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Identification of Molecular Markers Linked to Yellow Mosaic Virus Resistance in Blackgram (*Vigna mungo* (L.) Hepper)

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A B S T R A C T

Blackgram (*Vigna mungo* (L.) Hepper) (2n=22) is one of the most highly valuable pulse crop, cultivated in almost all parts of India. It is a good source of easily digestible proteins, carbohydrates and other nutritional factors. Besidesvarious biotic and abiotic constraints, viral diseases mostly yellow mosaic disease is the prime threat for extensive economic losses in areas of production. The Yellow Mosaic disease (YMD) caused by Mungbean Yellow Mosaic Virus (MYMV), a Gemini virus transmitted by whitefly (*Bemesia tabaci* Genn) is one of the most downfall disease that has the ability to cause yield loss upto 85%. The advancements in the field of biotechnology and molecular biology such as marker assisted selection and genetic transformation can be utilized in developing MYMV resistance uradbeans. The investigation was carried out to find out the markers linked to yellow mosaic virus resistance gene, MYMV resistant parent T9 and MYMV susceptible parent LBG 759 were crossed to produce mapping population. A total of 50 SSR primers were used to study parental polymorphism. Of these 14 SSR markers were found polymorphic showing 28% of polymorphism between the parents. These fourteen markers were used to screen the F2 populations to find the markers linked to the resistance gene by bulk segregant analysis. The marker CEDG185 present on linkage group 8 clearly distinguished resistant and susceptible parents, bulks and ten F_2 resistant and susceptible plants indicating that this marker is tightly linked to yellow mosaic virus resistance gene.

Introduction

Blackgram (*Vigna mungo* (L.) Hepper) also known as Uradbean, is one of the important pulse crops of India.

India is the largest producer and also consumer of blackgram. It has surely marked itself as the most popular pulse crop and can be most consequently referred to as the "king of the pulses" due to its delicious taste and numerous other nutritional qualities. Blackgram is superb combination of all nutrients, which contains proteins (25-26%), carbohydrates (60%), fat (1.5%), minerals, amino acids and vitamins (Karamany 2006). Being a good leguminous crop, it is itself a mini-fertilizer depository, as it has special characteristics of maintaining and restoring soil fertility through fixing atmospheric

nitrogen in symbiotic association with *Rhizobium* bacteria, present in the root nodules. It is short duration pulse crop (Delic *et al.*, 2009), usually flowering within 30-50 days of sowing and maturing within 60-90 days.

Among various biotic and abiotic yield limiting factors, mungbean yellow mosaic disease (MYMD) caused by mungbean yellow mosaic virus (MYMV) is the most destructive limiting factor in blackgram. Infection of MYMV may cause up to 85–100% yield loss in uradbean (Singh *et al.,* 2011). The virus is transmitted by white flies (*Bemisia tabaci*).

Various molecular markers have been used for the molecular analysis of grain legumes. Among different DNA markers, microsatellites (or) Simple Sequence Repeats (SSRs) have occupied a vital place due to their reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage. Molecular markers and genetic linkage maps are essential for molecular breeding in any crop. Such tools would speed up the process of introgression of useful traits into preferred varieties. Keeping in view of the above statements, the present study was undertaken to identify molecular markers linked to yellow mosaic virus resistance through bulk segregant analysis (BSA) in blackgram.

Materials and Methods

Parent materials

We selected susceptible blackgram genotype LBG 759 and resistant genotype T9 as parent materials.

Mapping population

To study molecular markers linked to yellow mosaic virus resistance in blackgram sowing of parent material and crossing programm (LBG 759 × T9) were performed during kharif 2015-16 season. F_1 seeds are collected and raised to obtain segregating F_2 mapping population in rabi 2015-16 season.

DNA isolations

Genomic DNA of the parents, F_1'sand resistant and susceptible F_2 plants were isolated by CTAB method (Doyle and Doyle, 1987). The quality and quantity of isolated DNA samples was examined by using 0.8 % (w/v) agarose gel electrophoresis.

PCR analysis

Molecular analysis was carried out for parental polymorphism survey, conformation of F_1'sand bulk segregant analysis (BSA) by PCR analysis. PCR amplification was carried out on thermal cycler (AB Veriti, USA) with the components and cycles mentioned below in tables 1 and 2.

PCR product was mixed with 3µl of 6X loading dye and the sample was loaded in the gel carefully. 50 bp ladder was loaded and used as a reference marker. The gel was run at constant voltage of 80V for about 4 hours, until the ladder got properly resolved. After run gelwas photographed using gel documentation system (BIORAD GEL DOC XR + IMAGING SYSTEM).

Molecular Analysis

Molecular analysis includes study of parental polymorphism among parents by using SSR markers, conformation of F_1's with the polymorphic markers and performing bulk segregating analysis to find the marker that linked to YMV resistance trait by differentiating resistant bulk, susceptible bulk, ten resistant and ten susceptible F_2 individual plants selected by phenotypic screening.

Phenotyping of F_2 individual Plants

Sowing and phenotyping of F_2 individual plants was carried in summer 2015-16 season at ARS, MADHIRA which is hot spot for MYMV incidence. For evaluation of the test material against MYMV, pot sowing was done by following the infector row method of sowing two test rows alternating with spreader rows of highly susceptible check 'LBG 759' so as to adequately spread the inoculum at the test location which is hot spot for MYMV.

No insecticide was sprayed in a plan to maintain the whitefly population in experimental field. The MYMV occurrence was recorded on all the plants of F_2 population of the crose based on the visual scores on 50th day while the susceptible check LBG 759 recorded scale 9. The rating scale implied by Shad *et al.,* (2006), was used as given below table 3.

Preparation of DNA Bulks

DNA bulks of extreme phenotypes (Resistance and Susceptible) were used for the BULK SEGREGANT ANALYSIS (BSA). From ten resistant and ten susceptible F_2 plants equl quantities of DNA were bulked to form resistant and susceptible bulks.

Results and Discussion

In the present study, the major objective was to find the molecular markers (SSR's) linked to yellow mosaic virus resistance by bulk segregant analysis using F_2 population obtained from the cross between LBG 759 × T9 as follows:

Study of Parental Polymorphism

The LBG 759 (MYMV susceptible parent) and T9 (MYMV resistant parent) were initially screened with 50 SSR markers, to find out the markers showing polymorphism between the parents. Out of these 50 markers used for parental polymorphism survey, 14 markers (28%) showed polymorphism between the parents (Fig 2) and the remaining markers were showed monomorphic (Fig 1). The sequence of polymorphic primers, annealing temperature and amplification are represented in the table 4.

Conformation of F_1's

Similarly, the confirmation of F_1's progeny was carried out using 14 polymorphic markers (Fig 3).

Bulk Segregate Analysis (BSA)

Bulk segregant analysis was carried with these polymorphic markers to identify the markers linked to the gene conferring resistance to MYMV.

For the preparation of susceptible and resistant bulks equal amounts of DNA were taken from ten susceptible F_2 individuals (MYMV score 5) and ten resistant F_2 individuals (MYMV score 1) respectively. These parents and bulks were further screened with the 14 polymorphic SSR markers, which showed polymorphism in parental survey using same concentration of PCR ingredients under the same temperature profile.

Out of these 14 SSR markers, one marker CEDG185 showed the polymorphism between the bulks as well as parents (Fig 4). When tested with susceptible parent, ten individual susceptible F_2 plants and susceptible bulk CEDG185 marker amplified an allele of 160 bp in size (Fig 6).

Similarly this marker found to be amplified an allele of 190 bp in size with resistant parent, resistant bulk and ten individual resistant F2 plants (Fig 5).

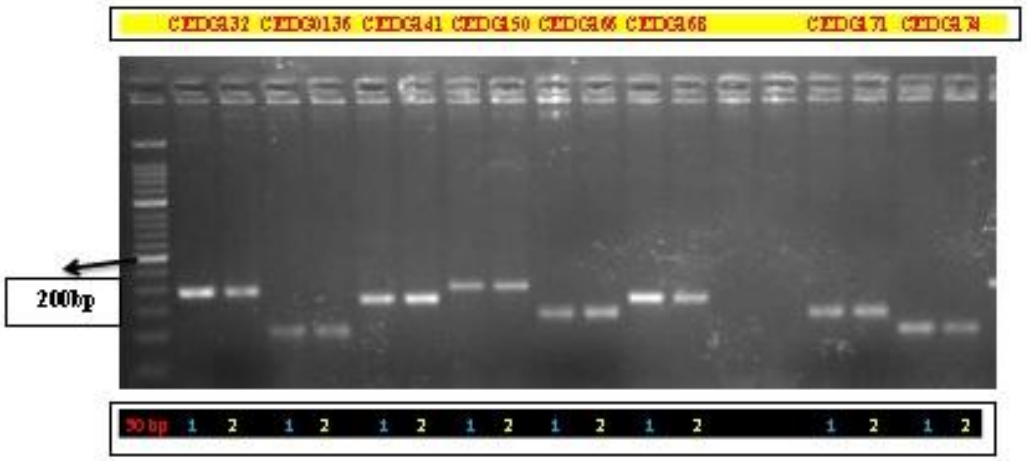

Figure 1: Parental survey of uradbean lines LBG759 (1) × T9 (2) with monomorphic SSR primers. The ladder used was 50bp.

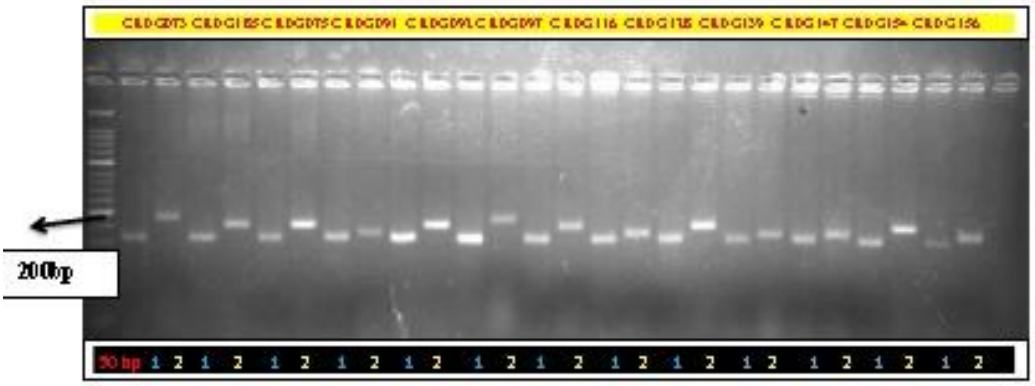

Figure 2: Parental survey of uradbean lines LBG759 (1) × T9 (2) with Polymorphic SSR primers. The ladder used was 50bp.

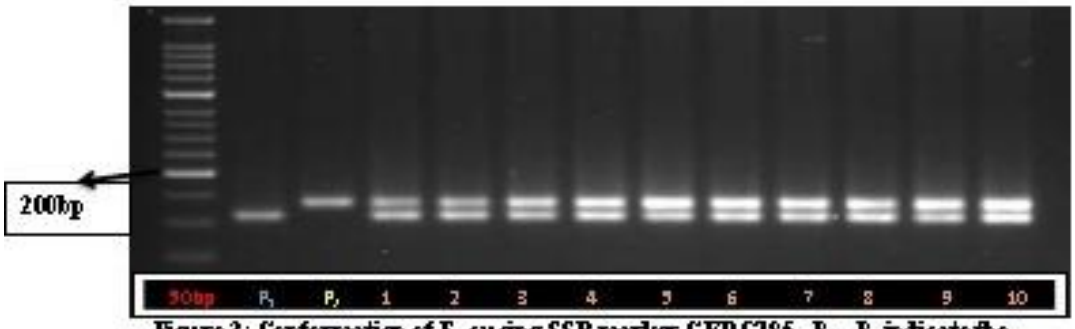

Figure 3: Conformation of F_1 s using SSR marker CEDG185. P_1, P_2 indicate the parents. Lanes 1-10 indicate F_1 plants. The ladder used was 50bp.

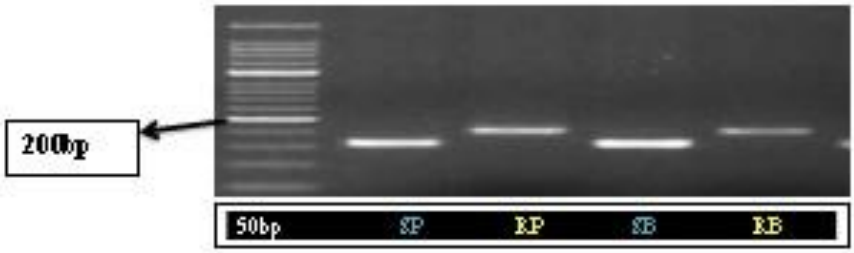

Figure 4: Bulk segregant analysis with SSR primer CEDG 185. SP, RP indicates susceptible and resistant parents. SB, RB indicates susceptible and resistant bulks. The ladder used is 50bp

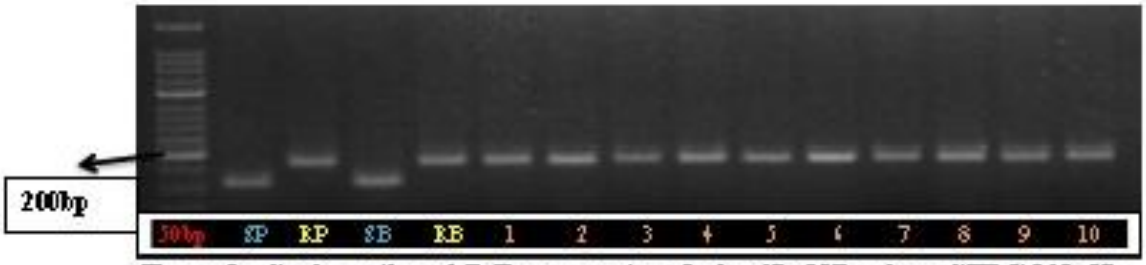

Figure 5: Conformation of Bulk segregant analysis with SSR primer CEDG 185. SP, RP indicates susceptible and resistant parents. SB, RB indicates susceptible and resistant bulks. The lanes 1-10 indicates F_1 resistant plants. The ladder used is 50bp.

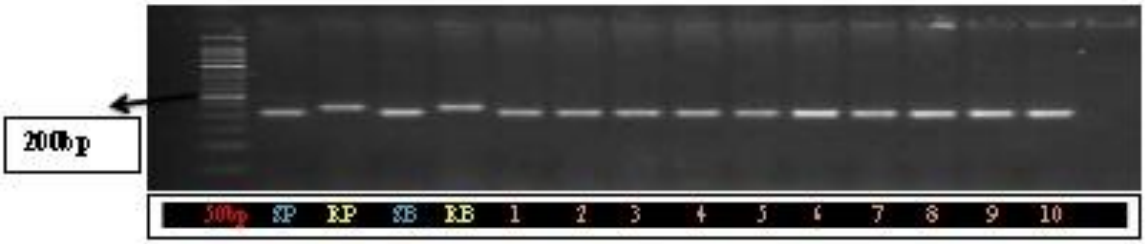

Figure 6: Conformation of Bulk segregant analysis with SSR primer CEDG 185. SP, RP indicates susceptible and resistant parents. SB, RB indicates susceptible and resistant bulks. The lanes 1-10 indicates F_1 suceptible plants. The ladder used is 50bp ladder.

Table.1 Components of PCR reaction

Component	Quantity	Reaction volume
Taq buffer (10X) with Mg Cl_2	1X	1.0 µl
dNTP mix	2.5 mM	1.0 µl
Taq DNA polymerase	3U/µl	0.2 µl
Forward primer	0.2 µM	0.5 µl
Reverse primer	0.2 µM	0.5µl
Genomic DNA	50 ng/µl	3.0 µl
Sterile distilled water		3.8 µl

PCR reaction was performed in a 10 µl volume of mix containing the following.

Table.2 PCR temperature regime

S.NO	STEP	TEMPERATURE	TIME	Cycles
1.	Initial denaturation	95° C	5 minutes	1
2.	Denaturation	94° C	30 seconds	35cycles
3.	Annealing	57-60 ° C	30 seconds	
4.	Extension	72° C	1 minute	
5.	Final extension	72° C	7 minutes	1
6.	4˚c		∞	

Table.3 Grouping of Genotypes into different categories based on 0-5 scale

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 Suseptible	MS
4	30-50% infection	Susceptible	S
5	More than 50%	Highly susceptible	HS

Table.4 List of polymorphic primers of the cross LBG 759 X T9

Sl. No	Primer name	Primer sequence	Annealing temperature(°c)	Allele size (bp)	
				S	R
1.	CEDG073	F- CCCCGAAATTCCCCTACAC R- AACACCCGCCTCTTTCTCC	60	150	250
2.	CEDG075	F- GCGACCTCGAAAATGGTGGTTT R- TCACCAACTCACTCGCTCACTG	60	150	200
3.	CEDG091	F- CTGGTGGAACAAAGCAAAAGAGT R- TGGGTCTTGGTGCAAAGAAGAAA	57	150	170
4.	CEDG092	F- TCTTTTGGTTGTAGCAGGATGAAC R- TACAAGTGATATGCAACGGTTAGG	57	150	210
5.	CEDG097	F- GTAAGCCGCATCCATAATTCCA R- TGCGAAAGAGCCGTTAGTAGAA	57	150	230
6.	CEDG116	F- TTGTATCGAAACGACGACGCAGAT R- AACATCAACTCCAGTCTCACCAAA	57	150	170
7.	CEDG128	F- CTGCCAAAGATGGACAACTTGGAC R- GCCAACCATCATCACAGTGC	60	150	180
8.	CEDG139	F- CAAACTTCCGATCGAAAGCGCTTG R- GTTTCTCCTCAATCTCAAGCTCCG	60	150	190
9.	CEDG147	F- CTCCGTCGAAGAAGGTTGAC R- GCAAAAATGTGGCGTTTGGTTGC	60	150	160
10.	CEDG154	F- GTCCTTGTTTTCCTCTCCATGG R- CATCAGCTGTTCAACACCCTGTG	58	150	180
11.	CEDG156	F- CGCGTATTGGTGACTAGGTATG R- CTTAGTGTTGGGTTGGTCGTAAGG	58	150	210
12.	CEDG176	F- GGTAACACGGGTTCAGATGCC R- CAAGGTGGAGGACAAGATCGG	60	150	180
13.	CEDG185	F- CACGAACCGGTTACAGAGGG R- CATCGCATTCCCTTCGCTGC	60	160	190
14.	CEDG199	F- CCTTGGTTGGAGCAGCAGC R- CACAGACACCCTCGCGATG	60	150	180

R=Resistant parent; S= Susceptible parent

The amplification of resistant parental allele in resistant bulk and susceptible parental allele in susceptible bulk indicated that this marker is associated with the gene controlling MYMV resistance in blackgram.

This marker CEDG185 can be effectively utilized for developing the YMV resistant genotypes thereby achieving substantial impact on crop improvement by marker assisted selection.

Similar results were found in mungbean using 361 SSR markers. Out of 361 markers used, 31 were found to be polymorphic between the parents. The marker CED 180 markers were found to be linked with resistance gene by the bulk segregant analysis (Gupta *et al.*, 2013). Shoba *et al.*, (2012) identified the SSR marker PM384100 allele for late leaf spot disease resistance by bulked segregant analysis. Identified SSR marker PM 384100 was able to distinguish the resistant and susceptible bulks and individuals for late leaf spot disease in groundnut.

In Blackgram several studies were conducted to identify the molecular markers linked to YMV resistance by using the RAPD marker 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 marker OPBB05 from azukibean which shows specific amplified size of 450 bp in susceptible parent, bulk and five individuals of F2 populations and another phenotypic (resistant) specific amplified size of 260 bp for resistant parent, bulk and five individuals of F2 population. One species-specific SCAR marker was developed for ricebean which resolved amplified size of 400bp in resistant parent and absent in the bulk (Sudha *et al.*, 2012). Karthikeyan *et al.*, (2012) studied the SSR markers linked to YMV resistance from azukibean in mungbean BSA. Out of 45 markers, 6 showed polymorphism between parents and not able to distinguish the bulks. Similar results were found in blackgram using 468 SSR markers from soybean, common bean, red gram, azuki bean. Out of which 24 SSR markers showed polymorphism between parents and none of the primer showed polymorphism between bulks (Basamma *et al.*, 2011).

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