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Evaluation of improved drought-tolerant parental lines of KMR3R for fertility restoration by molecular analysis

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Abstract

The present investigation was carried out to identify fertility restoration in improved drought tolerant parental lines with ideal agronomic trait performances and their utilization in the hybrid rice breeding program. Backcross derived lines obtained from a cross between a drought-tolerant Vandana NIL (donor, possessing *qDTY12.1* and *qDTY1.1*) and a drought susceptible restorer, KMR3R (recurrent parent, possessing *Rf3* and *Rf4* genes). Based on foreground and background selection, the backcross population was advanced to BC_2F_4 population by stringent Marker-Assisted Backcross Breeding (MABB). Molecular markers were utilized in the marker assisted restorer selection in WA-CMS from large source of nurseries to keep away from regular test cross nursery (TCN) in the hybrid rice breeding technology. The tightly linked/or gene-specific markers *viz.*, RM6100, RMS-PPR-9-1, DRCG-Rf4-14, for *Rf4* on chromosome 10, and DRRM Rf3-10, RM 10313, and RMS-SF21-5 for *Rf3* located on chromosome 1 were used to screen for the presence or absence of specific restorer allele in the population. 71 improved drought-tolerant backcross inbred lines (BILs), including checks, and parents were screened for their fertility restoration. Results were skewed in their frequency distribution by showing 48.48% of *Rf3* and *Rf4* genes (*Rf3Rf3/Rf4Rf4*). These double allelic combination containing genotypes exhibit better fertility restoration than any of single *Rf3Rf3/rf4rf4* or *rf3rf3/Rf4Rf4* individually. Ultimately ten genotypes were identified as complete restorers (RP6340 NPVR1, RP6340 NPVR3, RP6340 NPVR10, RP 6340 NPVR24, RP6340 NPVR27, RP6340 NPVR32, RP6340 NPVR48, RP6340 NPVR52, RP6340 NRR5 and RP6340 NRR11) with drought QTLs for drought-prone lowland ecosystems and can be utilized in the hybrid rice breeding programme under unfavorable drought ecologies.

Keywords: Rice, hybrid, drought tolerance, fertility restoration, molecular markers

Introduction

Rice is an essential food cereal crop for more than half of the world's population and livelihood. The utmost priority of rice production is to satisfy the hunger of the escalating population and to improve food security. The productivity is inadequate to meet the future demand of escalating population in India (Shidenur *et al.*, 2019). Among various genetic approaches available today to enhance the yield potential, hybrid rice technology is the most encouraging, and accepted strategy for improving rice productivity. Though rice is a self pollinated crop, exploitation of heterosis through male sterility system is a prerequisite and distinctly shown standard heterosis of 15–20% over the commercially cultivated indica genotypes/high yielding varieties in a similar growth environment (Virmani *et al.*, 2003; Gramaje *et al.*, 2020). The heterosis proportion depends on the possible course of existing hybrid rice parental lines.

India occupied second place after China in the production of hybrid rice. India covers an area of 2.8 Mha of hybrid rice production (Senguttuvel *et al.*,

2019). However, India reaches only about 5.6% of the total rice area; ten times lower than that of China (Katara *et al.,* 2017). Nevertheless, distinct heterosis and adaptability, the slow spread of hybrid rice technology is attributed to various biotic and abiotic stresses. Hence, the development of genetically potential and suitable hybrids for different ecologies with a high level of heterosis along with desirable grain and cooking quality is imperative to enlarge its cultivated area (Verma *et al.,* 2021).

In India, hybrid rice is primarily developed using a three-line system, which involves cytoplasmic male sterile line (A-line; male-sterile), an isonuclear maintainer line (B line; male fertile) and restorer line (R line; male fertile with fertility restorer gene). Drought is the primary and most important abiotic factor among all abiotic stresses. Exploitation of plant tolerance to drought stress is considered a viable strategy to enhance the potential of rice hybrids under drought-prone ecology. Cytoplasmic male sterility (CMS) is a maternally inherited trait that results in the plant lacking the capacity to produce fertile pollen. Nuclear-encoded genes restore pollen fertility called the fertility restorer (*Rf*) gene. Hence, to develop high-yielding heterotic hybrids, the first and foremost step is to identify restorers (R line) that can efficiently restore the fertility of CMS (A) lines.

The restorer lines employed to develop new rice hybrids remain susceptible to drought stress. Therefore, in the present study, we attempted to fortify the restorer line, KMR-3R (a restorer line), as a recurrent parent carrying major restorer genes (*Rf3* and *Rf4*) to develop hybrids tolerant to drought stress. The donor parent, Vandana NIL (drought tolerant), possessed major qDTYs *viz.,* qDTY12.1 and qDTY1.1. This approach was carried out by marker-assisted backcross breeding (MABB) and 71 (BC_2F_4) backcross inbred lines (BILs) were generated.

Two fertility restorer *Rf3* and *Rf4* genes are essential for viable pollen production in the WA-CMS type of CMS system (Bhati *et al.,* 2018). The use of molecular markers linked to *Rf* genes can enhance the selection efficiency and remove the impediments related to phenotype screening. The analysis of genetic linkage indicated that SSR markers RM6100 (Singh *et al.,* 2005) on the long arm of chromosome 10, is linked with the *Rf4* gene (at 1.2 cM) and RM10313 (Neeraja *et al.,* 2009) on the short arm chromosome 1 is linked with *Rf3* gene (at 4.2 cM). SSR marker RM6100 may facilitate MAS selection of WA-CMS-based restorer lines by avoiding regular testcross in large breeding genotypes in a hybrid rice breeding program (Sheeba *et al*., 2009; Kiani *et al.,* 2015). Other candidate gene markers (Suresh *et al.,* 2012; Pranathi *et al.,* 2016) have been utilized efficiently to screen the BIL population to identify superior drought tolerant restorers The MAS (Marker-assisted selection) program was carried out to introgress the drought-tolerant qDTYs, while maintaining fertility restoration (*Rf3* and *Rf4*) genes into popular restorer lines to develop agronomically superior hybrid rice parental lines for drought-prone lowland ecologies.

Materials and Methods

Plant genetic material

The study material comprises of 71 (BC_2F_4) improved Backcross Inbred Lines (BILs) (including parents and checks) derived from a drought-tolerant donor parent, Vandana NIL (*rf3rf3/rf4rf4*), and an elite restorer recurrent parent KMR3R (*Rf3Rf3/Rf4Rf4*). Along with parents, checks including two maintainers (B) lines, IR79156B and APMS-6B devoid of fertility restoration (*rf3rf3/rf4rf4)* and two restorers (R) lines, RPHR1005 and BK49-72 with complete fertility restoration (*Rf3Rf3/Rf4Rf4)*, were utilized as negative and positive controls respectively, for Rf3 and Rf4 alleles.

Experimental details

All the experiments were conducted during the wet season (*Kharif,* 2018) at Research Farm, ICAR-Indian Institute of Rice Research (IIRR, 17.3200˚ N, 78.3939˚ E), Hyderabad, India. Standard agronomical practices and plant protection measures were followed to ensure healthy crop. 21-day old seedlings were transplanted into the main field using a randomized complete block design (RCBD) with three biological replications. The stringent phenotypic and genotypic screening was employed to identify desired plants with restoring ability and drought tolerance at BC_2F_4

population using marker-assisted backcross breeding (MABB) approach.

Genotyping of parents and BILs

Genomic DNA was extracted from the young leaf tissues of parents collected at active tillering stage and BILs using the CTAB method (Dellaporta *et al.,* 1983). DNA quantification was done using 0.8% of agarose gel. For microsatellite assay, PCR reaction mix was prepared and carried out using 50 ng/l of isolated template DNA, containing 0.5µl of each forward and reverse primer, 1µl (2.5 mM of each) of dNTP, 0.2 µl of Taq DNA polymerase, 10µl of 10X PCR reaction buffer in a total volume of in thermal cycler (Eppendorf, USA). PCR Amplification was programmed by following steps: as 94℃ for 4 min, followed by 35 cycles of 94℃ for 30sec denaturation, 55℃ for 1min of annealing, and 72℃ for 1 min of extension and last step is 5min at 72℃ for the final extension. The amplified PCR products, along with 100 bp molecular marker (Bangalore Genie, India), were electrophoresed on a 3.0% agarose gel (Seakem® LE), stained with ethidium bromide, and the gel was documented using Gel documentation unit (Alpha Innotech). Unambiguous and resolved DNA bands were scored for their presence visually for each reported primer. BILs (BC_2F_4) were screened for the fertility restoration status and positive checks for restorer and maintainer. This was successfully done by closely linked reported SSR (Simple sequence repeat) fertility restoration markers for *Rf4* and *Rf3* locus (**Table 1**).

Results and Discussion

Hybrid rice technology has been introduced successfully in more than 40 countries, and India is the second largest country in the adoption and production of hybrid rice since 1989 (Yuan *et al.,* 2017). India has made substantial progress and released 127 hybrids for commercial cultivation which are mostly suitable to irrigated ecologies (Senguttuvel *et al.,* 2019). Due to the unambiguous specificity of hybrids released so far in India, *viz.,* lack of specific ecosystem, tolerance to several biotic and abiotic stresses (drought, salinity, submergence, etc.), and consumer's preference (Rout *et al.,* 2020) exploitation of genetic diversity of drought tolerant parental lines along with fertility restoration is a convenient way for the development of promising drought tolerant hybrids (Singh *et al.,* 2021). Hence, this study was undertaken to develop the parental lines suitable for drought-prone lowland and upland ecosystems besides the normal irrigated environment.

Screening for fertility restoration genes *Rf3* and *Rf4*

Identifying superior parental lines harboring restorer genes through molecular approach is desirable as phenotyping is a very time-consuming and tedious process, and spikelet sterility in testcross progeny needs to be determined (Ahmadikhah *et al.,* 2007). The success of hybrid rice is largely dependent on high pollen and spikelet fertility due to the high compatible interaction of both *Rf3* and *Rf4* genes and CMS cytoplasm (Shalini *et al.,* 20 15). Reported linked molecular markers may be effectively used in the marker assisted restorer selection in WA-CMS from a large source of nurseries to give a wide berth to usual test cross nursery in the hybrid rice breeding (Singh *et al.,* 2021). In the evaluation of testcross nursery, we conducted the present experiment with the help of reported gene-linked/specific markers. For fertility restoration, markers linked to *Rf3* and *Rf4* were used. The SSR marker RM 6100 (175 bp for restorer line), one candidate gene marker DRCG-RF4-14 for *Rf4* locus, and one SSR marker DRRM-RF3-10 (150 bp for restorer line) for Rf3 reported by Suresh *et al.,* (2012) were used for screening the population. The details of markers are given in **Table 1**. The plants were grouped as B (maintainer indicates allele), R (fertility restorer genes) and also partial maintainer and partial restorers, respectively.

The fertility restorer genes (*Rf3* and *Rf4*) reported to restore male fertility in the WA CMS system was mapped on chromosomes 1 and 10, respectively (Zhang *et al.,* 1997; Alavi *et al.,* 2009). Six markers, RM6100, RMS-PPR-9-1, DRCG-Rf4-14, DRRM Rf3-10, RM 10313, and RMS-SF21-5 were already validated as tightly linked with fertility restoration of WA-based cytoplasm by Rf3 and Rf4 genes in rice (Singh *et al.,* 2005, Neeraja *et al.,* 2009, Balaji *et al.,* 2012, Revathi *et al.,* 2013, Pranathi *et al.,* 2016).

Categorizations of BILs based on fertility restoration were represented in **Table 2.**

A total of 71 drought tolerant BILs (BC_2F_4) were screened without prior information of fertility restoration status and known restorers and maintainers linked to fertility restoration genes, namely *Rf3 & Rf4* located on chromosome 1 and chromosome 10, respectively. Possessing a single Rf4 gene in the genotype will not complete the pollen fertility restoration. So, other fertility restorer genes, such as the *Rf3* gene located on chromosome 1, are necessary for a restorer as they express fully restoring WA-CMS (Suresh *et al.,* 2012).

Table.1 Reported SSR markers linked to *Rf3* & *Rf4*genes

Marker	Linked gene	Chromo-some	Reference
DRRM Rf-3–10	*Rf3*	1	Balaji *et al.,* 2012
RM 10313	*Rf3*	1	Neeraja *et al.,* 2009
RMS-SF21-5	*Rf3*	1	Pranathi *et al.,* 2016
RM 6100	*Rf4*	10	Singh *et al.,* 2005
RMS-PPR-9-1	*Rf4*	10	Pranathi *et al.,* 2016
DRCG-Rf4-14	*Rf4*	10	Balaji *et al.,* 2012

Table 2. Advanced drought-tolerant backcross inbred lines (BILs)

S No	Genotype	*Rf3/Rf4*	Restorer/ maintainer
1.	RP6340-NPVR-1	*Rf3Rf3/Rf4Rf4*	Restorer
2.	RP6340-NPVR-2	*rf3rf3/rf4rf4*	Maintainer
3.	RP6340- NPVR-3	*Rf3Rf3/Rf4Rf4*	Restorer
4.	RP6340- NPVR-4	*Rf3Rf3/rf4rf4*	Partial Maintainer
5.	RP6340- NPVR-5	*Rf3Rf3/rf4rf4*	Partial Maintainer
6.	RP6340- NPVR-6	*rf3rf3/rf4rf4*	Maintainer
7.	RP6340- NPVR-7	*rf3rf3/Rf4Rf4*	Partial Restorer
8.	RP6340- NPVR-8	*Rf3Rf3/Rf4Rf4*	Restorer
9.	RP6340- NPVR-9	*Rf3Rf3/Rf4Rf4*	Restorer
10.	RP6340- NPVR-10	*Rf3Rf3/Rf4Rf4*	Restorer
11.	RP6340- NPVR-11	Rf3Rf3/rf4rf4	Partial Maintainer
12.	RP6340- NPVR-12	Rf3Rf3/Rf4Rf4	Restorer
13.	RP6340- NPVR-13	Rf3Rf3/Rf4Rf4	Restorer
14.	RP6340- NPVR-14	Rf3Rf3/Rf4Rf4	Restorer
15.	RP6340- NPVR-15	rf3rf3/rf4rf4	Maintainer
16.	RP6340- NPVR-16	Rf3Rf3/Rf4Rf4	Restorer
17.	RP6340- NPVR-17	rf3rf3/Rf4Rf4	Partial Restorer
18.	RP6340- NPVR-18	rf3rf3/Rf4Rf4	Partial Restorer
19.	RP6340- NPVR-19	rf3rf3/Rf4Rf4	Partial Restorer
20.	RP6340- NPVR-20	rf3rf3/Rf4Rf4	Partial Restorer
21.	RP6340- NPVR-21	Rf3Rf3/Rf4Rf4	Restorer
22.	RP6340- NPVR-22	rf3rf3/Rf4Rf4	Partial Restorer
23.	RP6340- NPVR-23	Rf3Rf3/Rf4Rf4	Restorer
24.	RP6340- NPVR-24	Rf3Rf3/Rf4Rf4	Restorer
25.	RP6340- NPVR-25	Rf3Rf3/Rf4Rf4	Restorer
26.	RP6340- NPVR-26	Rf3Rf3/Rf4Rf4	Restorer
27.	RP6340- NPVR-27	Rf3Rf3/Rf4Rf4	Restorer
28.	RP6340- NPVR-28	rf3rf3/Rf4Rf4	Partial Restorer
29.	RP6340- NPVR-29	Rf3Rf3/rf4rf4	Partial Maintainer
30.	RP6340- NPVR-30	rf3rf3/Rf4Rf4	Partial Restorer
31.	RP6340- NPVR-31	Rf3Rf3/Rf4Rf4	Restorer
32.	RP6340- NPVR-32	Rf3Rf3/Rf4Rf4	Restorer
33.	RP6340- NPVR-33	rf3rf3/Rf4Rf4	Partial Restorer
34.	RP6340- NPVR-34	Rf3Rf3/Rf4Rf4	Restorer
35.	RP6340- NPVR-35	Rf3Rf3/Rf4Rf4	Restorer
36.	RP6340- NPVR-36	Rf3Rf3/Rf4Rf4	Restorer
37.	RP6340- NPVR-37	rf3rf3/Rf4Rf4	Partial Restorer
38.	RP6340- NPVR-38	rf3rf3/Rf4Rf4	Partial Restorer
39.	RP6340- NPVR-39	Rf3Rf3/rf4rf4	Partial Maintainer
40.	RP6340- NPVR-40	Rf3Rf3/Rf4Rf4	Restorer
41.	RP6340- NPVR-41	Rf3Rf3/rf4rf4	Partial Maintainer
42.	RP6340- NPVR-42	Rf3Rf3/Rf4Rf4	Restorer
43.	RP6340- NPVR-43	rf3rf3/rf4rf4	Maintainer
44.	RP6340- NPVR-44	rf3rf3/rf4rf4	Maintainer
45.	RP6340- NPVR-45	Rf3Rf3/Rf4Rf4	Restorer
46.	RP6340- NPVR-46	rf3rf3/rf4rf4	Maintainer
47.	RP6340- NPVR-47	rf3rf3/Rf4Rf4	Partial Restorer
48.	RP6340- NPVR-48	Rf3Rf3/Rf4Rf4	Restorer
49.	RP6340- NPVR-49	Rf3Rf3/Rf4Rf4	Restorer
50.	RP6340- NPVR-50	Rf3Rf3/Rf4Rf4	Restorer
51.	RP6340- NPVR-51	Rf3Rf3/Rf4Rf4	Restorer
52.	RP6340- NPVR-52	Rf3Rf3/Rf4Rf4	Restorer
53.	RP6340- NPVR-53	rf3rf3/Rf4Rf4	Partial Restorer

S No	Genotype	*Rf3/Rf4*	Restorer/ maintainer
54.	RP6340- NPVR-54	rf3rf3/rf4rf4	Maintainer
55.	RP6340- NPVR-55	rf3rf3/rf4rf4	Maintainer
56.	RP6340- NPVR-56	rf3rf3/Rf4Rf4	Partial Restorer
57.	RP6340- NPVR-57	Rf3Rf3/Rf4Rf4	Restorer
58.	RP6340- NPVR-58	rf3rf3/rf4rf4	Maintainer
59.	RP6340- NPVR-59	Rf3Rf3/rf4rf4	Partial Maintainer
60.	RP6340- NPVR-60	Rf3Rf3/rf4rf4	Partial Maintainer
61.	RP6340- NPVR-61	Rf3Rf3/rf4rf4	Partial Maintainer
62.	RP6340- NPVR-62	rf3rf3/Rf4Rf4	Partial Restorer
63.	RP6340- NPVR-63	Rf3Rf3/Rf4Rf4	Restorer
64.	RP6340- NPVR-64	rf3rf3/rf4rf4	Maintainer
65.	RP6340- NPVR-65	Rf3Rf3/Rf4Rf4	Restorer
66.	RP6340- NPVR-66	Rf3Rf3/Rf4Rf4	Restorer
67.	BK-49-77 (Check)	Rf3Rf3/Rf4Rf4	Restorer
68.	IR79156B (Check)	rf3rf3/rf4rf4	Maintainer
69.	APMS-6B (Check)	rf3rf3/rf4rf4	Maintainer
70.	RPHR-1005 (Check)	Rf3Rf3/Rf4Rf4	Restorer
71.	KMR-3R	Rf3Rf3/Rf4Rf4	Restorer
72.	Vandana NIL	rf3rf3/rf4rf4	Maintainer

Genotypes were identified as restorers *(Rf3Rf3/ Rf4Rf4*), partial restorers (*rf3rf3/Rf4Rf4)*, partial maintainers (*Rf3Rf3/rf4rf4)*, and maintainers (*rf3rf3/ rf4rf4)* based on the existence of specific allele band. The amplification of *Rf3 & Rf4* gene-specific/linked markers showed a noteworthy difference in their allelic pattern and represented in **Figures 1 & 2.** Of the total, 42 homozygous plants were identified as positive for fertility restoring gene Rf3 by carrying a single dominant gene (Rf3Rf3/rf4rf4), and a total of 48 genotypes were identified as positive for Rf4 single dominant gene (*rf3rf3/Rf4Rf4)* using gene-specific/linked markers represented in **Table 1**. 32 genotypes were identified as positive (restorers) for both *Rf3* and *Rf4* fertility restorer genes by carrying both dominant non-allelic combinations (*Rf3Rf3/ Rf4Rf4)*. The number of genotypes used for fertility restoration using gene-specific/linked markers in BC_2F_4 generation is provided in **Table 2.**

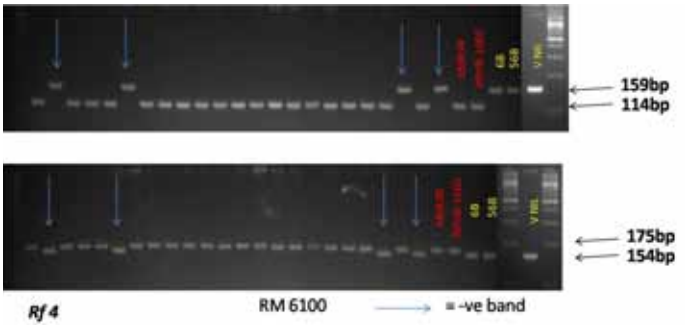

Figure 1: Showing the presence and absence of *Rf4* gene in BILs

Figure 2: Showing the presence and absence of Rf3 gene in BILs

The result out turned in the experiment was skewed by their distribution of allelic frequencies. The presence of *Rf3* (*Rf3Rf3/rf4rf4*) dominant functional gene alone (13.63%) is relatively less than *Rf4* (*rf3rf3/Rf4Rf4*) gene (22.72%). Both fertility restoring genes *Rf3* & *Rf4 (Rf3Rf3/ Rf4Rf4)* were notched with 48.48% with more efficient reported markers. Traditionally, crossing the test genotypes with CMS lines has been reported as a standard protocol to ensure restorer or maintainer lines (Virmani 1996, Singh *et al.,* 2022), which is tedious, laborious, and time-consuming. Hence, molecular analysis of drought-tolerant BILs for the presence of *Rf3* and *Rf4* genes was espoused as a primary selection criterion in this current experiment and can help to reduce number of test crosses for final hybrid development programme.

Revathi *et al.,* (2013) evaluated and reported that 85–92% of efficiency would be there with tightly linked markers of *Rf3* and *Rf4* genes for fertility restoration, and *Rf3Rf3/rf4rf4* genotypes mainly behave as partial maintainers or partial restorers (less than 30% fertility). In the same way, *rf3rf3/Rf4Rf4* genotypes were partial or effective restorers (up to less than 70% fertility). However, double dominant genotypes (*Rf3Rf3/Rf4Rf4*) appeared with greater fertility restoration than the single *Rf3* or *Rf4* genotypes (Katara *et al.,* 2017). Based on the molecular survey for fertility restoration, the results revealed that thirteen genotypes with the absence of *Rf3* and *Rf4* (*rf3rf3/rf4rf4)* allelic combinations were identified

maintainers, and they were categorized in group-1. Nine genotypes with only one *Rf3* dominant allele (*Rf3Rf3/rf4rf4*) were nominated as partial maintainers and categorized as group-2. Of them, 15 genotypes were identified as partial restorers or effective restorers with a single *Rf4* functional dominant allele (*rf3rf3/Rf4Rf4*) and categorized as group-3. Of the total, 35 genotypes were identified as restorers with *Rf3* and *Rf4* dominant fertility restoration allelic combination (*Rf3Rf3/Rf4Rf4*) and were categorized as group-4 (**Figure 3**). Of the identified restorers, ten promising and phenotypically desirable (RP 6340 NPVR1, RP6340 NPVR3, RP6340 NPVR10, RP 6340 NPVR24, RP6340 NPVR27, RP 6340 NPVR32, RP 6340 NPVR48, RP 6340 NPVR52, RP 6340 NRR5 and RP6340 NRR11) restorer lines with qDTY 12.1 and 1.1 in combination or individually were utilized for station trial and on AICRIP trials.

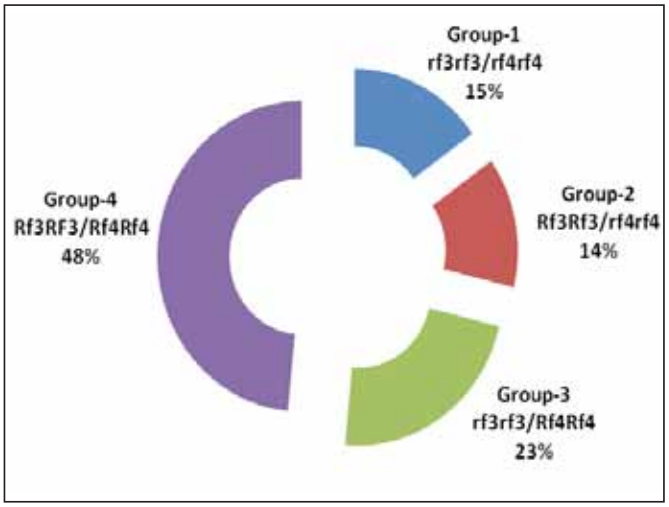

Figure 3: Frequency distribution of Rf3 and Rf4 allelic combination in BILs

Based on a molecular survey undertaken, the identified maintainers can be utilized in the development of a new CMS line for the drought-prone lowland ecology as the genotypes possessing drought-tolerant qDTYs as well as normal irrigated ecology. Besides, identified restorers *viz.*, RP6340 NPVR1 and RP6340 NPVR32 possessing qDTY12.1 and 1.1 with fertility restoration genes (Rf3 and Rf4) may be effectively used as genetic stocks for drought-tolerant parental lines. And also to develop new hybrids in hybrid rice improvement programs for drought-prone lowland environments.

Conclusions

To fulfill the future demand for rice grain, research needs to be intensified in breeding for unfavorable ecologies as an alternative method to substantiate the yield plateauing in rice production. Among all the options available for yield enhancement, exploitation of heterosis through hybrid rice technology is the most feasible one. From this study, a remarkable set of drought tolerant restorers were identified with the help of promising gene-linked/specific markers. This is first of its kind in improvement of restorers and useful in further development of hybrids for drought prone lowland and upland ecologies.

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