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Construction of Full-length Dimer Clones of Yellow Mosaic Virus and Screening of Blackgram Germplasm using Agroinoculation

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ABSTRACT

Background: *Vigna mungo* (Blackgram) is the major pulse crop cultivated in the Indian sub-continent. It is highly prone to yellow mosaic disease (YMD), which is widespread problem because all cultivated varieties are susceptible to the YMD in Andhra Pradesh state. The presence of two species of begomovirus *i.e.* Mungbean yellow mosaic virus (MYMV) and Mungbean yellow mosaic India virus (MYMIV) with YMD was reported in Andhra Pradesh. The current study was aimed to develop simple method of viral dimer clone construction and identifying the resistant blackgram genotypes against two species of begomovirus (MYMV and MYMIV) by agroinoculation technique.

Methods: In this study, we standardize the rolling circle amplification (RCA) based viral dimer clone construction (MYMV DNA-A, MYMV DNA-B and MYMIV DNA-A) and constructed viral dimer clones were used to transform *Agrobacterium tumefaciens* EHA105 cells through freeze-thaw technique. Sprouted seed method of agroinoculation screening was conducted.

Result: Total 45 blackgram genotypes were screened by sprouted seed method of agroinoculation with two combinations of viral dimeric constructs (MYMV-TPT-A+MYMV-TPT-B and MYMIV-TPT-A+MYMV-TPT-B). Twenty four genotypes offered resistant against MYMV infection and twenty three genotypes were resistant to MYMIV infection. Total 17 genotypes offered resistance to both species of virus (MYMV andMYMIV) associated with YMD.

Key words: Agroinoculation, Blackgram, *Mungbean yellow mosaic virus*, Screening.

INTRODUCTION

Blackgram [*Vigna mungo* (L.) Hepper] is the third major pulse crop of India and contributes 70% of the world's total blackgram production. The India, Black gram crop is cultivated in an area of 29.03 Mha with a total production of 23.4 Mt with a productivity of 806 kg/ha during 2018-19 (Directorate of Economics and Statistics, 2019). Yellow mosaic disease (YMD) is the major constraint to the productivity of grain legumes across the Indian subcontinent and the estimated annual yield loss in three legumes *i.e.* blackgram, greengram and soyabean was $ 300 million (Varma and Malathi, 2003). The reduction in seed yield was due to YMD in blackgram was 100% (Vadivel *et al.,* 2021) and it was 20-70% in green gram (Ramarao *et al.,* 2021). The YMD in southern Asia is caused by four distinct begomoviruses collectively known as the yellow mosaic viruses (YMVs): *Mungbean yellow mosaic virus* (MYMV), *Mungbean yellow mosaic India virus* (MYMIV), *Dolichos yellow mosaic virus* (DoYMV) and *Horsegram yellow mosaic virus* (HgYMV) (Qazi *et al.,* 2007). Of these MYMIV and MYMV are most important as it infect a large number of legumes in India.

The existence of two species of begomoviruses (MYMIV and MYMV) with YMD of blackgram in Andhra Pradesh was reported (Reddy *et al.,* 2014). The diverse virus isolates of MYMV have different levels of stability or virulence, as reflected by the symptom severity in each genotype of mungbean (Sathya *et al*., 2013). The concomitant existence of both species with YMD of blackgram results in the variation of symptoms in blackgram. As a result of this, each genotype exhibits a different level of resistance against diverse viral isolates and it is also difficult to confirm the resistance to which species of begomovirus under field conditions. Even the success of screening under field conditions for YMV resistance would depend upon the season, climate and vector population.

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To accomplish this, it is necessary to establish a simple inoculation technique for the efficient evaluation of resistance against particular species of begomovirus. Here, we applied the rolling circle amplification (RCA) method for the construction of infec-tious MYMV-TPT-A, MYMIV-TPT-A and MYMV-TPT-B dimer clones. This technique will allow screening germplasm throughout the year with various isolates/strains of virus which is not possible by natural field screening. The objective of this study was to develop a simple method of viral dimer clone construction and identify the resistant blackgram genotypes against two species of begomovirus (MYMV and MYMIV) by agroinoculation technique.

MATERIALS AND METHODS

Blackgram genotypes

Total forty-five blackgram genotypes were collected from pulse breeder, Regional Agricultural Research Station, Tirupati. These genotypes were screened against MYMV and MYMIV through the agroinoculation technique during 2017-19. The details of blackgram genotypes are as follows.

IVT entries

COBG 13-04, GBG-12, LBG-888, ADBG-13023, COBG-653, TJU-258, OBG-38, TJU-103, OBG-39, DKU-95 and IPU 12-30.

AVT entries

NDUK 15-222, PU 13-15, RU 03-22, VBG 12-034, KPU 12-213, VBG 12-111, TJU 24, DKU 82, MDBGV 06, KPU12-1730, KU 16-07, IPU 13-3, DKU 99, IPU 2-43, AKU 1316, PU 11-25, COBG 13-14, DKU 116 and KUG 718.

Advanced breeding lines

TBG-123 TBG-125 TBG-129 TBG138.

Other genotypes

ABG-1, ABG-3, PU-31, TBG-104, GBG-1, LBG-787, LBG-752, LBG-623, LBG-645, PBG-1 and PBG-32.

Construction of infectious MYMV and MYMIV dimer clones

The rolling circle amplification (RCA) was done with blackgram genomic DNA isolated from the YMD infected samples from the Tirupati region (Packialakshmi *et al.,* 2010). The viral dimers were generated by partial restriction digestion (*Bam*HI) product of multimeric viral genomes produced by RCA with *phi* 29 DNA polymerase. The viral dimer clone construction (MYMV-TPT-A, MYMIV-TPT-B and MYMV-TPT-B) was standardized in our study and described in results and discussion part.

Sprout- seed method of agroinoculation

EHA 105 *Agrobacterium* cells harboring full-length dimers of MYMV DNA-A and MYMV DNA-B were grown to OD_{600} of 0.8 and mixed in equal proportion. Bacterial cells were collected by low-speed centrifugation (5000RPM) and cells were resuspended in a small volume of Luria broth with 100µm acetosyringone and used for inoculation. Seeds of blackgram plants were surface sterilized and soaked in sterile water for 2-3 hrs and kept for germination overnight at 37°C, seed coat of sprouted seeds were removed by using forceps and pinpricked around the hypocotyls region with a fine needle and were immediately immersed in *Agrobacterium* cells containing DNA-A of MYMV and DNA-B of MYMV. After 2 hrs incubation seeds were washed and sown in pots. These pots were maintained in a plant growth chamber at 25±2°C, 60-70% RH with a 16 hr photoperiod (Jacob *et al.,* 2003). The percent disease incidence was calculated and the presence of the virus associated with infected plants was confirmed by PCR.

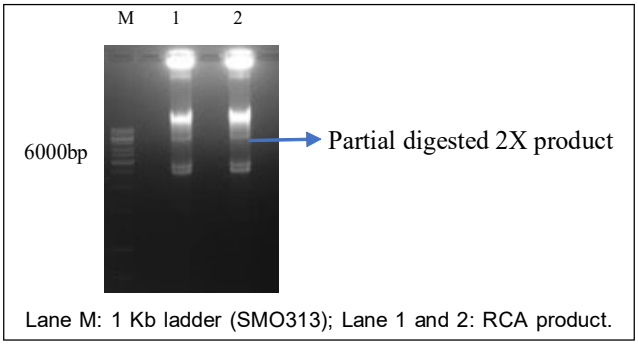
Polymerase chain reaction

PCR was performed in 25 µl reaction mixture containing 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 10 mM each dNTPs, 10 pmoles of forward and reverse primer, 2.5 U/µl Taq polymerase and 100 ng of DNA template.The amplification of specific gene was performed in PCR machine. The PCR products were analyzed on 1% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Construction of full-length viral dimer clones

In order to get dimer length fragment of the viral genome, the RCA product was partially digested with EcoRI/ HindIII/ BamHI restriction enzymes with three concentrations each (0.1U, 0.2U and 0.3U) at different incubation periods (5min/ 10 min/15 min). Finally with repeated attempts, partial digestion of RCA product (dimer-2x) was yielded successfully with 0.2U of BamH1 restriction enzyme for 10min of the incubation period. This dimer length (5.9kb) fragment of the viral genome was eluted and ligated into a binary vector pCAMBIA 2301 at BamHI site (Fig 1). Ligated products were transformed into *Escherichia coli* (TOP 10) cells. Confirmation of recombinant clones was done by PCR using specific primers designed to DNA-A (Chattopadhyay *et al.,* 2010; F- GGATCCATTGTTGAACGACTTTCC / R-GGATCCCACATTGTT AGTGGGTTC) and DNA-B of begomoviruses (Naimuddin *et al.,* 2011; F-ATGGAGAATT ATT CAGGCGCA/ R-TTACAACGCTTTGTTCACATT) and further confirmation was done by restriction digestion analysis with two combinations of restriction enzymes (HindIII / SacI and Pst1/ EcoRI). In our study total of three dimer clones were constructed (MYMV-TPT-A, MYMIV-TPT-A and MYMV-TPT-B) in the pCAMBIA 2301 vector (Fig 2).



Lane M: 1 Kb ladder (SMO313); Lane 1 and 2: RCA product.

Fig 1: Partial digestion of RCA product with BamH1 (0.2U) for 10 min.

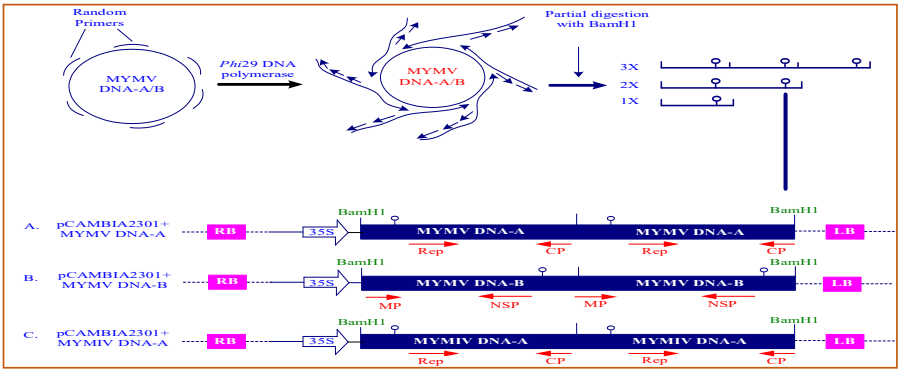


Fig 2: Linear map of dimeric constructs of MYMV DNA-A (A); MYMV DNA-B (B) and MYMIV DNA-A(C).

Table 1: Infectivity response of blackgram susceptible variety to agroinoculation.

Experiment details	Total no of agroinoculated seeds sown	Total no of plants which shown characteristic YMD symptoms	Per cent of infectivity by agroinoculation (%)
Agroinoculation with MYMV-TPT-A and MYMV-TPT-B	20	18	90
Agroinoculation with MYMIV-TPT-A and MYMV-TPT-B	20	19	95

Infectivity analysis of constructed viral clones

To test the infectivity, MYMV-TPT-A, MYMIV-TPT-A and MYMV-TPT-B dimer clones were introduced into competent cells of *Agrobacterium tumefa-ciens* (EHA 105) by freeze-thaw method. The pres-ence of the binary vector in *A. tumefaciens* was confirmed by PCR using specific primers and restriction digestion analysis.

To confirm the infectivity/ to prove Koch's postulates for constructed dimer clones, agroinoculation was done in two sets using known susceptible genotype PBG-32. One set of agroinoculation was carried out using *Agrobacterium* strains containing MYMV-TPT-A and MYMV-TPT-B dimeric clones and the second set of agroinoculation was done with *Agrobacterium* strains containing MYMIV-TPT-A and MYMV-TPT-B dimeric clones. In two experiments, we observed characteristic yellow mosaic symptoms on first emerged trifoliate at 8-12 days after inoculation (DAI). It was confirmed that both DNA-A components (MYMV and MYMIV) are infectious when agroinoculated with one DNA-B component (MYMV) and successfully express the viral genome in the blackgram plant.

The genotypes were observed up to 25-30 days for symptoms expression and percentage incidence of disease was recorded (Table 1). The accumulation of virus was detected by PCR using coat protein and movement protein specific primers.

In the PCR analysis, virus presence was observed in symptomatic and asymptomatic agroinoculated plants as well as in a few control plants. The differences in band intensity were observed between symptomatic, asymptomatic agroinoculated and control blackgram plants. It is necessary to investigate the fate of the virus in asymptomatic and control plants as there was no symptoms were observed in the total growth period of blackgram plants. It was reported that the presence of MYMV in various parts of blackgram seed like seed coat, cotyledon and embryonic axis was confirmed by different techniques like PCR, southern blot analysis and sequencing. When the growing test was performed with same batch seeds, no symptom development was reported in the seedlings. These results indicate the seed borne nature of the MYMV and it was hypothesized that the robust metabolic environment of a growing seedling may not be favourable to efficient build-up and translocation of the virus (Kothandaraman *et al.,* 2016). But in greengram, seed borne nature of YMD was contradicted by Naimuddin *et al.* (2016). Thus, detailed analysis is still needed to confirm the exact mechanism of the seed-borne nature of YMVs in different *Vigna* species (Mishra *et al.*, 2020). The concentration of virus particles needed for symptom expression in blackgram should be analyzed. Similarly, the relation between virus load and resistance level in plants is to be studied.

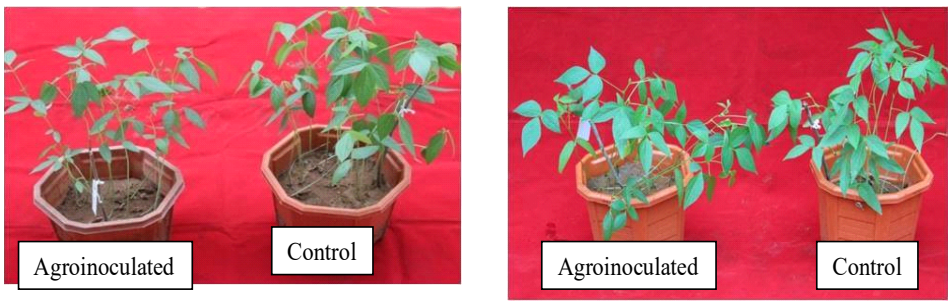
Most of the infectious clones of begomovirus were constructed by inserting a fragment containing intergenic region into the end of full-length genome (partial tandem repeat) which usually involve the selection and design of cloning sites and several subcloning procedures, which is a laborious process. Recently, rolling circle amplification (RCA) technology has been developed for amplification of any circular DNA for research and diagnostic purpose without using PCR. This method generates tandemly repeated conctamers/ viral multimers and partial digestion of these viral multimers with restriction enzymes will generate infectious dimers that can clone into any plant transformation vector without any further sub cloning procedures (Inoue-Nagata *et al.,* 2004).

Several workers were made infectious partial dimeric clones of geminivirus by RCA method (Balaji *et al.,* 2004; Bag *et al.,* 2014; Karthikeyan *et al.,* 2004 and Jacob *et al.,* 2003). In this study, we successfully generated infectious full dimeric clones (MYMV-TPT-A, MYMIV-TPT-A and MYMV-TPT-B) and further used in the screening experiment. Similarly, infectious complete tandem dimeric construct of Chickpea chlorotic dwarf Pakistan virus associated with chickpea stunt disease was constructed by Kanakala *et al.* (2013).

Total of 45 genotypes were screened by the sprouted seed method of agroinoculation. The screening experiment was carried out separately with two combinations of viral



(a) Highly susceptible reaction of blackgram genotypes (DKU-99 and PU 11-25) in agroinoculation with MYMIV-TPT-A + MYMV-TPT-B.



(b) Resistant reaction of DKU-99 and PU 11-25genotypes in agroinoculation with MYMV-TPT-A + MYMV-TPT-B.



(c) Resistant reaction of CO 13-14 and LBG-888 genotypes in agroinoculation with MYMIV-TPT-A + MYMV-TPT-B.



(d) Susceptible reaction of CO 13-14 and LBG-888 genotypes in agroinoculation with MYMV-TPT-A + MYMV-TPT-B.

Fig 3: (a-d) Differential response of the blackgram genotypes to agroinoculation with respective DNA-A of MYMV and MYMIV.

Table 2: Disease response of blackgram genotypes to agroinoculation.

Genotype	Mean per cent disease incidence		Disease scale		No. of days for symptom development	
	Ist screening experiment (I)	IInd screening experiment (II)	I	II	I	II
NDUK 15-222	-	-	1	1	NI	NI
PU 13-15	-	-	1	1	NI	NI
RU 03-22	14.6(MS)	23.85(S)	5	5	12	14
VBG 12-034	14(MS)	-	5	1	17	NI
KPU 12-213	-	-	1	1	NI	NI
VBG 12-111	-	-	1	1	NI	NI
TJU 24	-	-	1	1	NI	NI
DKU 82	4.35(MR)	-	3	1	23	NI
MDBGV 06	16(MS)	23.45(S)	5	5	17	12
KPU12-1730	-	-	1	1	NI	NI
KU 16-07	-	-	1	1	NI	NI
IPU 13-3	-	-	1	1	NI	NI
DKU 99	-	100(HS)	1	9	NI	17
IPU 2-43	6.25(MR)	-	3	1	17	NI
AKU 1316	4.25(MR)	83.65(HS)	3	9	17	14
PU 11-25	-	59.15(HS)	1	9	NI	14
COBG 13-14	26.5(S)	-	7	1	23	NI
DKU 116	-	-	1	1	NI	NI
KUG 718	-	-	1	1	NI	NI
COBG 13-04	35.4(S)	27.05(S)	7	7	16	17
GBG-12	27.05(S)	52.05(HS)	7	9	19	17
LBG-888	23.9(S)	-	7	1	22	NI
ADBG-13023	41.3(S)	63.7(HS)	7	9	16	17
COBG-653	79.15(HS)	72.95(HS)	9	9	16	19
TJU-258	57.15(HS)	55.5(HS)	9	9	16	20
OBG-38	-	42.8(S)	1	7	NI	24
TJU-103	-	15.15(MS)	1	5	NI	22
OBG-39	15.85(MS)	65.9(HS)	5	9	19	24
DKU-95	-	36.75(HS)	1	5	NI	20
IPU 12-30	-	-	1	1	NI	NI
TBG138	-	-	1	1	NI	NI
PU-31	-	-	1	1	NI	NI
TBG-104	-	-	1	1	NI	NI
GBG-1	-	-	1	1	NI	NI
LBG-787	-	12.25(MS)	1	5	NI	16
ABG-1	4.25(MR)	25.55(S)	3	7	22	24
ABG-3	-	19.1(MS)	1	5	NI	24
TBG-123	14.55(MS)	-	5	1	22	NI
TBG-129	-	-	1	1	NI	NI
TBG-125	-	-	1	1	NI	NI
LBG-645	79.6(HS)	85.4(HS)	9	9	14	15
LBG-623	80.9(HS)	100(HS)	9	9	16	15
LBG-752	64.6(HS)	100(HS)	9	9	15	17
PBG-1	85.4(HS)	100(HS)	9	9	9	10
PBG-32	89.8(HS)	89.45(HS)	9	9	10	12

Note: Ist Screening experiment: Agroinoculation with MYMV-TPT-A + MYMV-TPT-B.

IInd Screnning experiment: Agroinoculation with MYMIV-TPT-A + MYMV-TPT-B.

R- Resistant, MR- Moderately resistant, MS- Moderately susceptible, S- Susceptible, H- Highly susceptible and NI- No infection.

clones (MYMV-TPT-A+MYMV-TPT-B and MYMIV-TPT-A+MYMV-TPT-B) to know the resistant response of blackgram genotypes against MYMV and MYMIV infection. The disease rating scale 0-9 was used to categorize the genotypes (Sudha *et al.,* 2013).

Total of 45 blackgram genotypes were subjected to agroinoculation in two replications with two combinations of viral clones. The agroinoculated blackgram genotypes started showing characteristic yellow mosaic symptoms from 8-25 days of agroinoculation and no symptoms were observed in control plants. The average per cent infection of agroinoculated blackgram genotypes ranged between 0-100%. The genotypes from each category (S, HS, MS, MR and R) or disease response of each genotype are given in Table 2. It was observed that, among 45 genotypes, 24 genotypes offered resistance against MYMV infection and 23 genotypes were resistant to MYMIV infection. On the 25th day, diseased leaf samples were collected for DNA isolation. The isolated DNA samples were analyzed for the presence of viral genome by PCR by coat protein and movement protein specific primers. As described earlier, presence of viral genome was noticed even in a few control plants.

Interestingly, thirteen genotypes namely VBG 12-034, DKU 82, DKU 99, IPU 2-43, PU 11-25, COBG 13-14, LBG-888, OBG-38, DKU-95, TJU 103, ABG-1, ABG-3 and TBG123 showed differential resistant response to agroinoculation with respective DNA-A of MYMV and MYMIV. Among thirteen genotypes, DKU 99 and PU11-25 genotypes were resistant to MYMV infection, but they were highly susceptible to MYMIV infection (Fig 3a-b). Whereas COBG 13-14 and LBG 888 genotypes were susceptible to MYMV infection and showed resistance to MYMIV infection (Fig 3c-d). Finally,17 genotypes *i.e.* NDUK 15-222, PU 13-15, KPU 12-213, VBG 12-111, TJU 24, KPU12-1730, KU 16-07, IPU 13-3, DKU 116, KUG 718, IPU 12-30, TBG-138, PU-31, TBG-104, GBG-1,TBG-129 and TBG-125 offered resistance to both species of virus (MYMV and MYMIV). Among these 17 genotypes, TBG 129 and TBG-125 are advanced breeding germplasm developed from RARS, Tirupati. To confirm the results the experiment had repeated once and comparable results were observed. The above results clearly indicated that there is a differential resistant response of genotypes against MYMV and MYMIV infection was noticed. The sequence information of constructed clones might give a clue for this differential resistance response of the genotypes.

A similar type of artificial screening of germplasm by agroinoculation technique was previously done in different hosts like blackgram, greengram, soyabean and cowpea by several workers (Jacob *et al*., 2003; Usharani *et al*., 2004; Karthikeyan *et al*., 2011; Sudha *et al.,* 2013; Bag *et al.,* 2014; Madhumitha *et al*., 2022), But all these screening trials were evaluated with DNA-A and DNA-B clones of MYMV. In this study, first time we attempted the screening experiment with clones of MYMIVDNA-A and MYMVDNA-B along with MYMV DNA-A and DNA-B clones.

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