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 Rf4 and Rf3 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 varieties 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, identifying restorer lines is a fundamental step in developing high-yielding heterotic rice hybrids (Venkanna et al., 2022). The genes *Rf*3 and *Rf*4, located on chromosomes 1 and 10 respectively, are crucial for the fertility restoration of the WA-CMS system (Yao et al., 1997). RM6100 marker is linked to the *Rf* gene in the restorer lines PRR78 R, IR40750, and MTU9992, positioned 6–7 cM from chromosome 10 (Singh et al., 2005). The presence of RM6100 on chromosome 10 is confirmed by Singh et al., 2005 and *Rf*4 is the key locus for WA-CMS fertility restoration (Balaji Suresh et al., 2012), while RM10313 for *Rf*3 is located on chromosome 1 (Balaji Suresh et al., 2012; Neeraja, 2008). Balaji Suresh et al., (2012) developed markers DRRM-RF3-5, DRRM-RF3-10 (*Rf*3) and DRCG-RF4-14, DRCG-RF4-8 (*Rf*4) for fertility restoration in WA-CMS. Pranathi et al., (2016) developed gene-based functional markers, namely RMS-PPR9-1 for Rf4 and RMS-SF21-5 for Rf3. Screening for fertility restoration genes (*Rf*3 and *Rf*4) using markers is essential for developing high-yielding rice hybrids (Surender et al., 2021). Phosphorus (P) is an important

macronutrient for the growth and development of rice plants, but it is often deficient in rice soils, substantially limiting rice production (Wissuwa et al., 1998). Phosphorus uptake (*Pup*1) is a major QTL identified in Kasalath on chromosome 12 linked with tolerance to phosphorus deficiency in the soil (Wissuwa et al., 1998, 2002). Developing new rice varieties with the *Pup*1 gene helps in maintaining high yields in phosphorus-poor soils, enhancing food security and agricultural sustainability in low-fertility regions (Chin et al., 2011). Rice production is frequently challenged by various abiotic stresses, including submergence and phosphorus-deficient soil. Rice varieties that can withstand submergence stress and low phosphorus soil is necessary for ensuring stable yields. KMR-3R is a popular restorer line known for its high yield potential, but its performance can be significantly affected by submergence. To address this, *Sub*1 (a submergence tolerance gene) from Swarna-Sub1 is introgressed into KMR-3R. As Swarna-Sub1 carries *Pup*1 genes along with *Sub1* (Barik et al., 2023), 17 BILs were selected and evaluated with the functional dominant markers - K46-1 and K46-2 (Chin et al., 2011) for *Pup*1 along with fertility restoration markers.

2. MATERIAL AND METHODS

Thirty-two BILs (BC_2F_4) viz., RP-6342-VTCP1, RP-6342-VTCP2, RP-6342-VTCP3, RP-6342-VTCP4, RP-6342-VTCP5, RP-6342-VTCP6, RP-6342-VTCP7, RP-6342-VTCP8, RP-6342-VTCP9, RP-6342-VTCP10, RP-6342-VTCP11, RP-6342-VTCP12, RP-6342-VTCP13, RP-6342-VTCP14, RP-6342-VTCP15, RP-6342-VTCP16, RP-6342-VTCP17, RP-6342-VTCP18, RP-6342-VTCP19, RP-6342-VTCP20, RP-6342-VTCP21, RP-6342-VTCP22, RP-6342-VTCP23, RP-6342-VTCP24, RP-6342-VTCP25, RP-6342-VTCP26, RP-6342-VTCP28, RP-6342-VTCP29, RP-6342-VTCP30, RP-6342-VTCP31, RP-6342-VTCP32 and RP-6342-MB44 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). For readability, the above BILs were referred as TCP and MB44 in this manuscript. Whereas, 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 Rf4, 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 1. 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 (32) 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 2. 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 3). 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 shown the identification and use of fertility restoration genes *Rf*3 and *Rf*4 that are vital for hybrid rice breeding. Sheeba et al., (2009) reported a high selection accuracy of 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) screened 570 Indian rice varieties for fertility restorer genes *Rf*3 and *Rf*4, found various allelic combinations and stated that double dominant genotypes showed better fertility restoration than individual *Rf* genes. Bhati et al., (2018) identified eight restorers among 40 breeding lines using RM171 and RM6100 linked to *Rf* genes (*Rf*3 and *Rf*4). 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 introgressing 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) stated that the Introgression lines (ILs) enhance genetic diversity and improves rice traits by identifying and utilizing elite alleles and QTLs. These studies show the importance of molecular markers in identifying fertility restorers and Pup1, aiding in the development of robust rice hybrids under diverse environmental conditions like submergence and phosphorous deficient soils.

Table 1. 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 2. 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	+	+	+
KMR3R	+	+	+
IR58025B	-	-	-
RPHR1005	+	+	+
IR68897B	-	-	-

Table 3. 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	KMR3R	-	-
19	Swarna-Sub1	+	+

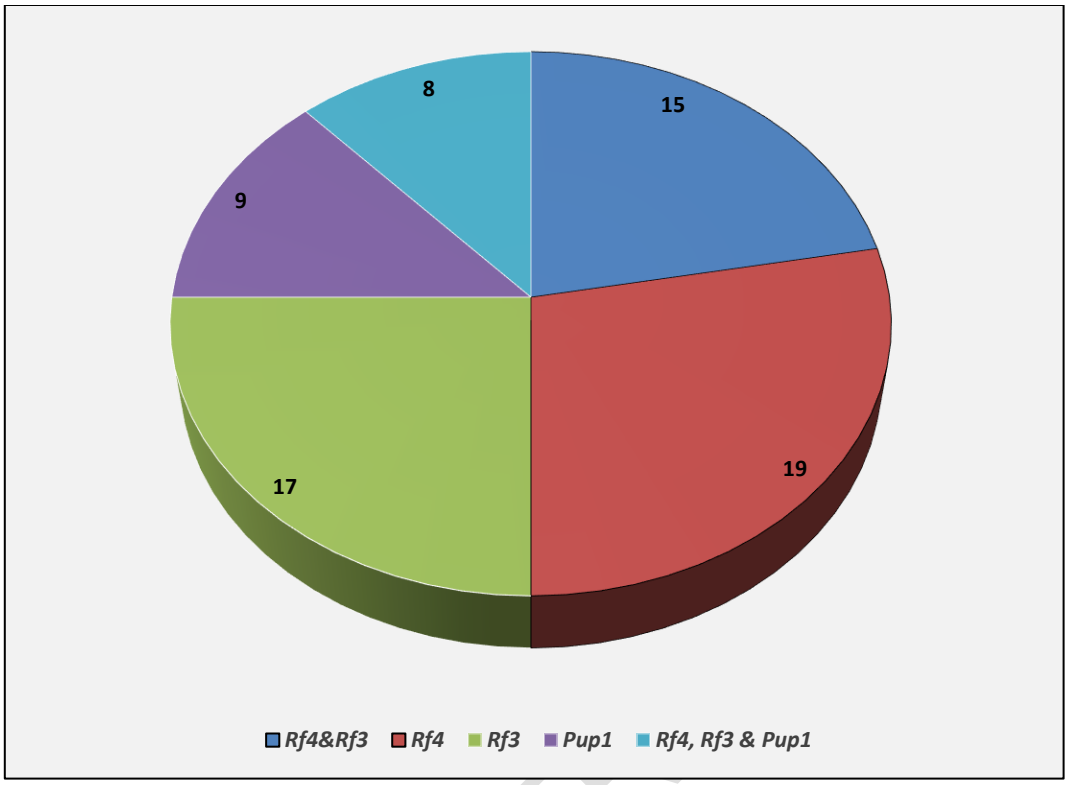

Fig. 1. Representation of positive BILs for respective markers

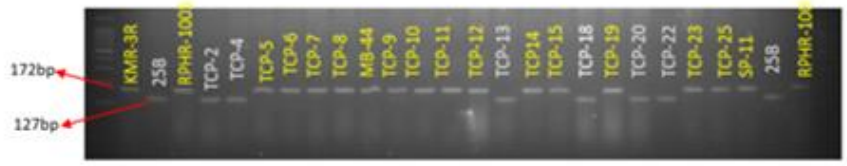

Fig: 2a, Screening of BILs for Rf3 using RMS-SF21-5, positives indicated in yellow

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

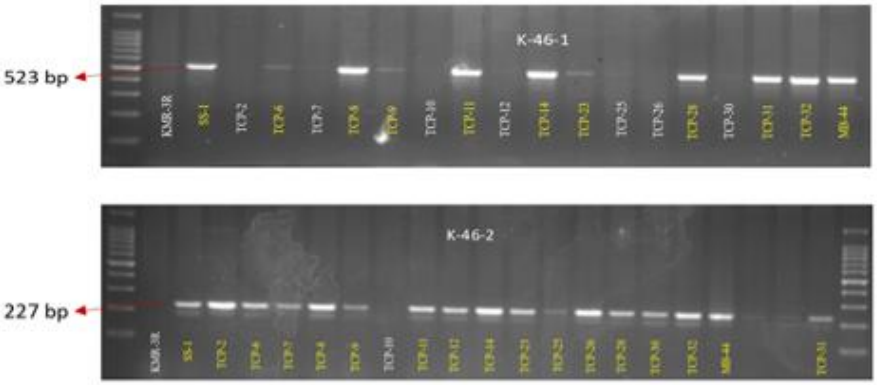

Fig. 2c, BILs screened with functional markers K46-1 and K46-2 for *Pup1*, positives indicated in yellow

4. CONCLUSION

Genotyping of BILs with *Sub*1 and fertility restoration (*Rf*) markers resulted in the identification of 15 Restorers viz., TCP6, TCP7, TCP8, TCP9, TCP10, TCP11, TCP12, TCP14, TCP15, TCP23, TCP25, TCP26, TCP28, TCP32, and MB44. These BILs were found to possess *Sub*1+*Rf*3+*Rf*4. Eight BILs (TCP6, TCP8, TCP11, TCP14, TCP23, TCP28, TCP32 and MB44) showed positive for *Sub*1+*Rf*4+*Rf*3+*Pup*1 (both K46-1 and K46-2). The improved restorers can be used for developing submergence and low phosphorous tolerant rice hybrids in flood prone and phosphorus deficient soil regions.

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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