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Research Article

Validation of molecular markers linked to yellow mosaic virus disease resistance in diverse genetic background of black gram [*Vigna mungo* (L.) Hepper]

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Abstract
Yellow mosaic virus (YMV) disease is a serious disease which affects the black gram productivity. In this study, 10 molecular markers reported to be linked to YMV resistance in black gram and mungbean were validated on 19 diverse black gram genotypes for their utility in marker assisted selection. Three molecular markers ($ISSR811_{1357}$, YMV1-FR and CEDG180) differentiated the YMV resistant and susceptible black gram genotypes. Other seven molecular markers were either monomorphic or failed to amplify the marker fragment in black gram genotypes. Inter Simple Sequence Repeat (ISSR) marker $ISSR811_{1357}$ and its derived Sequence Characterized Amplified Region (SCAR) marker YMV1-FR amplified the respective marker fragments in all YMV resistant genotypes except DPU 88-31, IPU 02-43, IPU 94-1 and IPU 07-3. A 136-bp allele of Simple Sequence Repeat (SSR) marker CEDG180 linked to YMV resistance was amplified in the aforementioned four genotypes in addition to PU 31. $ISSR811_{1357}$ and CEDG180 are located on different linkage groups in the black gram genetic linkage map, suggesting that two independent resistance genes may be governing resistance to YMV in Indian black gram cultivars with marker $ISSR811_{1357}$ linked to one resistance gene and marker CEDG180 linked to other resistance gene. Therefore, both markers can be used for marker assisted selection of YMV resistance. Among the resistant genotypes, PU 31 was the only genotype where both markers were amplified suggesting that PU 31 might be carrying both YMV resistance genes. Therefore, genotype PU 31 would be a valuable donor of YMV resistance and should be actively used in black gram breeding programs for incorporating YMV resistance.

Key words
Microsatellite marker, *Vigna mungo*, validation, yellow mosaic disease

Introduction

Yellow mosaic virus (YMV) disease caused by geminivirus and transmitted by whitefly is a major disease in a number of leguminous crops in India and South East Asia. The most seriously affected leguminous crops by this disease are mungbean, black gram, and soybean. Yellow mosaic disease causes 10–100% yield losses depending on the crop stage at which the plants being infected (Nene, 1972; Marimuthu *et al*., 1981). An annual loss of over US$300 million is incurred due to YMV infection in these crops (Varma *et al*., 1992). Several genotypes resistant to YMV have been identified in black gram germplasm. However, progress in breeding is hampered because of inconsistencies in scoring of infection phenotype and existence of different viral strains. It has been confirmed that at least two virus species causing yellow mosaic disease are prevalent in Indian sub continent. One of the species, *Mungbean Yellow Mosaic India Virus* (MYMIV) is commonly occurring in northern part of Indian sub-continent, while *Mungbean Yellow Mosaic Virus* (MYMV) is mostly confined to peninsular region of India (Varma and Malathi, 2003). Moreover, YMV being transmitted through whitefly, lack of uniform screening procedures and in many cases resistance is being governed by recessive genes have significantly delayed the introgression of YMV resistance genes into elite black gram lines. In such circumstances, indirect selection using molecular markers linked to resistant genes should be an effective approach as they can enable marker assisted selection to overcome inaccuracy in the field evaluation (Tanksley *et al*., 1989).

Molecular markers can be used as a diagnostic tool to predict the presence of a specific gene with high accuracy and to efficiently transfer the genes in different genetic backgrounds. The use of molecular markers for resistance genes is particularly powerful as it may avoid the delay in breeding programme associated with the phenotypic analysis (Paran and Michelmore, 1993). During pyramiding of resistance genes, it is difficult to select plants with multiple resistance genes based on phenotype alone as the action of one gene may mask the action of another. In such situations, the molecular markers help in identifying individual genes that could be used in gene pyramiding without pathogen inoculation and/or progeny testing. 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). Validation of molecular marker linked to disease resistance genes has been reported for *Fusarium* wilt in chickpea (Mayers *et al*., 1997), bud blight resistance in soybean

(Fasoula *et al*., 2003), and leaf rust in wheat (Gupta *et al*., 2005; Gupta *et al*., 2006).

To transfer the YMV resistance to susceptible cultivars, many Polymerase Chain Reaction (PCR) based molecular markers has been identified in black gram. This included ISSR and SCAR markers (Souframanien and Gopalakrishna, 2006), random amplified polymorphic DNA (RAPD) markers (Prasanthi *et al*., 2013), resistance gene analogue (RGA) markers (Basak *et al*., 2004; Maiti *et al*., 2011) and SSR markers (Gupta *et al*., 2013). In addition, molecular markers linked to YMV resistance also have been reported in mungbean which is a close relative of black gram (Selvi *et al*., 2006; Chen *et al*., 2013; Dhole and Reddy, 2013). However, most of these markers were identified using specific mapping populations and therefore, there is a need to validate these markers in different black gram genotypes before applying these markers for marker assisted selection (MAS) of YMV resistance in black gram. In this study, we used 10 molecular markers reported to be linked to YMV resistance in black gram and mungbean for validation on different black gram genotypes for their usefulness in MAS of YMV resistance in black gram breeding programs.

Materials and Methods

A total of 19 diverse black gram genotypes differing for their response to YMV disease were used in the study. The details of the genotypes used in the study are given in Table 1. The material was screened for the YMV reaction in the field under natural conditions at Trombay, Mumbai over a period of two years. Under field conditions, resistant plants did not show any yellowing of leaves or pods during the growth period, while susceptible plants showed yellowing on the leaves and pods. Normal cultural practices were followed, except that there was no insecticide spraying. LBG17, a highly susceptible cultivar to YMV, was used as an insect refuge after every five rows. Genomic DNA was extracted from seedlings using DNeasy Plant Mini kit (Qiagen, USA) using the manufacturer's protocol and DNA concentration was determined on Nanodrop ND 1000 spectrophotometer (Thermo Scientific, USA). The DNA samples were diluted to 10 ng μl^{-1} for PCR amplification.

PCR amplification: A total of 10 molecular markers reported to be linked to YMV resistance in black gram and mungbean were used for the validation study. The details of the markers used are given in Table 2. PCR amplification were performed in a 25 µl reaction volume containing 10 mM Tris - HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dNTP, 20 pmoles of RAPD primer, 50 ng of genomic DNA and 0.5 units of *Taq* DNA polymerase (Fermentas Life Sciences). Amplifications were performed in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). Amplification conditions were an initial denaturation at 94^{o}C for 4 min and 40 cycles at 94^{o}C for 1 min, 37-60^{o}C (depending on marker type) for 1min, 72^{o}C for 1 min followed by 7 min at 72 ^{o}C. The annealing temperatures of different markers used in the study are given in Table 2. Amplified products were separated on 2% agarose gel in 1X TBE (Tris-Borate-EDTA) buffer. SSR products were resolved on 4% Metaphor agarose gel. The gels were stained with 0.5 µg/ml ethidium bromide solution and were photographed on a gel image analysis system (Syngene, U.K.).

Results and Discussion

Many commercial black gram varieties are susceptible to YMV and there is a need to identify tightly linked molecular markers that could facilitate the transfer of the resistant genes in to popular cultivars using marker assisted breeding. In this study, 10 molecular markers which included 7 markers identified as linked to YMV resistance in black gram (Basak *et al*., 2004; Souframanien and Gopalakrishna, 2006; Maiti *et al*., 2011; Prasanthi *et al*., 2013, Gupta *et al*., 2013) and 3 markers reported to be linked to YMV resistance in mungbean (Selvi *et al*., 2006; Chen *et al*., 2013; Dhole and Reddy, 2013) were validated for their usefulness in marker assisted breeding.

Out of 10 molecular markers tested, only three markers ($ISSR811_{1357}$, YMV1-FR and CEDG180) were found polymorphic between resistance and susceptible black gram genotypes studied (Table 3). ISSR marker $ISSR811_{1357}$ reported to be linked to YMV resistance (6.8 cM) in black gram (Souframanien and Gopalakrishna, 2006) amplified the 1357 bp marker fragment in 9 of the 13 YMV resistant black gram genotypes and marker fragment was absent in all six YMV susceptible black gram genotypes (Fig. 1). Four YMV resistant black gram genotypes such as DPU 88-31, IPU 02-43, IPU 94-1 and IPU 07-3 failed to amplify the 1357 bp marker fragment of $ISSR811_{1357}$. Similarly, a SCAR marker (YMV1-FR primer) developed from ISSR marker $ISSR811_{1357}$ (Souframanien and Gopalakrishna, 2006) was able to distinguish 9 of the 13 YMV resistant genotypes and failed to amplify the marker fragment in four YMV resistant genotypes, DPU 88-31, IPU 02-43, IPU 94-1 and IPU 07-3. All susceptible black gram genotypes lacked the SCAR marker fragment. In contrary to marker $ISSR811_{1357}$, SSR marker CEDG180 (Gupta *et al*., 2013) amplified the 136 bp allele linked to MYMIV resistance only in five resistant genotypes (PU 31, DPU 88-31, IPU 02-43, IPU 94-1, IPU 07-3) and in one susceptible genotype *viz*., EC168058 (Fig. 2). In rest of the resistant and susceptible genotypes, about 163 bp amplicon linked to susceptibility was amplified. Gupta *et al*. (2013)

has used genotype DPU 88-31 as MYMIV resistance source for establishing the linkage of SSR marker CEDG180 to MYMIV resistance. Based on pedigree, we found that genotype DPU 88-31 is also the MYMIV resistance donor to three other resistant genotypes, PU 31, IPU 02-43 and IPU 07-3. Therefore, resistance linked 136 bp allele of SSR marker CEDG180 was faithfully amplified in these four resistant genotypes. The 136 bp allele of marker CEDG180 was also amplified in one susceptible genotype, EC168058 and in rest of the resistant and susceptible genotypes, approximately 163 bp amplicon linked to susceptibility was amplified. The amplification of resistance linked allele in susceptible genotypes and susceptibility linked allele in resistance individuals may be attributed to lose linkage of this marker to YMV resistance gene (12.9 cM) and hence should be cautiously used for MAS.

The absence of $ISSR811_{1357}$ marker fragment and presence of 136 bp allele of SSR CEDG180 in genotypes, DPU 88-31, IPU 02-43, IPU 94-1 and IPU 07-3 indicated that these genotypes may be carrying different source of YMV resistance than the one targeted by marker $ISSR811_{1357}$ and thus two independent genes governing resistance to YMV are present in Indian black gram cultivars. This was further confirmed by the position of these two molecular markers on the genetic linkage map developed by Gupta *et al*. (2008). The marker $ISSR811_{1357}$ was mapped at a position of 61.2 cM on LG2, whereas, SSR marker CEDG180 was present on LG10 at a position of 51.9 cM in black gram genetic linkage map, suggesting that both the markers had targeted different YMV resistance genes during gene mapping studies. Existence of two independent resistance genes for YMV resistance has been reported in black gram (Singh, 1980) and mungbean (Dhole and Reddy, 2012).

The genotype PU 31 was the only resistant genotype where 1357 bp amplicon of $ISSR811_{1357}$ as well as 136 bp allele of SSR CEDG180 were amplified. Based on pedigree information, it was found that genotype PU 31 is a selection of the cross UPU97-10 x DPU88-31. Parent line UPU97-10 is resistant to MYMV in Central India (Gupta, 2003) and may be carrying the resistance gene targeted by marker $ISSR811_{1357.}$ Presence of both these markers $ISSR811_{1357}$ and CEDG180 in genotype PU 31 suggested that PU 31 may be carrying two YMV resistance genes from two resistant parents. Therefore, genotype PU 31 could be a valuable donor of YMV resistance and should be actively used in black gram breeding programs for incorporating YMV resistance in India.

RGA marker VMYR1 developed by Basak *et al*. (2004) failed to reveal polymorphism and amplified the 445 bp marker fragment in all resistant and susceptible black gram genotypes (Fig. 3). Similarly, other two RGA markers YR4 and CRY1 (Maiti *et al*., 2011) reported to be linked to MYMIV resistance amplified the respective marker fragment of 456 bp and 1236 bp in all black gram genotypes and were monomorphic. The source of MYMIV resistance used in developing these RGA markers is a MYMIV tolerance mutant derived from a highly MYMIV susceptible cultivar T9. Therefore, there is a possibility that these RGA markers are specific to mutant gene for MYMIV tolerance and may not be useful in other genetic backgrounds. In a previous study, Gupta *et al*. (2013) had also reported the failure of these RGA based markers in black gram. In other studies also, it has been observed that molecular markers reported as linked to target resistance genes in specific mapping populations may not be polymorphic in other genetic backgrounds and hence may not be suitable for MAS (Prabhu *et al*., 2004; Bernardo *et al*., 2013). RAPD marker identified by OPQ1 (Prasanthi *et al*., 2013) failed to amplify the 532-bp marker fragment in black gram genotypes studied.

Mungbean is a close relative of black gram and therefore molecular markers linked to YMV resistance in mungbean were also included in the validation study to see their usefulness in black gram. However, all three molecular markers, DMB-SSR158, MYMVR-583 and $OPS7_{900}$ linked to YMV resistance in mungbean failed to produce any polymorphism between YMV resistant and susceptible black gram genotypes in this study. SSR marker DMB-SSR158 linked to a QTL for MYMIV resistance in mungbean (Chen *et al*., 2013) was found to be monomorphic in this study. SCAR marker MYMVR-583 (Dhole and Reddy, 2013) linked to MYMV resistance (6.8 cM) and RAPD marker $OPS7_{900}$ (Selvi *et al*., 2006) failed to amplify the marker fragment in black gram genotypes. However, marker fragments were successfully amplified in the MYMV resistant mungbean genotype used as a positive control suggesting that these markers are mungbean specific and cannot be used for MAS in black gram.

In summary, three markers, $ISSR811_{1357,}$ YMV1-FR and CEDG180 were found to discriminate the YMV resistant and susceptible black gram genotypes and can be used for MAS. Validation studies also indicated that two independent resistance genes may be governing resistance to YMV in Indian black gram cultivars with marker $ISSR811_{1357}$ linked to one resistance gene and marker CEDG180 linked to other resistance gene. However, there is a need to identify tightly linked marker to the gene conferring resistance to different races of yellow mosaic viral disease.

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Table 1. List of black gram genotypes with pedigree used in the study

S. No.	Genotypes	Pedigree	Reaction to YMV
1.	TU94-2	TPU-4 x TAU-5 (Mutant of EC-1682000)	Resistant
2.	Nayagarh	Local selection	Resistant
3.	Pant-U19	UPU-1 x UPU-2	Resistant
4.	KU 96-3	PU19 x NP21	Resistant
5.	No. 55	Local selection from Satpur Plateau	Resistant
6.	Pusa-3	L-151 x T-9	Resistant
7.	TU-40	TU94-2 x *V. mungo* var. *silvestris*	Resistant
8.	PU-31	UPU97-10 x DPU88-31	Resistant
9.	DPU88-31	PLU131 x T-9	Resistant
10.	IPU02-43	DPU88-31 x DUR-1	Resistant
11.	IPU94-1	NP19 x T-9	Resistant
12.	IPU07-3	DPU88-31 x PDU-1	Resistant
13.	EC-168200	Exotic collection from AVRDC, Taiwan	Resistant
14.	TAU-1	T-9 x UM-196 (Mutant of No. 55)	Susceptible
15.	T-9	Local selection from Bareilly U.P.	Susceptible
16.	LBG-17	Netiminumu x Chikkuduminumu	Susceptible
17.	TPU-4	UM-201 (Mutant of No. 55) x T-9	Susceptible
18.	Trombay wild	*Vigna mungo* var. *silvestris*	Susceptible
19.	EC-168058	Exotic collection from AVRDC, Taiwan	Susceptible

Table 2. Details of molecular markers linked to YMV resistance used for validation study

S. No.	Marker name	Marker type	Crop	Primer sequences (5′-3′)	Annealing temperature (^{o}C)	Expected product size (bp)	Reference
1	$ISSR811_{1357}$	ISSR	Black gram	GAGAGAGAGAGAGAGAC	50-55	1357	Souframanien and Gopalakrishna, 2006
2	YMV1-FR	SCAR	Black gram	F:GAGAGAGAGAGAGAGACAAAG R: GAGAGAGAGAGAGAGACAGGA	64	1357	Souframanien and Gopalakrishna, 2006
3	CEDG180	SSR	Black gram	F:GGTATGGAGCAAAACAATC R:GTGCGTGAAGTTGTCTTATC	55	136/163	Gupta *et al*., 2013
4	VMYR1	RGA	Black gram	F: AGTTTATAATTCGATTGCT R: ACTACGATTCAAGACGTCCT	45	445	Basak *et al*., 2004
5	YR4	RGA	Black gram	F:GGNAAGACGACACTCGCNTTA R:GACGTCCTNGTAACNTTGATCA	60	456	Maiti *et al*., 2011
6	CYR1	RGA	Black gram	F:GGGTGGNTTGGGTAAGACCAC R:NTCGCGGTGNGTGAAAAGNCT	58	1236	Maiti *et al*., 2011
7	OPQ-01	RAPD	Black gram	GGGACGATGG	36	532	Prasanthi *et al*., 2013
8	DMB-SSR158	SSR	Mungbean	F: TGGAAAATTTGCAGCAGTTG R: ATTGATGGAGGGCGGAAGTA	55	-	Chen *et al*., 2013
9	MYMVR-583	SCAR	Mungbean	F:GTGATGCACACGGTTACGGT R:GGTGACGCAGTCCATACAAATTT	60	583	Dhole and Reddy, 2013
10	OPS-7	RAPD	Mungbean	TCCGATGCTG	36	900	Selvi *et al*., 2006

Table 3. Amplification profile of molecular markers linked to YMV resistance on diverse black gram genotypes

Genotype	Reaction to YMV	ISSR 811_{1357}*	YMV1-FR	CEDG-180	VMYR1	YR4	CYR1	OPQ-01	DMB-SSR158	MYMVR-583	OPS-7
TU94-2	Resistant	+	+	-	+	+	+	NA	+	NA	NA
Nayagarh	Resistant	+	+	-	+	+	+	NA	+	NA	NA
Pant-U19	Resistant	+	+	-	+	+	+	NA	+	NA	NA
KU 96-3	Resistant	+	+	-	+	+	+	NA	+	NA	NA
No. 55	Resistant	+	+	-	+	+	+	NA	+	NA	NA
Pusa-3	Resistant	+	+	-	+	+	+	NA	+	NA	NA
TU-40	Resistant	+	+	-	+	+	+	NA	+	NA	NA
PU-31	Resistant	+	+	+	+	+	+	NA	+	NA	NA
DPU88-31	Resistant	-	-	+	+	+	+	NA	+	NA	NA
IPU02-43	Resistant	-	-	+	+	+	+	NA	+	NA	NA
IPU94-1	Resistant	-	-	+	+	+	+	NA	+	NA	NA
IPU07-3	Resistant	-	-	+	+	+	+	NA	+	NA	NA
EC-168200	Resistant	+	+	-	+	+	+	NA	+	NA	NA
TAU-1	Susceptible	-	-	-	+	+	+	NA	+	NA	NA
T-9	Susceptible	-	-	-	+	+	+	NA	+	NA	NA
LBG-17	Susceptible	-	-	-	+	+	+	NA	+	NA	NA
TPU-4	Susceptible	-	-	-	+	+	+	NA	+	NA	NA
Trombay wild	Susceptible	-	-	-	+	+	+	NA	+	NA	NA
EC-168058	Susceptible	-	-	+	+	+	+	NA	+	NA	NA

* + = Marker fragment present; - = marker fragment absent; NA = marker not amplified

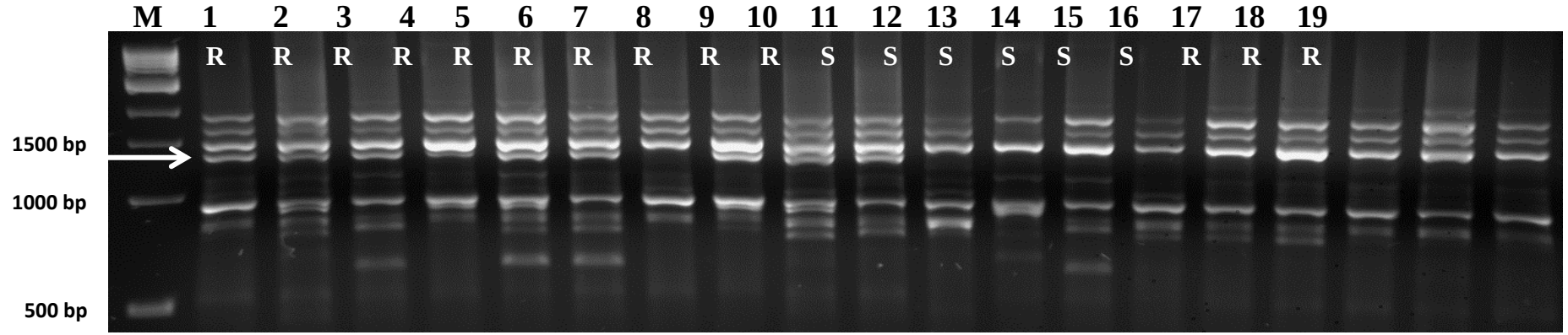

Figure 1. Amplification profile of ISSR marker $ISSR811_{1357}$ on 19 black gram genotypes. Lane M: 1Kb DNA MW marker; Lane 1: KU96-3; 2: TU94-2; 3: TU-40; 4: Pant U19; 5: Nayagarh; 6: PU 31; 7: DPU 88-31; 8: No.55; 9: EC-168200; 10: Pusa 3; 11: TAU-1; 12: EC-168058; 13: LBG-17; 14: T-9; 15: TPU-4; 16: Trombay wild; 17: IPU 02-43; 18: IPU 94-1; 19: IPU 07-3. R: YMV resistant and S: YMV susceptible. *Arrow* indicates the 1357 bp fragment linked to MYMV resistance.

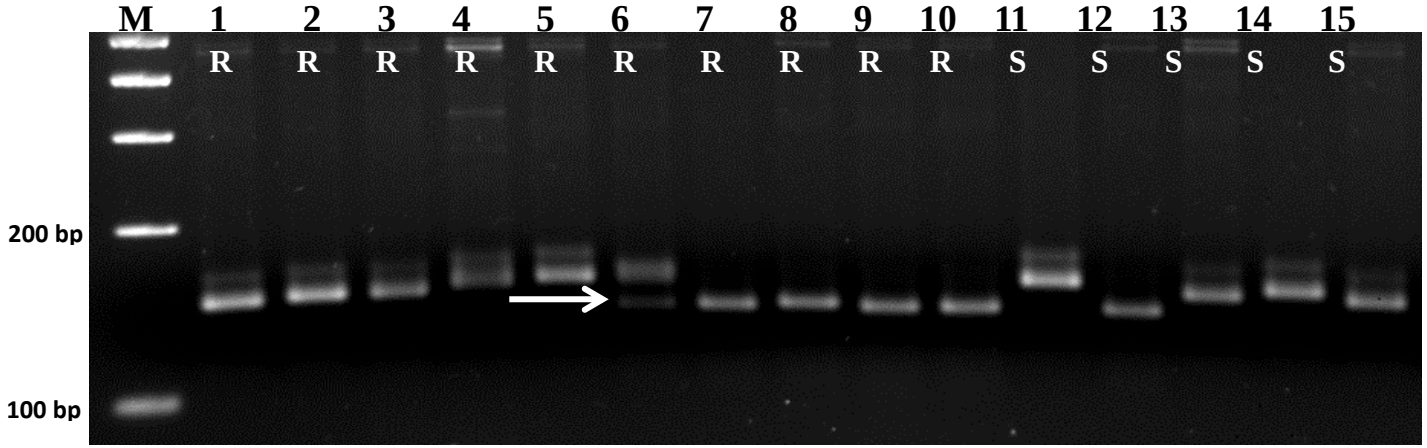

Figure 2. Amplification profile of SSR marker CEDG180 on black gram genotypes. Lane M: 100 bp DNA MW marker; Lane 1: KU96-3; 2: TU94-2; 3: TU-40; 4: Pant U19; 5: Nayagarh; 6: PU 31; 7: DPU 88-31; 8: IPU 02-43; 9: IPU 07-3; 10: IPU 94-1; 11: TAU-1; 12: EC-168058; 13: LBG-17; 14: T-9; 15: TPU-4). R: YMV resistant, S: YMV susceptible. *Arrow* indicates the 136 bp allele linked to MYMIV resistance.

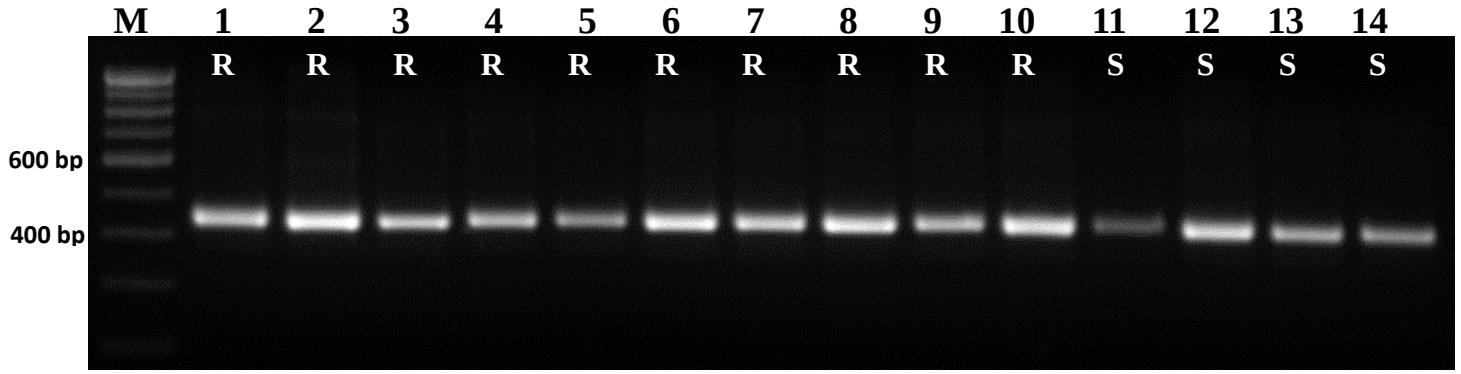

Figure 3. Amplification profile of RGA marker VMYR1 on black gram genotypes. Lane M: 100 bp DNA MW marker; Lane 1: KU96-3; 2: TU94-2; 3: TU-40; 4: Pant U19; 5: Nayagarh; 6: PU 31; 7: DPU 88-31; 8: IPU 02-43; 9: IPU 07-3; 10: TAU-1; 11: T-9; 12: EC-168058; 13: LBG-17; 14: TPU-4). R: YMV resistant, S: YMV susceptible.