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Evaluation of Salinity-Tolerant Backcrossed Inbred Lines (BILs) For Fertility Restoration Using Molecular Markers

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Abstract

Fertility restoration is a very important trait for the WA-CMS system in hybrid rice. Fifty-five BILs derived from KMR3 and FL478 cross combination possessing *Saltol1* generated through Marker Assisted Backcross Breeding (MABB) approach were screened using *Rf4* and *Rf3* markers during Rabi 2021. The use of molecular markers tightly linked with fertility restoration aided in identifying promising groups of restorers, maintainers, partial restorers, and partial maintainers. The presence of both *Rf4* and *Rf3* alleles among the nineteen BILs was 35% which showed a clear difference in the marker distribution among the BILs. The BILs namely TCP32, TCP34, TCP35, TCP36, TCP36-3, TCP37-2, TCP38, TCP13, TCP18, TCP19, TCP45, TCP46, TCP48, TCP54, TCP56, TCP57-1, TCP60, TCP61, TCP61-3 were identified as promising restorers possessing both *Rf4* and *Rf3* genes. The current study reflects that *Rf4 and Rf3* genes in combination help in the breeding of WA-CMS-based hybrids in *Saltol* introgressed restorers with better heterosis under various ecologies, especially coastal saline areas.

Keywords: WA-CMS, MABB, Rf3, Rf4, Saltol, Heterosis

Introduction

Rice is the largest consumed food grain in the world and its consumption was estimated to increase by 3% to 108 million tonnes (USDA, 2021; https://www.fas.usda.gov/ commodities/rice). The worldwide production of rice for 2021-2022 is estimated to be around 515.05 million tonnes, whereas India's production for the year 2021-22 was 126500 metric tonnes (https://www.worldagriculturalproduction.com/crops/rice.aspx). Achieving food security is the most important criterion to meet the food demands of the increasing global population and this is possible through adopting hybrid rice cultivation. Utilization of hybrid rice technology for greater heterosis is vital for increasing rice production all over the world (Anis, 2019). Identification of restorer lines (that restore the fertility of CMS lines) is the foremost step for superior-yielding heterotic rice hybrids (Venkanna *et al.,* 2022). The production of fertile pollen is supposed to be restored by a nuclear gene called the restorer of fertility (*Rf*) by modifying the male sterility effect (Katara *et al.,* 2017). Previous studies on fertility restoration were confirmed to be governed by two nuclear genes which are dominant and independent as well (Venkanna *et al.,* 2022). The two nuclear genes *Rf3* and *Rf4* on chromosomal locations 1 and 10 respectively were reported for the fertility restoration of the WA-CMS system (Katara *et al.,* 2017). The major locus for WA-CMS fertility restoration is identified as *Rf4* from previous studies (Balaji Suresh, 2012). RM6100 (Singh *et al.,* 2005) at 1,837,2167 bp for *Rf4* has been confirmed on Chromosome 10 of Nipponbare (NC_008403) while RM10313 for *Rf3* gene has been identified by Neeraja (2008) at a distance of 4.2 cM on the short arm of chromosome 1.

Markers for candidate genes have been developed and validated with the aid of marker-assisted breeding and molecular mapping (Suresh *et al.,* 2012). Gene-based functional markers like RMS-PPR9-1 and RMS-SF21-5 for *Rf4* and *Rf3* respectively were developed by Pranathi *et al.,* (2016). A few of the reported markers for *Rf4* and *Rf3* are represented in **Table 1**. Screening for *Rf*3 and *Rf*4 fertility restoration genes based on markers fetch in quick identification of restorers within bulk genetic stock (Nagamani *et al.,* 2022). In the context of the identification of superior restorers along with fertility restorer genes, we have attempted to improve the parental line KMR3 whose genetic background has *Rf3* and *Rf4* to develop a salt-tolerant hybrid for saline-prone ecosystems. In the present study, an advanced BC_2F_4 population derived from KMR3 and FL478 (donor for *Saltol1*) using a marker-assisted breeding approach (MABB), was developed and these BILs were evaluated for the presence of *Rf3* and *Rf4* genes.

Materials and Methods

The backcrossed inbred lines (55 BILs) at BC_2F_4 generation derived using MABB approach for salt tolerance were evaluated for the presence of fertility restoration genes *Rf4* and *Rf3*. The recurrent parent KMR3 is a popular restorer, containing *Rf4* and *Rf3*, and APMS6B, a negative check for fertility restoration was used along with the BILs. 21-day-old healthy seedlings were raised in the nursery and transplanted to the field using a randomized complete block design (RCBD) with two biological replicates. The genomic DNA was isolated using the CTAB method from the leaves of the established BILs after 21-days after transplanting. The genotyping was done in the molecular laboratory, Crop improvement section, Hybrid Rice, Indian Institute of Rice Research, Hyderabad. The primers used for genotyping of the improved BILs were RM6100, DRCG-RF4-14, RMS-PPR-9-1 for *Rf4* and RM10313, DRRM-RF3-10, RMS-SF-21-5 for *Rf3* (**Table 1**). The PCR was run at 94°C for 5 min, 94°C for 30 secs, 55°C for 1 min, 72°C for 1 min, and 72°C for 10 minutes in a thermal cycler (*BIO-RAD, T100™* Thermal Cycler, USA); the amplified product was stored at 4°C. The components were resolved in the 3% agarose gel (*Seakem®LE Agarose*) and visualized under the UV documentation system (IGENE ®LABSERVE) and scored accordingly.

Table 1. Molecular markers reported for *Rf3 and Rf4*

S.No.	Reported markers	Genes	Chromosome number	Reference
1	RM6100	*Rf4*	10	Singh *et al.,* 2005; Sheeba *et al.,* . 2009
2	RMS-PPR9-1	*Rf4*	10	Pranathi *et al.,* . 2016
3	DRCGRF4-14	*Rf4*	10	Balaji Suresh *et al.,* . 2012
4	DRCG-RF4-8	*Rf4*	10	Balaji Suresh *et al.,* . 2012
5	TMPPR3	*Rf4*	10	Balaji Suresh *et al.,* . 2012
6	RM10313	*Rf3*	1	Neeraja 2009
7	DRRM-RF3-5	*Rf3*	1	Balaji Suresh *et al.,* . 2012
8	DRRM-RF3-10	*Rf3*	1	Balaji Suresh *et al.,* . 2012
9	RMS-SF21-5	*Rf3*	1	Pranathi *et al.,* . 2016

Results and Discussion

Fifty-Five (55) BILs conferring salinity tolerance were genotypically screened for the presence/absence of fertility restoration genes *Rf4* and *Rf3*. The primers used for screening are RM6100, DRCG-RF4-14, RMS-PPR-9-1 for *Rf4* and RM10313, DRRM-RF3-10, RMS-SF-21-5 for *Rf3*. The genotypes were classified into four groups *viz.*, restorers, partial restorers, maintainers, and partial maintainers based on the presence/absence of the desired allelic pattern.

The primer RMS-PPR9-1 for *Rf4* has a positive allele for restorer at 114 bp and a non-restorer had band size at 159 bp. Similarly, the candidate gene DRCG-RF4-14 had a positive allele at 782 bp for the R line and 887 bp for the B line. The functional marker for *Rf3*, RMS-SF21-5 had positive alleles at 172 bp and 127 bp for restorer and non-restorer respectively. The gel pictures for *Rf4* and *Rf3* screened were represented in **Figure 1**.

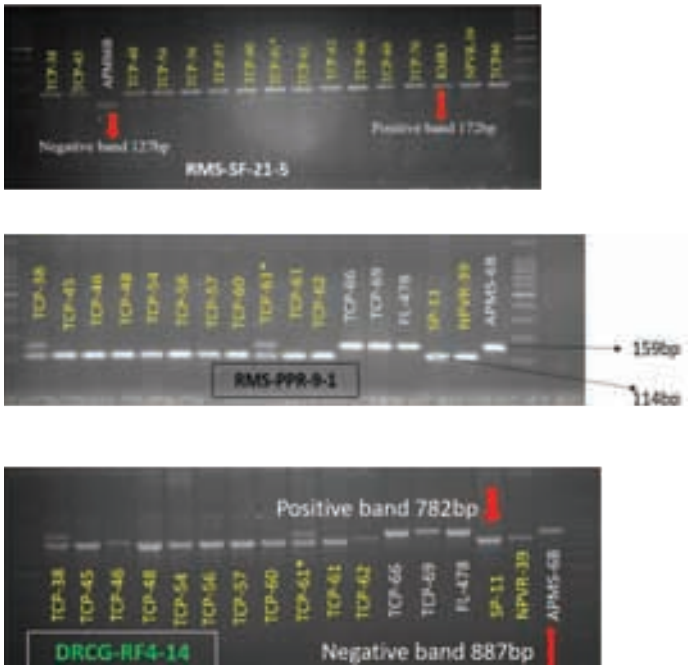

Figure 1: BILs screened for the presence of *Rf3* and *Rf4* a) functional marker for *Rf3,* RMS-SF-21-5; b) functional marker for *Rf4,* RMS-PPR9-1; c) candidate gene DRCG-RF4-14 for *Rf4*

Out of 55 BILs, nineteen BILs (19) were reported to be positive for both *Rf4* and *Rf3* considered as restorers along with the parental line KMR3 which are TCP32, TCP34, TCP35, TCP36, TCP36-3, TCP37-2, TCP38, MB13, MB18, MB19, TCP45, TCP46, TCP48, TCP54, TCP56, TCP57-1, TCP60, TCP61, TCP61-3. Thirty-three (33) were found to be positive for only *Rf4* allele which includes TCP32, 34, 35, 36,36-3, 37, 37-2, 38, 39, 11, 12, 13, 14, 15,16, 17, 18, 19, 45, 46, 47, 48, 30, 54, 55, 56, 57-1, 60, 61, 61-3, 62, 63 and 67 along with KMR3 and twenty-nine (29) positive for *Rf3* alone that are TCP32, 34, 35, 36, 36-3, 37-2, 38, 13, 18,19, 20, 21, 45, 46, 3, 48, 54, 56, 57-1, 72, 74, 58,59,60, 61, 61-3, 64 and 68. The percentage of *Rf4* contribution alone was 60% while *Rf3*s was 52.72%. Both *Rf4*+*Rf3* were present in 34.54% among the 55 BILs. The number of BILs positive for their respective markers was graphically represented in the clustered column in **Figure 2**.

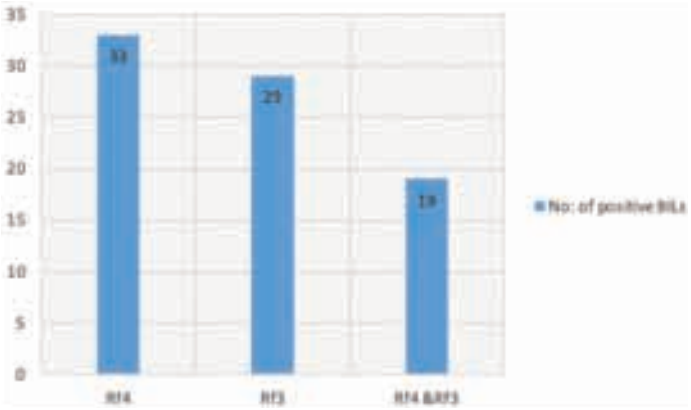

Figure 2: Clustered column of positive BILs against respective markers

The effectiveness of *Rf4* and *Rf3* markers genotypically was confirmed for fertility restoration based on various studies by the researchers. Screening of 310 NPT lines for fertility restoration targeting *Rf3* and *Rf4* using DRRM-*Rf3*-5 and DRRM-*Rf3*-10 and functional markers like RMS-SF21-5; RM6100 and functional marker RMS-PPR9-1 was reported by Shidenur *et al.,* (2020). Pranathi *et al.,* (2016) screened to distinguish 120 restorers and 44 non-restorers for fertility restoring ability genotypically and further developed functional markers for *Rf3* and *Rf4*. Similarly, a total of 51 genotypes were also screened for *Rf4* and *Rf3* and strong restorers and maintainers were identified using RM6100 and RM10313, respectively (Nath *et al.,* 2020). Nagamani *et al.,* (2022) screened 62 red-kernelled genotypes using RM6100, RMS-PPR9-1, and RMS-SF21-5 and identified restorer lines in combinations *Rf*3 and *Rf*4. Among the 24 genotypes screened by Rashid *et al.,* (2019) three genotypes were confirmed as complete restorers based on the screening with RM6100 and DRCG-RF4-14 for *Rf4* and DRRM-RF3-10 for *Rf3*. New markers (RM304, RM258 on Chromosome 10 and RM23598 on Chromosome 9) were found to be related to fertility restoration when screened with various SSR markers in an F2-derived population (Thakur *et al.,* 2021). Ramalingam *et al.,* (2020) screened *Pi54* introgressed BC_3F_2 lines for fertility restoration using DRRM-RF3-10, DRCG-RF4-8, and

RM6100 and identified potential restorers with *Pi54* target gene and the potential restorers were planned for hybrid development. Katara *et al.,* (2017) also screened 570 Indian-rice varieties for the identification of restorer genes using DRRM-RF3-10 and RM6100 and identified 40 potential restorers. In another study thirty-one (31) tropical *japonica-derived* rice hybrids were screened and distinguished into *Rf3, Rf4,* and *Rf3* + *Rf4* hybrids (Shidenur *et al.,* 2020).

Nagaraju *et al.,* (2021) also screened seventy-one (71) BILs derived from drought-tolerant parents for fertility restoration using *Rf4* and *Rf3* markers RM6100, RMS-PPR-9-1, DRCG-Rf4-14, for *Rf4* and DRRM-RF3-10, RM10313, and RMS-SF21-5 for *Rf3* respectively and identified ten restorers with *Rf4* and *Rf3* alleles in combination. Several other findings from various rice accessions screened for fertility restoration have reported the efficiency of *Rf4* and *Rf3* frequencies. Based on all the above outcomes these markers for *Rf4* and *Rf3* can be considered to speed up the breeding program of restorer lines in rice (Rashid *et al.,* 2019; Balaji Suresh *et al.,* 2012).

Conclusion

Based on genotyping for Saltol and fertility restoration the BILs TCP38, TCP45, TCP46, TCP48, TCP54, TCP56, TCP57-1, TCP60, and TCP61 were found to possess Saltol+*Rf4*+*Rf3*. Therefore, *Rf4* and *Rf3* were found to be the major fertility-restoring genes based on many research findings including our experiment. These genes were proved to restore complete fertility and play a major role in the three-line breeding of rice. The *Saltol* introgressed hybrids may confer salinity tolerance, especially in saline-prone rice ecosystems with superior yield heterosis.

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