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NBS-LLR Marker Assisted Screening of Resistance Genotypes for Mungbean Yellow Mosaic Virus (MYMV) in Mungbean (*Vigna radiata* (L.) Wilcezk) Genotypes

N. Jyothi*, B. R. Patil, Ramesh Bhat and B. M. Lokesh Kumar

Department of Genetics and plant breeding, University of agriculture science, Dharwad, Karnataka, India
**Corresponding author*

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A B S T R A C T

Mungbean is one of the most important pulse crops which is native to India. The yield of mungbean has been stagnant over years. Improvement in yield of mungbean is becoming difficult mainly due to the occurrence of pest and diseases. Among the various diseases Mungbean yellow mosaic virus (MYMV), which is a Begomo virus transmitted through white fly, *Bemesia tabaci*, causing significant yield losses in mungbean, leading to a yield loss. With this aspect the present study was carried to identify the resistance source employing NBS-LLR markers. A total of fifty-five NBS-LLR markers was screened in fifty-four genotypes of mungbean. Out of fifty-five markers seven polymorphic viz. XLRR, RGA-1TG, MTB-99, S1, CLRR-INV1, ptokiniIN and VURS02F16V markers were identified these markers were specifically linked with MYMV disease and also twenty-one resistance genotypes were observed these resistance lines can be further used for resistance breeding programme.

Introduction

Mungbean (*Vigna radiata* (L.) Wilcezk) is also known as green gram, is one of the important pulse crop India. It belongs to the family Leguminoceae having the chromosome number 2n=22, is warm season annual and self-pollinated crop. Currently, the global annual growing and production is about 6 million hectares worldwide and global 3 million tonnes, respectively. India leads the production of mungbean worldwide followed by China and Myanmar Nair *et al.*, (2014). In India, mungbean is grown on an area of about 3 million hectares with the production of about 1 million tonnes. The major mungbean grown states are Orissa, Maharashtra, Andhra Pradesh, Telangana, Rajasthan, Madhya Pradesh, Bihar, Karnataka, and Uttar Pradesh. It is an excellent and inexpensive source of vegetable protein and ranks high among the different pulse crops, grown in India. Mungbean contains about 23.9% protein; rich in lysine which is generally low or deficient in cereals. Mature seeds are rich in proteins, and cooked seeds form a valuable constituent of diet of considerable number of people in country.

The tender pods of mungbean are also eaten as vegetable. The ripe seeds serve as a source of pulse which is an important constituent of diet in Indian subcontinent.

Several fungal and viral diseases are reported which caused severe reduction inmungbean and urdbean yield Paul *et al.,* (2013). The mungbean yellow mosaic virus (MYMV) is the most devastating, especially in South Asian countries. MYMV can cause yield loss of about 75–100 per cent depending on disease incidence, virus strains, mungbean genotypes and interaction between these factors (Singh, 1980). MYMV is caused by different species of Begomovirus (family Geminiviridae these viruses are transmitted by whitefly (*Bemisiatabaci*).

The management of MYMV is focused mainly on whitefly control. However pesticides can provide temporary management of whiteflies, but do not give effective control of MYMV. A more efficient and environmentally safe long-term solution is the development of mungbean cultivars resistant to both virus and its vector *Bemisia tabaci*. Therefore, using resistant varieties is the most desirable means of managing the disease. For better identification of desired genotype in the breeding programme, there is a need to identify DNA markers linked to mungbean yellow mosaic virus (MYMV) in mungbean. Among the various molecular markers that are being used, the use of Resistant Gene Analogues and Resistant Gene Homologues is very efficient as they originate from the NBS-LRR disease resistant motifs and they can be conveniently designed from diagnostic motifs of known disease resistant genes (Kanazin *et al.,* 1996; Huang and Gill 2001; Yan *et al.,* 2003). Resistant genes (R-Genes) are genes in plant genomes that convey plant disease resistant against pathogens by producing R proteins. These large, abundant proteins are involved in the detection of diverse pathogens, including bacteria, viruses, fungi, nematodes, insects and oomycetes. With this background knowledge, the main aim of the present study was carried out to identify the resistance source for the resistance breeding programme.

Materials and Methods

Plant material

A total of fifty four mungbean (Table 1), used in this study were raised in Department of Genetics and plant breeding, University of agriculture science. Dharwad.

DNA extraction

All fifty-five genotypes of mungbean were sown in sowing trays which contained a mixture of coirepith and sand. When the plants were at two leaf stage, the DNA was isolated using a modified Saghai and Maro of (1984) CTAB method. Grind tissue with liquid nitrogen in a micro centrifuge tube. When liquid nitrogen has all sublimed away, add hot (65 o C) CTAB buffer (the volume of the buffer added should be approximately equal to the volume of the tissue. The mixture should now resemble thick, slimy soap) then incubated it for 15-20 min at 65 oC followed by cooling the tubes at room temperature. Then Centrifuge the tubes at 13,000 rpm for 15 min. Take out the supernatant add equal volume of Chloroform and Iso amyl alcohol (24:1) with gentle mixing. Again centrifuge the tubes at 13,000 rpm for 15 min. repeat the steps of CI treatment twice. Take supernatant and add twice amount of Iso Propanol. Keep for overnight incubation at -20 o C. After 24hthe tubes were centrifuged at 10,000 rpm for 10 min at cool condition. After the centrifuge supernatant was discarded followed by ethanol wash to pellet at 8000 rpm for 5 min and repeat the same procedure 2-3 times. Finally pellet were draied the at

room temperature for 2-3 hrs then dissolve the pellet in T 10 E 1 (based on the pellet obtained) Store at -20 o C. the DNA was purified from RNA by treating with RNAse (Bangalore Genei). The quality of DNA was assessed by taking Nano drop readings and concentration of the DNA was adjusted.

NBS-LRR assay

A standard PCR reaction were performed in 20.00 µl volume containing 2.0µl of 15ng of template DNA, 3 units/µl of Taq DNA polymerase (Bangalore Genei Ltd., Bangaluru, Karnataka, India) 2.00µl of 10X of assay buffer, 2.5 mM of 2 µldNTPs, 1 µl of 0.5 µM each of forward and reverse primers was performed in thermocycler (Eppendoff) with the following PCR conditions: DNA denaturation at 95 °C for 74min,35 cycles of 95 °C for 1min, primer annealing at 50-58 °C for 30s and a primer extension step of 72 °C for 1min and final elongation step was extended to 72 °C for 7 min. Further the PCR products (20 µl) were subjected to Electrophoresis on 3 per cent Agarose gel in 1X TAE buffer for 3 hours at 50 volts. A 100 bp ladder (Bangalore Genei) was used as a known standard size marker. The electronic image of ethidium bromide stained gels was captured using UVITEC Cambridge Doc.

Recording of the observations

Presence of a band was marked as "+" and absence of band was marked as "–". The allele sizes were determined by comparing with the 100 bp marker.

Statistical method of analysis

Allelic variation was calculated from the frequencies of genotypes at each locus as the polymorphic information content. Genetic parameters namely frequency of the abundant allele, genotype frequency and polymorphic information content (PIC) were estimated using the software program Power Marker version 3.25 (Liu and Muse, 2005).

Results and Discussion

PCR amplification

PCR approach was chosen as a first step in the identification of putative resistant genes in mungbean. A set of fifty-five Resistant Gene Homologous (RGHs) from cowpea (*Vigna unguiculata*), chickpea (*Ciceraritinum*), *Medicago truncatula* and were screened across fifty-five genotypes of green gram. Increasing the stringency of PCR condition by adopting higher annealing temperatures and "touch-down" protocols were not successful in obtaining specific amplicon, hence it was necessary to optimize the PCR conditions. The optimum annealing temperature, determined after testing the temperatures through gradient PCR approach, ranged between 43°C and 53°C (Table 3). Majority of primers which belonged to Vu series derived from NBS-LRR disease resistant motifs, generated amplification at 47°C and above, while the remaining primers produced amplification between 43°C and 49°C (Fig. 1).

Study NBS-LRR markers and study the polymorphism in the black gram and green gram genotypes

Specificity to resistance among a total of 54 genotypes of green gram was examined with 55 markers. Only 32 markers out of 55 amplified successfully in all the fifty four genotypes with twenty markers producing no amplification. Out of thirty two markers which produced amplification only seven markers generated polymorphism, remaining twenty-five were monomorphic. The seven polymorphic markers collectively yielded 15 alleles in green gram with an average of 2.1

polymorphic alleles per locus respectively. The characterization of these 15 polymorphic markers is provided in Table 2. Markers XLRR, Pto kin1, S1-INV, S1 and Pto-kin1IN produced three alleles while reaming markers produced two alleles each.

Table.1 Details of Mungbean/ green gram genotypes used in the study

Genotypes	Agro climatic zones/situation	Genotypes	Agro climatic zones/situation
VGG4	Karnataka	PM5	Madhya Pradesh
PS 16	Karnataka, Assam, Delhi, Bihar,	HUM1	Karnataka, Gujarat, Madhya Pradesh, Tamilnadu,
K851	Andhra Pradesh, Himachal Pradesh, Rajasthan	AKM8803	Maharashtra
PB1	Karnataka, Maharashtra, Tamil nadu	KKM4	Karnataka
TAP7	Maharashtra	KM15	Karnataka cultivars
TARM2	Maharashtra (ikisan)	KM16	Karnataka cultivars
SML668	Himachal Pradesh, Punjab, Haryana, Western Uttar Pradesh.	KM 28	Karnataka cultivars
PSA9591	Haryana, Maharashtra	KM 30	Karnataka cultivars
PM1	North western zone, Haryana. (ikisan)	KM31	Karnataka cultivars
HUM12	North eastern plains	KM 39	Karnataka cultivars
SML348	Punjab, Haryana, Western Uttar Pradesh.	KM 45	Karnataka cultivars
LM182	Haryana	KM 46	Karnataka cultivars
KKM3	Punjab, Haryana, Western Uttar Pradesh.	KM 47	Karnataka cultivars
MAVT836	Haryana, Maharashtra	KM 48	Karnataka cultivars
NP36	Maharashtra	KM 51	Karnataka cultivars
MDV3156	Punjab	KM 59	Karnataka cultivars
PM103	Orissa, Andhra Pradesh, Assam, Karnataka	KM 60	Karnataka cultivars
M108	Punjab, Haryana, Western Uttar Pradesh.	KM 49	Karnataka cultivars
NDM1	Gujrat, Madhya Pradesh, Tamilnadu, Maharashtra. (ikisan)	KM 70	Karnataka cultivars
PM2	Orissa, Andhra Pradesh, Assam	KM 78	Karnataka cultivars
PRATHAP	Orissa, Andhra Pradesh, Assam, Karnataka	KM 4	Karnataka cultivars
SML134	Punjab	KM 3	Karnataka cultivars
PUSA9072	Maharashtra	KM 10	Karnataka cultivars
PDM11	Uttar Pradesh	MDV31	
KAPORGAON	Orissa, Andhra Pradesh, Assam, Karnataka	KKM3	Karnataka
PUSA9531	Bihar, Gujarat.	PRATHAP	Andhra Pradesh, Assam, Karnataka, Odissa
PUSA VISHAL	Uttar Pradesh, Madhya Pradesh.	PDM11	Andhra Pradesh, Assam, Karnataka

Table.2 List of RGA primers used in the study

Sl. No	Name	Forward sequence	Tm value
1	XLRR-F	CCGTTGGACAGGAAGGAG	47°C
	XLRR-R	CCCATAGACCGGACTGTT	
2	CLRR-F	TTTTCGTGTTCAACGACG	43°C
	CLRR-R	TAACGTCTATCGACTTCT	
3	RLRR-F	CGCAACCACTAGAGTAAC	47°C
	RLRR-R	ACACTGGTCCATGAGGTT	
4	NLRR-F	TAGGGCCTCTTGCATCGT	43°C
	NLRR-R	TATAAAAAGTGCCGGACT	
5	NPLOOP-F	TCAATTAATGTTTGAGTTATTGTA	43°C
	Nkin2-R	GTAACTAAGGATAGA	
6	Pto kin1-F	GCATTGGAACAAGGTGAA	43°C
	Pto kin2-R	AGGGGGACCACCACGTAG	
7	Pto kin3-F	TAGTTCGGACGTTTACAT	44°C
	Pto kin4-R	AGTGTCTTGTAGGGTATC	
8	NBS-F	GGAATGGGNGGNGTNGGNAARAC	44°C
	NBS-R	YCTAGTTGTRAYDATDAYYYTRC	
9	RLK-F	GAYGTNAARCCIGARAA	46°C
	RLK-R	TCYGGYGCRATRTANCCNGGITGICC	
10	S1-F	GGTGGGGTTGGGAAGACAACG	46°C
	AS1-R	CAACGCTAGTGGCAATCC	
11	S2-F	GGIGGIGTIGGIAAIACIAC	46°C
	AS3-R	IAGIGCIAGIGGIAGICC	
12	PtoFen-S-F	ATGGGAAGCAAGTATTCAAGGC	45°C
	PtoFen-AS-R	TTGGCACAAAATTCTCATCAAGC	
13	XLRR-INV1-F	TTGTCAGGCCAGATACCC	47°C
	XLRR-INV2-R	GAGGAAGGACAGGTTGCC	
14	CLRR-INV1-F	GCAGCAACTTGTGC	47°C
	CLRR-INV2-R	TCTTCAGCTATCTGC	
15	NLRR-INV1-F	TGCTACGTTCTCCGGG	45°C
	NLRR-INV2-R	TCAGGCCGTGAAAAATAT	
16	Pto-kin1IN-F	AAGTGGAACAAGGTTACG	45°C
	Pto-kin2IN-R	GATGCACCACCAGGGGG	
17	S1-INV-F	GCAACAGAAGGGTTGGGGTGG	45°C
	AS1-INV-R	CCTAACGGTGATCGCAAC	45°C
18	S2-INV-F	CAICAIAAIGGITGIGGIGG	
	AS3-INV-R	CCIGAIGGIGAICGIG	
19	wlrk-S-F	GAAAGATGAGTAAATTACTTG	45°C
	wlrk-AS-R	TGAGGGTCAGGCATGCAG	

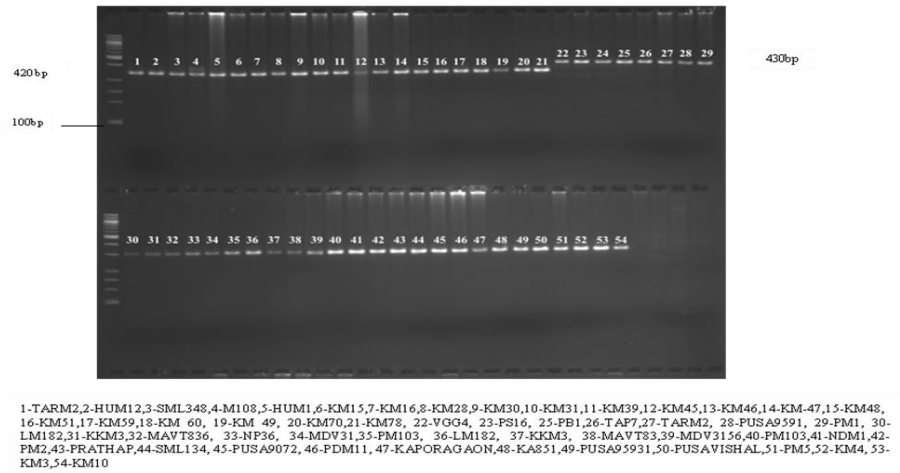
20	**Cre3Ploop-F**	**GCGGGTCTGGGAAATCTACC**	**46°C**
	Cre3-k3-R	CTGCAGTAAGCAAAGCAACG-	
21	Cre3LR-F	CACACACTCGTCAGTCTGCC	45°C
	Cre3LR-R	CAGGAGCCAAAAATACGTAAG	
22	Xa1NBS-F	GGCAATGGAGGGATAGG	46°C
	Xa1NBS-R	CTCTGTATACGAGTTGTC	
23	Xa1LR-F	CTCACTCTCCTGAGAAAATTAC	44°C
	Xa1LR-R	GAGATTGCCAAGCAATTGC	
24	RGA-1-F-CGa	AGTTTATAATTCGATTGC	49°C
	RGA-1-R	ACTACGATTCAAGACGTCCT	
25	RGA-1-F-CGb	AGTTTATAATTCGATTGCT	49°C
	RGA-1-R	ACTACGATTCAAGACGTCCT	
26	RGA-1-F-TG	AGTTTATAATTTGATTGCT	49°C
	RGA-1-R	ACTACGATTCAAGACGTCCT	
27	MtB331-F	GGCTTCCTGATGCTGGTTAG	48°C
	MtB331-R	ACAAGCAGGTTGGACACACA	
28	MtB99-F	CTTGGCAAAATGTCAACTCT	47°C
	MtB99-R	GGAAAGGGGTTAGGTGAGTA	
29	h2_119h6a-F	CGCACGAGTTGGATATGATG	48°C
	h2_13m22a-R	CGTCGCACGAGTTTACTGAT	
30	h2_13m22a-F	TCAAACTCAAGCCACCACAA	47°C
	h2_13m22a-R	GCTCGAGTCATGGAGGGTAA	
31	VuRS01N7V	GAACGGTGAAGATTGGAATTTG	49°C
		CTATGACCGAGTGTTGCATGAT	
32	VuRS01J7V	TCTCCAAAACCAGAGAGTTGC	48°C
		GGGCAGAATTGGAAATTTGA	
33	VuRS01N9V	GCCAATTCAGCACAAGGTTT	48°C
		TAGGTGGAGGATGTGCATTG	
34	VuRS02A2R	CCAGCGTAGTGATGTTCTTGAG	46°C
		GCAACCCTTGATAGCTTATGGA	
35	VuRS02F16V	CCAATGCCTTGAGGATTAAAA	51°C
		CGGTCTAAGTCGGTCATGAAG	
36	VuRS01A03R	TGAGCAATCTTTCCCCAATC	53°C
		CCACGCTCTCTCACCTCTCT	
37	VuRS01G15R	GCAGTGCACTCCAATTCCTA	51°C
		CGCCATTAAGCATAGCACAC	
38	VuRS01G18V	GGATGACAACGAGGCTTTATTC	45°C
		TACTGGAGTGGACAGAGTGTGG	
39	VuRS01G04R	CTGTTCTGCTTGTTGGTTTTCA	47°C
		AGGTTTGTTGATCGTCAGGAAG	

40	**VuRS01J11R**	**TCCGTGATTTTACGCCTTTC**	**47°C**
		AAAATAGGTTTAATTGGAACGGACT	
41	VuRS01K16R	TACCCACGGGTACGGGTATT	47°C
		TGAAGAATGAAGACAGAAGACAAGA	
42	VuRS01L21R	TGAGTTAGCCGTTTTGGGTA	47°C
		GCCTGGAATTGCAAAAATGT	
43	VuRS02H07V	CTCTTCGTTATCCCCTCTGCTA	47°C
		CAGGTCTCTGGTGCTCTACCTT	
44	VuRS02L24R	TGGCGAAATTGTACTAAGCAAG	50°C
		AAGGGGTTTAGAAAAGAGGGTG	
45	VuRS03A23V	CAGGCATGCAAGCTTCTCTT	47°C
		CAATTCTAGCCGGGTAAAGG	
46	VuRS03G17V	CAGAATACACGAAACGAAAGTG	47°C
		CCGATTGGACAGTTTAAGAAGA	
47	VuRS03O19V	TACGTGTGAAAATTGCTTGACC	51°C
		TAGAATTGGGAGATTTGGAACG	
48	VuRS01P18V	TTTGGATTCTTTCCCGTGTT	47°C
		ATTCTTGGCTGACTCGCAAT	
49	VuRS01P23V	TTTATGTTTTGAGAATCATTGCAG	47°C
		CGTGGATTTTGAAACCTCCATA	
50	VuRS02B24R	GTGGCTTGATGAGGATATGAAA	47°C
		GGAGAAGTGTATTTGTTGTTGAGAG	
51	VuRS02F19V	GGTGGAACAGACGACATGAA	47°C
		GTGATCTCTTTGGCCCATGT	
52	VuRS02H06R	AGTCAACAAGGGAAAGCAAGAG	48°C
		CCATGAGTCTGTGAGTTTGCAT	
53	VuRS02M19V	GAACCCACAACCCTGAAATG	48°C
		TGAGAGGACTTGGGTTCGAG	
54	VuRS02M22R	CACAAGCCCTAGCACTCCTC	48°C
		CTAGTTTTGCCCCCTGTTTG	
55	RGA1-F	GGNTGNATNGGNTANGANCAN	48°C
	RGA1-R	GANCTNTGNAANGANATNAAN	

Table.3 Number of alleles, allele frequency, gene diversity, polymorphic information content for fifty four genotypes of green gram

Marker	Frequency of abundant Allele	Sample Size	Allele No	Gene Diversity	PIC
XLRR	0.596	54	3	0.493	0.463
Pto kin1	1	54	1	0	0
NBS	1	54	1	0	0
S2	1	54	1	0	0
S1-INV	0.5	54	3	0.664	0.61
Wlrk-S	1	54	1	0	0
Cre3Ploop	1	54	1	0	0
Xa1LR	1	54	1	0	0
RGA-1CGa	1	54	1	0	0
RGA-1CGb	1	54	1	0	0
RGA-1TG	0.592	54	2	0.4753	0.36
MtB331	1	54	1	0	0
MtB99	0.59	54	2	0.482	0.366
h2119h6a	1	54	1	0	0
h213m22a	1	54	1	0	0
S1	0.518	54	3	0.55	0.46
CLRR-INV1	1	54	1	0	0
Pto-kin1IN	0.49	54	3	0.59	0.51
VuRS01N7V	1	54	1	0	0
VuRS01N9V	1	54	1	0	0
VuRS02A2R	1	54	1	0	0
VuRS01A03R	1	54	1	0	0
VuRS01G04R	1	54	1	0	0
VuRS01L21R	1	54	1	0	0
VuRS02H07V	1	54	1	0	0
VuRS03A23V	1	54	1	0	0
VuRS03G17V	1	54	1	0	0
VuRS03O19V	1	54	1	0	0
VuRS02H06R	1	54	1	0	0
VuRS02F16V	0.53	54	3	0.614	0.54
VuRS02L24R	1	54	1	0	0
VuRS02B24R	1	54	1	0	0

Fig.1 PCR amplification generated by MTB99 marker in mungbean in 3% agarose gel electrophoresis



1-TARM2,2-HUM12,3-SML348,4-M108,5-HUM1,6-KM15,7-KM16,8-KM28,9-KM30,10-KM31,11-KM39,12-KM45,13-KM46,14-KM-47,15-KM48, 16-KM51,17-KM59,18-KM 60, 19-KM 49, 20-KM70,21-KM78, 22-VGG4, 23-PS16, 25-PB1,26-TAP7,27-TARM2, 28-PUSA9591, 29-PM1, 30-LM182,31-KKM3,32-MAVT836, 33-NP36, 34-MDV31,35-PM103, 36-LM182, 37-KKM3, 38-MAVT83,39-MDV3156,40-PM103,41-NDM1,42-PM2,43-PRATHAP,44-SML134, 45-PUSA9072, 46-PDM11, 47-KAPORAGAON,48-KA851,49-PUSA95931,50-PUSAVISHAL,51-PM5,52-KM4, 53-KM3,54-KM10

Out of 32 markers seven markers generated polymorphic amplicones and 25 markers generated monomorphic amplicons. Total of 40 alleles were generated with an average of 2 alleles per markers for the genotypes evaluated. Majority of markers amplified one allele per marker. Number of alleles ranged from 1 to 3. The allele frequency was least for Pto-kin1IN (0.49).The Polymorphic Information Content (PIC) (Table 3) of individual loci ranged from 0.00 to 0.61 with a mean value of 0.07 in green gram, the highest value (0.61) belonged to S1-INV followed by VuRS02F16V (0.54).

Out of the 55 markers used, 32 markers generated amplification. Out of the 32 primers amplified, only 7 found to be polymorphic. However, these 7 polymorphic markers collectively yielded 15 in mungbean, with an average of 2.1polymorphic alleles per locus respectively, which was comparable to 3.9 alleles perlocus obtained by Gupta and Gopalakrishna (2010) in a study using EST derived SSR markers in cowpea. These results were also comparable to studies done using genomic SSR markers in *Vigna* species including cowpea (4.6 alleles per locus; Li *et al.,* 2001), urdbean (4.1 alleles per locus; Gupta and Gopalakrishna 2009) and azuki bean (4 alleles perlocus; Wang *et al.,* 2004). As the resistant gene homologues are derived from the NBS-LRR disease resistant motifs it offers resistant to fungal, bacterial and viral pathogens (Gupta and Gopalakrishna, 2010). Number of reasons could be attributed to his. Firstly, the RGH's are generally considered less polymorphic as compared to genomic SSR markers (Eujayl *et al.,* 2001; Gupta *et al.,* 2003). As the RGH markers are conserved across generations and also across species, the degree of polymorphism that can be expected using them is very low. Secondly, low polymorphism obtained may be because of the use of makers belonging to cowpea, chickpea, red gram and Medicago (Gupta and Gopalakrishna, 2010). In 2017 Sagi *et al.,* studied the Genetic Analysis of NBS-LRR Gene Family and their Expression Profiles in Chickpea in response to ascochyta blight infection. Recently Wu *et al.,* 2017 reported the 178 NBS-LRR-type genes and 145 partial genes were associated with Anthracnose and Common Bacterial Blight in the Common Bean.

Not many markers are developed specifically for mungbean; hence researchers use the markers that are designed from cowpea, common bean and soya bean extensively in

these two crops. Though recently, SSR markers have been developed from mungbean, the number of these SSR's is still very limited. The genotypes TARM 2, HUM12, SML348, HUM1 VGG4, PS16 PB1, TAP 7 KM 15 KM 16 KM 30 are found resistance to mungbean yellow mosaic virus these genotypes can be used as a resistance source for further resistance breeding programme

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