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Screening of MYMV Resistant Mungbean (*Vigna radiata* L. Wilczek) Progenies through Agroinoculation

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ABSTRACT

Yellow mosaic disease is one of the most important viral diseases in mungbean caused by Mungbean Yellow Mosaic Virus (MYMV) which leads to severe yield reduction and it necessitates developing MYMV resistant lines for improved crop yield. The present investigation was carried out with an objective of identification of MYMV resistant progenies through Agroinoculation. This technique was employed to examine the F_2 individuals, which were derived from a cross between Vamban (Gg) 2 (susceptible) x KMG 189 (resistant) to screen for the MYMV resistant progenies. In the field condition, MYMV infection can be evaluated by MYMV disease rating scale (1-9). Out of the 203 F_2 individuals, 30 individuals were identified as resistant to MYMV and subjected to agroinoculation. The two tandem viral constructs of MYMV, VA 221 (KA30 DNA A + KA22 DNA B) and VA 239 (KA30 DNA A + KA27 DNA B) mobilized in *Agrobacterium tumefaciens* strains Ach 5 and C 58 were used for Agroinoculation. The results shows that among the thirty individuals, only five individuals namely MYMVR 10, MYMVR 13, MYMVR 15, MYMVR 17 and MYMVR 37 exhibited resistance to VA 221 strain and found to be susceptible to VA 239 strain. Other twenty five individuals are susceptible to both strains, VA 221 and VA 239. The presence of viral DNA in agroinoculated plants were confirmed by Reverse Transcriptase-Polymerase Chain Reaction (PCR) analysis. For additional corroboration, these five resistant individuals are forwarded to F_3 generation and confirmed for resistance in field condition.

Key words: Agroinoculation, mungbean, mungbean yellow mosaic virus, reverse transcriptase-PCR analysis

INTRODUCTION

Mungbean (*Vigna radiata* L.) is an important pulse crop in developing countries of Asia, Africa and Latin America where it is consumed as dry seeds, fresh green pods. (Karuppanapandian *et al*., 2006). Mungbean serves as vital source of vegetable protein (19.1-28.3%), mineral (0.18-0.21%) and vitamins. It is a native of India-Burma and is cultivated extensively in Asia (Khattak *et al*., 2007). India is the leading mungbean cultivator, covers up to 55% of the total world acreage and 45% of total production (Rishi, 2009). Among the biotic agents plant viruses are responsible for a significant proportion of crop disease (Prajapat *et al*., 2011). It cause serious economic losses in

many major crops by reducing seed yield and quality (Kang *et al.*, 2005). Yellow Mosaic Disease (YMD) is reported to be the most destructive viral disease among the various viral diseases, caused by Yellow Mosaic Virus. It causes severe yield reduction in all mungbean growing countries in Asia including India (Biswass *et al.*, 2008). MYMV belongs to the family Geminiviridae (Fauquet *et al.*, 2003). The family Geminiviridae is divided into four genera (Mastrevirus, Curtovirus, Topocuvirus and Begomovirus) based on genome structure, type of insect vector and host range (Medina-Ramos *et al.*, 2008). Begomovirus is the largest genus of the family Geminiviridae (Dhakar *et al.*, 2010) which is characterized by a bipartite genome (DNA-A and DNA-B) or monopartite genomes that were transmitted in a circulative persistent manner by white fly *Bemisia tabaci* (Sidhu *et al.*, 2009). Conventional breeding methods are unsuccessful in developing Yellow Mosaic Virus (YMV) resistant mungbean lines due to rapid explosion of new isolates and also the complexity of mechanism in controlling the resistance to MYMV (Selvi *et al.*, 2006). The major problem encountered by scientists to develop the MYMV resistant variety is the identification of MYMV resistant lines in segregating population. Identifying the resistant lines is very complicated task due to the lack of reliable screening protocol for assessing the resistance/susceptibility against MYMV. Hence the scientists are in need of any biological/molecular tool that can lead to screening of the resistant or susceptible lines for MYMV. Developing host resistance to the disease or the vector has therefore been considered as the only solution to control this disease (Kang *et al.*, 2005). Plant genetic transformation is of particularly benefit to molecular genetic studies and crop improvement programmes (Darbani *et al.*, 2008). The emerging field of genetic engineering endows with a new technique agroinoculation was successful for screening virus resistant plants (Usharani *et al.*, 2005; Bi *et al.*, 2010). Jacob *et al.* (2003) demonstrated the feasibility of using an *in vitro* molecular protocol to screen for resistance/susceptibility against MYMV and proved that Agroinoculation can be successfully adopted for screening MYMV resistant mungbean genotypes. Exploitation of this reproducible and less expensive technique may lead to the development of a MYMV resistance genotype. With this background knowledge this present investigation was carried out with the aim of identifying the MYMV resistant progenies through agroinoculation.

MATERIALS AND METHODS

Plant materials: The experimental material for the present investigation consisted of 203 F_2 individuals, ten F_3 progenies and parents KMG 189, VBN (Gg) 2, which were obtained from National Pulse Research Centre, Tamil Nadu Agricultural University and Vamban. VBN (Gg) 2 developed at Vamban is an agronomically superior high yielding variety but highly susceptible to MYMV. KMG 189 is a field resistant genotype to MYMV. The field experiment was conducted in two consecutive seasons, namely kharif 2009 and summer 2010 at the National Pulse Research Centre.

Phenotyping of mapping population: In the field condition, the MYMV infection can be evaluated by infector row method as described by Selvi *et al.* (2006). The test materials were scored after 80% of plants showed MYMV incidence. The 203 individuals and progenies in the F2 and F_3 generation respectively were scored for MYMV infection using 1-9 rating scale suggested by Singh *et al.* (1988) is adopted.

Agroinoculation: Agroinoculation study was conducted in the Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore. The tandem viral constructs of MYMV, VA 221

(KA30 DNA A+KA22 DNA B) and VA 239 (KA30 DNA A+KA27 DNA B) mobilized in *Agrobacterium tumefaciens* strains Ach 5 and C 58 were collected from Madurai Kamaraj University, Madurai and used for further studies. Agroinoculation was done on surface sterilized overnight sprouted seeds of the parents (VBN (Gg)2 and KMG189) and F_2 individuals. *Agrobacterium tumefaciens* strains harbouring the appropriate partial tandem repeat clone were grown to 1 Optical Density at 600 nm in 2 mL AB minimal medium pH 7.0 containing the antibiotics like streptomycin (150 mg L^{-1}), spectinomycin (50 mg L^{-1}) and tetracycline (5 mg L^{-1}) at 28°C at 220 rpm. From this, 1 mL of the culture was taken to inoculate, another 50 mL of AB minimal medium (pH - 7.0) containing the above mentioned antibiotics and grown to 1 OD at 600 nm at 28°C at 220 rpm. The culture was spinned at 4000 rpm for 10 min at 25°C. Cells obtained were re-suspended in 50 mL of AB minimal medium (pH - 5.6) with 100 μL acetosyringone (100 μm). Seed coat of the sprouted seeds was removed by using forceps and pricked around the hypocotyl region and were immediately immersed in the appropriate culture of *A. tumefaciens*. After the overnight incubation, seeds were washed with distilled water and sown in pots containing autoclaved sand and vermiculite in the ratio of 1:1. Agroinoculated plants were maintained in a growth chamber at 25°C, 60-70% relative humidity and a photoperiod of 16/18 h. The Hoagland's solution was applied twice in a week for proper growth of the plants and transferred to green house after 15 days for symptom observation (Balaji *et al*., 2004).

RT-PCR analysis: After 25 days from agroinoculation, when the mungbean yellow mosaic symptoms were clearly seen in the leaves of mungbean plants, the leaf samples were collected. Total RNA was extracted by TRIZOL method using SV total RNA isolation kit (Promega, USA), according to user manual guide. The quality of the RNA was checked in 1.5% agarose gel by visualizing the intactness of RNA bands by performing agarose gel electrophoresis and the quantification of RNA was done by Nanodrop Spectrophotometer (Thermo scientific Inc.). The extracted RNA was converted into cDNA using RevertAid™ H minus First Strand cDNA Synthesis Kit (Fermentas cat# R0081). The first strand (cDNA) was used as a template for the synthesis of second strand through RT-PCR with the help of coat protein gene specific primer. The PCR reaction mixtures were prepared with the volumes of 20 μL containing, 2.00 μL of the cDNA, 5x HF phusion buffer -4 μL, 25 mM dNTPS -0.4 μL (Bangalore Genei Ltd., India), 10 mM Forward primer -1.0 μL, 10 mM Reverse primer -1.0 μL, Phusion enzyme (2 u/μL -0.2 μL and 11.4 μL MilliQ water. The PCR reaction was carried out in a Eppendorf Mastercycler-Personal 5332® programmed to run the following specific temperature profile: 94°C for 5 min, initial denaturation for a minute, 35 cycles consisting each of a denaturation step for 1 min at 94°C, an annealing step for 1 min at 55°C, an extension step for 2 min at 72°C and the final extension for 5 min at 72°C. Agarose gel (1%) electrophoresis was performed to separate the amplified products. Seven micro litre of PCR amplified product was loaded with 3 μL of loading dye. The voltage was maintained at 100 volts for 1 h. The staining is done with ethidium bromide solution separately after agarose gel electrophoresis and the bands were visualized and documented in gel documentation system (Alpha Imager™1200, Alpha Innotech Corp., CA and USA).

RESULTS AND DISCUSSION

Most of the commercially accepted mungbean varieties are susceptible to MYMV. It necessitates developing MYMV resistant varieties because mungbean is important pulse crop in Asia. Most of previous screening studies for YMD resistance in Asia were conducted in field condition under high

YMD epidemic pressure. Identification of MYMV resistant lines through conventional breeding method relies on field screening. It is a time consuming one and requires evaluation at hot spot area (Selvi *et al*., 2006). Sometimes the screening based on natural occurrence in the hot spot areas also does not give consistent results. A combination with plant breeding approaches will likely to be needed for the improvement of crops (Roy *et al*., 2011). Pathogen-derived resistance (PDR) is a very effective genetic engineering approach to control plant viruses (Chellappan *et al*., 2004). Advancements in the field of genetic engineering provide a new technique called agroinoculation. It is an effective method by which infectious viral clones can be introduced into plants using *A. tumefacians* (Grimsley *et al*., 1986). Usharani *et al*. (2005) reported agroinoculation is an efficient method to employ the virus resistant plants. In earlier report successful agro-inoculation of mungbean with MYMV was recorded by Jacob *et al*. (2003) and Balaji *et al*. (2004) proved for YMD studies in mungbean. Genetic transformation in combination with conventional breeding increases the efficiency of the breeding programme especially the incorporation of the disease resistance into the varieties and is the most predominant and a powerful tool to achieve the goal.

The parents VBN (Gg) 2 and KMG 189 initially subjected to agroinoculation, revealed that KMG 189 did not develop any mosaic or leaf curling symptoms upon inoculation with VA 221 strain but at the same time they exhibited susceptibility against VA 239 strain (Fig. 1) VBN (Gg) 2, the susceptible mungbean, developed typical yellow mosaic and leaf curling symptoms in the trifoliate leaves upon agroinoculation with both the *Agrobacterium* strains (VA 221 and VA 239) (Fig. 2). Similar to the present finding, Balaji *et al*. (2004) also observed the leaf curling and mild yellow mosaic symptoms from agroinoculation in *Vigna* sp using two different MYMV isolates. The 203 F_2 individuals with MYMV infection, evaluated under field condition indicated that 30 individuals were identified as resistants and their seeds were harvested and subjected to agroinoculation which

Fig. 1: Symptom expression by resistant mungbean KMG 189 through Agroinoculation. (1) Resistant symptom expression to *Agrobacterium tumafaciens* strain VA 221, (2) Uninoculated control and (3) Susceptible symptom expression to *Agrobacterium tumafaciens* strain VA 239

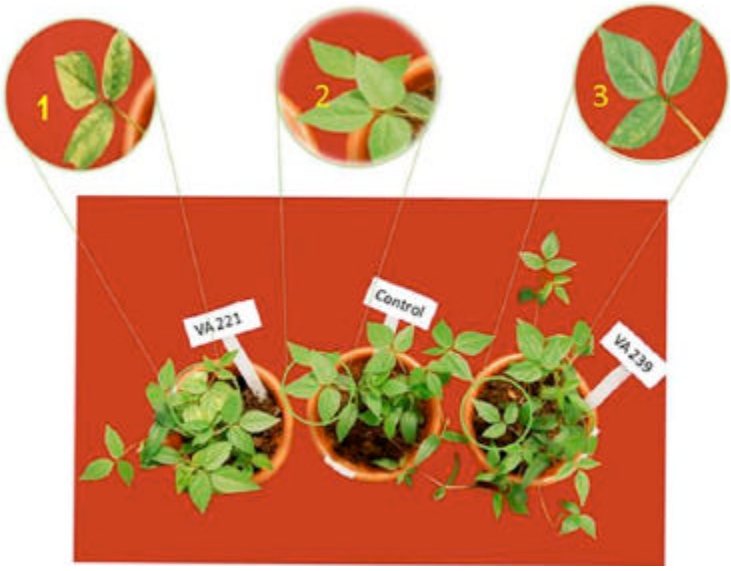

Fig. 2: Symptom expression by susceptible mungbean VBN (Gg) 2 through Agroinoculation. (1) Susceptible symptom expression to *Agrobacterium tumafaciens* strain VA 221, (2) Uninoculated control and (3) Susceptible symptom expression to *Agrobacterium tumafaciens* strain VA 239

aims to identify the MYMV resistant mungbean progenies in the segregating population. The list of the MYMV resistant individuals subjected to agroinoculation screening is presented in Table 1. The results of the agroinoculation showed that, among the 30 individuals only five individuals showed to be resistant. The five individuals namely MYMVR 10, MYMVR 13, MYMVR 15, MYMVR 17 and MYMVR 37 did not develop any mosaic or leaf curling symptoms upon inoculation with VA 221 strain. But at the same time they exhibited susceptibility reaction against the strain V A239 (Fig. 3, 4). The remaining twenty five individuals developed typical yellow mosaic symptom for both the strains (VA 221 and VA 239). The five resistant individuals were found to have behaved the same way as that of their resistant parent KMG 189.

The agroinoculated mungbean plants started developing yellow mosaic symptoms from the 17th day to 25th day and there were no symptoms in the control plants. At the 25th day, the yellow mosaic symptoms were clearly seen on the leaves. The average infectivity of MYMV strains through agroinoculation in mungbean ranged from 0 to 94%. Average per cent infection of resistant KMG 189 in the strain VA 221 is 0.00 and VA 239 strain is 42.50. The susceptible parent VBN (Gg) 2 recorded an average infection of 99.00 and 97.50% in VA 221 and VA 239, respectively. No infection was recorded on MYMVR-10, MYMVR-13, MYMVR-15, MYMVR-17 and MYMVR-37 in VA-221 strain, however infection was recorded in these five individuals ranging from 39.50 to 63.50% in VA 239 strain. Among the individuals the highest average infection was recorded in MYMVR-43 (91.00%) followed by MYMVR -30 (90.00%) and MYMVR-23 (94.00%) followed by MYMVR-51 (93.00) for VA 221 and VA 239 strains, respectively. The individuals MYMVR 45 (32.50%) followed by MYMVR-24 (48.75%) and MYMVR -15 (36.50%) followed by MYMVR -13

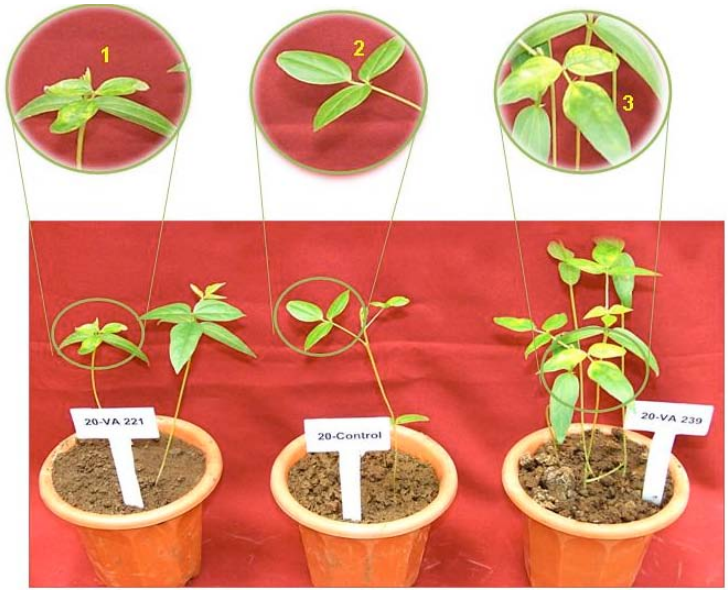

Fig. 3: Symptom expression by resistant F2 individual MYMVR 20 through agroinoculation. (1) Susceptible symptom expression to *Agrobacterium tumafaciens* strain VA 221, (2) Uninoculated control and (3) Susceptible symptom expression to *Agrobacterium tumafaciens* strain VA 239

Fig. 4: Symptom expression by resistant F2 individual MYMVR 37 through agroinoculation. (1) Resistant symptom expression to *Agrobacterium tumafaciens* strain VA 221, (2) Uninoculated control and (3) Susceptible symptom expression to *Agrobacterium tumafaciens* strain VA 239

Table 1: List of MYMV field resistant F_2 individuals

S. No.	MYMVR F2 individuals	MYMV score
1	MYMVR 01	1.0-2.0
2	MYMVR 09	1.0-2.0
3	MYMVR 10	1.0-2.0
4	MYMVR 11	1.0-2.0
5	MYMVR 12	1.0-2.0
6	MYMVR 13	1.0-2.0
7	MYMVR 15	1.0-2.0
8	MYMVR 16	1.0-2.0
9	MYMVR 17	1.0-2.0
10	MYMVR 20	1.0-2.0
11	MYMVR 22	1.0-2.0
12	MYMVR 23	1.0-2.0
13	MYMVR 24	1.0-2.0
14	MYMVR 27	1.0-2.0
15	MYMVR 29	1.0-2.0
16	MYMVR 30	1.0-2.0
17	MYMVR 34	1.0-2.0
18	MYMVR 35	1.0-2.0
19	MYMVR 37	1.0-2.0
20	MYMVR 38	1.0-2.0
21	MYMVR 40	1.0-2.0
22	MYMVR 41	1.0-2.0
23	MYMVR 42	1.0-2.0
24	MYMVR 43	1.0-2.0
25	MYMVR 45	1.0-2.0
26	MYMVR 47	1.0-2.0
27	MYMVR 48	1.0-2.0
28	MYMVR 50	1.0-2.0
29	MYMVR 51	1.0-2.0
30	MYMVR 53	1.0-2.0

MYMVR: Mungbean yellow mosaic virus Resistant F2 individual

(39.00%) recorded the lowest average infection in the strains VA 221 and VA 239, respectively (Table 2). These findings are nearly in close conformity with the repots Usharani *et al.* (2005) in agroinoculating mungbean with 71 to 95% of MYMV.

To verify the presence of viral DNA inside the host genome Reverse Transcriptase-PCR confirmatory studies were carried out using oligonucleotide primers, specific to MYMV coat protein gene of DNA A (the expected amplicon size being 632 bp) in all the inoculated samples. cDNA from the total RNA of agroinoculated leaves were used as template for PCR reaction (Fig. 5). These results are in accordance with the reports of Usharani *et al.* (2005) indicating the presence of viral DNA in agroinoculated symptomatic plants and their absence in asymptomatic plants with coat protein specific primers for the DNA A and B components.

The identified resistant individuals namely, MYMVR 10, MYMVR 13, MYMVR 15, MYMVR 17 and MYMVR 37 were similar to their resistant parent, exhibiting the presence of viral coat protein gene for both the strains. This clearly depicts their pure inheritance pattern of the resistance for MYMV. For further confirmation, the individuals which showed resistance in field (F_2 generation) and agroinoculation, were alone forwarded to next generation (F_3) and as per this, the five

Table 2: Average infectivity of Mungbean parents and 30 MYMV resistant F_2 individuals Agroinoculation for MYMV

S. No.	Mungbean parents and MYMV resistant F2 individuals	Agrobacterium tumefaciens strains	No. of days taken for symptom development	Infectivity (%) (Replication 1)	Infectivity (%) (Replication 2)	Average infectivity (%)
1	KMG 189	VA 221	-	0.00	0.00	0.00
		VA 239	23	40.00	45.00	42.50
2	VBN(Gg)2	VA 221	23	100.00	98.00	99.00
		VA 239	24	100.00	95.00	97.50
3	MYMVR 01	VA 221	23	78.00	100.00	89.00
		VA 239	20	85.00	65.00	75.00
4	MYMVR 09	VA 221	24	80.00	58.50	69.25
		VA 239	22	60.00	100.00	80.00
5	MYMVR 10	VA 221	-	0.00	0.00	0.00
		VA 239	23	65.00	60.00	62.50
6	MYMVR 11	VA 221	23	85.60	78.00	81.80
		VA 239	22	94.00	70.00	82.00
7	MYMVR 12	VA 221	22	52.50	100.00	76.25
		VA 239	19	65.00	85.00	75.00
8	MYMVR 13	VA 221	-	0.00	0.00	0.00
		VA 239	20	35.00	43.00	39.00
9	MYMVR 15	VA 221	-	0.00	0.00	0.00
		VA 239	23	33.00	40.00	36.50
10	MYMVR 16	VA 221	20	78.00	68.00	73.00
		VA 239	22	75.00	74.00	74.50
S. No.	MYMV resistant F2 individuals	Agrobacterium tumefaciens Strains	No. of days taken for symptom development	Infectivity (%) (Replication 1)	Infectivity (%) (Replication 2)	Average infectivity (%)
11	MYMVR 17	VA 221	-	0.00	0.00	0.00
		VA 239	22	44.00	39.50	41.75
12	MYMVR 20	VA 221	24	55.50	71.50	63.50
		VA 239	23	65.00	78.00	71.50
13	MYMVR 22	VA 221	18	86.50	90.00	88.25
		VA 239	19	87.00	75.00	81.00
14	MYMVR 23	VA 221	20	88.00	90.00	89.00
		VA 239	20	90.00	98.00	94.00
15	MYMVR 24	VA 221	22	52.50	45.00	48.75
		VA 239	20	65.00	72.50	68.75
16	MYMVR 27	VA 221	24	80.00	75.00	77.50
		VA 239	19	58.00	85.00	71.50
17	MYMVR 29	VA 221	24	98.00	75.60	86.80
		VA 239	23	74.00	85.00	79.50
18	MYMVR 30	VA 221	24	90.00	90.00	90.00
		VA 239	17	45.00	90.00	67.50
19	MYMVR 34	VA 221	25	52.00	65.00	58.50
		VA 239	26	87.00	60.00	73.50
20	MYMVR 35	VA 221	25	80.00	78.00	79.00
		VA 239	24	75.00	74.00	74.5
21	MYMVR 37	VA 221	-	0.00	0.00	0.00
		VA 239	23	70.00	63.50	66.75
22	MYMVR 38	VA 221	24	75.00	93.50	84.25
		VA 239	26	85.00	84.00	84.50

Table 2: Continued

S. No.	MYMV resistant F2 individuals	Agrobacterium tumefaciens Strains	No. of days taken for symptom development	Infectivity (%) (Replication 1)	Infectivity (%) (Replication 2)	Average infectivity (%)
23	MYMVR 40	VA 221	25	78.00	69.00	73.50
		VA 239	20	90.00	78.00	84.00
24	MYMVR 41	VA 221	21	56.00	90.00	73.00
		VA 239	22	65.00	85.00	75.00
25	MYMVR 42	VA 221	20	78.00	96.00	87.00
		VA 239	20	94.00	90.00	92.00
26	MYMVR 43	VA 221	24	90.00	92.00	91.00
		VA 239	24	95.00	90.00	92.50
27	MYMVR 45	VA 221	24	25.00	40.00	32.50
		VA 239	25	56.00	49.50	52.75
28	MYMVR 47	VA 221	26	75.00	85.00	80.00
		VA 239	27	80.00	78.50	79.25
29	MYMVR 48	VA 221	24	65.00	70.00	67.50
		VA 239	23	70.00	85.00	77.50
30	MYMVR 50	VA 221	22	78.00	85.00	81.50
		VA 239	20	70.00	94.00	82.00
31	MYMVR 51	VA 221	25	85.00	90.00	87.50
		VA 239	20	94.00	92.00	93.00
32	MYMVR 53	VA 221	23	79.00	87.00	83.00
		VA 239	26	66.00	92.00	79.00

MYMVR: Mungbean yellow mosaic virus resistant F2 individual

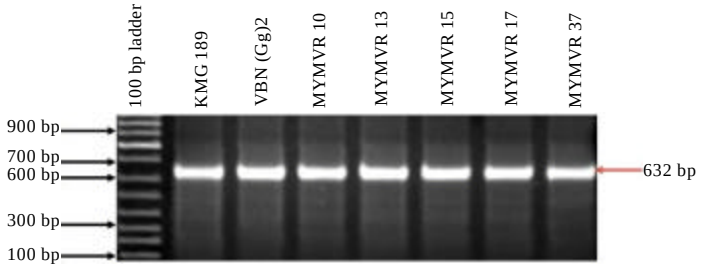

Fig. 5: RT-PCR amplification of MYMV coat protein primer of DNA A in Agroinoculated Mungbean plants *Agrobacterium tumafaciens* strain VA 221

individuals were forwarded to next generation along with five susceptibles. In F_3 generation, MYMV infection can be evaluated by MYMV disease rating scale. Interestingly, the five individuals which showed resistance in field (F_2 generation) and agroinoculation, are found to be resistant in F_3 generation under field conditions also.

CONCLUSION

The results of this study show that the five progenies namely MYMVR 10, MYMVR 13, MYMVR 15, MYMVR 17 and MYMVR 37 are confirmed for resistance in both agroinoculation and field screening. From these five progenies, a MYMV resistant mungbean genotype can probably be developed by adopting future breeding programmes.

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