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IDENTIFICATION OF RAPD AND ISSR MAKERS FOR RESISTANCE AGAINST MUNGBEAN YELLOW MOSAIC VIRUS IN MUNGBEAN (*VIGNA RADIATA* L.) UNDER SOUTH GUJARAT AGRO CLIMATIC CONDITION OF INDIA

R. K. KALARIA[1], DIGVIJAY CHAUHAN[2], MAHESH KUMAR MAHATMA[3] AND LALIT MAHATMA*[4]
[1]Department of Plant Mol. Bio. and Plant Biotech. ,Navsari Agricultural University,Navsari - 395 007
[2]Pulses Research Station, N. M. College of Agriculture, Navsari Agricultural University, Navsari - 396 450 (INDIA)
[3]Directorate of Groundnut Research, ICAR, Junagadh - 362 001 (INDIA)
[4]Department of Plant Pathology, N. M. College of Agriculture,
Navsari Agricultural University, Navsari - 396 450 (INDIA)
e-mail: mahatmalalit@yahoo.co.in

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***Corresponding author**

ABSTRACT

The present study was undertaken to identify genetic variation and tightly linked marker associated with mungbean yellow mosaic virus (MYMV) resistance from different mungbean lines. Out of 200 RAPD markers, OPG-5, OPJ-18 and OPM-20 were proved to be the best markers for the study of polymorphism as it produced 28, 35, 28 amplicons respectively with overall polymorphism was found to be 70.17 %. Out of 17 ISSR markers used, DE-16 proved to be the best marker as it produced 61 amplicons and 15 scorable bands and showed highest polymorphism among all. The combined dendrogram obtained from these RAPD and ISSR indicated two clearly visible clusters where all resistant lines viz., NAUMR1, NAUMR2, NAUMR3 and MEHA clearly separated from susceptible GM4. Here validation of these data could be compare with phenotypic character in the field. Once these markers are identified, they can be used to detect the QTLs linked to MYMV resistance in mungbean breeding programs, as a selection tool in early generations and further use in developing segregating material.

INTRODUCTION

Pulses or legumes are important in our diet and constitute a major source of protein to the vegetarian people. It is the second most important part of human diet after cereals. Pulses belong to the family *Leguminoceae* (Fabaceae) and subfamily *Papilioniceae* (Kannaiyan, 1999). Among the different pulses, mungbean [*Vigna radiata* (L.)Wilczek] which is also known as mungbean, green gram, golden gram, chicksaw pea or Oregon pea is the most common legume and contains about 24 per cent protein of high digestibility and quantity which is about two third of the protein content of soybean, twice that of wheat and thrice that of rice (Thirumaran & Seralathan, 1988; Singh *et al.*, 1988). Yellow Mosaic Disease (YMD) causes by Mungbean Yellow Mosaic Virus (MYMV), a whitefly (*Bemisia tabaci*, Gennadius) transmitted *Begomovirus*, is most severe disease among all the viral diseases. Among other diseases, MYMV has given special attention due to its severity and ability to causes yield losses up to 85 per cent (Shanmugasundaram, 1998). YMD in mungbean was first reported by Nariani (1960) from New Delhi. This disease is caused by different strains and variants of two species of *Begomovirus* viz., Mungbean Yellow Mosaic Virus (MYMV) and Mungbean Yellow Mosaic India Virus (MYMIV) (Fauquet *et al.*, 2008).

Due to limitation of chemical methods and vector control in field, alternate is to the development MYMV resistant varieties by introgression of MYMV resistant genes into agronomic superior cultivars. Further Identification of resistant lines through conventional breeding method is time consuming and required evaluation at 'hot spot' regions. Therefore, the indirect selection using molecular marker like RAPD, ISSR etc. linked to MYMV resistant genes will be helpful in rapid identification of genotypes carrying these genes without screening them for MYMV in field (Selvi *et al.*, 2006; Karthikeyan *et al.*, 2012).

RAPD and ISSR are most preeminent molecular markers that are being used to screen resistant genes and for exploiting genetic polymorphism within and among in various crops including tomato, potato, rice, grapevine and soybean exhibiting relatively low genetic diversity (Prevost and Wilkinson 1999; Pradeep *et al.*, 2002; Kochieva *et al.*, 2002; Souframanien and Gopalkrishna 2004). These markers have been more effectively for tagging disease resistant (Hu *et al.*,

1997; Sartorato *et al.*, 1999; Alzate-Marin *et al.*, 1999) and virus resistant genes (Stevens *et al.*, 1995; Ohmori *et al.*, 1995; Ohmori *et al.*, 1996; Gmitter *et al.*, 1996; Selvi *et al.*, 2006; Karthikeyan *et al.*, 2012; Holeyachi and Savithramma 2013). Studies on disease resistance genes have indicated a high level of polymorphism and the presence of SSRs at certain loci (Yu *et al.*, 1996). Thus it was felt that molecular markers methodology which involves the use of specific primers for amplifications in the polymerase chain reaction (PCR) (Zietkiewicz *et al.*, 1994) would identify polymorphism around the disease resistance locus. Studies on disease resistance genes have indicated a high intensity of polymorphism and the incidence at certain loci (Yu *et al.*, 1996). Once these markers are identified, they can be used to detect the QTLs linked to MYMV resistance in mungbean breeding programs, as a selection tool in early generations and further use in developing segregating material. Considering the potentials of the molecular marker based genetic diversity from mungbean under south Gujarat agro climatic conditions the present investigation has been carried out for identification of potential molecular marker that can differentiate different lines of susceptible and resistant for MYMV resistant gene in *Vigna radiata* and that can be further used for the marker assistant breeding from segregating population for YMV-reaction.

MATERIALS AND METHODS

Mungbean material against MYMV

Vigna radiata cv. GM4 (Gujarat Mung-4), a MYMV-susceptible cultivar, was collected from Pulse Research farm, Navsari Agricultural University. Three MYMV- resistant lines NAUMR1, NAUMR2, NAUMR3 were screened from large population of the susceptible cultivar GM4.Here MEHA variety taken as check (Singh *et al.*, 1988, Paul *et al.*, 2013). The test materials were screened in field and leaves were harvested after infection from one month old seedlings.

Isolation of DNA

Genomic DNA was isolated from fresh leaves using CTAB with modified method of Karuppanapandiyan *et al.*, 2006. The pallet was collected dried at room temperature, 50 µl TE buffer and incubated over night at 4^{0}C. DNA was quantified using Nano spectrophotometer and diluted to 50ng/µl for PCR amplification.

Screening of RAPD and ISSR primers

To detect polymorphism between the MYMV-susceptible cv. GM4, resistant lines NAUMR1, NAUMR2, NAUMR3 and MEHA, 200 RAPD (Operon) primers including OP-(E,F,G,H,I,J,K,L,M,N) series and 17 ISSR (Operon) primers including DE-(1to17), were used in PCR (Kanazin *et al.*, 1996). Amplification was carried out in 50μl reaction mixture containing 50ng of genomic DNA, 100ng of each primer, 1X PCR buffer containing 1.5mM $MgCl_2$, 25mM of each dNTPs and 1unit of Taq DNA polymerase (Bangalore Genei, India), using a Eppendorf thermocycler. The following cycling programme was used: a first cycle of 5min at 94°C, followed by 35 cycles of 45s at 94°C, 45s at the annealing temperature, followed by 1min at 72°C, and a final extension of 10 min at 72°C. The annealing temperature for RAPD primers was in range of 35-38 °C and for ISSR primers 50-65 °C. PCR products were electrophoreses on 1.5% (w/v) agarose gels, in 0.5X TBE Buffer at 80V for 1 hr and then stained with ethidium bromide (0.5μg/ml). Gels with amplified fragments were visualized and photographed under UV light.

Reproducibility of Amplification Patterns.

DNA amplifications with each ISSR and RAPD primers were repeated at least thrice to ensure reproducibility. The bands were considered reproducible and scorable only after observing and comparing them in three separate amplifications for each primer. Clear and intense bands were scored while faint bands against background smear were not considered for the further analysis.

Data Analysis

For each sample, each band that was amplified using RAPD and ISSR primers was treated as unit character. Unequivocally scorable and consistently reproducible amplified DNA fragments were transformed into binary character matrices (1 for presence, 0 for absence). These data were then used to construct dendrogram for cluster analysis based on the distance matrix using Jaccard's (Jaccard, 1908) coefficients. Data analysis was done using NTSYSpc software 2.02 (Rohlf, 1998). Using distance matrix a principal coordinate analysis to construct a three-dimensional array of eigenvectors was performed using DCENTER module of NTSYS 2.2 program.

RESULTS AND DISCUSSION

Many commercial mungbean varieties are susceptible to MYMV and there is a need to identify tightly linked markers that could assist the transfer of the resistant genes to superior cultivars using marker-assisted breeding. The exploit of molecular markers for resistance genes is predominantly potent as it save time in breeding programmes associated with the phenotypic analysis. Inconsistencies in scoring infection phenotypes have significantly delayed the introgression of MYMV into elite genotypes.

Molecular markers are extremely used for the diversity analysis, generation of genetic map and subsequent development of resistant varieties since1980s (Singh, 2009). Molecular markers, revealing polymorphism at the DNA level, has been playing an increasing part in mungbean for screening genotypes against MYMV. In this work, the utility of RAPD and ISSR markers for screening of mungbean lines for MYMV resistant was carried out. Four resistant germplasm/lines identified resistant in the field were found morphological distinct from each other. Validation of the phenotypic resistant and distinctness found necessary to check at genetic level. The another question raised was to find whether all the four germplasm have different resistant gene or the same gene pool might have been involved in the imparting resistant reaction. Thus, Different molecular markers linked to MYMV resistance gene in mungbean could be an effective approach to expedite the breeding programme and overcome the inaccuracies associated with the phenotypic selection against YMD.

Polymorphism in mungbean lines using RAPD and ISSR markers

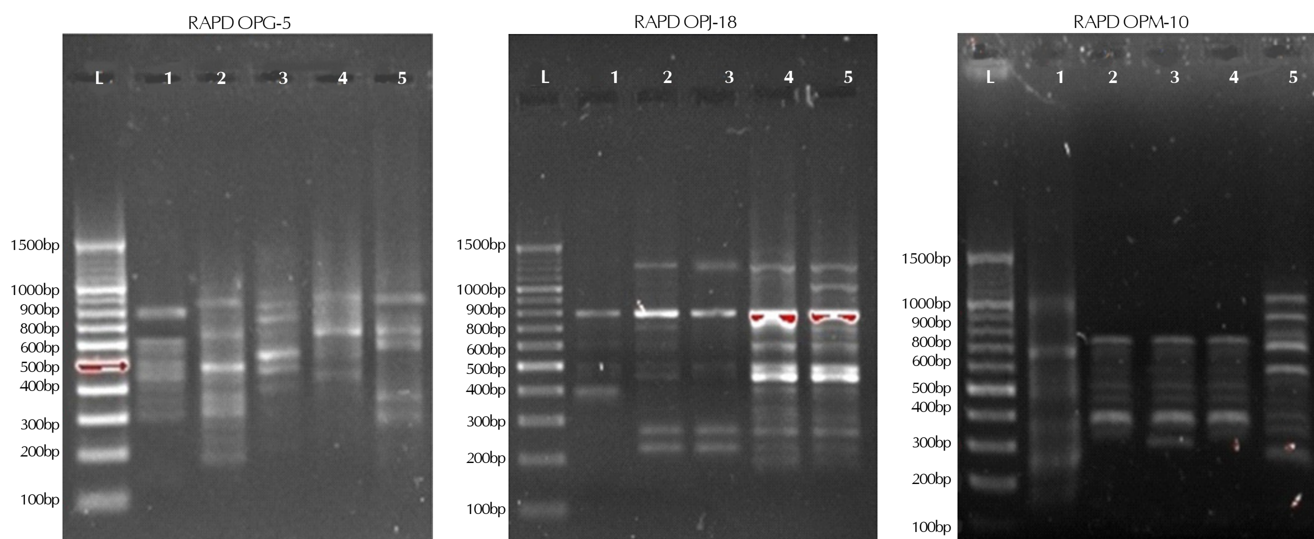
Out of 200 RAPD markers used in the present investigation,

Table 1: Amplification obtained with different RAPD markers

Sr.No	RAPD markers	Marker sequence (5'-3')	Total no. of Bands(a)	Total no. of polymorphic band (b)	%polymorphism %((b/a) × 100)
1	OPE-18	GGACTGCAGA	8	4	50.00
2	OPF-13	GGCTGCAGAA	7	6	85.71
3	OPF-14	TGCTGCAGGT	10	5	50.00
4	OPG-5	CTGAGACGGA	10	9	90.00
5	OPH-9	TGTAGCTGGG	7	2	28.57
6	OPH-13	GACGCCACAC	11	8	72.72
7	OPH-15	AATGGCGCAG	11	9	81.81
8	OPH-16	TCTCAGCTGG	9	6	66.66
9	OPI-12	AGAGGGCACA	8	5	62.50
10	OPI-13	CTGGGGCTGA	10	7	70.00
11	OPI-14	TGACGGCGGT	9	7	77.77
12	OPJ-18	TGGTCGCAGA	10	9	90.00
13	OPM-20	AGGTCTTGGG	10	9	90.00
14	OPN-16	AAGCGACCTG	6	4	66.66
	Total		126	90	70.17

Table 2: Details of amplification obtained with different ISSR markers

Sr. No.	ISSR marker	Sequence(5'-3')	Total no. of Bands(a)	Total no. of polymorphic band(b)	%Polymorphism %(b/a × 100)
1	DE-3	AGAGAGAGAGAGAGAGT	15	4	26.66
2	DE4	GAGAGAGAGAGAGAGAT	13	7	53.84
3	DE-5	CACACACACACACACAT	13	2	15.38
4	DE-16	GACAGACAGACAGACA	15	11	73.33
	Total		56	24	42.30



L-100bp Ladder, 1-GM4, 2-NAUMR1, 3-NAUMR2, 4-NAUMR3, 5-MEHA

Figure 1: Testing of different RAPD markers on different mungbean germplasm

Table 3: Jaccard's similarity coefficient between different mungbean lines based on RAPD and ISSR data

	GM4	NAUMR1	NAUMR2	NAUMR3	MEHA
GM4	1.000				
NAUMR1	0.494	1.000			
NAUMR2	0.515	0.790	1.000		
NAUMR3	0.489	0.794	0.840	1.000	
MEHA	0.509	0.761	0.774	0.822	1.000

only fourteen markers viz., OPE-18, OPF-13, OPF-14, OPG-5, OPH-9, OPH-13, OPH15, OPH16, OPI-12, OPI-13, OPI-14, OPJ-18, OPM-20, OPN-16 could clearly amplified genome of different mungbean germplasm, whereas 186 markers failed to show any amplification. All 14 markers showed 126 bands out of which 90 were polymorphic band with variable degree of polymorphism (Table1). Overall polymorphism was found to be 70.17 per cent. Among the entire marker tested, OPG-5, OPJ-18 and OPM-20 were proved to be the best markers for

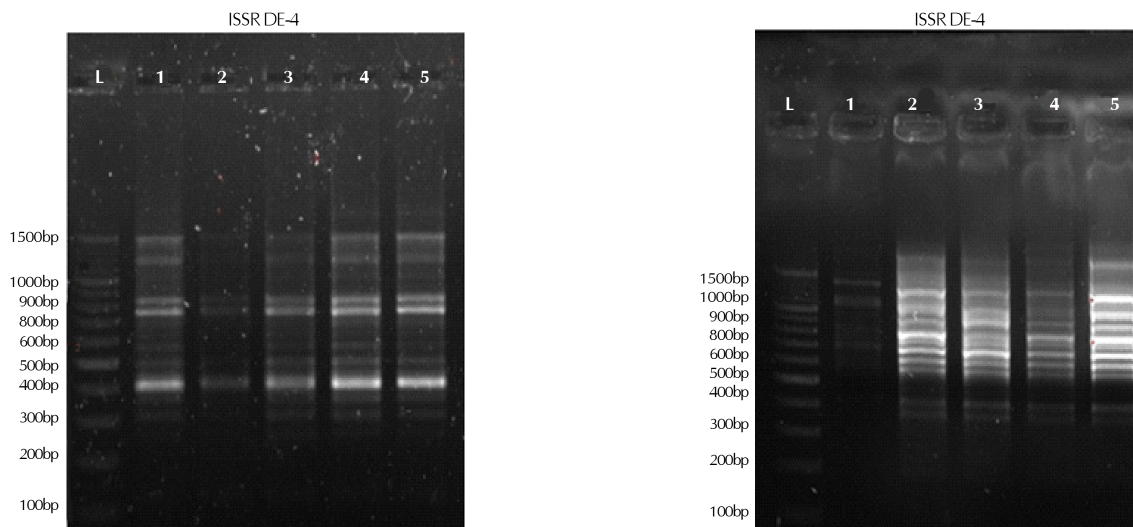


Figure 2: Testing of different ISSR markers on different mungbean germplasm

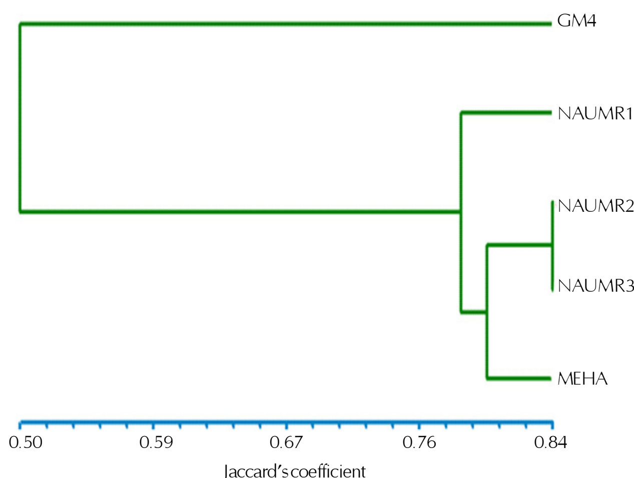


Figure 3: Combine dendogram generated by using RAPD and ISSR data of mungbean germplasm

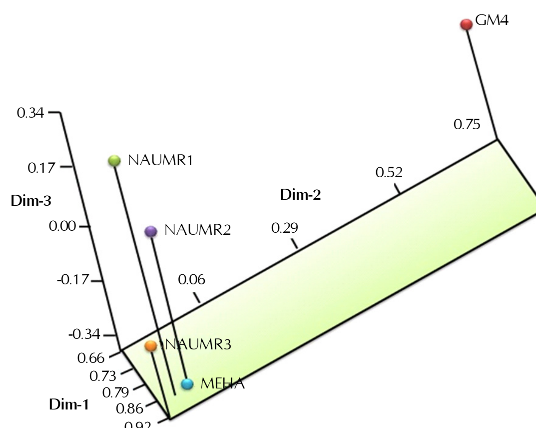


Figure 4: Genetic relationship among different mungbean lines (GM4, NAUMR1, NAUMR2, NAUMR3 and MEHA) based on Principle Coordinate Analysis

the study of polymorphism as it produced 28, 35 and 28 amplicons respectively (Fig 1). Average 10 scorable bands were observed in all markers, out of which 9 bands were polymorphic. Average polymorphism was 70.17 per cent (Table1). Agarose gel electrophoresis pattern obtained using the marker OPG-5 showed the presence of 10 scorable bands in the size ranged from ~200bp to ~850bp. In all mungbean lines exhibited one common band as monomorphic of size ~600bp. Nine polymorphic bands were of approximately 200bp, 250bp, 400bp, 450bp, 500bp, 550bp, 650bp, 800bp and 850bp size. A band of size ~850bp was absent in susceptible cultivar GM4 while present in all the resistant lines. In the marker OPJ-18, total numbers of 10 scorable bands in the size range from ~250bp to ~1250bp were observed. Out of which 9 bands were of approximately 250bp, 300bp, 350bp, 450bp, 500bp, 600bp, 750bp, 1000bp and 1250bp size were polymorphic and one band of approx 800bp was monomorphic. Total 6 unique band of approximately 250bp, 300bp, 450bp, 500bp, 850bp and 1250bp size were present in all resistant lines while absent in susceptible one. Similar attempts to find RAPD markers linked MYMV resistance were made by several researchers (Lambrides et al 2000; Selvi *et al.*, 2006; Karthikeyan *et al.*, 2012; Holeyachi and Savithramma, 2013). RAPD markers can give clear picture of genetic variability, however, are least reproducible than the other marker. Therefore, reconfirmation of genetic makeup was done by using ISSR markers.

Out of 17 ISSR marker used, amplification could be obtained with 4 markers viz., DE-3, DE-4, DE-5 and DE-16 only. All the 4 markers showed 56 bands out of which 24 were polymorphic variable degree of polymorphism ranging from 15.84 per cent (DE-5) to 73.33 per cent (DE-16). Overall polymorphism was found to be 42.30 per cent (Table 2). The maximum 73 amplicons were produced by the marker DE-3

followed by 61 amplicons in DE-16 and Minimum 55 amplicons were produced by the marker DE-4. Among the entire marker tested, DE-16 proved to be the best marker as it produced 61 amplicons and 15 scorable bands (Fig.2). Four bands of approximately 400bp, 750bp, 800bp and 1500bp were monomorphic. Polymorphic bands were 360bp, 550bp, 600bp, 650bp, 700bp, 900bp, 1000bp, 1100bp, 1300bp size. Parts from these two bands of more than 1500bp size were observed however, could not be scaled as the ladder had maximum 1500bp only. Average polymorphism was 73.33 per cent. Marker DE-4 produced 13 scorable bands of which 7 bands were polymorphic of approximately 250bp, 300bp, 350bp, 500bp, 600bp, 1000bp size and one band of size >1500bp contributing 53.84 per cent polymorphism(Fig. 2). Whereas 6 bands were monomorphic of approx 400bp, 550bp, 750bp, 850bp, 1200bp and 1500bp size. Unique band of size 600bp was present in all resistant line while absent in susceptible one. ISSR markers are considered more reliable by virtue of their primer sequence. The results showed that genetic variation through RAPD and ISSR in different mungbean line for MYMV can be used for evaluating the molecular variation.

Combined dendrogram obtained using RAPD and ISSR data

Amplification data obtained from RAPD and ISSR markers were used for obtaining combined similarity matrix (Table 3) and dendrogram (Fig.3) of RAPD and ISSR. Data suggested that Variation in the amplification and banding pattern in all the four resistant (NAUMR1, NAUMR2, NAUMR3 and MEHA) and one susceptible line GM4 clearly indicated the genotypic variability in all the five lines.

Principle Coordinate analysis based on RAPD and ISSR data

Principle components were derived for each line using Eigen vectors and Eigen values extracted from correlation matrix among markers those were obtained from standardized data matrix (Fig.4). Only polymorphic bands were used to construct a binary matrix. PCA also indicate that data were in accordance with the data obtained from the dendrogram analysis by the Jaccard's similarity coefficient. The data were in accordance with the data obtained from the dendrogram analysis by the Jaccard's similarity coefficient. These RAPD and ISSR markers can be used in screening mungbean genotypes for resistance to MYMV disease.

In the present study, markers linked to the resistance gene has been identified and more prominently validated using diverse mungbean genotypes. This linked marker will boost the efficiency and precision of MYMV resistance breeding in mungbean. Work on identifying more Potential markers around the resistance locus is in progress and would assist in isolating the MYMV resistance gene and characterizing the mechanism of resistance at a genetic level. It would also be interesting to study whether these markers are linked to the resistance gene in blackgram which is a close relative of mungbean.

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