Indian Journal of Experimental Biology
Vol. 60, July 2022, pp. 481-489

Genetic diversity and marker trait association for yellow mosaic virus disease in Green gram, *Vigna radiata* (L.) Wilczek

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Received 14 December 2021; *revised 17 May 2022*

Yellow mosaic virus (YMV) disease is known to cause severe damage in green gram in terms of yield loss. As the resistance is often governed by recessive genes, introgression of such resistance faces some difficulty. DNA molecular markers are reported to be effective in this process. However, validation of such markers is important. Here, we have made an attempt to validate DNA markers associated with YMV disease resistance gene from a diverse group of 26 green gram genotypes. A total of 19 molecular markers were used to assess the susceptibility or resistance against YMV disease. Results show that among the amplified 31 alleles, 21 were polymorphic, with a mean of 1.1.0 per locus. The polymorphism information content (PIC) values ranged from 0.32 to 0.80. Only five markers exhibited higher PIC value (>6.0) and were revealed to be polymorphic, suggesting its utility in marker assisted selection for breeding YMV resistant genotypes in green gram. Dice dissimilarity coefficient among the genotypes exhibited a range of 0.07 to 1.0 which show a wide genetic variation among the genotypes for YMV tolerance. Neighbor-joining cluster analysis has grouped 26 green gram genotypes into 4 main clusters which revealed the existence of genetic dissimilarities among the genotypes. The genotypes AUGG 6, VBN (Gg) 2 and CO (Gg) 8 carried the positive alleles for YMV disease resistance and the allele for susceptibility were found in the genotypes AUGG 12, AUGG 15, AUGG 17 and AUGG 19. Single marker analysis indicated that there was correlation between the markers and the disease reaction in the field with exceptions. The findings revealed that the SSR markers CEDG180 and YR4 could be used to screen germplasm in order to discriminate the YMV resistant genotypes from the susceptible genotypes in marker assisted selection.

Keywords: Biotic stress, Mungbean, Polymorphism information content, YMV tolerance

Pulses are one among the important crops in the world. They are grown on 5.9 million hectares of land globally with an annual production of 4.4 million tons[1]. Among the pulses, pigeon pea, black gram and green gram makes significantly large contribution to the pulses production in India. Green gram or mung bean is a member of the genus *Vigna* and the family Leguminaceae. It is a short duration, self-pollinated, diploid (2n = 22) with a genome size of 0.56pg/1C (574 Mbp)[2]. India ranks number one in the world for production and consumption of pulses. It is cultivated in an area about 2.2 million hectares with a production of 0.93 million tones[3]. Increased consumption and thereby the growing global demand has encouraged researchers to develop new varieties adapted to different conditions, particularly water stress[4,5].

Many factors that can lead to low productivity of green gram ranging from plant ideotype to various biotic and abiotic stresses. Yellow mosaic virus disease (YMD) is most destructive disease caused by Yellow mosaic virus (YMV)[6,7]. YMV belongs to the genus Begomovirus[8]. French bean, dolichos, horsegram, soybean, black gram, green gram, mothbean, Lima bean, cowpea and a few other leguminous species are also affected by this virus[9]. White fly (*Bemisia tabaci)* is the vector which transmits YMD to green gram[10]. The YMV disease causes loss varying from 5 to 100%[11].

The YMV disease causes more severe damage in green gram than in black gram in South Asia. In India, there are two species of this virus *viz*., Mungbean Yellow Mosaic India Virus (MYMIV) and Mungbean Yellow Mosaic Virus (MYMV). The first one is prevalence in northern India and the latter is predominant to Southern India[12,13].

Hence, the nature of resistance exhibited by green gram from southern to northern parts of India varies. There is no uniform screening procedure and also observed that frequently resistance is governed by recessive genes and hence there is difficulty in introgression of YMV resistance in green gram. Therefore, we need to utilize modern biotechnological

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tools that can identify MYMV resistant genotypes reliably.

DNA markers molecular markers can be effectively used as a tool to predict the presence of a specific gene with high accuracy and to transfer the genes into desirable agronomic backgrounds. The use of DNA markers can accelerate the resistance breeding cycles associated with the phenotyping and also to overcome the inaccuracies in the field evaluation because of their property and environmental neutrality[14]. Marker assisted selection (MAS) also offer the opportunity for non-transgenic transfer enabling introgression of disease resistance from wild species[15]. Since YMV is a complex disease, experimental validation of molecular markers for YMV resistance becomes very important. It is more important in the Indian context, since there are different species of yellow mosaic viruses and variability in the nature of disease resistance from southern to northern parts of India.

The currently available screening procedures lack uniformity and many times the resistance is governed by recessive gene which causes significant delay in transfer of YMV resistance in superior genotypes. The white fly vector exhibits variable transmission efficiency depending on the host genotypes, vector biotypes and growth conditions available. Screening for YMV resistance under hot spot conditions has also not given consistent results. Hence, development of resistant cultivars needs molecular tools that can lead to the identification of MYMV resistant and distinguish them from susceptible genotypes reliably. Marker assisted selection can accelerate the breeding cycle for disease resistance to a great extent when compared to conventional backcross method of transfer.

Validation of Polymerase Chain Reaction (PCR) based DNA marker system linked to disease resistance genes have been reported in earlier studies for YMV disease tolerance in urad bean, such as Random Amplified Polymorphic DNA (RAPD) markers[16,17], Simple Sequence Repeat (SSR) markers[18], Inter Simple Sequence Repeat (ISSR) and Sequence Characterized Amplified Regions (SCAR) markers[19], Resistance Gene Analogue (RGA) markers[20-22]. Among various DNA marker systems, SSR markers are considered the most ideal marker for genetic studies because of their multi-allelic, abundant, random and wide distribution throughout the genome, co-dominant, simple to assay, highly reliable, reproducible and amenable for automation[23-26]. Hence, in the present investigation, we have analyzed 19 molecular markers which have been reported to be linked to YMV resistance in green gram and black gram genotypes. Of which16 SSR markers, one SCAR marker and two RGA markers were engaged to reveal the genetic diversification among green gram genotypes for YMV resistance.

Materials and Methods

Plant materials

Twenty six green gram genotypes were taken for investigation of YMV resistance or tolerance. Of these, twenty genotypes were cultures (AUGG 1, AUGG 2, AUGG 3, AUGG 4, AUGG 5, AUGG 6, AUGG 7, AUGG 8, AUGG 9, AUGG 10, AUGG 11, AUGG 12, AUGG 13, AUGG 14, AUGG 15, AUGG 16, AUGG 17, AUGG 18, AUGG 19 and AUGG 20) developed in Department of Genetics and Plant Breeding, Annamalai University and six commercial cultivars (VBN (Gg) 3, VBN (Gg) 4, VBN (Gg) 5, VBN (Gg) 6, CO (Gg) 7 and CO (Gg) 8) were obtained from National Pulses Research Centre, Vamban, Pudukkottai district, Tamil Nadu, India (Table 1). Genetically pure seeds of each accession were raised in experimental field of Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University, Tamil Nadu, India.

Table 1 — List of green gram genotypes and their disease scoring for YMV in hotspot based on percentage disease incidence

Genotypes	Source	PDI (%)	Reaction to YMV
AUGG-1	Cross derivative	0.0	HR
AUGG-2	Cross derivative	20.0	MR
AUGG-3	Cross derivative	40.0	S
AUGG-4	Cross derivative	13.0	MR
AUGG-5	Cross derivative	80.0	HS
AUGG-6	Cross derivative	0.0	HR
AUGG-7	Cross derivative	15.0	MR
AUGG-8	Cross derivative	33.3	MS
AUGG-9	Cross derivative	16.0	MR
AUGG-10	Cross derivative	13.0	MR
AUGG-11	Cross derivative	60.0	HS
AUGG-12	Cross derivative	40.0	S
AUGG-13	Cross derivative	62.5	HS
AUGG-14	Cross derivative	20.0	MR
AUGG-15	Cross derivative	62.5	HS
AUGG-16	Cross derivative	41.6	S
AUGG-17	Cross derivative	53.3	HS
AUGG-18	Cross derivative	25.0	MS
AUGG-19	Cross derivative	62.5	HS
AUGG-20	Cross derivative	25.0	MS
VBN (Gg) -1	Vamban, Pudukkottai	40.0	S
VBN (Gg)-2	Vamban, Pudukkottai	10.0	R
VBN (Gg)-3	Vamban, Pudukkottai	60.0	HS
VBN (Gg)-4	Vamban, Pudukkottai	20	MR
CO (Gg)-7	Coimbatore	10.0	R
CO (Gg)-8	Coimbatore	10.0	R

Field screening for YMV tolerance/phenotypic variability

Disease resistance screening for YMV disease was done in the hotspot, Panboli village of Tirunelveli District, Tamil Nadu during summer, 2017 using infector row technique. Twenty six genotypes were sown in single row with spacing of 30×10 cm in two replications. One row of SML 1082 (infector line) was raised after every five test genotypes. Standard agronomic practices were made as per recommendation. We did not use insecticidal spray which enabled the vector to spread. Disease infection was noted at periodic intervals and the percentage disease incidence (PDI) was calculated using the formula-Percent disease incidence = [(Number of plants infected in a row)/(Total number of plants in a row)] × 100. The test genotypes were classified using 0-9 arbitary scale as Immune (HR), Resistant (R), Moderately resistant (MR), Moderately susceptible (MS), Susceptible (S) and highly susceptible (HS) as suggested by Humphry *et al.*[11] (Table 1).

DNA extraction

The genomic DNA was isolated from leaf tissue of 20 days old seedlings following the protocol of cetyl trimethyl ammonium bromide (CTAB) with slight modifications as described by Doyle[27] DNA concentration was quantified on Nanodrop ND 1000 spectrophotometer (Thermo Scientific, USA). The results were confirmed by staining DNA with ethidium bromide (intercalating dye) after electrophoresis on 0.8% agarose gel at 100V for 45 min in TBE buffer (0.4 M Tris-boric, 0.001 M EDTA, pH 8.0) using known DNA concentration standards (λ DNA, uncut).

PCR analysis

PCR amplification was carried out in a thermocycler (Mastercycler Personal, Eppendorf, USA). A total of 16 microsatellite SSR markers, one SCAR marker and two RGA markers (Table 2[14,17,28,29]) found to be

Table 2 — Details of SSR, SCAR and RGA markers linked to YMV resistance

Marker name	Marker type	Primer sequence	Product size (bp)	Annealing temperature (°C)
CEDG 008	SSR	F-AGGCGAGGTTTCGTTTCAAG R-GCCCATATTTTTACGCCCAC	110-140	55[28]
CEDG 011	SSR	F-GTCCGACTTTATGTGTGGAG R-TTTCTAGTTCCAGCCCCGAC	118	59[29]
CEDG 013	SSR	F-CGTTCGAGTTTCTTCGATCG R-ACCATCCATCCATTCGCATC	92-182	54[29]
CEDG 014	SSR	F-GCTTGCATCACCCATGATTC R-AAGTGATACGGTCTGGTTCC	176-116	58[29]
CEDG 020	SSR	F-TATCCATACCCAGCTCAAGG R-GCCATACCAAGAAAGAGG	143-149	56[29]
CEDG 022	SSR	F-AGGAATGTGAGATTTG R-AATCGCTTCAAGGTCAAGCC	189-145	49[28]
CEDG 030	SSR	F-CATCTCCCTGAAACTTGTG R-GCTATCAATCGAGTGCAG	105-107	59[28]
CEDG 044	SSR	F-TCAGCAACCTTGCATTGCAG R-TTTCCCGTCACTCTTCTAGG	172-210	58[28]
CEDG 056	SSR	F- TTCCATCTATAGGGGAAGGGAG R- GCTATGATGGAAGAGGGCATGG	172-220	61[28]
CEDG 059	SSR	F-AGAAAAGGGTGGCCTCGTTG R-GCAGGCATTTCCATCGCAG	217-215	60[28]
CEDG 067	SSR	F-AGACTAAGTTACTTGGGCAACCAG R-TGACGGCCCGGCTCTCC	64-76	62[28]
CEDG 092	SSR	F-TCTTTTGGTTGTAGCAGGATGAAC R-TACAAGTGATATGCAACGGTTAGG	150-170	55[29)]
CEDG 133	SSR	F-GCATACATAATGTGGTGAGATG R-GTCTCGTGCCTTTCACAC	200-210	54[29]
CEDG 139	SSR	F-CAAACTTCCGATCGAAAGCGCTTG R-GTTTCTCCTCAATCTCAAGCTCCG	190	58[29]
CEDG 198	SSR	F-CAAGGAAGATGGAGAGAATC R-CCTTCTAAGAACAGTGACATG	227-209	50[28]
CEDG 180	SSR	F-GGTATGGAGCAAAACAATC R-GTGCGTGAAGTTGTCTTATC	136-163	55[29]
YMV1	SCAR	F-GAGAGAGAGAGAGAGACAAAG R-GAGAGAGAGAGAGAGACAGGA	1357	58[14]
YR4	RGA	F-GGTAAGACGACACTCGCTTTA R-GACGTCCTTGTAACTTTGATCA	456	58[17]
CYR1	RGA	F-GGGTGGTTTGGGTAAGACCAC R-TTCGCGGTGTGTGAAAAGTCT	1236	58[17]

polymorphic and associated with YMV disease resistance genes in green gram and black gram were selected from previously published literatures[18,21]. Theses microsatellite primers were procured from Genei (Bangalore Genei Pvt. Ltd., Bangalore, India) were used for amplification purposes. The amplifications were performed in 25 µL reaction volumes containing 1 µL of genomic DNA (25 ng/µL), 2.5 µL of 10X Taq polymerase buffer, 1 µL of 10 mM dNTP mix (0.20 mM), 1.0 µL of primer (10 ng/µL), 1 U of Taq DNA polymerase and 18.5 µL of milliQ water. After initial denaturation at 94°C for 4 min, PCR was run for 40 cycles consisting of a denaturation step of 1 min at 94°C, annealing for 1 min at 50-62°C (depending on marker type) and an extension at 72°C for 1 min. The final extension was done at 72°C for 1 min. List of genetic markers and their annealing temperatures are given in Table 2. The amplified PCR product (10 µL) was resolved on 1.5% (w/v) agarose gels in 1X TBE buffer. Staining of the gels was through with ethidium bromide and also the sizes of the fragments were calculated by employing 100 bp ladder (Genei, Bangalore) as a size marker. The gel was run until the dye reached the top of the gel, then the gel pictures were photographed with Gel Documentation system (Vilber Lourmat, France). All the PCR amplifications were recurrent thrice so as to confirm dependableness of the results.

Data analysis

The amplified DNA fragments in gel images were transformed to binary data (0, 1), where 1 and 0 were scored for the presence and absence of alleles, respectively. Percent polymorphism for the markers was work out by the formula: (Number of polymorphic alleles/Total number of alleles) × 100. The polymorphic information content (PIC), a degree of polymorphism, was estimated by the formula PIC = $1-\sum (Pi)^2$, where P_i is the proportion of samples carrying the i^{th} allele of a particular locus. The clustering was done by using the dissimilarity coefficient matrix for the 26 genotypes by employing the unweighted neighbour joining method bootstraped over 5000 times. The dendogram was constructed by using the joining pattern of the genotypes. The clads showing more than 70% of the bootstrap value where considered arbitrarily as a strong cluster. We used the DARwin v. 5.0.157 software[23] for clustering. Single marker analysis was conducted using ANOVA, regression in Microsoft excel. The fact that molecular marker genotypes were classified into groups means that marker genotypes can be used as classifying variables for a t-test or ANOVA, or as variables for regression analysis

Results and Discussion

Genetic variability for important agronomic traits is the basic requirement in the germplasm which can be used for crop improvement. Therefore, estimation of the genetic variation in the germplasm by conventional and molecular markers and identification of superior genotypes constitutes the foremost step in crop improvement. Many types of DNA markers have been developed and put to use in the past few decades in crop breeding program. The YMV virus infects most of the green gram varieties under commercial cultivation and hence we need to identify closely associated molecular markers that could be utilized in the transfer of the resistant genes into commercially grown cultivars.

Molecular marker statistics

A considerable level of variability was observed among tested green gram genotypes using SSR, SCAR and RGA markers. In the present study, all the 19 markers generated reproducible, clear and distinct DNA bands. Reproducibility of the PCR amplifications was verified by replicating the PCR reactions. Of these 19 markers, 12 (63.2%) markers were polymorphic and the remaining 7 (36.8%) produced monomorphic amplification products (Table 3). Allele sizes ranged from 64 bp (CEDG067) to 1357 bp (YMV1). The markers produced alleles in a range of 1 to 8, with a mean value of 1.75 alleles per locus. Eight markers

Table 3 — Genetic diversity analysis of 26 green gram genotypes using molecular markers

Primers	No. of alleles	No. of polymorphic alleles	% polymorphism	PIC Value
CEDG008	2	2	50.00	0.633
CEDG014	2	1	50.00	0.651
CEDG011	1	0	0.00	0.000
CEDG013	2	0	0.00	0.000
CEDG022	1	1	100	0.519
CEDG020	1	0	0.00	0.000
CEDG044	2	2	100	0.320
CEDG059	1	1	100	0.410
CEDG056	1	0	0.00	0.000
CEDG067	2	1	50.00	0.486
CEDG133	2	0	0.00	0.000
CEDG092	2	1	50.00	0.570
CEDG139	2	2	100	0.618
CEDG198	2	2	100	0.560
CEDG180	2	2	100	0.707
CEDG030	2	1	50.00	0.558
CYR1	1	0	0.00	0.000
YR4	1	1	100	0.809
YMV1	2	0	0.00	0.000
Total	31	21	95.0	7.195
Mean	1.63	1.10	50	0.378

were bialleic producing two distinct alleles among the genotypes while four markers were mono-allelic showing a dominant allele pattern. All the markers expect one (CEDG139) showed polymorphic allele pattern. In CEDG139 one of the allele was monomorphic. Further, in the present investigation, we observed per cent polymorphism ranges from 30.8 to 55.8. Among the 12 polymorphic markers, CEDG067, CEDG092 and CEDG198 scored 48.1% polymorphism. Only two markers showed highest polymorphism such as 50% (CEDG044) and 55.8% (CEDG059). The marker YR4 gave the lowest polymorphism (30.8%). The high level of polymorphism can be attributed to the genetically diverse nature of the genotypes. It has been well documented that individuals that are genetically dissimilar may have either same or different phenotype, but those individuals having similar genotype could only have similar phenotype[30].

Assessment of genetic polymorphism

PIC provides an estimate of the ability of any locus to discriminate the genotypes by considering the number of alleles that are present in a locus and their relative frequencies. The PIC values ranged from 0.192 (CEDG059) to 0.804 (YR4) with a mean of 0.464 (Table 3) which indicated that most of the markers were useful in germplasm characterization. However, 6 markers were more informative as they showed PIC values above 0.5. As per the PIC values, the most informative locus was YR4, which had the highest PIC value.

Dice dissimilarity co-efficient

Dice dissimilarity among the 26 green gram genotypes was found to vary from 0.07 to 1.0 with a mean of 0.535. The genetic distance calculated using Dice dissimilarity co-efficient showed AUGG13 and AUGG16 had lowest dissimilarity coefficient with AUGG12 followed by VBN (Gg) 3 with VBN (Gg) 4. The highest dissimilarity of 1.0 was between CO (Gg) 4 and AUGG17 and between CO (Gg) 8 and AUGG19 (Table 4).

Cluster analysis

Cluster analysis is a prominent tool which has been used most often to identify cluster of genotypes harboring desirable traits from the germplasm during selection[26]. By forming four groups, cluster analysis indicated divergence in 25 accessions. Clusters II and IV had the greatest inter-cluster distance, indicating that hybridization between genotypes from these two

Table 4 — Dice dissimilarity index using 12 SSR markers from 26 green gram genotypes

Units	AUGG1	AUGG2	AUGG3	AUGG4	AUGG5	AUGG6	AUGG7	AUGG8	AUGG9	AUGG10	AUGG11	AUGG12	AUGG13	AUGG14	AUGG15	AUGG16	AUGG17	AUGG18	AUGG19	AUGG20	VBN (Gg) 1	VBN (Gg) 2	VBN (Gg) 3	VBN (Gg) 4	CO (Gg)-7	CO (Gg)-8
AUGG-1	0.000																									
AUGG-2	0.273	0.000																								
AUGG-3	0.304	0.200	0.000																							
AUGG-4	0.238	0.217	0.083	0.000																						
AUGG-5	0.333	0.304	0.250	0.273	0.000																					
AUGG-6	0.565	0.360	0.308	0.417	0.250	0.000																				
AUGG-7	0.238	0.217	0.167	0.273	0.364	0.417	0.000																			
AUGG-8	0.500	0.455	0.478	0.429	0.429	0.478	0.714	0.000																		
AUGG-9	0.364	0.333	0.280	0.391	0.478	0.520	0.217	0.636	0.000																	
AUGG-10	0.545	0.250	0.200	0.304	0.304	0.360	0.391	0.364	0.250	0.000																
AUGG-11	0.455	0.333	0.120	0.217	0.391	0.360	0.304	0.455	0.250	0.167	0.000															
AUGG-12	0.417	0.231	0.333	0.440	0.280	0.333	0.280	0.500	0.231	0.231	0.308	0.000														
AUGG-13	0.364	0.167	0.280	0.391	0.304	0.360	0.217	0.455	0.167	0.167	0.250	0.077	0.000													
AUGG-14	0.652	0.440	0.385	0.500	0.333	0.231	0.500	0.304	0.440	0.280	0.360	0.259	0.280	0.000												
AUGG-15	0.652	0.440	0.385	0.500	0.333	0.231	0.417	0.391	0.440	0.280	0.360	0.185	0.280	0.154	0.000											
AUGG-16	0.455	0.250	0.360	0.478	0.304	0.360	0.304	0.455	0.250	0.250	0.333	0.077	0.083	0.200	0.280	0.000										
AUGG-17	0.579	0.429	0.455	0.500	0.500	0.455	0.400	0.579	0.429	0.429	0.429	0.217	0.333	0.364	0.182	0.333	0.000									
AUGG-18	0.647	0.684	0.700	0.667	0.556	0.700	0.778	0.529	0.684	0.684	0.684	0.524	0.579	0.400	0.600	0.474	0.500	0.000								
AUGG-19	0.600	0.364	0.391	0.429	0.429	0.478	0.429	0.600	0.545	0.364	0.455	0.333	0.364	0.478	0.304	0.455	0.368	0.647	0.000							
AUGG-20	0.619	0.565	0.333	0.455	0.545	0.583	0.545	0.429	0.478	0.391	0.304	0.520	0.478	0.333	0.500	0.478	0.600	0.333	0.524	0.000						
VBN (Gg) -1	0.556	0.500	0.429	0.368	0.474	0.619	0.579	0.444	0.500	0.400	0.400	0.455	0.400	0.429	0.524	0.400	0.529	0.333	0.333	0.368	0.000					
VBN (Gg)-2	0.700	0.545	0.478	0.429	0.619	0.565	0.714	0.500	0.636	0.455	0.364	0.583	0.545	0.478	0.652	0.545	0.684	0.412	0.500	0.333	0.222	0.000				
VBN (Gg)-3	0.294	0.368	0.500	0.444	0.444	0.700	0.444	0.647	0.474	0.579	0.579	0.429	0.368	0.700	0.700	0.474	0.625	0.429	0.412	0.556	0.333	0.529	0.000			
VBN (Gg)-4	0.375	0.444	0.579	0.529	0.529	0.684	0.529	0.625	0.556	0.667	0.667	0.500	0.444	0.684	0.684	0.556	0.600	0.385	0.500	0.529	0.429	0.625	0.077	0.000		
CO (Gg)-7	0.500	0.444	0.474	0.412	0.529	0.579	0.529	0.625	0.556	0.556	0.556	0.500	0.444	0.579	0.579	0.556	0.467	0.385	0.375	0.529	0.286	0.500	0.231	0.167	0.000	
CO (Gg)-8	0.750	0.889	0.684	0.765	0.765	0.684	0.765	0.875	0.556	0.778	0.667	0.800	0.778	0.684	0.895	0.778	1.000	0.692	1.000	0.529	0.857	0.625	0.846	0.833	0.833	0.000

clusters will utilise heterosis to a higher extent. For additional development, selection criteria based on pod length, pods per cluster, protein content, 100 seed weight, and seed yield per plant can be created[26]. The DICE dissimilarity co-efficient was used to construct the dendrogram and we observed four clusters based on marker allele distribution. The first cluster consisted of six genotypes (AUGG 2, AUGG 7, AUGG 9, AUGG 12, AUGG 13 and AUGG 16) and accommodated 23% of the total population based on allelic similarity. The second cluster consisted of four genotypes having a membership density of 15.4%. It consisted of AUGG 3, AUGG 4, AUGG 10 and AUGG 11.The third cluster consisted of eight genotypes accommodating 30.8% of total genotypes. It consisted of all VBN and CO genotypes VBN (Gg) 1, VBN (Gg) 2, VBN (Gg) 3, VBN (Gg) 4, CO (Gg) 7, and CO (Gg) 8 along with AUGG 18 and AUGG 20. The fourth cluster had remaining 27% of the population consisting of genotypes AUGG 5, AUGG 6, AUGG 8, AUGG 9, AUGG 14, AUGG 15 and AUGG 17. None of the groups indicated bootstrap esteem over 70% despite the fact that the neighbor joining methodology was bootstrapped 10000 times. The highest bootstrap value of 69% was shown by two genotypes VBN (Gg) 3 and VBN (Gg) 4 followed by their grouping with CO (Gg) 7 (54%).

Genetic divergence among green gram genotypes

Mahalanobis's D squares distances were estimated using cluster analysis. The genetic divergence of the test genotypes clustered them into more precise clusters and estimated the average distance between them. The D^2 value was calculated and genetic distance/divergence was estimated for the 26 genotypes under study. The cluster analysis produced into six clusters following Tocher's method as presented in Table 5. Cluster VI was the largest with 12 genotypes, AUGG 7, AUGG 8, AUGG 9, AUGG 12, AUGG 13, AUGG 14, AUGG 15, AUGG 17, AUBG 18, VBN (Gg) 1, VBN (Gg) 3, and VBN (Gg) 18. Cluster I with 5 AUGG 1, AUGG 2, AUGG 3, AUGG 4, AUGG 16 was the second largest cluster. Cluster IV was the third largest cluster with 3 genotypes, namely AUGG5, AUGG10, VBN (Gg) 2. The remaining clusters II, III and IV had two genotypes each. The intra and inter cluster D^2 values are presented in Table 6. Intra cluster values ranged from 12.86 (Cluster II) to 1188.15 (Cluster V). From the inter cluster values of six clusters, it can be seen that the highest divergence occurred between cluster IV and cluster V (1591.36) followed by cluster V and cluster VI (1578.54), cluster II and cluster V (1554.80), cluster 1 and cluster V (1186.67) in that order of magnitude. The minimum inter cluster distance was noticed between cluster III and cluster IV (130.24)) followed by cluster II and cluster IV (104.92) and cluster II and cluster III (51.12). In the present study there was a divergence in phenotypic and molecular genotypic diversity. The phenotypic diversity analysis produced six clusters while the molecular diversity analysis produced three clusters. All the VBN genotypes (developed in National Pulses Research Centre, Vamban, Tamil Nadu) were in same cluster (Cluster III) by molecular diversity while they were in three different clusters by phenotypic diversity. Similarly the genotypes AUGG 5 and AUGG 10, AUGG 19 and AUGG 11 which was grouped in the same cluster by phenotypic analysis were in different clusters by genotypic analysis. There were instances of correlation between both analyses. The genetic distance announced by DICE dissimilarity co-efficient indicated the lowest value of 0.007 between AUGG 12 with AUGG 13 and AUGG 16, and VBN (Gg) 3 with VBN (Gg) 4. This was in correlation with phenotypic diversity analysis were AUGG 12 and AUGG 13 were grouped in the same cluster. The highest divergence of 1.0 was between CO (Gg) 8 and AUGG 17 and between CO (Gg)

Table 5 — Clusters and their genotype distributions

Clusters	No. of genotypes	Genotypes
I	5	AUGG-1, AUGG-2, AUGG-3, AUGG-4, AUGG-16
II	2	VBN (Gg) 4, CO (Gg) 7
III	2	AUBG -6, AUGG-20
IV	2	AUGG-11, AUGG-19
V	3	AUGG-5, AUGG-10, VBN (Gg)-2
VI	1	AUGG-7, AUGG-8, AUGG-9, AUGG-12, AUGG-13, AUGG-14, AUGG-15, AUGG-17, AUGG-18, VBN (Gg) -1, VBN (Gg)-3, CO (Gg)-8

Table 6 — Average intra- cluster and (inter- cluster) D^2 values of green gram

Clusters	I	II	III	IV	V	VI
I	252.938 (15.165)	182.063 (14.658)	174.298 (13.458)	269.124 (15.467)	1158.590 (35.391)	276.752 (15.490)
II		12.69 (4.489)	132.243 (12.279)	106.898 (12.394)	1569.790 (10.423)	189.156 (36.362)
III			13.503 (3.724)	98.029 (10.015)	1052.232 (32.498)	236.796 (15.104)
IV				30.297 (5.603)	1588.264 (38.981)	176.762 (14.695)
V					1167.153 (35.387)	1563.487 (41.554)
VI						269.313 (16.695)

8 and AUGG 19. In the phenotypic analysis CO (Gg) 8 and AUGG 19 were grouped in different clusters.

The results showed that the pattern of genetic diversity in the genotypes depended on whether the markers used were molecular or phenotypic. Phenotypic clustering pattern obtained by screening under hotspot condition didn't correlate with the clustering obtained by DNA markers specific for YMV resistance in most of the occasions. Earlier studies have found fewer relationships between molecular genotypic analysis and divergence based on morphological data[17,18,24]. In the present investigation, the molecular markers produced close relationship among the genotypic pairs than the association based on phenotypic data. This finding suggests that the state of genetic diversity in green gram may be better described when large numbers of different markers are used in a complementary manner. It also indicats that SSR markers effectively sample the genomic regions which may code for many simple physio-logical functions affecting a morphological trait that could be relevant for phenotypic classification of the accessions.

Single marker analysis

Single marker analysis is one of several quantitative trait locus (QTL) analysis approaches that can reveal relationship between molecular markers and phenotypic scores was analyzed by single marker analysis to identify SSR markers that are association with yellow mosaic virus disease scores in black gram. The single-marker analysis (SMA) study showed that SSR markers CEDG180 and YR4 were highly associated with the phenotypic characters, number of branches per plant, length of the pod, number of seeds per pod and seed yield per plant. Moreover, phenotypically these characters have more association with each other. YMV percentage of the disease incidence showed the target alleles R^2 value CEDG180 (21.80), YR4 (22.30) and P value is CEDG180 (0.016) and YR4 (0.0014) (Table 7). QTL analysis, MAS and QTL cloning are the major application for breeding program. Molecular markers linked with QTL/major genes for traits of interest are being routinely developed in several crops[31]. Therefore, markers identified during the present study may be useful for further marker assisted breeding program to develop the yellow mosaic virus resistant black gram.

Table 7 — Single Marker Analyse for PDI

Traits	Allele	R^2	Group Mean		P-Value
			Positive	Negative	
YMV percentage disease Incidence	CEDG180	21.80	2.33	34.10	0.016
	YR4	22.30	54.50	26.10	0.014

To detect associations between molecular markers and traits of interest, data analysis approaches include single marker analysis, simple interval mapping (SIM), multiple interval mapping (MIM), and composite interval mapping (CIM). Although these approaches are designated for QTL analysis, they are also typically employed whenever a trait's method of genetic control is unknown. This article focuses on single marker analysis[32].

Marker trait association

Marker and phenotypic trait relationship was assessed between all the twelve polymorphic markers and YMV resistance. The variation between groups defined by the allelic pattern of each of the marker was assessed for YMV resistance as indicated by the Percentage Disease Incidence. Two alleles, one from marker CEDG092 showed a significant correlation with the YMV disease resistance in which the presence of the allele averaged an infection rate of 2.33% while the absence of the allele increased the average infection to 34.2%. The allele defined a proportion of variation of 21.9% towards the YMV resistance. Genotypes AUGG6 (HR), VBN (Gg) 2, (R) and CO (Gg) 8 (R) carried the positive alleles for this marker among the germplasms. The three genotypes clearly exhibited high resistance for YMV in the field condition in the hotspot region. Similarly, another allele from CEDG044 also showed significant correlation with disease incidence percentage with the presence of marker defining an average of 54.6% infection while absence of marker showed an average of 26.1% infection and contributing towards 22.3% of total variation for the percentage disease incidence.

The allele for susceptibility was present in AUGG 12(S), AUGG 15(HS), AUGG 17 (HS), and AUGG 19 (HS) which showed high susceptibility reaction. There was a clear association between the marker and the disease reaction in the field. However, absence of the marker did not show a clear-cut resistant reaction in the remaining genotypes. These exceptions and disparity in the expression of disease resistance by the genotypes could be reasoned to the partial linkage with the YMV resistance gene. These findings are in accordance with the reports of Gupta *et al*[15]. The study discovered that the SSR markers CEDG044 and CEDG092 (Fig. 1) can be

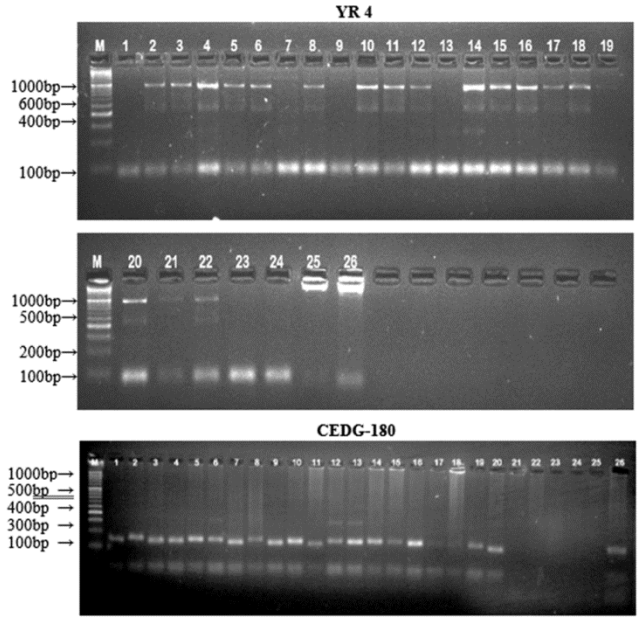

Fig. 1 — Microsatellite profiles of green gram genotypes differing with respect to YMV tolerance. Amplifications performed with the primer set, CEDG044 (a) and CEDG092 (b) along with a 50 bp ladder (M). The numbers in the gel corresponds to the genotypes number in Table 1.

utilized to screen large germplasm in order to discriminate the resistant and susceptible genotypes for YMV disease of green gram.

Conclusion

The present study revealed that the two SSR markers CEDG180 and YR4 were found to discriminate the YMV resistant genotypes from the susceptible genotypes and could be used to screen germplasm in MAS. These two markers might have been linked to two different resistance genes. Further validation studies are required to explore the tightly linked marker to gene conferring resistance to YMV.

Conflict of interest

The authors declare no competing interests.

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