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RESEARCH ARTICLE :

Identification of simple sequence repeats (SSR) markers linked to yellow mosaic virus (YMV) resistance in blackgram [*Vigna mungo* (L). Hepper]

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SUMMARY : YMV transmitted by whitefly is the major disease in pulses that causes the severe yield loss in India as well as in other blackgram growing countries in Asia. Molecular markers linked with YMV can improve the process of identification of resistant genotypes. In the present investigation Simple Sequence Repeats (SSR) and Bulk segregant analysis (BSA) techniques were used to analyse the F_2 individuals of T_9 (resistant) × LBG-759 (susceptible) to screen and identify the yellow mosaic virus (YMV) resistant gene in urdbean. Two DNA bulks, namely resistant and susceptible bulks were setup by pooling equal amount of DNA from ten extreme phenotypes, resistance and susceptible plants. Parental survey study was carried out by using 59 SSR primers. This study revealed that 12 SSR markers showed polymorphism between the parents. These polymorphic markers were utilized for bulk segregant analysis (BSA). Among the polymorphic SSR markers, one primer *viz.*, VR9 were able to distinguish the resistant and susceptible bulks and individuals indicating that this marker is tightly linked to yellow mosaic virus resistance gene and report of YMV-resistance linked marker in blackgram. This marker VR9 could be utilized in the marker assisted breeding programme.

KEY WORDS :
Marker assisted breeding, Urdbean, yellow mosaic virus, Simple sequence repeats, Bulk segregant analysis

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BACKGROUND AND OBJECTIVES

Black gram [*Vigna mungo* (L.) Hepper] popularly known as urdbean in India. It is an important short duration, self-pollinating, diploid grain legume (2n=2x=22) crop belonging to the family Leguminaceae with a small genome size estimated to be 0.56pg/1C (574 Mbp) (Gupta *et al*., 2008). It is one of the most highly prized pulse crop, cultivated in almost all parts of India. It has inevitably marked itself as the most popular pulse and can be most appropriately referred to as the "king of the pulses" due to its mouth watering taste and numerous other nutritional qualities. Whether it is the very special "Dal makhni" of Punjab or the "Vada Sambhar" of South India.

Improvement in yield of urdbean is becoming difficult mainly due to the occurrence of pest and diseases (Karthikeyan *et al*., 2012). Among the various diseases yellow mosaic virus (YMV), which is a Begomovirus transmitted through white fly, *Bemesia tabaci*, Causing significant yield loss upto 85% was reported in blackgram (Nene, 1972 and Varma and Malathi, 2003). The disease occurs throughout the Asian countries. It remains unsuccessful in controlling the disease and developing resistant variety for MYMV through conventional breeding methods due to rapid explosion of new isolates of MYMV and also to the complexity of mechanism in controlling MYMV resistance (Selvi *et al*., 2006 and Karthikeyan *et al*., 2012). Identifying resistant donors is a very complicated task due to the lack of reliable screening protocol for assessing the resistance/ susceptibility of existing varieties. The advancements in the field of biotechnology and molecular biology such as genetic transformation and marker assisted selection could be utilized in developing YMV resistance urdbean (Xu *et al.,* 2000). Different molecular markers have been used for the molecular analysis of grain legumes (Gupta and Gopalakrishna, 2008). Among different DNA markers, microsatellites (or) Simple Sequence Repeats (SSRs) have occupied a crucial place because of their reproducibility, multiallelic nature, co dominant inheritance, relative abundance and good genetic coverage. Molecular markers and genetic linkage maps are pre- requisites for molecular breeding in any crop. Such tools would speed up the process of introgression of beneficial traits into preferred varieties.

RESOURCES AND METHODS

Mapping population :

T9 (YMV resistant), LBG 759 (YMV susceptible) urd beans were selected as parents and crosses were carried out during *Rabi* 2013-14. The F_1 of the cross was selfed to raise the F_2 population during *Kharif* 2014-15. The present investigation was carried out with 112 F_2 individuals. F_2 populations along with parents, F_1 were raised without any replications during late *Rabi* 2014-15 at Agricultural Research Station, PJTSAU, Madhira, khammam. The F_2 population was used for genotyping and phenotyping.

Phenotyping of F_2 individuals :

All the parents along with $F_{1,}$ and F_2 population were evaluated for mungbean yellow mosaic virus resistance under field conditions using the disease screening methodology. The F_2 (mapping population) was sown in pots filled with soil. Two rows of the susceptible variety check pots were raised all around the experimental pots in order to attract white fly and enhance infection of MYMV under field conditions. To maintain the experiment except that insecticide sprays were not given to encourage the white fly population for spread of the disease. The crop was regularly monitored for the presence of whitefly and development of YMV. Whitefly started landing on the plants soon after germination and the disease made its first appearance 3rd week after planting. Infection and disease severity of MYMV progressed in the next 6 weeks. The disease was scored on 0-5 arbitrary scale, as suggested by Bashir *et al.* (2005) which is described in Table A. The disease scoring was recorded from initial flowering to harvesting by weekly intervals.

Table A : Scale used for YMV reaction (Bashir *et al.*, 2005)

Severity	% Infection	Infection category	Reaction group
0	All plants free of virus symptoms	Highly resistant	HR
1	1-10% infection	Resistant	RR
2	11-20% infection	Moderately resistant	MR
3	21-30% infection	Moderately susceptible	MS
4	30-50% infection	Susceptible	S
5	More than 50%	Highly susceptible	HS

Genotyping for yellow mosaic virus

SSR analysis :

Extraction of two parents DNA was carried by CTAB method (Doyle and Doyle, 1987) and the quantification done by using 0.8 % (w/v) agarose gel electrophoresis. A total of 59 SSR primers were used in this experiment. PCR reaction mixtures were prepared with the volumes of 10 µL containing 2.00 µL of the extracted DNA, (50 ng/µL), 1.00 µL (10X) assay buffer, 1.00 µL (0.2µm) forward and reverse primers, 1.0 µL (2.5 mM) dNTPs, 0.30 µL (3 units/µL) and 3.70 µL Sterile distilled H_2O. The PCR reaction was carried out in thin walled PCR plates (96 wells/plate) in a thermal cycler (Applied Biosystems) programmed to run the following temperature profile: 94°C for 5 minutes Initial denaturation then 35 cycles consisting each of a denaturation step for 30 seconds at 94°C, an annealing

step for 30 seconds at 57-59°C: an extension step for 1 min at 72°C and the final extension for 7 min at 72°C. The amplified products were loaded on ethidium bromide stained agarose gels (3 %) and polymorphic primers were noted. PCR product was mixed with 3 µl of 6X loading dye and the sample was loaded in the well carefully. 50 bp ladder was loaded as reference marker. The gel was run at constant voltage of 90V for about 3 hours, until the ladder got properly resolved. Gel was photographed using Gel Documentation system. (BIORAD GEL DOC XR + Imaging system).

DNA bulks for BSA :

Two bulks of extreme phenotypes (resistance and susceptible) were used for the BSA analysis. Equal quantities of DNA were bulked from susceptible and resistant F_2 plants to give two DNA bulks. Ten plants from resistant and susceptible progenies were pooled for BSA analysis.

OBSERVATIONS AND ANALYSIS

The results obtained from the present study as well as discussions have been summarized under following heads:

Polymorphism of SSR markers :

The YMV susceptible LBG 759 and T9 YMV resistant parents were initially screened with 59 SSR primers, to detect the primers showing polymorphism between the parents. Out of these 59 primers used for parental survey, 12 markers showed the polymorphism between the parents (Fig. 1,) and the rest of markers are monomorphic (Fig. 2). 20.33% of polymorphism was observed in urdbean. The sequence of polymorphic primers, annealing temperature and amplification are represented in the Table 2. The confirmation of F_1 was done with polymorphic prmers (Fig. 3).

Identification of SSR markers linked to yellow mosaic virus resistance through BSA :

The F_1s along with their parents for the cross T_9, LBG 759 were raised for studying F_2 performance during *Kharif* 2014-15. Thus the mapping population comprised of F_2 generation was developed. A total of 112 F_2 individuals along with the parents and F_1 were screened for mungbean yellow mosaic virus resistance during *Rabi* 2014-15. Ten plants each of susceptible and resistant progenies for YMV were selected and pooled separately to form disease susceptible bulk and disease resistant bulk. 12 polymorphic SSR primers were used to survey the bulk. Among the polymorphic SSR markers, one SSR primers *viz.*, VR9 was able to distinguish the bulks. Differences between the bulked extremes and the respective individuals were very clear in all the Fig. 4, 5,

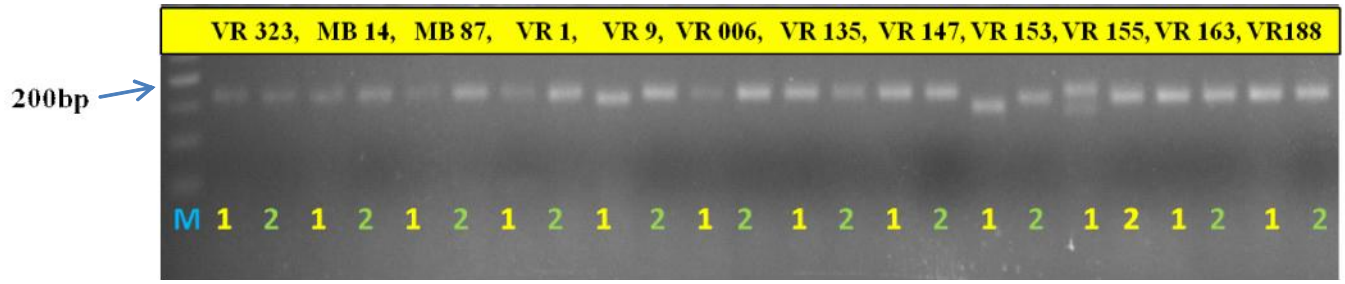

Fig. 1: Parental Polymorphism of urdbean lines LBG 759 (1) x T_9 (2) with 12 polymorphic SSR markers. The marker used is 50bp ladder

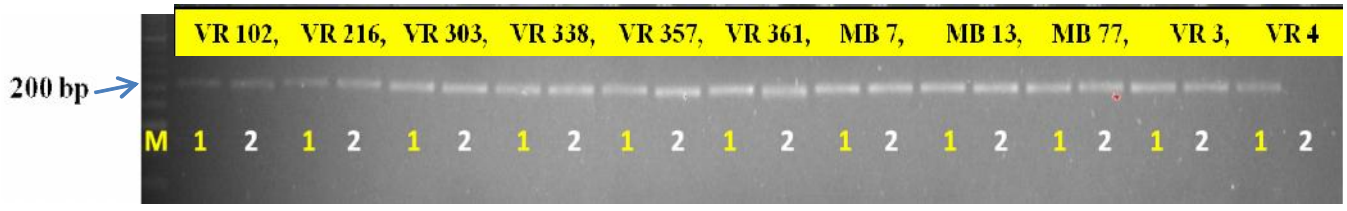

Fig. 2: Parental survey of mungbean lines LBG 759 (1) X T_9 (2) with monomorphic SSR

6.

The amplification of resistant parental allele in resistant bulk and susceptible parental allele in suseptible bulk indicated that this marker is associated with the gene controlling MYMV resistance in blackgram. Similar results were found in mungbean using 361 SSR markers. Out of 361 markers used, 31 found polymorphism between the parents. The marker CED 180 was found to be linked with resistance gene following the bulk segregant analysis (Gupta and Gupta, 2013). Shoba *et al*. (2012) identified the SSR primer PM 384_{100} allele for late leaf spot disease resistance by bulked segregant analysis in groundnut. PM 384100 was able to distinguish the resistant and susceptible bulks and individuals for late leaf spot disease.

Several studies were conducted in blackgram to identify the molecular markers linked to YMV resistance by using the RAPD primer from azukibean which shows the specific fragment in resistant parent and resistant bulk which were absent in susceptible parent and susceptible bulk (Selvi *et al*., 2006). Karthikeyan *et al*. (2012) reported that RAPD primer OPBB05 from azukibean which shows specific band size of 450 bp for susceptible parent, bulk and five individuals of F_2 population and another phenotypic (resistant) specific band size of 260 bp for resistant parent, bulk and five individuals of F_2 population. One species-specific SCAR marker was developed for ricebean which resolved a band of 400bp in resistant parent and not in the bulk (Sudha *et al*., 2012) Using SSR markers from azukibean Karthikeyan *et al*. (2012) studied the markers linked to YMV resistance in mungbean by BSA. Out of 45 primers, 6 showed polymorphism between parents and not able to distinguish the bulks. Similar results were found in blackgram using 468 SSR primers from soyabean, commonbean, redgram, azuki bean. Out of which 24 shows polymorphism between parents and none of the primer showed polymorphism between bulks (Basamma, 2011).

In several, such studies conducted earlier, molecular markers have been used to tag YMV resistance in many legumes like peanut (Shoba *et al*., 2012). Gioi *et al*. (2012) identified and characterized SSR markers linked to YMV resistance gene in cowpea by using 60 markers. The interval QTL mapping showed 98.4 per cent of the resistance trait mapped in the region of three loci AGB1, VM31 and VM1 covered 32.1 cM, in which 95%

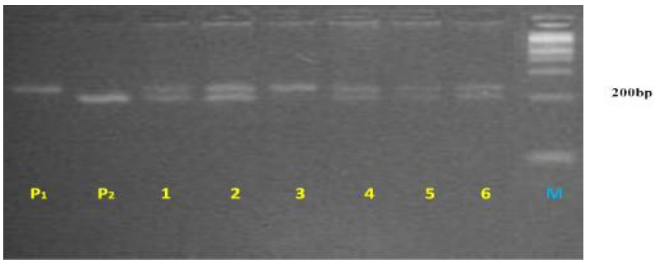

Fig. 3: Confirmation of F_1s using SSR marker VR9. P_1, P2, indicates the parents. Lanes 1-6 indicate F_1 plants.The marker used was 50 bp ladder

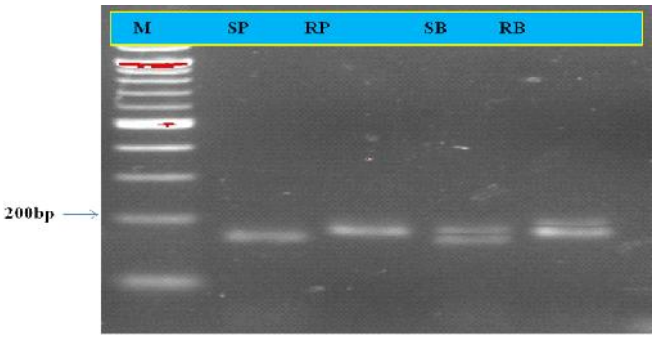

Fig. 4 : Bulk segregant analysis with SSR primer VR9. SP, RP indicates susceptible and resistant parents. SB, RB indicates susceptible and resistant bulks. The marker used in 50bp ladder

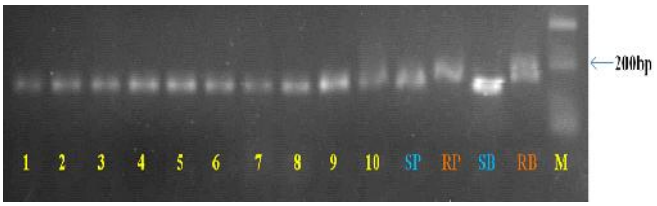

Fig. 5 : Confirmation of bulk segregant analysis with SSR primer VR9. SP, RP indicates susceptible and resistant parents. SB, RB indicates susceptible and resistant bulks. The lanes 1-10 indicate the F_2 resistant plants. The marker used in 50bp ladder

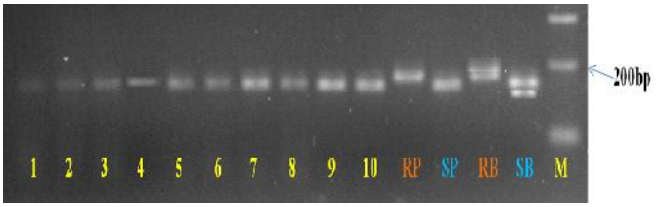

Fig. 6 : Confirmation of bulk segregant analysis with SSR primer VR9. SP, RP indicates susceptible and resistant parents. SB, RB indicates susceptible and resistant bulks. The lanes 1-10 indicate the F_2 susceptible plants. The marker used in 50bp ladder

Table 1 : SSR primers used for molecular analysis of MYMV disease in Blackgram

Sr. No.	Marker name	Primer sequence	Annealing temperature (°C)
1.	VR073	F – GGTAGTTCATTTCGGCCACTT	
		R –GGTAGTTCATTTCGGCCACTT	59
2.	VR102	F – CATGTGAGCTACCCTTTCAACA	
		R- CAAGGACTGCTATATCCAAGGC	58
3.	VR216	F – TTCCCTGTGTCCTTATATGTCC	
		R- GAGGATAGTGAATTTTGAAGGC	58
4.	VR303	F – AGACGAAGAAGAAAACGCAGAC	
		R- CCTCACACACAACACAACAGAA	59
5.	VR304	F - GAAGCGAAGAAGCCATAGAAAA	
		R- CCTCACACACAACACAACAGAA	58
6.	VR323	F - ATATCAGCCATTGTTGCTTTCC	
		R- TTCCCAGTTCAGACAACCAAGT	59
7.	VR326	F – GATGGCTCTGCATTGAAACC	
		R- GATCTTCCCAACTTTCCCTCTC	58
8.	VR357	F – GCCCGATGTCCTAGCTTTTAG	
		R- CCTCAAAACAATCAGAACTCTCG	59
9.	VR361	F – CTTGGACTTCGTCTCTGCG	
		R- CAAAACAACCAACGCCATTAC	59
10.	MB 7	F – CTTGCTTGCGAGGATGAC	
		R- TCCAGTGCAGCAGATTGA	57
11.	MB 13	F – GCAGCAACAACAGCAACA	
		R- GCAGGTTTTGTGGCTCAG	57
12.	MB 14	F – TGGAATTTGGAAGGAAGGA	
		R- GATGCAGGTGTTTGGGAG	57
13.	MB 17	F – ACCTGCAAGTTGGCAAGA	
		R- TATGTGCACGCATGGAAG	57
14.	MB 77	F – GGAGAGGAAGGAACAGGG	
		R- GGCAGAGCATAAACATGGC	57
15.	MB 87	F – TCCCTTGTGGGAGATCCT	
		R- CTTTGCCACACTCCTTGC	57
16.	MB 91	F – GAGGCCAATCCCATAACTTT	
		R- AGCACCACATCAGAGATTCC	58
17.	VR 1	F – AGCCCTTCGTGCTAGGAAAT	
		R- CCCTACCGGTTGGTTGGT	59
18.	VR 2	F – CGCCCCTCTAGGTTGGTTGG	
		R- GGGAAAGACGAAGGGTAGAA	59
19.	VR 3	F – GCCCCCCTTAGGTTGGTTGG	
		R- CCTTGTATTTGGATTCACAAGA	56
20.	VR 4	F – TGGTTGGTTGGTTCACAAGA	
		R- CACGGGTTCTGTCTCCAATA	58
21.	VR 5	F – TCACAAAGGGAGGGAAGAGA	
		R- CCCCAGGTTTGGTTGGTTGGA	59
22.	VR 9	F - TGACGGAGAGAGAGAGAGAGAG	
		R- TGCTTCCTTTTGTCTGAGTTAGAA	57
23.	VR006	F - CCTTCTATCTCATGTTCACCGTC	
		R- TGGAATAGGGACAAAATGGACT	59
24.	VR029	F -GAAAGAAGCCAAACAAAACAGG	
		R- TGGCAGAGAAGGTAAATAAGGG	59
25.	VR040	F - TGACAACATGGGAAGAAGAAGA	
		R- ACACCAACACAAAAGCAAACAC	58
26.	VR044	F - CCCATGAAGGTATGAGACAACA	
		R- GACTGAGAAAGAGAGAGAAGCATTT	59
27.	VR062	F - CGAAGACGAAATCTGAAGACAA	
		R- TTACTTCTCCCAGCACTCCAAT	58

Table 1 contd…

Table 1 contd…

29.	VR078	F – CATGTGGCAACGCAGAAG	
		R- TCAACTTATTCCTCCTTTCTCTCAC	58
30.	VR084	F – GAGCCACTTTGCCATATTTCT	
		R- ATTCTCCATTGTTCTCGTTCTC	57
31.	VR086	F - GAGATCCTCCTACGGATTTGC	
		R- TTTCCTTCTCCAATTCTTGCTC	59
32.	VR095	F - GAAATGGGAGTTCAAAGAGGAA	
		R- TGGAGAAGTCTGGAAGAGAACC	58
33.	VR099	F - ATACTTCGATCCGACCACTAGG	
		R- CAAAGACAGGAGGAGAACAAGG	58
34.	VR108	F - GCTCCAACACTCACTCACAAAC	
		R- CAGAAATGCAGGAAAAGAGAGG	59
35.	VR111	F - TGCATCTTTATTGAGTTCCGTG	
		R- GTTTTGGGGTGAATGTTGGATA	59
36.	VR133	F - GAAGTGGCGGAAGATTGATAAG	
		R- GGTAGATGGAAGGTAGAGGAATGA	59
37.	VR135	F - GCCCAGATTTGTTCATCCTAGA	
		R- ACTGTTTTGAGTGGGGAAAAGA	59
38.	VR140	F - GGTGTTGTTGTTGAGGAATGAA	
		R- AACATTGAGGACCCACATATCC	59
39.	VR147	F - CCATGTGTGTGAATGTGAGTGA	
		R- CCTTTGATTTTGTGGGATGTGT	58
40.	VR148	F – CCGTTGTTGTTGCTGTTGTG	
		R- GAGCTTGCTAACCCTCTCCAAT	59
41.	VR153	F - AATTGTGAAGCAACAGAAAGCC	
		R- AGAAATAGGCAGGCAGTTTTCA	59
42.	VR155	F - AAGATCACACACAACCAACCC	
		R- AATTAGTTCCACAGGCCAGATT	58
43.	VR163	F -AGGAGAAATTGTTGTTGTTCGG	
		R- GTGTTGATTGTTAGGGAGGGAG	59
44.	VR169	F - GGAAGATAGCGGAGATGAAGAG	
		R- CACCATACACCATAACATTCCTG	58
45.	VR188	F -ATACAAGGGCAGGTGTAGCATC	
		R- CAGAAAACTTCATCCCCAGCTA	59
46.	VR198	F -AAGAAGAATGCGAGAAAGAAGC	
		R- GTCCTAGAAGTTAGGGTTTGTGATT	58
47.	VR200	F - TGGGAAATAAAGAAAGCGTAGG	
		R- CTCTTCTCCTTTGCCTCTACAAA	58
48.	VR212	F - AAACCAAAACGTAAGATCAGGG	
		R- ATAGAAAGAAGTTGGCGCAGAA	58
49.	VR222	F - TCTCTTCTCTCTTCTCTCTTCTTCTTC	
		R- TTGTGTCTGAGGCTATGTTGGT	57
50.	VR223	F – GCGTGATCGAGGCAGACTAT	
		R- GTGGGTAGCTCGGTAATAGCAC	59
51.	VR225	F - CAGCAACAGAACTACAATCCCA	
		R- CGGCAATCCTCCTATATTCATT	56
52.	VR226	F - GCTTCTCTTTCTTGCATTCATC	
		R- GACTAGGCGCTGGGAAAA	57
53.	VR238	F –ATTCTCTGCCTGCCATTTT	
		R- ACGATTGTGTTTGTTGATGC	58
54.	VR244	F -GCTCTAAAACACGAAAGGGGT	
		R- TCATGGTGGAAGAAAAGCAA	58
55.	VR248	F - ATTCGGTTCCAGTGCTAAGAAG	
		R- AGCAGAAGTGCTTATCCCAGAG	58
56.	VR256	F -GCTGTGGTGTATTTACCTTGGG	
		R- ATCCTCCGGTCATTATCTTGTG	59
57.	VR257	F - AGGAAGATGAGGGGAAAGTGA	
		R- TATTCTATACCTGCCACCCCAC	59
58.	VR274	F - ATTCGGGTAAAGTTCTGCATCT	
		R- AATGTTCACACACGTCATAGCA	58
59.	VR293	F -GTGGCTCACAAGGTAGTGCTAA	58

Table 2 : List of polymorphic primers of the cross LBG 759 X T9

Sr. No.	Primer name	Primer sequence	Ta (°c)	Allele size (bp)	
				S	R
1.	VR 155	F- AAGATCACACACAACCAACCC			
		R- AATTAGTTCCACAGGCCAGATT	58	180	210
2.	VR 1	F – AGCCCTTCGTGCTAGGAAAT			
		R- CCCTACCGGTTGGTTGGT	59	220	230
3.	VR006	F - CCTTCTATCTCATGTTCACCGTC			
		R- TGGAATAGGGACAAAATGGACT	58	180	190
4.	VR 323	F- ATATCAGCCATTGTTGCTTTCC			
		R- TTCCCAGTTCAGACAACCAAGT	59	180	190
5.	MB 14	F- TGGAATTTGGAAGGAAGGA			
		R- GATGCAGGTGTTTGGGAG	57	240	250
6.	MB 87	F- TCCCTTGTGGGAGATCCT			
		R- CTTTGCCACACTCCTTGC	57	150	180
7.	VR135	F - GCCCAGATTTGTTCATCCTAGA			
		R- ACTGTTTTGAGTGGGGAAAAGA	58	200	210
8.	VR 9	F- TGACGGAGAGAGAGAGAGAGAG			
		R- TGCTTCCTTTTGTCTGAGTTAGAA	57	180	200
9.	VR163	F -AGGAGAAATTGTTGTTGTTCGG			
		R- GTGTTGATTGTTAGGGAGGGAG	58	210	230
10.	VR147	F - CCATGTGTGTGAATGTGAGTGA			
		R- CCTTTGATTTTGTGGGATGTGT	59	200	210
11.	VR153	F - AATTGTGAAGCAACAGAAAGCC			
		R- AGAAATAGGCAGGCAGTTTTCA	59	110	130
12.	VR 188	F- ATACAAGGGCAGGTGTAGCATC			
		R- CAGAAAACTTCATCCCCAGCTA	59	180	220

R=Resistant parent; S= Susceptible parent; Ta = Annealing temperature(°c)

confidence interval for the CYMV resistance QTL associated with VM31 locus was mapped within only 19 cM.

Linkage of a RGA marker of 445 bp with YMV resistance in blackgram was reported by Basak *et al.* (2004). The resistance gene for yellow mosaic disease was identified to be linked with a SCAR marker at a map distance of 6.8 cm (Souframanien and Gopalakrishna 2006). In another study, a RGA marker namely CYR1 was shown to be completely linked to the MYMIV resistance gene when validated in susceptible (T_9) and resistant (AKU9904) genotypes (Maiti *et al*., 2011). Prashanthi *et al*. (2011) identified random amplified polymorphic DNA (RAPD) marker, OPQ-1, linked to YMV resistant among 130 oligonucleotide primers. Dhole *et al.* (2012) studied the development of a SCAR marker linked with a MYMV resistance gene in Mungbean. Three primers amplified specific polymorphic fragments *viz*., $OPB\text{-}07_{600,}$ $OPC\text{-}06_{1750}$ and $OPB\text{-}12_{820}$. The marker $OPB\text{-}07_{600}$ was more closely linked (6.8 cM) with a MYMV resistance gene.

In the present study the marker VR9 showed the polymorphism between the parents and bulks and amplified an allele of specific bp in ten individual of both resistant and susceptible plants which were taken as bulks. This marker VR9 can be effectively utilized for developing the MYMV resistant genotypes thereby achieving substantial impact on crop improvement by marker assisted selection resulting in sustainable agriculture. Such cultivars will be of immense use for cultivation in the northern and central part of India, which is the major blackgram growing area of the country.

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