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Co-segregation study of Marker locus with Restoring fertility (R*f*) gene in Rice

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Abstract
The experiment was conducted to study the co-segregation pattern of markers linked to restoring fertility (R*f*) gene in HYV of Kerala. Bulk segregation analysis (BSA) was carried out in segregating population of cross between male sterile line UPRI95-17A and Remya, Jayathi, Neeraja and Pavizham to study co-segregation of SSR marker RM 1 and RM 171 associated with R*f3 and Rf4* genes and the trait of restoration of fertility. It was observed that the amplicon size of RM 1 and RM 171 markers in fertile and sterile bulk is similar to their respective fertile and sterile parents. The result indicates that the marker loci and fertility restoration genes R*f*3 and R*f*4 are tightly linked. Closely linked DNA markers RM1 and RM171 will facilitate effective selection in plant breeding and proper management of genetic purity of hybrid seed.

Keywords: Co-segregation, R*f* loci, molecular marker, linkage

Introduction
Bulk segregant analysis (BSA) is a marker assisted breeding strategy, which significantly reduces the sample size by aggregating plants based on the expression of particular trait. BSA assess variation present in pools of segregants that have been classified according to the phenotypic expression of a trait and assign a specific location in genetic map based on correlation. So through BSA technique gene linked to R*f* loci can be identified (Ahmadikhah *et al.*, 2007) [7].

This technique involves formation of 2 groups that display identifiable morphological difference for a trait of interest. DNA samples are then prepared by mixing equal amount of DNA of all the individual plant in a particular group. Pool of DNA samples are then analysed to determine resemblance and dissimilarity throughout the genome. Pooled DNA samples will have a uneven distribution of different alleles in all loci of the genome other than the loci associated with mutation (McClean, 1992) [5]. A persistence difference on a locus between the two bulked samples would indicate that a particular locus has a close association with a specific mutation.

Hybrid rice technology based on Cytoplasm Genic Male Sterility (CGMS) system has been widely adopted across around the world, especially in Asia and USA. The essential requirements in development of hybrid rice is highly heterotic parents. Male sterility in Wild abortive (WA) cytoplasm is governed by cytoplasmic factor and its contrasting fertility restoration genes are present in the nuclear genome. Different cytoplasm in rice have different mechanism for the cause of male sterility and they have different fertility restorer genes governed by nuclear genome. Anandakumar and Subramaniam (1992) [8] reported that a single dominant gene governed fertility restoration in WA-cytoplasm. Though, *Rf3* and *Rf4*, two major fertility restorer genes for WA-type CMS in rice had been mapped in chromosomes 1 and 10, respectively (Ahmadikhah and Karlov 2006 [6]; Tang *et al.* 2014 [9]; Zhang *et al.* 1997 [10]). Considering above facts, in the present experiment we aim to analyse co-segregation of Rf_3 and Rf_4 gene in restorer lines with the linked SSR markers.

Materials and Methods
To find markers that are associated to the *Rf* gene, bulk segregation analysis is used which is essentially method reported by Zhang *et al.*, 1997 [10]. Bulks were made by selecting fertile and sterile plants in F_2 population to identify marker linked to R*f* gene through co-segregation of allele and restorer gene. DNA was pooled in fertile and sterile bulk by mixing equal amount of DNA from 10 plants in each group in the F_2 population of cross between male sterile line UPRI95-17A and fertile restorers such as Remya, Jayathi, Neeraja and Pavizham.

Genomic DNA from sterile and fertile plants were extracted using the procedure of QIAGEN DNeasy plant mini kit. DNA was pooled from each fertile and sterile plant to form fertile and sterile bulk. Bulk DNA are then amplified using SSR marker's RM1 and RM171 linked to fertility restoration gene. Volume of the PCR reaction mixture 25 µl contained 50 ng genomic DNA, 10 pico moles of each marker, 10 nM dNTP, 10x PCR buffer and 1 Unit of Taq DNA polymerase. Amplifications were carried out in gradient PCR following 94°C for initial denaturation for 4 min followed by 35 cycles of PCR amplification with the following parameters: 94°C denaturation temperature for 30 second, a 30sec annealing at standerdised temperature for each primer, 72°C primer extension for 1 min and final extension 7 min at 72°C. The amplified product was resolved on a 1.8% agarose gel containing 0.5 µg/ml of ethidium bromide and visualized under documentation unit.

Table 1: SSR marker used in the study

SL. No	Marker	Gene	Chromosome No	Primer sequence	Annealing temp	Reference
1	RM 1	R*f3*	1	F: GCGAAAACACAATGCAAAAA R:GCGTTGGTTGGACCTGAC	53.8	Ahmadikhah *et al.*, 2007 [7]
7	RM 171	R*f4*	10	F: AACGCGAGGACACGTACTTAC R: ACGAGATACGTACGCCTTTG	58.7	Jing *et al.*, 2001 [11]

Agarose gel electrophoresis was carried out in a BIO-RAD, gel electrophoresis Unit. 0.8g of agarose was weighed and melted in 1x TAE buffer. After cooling the solution to 42-45 ºC, ethidium bromide was added at the rate of 3µl for 100ml. The solution was then poured on to a preset, sealed gel casting tray with a comb fixed in position, to a height of 3mm-5mm. The gel was allowed to solidify for 15-20 min. The comb and sealing tapes were then removed and tray was submerged in electrophoresis tank filled with 1x TAE buffer ensuring that the buffer covered the gel to height of 1mm. Required volume of DNA sample and loading dye (glycerol 30% + bromophenol blue) were mixed in the ratio 5:1 and loaded into the slots of gel using a micropipette near the negative terminal. The electrophoresis unit were attached to the power supply and a constant voltage of 60V was used for the run. The power supply was turned off when the loading dye moved about 3/4th of the gel. The gel was then documented using gel documentation system.

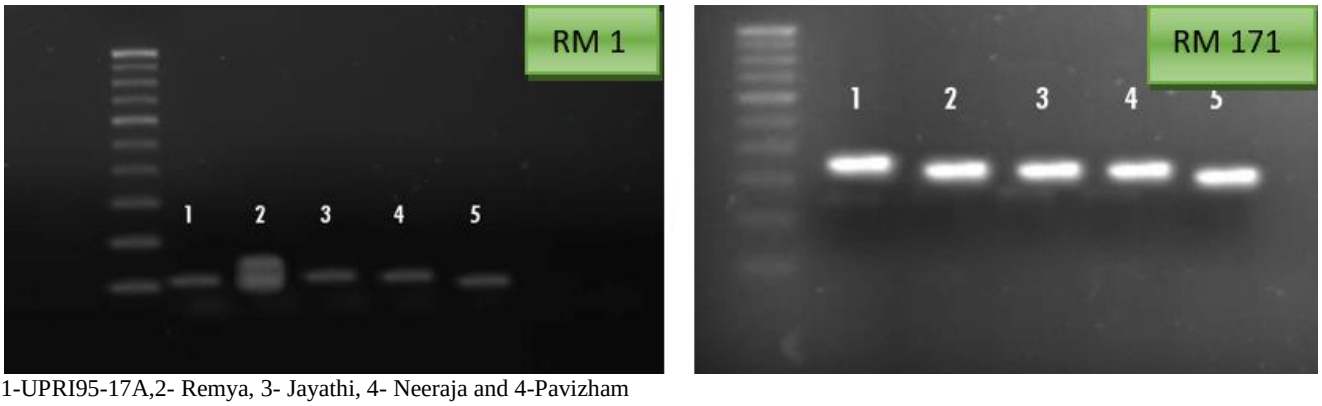
Results

Bulks were made by selecting fertile and sterile plants from the F_2 population of cross between CMS line UPRI95-17A and restorers, Remya, Jayathi, Neeraja and Pavizham. For genotyping PCR amplification of the Bulk sample, identified putative restorer lines, (Remya, Jayathi, Neeraja and Pavizham) as well as CMS line (UPRI95-17A) were carried out using RM 1 and RM 171 primers having a tight linkage with *Rf3* and *Rf4* genes. Markers were selected based on the research report that SSR markers RM1 is linked with *Rf3* gene on the short arm of 1st chromosome (He *et al.*, 2002) [12], and RM171 with *Rf*4 on the long arm of 10th chromosome (Jing *et al*. 2001) [11].

Polymorphism study between male sterile and restorer lines: Polymorphism was observed between CMS line (UPRI95-17A) and 4 fertile lines for both primer pairs (Figure 1). Though the marker loci detect polymorphism between CMS line and four restorers', the existence of a polymorphism alone is inadequate to relate this polymorphism with possible linkage between marker loci and the given genes. It is established fact that SSR markers are not specific for a gene, but are located in a short distance from the R*f* loci, so to study possible association of RM1 and RM 171 marker with Rf3 and Rf4 gene co-segregation analysis of the marker and the given trait in a segregating population is carried out using bulked segregant analysis (BSA).

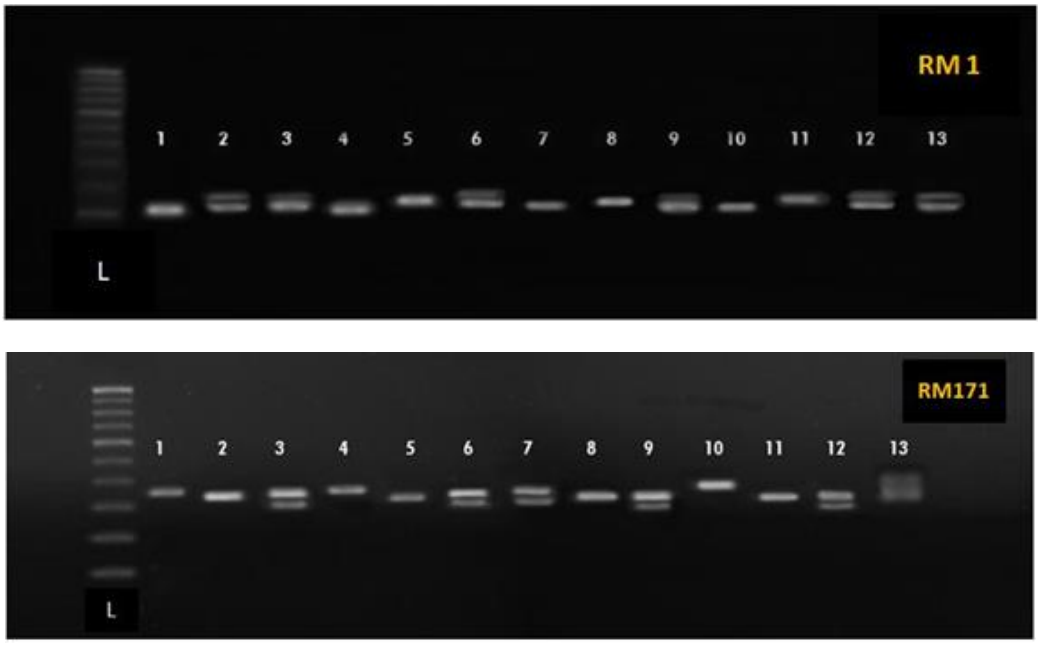
Co-segregation study of *Rf* gene with marker locus

To proceed with BSA analysis, F_2 plants were divided into two groups such as fertile and sterile based on the expression of the trait fertility. As a result, plants in two groups differed among themselves for the alleles of the R*f* genes. When five parents and bulk DNA was polymerised in PCR with RM 1 and RM 2 it has been observed that all the fertile bulk and sterile bulk producing heterozygous and homozygous band respectably. The amplicon size of fertile and sterile bulk is similar to their respective fertile and sterile parents (figure 2), except for Pavizham with RM 1 and Jayathi with RM 171 where the F_2 bulks producing heterozygous band with amplicon size almost equal to the fertile parent.



1-UPRI95-17A,2- Remya, 3- Jayathi, 4- Neeraja and 4-Pavizham

Fig 1: Polymorphism between male sterile line and restorer lines used for BSA analysis



1-UPRI95-17A, 2-Remya, 5-Jayathi, 8-Neeraja, 11- Pavizham 3, 6, 9, 12-Fertile bulk, 4, 7, 10, 13-Sterile

Fig 2: Gel picture of 5 parents and their respective Fertile Bulk and Sterile Bulk in F_2 generation

Co-segregation was not observed between the *Rf* gene and the marker locus of RM1 and RM171 in the F_2 generation of crosses UPRI95-17A x Pavizham and UPRI95-17A x Jayathi. In the crosses UPRI95-17A x Remya, UPRI95-17A x Jayathi and UPRI95-17A x Neeraja co-segregation between marker RM 1and the trait was observed. While co-segregation between marker RM 171and the trait was observed in F_2 generation of cross between UPRI95-17A and Remya, Neeraja and Pavizham.

Discussion

Co-segregation between marker RM 1 and the trait of interest is detected in the crosses *viz.*, UPRI95-17A x Remya, UPRI95-17A x Jayathi and UPRI95-17A x Neeraja, indicates *Rf* present in these lines can be tracked on short arm of chromosome 1. Similarly, co-segregation between marker RM 1 and the trait was detected in cross between sterile UPRI95-17A and restorers, Remya, Neeraja and Pavizham, designates fertility restoration gene carried by these lines is located in chromosome 10. In F_2 generation of crosses UPRI95-17A x Pavizham and UPRI95-17A x Jayathi co-segregation *Rf* gene with the marker locus of RM1 and RM 171 is not detected. This implies that *Rf* gene in these lines is located on a chromosome apart from chr. 1 or away from that locus.

In the previous experiment, we have found that rice varieties Remya, Jayathi and Neeraja had restoring fertility gene *Rf*3 where as Remya, Neeraja and Pavizham had restoring fertility gene *Rf4* when screened with SSR marker RM 1 and RM171 respectively. Bazrkar *et al.*, in the year 2008 and Kiani, 2015 had reported that microsatellite marker RM 1 and RM 171 is linked to *Rf3* gene and *Rf4* gene respectively. Results of this experiment also emphasized that fertility restoration is under the control of two major genes. In the present investigation it has been seen that *Rf3* and *Rf4* gene is co-segregating in the F_2 generation of cross between UPRI95-17A x Remya, UPRI95-17A x Jayathi, UPRI95-17A x Neeraja and UPRI95-17A x Remya, UPRI95-17A x Neeraja and UPRI95-17A x Pavizham respectively, this implies that marker locus and R*f* gene are tightly linked in those fertile parental lines. Similar result had also been reported by Ahmadikhah *et al.*, (2007) [7], Alavi *et al.*, (2009) [1] and Boopathi *et al.*, (2013) [2] while studying inheritance pattern of *Rf* gene in rice.

Conclusion

Bulk segregation analysis (BSA) was carried out in segregating population of cross between male sterile line UPRI95-17A and Remya, Jayathi, Neeraja and Pavizham to study co-segregation of SSR marker RM 1 and RM 171 associated with R*f3 and Rf4* genes and the trait of restoration of fertility. It was observed that the amplicon size of RM 1 and RM 171 markers in fertile and sterile bulk is similar to their respective fertile and sterile parents. The result indicates that the marker loci and fertility restoration genes R*f*3 and R*f*4 are tightly linked. Closely linked DNA markers RM1 and RM171 will facilitate effective selection in plant breeding and proper management of genetic purity of hybrid seed.

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