recently, the MTSI value was employed by Zuffo et al. (2020) for selecting the best genotypes for both drought and saline stresses; Abdelghany et al. (2021) studied phenotypic stability of seed composition traits, protein, oil and fatty acid contents in soybean by using MTSI; Koundinya et al. (2021) employed MTSI to select cassava genotypes for rainy and water stress conditions for multiple traits.

Choosing multi-trait stable genotypes or restorers is highly crucial for the consistent performance of hybrids. Stable performance is usually associated with genetic diversity and shows a higher degree of buffering capacity for various stresses (Allard, 1961; Roy & Kharkwal, 2004). The genotypes RP6376-28 (*Rf3/Rf4*), RP6375-33 (*Rf3/Rf4*), RP6368-38 (*Rf3/Rf4*), RP6382-49 (Rf4), RP6370-16 (*Rf3/Rf4*), RP6376-30 (*Rf3/Rf4*), RP6375-31 (*Rf3/Rf4*), RP6375-81(*Rf3/Rf4*) and RP6388-90 (*Rf3/Rf4*) were having good fertility restoration potential and can be served as a valuable resource for the future hybrid breeding programme with tropical *japonica* genome segments in their genome. Certain combinations of stable traits are also highly required for successful hybrid seed production, for example, the genotypes RP6380-46, RP6385-56 and RP6389-85 were identified as highly unstable for their flowering duration in our seed production plots; in that case, the synchronization for flowering will become very difficult and with fails in hybrid seed production. Sometimes, even though the combinations (A × R) have heterotic potential, because of their highly variable behaviour for flowering/maturity, the heterotic combinations cannot be even entered into a hybrid evaluation system (All India Coordinated Rice Improvement Project [AICRIP] for Indian context).

In the present study, two wet seasons and one dry season were considered for MTSI analysis. During the dry season, for traits, PH, PL, FG and SF considerable reduction in the performance was observed, and for DFF considerable increase in the duration, and PT a slight increase in the average was observed during the dry season. The majority of the traits showed an increase in performance during the wet season; wet season (*Kharif*) is considered the best season to harvest maximum yield as the majority of the yield-related traits performed well during the wet season. In the present study, especially for TGW, the many *indica*/tropical *japonica* derived restorers showed significantly superior performance compared to checks (RPHR1005 and KMR3R).

5 | CONCLUSION

In the present study, our major efforts focused mainly to address the challenges of the low magnitude of heterosis and inconsistent performance. We aimed at developing genetically diverse superior, stable parental lines with a broad spectrum of compatibility and with wider adaptation from *indica* tropical *japonica* crosses to realize greater heterosis with consistent performance. In this direction, 54 restorers were developed, 46 were validated for their fertility restoration potential, and 31 restorers were studied for their stability. Functional marker-assisted screening helped in identifying the potential restorers for the WA-CMS system with maximum efficiency (90%–100%) and could shortlist the genotypes. This has reduced the number of crosses by half in attempting unnecessary test crosses and studying their pollen and spikelet fertility and saved a lot of time and labour. Further extensive heterotic grouping, combining ability and heterosis studies need to be conducted to identify the best performing heterotic hybrid/successful combinations. The present set of new restorers developed from inter-sub-specific hybridization will serve as a great resource for future hybrid rice breeding programmes to broaden the genetic base of the hybrid rice parental pool and for developing heterotic hybrids in India.

AUTHOR CONTRIBUTIONS

A. S. Hari Prasad, K. B. Eswari and K. Sruthi conceptualized the study; A. S. Hari Prasad, P. Koteswararao, M. Sadath Ali, P. Senguttuvel, P. Revathi and K. B. Kemparaju developed the breeding material; K. Sruthi conducted the lab and field experiments; K. Sruthi, M. Bala

Satya Sree, K. Sri Krishna Latha, P. Beulah, P. Nagaraju and Y. Manasa collected the data; K. Sruthi wrote the first draft of the manuscript; A. S. Hari Prasad, R. M. Sundaram, B. Divya, A. Dhandapani, K. B. Eswari, C. Damodhar Raju, M. Sheshu Madhav and B. Jyothi corrected the manuscript; A. S. Hari Prasad received the funds.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare that are relevant to the content of this article.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material

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