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Molecular Cloning of MYMV Genome and Infectivity of Yellow Mosaic Virus in Green Gram Using Different Viral Transmission Tools

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Mungbean yellow mosaic virus (MYMV) causes massive crop losses in green gram. MYMV is a member of begomovirus with bipartite genome comprising DNA-A and DNA-B components, which is transmitted by whiteflies. Cloning and preparation of infectious clone is very much essential for screening germplasm or transgenic material of pulse crops since viruliferous whiteflies may not be available throughout the year. In the current work, we have amplified rolling circle mediated viral genome of MYMV using F29 DNA polymerase. The amplified products was digested and cloned into the plant expression vector pCAMBIA2301. The cloned constructs was then transformed into Agrobacterium LBA4404 through freeze thaw method. Further, three viral transmission techniques including mechanical rubbing, Agroinfiltration and Agroinoculation, were employed for assessing the mosaic symptoms in green gram. The molecular confirmation through polymerase chain reaction (PCR) indicated that the yellow mosaic symptoms were formed due to infectivity of MYMV in the green gram.

Keywords: Agroinfiltration; Agroinoculation; F29 DNA polymerase; Yellow mosaic disease.

Yellow Mosaic Disease in greengram is caused by Mungbean Yellow Mosaic Virus (MYMV), devastating large acres of crops, particularly in tropical and subtropical countries[1]. The MYMV belongs to the *Geminiviridae* family and two isolates of MYMV were evolved in Indian sub-continent. The MYMV possesses a circular and single-stranded DNA genome encapsulated with icosahedral particles. Generally, Begomovirus have either monopartite DNA-A or DNA-B (Concatomeric 2.6 or 2.7 kb) or bipartite (Concatomeric 5.2 kb) circular ssDNA genome. The DNA-A genome has five open reading frames (ORFs), encoding for functional proteins *i.e*., AV1 coat protein (CP), AC1 viral replication initiation protein (Rep), AC2-Transcriptional activator (Trap), AC3-replication enhancer (Ren), and AC4. The genome also has two ORFs in DNA-B encoding i.e., movement or mobility protein (MP) and a nuclear shuttle protein (NSP)[2]. The predominant geminiviruses are causative entities in leguminous species including greengram, black gram, french gram, pigeon pea, and soybean[3]. MYM virus is transmitted by whitefly, *Bemisia*

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tabaci Genn. (Hemiptera: Aleyrodidae). It is a polyphagous insect, completes its life cycle in less than 2 weeks to more than 10 weeks depending on temperature and host plant where it has greater ability to transmit disease[4].

Geminiviruses are circular single-stranded (ss) DNA plant pathogens that can replicate double- stranded (ds) DNA *via* rolling circle mode of amplification (RCA) in host plants[5]. RCA is a process in which a circular DNA or RNA molecule is replicated in one direction through strand displacement activity of Ö29 DNA polymerase. First DNA molecule is displaced by newly synthesized DNA and releases the single-stranded DNA (ssDNA). The primer enzymatic extension combined with strand displacement generates a long ssDNA complementary to the DNA template. RCA is recognized as an important diagnostic tool to amplify the complete genome of viruses artificially under *in vitro* conditions. This technique is used to detect many ss, ds DNA viruses infecting different crops[6]. The double stranded circular papilloma viral genome sample of infected leaf tissue was efficiently amplified using Ö29 DNA polymerase *via* RCA technique[7, 8]. Similarly, Inoue-Nagata et al.[9] used cloning of single circular DNA method in tomato for *Tomato Chlorotic Mottle Virus*.

The whiteflies act as natural vectors carrying viruses that spread viral diseases[10]. The host phloem cells are used by virus to enter and their viral aggregates pass on through it. The symptoms appear within two days as a scattered yellow spot on the young leaves turning to mosaic appearance, infected plant pods size decreases, leaf yellowing decreasing the efficiency of photosynthesis and causing a severe crop losses[11]. The insect would not persist in all seasons; hence, researchers are encountering issues with screening germplasm and assessing resistant varieties. *In vitro* cloning of the complete viral genomes through RCA is possible to develop screening techniques like Agro-inoculation, Agroinfiltration. Cloning a viral genome in a suitable vector is the possible solution for screening and challenging the testing material in the laboratory without any climatic barrier and escaping the transfer of viral components.

Viral transmission studies help in understanding *inplanta* gene – gene interaction, gene expression and functional analysis. Further, the Agroinoculation of viral genome into plants would be useful for understanding viral replication, assembly and their movement. Similar technique was followed using soyabean isolate to infect in greengram and blackgram[12]. In another study the tobacco leaves were infiltrated with Agro-clone for multiplication and propagation of *Potato virus X* (PVX)[13]. Madhuitha et al [14] has evaluated Vigna germplasm was to detect the level of resistance/ susceptibility against yellow mosaic virus.

Agroinoculation and Agroinfiltration methods were first applied for the of both viral DNA/RNA genomes such as *Cauliflower Mosaic Virus* (CaMV) in turnip plant *Maize Streak Virus* (MSV) in maize[15, 16]. Vaghchhipawala et al. [17] reported that by using virus-induced gene silencing (VIGS) method to study plant-virus interactions, functional analysis of viral genomes and also for genetic screening with forward and reverse approaches. These techniques are applicable to investigate transient gene expression effects, analyze its protein localization and protein-protein interactions in plant-pathogen studies. Agroinfiltration method was used for delivering viral vectors carrying *Tobacco Mosaic Virus (*TMV) and *Potato Virus X* (PVX) in leaf part or whole plant for gene expression studies[17]. Through Agroinfiltration method, sGFP tagged viruses of *Tomato Torrado virus* was inoculated in tomato and tobacco for studying molecular determinants during viral movement for analyzing plant-virus interactions.[18] A cDNA clone of *tomato mottle moasic virus* (ToMMV) was used for agroinfiltration of tomato leaves, where pathogenicity and virus host interactions were studied through electron micrographs[19].

In present study, we report a simple procedure for construction of agro-infectious genomic clones of MYMV. The complete genome of MYMV was amplified using Ö 29 DNA polymerase and digested genome was cloned in pCAMBIA2301 plant expression vector. This vector was used for Agroinfiltration and Agro-inoculation studies in greengram. The yellow mosaic symptoms in infected samples were assessed and screened through PCR using genes specific markers encoding DNA-A and DNA-B in plants. .

MATERIALS AND METHODS

Extraction of genomic DNA from infected leaves of greengram

The greengram plants exhibiting mosaic symptoms with irregular green and yellow spots were collected from greengram fields in Acharya N. G. Ranga Agricultural University, LAM farm, AP, India (Fig 1A). Genomic DNA was extracted from infected leaf samples and control plants by following CTAB method. Briefly, infected leaves were ground with CTAB buffer (10 mM EDTA, 100 mM Tris, 1.4-2.0 M NaCl, 2% CTAB, 2-5% ß-mercaptoethanol). The leaf slurry incubation was done at 60º C for 30 min, equal volumes of Phenol: Chloroform : Iso-amylalcohol (25: 24: 1) was added to the samples supernatant and centrifuged for 10 min at 13,000 rpm. The supernatant was then added with Chloroform: Iso-amylalcohol (24: 1) followed by centrifugation. Further the supernatant was precipitated with the addition of 100 % isopropanol and 7.5 M Sodium acetate. Finally, the pellet was washed with 70 % ethanol and DNA pellet was dissolved in sterile water [20]. The extracted DNA was qualitatively measured by resolving in 0.8 % agarose gel, then purity was analyzed at OD of 260 and 280 nm by NanoVue spectrophotometer (GE healthcare, USA).

Rolling circle amplification of MYMV genome

The genomic DNA from infected and control samples was subjected to RCA using Ö 29 DNA polymerase (Thermo Scientifics, USA) according to the instructions given by manufacturer. Briefly, reaction mixture was prepared by the addition of 100 ng of RCA DNA, 500 µM Exo-Resistance Random primer and adjusting reaction volume for 10 µl with water. Following with incubation of the reaction mixture was done at 5 min at 95ºC and chilled on ice for 2 min. To this reaction mixture, 10 mM dNTPs and 1 µl Ö29 DNA polymerase (10 U/µl) and Pyrophosphate inorganic (0.1 U/µl) was added and the final volume was made up to 15 µl - 20 µl based on DNA concentration. The above mixture was then incubated at 30 ºC for 18 hrs and the reaction was terminated by keeping at 65 ºC for 10 min.

Confirmation of presence of DNA-A and DNA-B components in MYMV genome

To confirm whether the RCA possessing, DNA-A and B, was processed using gene specific primer sets such as *AC1, VCP2 (AV1), BC1* and *BV1* through PCR. The reaction performed in 10 ìl containing 10 x PCR reaction buffer, 2.5 mM dNTPs, 2.5 mM $MgCl_2$, each primer 10 pmols, 2.5 U/il Taq DNA polymerase (Takara) and 100 ng RCA-DNA. The primer details are given in (Table 1). The PCR program was followed as 94ºC for 5 min for initial denaturation; 94 ºC for 40s; 54 ºC for 40s for annealing of *AC1, VCP2 (AV1), BC1* and *BV1,* extension at 72ºC for 40s and 72ºC for 7 min for final extension. After the amplification, the PCR products were resolved on 1% agarose gel in 1x TAE buffer at 100v through electrophoresis. The gel was analyzed with gel documentation system (Syngene, USA).

Construction of pCAMBIA2301 vector with MYMV genome RCA product

RCA products were digested with *Bam* HI and *Hind* III by following manufacturer's instructions (Thermo Scientific, USA). RCA products were digested individually with *Bam* HI and *Hind* III using about 2 µg of RCA product, 10 µl buffer, 10 U/µl of *Bam* HI and *Hind* III restriction enzyme**,** adjusting reaction volume with water to 50-100 µl, incubated for 25 min at 37ºC and were inactivated by an enzyme at 85ºC for 10 min. Further, 0.8% Agarose gel was used to resolve the digested products. The digested DNA fragments were excised from the gel was eluted with Qiagen Gel Extraction kit (Qiagen, USA). The eluted DNA fragments (300 ng) were ligated with pCAMBIA2301 using T_4 ligase (Thermo scientific, USA) individually (Fig 1B).

***E. coli* and *Agrobacterium* transformation**

The ligated products that contain pCAMBIA2301 vector and MYMV genome (2.6 kb and 2.7 kb) fragments separately was transferred into *E. coli* (DH5á) by heat shock method[21]. About 10 µl ligated product was added into competent DH5á cells. The mixture was incubated on ice for 5 min, and given heat shock at 42°C for 90 s and incubated on ice for 1 min. To the mixture, 1 ml of LB broth was added and incubated for 1 hr at 37°C, 180 rpm in the incubator. After the incubation, 50 µl cell suspension was spread onto LB agar medium supplemented with kanamycin (50 mg/L) and incubated at 37°C for overnight (Fig 2A). Then the colony PCR was performed for detecting the cloned fragments such as DNA-A *i.e.,* AC1, AV1 (VCP2), and DNA-B *i.e*., BC1, BV1 using gene

specific primers. Plasmid DNA was isolated from positive colonies using Qiagen Plasmid isolation kit (Qiagen, USA). The sequencing of amplified fragments was done at DNA sequencing facility (Eurofins Genomics India Pvt, Ltd, Bangalore). Analysis of sequenced data of MYMV DNA was carried out by multiple sequence alignment.

The two plasmids DNA-A and DNA-B were then transferred separately into *Agrobacterium* through freeze thaw method. About 1 µg of plasmid DNA was added into 200 µl of *Agrobacterium LBA4404 cells,* and snap freezed in liquid nitrogen, 1 ml of LB broth was and incubated at 28°C for 4 hr. The suspension was then plated on LB agar plates supplemented with streptomycin (50 mg/L) kanamycin (50 mg/L), and rifamycin (20 mg/L) followed by incubation at 28°C for 48 -72 hr. Plasmid DNA was isolated from positive colonies using Qiagen Plasmid isolation kit (Qiagen, USA) and glycerol stocks were prepared and used for *Agrobacterium* transmissions.

Viral transmission studies in green gram

Rubbing

Mechanical transmission of MYM in greengram was performed using rubbing method. The sap was prepared from MYMV infected leaves of greengram by macerating in the cold sodium phosphate buffer (300 mg of powdered tissue with 1.5 ml of sodium phosphate buffer) and centrifuged at 4°C at 13,000 for 5 min. About 100 µl of supernatant was rubbed on the adaxial surface of 3-4 fully expanded leaves per plant. Symptoms were scored after 2 days of rubbing. The genomic DNA was extracted from infected leaves and analyzed through the PCR using AC1 gene specific primers.

Agro-infiltration

Agrobacterium containing pCAMBIA2301 vector harbouring DNA-A and DNA-B was grown till the OD_{600} reaches 0.4 - 0.6 at 28°C for 48 hrs. Then, the cultures was centrifuged at 5,000 rpm and each culture was diluted in three volumes of Agro-infiltration medium (10 mM $MgCl_2$, 10 mM MES, pH 5.6) until culture OD_{600} reached to 0.5. Further, culture was incubated at room temperature with gentle shaking for 1-2 hrs. Both cultures were mixed together along with 100 µM acetosyringone and incubated for 2 hrs for *vir* gene induction[13]. Culture was infiltrated on abaxial surface of leave without any needle to infiltrate leaves of 4 to 5-week-old plants of greengram. After post infection, occurrence of yellow mosaic symptoms (4 to 8 days), the genomic DNA was extracted from infected leaves and analyzed through the PCR using AC1 gene specific primers (Figure 3).

Agro-inoculation

The greengram seeds were surface sterilized with 70% ethanol and imbibed in water for overnight. The seeds were pricked with fine syringe and inoculated with medium along with 100 µM acetosyringone and seeds were maintained at 28°C for 3 hrs and excess culture was removed and seeds were sown in soil. Post 15-21-days of infection, the MYMV symptoms were phenotypically characterized and the symptoms were recorded in the trifoliate leaves. The uninoculated seeds of each line were maintained as control.

RESULTS AND DISCUSSION

Detection and Confirmation of DNA-A and DNA-B components of MYMV genome

Yellow mosaic disease (YMD) of green gram and black gram is economically very important and it is caused by two species of virus, MYMV and MYMIV and are transmitted by white flies. Incidence of this disease leads to cent percent yield loss of the crop. Hence it is necessary to develop the MYMV resistant varieties through Marker Assisted Breeding. To identify the new sources of resistant variety, we need to screen the germplasm against MYMV. Screening under the field conditions was always influenced by the different factors like i.e., environmental changes, whitefly genotypes etc.. Advancement in the molecular biology provides us three different method of screening methods: Rubbing method, Agroinfiltration and Agroinoculation (Aguilar et al[13] ; Usharani et al. 2006[23] ; Biswas and Varma [24] ;Mandal et al.[25]). In the present study, we have followed these three techniques for greengram screening against YMD. Greengram plants with typical characteristics of mosaic symptoms including green and yellow patches (Fig 1A) with lesser flowers and pods in the fields were chosen for this experiment. The infected and control leaf samples were collected for DNA extraction. About 20 µg of DNA was extracted from those

Table 1. Primers used for amplifying selected regions and full-length DNA-A and DNA-B components of yellow mosaic viruses infecting greengram

MYMV Genome	Gene	MYMV isolates of Vigna Species Accession No	Primers: Nucleotide Sequence	Primer Size	Annealing Temp	Product Size (kb)
DNA –A	AC1	AB017341.1	FP: 5' ATGCCTAGACTCGGTCGTTTTG 3'	22 bp	54°C	0.7 kb
			RP: 5' CGTGCGACTATCGCCTTCAATC 3'	22 bp		
	AV1(VCP2)	DQ400848.1	FP: 5' GCCAAAGCGGAATTACGA 3'	18 bp		0.7 kb
			RP: 5' GCCTCTTGGTGGTTGTAAAC 3'	20 bp		
DNA-B	BC1	AJ439059.1	FP: 5'ATGGAGAATTATTCAGGCGCAG 3'	22 bp		0.8 kb
			RP: 5' TTACAACGCTTTGTTCACATTG 3'	22 bp		
	BV1	DQ400849.1	FP: 5' ATGTTTAACCGCAATTATCGCA 3'	22 bp		0.7 kb
			RP: 5' TTATCCCACGTATTTCAATTCA 3'	22 bp		

infected leaves. The DNA contains both plant and viral genomic DNA. The purity of the DNA was analyzed by NanoVue at OD 260/280 with 1.8. The complete genome of MYMV was amplified from 200 ng of genomic DNA as a template by using Ö29 DNA polymerase (Fig 1B). In this method, the DNA fragments amplifies as mini circles through rolling circle amplification by random priming for generating high concentration of concatermerized DNA Dean et al.[22] . Then the RCA product was partially digested with *Bam* HI and *Hind* III, obtained a fragments with a size of 2.6 kb and 2.7 kb each. The digested product was confirmed by amplifying coat protein by using gene specific primers and obtained 0.7 kb fragment (Fig 1C). Similar to our work, Kumar et al.[26] reported 2.6 kb and 2.7 kb fragments of DNA A and DNA B isolated from cowpea against MYMV. Similarly, in the other report Jyothsna et al [27] reported 2.7 kb and 2.6 kb of viral genomes isolated from *Rhynchosia minima weed.*[28] In contrast, Haq et al.[28] reported 2 kb and 1.5 kb fragments of DNA-A and B from blackgram.. Based on the reports to avoid the confusion we have amplified the DNA-A and DNA-B with reported specific primers and sequenced.

RCA products cloning in pCAMBIA2301 expression vector and *Agrobacterium* transformation

Geminiviruses, damages many crop on several countries. To control the spread of the virus demands efficient diagnostic tools like specific antibodies, PCR and RFLP (Briddon and Markham[29]). Later Dean et al.[22] provided the feasible procedure by the uses of *Bacillus subtilis* bacteriophage Phi29 polymerase. By using this DNA polymerase we can able to do polymerase and stand displacement activity through RCA mechanism. Through this RCA approach we can detect and characterize geminiviruses and related subgenomic components (Haible et al.[1]). In the present study, we have used the RCA mechanism to characterize YMD in greengram. The digested DNA fragment was cloned in the *Bam* HI site of pCAMBIA2301 using T_4 DNA ligase and another DNA fragment was cloned in the *Hind* III site of pCAMBIA2301 using T_4 DNA ligase. Then, two plasmids were transferred into *Agrobacterium* for further viral transmission studies. The Agro colonies appeared in 48 hr of incubation (Fig 2A).

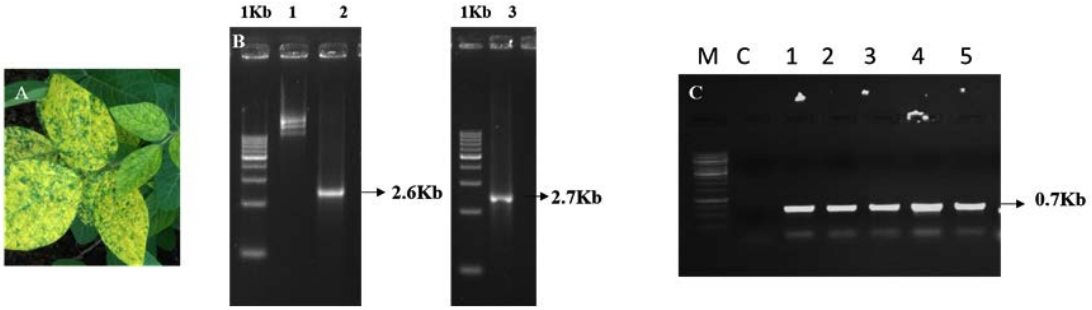
The colonies were confirmed by PCR. using a different set of primers including AC1 (replication protein), BC1 (mobility protein) and BV1 (shuttle protein), which showed expected amplification products of 0.7 kb, 0.8 kb and 0.7 kb respectively (Fig 2B and 2C). All these fragments were sequenced by Eurofins Genomics, Bangalore. About 97% of DNA-A sequence was matched with accession no DQ400847.1 isolated from blackgram. This sequence also matches 96-97% with the DNA-A of another crop like soyabean, French Bean, greengram infected with YMD. In case of DNA-B the sequences matched 97% with the accession no AJ439059.1. reported from mungbean. DNA-B sequences matched only with mungbean not with any other crops . Based on the blast and Clustal w analysis, we confirmed our isolated clone belongs to YMD DNA-A and DNA-B and used for agrotransformation. . The confirmed *Agrobacterium* LBA4404 clone with MYMV genome was used for further virus transmission studies.

Mechanical transmission with MYMV maintained *Agrobacterium* clones

Transmission may be due to rubbing of plants with liberated virus from damaged hair cells or epidermal cells [30]. The virus is transmitted by rubbing the viral sap on to the adaxial side of trifoliate leaves of greengram (Fig 3A). The greengram is highly susceptible to MYMV. The severe yellow mosaic symptoms appeared on greengram leaves in 7-10 days post infection (Fig 3B). Further, the infected and control plant were used for detecting viral particle accumulation in the plants through PCR. The PCR results indicated that the AC1 fragment with 0.7 kb was amplified in all infected samples. However, uninfected control did not showed any symptoms and failed to show amplification with AC1 primers(Fig 3C). . About 90±1 % of 10 leaflets rubbed with sap

Table 2. PCR screening based on appearance of symptoms in blackgram and greengram after challenging with MYMV through Agro inoculation

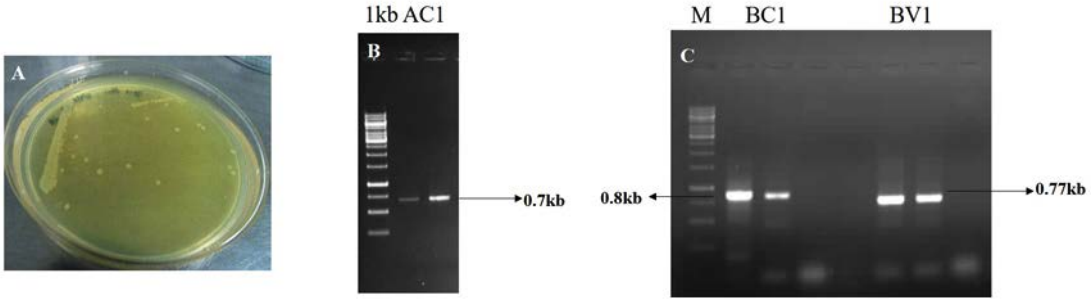
Variety	No of seedling inoculated	No of seedling germinated	No of plants showed YMD symptoms	No of plants showed AC1 amplification	Percentage of plants observed with symptoms
LGG460 (Gg-S)	15	10	9	10	90%
GG150 (Gg-R)	15	14	1	1	7.1%
LBG685(Bg-S)	15	13	11	11	84.6%
PU31(Bg-R)	15	15	1	1	6.6%



A. Greengram showing typical yellow mosaic symptoms collected and isolated genomic DNA. B. Rolling circle mediated amplification of complete viral genomic DNA using Ö 29 DNA polymerase (1), Restriction and digestion of viral genome by *Bam* HI (2.6 kb) and (3) Restriction and digestion of viral genome by *Hind* III (2.7 kb) C. RCA product confirmation of viral genome using Coat protein primers. 1 kb: ladder, C: Uninfected leaf sample, 1 to 5: infected samples of greengram.

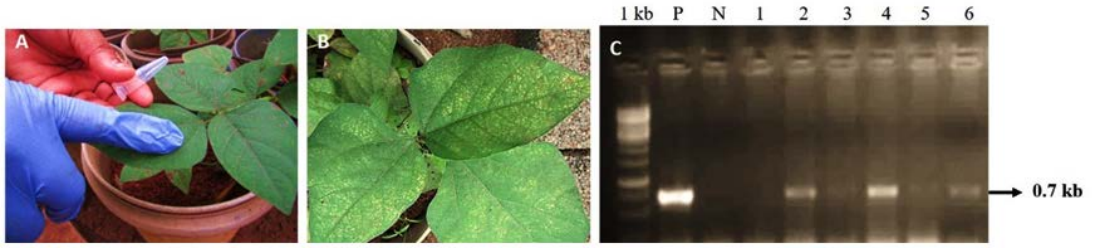
Fig. 1. Cloning of MYM viral genome using Ö 29 DNA polymerase

extracted from MYMV infected greengram plants in triplicates, developed yellow mosaic symptoms after 5 days of post infection. During this period, it allowed the virus particles penetrate the leaf surface. Aguilar et al.[13] reported that the mechanical rubbing of PVX sap inoculum on the surface of



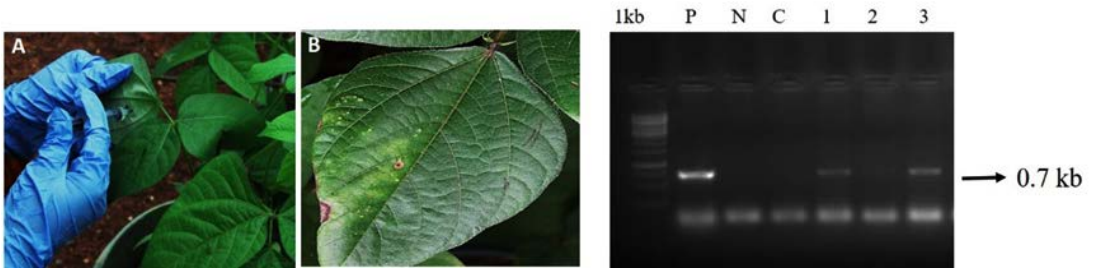
A. The digested samples were cloned into plant expression pCAMBIA2301 vector and transferred into DH5á cells and then into *Agrobacterium* through electroporation. B. Agarose gel picture showing confirmation of MYMV DNA-A using AC1 representing 0. 7 kb fragment and C. DNA-B confirmation with BC1 and BV1 specific primers showing 0.89 kb and 0.77 kb fragments respectively. 1 kb: ladder

Fig. 2. Cloning of MYM viral genome into pCambia2301 and confirmation of viral genes using gene specific primers:



A. Viral inoculum prepared from infected leaf samples and ruptured on adaxial side of greengram. B. *Agrobacterium* culture harboring MYMV clone infiltration using syringe. The mosaic symptoms were appeared in 7 dpi. C. The gel picture showing the amplification of AC1 representing 0.7 kb fragment. 1 kb: ladder, P: positive control, N, negative control; 1 to 6; test samples

Fig. 3. Viral transmission studies using mechanical rubbing



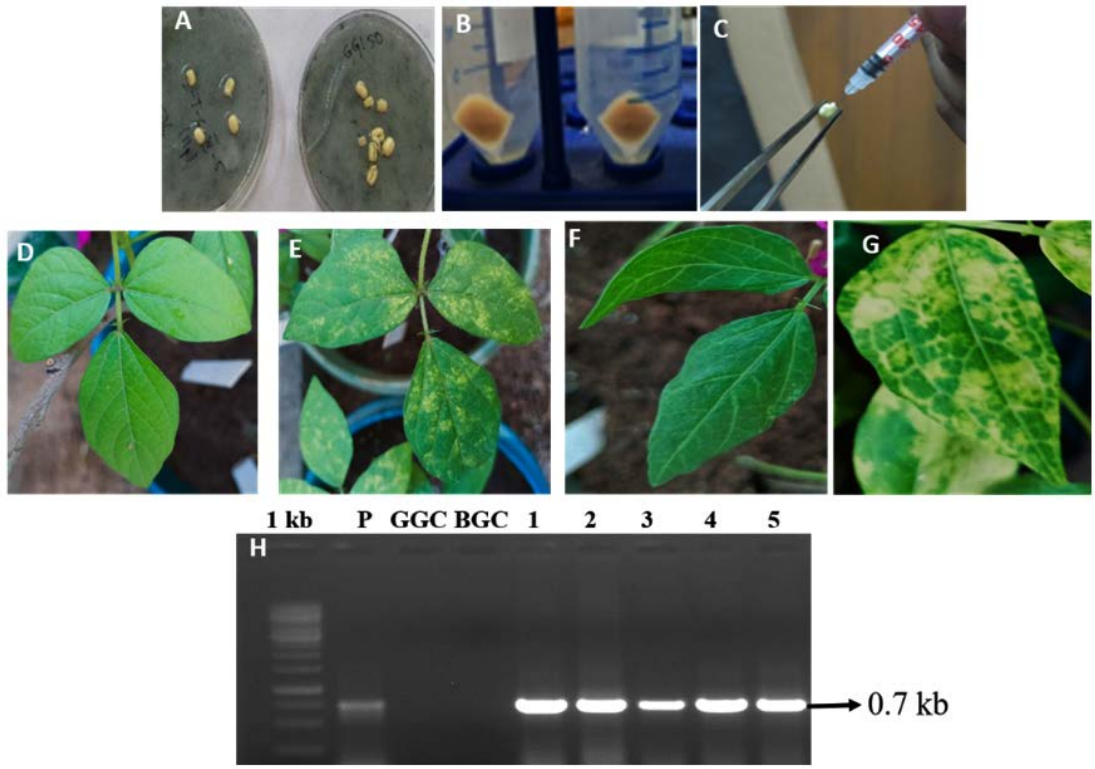
A. 45 day old leaf samples were infiltrated with syringe containing *Agrobacterium* culture. B. Appearance of mosaic symptoms at infiltrated region in 5 dpi. C. Agarose gel picture showing the PCR amplified products with expected amplification of AC1 gene with 0.7 kb.

Fig. 4. Agro infiltration using MYMV clone.

Nicotiana benthamiana allowed penetration and propagation of viral particles and the symptoms appeared in 7 days of post infection. Similarly, rubbing method was reported for transmission of sugarbeet virus in Beta vulgaris and Spinacia oleracea crops (Kassanis[31]). Another Begomovirus, Curly top virus which affects the different crops i.e., tomatoes, beans, cassava, squash and cotton also transmitted by rubbing method (Saad et al.[32]). Singh and Awasthi [33] reported different artificial mechanical transmission of virus like TMV, CMV, TSV, Okra mosaic virus, Papaya ringspot virus, Cowpea mosaic virus and alfalfa mosaic virus in tobacco, tomato, sunflower, okra, papaya, cowpea and potato (Sacrista´n et al.[34]; Jalender et al.[35]; Sundaresha et al.[36]; Givord and Hirth [37]; Gonsalves [38]; Surekha et al.[39]; El-Abhar et al. [40]).

Agroinfiltration using MYMV Agrobacterium clone

Agro culture was infiltrated on the abaxial side of the trifoliate stage of greengram. The severe yellow mosaic symptoms were observed in 21 days of post infection (dpi) in greengram leaves. None of the symptoms observed in control leaves. The viral DNA was detected in in Agroinfiltrated leaves using AC1 gene specific primers. The expected amplicon size of 0.7 kb was obtained in two out of three symptomatic plants (Fig 4C). However, the uninfected control plants didn't show any amplification. Zhang et al.[41] constructed cDNA clone of *Wheat yellow mosaic virus* (WYMV) genomic RNA and used Agroinfiltrated for viral multiplication and systemic infection in tobacco



A. overnight soaked greengram (LGG460) and black gram (LBG685) seeds B. *Agrobacterium* pellet after centrifugation C. Pricking of seedling for effective infection with *Agrobacterium*. D. Control leaves of greengram E. Spread and appearance of mosaic symptoms on 5 dpi leaves of greengram F. control leaves of blackgram G. typical yellow mosaic symptoms on 21 dpi leaves of blackgram H. Agarose gel picture showing the amplification of AC1 gene which accumulated in virus infected greengram (1 to 3 lanes) and blackgram (4 & 5) representing 0.7 kb fragment. 1 kb: ladder, GGC: Greengram control, BGC: Blackgram control.

Fig. 5. Viral transmission studies through Agroinoculation on greengram and blackgram.

and wheat. Interestingly synergic effect was detected between WMYV and *Chinese wheat mosaic virus* (CWMV).

Liu et al [42] reported that through agroinfiltration tobacco rattle virus (TRV) was transmitted in tomato and studies the functional analysis in detail (Liu et al., [42,43,44]; Dong et al., [45]; and Anand et al., [46]). In another study, Gao et al., [47] reported that agroinfiltration studies had typical mosaic symptoms in *J.curcas* with *Jatropha curcas mosaic disease* and used for screening the cultivars to identify the virus resistant plants. Wieczorek et al.,[48] developed a construct of infectious cones of *tomato torrado virus* and transmitted to plants through agroinfiltration.. Similarly, Usharani et al., [49] studied AV promoter expression *via*., infiltration in tobacco and different legumes by a reporter gene *i.e.,* GUS.

Transmission of MYMV using Agro-inoculation technique

Agroinoculated plants were assessed for yellow mosaic symptoms in Greengram and Blackgram. For this experiment, LGG460 (greengram) and LBG685 (blackgram) lines were subjected to Agro-inoculation in five replications. Severe yellow mosaic symptoms were observed in 14 days after inoculation in trifoliate leaves of blackgram and greengram (Fig 5D and 5F), however, no symptoms were developed in the control uninoculated plants. The genomic DNA was extracted from the leaves showing yellow mosaic symptoms. The PCR confirmation was done for the viral DNA presence i.e., MYMV AC1 gene and all inoculated samples showed expected amplification of 0.7 kb fragment (Fig 5H). MYMV symptoms were appeared 90% of greengram and 84.6% in blackgram plants inoculated with Agro infectious clone. However, the resistant lines in greengram and blackgram have shown minimal symptoms (Table 2). Jacob et al [50] reported MYMV which infects blackgram, mungbean and soyabean. Usharani et al.[12] demonstrated viral DNA presence in symptomatic plants agro-inoculate. Asymptomatic of resistant genotypes compared to control plants of were compared with controls through PCR.Advantage of agroinoculation is to observe the uniform symptoms in all infected leaves, which would make easier to compare with control plants[24]. MYMV symptoms are analysed based on the scattered appearance of yellow-color spots on young leaves which turns into mosaic pattern[11]. Sudha et al.[51] screened germplasms of mungbean to identify the resistant/susceptible against MYMV and analysed its viral load by PCR with coat protein gene primers.

Agroinoculation studies were used to screen Tomato yellow leaf curl virus in Sweet pepper, and Pepper yellow leaf curl virus in capsicum (Kil et al., [52]; Koeda et al., [53]). In another study, Overcoming a resistance against Tomato yellow leaf curl virus in Lycopersicon species was observed by Kheyr et al.[54].

CONCLUSION

The MYMV genome was amplified using Ö29 DNA polymerase from infected samples of greengram. The cloned MYMV construct is very useful in bioassay experiments for screening different genotypes of greengram, blackgram and other crops. These viral transmission tools would be employed for screening the CRISPR mediated yellow mosaic virus resistance in pulse crops.

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Conflicts of interest

The authors declare no conûicts of interest

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