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Validation of Molecular Markers Linked to Yellow Mosaic Virus Disease Resistance in Diverse Genotypes of Green Gram (*Vigna radiata*)

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Marker assisted indirect selection of resistant genotypes using linked markers has been reported as an effective breeding approach for developing yellow mosaic disease resistant cultivars. Seven genotypes of greengram with different genetic makeup showing resistance reaction to yellow mosaic virus (YMV) were selected along with a susceptible one for molecular confirmation with respect to YMV reactions. These were screened with PCR-based technique employing primer pairs to validate efficiency and reliability of identified markers loci (CYR1 and YMV1). Both CYR1 and YMV1 marker showed consistent polymorphism with respect to disease reaction in seven resistant genotypes. CYR1 was produced an allele size of approximately 90 bp. which concluded that seven genotypes (OBGG-2013-8, OBGG-2013-12, OBGG-2013-11, OBGG-2013-16, OBGG-2013-20, OBGG-2013-21 and OBGG-2013-39) have yellow mosaic virus resistance gene and both the markers are efficient and ubiquitous for genotyping of YMV reaction. OBGG 2013-20 was an YMV resistance and high yielding line which can be used as YMV donor or can be released as a variety after further multi-location evaluations.

Introduction

Greengram (*Vigna radiata* L. Wilczek) is one of the important short duration pulse crops of India and an excellent source of good quality protein but the productivity is comparatively low i.e. 499 kg/ha (Directorate of Economics and statistics, 2016). This is due to various biotic and abiotic stresses and lack of suitable genotypes/varieties. Among the biotic constraints, yellow mosaic disease (YMD) caused by the mungbean yellow mosaic virus is the major threat for huge economic losses in the Indian subcontinent (Nene, 1973). The disease is caused by white fly (*Bemicia tabaci*) transmitted begomo gemini viruses with bipartite genomes (Varma and Malathi, 2003). Begomoviruses are a group of plant viruses containing single-stranded circular DNA encapsidated in geminate particles (Khattak *et al.,* 2000; Karthikeyan *et al.,* 2004). Greengram plants infected with YMD generally show yellowing or chlorosis of leaves followed by necrosis, shortening of internodes, and severe stunting of plants with no yield or few flowers and deformed pods produced with small, immature and shrivelled seeds (Akhtar *et al.,* 2009). This disease

causes 10–100 percent yield losses depending on the crop stage at which the plants being infected (Marimuthu *et al.,* 1981; Bashir *et al.,* 2006; Pandey *et al.,* 2009).

The recent era of advanced biotechnology provides various molecular markers that can facilitate conventional breeding as marker assisted breeding (Ashraf and Foolad, 2013). Marker assisted indirect selection of resistant genotypes using linked markers has been reported as an effective breeding approach for developing cultivars resistant to yellow mosaic disease. Marker assisted selection (MAS) is assuming increased importance due to lack of uniform field screening procedure as well as indirect selection due to complex virus vectors, host and environmental interaction. Thus resistance genotype may be confirmed by molecular analysis and these resistance genotypes can be utilized in hybridization programme for transfer of resistance gene to the high yielding genotype susceptible to YMV for development of a high yielding YMV resistant varieties in a comparatively shorter time than conventional breeding. Michelmore *et al.,* (1991) developed SCARs marker which are DNA fragments amplified by the PCR using specific 15–30 bp primers, designed from nucleotide sequences established from cloned RAPD fragments linked to a trait of interest. These are locus specific and have been applied in gene mapping studies and marker assisted selection (Paran and Michelmore, 1993). The $ISSR811_{1357}$ marker was sequenced and sequence characterized amplified region (SCAR) primers were designed (YMV1-F and YMV1-R) to amplify the marker and validated using diverse blackgram genotypes differing in their MYMV reaction by Souframanien and Gopalkrishna (2006). Use of RGA-markers is comparatively recent and can be conveniently designed from the diagnostic motifs of known disease resistance genes (Yan *et al.,* 2003). Resistance gene analogous (RGA) based molecular markers can significantly enhance the breeding efficiency for disease resistance in various crops (Wang *et al.,* 2014). Among RGA markers, CYR-1 marker better linked with yellow mosaic virus disease resistance genes in blackgram have been earlier reported (Basak *et al.,* 2004; Maiti *et al.,* 2011; Sowami and Jayamani, 2014; Panigrahi *et al.,* 2016). Development of markers to identify YMV resistance in greengram and deploying them through marker-aided selection in breeding programme would fasten the process of developing resistant lines (Tanksley *et al.,* 1989).

Molecular markers identified in one population should be validated by testing for the presence of the marker in a range of cultivars and other important genetic backgrounds (Gupta *et al.,* 1999). Thus the present study was undertaken to link the available SCAR and RGA markers with YMV resistance and identify the YMV resistant genotypes of greengram.

Materials and Methods

Plant materials

The best seven greengram genotypes were selected from twenty one newly developed genotypes on the basis of resistance to YMV under field condition and yield performance (Table 1). These selected genotypes were grown in pot along with a check variety (OUM 11-5, a popular and widely adopted variety of Orissa) for YMV reaction study. Fresh and young leaf samples were collected from 5 – 20 days old seedlings for the isolation of genomic DNA.

Extraction and purification of genomic DNA

Genomic DNA was isolated from tender young leaves which were harvested freshly

before sunrise and washed thoroughly with cold autoclaved, distilled water and then blotted to dry. About two grams of young leaves were excised from the upper tip portion and DNA was extracted on the same day of collection. Total genomic DNA from the leaves was isolated by using standard CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, l990). After DNA extraction, the quality of DNA was checked by gel electrophoresis on a 0.8% gel. In the well, 4µl of bromophenol blue and 4µl of DNA sample were loaded and run at 70v for 20-25 min. After that, gel was checked in Gel documentation System. The band which appeared near the well was identified as DNA and the band which appeared distant to the well was identified as RNA and these corresponding sample required purification.

The dissolved DNA is the crude DNA and requires further purification. The RNA was removed by giving RNase treatment. Quantity of RNase was added to DNA depending on the quantity of RNA present in the sample. Quantity of RNase treated varies from 3 µl - 30 µl. and it is known after gel run. The solution was incubated in water bath at 37ºC for 1 hour. After 1 hour it was removed from the water bath and equal volume of phenol about 500 µl was added and mixed gently for about 10 min.

The solution was then centrifuged in l0, 000 rpm for 10 minutes with a medium speed centrifuge and upper aqueous phase was pipetted out to another centrifuge tube. After that equal volume of chloroform-isoamylalcohol (24:1) was added to sample and shaken for 10 min as phenol treatment and centrifugation was also done for 10 min at 10,000 rpm. For further purification, the DNA solution was washed with phenol: chloroform: isoamyl alcohol (25: 24: l). The upper aqueous phase was separated after centrifugation as per the procedure described earlier and mixed with l/10th volume of 3M sodium acetate (pH 4.8). DNA was precipitated by adding equal volume of chilled isopropanol and pelleted by spinning at 10, 000 rpm for 10 min. The pellet was washed twice with 70% ethanol carefully and dried under air.

The quality as well as quantity of DNA was also checked by UV-Vis spectrophotometer (Jasco V 350, Japan). The absorbance at 260 nm wave lengths gave the quantity of the total DNA and the ratio of the absorbance at 260 and 280 nm indicated the quality of the purified DNA. The DNA was loaded in 2% agarose gel alongside diluted uncut lambda DNA as standard to recheck the quality and quantity and it was observed that the DNA from all the samples were qualitatively good. The quantification was done in comparison with the known standard. After quantification, the DNA was diluted in $T_{10}E_1$ buffer to a working concentration of 25 ng/µl for PCR analysis.

Polymerase chain reaction (PCR) analysis

For the PCR, two selected primers of MERCK Specialties Private Limited *i.e.* SCAR (sequence characterized amplified region) and RGA (Resistance gene analog) were used for PCR amplification. The characters of these primers are described in table 2. Each amplification reaction mixture of 25 µl contain 2µl primer (each forward and reverse), 0.5µl dNTP, 0.5µl of 3 unit Taq DNA polymerase, 2.5µl Taq buffer, 17.5µl PCR water and 2µl of DNA sample. Amplification condition were 1 cycle at 94^oC for 5 min. followed by 45 cycles each consist of denaturation at 94^oC for 1 min, annealing at 60^0C for 1 min. elongation at 72^0C for 2 min. The final extension setup was carried out at 72^0C of 12 min which is a slight modification as followed by Kalaria *et al.*, (2014). The PCR- amplified products were resolved with 1.5 % agarose gel electrophoresis.

Agarose gel electrophoresis

Three grams of agarose was added to 200ml 1XTBE buffer boiled for complete melting of agarose, then cooled to 50ºC. Ethidium bromide (EtBr) (2µl/50ml of gel solution) was added to the gel and casted on the gel-casting tray.

Then the gel was transferred to the submarine gel tank containing lX TBE buffer. Prior to loading the samples. In the submerged gel 25µl of the PCR samples were loaded in each well along with a single well loaded with standard DNA ladder (l00 bp-3kb DNA ladder).

The electrophoresis was performed at a constant voltage (80v) for 3 hours. Then these gels were placed on the Gel doc system (UVITECH, Cambridge, UK) and were photographed under U.V. light for scoring the bands. The sizes of the amplicons were determined by comparing them with that of the ladder banding pattern.

Field evaluations

Disease score was recorded at maturity stage by counting number of infected plants in each line and the per cent disease incidence was calculated. The disease intensity was scored adopting the following 1-9 score (Singh *et.al.*, 1988) (Table 3). For yield evaluation, one hundred random mature plants of each entry selected and harvested separately from which the average plant yield of each entry calculated.

Results and Discussion

Both CYR1 and YMV1 showed consistent polymorphism with respect to disease reaction. The result confirmed that the band appeared in the resistant genotypes only and was absent in susceptible one (Lane.no.2). Both CYR1 and YMV1 showed amplifications in seven genotypes i.e OBGG-2013-8, OBGG-2013-21, OBGG-2013-16, OBGG-2013-11, OBGG-2013-20, OBGG-2013-39, OBGG-2013-12 (Fig. 1 and 2). RGA22F2/RGA24R2 (CYR1) markers produced an allele size of approximately 90 bp in contrast to that of findings obtained by Maiti *et al.*, (2010) i.e.1236bp in blackgram. In case of YMV1 two bands were observed in four genotypes (OBGG-2013-11, OBGG-2013-16, OBGG-2013-20, OBGG-2013-21) at place of 200bp and 90 bp whereas only one band was observed in the remaining three genotypes (OBGG-2013-8,OBGG-2013-12and OBGG-2013-39) at 90 bp.

Average yield (based on two year yield data) of the tested genotypes ranged from 3.00 g/plant to 4.73 g/plant (Table 4). Although all the new genotypes yield higher than the control (OUM 11-5), it was observed that four out of seven genotypes are more than 20% higher yield than the control (OUM 11-5).

Among all, OBGG 2013-11 and OBGG 2013-20 were found to be promising genotypes with high yielding potential however OBGG 2013-20 having high yielding potential with YMV resistance character can be released as a variety after further multi-location yield evaluations.

The YMV resistance lines (OBGG-2013-21, OBGG-2013-16, and OBGG-2013-20) confirmed by field and laboratory studies will be useful for donor in future breeding process.

In conclusion, both these markers validated in this study, endowed with features of resistance gene candidate may be useful for generating superior genotypes with durable YMV resistance. The markers will be of use in marker assisted selection and will hopefully aid in the development of resistant cultivars in relatively shorter time span.

Table.1 Yield potential and reaction to MYMV of different greengram genotypes

Sl No.	Genotypes	Yield/plant (g)	Disease Score	Disease Reaction
1	OBGG-2013-2	2.73	5	Moderately susceptible
2	OBGG-2013-3	2.10	9	Highly susceptible
3	OBGG-2013-5	1.40	9	Highly susceptible
4	OBGG-2013-7	4.15	9	Highly susceptible
5	OBGG-2013-8	3.83	3	Moderately Resistant
6	OBGG-2013-9	2.07	5	Moderately susceptible
7	OBGG-2013-10	3.53	5	Moderately susceptible
8	OBGG-2013-11	4.54	3	Moderately Resistant
9	OBGG-2013-12	3.56	3	Moderately Resistant
10	OBGG-2013-14	2.09	5	Moderately susceptible
11	OBGG-2013-15	4.37	9	Highly susceptible
12	OBGG-2013-16	3.89	1	Resistant
13	OBGG-2013-20	4.75	1	Resistant
14	OBGG-2013-21	3.45	1	Resistant
15	OBGG-2013-22	2.22	7	Susceptible
16	OBGG-2013-23	2.94	9	Highly susceptible
17	OBGG-2013-24	4.36	7	Susceptible
18	OBGG-2013-34	5.55	5	Moderately susceptible
19	OBGG-2013-39	2.77	3	Moderately Resistant
20	OBGG-2013-40	2.32	5	Moderately susceptible
21	OBGG-2013-42	4.02	7	Susceptible
22	IPM-02-03 (C)	2.86	7	Susceptible
23	OUM-11-5(C)	2.75	7	Susceptible
24	Tarm-1(C)	4.48	5	Moderately susceptible
25	Dhauli (C)	1.65	7	Susceptible
26	Sujata(C)	2.08	5	Moderately susceptible

Table.2 Characters of primers for YMV resistance gene used in PCR

Primers	Primer sequences (5′-3′)	% GC	Annealing temp.	Expected product	Reference
RGA22F2	GGGTGGTTTGGGTAAGACCAC	57.1	60	90	Maiti *et al.*, 2011
RGA24R2	TTCGCGGTGTGTGAAAAGTCT	47.6	58	90	Maiti *et al.*, 2011
YMV1-F	GAGAGAGAGAGAGAGACAAAG	47.6	64	90 and 200	Souframanien and Gopalakrishna 2006.
YMV1-R	GAGAGAGAGAGAGAGACAGGA	52.4	64	90 and 200	Souframanien and Gopalakrishna 2006.

Table.3 Rating scale for MYMV (Singh *et al.*, 1988)

Scale	Plants/foliage affected	Reaction
1	0.1-5.0%	Resistant (R)
3	5.1- 10.0%	Moderately Resistant (MR)
5	10.1 – 25.0%	Moderately susceptible (MS)
7	25.1 -50.0%	Susceptible (S)
9	50.1 – 100%	Highly susceptible (HS)

Table.4 Reaction of greengram genotypes to YMD

Lane No.	Entry	CYR1 Status (90bp)	YMV1 status		Field Disease Score	Disease Reaction	Av. Seed yield / plant (g)
			90bp	200bp			
1	OBGG-2013-8	P	P	A	3	MR	3.60
2	OUM 11-5 (C)	A	A	A	7	S	2.99
3	OBGG-2013-21	P	P	P	1	R	3.48
4	OBGG-2013-16	P	P	P	1	R	3.94
5	OBGG-2013-11	P	P	P	3	MR	4.73
6	OBGG-2013-20	P	P	P	1	R	4.47
7	OBGG-2013-39	P	P	A	3	MR	3.00
8	OBGG-2013-12	P	P	A	3	MR	3.48

Fig.1 PCR amplification of CYR1 marker in resistant genotypes

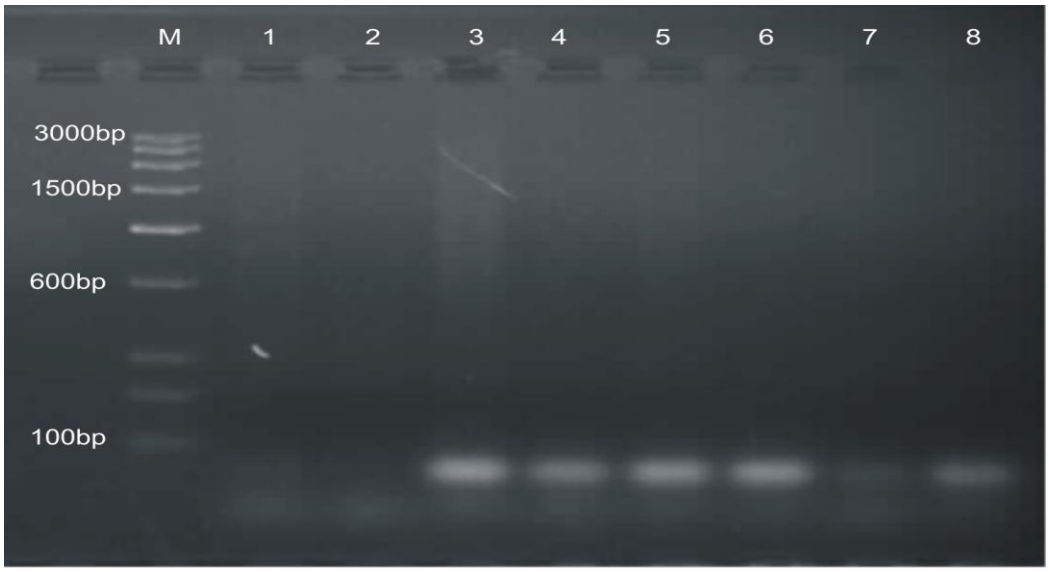
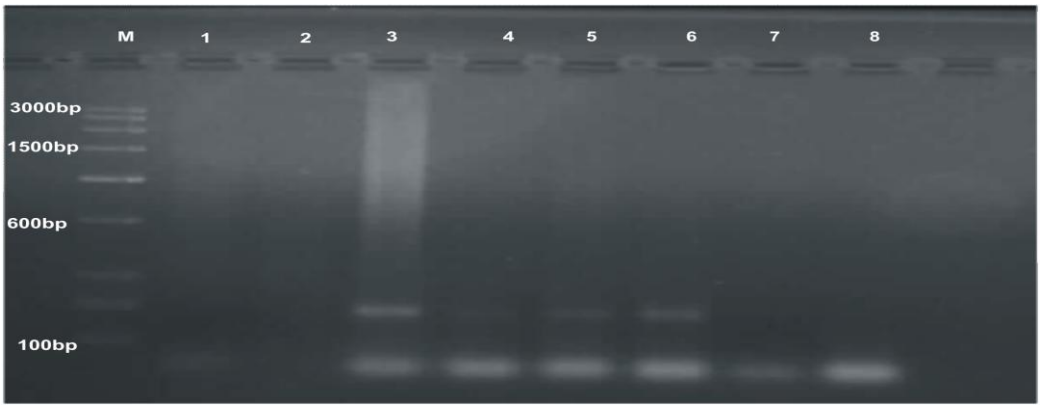


Fig.2 PCR amplification of YMV1 marker in resistant genotypes



[M: marker, Lane 1: OBGG-2013-8; 2: OUM 11-5 (control); 3: OBGG-2013-21; 4: OBGG-2013-16; 5: OBGG-2013-11; 6: OBGG-2013-20; 7: OBGG-2013 -39; 8: OBGG-2013-12]

Advantage of both markers are that these markers are completely linked with YMV resistance and these would assist in identifying plants endowed with resistance locus conferring YMV resistance straight away without challenging the plants with the pathogen for phenotyping, giving plant breeders the advantage to carry out repeated genotyping throughout the growing season in absence of any disease incidence. Additionally, screening of germplasms for virus resistance is possible directly from the seed stock. Identification of a linked marker with the desired trait is an essential requirement for Marker Assisted Selection (MAS) in advance breeding programme. Validation of marker by efficient screening is the process of unambiguous association of marker with the trait of interest. The YMV resistance lines (OBGG-2013-21, OBGG-2013-16, and OBGG-2013-20) confirmed by field and laboratory studies will be useful for donor in future breeding process. Genotype OBGG 2013-20 can be released as a high yielding YMV resistance variety or can be used as a source of parent for YMV resistance in crop improvement programme. Proper selections of single plants in OBGG2013-11 & OBGG2013-20 are suggested for further improvement. Recombination breeding between the genotype OBGG2013-34 and OBGG2013-20 followed by single plant selection may produce more desirable genotypes.

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