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Research Article
Assessment of Genetic Variability and Identification of Fertility Restoration Genes *Rf3*, *Rf4* of WA-CMS in RILs Population of Rice

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Abstract

Background and Objective: Development of multipurpose high-yielding rice lines is a good source of genetic variability, gene discovery and be used in two ways inbred and hybrid rice breeding. This investigation was conducted at the Experimental Farm of Sakha Agricultural Research Station, Sakha, Kafr El-Sheikh, Egypt during three seasons to study the genetic variability and identify fertility restoration genes in rice. **Materials and Methods:** Eighty-eight recombinant inbred lines (RILs) derived from across between PR6 (restorer) and G46B (maintainer) were evaluated for agronomic and yield-associated traits. Out of them, 20 lines as the best were selected based on their phenotypic and yield acceptability to confirm the evaluation for yield traits. Moreover, evaluation was extended to identify the fertility restoration genes *Rf3* and *Rf4* where located on chromosome 1 and 10, respectively. **Results:** Genetic variability determined in the studied traits revealed that the PCV was higher than GCV for all traits indicating that the apparent variation is not only genetic but also influenced by the growing environment in the expression of these traits. The highest broad-sense heritability values were found for growth duration, grain yield/plant and plant height. The strongest positive correlation coefficients were recorded between tillers/plant and panicles/plant and between grain yield/plant and tillers/plant, panicles/plant, spikelet fertility (%) indicating the importance of these traits as a selection criterion for yield improvement program in rice. The cluster analysis divided the 20 selected RILs into five main groups based on the mean values of the studied traits. Ten earlier reported SSR markers linked to *Rf3* and *Rf4* have been utilized to screen the 20 selected lines. The SSR markers RM490 and RM1108 linked to *Rf3* and *Rf4*, respectively, identified that eight lines carry both *Rf3* and *Rf4* genes. **Conclusion:** It could be recommended that these lines can be utilized in hybrid rice breeding program as restorer lines of wild-abortive cytoplasmic male sterile lines (WA-CMS).

Key words: Rice, genetic variability, fertility restoration, *Rf3*, *Rf4* genes

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rice (*Oryza sativa* L.), is one of the principal food grain crops and the main energy resource for almost 50% of the world's population[1]. Certainly, the world's population increased dramatically, at an alarming rate and making the food security the major challenge in the future. Thus, increasing the population requires an increase in rice production at least 40% in 2030 to meet ever-increasing demands[2]. The Egyptian rice production is one of the highest yield/units in the world and Egypt produces about 5.8 Mt of rough rice per year[3]. The yield of inbred rice varieties in the last decades was not considerably improved and appeared to reach a plateau, so enhancing rice yield is one of the top most priorities in rice breeding program for developing super rice lines (high yielding, excellent grain quality and tolerance of multiple biotic and abiotic stress). Recombinant inbred lines (RILs) produced from hybridization and repeated selfing using single seed descent (SSD) method will be able to break the undesirable linkages and give high yielding recombinants and producing transgressive segregants superior lines than parents. Development of recombinant inbred lines (RILs) from two genetically diverse parents will be a good source of genetic variability, enable to rice breeders for practicing gene discovery and the possibility of using these lines in two-way inbred and hybrid rice breeding like the Egyptian rice variety Giza 178 (commercial and restorer).

Hybrid rice is considered as one of the most practical, promising and sustainable eco-friendly options to break the yield ceiling witnessed in rice[4]. Exploitation of hybrid rice technology or heterosis breeding is the key approach for the increase of global rice production, it has a 15-30% higher yield potential over inbred varieties in the same environment[5]. In hybrid rice, wild abortive (WA)-type CMS has been almost predominantly and exclusively used CMS source in hybrid rice breeding and contributes to 10% of the total rice cultivated area worldwide[6,7]. Therefore, several studies have attempted to elucidate and determine the fertility restoring genes responsible for WA-CMS. For the production of viable pollen in wild-abortive (WA) CMS, two independent and dominant genes (*Rf3* and *Rf4*) are required. These two major genes *Rf3* and *Rf4* have been mapped on the chromosomes 1 and 10, respectively[8-14]. According to the previous studies, the genetic linkage analysis indicated that, the SSR markers RM490 on the short arm of chromosome 1 linked with *Rf3* gene, while RM1108 with located on the long arm of chromosome 10 linked with the *Rf4* gene[15-17]. Marker-assisted selection (MAS) has been proved to be an efficient selection tool for phenotypic selection in inbred and hybrid rice breeding. Therefore, SSR markers RM490 and RM1108 have been utilized to screen the rice genotypes and identify the lines which carry both *Rf3* and *Rf4* genes and these lines can be utilized in hybrid rice breeding as restorers in wild-abortive (WA) CMS.

The rice genotype PR6 has strong restoring ability to WA-cytoplasmic male sterility system[18]. For developing new restorer lines with high yielding and good floral traits in rice, specific hybridization between PR6 as a strong restorer genotype and G46B as a maintainer possessed very good floral traits has been done to achieve this purpose. wherefore, the objectives of this study were to perform phenotype screening and discrimination of a set of developed recombinant inbred lines of rice in terms of their agro-morphological traits and genetic variability. Moreover, screening of the distinctive lines for fertility restoration genes by using SSR markers.

MATERIALS AND METHODS

Plant materials and field experiments: The field investigation was carried out at the Experimental Farm of Sakha Research Station, Sakha, Kafr-El-sheikh, Egypt, during 2015 and 2016 summer seasons. The experimental materials for the present study consisted of 88 Recombinant Inbred Lines (RILs) population derived from a cross between two diverse genotypes PR6 (restorer) as female and G46B (maintainer) as male. Growing the F_1 plants and selfing to produce the F_2 generation was done in the nursery and the F_2 plants until F_6 lines were selected and selfed by Single Seed Descent Method (Fig. 1). A set of 88 RILs population along with the parental lines were evaluated for phenotypic performance and yielding ability during 2015. Out of 88 RILs, 20 lines based on their phenotypic and yield acceptability were selected for evaluation in randomized complete block design (RCBD) with three replications compared with the two parents during 2016. The standard package of recommendation practices were adopted for a good crop growth. Five randomly plants from the central rows in each replication were selected and evaluated for yield and its component traits. Data was collected on growth duration (day), plant height (cm), tillers/plant, panicles/plant, flag leaf area (cm^2), panicle length (cm), 1000-grain weight (g), filled grains/panicle, spikelet fertility (%) and grain yield/plant. All the measurement techniques were based on IRRI standard evaluation system of rice[19].

Table 1: Marker name, chromosome number and sequences of SSR markers used in this study

Marker	Chr.	Gene	F primer	R primer
RM1	1	*Rf3*	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC
RM265	1	*Rf3*	CGAGTTCGTCCAAGTGAGC	CATCCACCATTCCACCAATC
RM490	1	*Rf3*	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG
RM582	1	*Rf3*	TCTGTTGCCGATTTGTTCG	AAATGGCTTACCTGCTGTCTC
RM10353	1	*Rf3*	GGACACTTTGAATGAAGGCAACC	TTGTTAGTTGGCGAAAGGAAGC
RM171	10	*Rf4*	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG
RM228	10	*Rf4*	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC
RM244	10	*Rf4*	CCGACTGTTCGTCCTTATCA	CTGCTCTCGGGTGAACGT
RM1108	10	*Rf4*	CACACTGGATCCTGGACAGACG	CAGCACACACCTCCGTGAAGC
RM6100	10	*Rf4*	TTCCCTGCAAGATTCTAGCTACACC	TGTTCGTCGACCAAGAACTCAGG

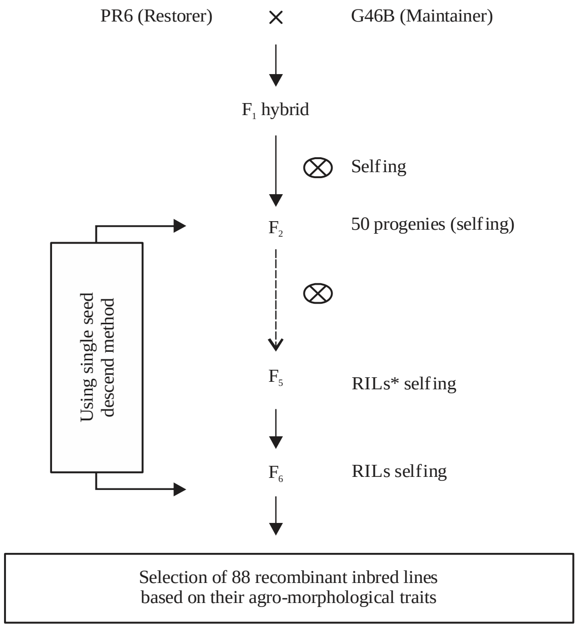


Fig. 1: Scheme for the development of 88 recombinant inbred lines based on their morphological and yield-related traits

*RILs: Recombinant Inbred Lines

DNA extraction, PCR and SSR molecular markers used: During the 2016-2017 winter season, 10 seeds from each line (Best 20 selected lines) and their parents were soaked, germinated and placed in an incubator at 28°C for 20 days. DNA was extracted from the fresh seedlings of each line and their parents using the CTAB methods[20] to evaluate with the help of molecular markers to establish the presence of fertility restoring genes (R*f3* and R*f4*). For detection of polymorphism between parental lines, their DNAs were supposed to the PCR-amplification by 18 SSR markers according to previous studies[17,21,22]. Out of 18 SSR molecular markers, 10 markers gave polymorphic between the parents and listed in Table 1.

Statistical analysis for field experiments: The observations and mean values were recorded and subjected to statistical analysis. The descriptive statistical analysis, analysis of variance (ANOVA) of yield and its component traits and correlation coefficients were worked out using SPSS software (version 20.0). The estimates of genotypic variance (GV), phenotypic variance (PV), phenotypic coefficient of variations (PCV%) and genotypic coefficient of variations (GCV%) were computed according to the formula suggested by Burton[23]. For the estimation of heritability the method outlined by Hanson *et al.*[24]. The genetic distance tree construction was expressed by using the Paleontological Statistics (PAST) software package using the mean performance of the studied genotypes[25].

RESULTS AND DISCUSSION

Agronomic traits of 88 RILs population and their parents: The analysis of phenotypic data of parents and 88 RILs for six studied traits such as growth duration (day), plant height (cm), panicles/plant, 1000-grain weight (g), filled grains/panicle and grain yield/plant (g) in regard to the parameters, mean, range, skewness and standard deviation are presented in Table 2. Among these six traits, the parent of G46B showed desirable and higher values than those of PR6 for earliness, shorte stature and panicles/plant traits. Duration was 124 days in G46B and 127 days in PR6 and it ranged from 118-141 days among the 88 RILs. Plant height values were 97.0 and 107.0 cm for G46B and PR6, respectively and RILs population ranged between 84.0 and 116.0 cm. The parental line G46B recorded 23.0 panicles/plant and PR6 recorded 22.0 panicles, while the 88 RILs were ranged from 16.0-26.0 for panicles/plant. On the other hand, the parental line PR6 was higher than those of G46B in 1000-grain weight, filled grains/panicle and grain yield/plant. The mean value of grain yield was 51.6 g for PR6 and 47.2 g for G46B and it ranged from 37.8-67.5 among the RILs population.

Histograms showing the distribution of the phenotypic variation for the 6 studied traits in the 88 RILs population are presented in Fig. 2. The phenotypic variation of the six traits displayed a continuous distribution and the skewness values ranged from -2.09 to 0.83 with fewer values, implying and suggesting near normal distributions. It also illustrated that

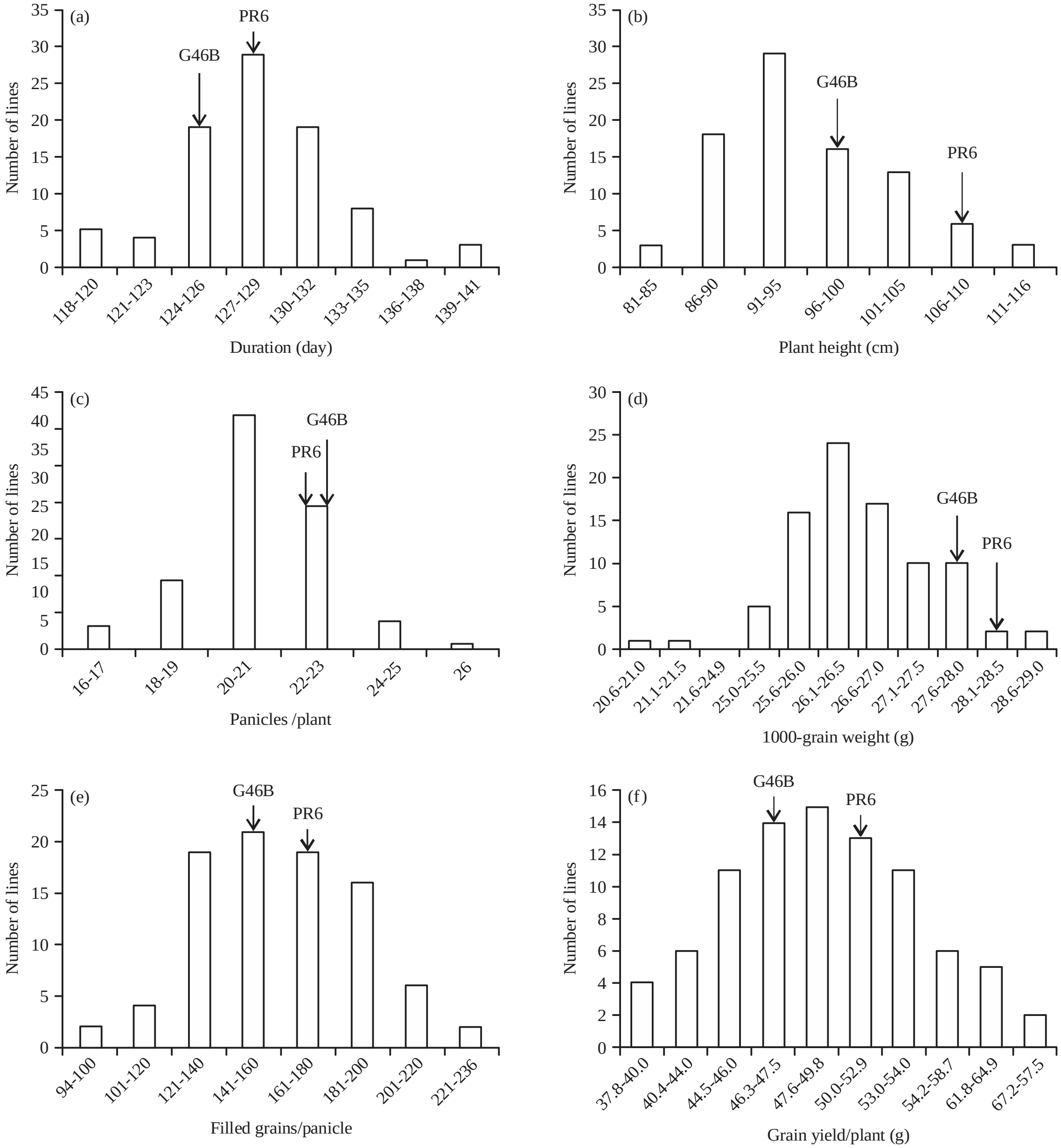


Fig. 2(a-f): Frequency distribution of 6 yield-related traits in 88 RILs and mean values of G46B and PR6 grown in 2015 season

Table 2: Phenotypic analysis of 88 recombinant inbred lines (RILs) and their parents during 2015 season

	Parents		RILs population				
Traits	G46B	PR6	Mean	Minimum	Maximum	Skewness	SD (%)
Duration (day)	124.0	127.0	128.2	118.0	141.0	0.20	4.44
Plant height (cm)	97.0	107.0	95.6	84.0	116.0	0.68	6.73
Panicles/plant	23.0	22.0	20.9	16.0	26.0	-0.08	1.90
1000-grain weight (g)	27.6	28.1	26.5	20.9	28.7	-2.09	1.15
Filled grains/panicle	142.0	163.0	160.0	94.0	236.0	0.19	29.80
Grain yield/plant (g)	47.2	51.6	49.9	37.8	67.5	0.83	6.25

the frequency distribution of the 6 traits varied widely In addition, transgressive segregation was observed higher for all traits indicating reflecting quantitative and polygenic inheritance. The range and mean values of the studied traits indicating transgressive segregation nature in the recombinant inbred lines and this result is agreement with these obtained by Tehrim *et al.*[26] and Chaudhari *et al.*[27].

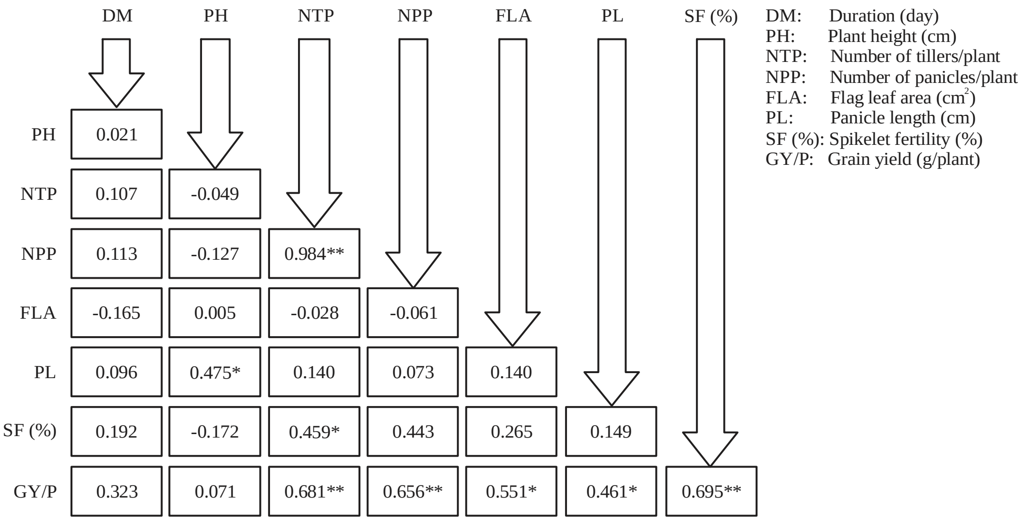
Table 3: Mean performance and variance analysis of agro-morphological traits for the best selected lines (20 RILs)

No.	Lines	Duration (day)	Plant height (cm)	Tillers/plant	Panicles/plant	Flag leaf area (cm^2)	Panicle length (cm)	Spikelet fertility (%)	Grain yield/plant (g)
1	RIL-7	126.00	91.30	22.00	21.30	30.90	21.90	97.70	48.20
2	RIL-9	120.00	95.30	24.30	23.70	29.30	20.50	92.00	45.30
3	RIL-23	136.00	99.00	17.00	16.70	29.60	20.30	90.90	41.50
4	RIL-26	124.00	90.00	19.00	18.70	42.70	23.30	94.80	43.10
5	RIL-31	126.00	106.30	21.30	20.70	34.80	24.80	95.00	46.50
6	RIL-33	127.00	97.00	22.30	21.70	30.00	20.50	93.90	40.20
7	RIL-34	123.00	105.70	18.70	18.30	24.40	23.90	92.00	41.30
8	RIL-41	125.00	99.30	24.70	23.30	29.30	23.30	94.50	49.60
9	RIL-45	124.00	105.00	20.70	20.00	41.70	24.80	94.10	45.30
10	RIL-55	127.00	100.30	21.70	20.30	43.80	23.80	92.00	42.30
11	RIL-57	126.00	96.70	23.00	22.00	46.70	23.30	93.50	41.70
12	RIL-59	124.00	103.30	22.30	22.00	40.50	25.00	92.90	42.90
13	RIL-62	123.00	97.00	24.70	24.00	33.60	25.30	97.30	53.20
14	RIL-67	125.00	91.30	24.30	23.70	40.00	22.00	95.50	50.00
15	RIL-71	124.00	104.00	20.70	19.30	32.40	28.80	90.80	40.60
16	RIL-72	126.30	86.70	21.00	20.70	28.30	24.80	93.90	41.70
17	RIL-79	136.00	95.00	22.00	22.00	26.90	23.80	89.30	39.90
18	RIL-82	123.00	98.00	24.70	24.00	24.10	24.90	94.30	59.80
19	RIL-84	125.00	97.70	20.30	19.70	26.30	23.70	95.40	49.60
20	RIL-86	135.00	103.70	26.70	25.00	29.60	26.10	97.60	61.30
LSD 0.05%		0.71	2.88	1.94	1.96	4.95	1.45	2.30	3.23
0.01%		1.02	4.14	2.79	2.82	7.13	2.09	3.31	4.65
Replications (2[#])		16.21	17.51	0.62	0.65	1.63	2.35	10.22	56.64
Genotypes (19)		57.42**	92.04**	17.07**	14.57**	143.02**	12.55**	15.53**	115.89**
Error (38)		0.27	4.39	2.00	2.03	13.00	1.12	2.81	5.52

[#]Degree of freedom, **Significant at 0.01 level

Mean performance and genetic variation for the best 20 selected RILs: Eight traits related to rice yield and its components were analyzed for variation assessment among the best 20 selected RILs (Table 3). From the data analysis, there were significant differences among the rice genotypes for yield and its components. Developing early maturing rice lines with high yielding may be an important objective in many cases. The most desirable mean values towards the earliness were obtained from the promising lines RIL-9 (120 days), RIL-34, RIL-62 and RIL-82 with the same value (123 days) which recorded the earlier lines for the growth duration (days to maturing). The results revealed that the rice genotypes RIL-23, RIL-79 and RIL-86 were the later lines for days from sowing to maturing with values of 136, 136 and 135 days, respectively. For plant height, the varietal impact was significant for all the genotypes. The results varied from 86.7-106.3 cm. The tallest line was RIL-31 (106.3 cm), whereas, the shortest line was RIL-72 (71.74 cm). The genotypes RIL-23, RIL-33, RIL-41, RIL-57, RIL-62, RIL-82 and RIL-84 were similar in plant height, where their height was an intermediate one. In the case of a number of tillers and panicles/plant, the results revealed highly significant differences (< 0.01) among the RILs genotypes and the ranges were from 18.7-26.7 for tillers/plant and 18.3-25.0 for the panicles/plant. The highest numbers of tillers and panicles/plant were from RIL-86 whereas, the lowest numbers were from RIL-34. The values for flag leaf area ranged from 24.1-46.7 cm^2. The RIL-57 genotype had the highest value, whereas, RIL-82 had the lowest value. Panicle length varied significantly among the RILs genotypes and ranged from 20.3-28.8 cm. The highest values (28.8 cm) was observed in RIL-71 followed by RIL-86 (26.1 cm), while the shortest panicle length (20.3 cm) was recorded in RIL-23 which was statistically similar to RIL-9 and RIL-33. The spikelet fertility (%) varied significantly among the RILs genotypes and ranged from 89.3-97.7%. The RIL-7 had the highest value of spikelet fertility (%), which was not significantly different from RIL-62 and RIL-86. The lowest spikelet fertility (89.3%) was recorded in RIL-79 (Table 3). Highly significant difference was observed among RILs genotypes for grain yield trait. The highest grain yield (61.3 g/plant) was found in rice genotype RIL-86, followed by RIL-82 (59.8 g/plant), whereas the lowest value (39.9 g/plant) was observed in RIL-79.

Estimation of genetic variability and heritability: Genetic variability in the investigation genotypes measured by genotypic and phenotypic variation and their coefficient in order to heritability (%). These parameters for the studied traits are shown in Table 4. In this study, the phenotypic variance was higher than the genotypic variances for all traits, indicating the influences of the environmental factor on these



	DM	PH	NTP	NPP	FLA	PL	SF (%)
PH	0.021						
NTP	0.107	-0.049					
NPP	0.113	-0.127	0.984**				
FLA	-0.165	0.005	-0.028	-0.061			
PL	0.096	0.475*	0.140	0.073	0.140		
SF (%)	0.192	-0.172	0.459*	0.443	0.265	0.149	
GY/P	0.323	0.071	0.681**	0.656**	0.551*	0.461*	0.695**

Fig. 3: Phenotypic correlation coefficients between 8 agro-morphological traits in 20 RILs of rice

Table 4: Genetic variability, heritability and genetic advance of the 8 agro-morphological traits in the best 20 RILs population

		Genetic parameters				
Traits	Mean	G.V	P.V	G.C.V	P.C.V	Hbs
Duration (day)	126.32	19.05	19.32	3.46	3.48	98.61
Plant height (cm)	98.14	29.22	33.61	5.51	5.91	86.93
Tillers/plant	22.16	5.02	7.03	10.16	12.01	71.50
Panicles/plant	21.40	4.18	6.22	9.58	11.68	67.25
Flag leaf area (cm^2)	33.24	43.34	56.34	19.81	22.58	76.93
Panicle length (cm)	23.72	3.81	4.93	8.23	9.37	77.27
Spikelet fertility (%)	93.86	4.24	7.05	2.19	2.83	60.11
Grain yield/plant (g)	46.20	36.79	42.31	13.13	14.08	86.94

studied traits and results were similar with Devi *et al.*[28] and El-Shafey *et al.*[29]. In addition, the genotypic and phenotypic coefficient of variation were ranged from low to high among the traits. High GCV and high PCV was recorded for flag leaf area with values 19.81 and 22.58, respectively. Moderate GCV and moderate PCV were recorded for the number of tillers/plant (10.16 and 12.01) and grain yield/plant (13.13 and 14.08), respectively. The number of panicles/plant is the only trait recorded low GCV and moderate PCV with values 9.58 and 11.68, respectively. On the other hand, low estimates of GCV and PCV were observed for the duration (3.46 and 3.48), plant height (5.51 and 5.91), panicle length (8.23 and 9.37) and spikelet fertility (%) (2.19 and 2.83), respectively. Low values of genotypic and phenotypic coefficient of variation indicate the need for creation of variability either by hybridization or mutation followed by selection. El-Shafey *et al.*[29] and Tiwari *et al.*[30] were found similar results in their studies. Heritability is the most important genetic component to the rice breeder, as it indicates the total variance that is inherited from the parental lines. In this investigation, broad sense heritability was high (>75%) for all the traits except the number of tillers/plant (71.50%), number of panicles/plant (67.25%) and spikelet fertility (%) (60.11%). The highest broad sense heritability value (98.61%) was found in duration, whereas, grain yield/plant, plant height, panicle length and flag leaf area had 86.94, 86.93, 77.27 and 76.93% values, respectively. This was also in confirmation with the findings of El-Shafey *et al.*[29], Tiwari *et al.*[30], Nandini *et al.*[31] and Oladosu *et al.*[32].

Phenotypic correlation coefficient between the traits: Estimates of phenotypic correlation coefficient were done between the studied traits in this study and the results are shown in Fig. 3. Correlation analysis of agro-morphological, yield and its components traits revealed that all the traits measured had a positive correlation with grain yield, except for duration and plant height. The strongest positive correlation coefficients were recorded between number of tillers and number of panicles (r = 0.984), grain yield and number of tillers (r = 0.681), grain yield and number of panicles (r = 0.656) and grain yield and spikelet fertility (%) (r = 0.695) indicating the importance of these traits as selection criterion in yield improvement programmes. Moderate positive correlation estimates were observed among grain yield and all of the flag leaf area and panicle

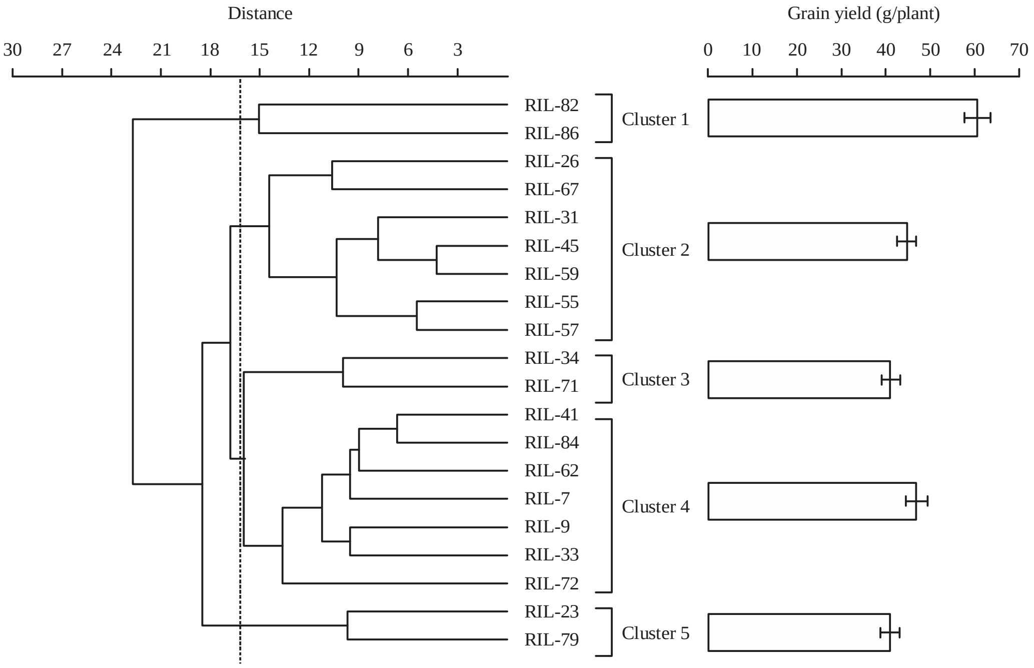


Fig. 4: Dendrogram of 20 rice RILs based on 8 agro-morphological traits constructed by means of paired group and genetic distance

length with values r = 0.551 and r = 0.461, respectively. The positive correlations coefficients between grain yield and number of panicles and spikelet fertility (%) indicated that better exploration of these traits along with flowering and plant height could be used to develop desired rice varieties/lines. This was also in confirmation with the findings of Mustafa and Elsheikh[33], Sabesan *et al.*[34], Jayasudha and Sharma[35] and Anis *et al.*[36] for yield and yield component traits. In the same context, positive and significant correlations were exhibited for plant height with panicle length (r = 0.475) and the number of tillers with spikelet fertility (%) (r = 0.459). Similar results were also reported by Oladosu *et al.*[32].

Cluster analysis based on mean performance of traits: The mean performances of eight agro-morphological traits were employed to calculate the Euclidean distances among the best 20 RILs and the dendrogram was constructed using these values as indicated in Fig. 4. This dendrogram was divided the 20 RILs into five main clusters based on the values of 8 agro-morphological traits. Among the five clusters, cluster 1 was composed of two genotypes, cluster 2 had 7 genotypes, cluster 3 had two genotypes, cluster 4 had seven genotypes and cluster 5 had two genotypes. Generally, the first cluster was contained 2 lines and characterized by higher grain yield/plant. The second cluster included 7 RILs and these lines were characterized by higher flag leaf area and almost similar in the number of tillers, number of panicles and grain yield. The third cluster was composed of 2 genotypes (RIL-34 and RIL-71) and characterized by higher in plant stature. The cluster 4 was consisting of seven lines, members of this cluster were higher in spikelet fertility (%) and grain yield/plant. Like cluster 1 and cluster 3, the 5th cluster was composed of two RILs (RIL-23 and RIL-79) and these two lines were characterized by late in maturing. Similar findings were reported in rice by Chandra *et al.*[37], Rajesh *et al.*[38] and Anis *et al.*[39].

Identification of fertility restoring genes *Rf3* and *Rf4*: This study was employed for identification of fertility restoring genes (*Rf3* and *Rf4*) in a developed RILs population derived from a cross between PR6 and G46B as two parents of a new promising hybrid combination. A total of 18 DNA markers were reported to be linked with two *Rf* genes (*Rf3* and *Rf4*) which were screened among the best 20 selected lines. Genotypic data showed that 10 markers were polymorphic for parents, out of which two markers viz., RM490 and RM1108 that were reported to be linked with fertility restoring genes (*Rf3* and *Rf4*) in different chromosomal locations on chromosome 1 and 10, respectively were further used for screening of fertility restorer genes (Fig. 5, 6). The detected polymorphism reflects the amount of diversity among the tested genotypes and thus the possibility of genetic improvement using a set of genotypes in hybrid rice breeding programs.

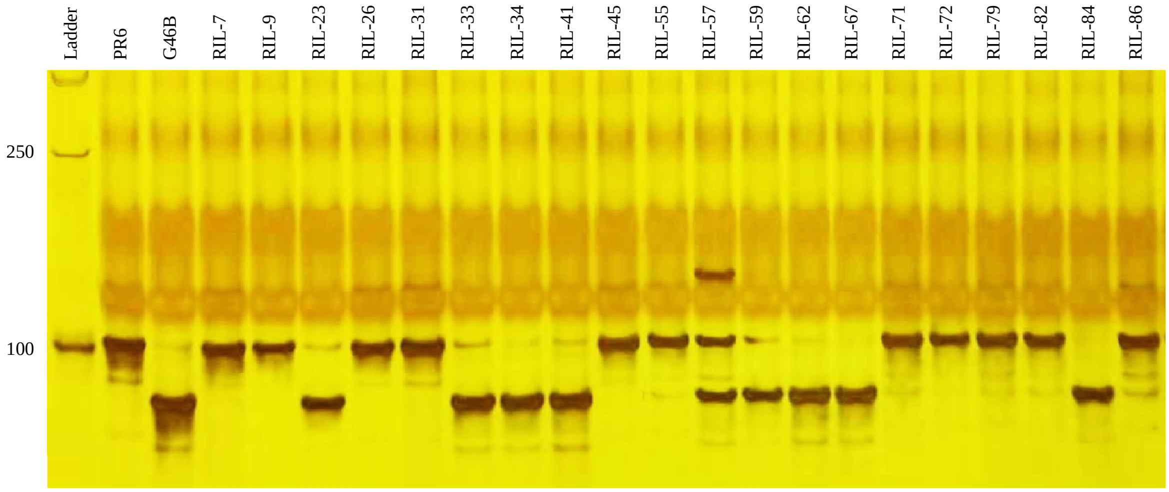


Fig. 5: Banding pattern for 20 recombinant inbred lines and their parents (PR6 and G46B) amplified by the RM490 (*Rf3*) marker

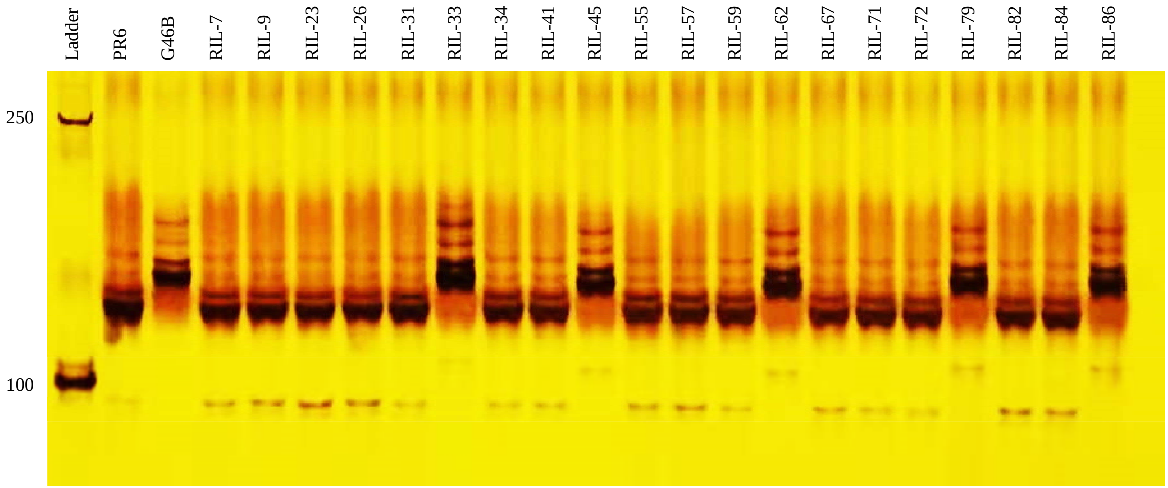


Fig.6: Banding pattern for 20 recombinant inbred lines and their parents (PR6 and G46B) amplified by the RM1108 (*Rf4*) marker

Out of 88 RILs, 20 lines were selected on the basis of their acceptability and superiority in agronomic traits. This part attempts to use the marker assisted selection to identify the restoring RILs possessing *Rf* genes (*Rf3* and *Rf4*) for WA-CMS to accelerate phenotype based screening. The results in Fig. 5 showed that RM490 marker that linked to *Rf3* on chromosome 1 gave three bands (alleles) with sizes of 101bp for restorer parent PR6 and twelve RILs (RIL-7, RIL-9, RIL-26, RIL-31, RIL-45, RIL-55, RIL-71, RIL-72, RIL-79, RIL-82 and RIL-86) while the band with size 55bp was presented in the maintainer parent G46B and six RILs (RIL-23, RIL-33, RIL-34, RIL-41, RIL-59,RIL-62, RIL-67 and RIL-84). Simultaneously, only one line (RIL-57) gave three bands indicating as heterozygous amplification pattern or off-type plant (Fig. 5). In same way breeding lines were screened with the help of SSR marker RM490 linked to *Rf3* gene reported by Singh *et al.*[17] and Ahmadikhah *et al.*[21]. On the other hand, Fig. 6 shows the amplification pattern of *Rf4* gene located on chromosome 10, with the help of SSR marker RM1108. According to the results, this marker gave two bands (alleles) through 20 RILs screening for the presence of fertility restorer gene R*f4*. Fifteen lines (RIL-7, RIL-9, RIL-23, RIL-26, RIL-31, RIL-34, RIL-41, RIL-55, RIL-57, RIL-59, RIL-67, RIL-71, RIL-72, RIL-82 and RIL-84) showed the presence of *Rf4* by amplifying 124 bp size fragment and five lines showed the absence of *Rf4* by amplifying 135 bp size (Fig. 6). Singh *et al.*[15] screened 100 rice lines for the presence of fertility restorer gene *Rf4* located on chromosome 10, with the help of SSR marker RM1108.

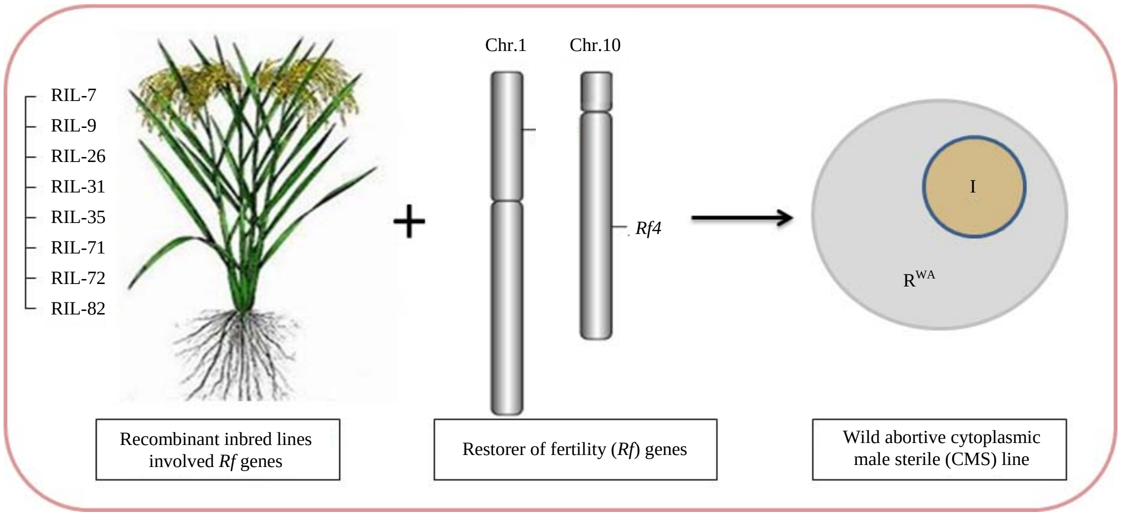


Fig. 7: Schematic showing the most important RILs can be utilized in hybrid rice breeding as restorers of wild-abortive cytoplasmic male sterile lines (WA-CMS)

Based on our results we can confirm that, out of the best twenty RILs breeding lines, 8 lines (RIL-7, RIL-9, RIL-26, RIL-31, RIL-55, RIL-71, RIL-72 and RIL-82) are restorer and including both fertility restorer genes *Rf3* and *Rf4* (Fig. 7). In addition, these superior lines performed higher for spikelet fertility (%) and grain yield/plant. Identification of like these high-yielding restorer lines could be effectively utilized in inbred and hybrid rice breeding programs to develop new hybrid rice varieties. Singh *et al.*[40] used SSR markers RM6 100 and RM 10313 linked to *Rf4* and *Rf3*, respectively to screen a set of breeding lines and identified that 61 lines to carry both *Rf3* and *Rf4* genes and reported that these lines can be utilized in hybrid rice breeding as restorers.

CONCLUSION

Development of multipurpose high-yielding rice lines is a good source of genetic variability, gene discovery and be used in two ways inbred and hybrid rice breeding. In this investigation, we successfully improved 88 Recombinant Inbred Lines (RILs) derived from a cross between PR6 as a restorer and G46B as maintainer. Significant variation was observed among these lines for most of the studied traits. Finally, we identified 20 elite lines combined with high yielding potential. Ten earlier reported SSR markers linked to *Rf3* and *Rf4* have been utilized to screen the best-selected lines. The SSR markers RM490 and RM1108 linked to *Rf3* and *Rf4*, respectively, identified 8 lines (RIL-7, RIL-9, RIL-26, RIL-31, RIL-55, RIL-71, RIL-72 and RIL-82) carry both *Rf3* and *Rf4* genes and these lines can be utilized in hybrid rice breeding program as restorer lines of wild-abortive cytoplasmic male sterile lines (WA-CMS).

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