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Morphological and Molecular Approaches in Identification of Fertility Restorers Among Aerobic Lines of Rice (*Oryza sativa* L.)

Srijan A., Senguttuvel P. Sundaram R. M., Jaldhani V. Sudheer Kumar S., Srinivasa Chary D., Kuldeep Singh Dangi

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ABSTRACT

Successful exploitation of hybrid vigor primarily depends on identification of effective fertility restoration for CMS based hybrid breeding. A study was conducted in identification of restorers from promising aerobic adapted breeding lines utilizing SSR based molecular markers and test crossing with WA based CMS lines for utilizing in hybrid breeding program. The present study was conducted at Indian Institute of Rice Research (ICAR–IIRR) during wet season (*kharif* 2016) with forty-nine aerobic adapted genotypes and initially screened using SSR markers (RM 6100 and DRRM *RF* 3–10) for presence of *Rf4* and *Rf3* fertility restorer genes. Out of forty nine genotypes, twenty-seven genotypes showed at least one of the fertility restoration genes were identified were simultaneously test crossed with IR 79156A CMS line and the derived F_1's were raised for evaluation during dry season (*rabi* 2017). The resultant F_1's expressed different fertility restoration reactions and accessed through pollen and spikelet fertility. Out of which 10 genotypes behaved as complete restorers, 10 as partial restorers and 7 as partial maintainers. The present study has a pivotal role in identification of potent restorer lines for aerobic situation and development of rice hybrids for water limited conditions.

Keywords Restorer, Pollen fertility, Spikelet fertility, Marker screening, *Rf* genes.

INTRODUCTION

Rice is the most staple food across majority parts of India providing calorie requirement of 43% for about 70% of India's population and increase production and productivity of rice is the need of the hour. India having largest acreage under rice crop, spread over 44 M ha (22% of cropped area) recorded annual production of 115.60 MT in the year 2018-19 (Anonymous 2019). Substantial increase in production and productivity of rice from 20 MT (1950) to 115 MT (2018) with the adoption of semi-dwarf type of high yielding varieties, however the genetic gain of recent release varieties since last two decades has come to stagnation and efforts to give tangible results in breaking the genetic yield

Srijan A.[1]*, Senguttuvel P.[2], Sundaram R.M.[2], Jaldhani V.[2], Sudheer Kumar S.[1], Srinivasa Chary D.[1], Kuldeep Singh Dangi[1]
[1, 2]Professor Jayashankar Telangana State Agricultural University, Hyderabad 500001, India
[2]Indian Institute of Rice Research (ICAR-IIRR), Rajendranagar 500030, India
Email : srijan.ambati1@gmail.com
*Corresponding author

Table 1. Summary of molecular screening of genotypes for the presence of *Rf3* and *Rf4* Genes.

Sl. No.	Genotypes	Fertility status	Total
1	ATR - 177, ATR - 186, ATR - 192, ATR - 375 and AR-19-18	*Rf3/Rf4*	5
2	AS - 5, ATR - 187, ATR - 222, ATR - 346, ATR - 356, ATR - 374 and KS-16	*Rf3/Rf4*	7
3	ATR - 189, ATR - 190, ATR - 216, ATR - 278, ATR - 279, ATR - 281, ATR - 372, ATR - 394, KS - 8, KS - 10, KS - 15, KS - 22, KS - 24, KS - 49 and HRSV - 7	*Rf3/Rf4*	15
4	AS - 17, ATR - 165, ATR - 170, ATR - 173, ATR - 174, ATR - 175, ATR - 178, ATR - 179, ATR - 217, ATR - 218, ATR - 285, ATR - 331, ATR - 342, ATR - 343, ATR - 344, ATR - 345, ATR - 351, ATR - 352, ATR - 363, ATR - 364, ATR - 373 and KS - 26	*Rf3/Rf4*	22

barrier in rice failed. In order to keep up the pace with this growing population, the rice requirement by 2025 was estimated to be about 130 MT (Kumar *et al.* 2016). Rice production system are also likely to undergo major changes due to plateauing trend in the yield of high yielding varieties, declining and degrading natural resources like water, land and acute labor shortage making this task of increase of rice production quite challenging (Katara *et al.* 2020).

Following China's success in the commercialization of hybrid rice, India was one of the countries to start applied strategic research program on hybrid rice. Up to date, WA-CGMS (Wild abortive-Cytoplasmic Genetic Male sterility) based sporophytic breeding system is commercially utilized in production of three-line rice hybrids resulted in release of 107 rice hybrids with a substantial increase in rice area and production. Hybrid rice technology gained momentum because of its yield heterosis by 15–30% over commercial varieties (Virmani 1996).

The fertility restoration trait of WA-CMS was consistently explained to be governed by two independent dominant major genes (Govindaraj and Virmani 1988, Teng and Shen 1994) and have been mapped on chromosome 10 and 1 as *Rf4* and *Rf3* respectively (Yao *et al.* 1997, Zhang *et al.* 1997). Studies indicated that *Rf4* has maximum effect in restoration than *Rf3* (Katara *et al.* 2017). The phenotypic screening for restorer identification is a time consuming and laborious event since it involves test crosses using a set of CMS lines and evaluating F_1s for per cent spikelet and pollen fertility. With the advent of molecular markers are becoming increasingly useful in identification of trait of interest and thereby enhancing the efficiency in crop improvement.

With increasing population and scarcity of water, labor, time and cultivable land forced breeders to look for a viable water saving technologies to cope up for the prevailing climate change scenario. Recent years, direct seeded aerobic rice gaining momentum for water limited ecologies. Nevertheless, yield heterosis of the present day hybrids bred for irrigated ecologies and not adapted to dry direct seeded aerobic system. Keeping in above needs, the present study focused in identification of potent restorer lines from forty-nine aerobic adapted breeding lines and its probable utilization in development of hybrids for water limited conditions.

MATERIALAS AND METHODS

The present experiment was carried out in *kharif* 2016 at Indian Institute of Rice Research (ICAR-IIRR) farm and marker work at Molecular Lab of Hybrid Rice section (Crop Improvement Division). Forty nine genotypes were first subjected to molecular screening using SSR markers (RM 6100) and (DRRM *Rf3*-10) for restorer identification. Further test cross nursery was raised to test cross those lines which expressed at least one of the genes to identify the restorers lines.

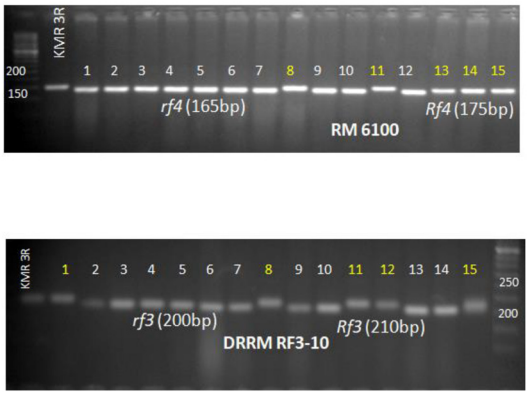

Fig. 1. Amplification profile of the gene Linked markers (RM 6100 and DRRM *Rf3*-10) for fertility restoration. [KMR-3 : Positive control ; Genotypes 1–15 are AS – 5, AS – 17, ATR – 165, ATR – 170, ATR – 173, ATR – 174, ATR – 175, ATR – 177, ATR – 178, ATR – 179, ATR – 186, ATR – 187, ATR – 189, ATR – 190 and ATR – 192].

DNA isolation and PCR analysis

DNA was isolated from forty-nine genotypes (Table 1) of 15–20 days old young leaves by Mini-preparation method (Dellaporta *et al.*1983). Screening for the major fertility restorer genes, *Rf3* and *Rf4* was carried out using tightly linked molecular markers viz., DRRM *Rf3*-10 and RM 6100, respectively. The polymerase chain reactions and cycling conditions were followed (Balaji *et al.* 2012) for the both linked primers of *Rf* genes. The PCR products which are amplified, along with 100 bp ladder (Bangalore Genie, India) were separated on a 3.0% Seakem ® LE agarose gel (Lonza, USA) and stained with ethidium bromide (Revathi *et al.* 2013) and documented using Gel documentation system (Alpha Innotech, USA).

Pollen fertility

Pollen fertility studies were carried out using anthers collected from spikelets at 2 days before anthesis. The anthers from each spikelet were smeared out into a drop of 1% Iodine-potassium iodide (I-KI) solution, placed on a glass slide. Fertile as well as sterile pollen were counted in the three randomly selected microscopic fields. Those pollen which are stained, round and well filled were counted as fertile, whereas unstained, shriveled and empty were considered sterile (Ponnuswamy *et al.* 2020). Pollen fertility was calculated as percentage is given below :

$$\text{Pollen fertility (\%)} = \frac{\text{No. of fertile pollen grains}}{\text{Total no. of pollen grains}} \times 100$$

Estimation of spikelet fertility

Estimation of spikelet fertility per cent was done based on the observations from three panicles per plant (two randomly selected and one panicle from the main culm) from a total of five plants randomly selected for every test cross hybrid while at maturity. Spikelet fertility of hybrids was estimated by counting number of well filled and chaffy spikelets in every panicle (Ramesh *et al.* 2018).

$$\text{Spikelet fertility (\%)} = \frac{\text{No. of filled spikelets/ panicle}}{\text{Total no. of spikelets/ panicle}} \times 100$$

Classification of pollen parents

The pollen parents were classified into four categories - Restorers (R), Partial Restorer (PR), Partial Maintainers (PM) and Maintainers (M) depending upon the their percent pollen fertility and spikelet fertility.

RESULTS

Molecular marker screening was taken up, looking for the presence of *Rf4* and *Rf3* gene, using SSR markers namely RM 6100 (Singh *et al.* 2005) and DRRM RF3-10 (Balaji-Suresh *et al*. 2012). In this study KMR-3 was used as positive control (Fig. 1). Based on molecular screening fifteen genotypes were identified to carry *Rf4* gene, seven genotypes were having *Rf3* gene and five genotypes showed the presence of both *Rf3* and *Rf4* genes (Table 1).

Further, all the genotypes those carrying at least

Table 2. Study of fertility restoration for restorers and maintainer identification among 27 lines test crossed with IR -79156A. *R - Restorer ; PR – Partial Restorer ; PM – Partial Maintainer.

Sl. No.	Test cross	Days to 50% flowering	Spikelet fertility %	Pollen fertility %	Fertility restorer gense *Rf3*	*Rf4*	Fertility reaction
1	IR-79156A × AS - 5	99.00	38.89	41.88	1	0	PM
2	IR-79156A × ATR - 177	87.00	85.53	88.7	1	1	R
3	IR-79156A × ATR - 186	90.00	82.64	86.25	1	1	R
4	IR-79156A × ATR - 187	91.00	47.87	49.64	1	0	PM
5	IR-79156A × ATR - 189	92.00	70.78	73.26	0	1	PR
6	IR-79156A × ATR - 190	97.00	45.76	48.32	0	1	PM
7	IR-79156A × ATR - 192	95.00	51.69	54.78	1	1	PR
8	IR-79156A × ATR - 216	72.00	86.73	89.66	0	1	R
9	IR-79156A × ATR - 222	90.17	44.62	47.86	1	0	PM
10	IR-79156A × ATR - 278	95.00	71.74	73.38	0	1	PR
11	IR-79156A ×ATR - 279	98.00	72.37	74.8	0	1	PR
12	IR-79156A × ATR - 281	99.00	68.82	72.33	0	1	PR
13	IR-79156A × ATR - 346	88.00	46.88	49.22	1	0	PM
14	IR-79156A × ATR - 356	94.00	40.77	44.75	1	0	PM
15	IR-79156A × ATR - 372	88.00	91.08	94.52	0	1	R
16	IR-79156A × ATR - 374	101.00	78.77	82.44	1	0	R
17	IR-79156A × ATR - 375	98.00	77.87	81.36	1	1	R
18	IR-79156A × ATR - 394	97.00	70.43	73.98	0	1	PR
19	IR-79156A × KS - 8	92.00	51.80	54.25	0	1	PR
20	IR-79156A × KS - 10	101.00	49.59	52.82	0	1	PR
21	IR-79156A × KS - 15	97.00	60.83	65.36	0	1	PR
22	IR-79156A × KS - 16	96.00	72.17	75.66	1	0	PR
23	IR-79156A × KS - 22	95.00	76.09	80.84	0	1	R
24	IR-79156A × KS - 24	98.00	80.52	83.96	0	1	R
25	IR-79156A × KS - 49	103.00	44.79	48.52	0	1	PM
26	IR-79156A × AR-19 -18	96.00	78.83	82.58	1	1	R
27	IR-79156A × HRSV - 7	86.00	85.42	89.12	0	1	R

one of these two genes were test crossed to identify restorer lines phenotypically. There sults pertaining to the attempted test crosses, 27 test crosses progeny were successfully evaluated (Table 2). Data pertaining to the test cross progeny viz., days to 50% flowering, per cent pollen and spikelet fertility were recorded. For days to 50% flowering, test cross hybrids ranged from 86 days to 103 days. The per cent pollen fertility of test cross hybrids ranged from 41.88% to 94.52% while, spikelet fertility ranged from 38.89% to 91.08%.

Ten genotypes have behaved as complete restorers, ten partial restorers and seven partial maintainers (Table 3). These 10 restorers (ATR - 177, ATR - 186, ATR - 216, ATR - 372, ATR - 374, ATR - 375, KS-22, KS-24, AR-19-18 and HRSV-7) can be used for the development of novel hybrids, as parental lines.

DISCUSSION

Variation in per cent pollen and spikelet fertility ranges across the test cross combinations was also reported by previous workers (Ali *et al.* 2014, Srijan *et al.* 2015). This variation may be attributed to occurrence of different pollen fertility - restoring genes or their differential penetrance or expressivity in different genotypes (Umadevi *et al.* 2010) or due to existence of modifiers genes (Pande *et al*. 1990). Experiments for screening for fertility restoration, documented that efficiency of combination of tightly linked molecular markers viz., *Rf3* and *Rf4* in fertility restoration was in range of 85–92% (Revathi *et al*. 2013). Therefore, further evaluation is carried out without confining ourselves to move forward with lines confirming to the above combination of dominant restorer genes. It is because there is no one combination of markers reported till today, that can

Table 3. Classification of the genotypes based on pollen and spikelet fertility per cent.

Sl. No.	Class	Spikelet fertility %	Pollen fertility %	No. of genotypes identified	List of genotypes
1	Restorers	>75	>80	10	ATR - 177, ATR - 186, ATR - 216, ATR - 372, ATR - 374, ATR - 375, KS-22, KS-24, AR-19-18 and HRSV-7
2	Partial Restorers	50-75	50.1- 80	10	ATR - 189, ATR - 192, ATR - 278, ATR - 279, ATR - 281, ATR - 394, KS-8, KS-10, KS-15 and KS-16
3	Partial Maintainers	0.1-50	1.1- 50	7	AS-5, ATR - 187, ATR - 190, ATR - 222, ATR - 346, ATR - 356 and KS-49
4	Maintainers	0	0- 1	0	Nil

identify restorer with 100% efficiency. These results were in accordance with those reported previously (Ahmadikhah *et al.* 2007, Balaji-Suresh *et al.* 2012, Arun Kumar *et al.* 2014, Namaky *et al.* 2016). The Line ATR-374 is one such line that found positive for *Rf3* only but yet succeeded in getting identified as restorer which was in agreement with earlier reports (Katara *et al.* 2017).

Though using diverse CMS lines in test crosses can give much validity to such fertility restoration studies (Das *et al.* 2013), there is great need to develop a panel of markers that can give 100% efficiency in identifying a restorer, without losing valuable germplasm as failing in molecular screening with limited markers.

CONCLUSION

The findings of present study, throws light that genotype's fertility restoration reaction differ with their genetic background. In molecular screening using any combination of *Rf3* and *Rf4* candidate gene based or gene linked markers have till now not found to identify restorers with 100% efficiency. The restorer lines, identified in this experiment can be a choice material as pollen parent in developing new commercial aerobic rice hybrids. Novel restorers can be developed by utilizing them in crossing programs that helps to expand the genetic base of restorer by means of pyramiding complementary traits out of diverse sources as per the framed breeding objectives.

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