Original Research Article

Evaluation of submergence tolerant improved restorer lines for fertility restoration and phosphorus use efficiency using gene-specific markers in rice (*Oryza sativa* L.)

ABSTRACT

Restoring fertility in hybrid rice to maximize yield potential mainly involves identification and utilization of fertility restorer (*Rf*) genes. Thirty-two backcross inbred lines (32 BILs) derived from KMR-3R and Swarna-Sub1 possessing *Sub*1 generated through marker-assisted backcross breeding (MABB) were screened using *Rf*4 and *Rf*3 markers during *Rabi*-2021. 13 BILs showed both *Rf*4 and *Rf*3 alleles, indicating significant variation in marker distribution. Remarkably, the BILs TCP2, TCP10, TCP11, TCP12, TCP14, TCP15, TCP18, TCP23, TCP25, TCP26, TCP28, TCP32, and MB44 were identified as promising restorers possessing both *Rf*4 and *Rf*3 genes along with *Sub1*. Additionally, the BILs TCP11, TCP14, TCP23, TCP28, TCP32, and MB44 were screened for the presence of *Pup1* and noted positive for *Sub1*+*Rf*4+*Rf*3+*Pup*1 (both K46-1 and K46-2). This study demonstrates the combination of *Rf*4, *Rf*3 and *Pup*1 genes in *Sub*1 introgressed restorers can be used for developing submergence tolerant hybrids under phosphorous deficient soils and flood prone eco-systems.

Keywords: Rice, Fertility restoration genes, Pup1, BILs, Submergence.

1. INTRODUCTION

Rice (*Oryza sativa* L.) sustains billions globally as a primary dietary staple, making its productivity crucial for global food security. Worldwide, 527.31 MMT of rice is produced on an area of 167.14 Mha whereas, India significantly contributes to this production, with 139 MMT cultivated on 48.5 Mha (FAS/USDA, 2024). Hybrid technology is a highly efficient, productive, and sustainable method to enhance rice yield (Peng et al., 2004). Hybrid rice produce 15-20% more yield than inbred varieties (Virmani et al., 1997). Restoring fertility in rice CMS lines involves complex nuclear-cytoplasmic interactions, resulting in varying levels of fertility (Awad-Allah et al., 2022). Therefore, the identification of restorer lines is a crucial step in the development of heterotic rice hybrids aimed at realizing targeted grain yield (Venkanna et al., 2022). The genes *Rf3* (on chromosome 1) and *Rf*4 (on chromosome 10) play critical roles in fertility restoration for the WA-CMS system (Yao et al., 1997). The molecular marker RM6100, linked to *Rf*4, has been mapped in restorer lines such as PRR78 R, IR40750 and MTU9992, positioned 6–7 cM from chromosome 10 (Singh et al., 2005). Singh et al. (2005) confirmed the presence of RM6100 on chromosome 10, identifying *Rf*4 as the key locus for WA-CMS fertility restoration (Balaji Suresh et al., 2012). Similarly, RM10313 is linked to *Rf*3 on chromosome 1 (Balaji Suresh et al., 2012; Neeraja, 2008). To advance marker-assisted breeding, Balaji Suresh et al., (2012) developed markers DRRM-RF3-5 and DRRM-RF3-10 for *Rf*3 and DRCG-RF4-14 and DRCG-RF4-8 for *Rf*4. Moreover, Pranathi et al., (2016) introduced gene-based functional markers, RMS-PPR9-1 for *Rf*4 and RMS-SF21-5 for *Rf*3, further enhancing the precision of fertility restoration screening. The application of these

markers for *Rf*3 and *Rf*4 is pivotal for developing high-yielding rice hybrids, particularly in WA-CMS systems (Surender et al., 2021). Phosphorus (P) is a vital macronutrient for the growth and development of rice plants; however, its deficiency in rice soils often poses a significant limitation to rice productivity (Wissuwa et al., 1998). The *Pup1* QTL, located on chromosome 12 and identified in the rice variety Kasalath, is strongly associated with enhanced phosphorus uptake and tolerance to phosphorus-deficient soils (Wissuwa et al., 1998, 2002). The integration of the *Pup1* into new rice varieties is instrumental in maintaining high yields under phosphorus-deficient soil conditions, thereby contributing to enhanced food security and agricultural sustainability (Chin et al., 2011). Rice production is often constrained by various abiotic stresses, such as submergence and phosphorus deficiency, demanding the development of stress-resilient varieties to ensure stable yields. KMR-3R, a widely used restorer line with high yield potential, is vulnerable to submergence stress. To overcome this limitation, the *Sub1* gene, which confers submergence tolerance, was introgressed from the donor variety Swarna-Sub1 into KMR-3R. Notably, Swarna-Sub1 possesses both the *Pup1* and *Sub1* genes, making it a valuable genetic resource for breeding (Barik et al., 2023). A total of 17 backcross introgression lines (BILs) were selected and evaluated using functional dominant markers (K46-1 and K46-2) for *Pup1* (Chin et al., 2011) alongside markers for fertility restoration, facilitating the development of promising rice hybrids adapted to challenging environmental conditions.

2. MATERIAL AND METHODS

Thirty-two BILs (BC_2F_4) derived using MABB for submergence tolerance were assessed for the presence of fertility restoration genes (*Rf*3 and *Rf*4) along with KMR-3R (recurrent parent having *Rf*4 and *Rf*3 genes) and APMS6B (negative check). The details of BILs were indicated in Table 1. Out of 32 BILs, 17 BILs namely, TCP2, TCP6, TCP7, TCP8, TCP9, TCP10, TCP11, TCP12, TCP14, TCP23, TCP25, TCP26, TCP28, TCP30, TCP31, TCP32 and MB44 were screened for the presence of *Pup*1 along with Swarna- Sub1 (donor parent) and KMR-3R (recurrent parent). Two biological replicates of 21- days- old seedlings were transplanted in the field using a randomized complete block design (RCBD). After 21 days of transplanting, the genomic DNA was isolated from the leaves of BILs and checks using CTAB method (Murray and Thompson, 1980). The genotyping was conducted in the molecular biology laboratory, CIS, Hybrid Rice, Indian Council of Agricultural Research (ICAR) - Indian Institute of Rice Research (IIRR), Hyderabad. The genotyping of the BILs was done by using the primers RMS-PPR9-1 and DRCG-RF4-14 for *Rf*4, RMS-SF21-5 for Rf3, K46-1 and K46-2 for *Pup*1 (17 BILs only). Some of the reported markers for *Rf*3, *Rf*4 and *Pup*1 were represented in the Table 2. The PCR was carried at 94°C (5 min), 94°C (30 secs), 55°C (1 min), 72°C (1 min), and 72°C (10 min) in a thermal cycler (BIO-RAD, T100TM Thermal Cycler, USA). The amplified products were resolved in the 3% agarose gel (Seakem® LE Agarose) and the bands were visualized under the UV light using a gel documentation system (IGENE® LABSERVE) and scored.

3. RESULTS AND DISCUSSION

Thirty-two submergence tolerant BILs (BC_2F_4) were genotypically screened for the presence of genes for fertility restoration (*Rf*3 and *Rf*4) and 17 BILs for phosphorous uptake efficiency (*Pup*1). The genotypes were categorized into restorers, partial restorers, maintainers and partial maintainers based on the desired alleles. The SSR primer RMS-PPR9-1 has positive allele at 114bp and negative at 159bp (Fig 2b) and the candidate gene DRCG-RF4-14 had positive alleles at 887bp for non-restorer and 782bp for the restorer line. The functional marker RMS-SF-21-5 had positive alleles at 172bp for *Rf*3 and negative alleles at 127bp (Fig 2a). The dominant markers K46-1 and K46-2 had positive alleles at 523bp and 227bp for *Pup*1 respectively (Fig. 2c).

Among the 32 BILs, fifteen BILs viz., TCP6, TCP7, TCP8, TCP9, TCP10, TCP11, TCP12, TCP14, TCP15, TCP23, TCP25, TCP26, TCP28, TCP32 and MB44 showed positive for both *Rf*4 and *Rf*3, identified as restorers along with the recurrent parent KMR-3R. Nineteen BILs (19) TCP2, TCP6, TCP7, TCP8, TCP9, TCP10, TCP11, TCP12, TCP14, TCP15, TCP18, TCP23, TCP25, TCP26, TCP28, TCP30, TCP31, TCP32 and MB44 along with KMR-3R were found to be positive for only Rf4 allele. Seventeen BILs TCP5, TCP6, TCP7, TCP8, TCP9, TCP10, TCP11, TCP12, TCP14, TCP15, TCP19, TCP23, TCP25, TCP26, TCP28, TCP32 and MB44 were positive for *Rf*3 only. The screening of BILs for *Rf*3 and *Rf*4 is represented in Table 3. The percentage of *Rf*4 contribution alone was 57.8% while *Rf*3 was 53.12%. Both *Rf*3 and *Rf*4 were present in 55.5% among the 32 BILs. Among the 17 BILs screened, 9 BILs-TCP6, TCP8, TCP11, TCP14, TCP23, TCP28, TCP31, TCP32 and MB44 showed positive for both K46-1 and K46-2 markers (Table 4). 8 BILs TCP6, TCP8, TCP11, TCP14, TCP23, TCP28, TCP32, MB44 showed positive for *Rf*4, *Rf*3 and *Pup*1 (both K46-1 and K46-2). The number of BILs positive for their respective markers were represented in Fig. 1.

Numerous studies have demonstrated that the identification and utilization of fertility restoration genes are crucial for successful hybrid rice breeding. Sheeba et al., (2009) reported a high selection accuracy (94.9%) using RM6100 for *Rf*4. Pranathi et al., (2016) developed candidate gene-specific markers RMS-PPR9-1 for *Rf*4 and RMS-SF21-5 for *Rf*3, differentiating 120 restorers and 44 non-restorers. Katara et al. (2017) conducted a comprehensive screening of 570 Indian rice varieties for the fertility restorer genes *Rf*3 and *Rf*4. Their study revealed diverse allelic combinations and highlighted that genotypes possessing both dominant alleles (*Rf*3*Rf*3*Rf*4*Rf*4) exhibited superior fertility restoration compared to genotypes with only one dominant *Rf* gene. Bhati et al., (2018) identified eight restorers among 40 breeding lines using RM171 and RM6100 linked to *Rf* genes. Surender et al., (2021) identified ten potential restorers in rice by screening 43 parental lines using RM6100 and RMS-SF21-5 linked to *Rf*4 and *Rf*3. The identified restorers showed 100% efficiency, highlighting the importance of molecular markers in crop improvement programs. Nagaraju et. al., (2021) identified ten restorers by screening 71 BILs with drought QTLs by using gene-specific markers viz., RM6100, RMS-PPR-9-1, DRCG-RF4-14, for Rf4 on chromosome 10, and DRRM-RF3-10, RM10313, and RMS-SF21-5 for Rf3 located on chromosome 1. In studies of assessing the heat tolerance potential (Jaldhani et al., 2021a; 2021b); direct seeded aerobic adoption (Srijan et al., 2021); reproductive stage drought tolerance (Nagarju et al., 2022; Sravanraju et al., 2024); genetic divergence studies (Prasanna et al., 2023); early seedling stage salinity tolerance (Beulah et al., 2024); low P tolerance (Madhusudan et al., 2024) of restorer lines, the markers DDRM-Rf3-10 and RMS SF 21-5 were utilized for the *Rf*3 gene, while RM6100 and RMS PRR 9-1 were employed for the *Rf*4 gene. Beulah et al., (2023) screened 55 BILs with salinity tolerance by using the primers RM6100, DRCG-RF4-14, RMS-PPR-9-1 for Rf4 and RM10313, DRRM-RF3-10, RMS-SF21-5 for Rf3 and identified 19 restorers. Madhusudan et al., (2022) improved APMS6B for phosphorous (P) deficiency tolerance and bacterial blight (BB) resistance using K46-1, K46-2 and Xa21, Xa38 through MABB. Kavitha et al., (2022) screened 250 ILs and identified 26 lines positive for Pup1 and Rf4 in KMR-3R using K46-1, K46-2 and RM6100 through MABB for low phosphorous tolerance. Barik et al., (2023) developed Reeta-Panidhan (CR Dhan 413), an improved version of Reeta. This variant was developed by introgression three QTLs- *Sub*1+*Pup*1+GW5 from the donor parent Swarna-Sub1 using MABB for increasing yield, submergence and phosphorus stress tolerance, suitable for flood-prone areas in Odisha. Zhang et al., (2022) highlighted that introgression lines (ILs) enhance genetic diversity and improve rice traits by facilitating the identification and utilization of elite alleles and QTLs. These findings underscore the critical role of molecular markers in identifying fertility restorer genes and *Pup1*, thereby aiding the development of resilient rice hybrids capable of thriving under challenging environmental conditions such as submergence and phosphorus-deficient soils.

Table 1. List of genotypes used in the study

S. No.	BIL ID	Readable name
1	RP-6342-VTCP1	TCP1
2	RP-6342-VTCP2	TCP2
3	RP-6342-VTCP3	TCP3
4	RP-6342-VTCP4	TCP4
5	RP-6342-VTCP5	TCP5
6	RP-6342-VTCP6	TCP6
7	RP-6342-VTCP7	TCP7
8	RP-6342-VTCP8	TCP8
9	RP-6342-VTCP9	TCP9
10	RP-6342-VTCP10	TCP10
11	RP-6342-VTCP11	TCP11
12	RP-6342-VTCP12	TCP12
13	RP-6342-VTCP13	TCP13
14	RP-6342-VTCP14	TCP14
15	RP-6342-VTCP15	TCP15
16	RP-6342-VTCP16	TCP16
17	RP-6342-VTCP17	TCP17
18	RP-6342-VTCP18	TCP18
19	RP-6342-VTCP19	TCP19
20	RP-6342-VTCP20	TCP20
21	RP-6342-VTCP21	TCP21
22	RP-6342-VTCP22	TCP22
23	RP-6342-VTCP23	TCP23
24	RP-6342-VTCP24	TCP24
25	RP-6342-VTCP25	TCP25
26	RP-6342-VTCP26	TCP26
27	RP-6342-VTCP28	TCP28
28	RP-6342-VTCP29	TCP29
29	RP-6342-VTCP30	TCP30
30	RP-6342-VTCP31	TCP31
31	RP-6342-VTCP32	TCP32
32	RP-6342-VTCPMB44	MB44

Table 2. Molecular markers reported for Rf3, Rf4 and Pup1

Marker	Linked gene	Chromosome	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, Sheeba et al., 2009
RMS-PPR-9-1	*Rf4*	10	Pranathi et al., 2016
DRCG-RF4-14	*Rf4*	10	Balaji et al., 2012
K46-1	*Pup1*	12	Chin et al., 2011
K46-2	*Pup1*	12	Chin et al., 2011
K48	*Pup1*	12	Chin et al., 2011

Table 3. Screening of BILs for Rf4 and Rf3 using markers.

Genotypes	*Rf4*		*Rf3*
	RMS-PPR9-1	**DRCG-RF4-14**	**RMS-SF21-5**
TCP1	-	-	-
TCP2	+	+	-
TCP3	-	-	-
TCP4	-	-	-
TCP5	-	-	+
TCP6	+	+	+
TCP7	+	+	+
TCP8	+	+	+
TCP9	+	+	+
TCP10	+	+	+
TCP11	+	+	+
TCP12	+	+	+
TCP13	-	-	-
TCP14	+	+	+
TCP15	+	+	+
TCP16	-	-	-
TCP17	-	-	-
TCP18	+	+	-
TCP19	-	-	+
TCP20	-	-	-
TCP21	-	-	-
TCP22	-	-	-
TCP23	+	+	+
TCP24	-	-	-
TCP25	+	+	+
TCP26	+	+	+
TCP28	+	+	+
TCP29	-	-	-
TCP30	+	+	-
TCP31	+	+	-
TCP32	+	+	+
MB44	+	+	+
KMR-3R	+	+	+
IR58025B	-	-	-
RPHR-1005R	+	+	+
IR68897B	-	-	-

Table 4. Screening of BILs for Pup1 using markers.

S. No	TCP CODE	*Pup1*	
		K46-1	**K46-2**
1	TCP2	-	+
2	TCP6	+	+
3	TCP7	-	+
4	TCP8	+	+
5	TCP9	-	+
6	TCP10	-	-

7	TCP11	+	+
8	TCP12	-	+
9	TCP14	+	+
10	TCP23	+	+
11	TCP25	-	+
12	TCP26	-	+
13	TCP28	+	+
14	TCP30	-	+
15	TCP31	+	+
16	TCP32	+	+
17	MB44	+	+
18	KMR-3R	-	-
19	Swarna-Sub1	+	+

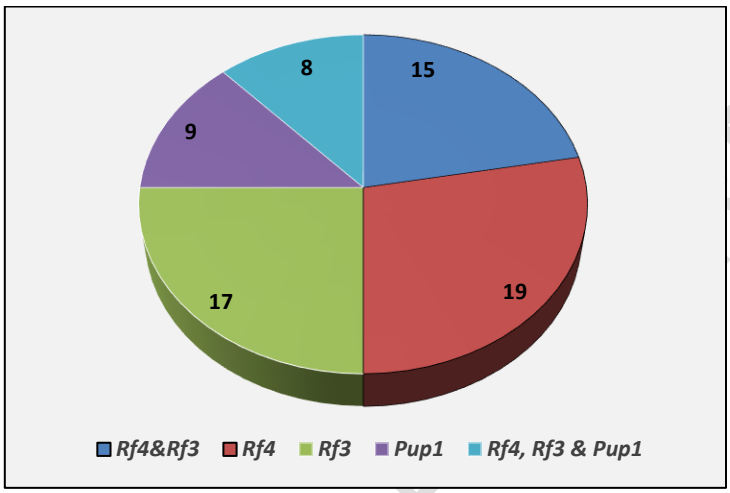

Fig. 1. Representation of positive BILs for respective markers

Fig: 2a, Screening of BILs for Rf3 using RMS-SF21-5, positives indicated in yellow

Fig. 2b, Screening of BILs for Rf4 using RMS-PPR9-1, positives indicated in yellow

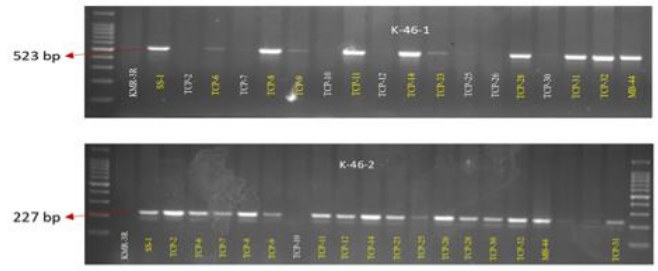

Fig. 2c, BILs screened with functional markers K46-1 and K46-2 for *Pup1*, positives indicated in yellow

4. CONCLUSION

Genotyping of BILs with *Sub*1 and fertility restoration (*Rf*) markers led to the identification of 15 restorers: TCP6, TCP7, TCP8, TCP9, TCP10, TCP11, TCP12, TCP14, TCP15, TCP23, TCP25, TCP26, TCP28, TCP32, and MB44. These BILs were found to possess the *Sub*1+*Rf*3+*Rf*4 combination. Notably, eight BILs (TCP6, TCP8, TCP11, TCP14, TCP23, TCP28, TCP32, and MB44) tested positive for *Sub*1+*Rf*4+*Rf*3+*Pup*1 (with both K46-1 and K46-2 markers). These improved restorers hold promise for developing rice hybrids with tolerance to both submergence and phosphorus deficiency, making them particularly suitable for flood-prone areas and phosphorus-deficient soils.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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