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Development and validation of candidate gene-specific markers for the major fertility restorer genes, *Rf4* and *Rf3* in rice

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Abstract Two major nuclear genes, *Rf3* and *Rf4*, are known to be associated with fertility restoration of wild-abortive cytoplasmic male sterility (WA-CMS) in rice. In the present study, through a comparative sequence analysis of the reported putative candidate genes, viz. *PPR9-782-(M,I)* and *PPR762* (for *Rf4*) and *SF21* (for *Rf3*), among restorer and maintainer lines of rice, we identified significant polymorphism between the two lines and developed a set of PCR-based codominant markers, which could distinguish maintainers from restorers. Among the five markers developed targeting the polymorphisms in *PPR9-782-(M,I)*, the marker RMS-PPR9-1 was observed to show clear polymorphism between the restorer ($n = 120$) and maintainer lines ($n = 44$) analyzed. Another codominant marker, named RMS-PPR762 targeting *PPR762*, displayed a lower efficiency in identification of restorers and maintainers, indicating that *PPR9-782-(M,I)* is indeed the candidate gene for *Rf4*. With respect to *Rf3*, a codominant marker, named RMS-SF21-5 developed targeting *SF21*, displayed significantly lower efficiency in identification of restorers and non-restorers as compared to the *Rf4*-specific markers. Validation of these markers in a F_2 mapping population segregating for fertility restoration indicated that *Rf4* has a major influence on fertility restoration and *Rf3* is a minor gene. Further, the functional marker RMS-PPR9-1 was observed to be very useful in identification of impurities in a seed lot of the popular hybrid, DRRH3. Interestingly, when RMS-PPR9-1 and RMS-SF21-5 were considered in conjunction with analysis, near-complete, marker–trait co-segregation was observed, indicating that deployment of the candidate gene-specific markers both *Rf4* and *Rf3*, together, can be helpful in accurate identification of fertility restorer lines and can facilitate targeted transfer of the two restorer genes into elite varieties through marker-assisted breeding.

Keywords Fertility restoration · *Rf4* · *Rf3* · WA-CMS · Gene-specific markers · Hybrid seed purity

K. Pranathi and R. M. Sundaram have contributed equally to this work.

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Introduction

Breeding rice for higher yield remains the key priority for developing nations such as India, which needs to produce ~125 million tonnes of rice by 2030 to feed its burgeoning population. Hybrids in rice have yield superiority of about 15–20 % over the best commercial inbred varieties under similar conditions (Virmani 1996) and large-scale adoption of hybrid rice production is one of the feasible options to meet the food security challenges in India. Hybrid based on wild-abortive cytoplasmic male sterility (WA-CMS) system has been extensively used in commercial rice hybrids production in most of the Asian countries including India (Lin and Yuan 1980; Virmani and Wan 1988), and so far, 75 hybrids based on WA-CMS system have been released for commercial cultivation in India (AS Hariprasad, personal communication). The utility of the CMS lines in hybrid rice breeding is determined by the availability of characterized and effective fertility restoration lines. A total of 17 alleles for fertility restoration have been identified in rice, and all except *rf17* are dominant in rice. Among these, at least two genes, viz. *Rf3* (located on chromosome 1) and *Rf4* (located on chromosome 10), are known to control fertility restoration of WA cytoplasm (Zhang et al. 1997; Yao et al. 1997). Various attempts have been made to fine-map and characterize the candidate genes underlying *Rf4* and *Rf3* (Ahmadikhah and Karlov 2006; Sheeba et al. 2009; Ngangkham et al. 2010; Balaji et al. 2012). Ngangkham et al. (2010) proposed that a gene encoding a pentatricopeptide repeat (PPR) motif-containing protein, named *PPR3*, located on the long arm of chromosome 10 is the candidate gene for *Rf4*, while recently, another study (Tang et al. 2014) identified another candidate, *PPR9-782 (M,I)*, located in the same region as *PPR3* as the candidate for *Rf4* gene. With respect to *Rf3*, Balaji et al. (2012) reported that a gene, named *SF21*, encoding a pollen-specific protein to be putative candidate for the gene.

In WA-CMS-based hybrid breeding (also called three-line system of hybrid rice breeding), identification of potential restorers among the diverse rice germplasm lines is of significant importance, as genetically diverse restorer lines can be helpful in breeding hybrids with higher magnitude of heterosis. The traditional method of identifying restorers by breeders involves test crossing the prospective lines with selected WA-CMS lines and evaluating the F_1 progenies for pollen and spikelet fertility. Lines with progenies showing >70 % pollen and spikelet fertility are then designated as restorers (Govinda Raj and Virmani 1988). Molecular mapping of *Rf3* and *Rf4* can reduce the time and effort involved in identification of fertility restorer lines (Sattari et al. 2007; Sheeba et al. 2009). Further, molecular markers specific for *Rf3* and *Rf4* can aid in targeted transfer of the two *Rf* genes into elite genetic backgrounds and also facilitate accurate estimation of genetic impurities in hybrid seed lots (Nandakumar et al. 2004; Sundaram et al. 2008). Many markers have been developed for *Rf4* (Ahmadikhah and Karlov 2006; Ngangkham et al. 2010; Balaji et al. 2012), and a few have been developed for *Rf3* (Nas et al. 2003). However, these markers display limited efficiency in accurate identification of restorers, as all of them are linked markers and not specific for the putative candidate genes underlying either *Rf3* or *Rf4*. The present study was carried out with the objective to analyze the sequence polymorphism in the genomic region underlying the reported candidate genes for *Rf3* and *Rf4*, develop candidate gene-specific, PCR-based codominant markers, validate them among a large set of known maintainer and restorer lines and a mapping population segregating for the trait of fertility restoration and finally demonstrate the utility of the candidate gene-specific marker in accurate identification of impurities in seed lot of a commercial rice hybrid.

Materials and methods

Plant materials

The plant materials in the study included a total of 120 restorer and 44 non-restorer lines (i.e., maintainers) of *indica*-type rice for WA-CMS cytoplasm (Table 1), which were used for validation of the gene-specific markers developed for *Rf3* and *Rf4*. The developed markers were also validated in a segregating population consisting of 1252 F_2 individuals derived from the cross between the WA-CMS line, IR58025A and the restorer line, KMR3R, which were phenotyped for spikelet fertility. A set of 71 wild rice lines (Supplementary Table 1) was analyzed for their amplification pattern with respect to the gene-specific marker for *Rf4*. In addition, a seed lot of the popular rice hybrid

Table 1 List of plant materials used in the study

S. no.	Rice genotype	Classification of genotype along with its source	S. no.	Rice genotype	Classification of genotype along with its source	S. no.	Rice genotype	Classification of genotype along with its source
1	RPHR-612-1	Restorer-indica	61	RNR-17420	Restorer-indica	1	APMS6B	Maintainer-indica
2	EPLT 104	Restorer-indica	62	RNR-17472	Restorer-indica	2	IR58025B	Maintainer-indica
3	EPLT 109	Restorer-indica	63	JGL-11118	Restorer-indica	3	IR68897B	Maintainer-indica
4	EPLT 107	Restorer-indica	64	RNR-2465	Restorer-indica	4	IR68888B	Maintainer-indica
5	RPHR 118	Restorer-indica	65	JGL-1798	Restorer-indica	5	PUSA5B	Maintainer-indica
6	RPHR 124	Restorer-indica	66	IR 40750	Restorer-indica	6	IR79156B	Maintainer-indica
7	RPHR-611-1	Restorer-indica	67	IR66	Restorer-indica	7	IR80555B	Maintainer-indica
8	RPHR 517	Restorer-indica	68	IR36	Restorer-indica	8	IR80561B	Maintainer-indica
9	RPHR-619-2	Restorer-indica	69	CN 1966-4-9	Restorer-indica	9	DRR5B	Maintainer-indica
10	RPHR619	Restorer-indica	70	Pusa 5001-6-3-2	Restorer-indica	10	DRR6B	Maintainer-indica
11	AYT-21	Restorer-indica	71	GQ-25	Restorer-indica	11	DRR9B	Maintainer-indica
12	RPBIO-4919-50-10	Restorer-indica	72	RPBIO-50-13	Restorer-indica	12	DRR10B	Maintainer-indica
13	RPBIO-4919-5-1	Restorer-indica	73	IR46	Restorer-indica	13	DRR3B	Maintainer-indica
14	IR48725	Restorer-indica	74	NDR 3026	Restorer-indica	14	CRMS32B	Maintainer-indica
15	BR 827-35	Restorer-indica	75	RPHR1005	Restorer-indica	15	CR 2655-18-2-3	Maintainer-indica
16	RPHR2	Restorer-indica	76	MTU-9992	Restorer-indica	16	SN 244	Maintainer-indica
17	BCW56	Restorer-indica	77	NRI-38	Restorer-indica	17	CRR 575-38-1-1	Maintainer-indica
18	C20R	Restorer-indica	78	DR714-1-2R	Restorer-indica	18	SM-156	Maintainer-indica
19	AJAYA	Restorer-indica	79	RPBIO-4919-363-5	Restorer-indica	19	RNSK-1054-1	Maintainer-indica
20	RPBIO50-10	Restorer-indica	80	PNR 3158	Restorer-indica	20	RSK 1046	Maintainer-indica
21	SG-22-289-3	Restorer-indica	81	RPHR 1096	Restorer-indica	21	DR714-1-2R X TJ-53	Maintainer-indica
22	SG-25-74	Restorer-indica	82	IR72	Restorer-indica	22	WR-37-1-1-2	Maintainer-indica
23	SG-27-177	Restorer-indica	83	KMR-3R	Restorer-indica	23	CR3818-1-1-1-1-2	Maintainer-indica
24	NLR-33358	Restorer-indica	84	RPHR 1004	Restorer-indica	24	CHIR 5	Maintainer-indica
25	Akshayadhan	Restorer-indica	85	RPHR 111-3	Restorer-indica	25	TTB 404	Maintainer-indica
26	RNR-2781	Restorer-indica	86	GQ70	Restorer-indica	26	RPHR-1096 X TJ-4	Maintainer-indica
27	IR64	Restorer-indica	87	IR 10198	Restorer-indica	27	UPR 3786-17-2-1	Maintainer-indica
28	RNR-6378	Restorer-indica	88	IR 24	Restorer-indica	28	PUSA 1557-06-28-188-1-17	Maintainer-indica

Table 1 continued

S. no.	Rice genotype	Classification of genotype along with its source	S. no.	Rice genotype	Classification of genotype along with its source	S. no.	Rice genotype	Classification of genotype along with its source
29	Bhadrakali	Restorer-indica	89	IR29723	Restorer-indica	29	RPHR 1005 X FBR-1	Maintainer-indica
30	RNR-17438	Restorer-indica	90	BK-64-116	Restorer-indica	30	PUSA 1557-06-2-9-159-3-2	Maintainer-indica
31	NP-6226	Restorer-indica	91	BK-36-167	Restorer-indica	31	SM-75	Maintainer-indica
32	WGL-640	Restorer-indica	92	BK 39-179	Restorer-indica	32	SN-15	Maintainer-indica
33	6527	Restorer-indica	93	BK-52-104	Restorer-indica	33	CRR 574-38-1-1	Maintainer-indica
34	NLR-145	Restorer-indica	94	BK-44-78	Restorer-indica	34	FGK-1	Maintainer-indica
35	CSR-23	Restorer-indica	95	BK-49-76	Restorer-indica	35	SN-322 T	Maintainer-indica
36	WGL-665	Restorer-indica	96	BK-49-43	Restorer-indica	36	CR 3818-1-1-1-1-2-1	Maintainer-indica
37	RNR-898	Restorer-indica	97	BK-49-77	Restorer-indica	37	TTB 404-1	Maintainer-indica
38	WGL-573	Restorer-indica	98	BK-49-120	Restorer-indica	38	CHIR 5-2	Maintainer-indica
39	NLR-40058	Restorer-indica	99	BK-49-80	Restorer-indica	39	RSK 1046-1	Maintainer-indica
40	Pushyami	Restorer-indica	100	BK-35-155	Restorer-indica	40	RMSB-514	Maintainer-indica
41	TM07275	Restorer-indica	101	BK-49-78	Restorer-indica	41	KAUMK157	Maintainer-indica
42	TCP 349	Restorer-indica	102	BK-49-76	Restorer-indica	42	TCP 1193	Maintainer-indica
43	KCD-1	Restorer-indica	103	SG-27-105	Restorer-indica	43	SM-202	Maintainer-indica
44	PNR-89	Restorer-indica	104	RNR-15351	Restorer-indica	44	KSLRV-221	Maintainer-indica
45	PNR-72	Restorer-indica	105	RNR-15038	Restorer-indica			
46	PNR-71	Restorer-indica	106	RNR-10291	Restorer-indica			
47	PNR-79	Restorer-indica	107	WGL-347	Restorer-indica			
48	PNR-80	Restorer-indica	108	WGL-283	Restorer-indica			
49	PNR-81	Restorer-indica	109	RNR-15048-1	Restorer-indica			
50	PNR-82	Restorer-indica	110	RNR-15038-1	Restorer-indica			
51	PNR-83	Restorer-indica	111	MTU-1081	Restorer-indica			
52	PNR-84	Restorer-indica	112	WGL-285	Restorer-indica			
53	PNR-85	Restorer-indica	113	RNR-11636	Restorer-indica			
54	PNR-86	Restorer-indica	114	KAVYA	Restorer-indica			
55	PNR-87	Restorer-indica	115	TCM-80-M	Restorer-indica			
56	PNR-88	Restorer-indica	116	PRR78	Restorer-indica			
57	PNR-74	Restorer-indica	117	HHZ5-SAL10-DT3-Y2	Restorer-indica			
58	RNR-15028	Restorer-indica	118	PNR-73	Restorer-indica			

Table 1 continued

S. no.	Rice genotype	Classification of genotype along with its source	S. no.	Rice genotype	Classification of genotype along with its source	S. no.	Rice genotype	Classification of genotype along with its source
59	C-26	Restorer-indica	119	HHZ12-Y4-Y1-DT1	Restorer-indica			
60	RNR-17494	Restorer-indica	120	vajram	Restorer-indica			

DRRH3 consisting of 400 seeds was also included for analysis of efficiency of gene-specific marker for *Rf4* in accurate identification of genetic impurities. All the plant materials utilized in the study were collected from Hybrid Rice Section of ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad.

Analysis of gene sequences of *Rf3* and *Rf4*

The candidate genes *PPR9-782-(M,I)* (Tang et al. 2014; Kazama and Toriyama 2014) and *PPR762* (Balaji et al. 2012) reported to be specific for *Rf4* on chromosome 10 were considered for sequence analysis. The reported restorer sequences (*PPR9-782-M* and *PPR9-782-I*) and non-restorer gene sequences (*PPR9-409* and *PPR9-782-ZH*) of *PPR9* gene (Tang et al. 2014) were downloaded from NCBI/GenBank public database. The coordinates of *PPR9-782-M* gene were identified in Nipponbare, a *japonica* cultivar from (19,287,680 to 19,295,473 bp; Pseudo molecule 6.1, http://rice.plantbiology.msu.edu/pseudomolecules) using BioEdit tool version 7.0.9 (Hall 2007). Using ClustalW multiple sequence alignment tool (Higgins et al. 1994), two functional restorer sequences and two non-restorer sequences were compared to identify different polymorphic regions (Supplementary Figure 1). Further, a 25-kb region upstream and a 25-kb region downstream of *PPR9-782-M* on chromosome 10 of Nipponbare (19,290,587–19,340,587 bp) and chromosome 10 of *indica* cultivar, 93–11 (17,749,895–17,799,895 bp) were also aligned using ClustalW tool in order to identify the polymorphic regions in the vicinity of the candidate gene. Similar sequence analysis was performed for another reported candidate gene *PPR762* specific for *Rf4* (Balaji et al. 2012). The reported amplicon sequences of DRCG-Rf4-14 marker (Balaji et al. 2012) targeting *PPR762* in restorer and non-restorer sequences were also considered for polymorphism analysis.

With respect to *Rf3*, a pollen-specific protein, *SF21*, located on chromosome 1 was identified earlier by fine-mapping analysis to be the putative candidate gene (Balaji et al. 2012). *SF21* gene sequence (LOC_Os01g09670) was downloaded from Gramene/NCBI public database. The coordinates of *SF21* gene were identified on chromosome 1 of Nipponbare, *japonica* cultivar (Pseudo molecule 6.1, http://rice.plantbiology.msu.edu/pseudomolecules)

and in *indica* cultivar 93–11 (Beijing Rice Information System http://rice.genomics.org.cn/rice) using BioEdit tool version 7.0.9 (Hall 2007). A 10-kb region upstream and 10-kb region downstream of *SF21* gene from both *japonica* (4,917,224–4,992,046 bp) and *indica* (5,350,773–5,371,596 bp) were aligned using ClustalW alignment tool (Higgins et al. 1994) (Supplementary Figure 2) to identify polymorphic regions in the vicinity of the *SF21* gene.

Primer designing and PCR analysis of the developed markers

The different polymorphic regions identified within the *PPR9-782-M* and *PPR-782-I* gene and also in the vicinity of gene (i.e., within 50 kb on either side) were targeted for designing of five PCR-based codominant markers specific for *Rf4*. Another codominant marker specific for *Rf4* was designed targeting the polymorphism within *PPR762* gene. In addition, different polymorphic regions identified based on alignment between *SF21* sequences from *japonica* and *indica* were targeted for designing of five PCR-based codominant markers specific for *Rf3*. All these primer pairs were designed using Primer 3 online tool (http://bioinfo.ut.ee/primer3-0.4.0), and the primer sequences of these markers specific to *Rf3* and *Rf4* are listed in Table 2. In addition, the reported SSR markers RM6100 (specific for *Rf4*) (Singh et al. 2005), DRCG-Rf4-14 (specific for *Rf4*) (Balaji et al. 2012) and DRRM-Rf3-10 (specific for *Rf3*) (Balaji et al. 2012) were also considered for analysis.

The total genomic DNA was isolated from young, healthy leaves of all the restorer lines, maintainer lines and individuals of the segregating F_2 mapping population by following the method of Dellaporta et al. (1983). The isolated DNA was used for PCR amplification with the codominant markers developed in the study. PCR was performed in 20 µl reaction volumes containing 1X PCR buffer [10 mM Tris·HCl (pH 8.3),

Table 2 List of *Rf3* and *Rf4* markers developed in the study

S. no.	Primer name	Primer sequence	Position in Japonica (bp)
List of markers specific to *Rf4*			
1	RMS-PRR9-1	GAGTTTTGAATAGATTTACGTGTGGA	19,294,526
		AGTGTCCAGATTCGTAGTAATGC	
2	RMS-PPR9-2	GAATGGAAGATCCACCGAAG	19,292,205
		ATGACATTGGGCTTCACACC	
3	RMS-PPR9-3	GGTGTGAAGCCCAATGTC	19,291,628
		GCAAAGCCCATGAAGGATTA	
4	RMS-PPR9-4	AACGTTACTATTCACCTC	19,335,773
		AGCTTTGCTAGTCTTCCAG	
5	RMS-PPR9-5	ATTGGTGTCTGAGGGGTCTG	19,318,121
		TTGGCAGGTTTGCTAATTTTG	
6	RMS-PPR762	TTGCCAGCATGTTCTCAGTT	19,394,606
		GCAAAGCCCATGAAGGATTA	
List of markers specific to *Rf3*			
1	RMS-SF21-1	ACAAAAGGCACACCCTG	4,985,388
		GTTTGAGGGACCTAAGGAATG	
2	RMS-SF21-2	ACGGAGGAGACATGGAGC	4,988,522
		GCAAAATACTACTCCCTATC	
3	RMS-SF21-3	GTCAGCCGTAGGATGATAT	4,979,591
		CCGACTCAATATTGCCACG	
4	RMS-SF21-4	GTCGTCAAGGTCGTCGTC	4,975,318
		GAGGCGGCGGGGAAAGGC	
5	RMS-SF21-5	GAGTTGGGGGTCGAGAAATC	4,977,751
		CGTACGTGCGGCTAGGATCAA	

50 mM KCI, 1.5 mM $MgCL_2$, 0.01 % (v/v) gelatin], 30–50 ng of template DNA, 5 pmol of each primer, 200 μM (each) deoxyribonucleotide and 1 unit of Taq polymerase (Merck, India). PCR conditions included an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 7 min. All amplified products were resolved in 2–3.5 % agarose gels (Lonza Inc., USA) along with 100-bp molecular marker (Merck, India). The codominant markers that showed clear polymorphism between restorers and maintainers were validated in the F_2 segregating populations. The scores 1, 2 and 3 were given to codominant markers for parent 1 type (P1) and parent 2 type (P2) and heterozygous (F1). The segregation of codominant markers in the F_2 population was studied by Chi-square test for the Mendelian segregation ratio 1:2:1 as outlined by Gomez and Gomez (1984).

Spikelet fertility analysis

About 20-day-old seedlings of F_2 individuals were transplanted in the field. At reproductive stage of growth, just before flowering, the panicles of main tiller and two side tillers of each individual plant were bagged with a paper bag to prevent cross-pollination. The seed set in each panicle was counted, and spikelet fertility was determined according to Sheeba et al. (2009). All the plants in the population were classified into four classes based on spikelet fertility percentage, namely fertile (more than 71 % spikelet fertility), partially fertile (31–70 %), partially sterile (1–30 %) and sterile (0 %).

Analysis of impurities in a seed lot of DRRH3 using *Rf4*-specific codominant marker

Four hundred seedlings of the popular rice hybrid DRRH3 from a seed lot were planted in a grow-out plot in the experimental farm of ICAR-Indian Institute of Rice Research, Hyderabad, India, during wet season 2015. DNA was isolated from 20-day-old seedlings of the 400 coded plants, individually as per the procedure of Zheng et al. (1995). Genotyping of the 400 seedlings was done using the *Rf4*-specific codominant marker, RMS-PPR9-1, which exhibited polymorphism among the female (APMS6A) and male (RPHR-1005) parents of DRRH3. The genotype inferred from the marker profile was compared with the phenotype at maturity to verify the results derived from marker analysis with grow-out test (as described in Yashitola et al. 2002 and Sundaram et al. 2008).

Sequencing of PCR fragments

Amplified PCR product of RMS-PPR9-1 marker from KMR3R and IR58025A was gel-purified (Wizard® SV PCR clean up kit, Promega), cloned into pDrive cloning vector (Qiagen, USA) and sequenced using an ABI Prism 3700 automated DNA sequencer (PerkinElmer, Wellesley, MA) as per the procedure suggested in Rajendrakumar et al. (2007). Homology search was performed by BLASTN algorithm (Altschul et al. 1990) through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast), and the amplicon sequences from IR58025A and KMR3R were aligned using the software ClustalW to validate the *in-del* polymorphisms which were identified through sequence analysis of *PPR9* genomic regions.

Results

Development and validation of candidate gene-specific markers for *Rf3* and *Rf4*

The sequence analysis of candidate gene *PPR9-782-M, PPR9-782-I* (which are specific for *Rf4*) and the non-restorer sequences *PPR9-409* and *PPR9-782-ZH* revealed the presence of three major *in-dels* within the gene (Supplementary Figure 1). These include a 42-bp *in-del*, identified in the first intronic region, a 105-bp *in-del* and a 1476-bp *in-del* identified within second exonic region. Targeting each of these *in-del* polymorphisms, codominant markers were designed and validated. Two other major *in-dels* were also identified in the upstream region of *PPR9-782-M* gene and targeted for development of codominant markers. Out of the five codominant markers specific for *in-dels* within *PPR9-782-M* or in its vicinity, three markers, viz. a marker targeting 42-bp *in-del* polymorphism within *PPR9* gene, i.e., RMS-PPR9-1, two codominant markers, viz. RMS-PPR9-4, RMS-PPR9-5 targeting polymorphisms in the upstream region of *PPR9-782-M* gene displayed clear polymorphism between IR58025A and KMR3R (Fig. 1; Table 3).

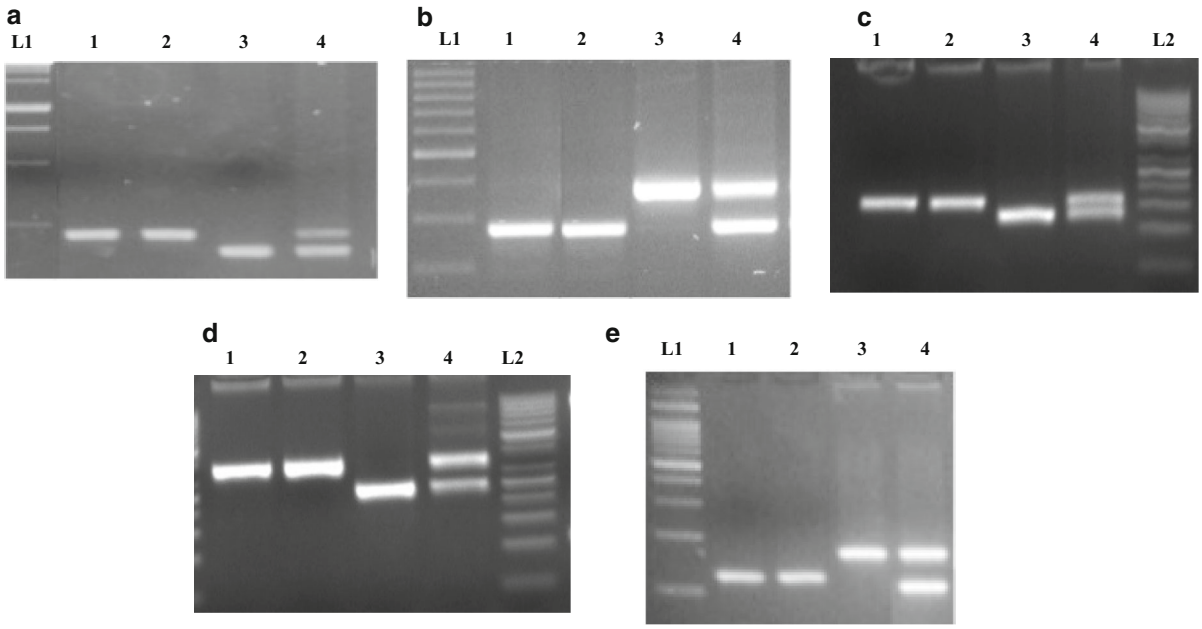

Fig. 1 Amplification pattern of markers developed targeting the candidate genes for *Rf4*, viz. *PPR9-78-M* and *PPR762:* **a** RMS-PPR9-1 (targeting *in-del* within *PPR9-782-M*); **b** RMS-PPR762 (targeting *PPR762*); **c** RMS-PPR9-4 marker (targeting *PPR9-782-M*); **d** RMS-PPR9-5 marker (targeting *PPR9-782-M*); **e** RMS-SF21-5 marker (targeting *SF21*). *L1* indicates 100-bp ladder, *L2* indicates 50-bp ladder, *1* indicates IR58025A, *2* indicates IR58025B, *3* indicates KMR3R, and *4* indicates KRH2 in the figure

Table 3 Expected amplification sizes of the markers developed in the study

S. no.	Primer name	Expected PCR amplicon size (bp)	Expected PCR product size (bp) in		
			Restorer	Non-restorer	Hybrid
1	RMS-PRR9-1	114/159	114	159	114,159
2	RMS-PPR9-2	447/1923	447	1923	447,1923
3	RMS-PPR9-3	365/470	365	470	365,470
4	RMS-PPR9-4	129/160	129	160	129,160
5	RMS-PPR9-5	178/360	178	360	178,360
6	RMS-PPR762	280/385	280	385	280/385
7	RMS-SF21-1	183/131	183	131	183,131
8	RMS-SF21-2	312/415	312	415	312,415
9	RMS-SF21-3	196/165	196	165	196,165
10	RMS-SF21-4	101/113	101	113	101,113
11	RMS-SF21-5	172/127	172	127	172,127

The analysis of restorer and non-restorer amplicon sequences of DRCG-RF4-14 marker targeting *PPR762* gene revealed existence of a 105-bp *in-del* polymorphism. Targeting this, a codominant marker RMS-PPR762 was designed and validated. RMS-PPR762 showed clear polymorphism between IR58025A and KMR3R (Fig. 1; Table 3). Thus, a total of four polymorphic markers were designed and validated in this study targeting the putative candidate genes for *Rf4* (i.e., *PPR9-782-M* and *PPR762*).

In addition to *Rf4*, the putative candidate gene for *Rf3* (another fertility restorer gene for WA-CMS), viz. *SF21* (Balaji et al. 2012), was analyzed through comparative sequence analysis of restorer and non-restorer genotypes and five major *in-del* polymorphisms were identified in the vicinity of gene.

Targeting each of these, a codominant marker was designed and validated. However, only one codominant marker, RMS-SF21-5, displayed clear polymorphism between the WA-CMS lines IR58025A and the restorer line, KMR3R (Fig. 1; Table 3).

Marker–trait co-segregation analysis

The candidate gene-specific markers for *Rf4* and *Rf3* which have shown clear polymorphism between IR58025A and KMR3R were analyzed for their co-segregation with the trait of fertility restoration in a F_2 population derived from the cross IR58025A/KMR3R (Supplementary Table 2). All the codominant markers displayed a Mendelian segregation ratio of 1:2:1 in the F_2 mapping population and the candidate gene-specific marker for *Rf4*, RMS-PPR9-1 was observed to be significantly associated with the trait at $P < 0.01$. The markers RM6100 (Singh et al. 2005) and DRCG-Rf4-14 (Balaji et al. 2012) were also observed to be associated with trait phenotype, but to a lesser extent. The earlier reported marker DRCG-Rf4-14 (Balaji et al. 2012) and the marker RMS-PPR762 developed in this study, targeting the same 105-bp polymorphism in *PPR762* gene, displayed identical association with the trait phenotype, but at a slightly lesser level of association as compared to RMS-PPR9-1 targeting *PPR9-782-M*. RMS-PPR762 showed clear and robust resolution of the restorer-specific and non-restorer-specific alleles when compared to the earlier designed marker, DRCG-Rf4-14. With respect to *Rf3*, the earlier reported SSR marker DRRM-Rf3-10 (Balaji et al. 2012) and RMS-SF21-5, the marker developed in this study, displayed same level of association with the trait phenotype (Supplementary Table 2) with the newly designed marker showing clear resolution of alleles as compared to DRRM-Rf3-10 (Supplementary Figure 3).

Assessment of prediction efficiency of the markers targeting *Rf4* and *Rf3*

To validate the efficiency of these markers in accurately predicting the fertility restoration trait, they were analyzed with a set of 120 known restorers and 44 known non-restorers. The selection efficiency of the candidate gene-specific markers, RMS-PPR9-1 and RMS-PPR762 developed in this study, was 91 and 82 %, respectively (Supplementary Table 3; Fig. 2). As expected, the earlier reported marker, DRCG-Rf4-14, and the newly designed marker, RMS-PPR762, displayed same selection efficiency of 82 %, as they targeted the same polymorphism. The selection efficiency of candidate gene-specific marker for *Rf3*, RMS-SF21-5 and the earlier reported marker DRRMRf3-10 in identification of restorers and non-restorers was identical (i.e., 57 %; Supplementary Table 3; Fig. 3). The combined selection efficiency of the best markers for *Rf4* and *Rf3*, viz. RMS-PPR9-1 + RMS-SF21-5, was as high as 94 %. Particularly, the candidate gene-specific marker for *Rf4*, RMS-PPR9-1, was observed to show polymorphism among all the male and female parents of commercial rice hybrids based on WA-CMS system analyzed in this study (Supplementary Figure 4). When these markers (viz. RMS-PPR9-1, RMS-SF21-5) were analyzed in a set of wild rice accession belonging to *O. nivara* and *O. rufipogon* (Supplementary Table 1), it was observed that many *O. nivara* accessions showed the presence of restoring allele with respect to *Rf4*.

Utility of gene-specific markers for *Rf4* in detection of impurities in hybrid/parental seed lots

The candidate gene-specific marker for *Rf4* locus, RMS-PPR9-1, was deployed for identification of impurities in a seed lot of the hybrid DRRH3. With the help of the marker, a total of seven impurities were identified in the seed lot (Supplementary Figure 5), and a perfect correlation was observed between the marker analysis data and grow-out test (GOT) data.

Discussion

Wild-abortive (WA)-type CMS-based hybrids contribute significantly to the total rice cultivated area worldwide. Inheritance of fertility restoration for the WA-CMS system has been extensively investigated and two major loci, *Rf4* and *Rf3* are known to control the trait (Young and Virmani 1984; Li and Yuan 1986; Virmani et al. 1986; Govinda Raj and Virmani 1988; Bharaj et al. 1991; 1995; Teng and Shen1994). However, efforts to delineate the candidate genes underlying *Rf4* and *Rf3* are very limited. Recently, Ngangkham et al. (2010) and Balaji et al. (2012) identified that *PPR3* and *PPR762* are the putative

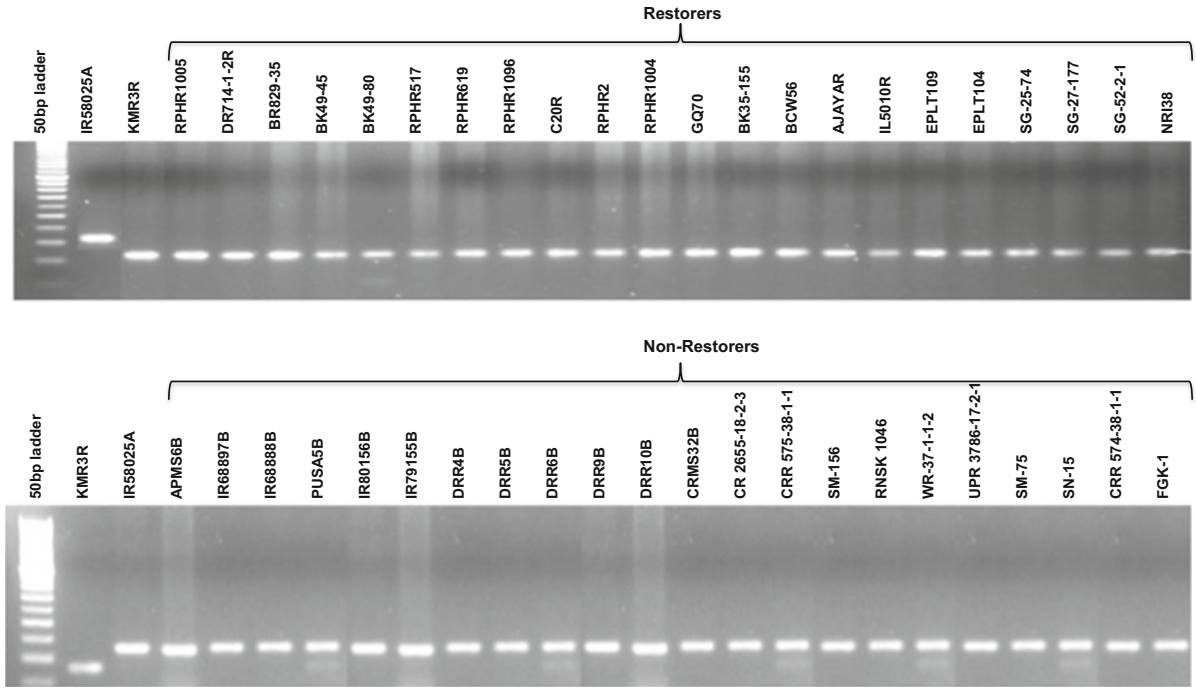

Fig. 2 Amplification pattern of RMS-PRR9-1, the candidate gene-specific marker for *Rf4* (targeting *in-del* within *PPR9-782-M*) in a set of restorers and non-restorers (i.e., maintainers) in the above lanes, IR58025A (WA-CMS) and KMR3R (restorers) are used to standard checks

candidate genes for *Rf4*, while two independent groups cloned and characterized another putative candidate gene *PPR9-782(M,I)*, for *Rf4* loci (Tang et al. 2014; Kazama and Toriyama 2014). According to the report of Tang et al. (2014), there are diverse functional *Rf4/rf4* alleles based on their donor source, *PPR9-782-M* allele from MH63 and *PPR9-782-I* from IR24 and two types of non-functional *rf4* alleles, *PPR9-409* (*rf4-i* from *indica*) and *PPR9-782-ZH* (*rf4-j* from *japonica*). With respect to *Rf3*, a putative candidate gene, *SF21* has been identified (Balaji et al. 2012).

In the present study, we analyzed the sequences of the above mentioned putative candidate genes, which have been earlier implicated with *Rf4* and *Rf3* controlled fertility restoration, identified sequence polymorphisms within the candidate genes and targeting these polymorphic regions, designed codominant markers and validated them in a mapping population and also in a large set of restorers and non-restorers lines. Based on marker–trait co-segregation analysis and analysis of selection efficiency of markers, we confirmed the candidacy of *PPR9-782-M* gene to be specific for *Rf4*. Our study is the first report on development of the candidate gene-specific marker, named RMS-PPR9-1 targeting *PPR9-782-M*, and *PPR9-782-I* gene specific for *Rf4* and another candidate gene-specific marker named RMS-SF21-5 targeting *SF21* gene specific for *Rf3*. Among these two candidate gene-specific markers developed in this study, RMS-PPR9-1, specific for *Rf4* has displayed higher selection efficiency of 91 % in terms of identification of all the known major restorer lines (Supplementary Table 2), as compared to the RMS-SF21-5 marker, which is specific for *Rf3* showing only 57 % selection efficiency. These findings support the general understanding that a good restorer would possess *Rf4* gene alone or *Rf4* gene along with *Rf3* gene, while lines possessing *Rf3* alone might not be good restorers. Thus, *Rf4* has a stronger influence on the trait than *Rf3* as observed earlier by several groups (Yao et al. 1997; Sattari et al. 2008; Cai et al. 2013, 2014). However, a few exceptions were also found in this study. Two of the known restorer lines IR66 and IR40750R were observed to possess only *Rf3* and not *Rf4* and another two known restorers PNR 3158 and AYT 21 do not possess both *Rf3* and *Rf4*. The possible explanation could be that IR66, IR40750R may not be very good restorers and/or may not possess

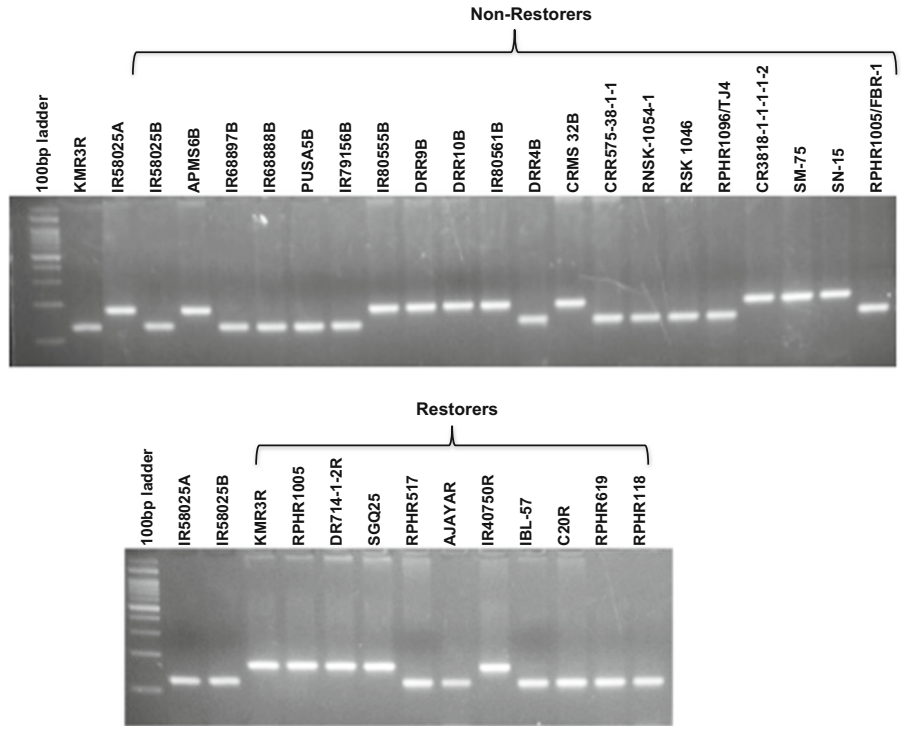

Fig. 3 Amplification pattern of RMS-SF21-5, the candidate gene-specific marker for *Rf3* (targeting *in-del* upstream of *SF21*) in a set of restorers and non-restorers (i.e., maintainers) in the above lanes, IR58025A (WA-CMS) and KMR3R (restorer) are used to standard checks

PPR9 gene (both *PPR9-782-M* and *PPR9-782-I* functional alleles) as RMS-PPR9-1 targets *PPR9-782 (M,I)* and might possess novel loci other than *Rf3* and *Rf4* for fertility restoration, as Kazama and Toriyama (2014) reported that other fertility restoration genes could be associated with restoration of WA-CMS.

The process of screening for the trait of fertility restoration is laborious and time-consuming as it involves test crossing with a set of WA-CMS lines followed by evaluation of the F_1s for pollen and spikelet fertility. Molecular markers targeting the candidate gene associated with the trait are more efficient in accurate identification of restorers among rice germplasm (Sheeba et al. 2009). Recently, our group reported development of a functional marker, targeting the candidate gene, *WA352* for WA-CMS trait (Pranathi et al. 2016). However, functional markers for the fertility restoration trait were not available, when this study was initiated and most of the markers available were either linked markers or markers targeting non-validated putative candidate genes. To develop candidate gene-specific markers for fertility restoration trait, we first attempted to identify candidate genes for *Rf4* and *Rf3*. In a recent study, Tang et al. (2014) delineated *Rf4* locus to a 137-kb region on chromosome 10 and identified three candidate genes, out of which *PPR9-782-M* derived from an elite restorer line Minghui 63 (MH63, with *Rf3* and *Rf4*) and *PPR9-782-I* from IR24 was confirmed as a causal gene through complementation assay. Further, the action of *Rf4* on *WA352* (*orf 352)* was confirmed by RNA blot analysis. The same study reported two *in-del* markers (M19288, with a 23-bp *in-del* and M19280 with a 6-bp *in-del*). However, it was observed that M19288 displays a dominant fashion of amplification, while M19280 amplified polymorphic fragments from restorers and maintainers in our study. However, the reported primer binding sites (F primer binding site-19,280,871 bp in *japonica*) of M19280 *in-del* marker was observed to be not located within the candidate gene (*PPR9* with genomic region from 19,287,680 to 19,295,473 bp) and the 6-bp *in-del* was located at a distance of 6.8 kb upstream from the gene. Further, as the marker targeted a 6-bp deletion, the polymorphism detected by it was not robust and required a higher percentage of agarose gels (>3.5 %)

for discrimination of the restorer and non-restorer alleles and could not identify many known restorer lines (data not shown), and hence, the marker may not be useful in routine breeding programs. Kazama and Toriyama (2014) identified the same gene (i.e., *PPR9-782-M,I*) through fine-mapping of *Rf4* locus that corresponded to a 213-kb region of Nipponbare genome in IR24 cultivar and demonstrated that the fertility restoration is controlled sporophytically. Interestingly, the sequence annotation study of reported candidate genes specific for *Rf4*, *PPR9-782(M,I)* and *PPR3* (Ngangkham et al. 2010) in Nipponbare genome shows that both genes encode the same 782 amino acid-containing protein (Os10g0495200 or LOC_Os10g35240), where as another reported putative candidate gene, *PPR762* encodes a different protein with 762 amino acids (BAD08213; protein sequence alignment file as Supplementary Figure 6). Even though we could observe a 105-bp polymorphism between restorers and non-restorers with respect to *PPR9-782-M*, on critical analysis of restorer and non-restorer sequences of the three putative candidate genes reported for *Rf4* (mentioned above), we identified that the 105-bp deletion is present in all the three putative candidates *PPR762*, *PPR9-782-(M,I)* and *PPR3* (alignment file as Supplementary Figure 7). Thus, this polymorphic region may not be unique to a particular candidate gene and hence is not be amenable for development of candidate gene-specific marker. We have identified a unique *in-del* region of 42 bp within the candidate gene (i.e., *PPR9-782-M*) (Supplementary Figure 1). Targeting this *in-del* polymorphism, we designed and validated a codominant marker named RMS-PPR9-1. The marker RMS-PPR9-1 displayed very significant association with the trait phenotype (of fertility restoration) and unequivocally distinguishes almost all the major restorer lines from the non-restorers of *indica* rice type (Supplementary Table 2). Thus, RMS-PPR9-1 marker targeting a unique 42-bp *in-del* within *PPR9-782(M,I)* gene can be considered as the ideal candidate gene-specific marker for *Rf4*. Further, we validated the 42-bp *in-del* polymorphism through sequencing of RMS-PPR9-1 candidate gene-specific marker amplicons from IR58025A (WA-CMS) and popular restorer KMR-3R (restorer) lines (Supplementary Figure 8).

Another loci known to be controlling the trait of fertility restoration in WA-CMS system is *Rf3*. Using RAPD and RFLP markers *Rf3* was earlier mapped on chromosome 1 (Yao et al. 1997; Zhang et al. 1997). Till now, there are no reports on cloning and characterization of the candidate gene(s) controlling *Rf3* loci. Balaji et al. 2012 reported a putative candidate, a pollen-specific protein (*SF21*) encoding gene to be specific for *Rf3*. Targeting the major deletion in the upstream region of *SF21* gene, RMS-SF21-5, a codominant, gene-specific marker, was designed and validated in our study. The newly developed marker, RMS-SF21-5, and the earlier reported SSR marker, DRRM-Rf3-10 (Balaji et al. 2012), displayed the same selection efficiency, as they target the same candidate gene, *SF21*. However, the newly designed marker RMS-SF21-5 was more robust, showing clear polymorphism as compared to DRRM-Rf3-10 (Supplementary Figure 3). Interestingly, when both the gene-specific markers RMS-PPR9-1 (specific for *Rf4*) and RMS-SF21-5 (specific for *Rf3*), used in conjunction, displayed increased selection efficiency of 94 % as compared to deploying them alone and also as compared to earlier reported markers for fertility restoration trait. The gene-specific markers developed in the study, notably RMS-PPR9-1 has higher efficiency in identifying all true F_1s hybrids in WA-CMS system (Supplementary Figure 4) and also highly efficient in detection of impurities in hybrid seed lots (Supplementary Figure 5), which was clearly demonstrated in this study through analysis of genetic impurities in a seed lot of the popular hybrid, DRRH3. When the marker RMS-PPR9-1 (specific for *Rf4*) was validated among 71 Indian accessions of *O. nivara* and *O. rufipogon* (Pranathi et al. 2016), many accessions displayed amplification of the restorer-specific allele and interestingly, most of the wild rice accessions, which possess wild-abortive cytoplasm and are still fertile, displayed *Rf4*-specific allele with respect to RMS-PPR9-1 marker indicating the possibility of coevolution of WA-CMS and fertility restoration traits in a few Indian wild rice accessions of *O. nivara* and *O. rufipogon.*

In conclusion, through the present study, we identified significant *in-del* polymorphisms within and around each putative candidate gene specific for *Rf4* and *Rf3* loci. Through marker–trait co-segregation analysis and higher selection efficiency of gene-specific marker targeting *in-del* polymorphism specific to *PPR9-782-M*, we confirmed the association of earlier reported gene *PRR9-782 (M,I)* with *Rf4* loci (Tang et al. 2014) and we report the first gene-specific

markers for major fertility restoration loci, *Rf4* and *Rf3*. The deployment of gene-specific marker for *Rf4* (RMS-PPR9-1) and another gene-specific marker for *Rf3* (RMS-SF21-5) in conjunction displayed higher selection efficiency compared to utilization of gene-specific marker alone. Further validation of gene-specific markers in F_2 population and germplasm established major influence of *Rf4* than *Rf3* on the trait. The gene-specific markers, particularly RMS-PPR9-1 marker, can facilitate marker-assisted selection and targeted transfer of *Rf4* gene into elite backgrounds and also provide highly accurate, rapid detection of impurities in hybrid seed lots. Further efforts are necessary for characterization of candidate gene(s) for *Rf3* loci.

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