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Testing the infectivity of a Begomovirus by particle bombardment method using a gene gun

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Abstract

This study was design to identify the causal agent of Horsegram yellow mosaic disease and to investigate the pathogenicity of *Horsegram yellow mosaic viruses* (HgYMV) infective clones. The samples were obtained using standard method from the two main horsegram growing areas of Bangalore, Karnataka State of India. The viral DNA from horsegram plants exhibiting severe symptoms was amplified by PCR. An isolate of HgYMV1 and HgYMV2 were associated with severe symptom phenotype from HgYMV. Full-length clones of DNA-A and DNA-B genomic components were constructed and attempts were made to introduce homologous (HgYMV1/HgYMV2) combinations of DNA-A and DNA-B genomic components into *Nicotiana benthamiana* plants. Inoculation of linearized constructs containing full-length clones or partial head-to-tail dimers of DNA-A and DNA-B genomic components resulted in the introduction of DNA-A genomic components into the host plant. However, these combinations of genomic DNA component were not detected in the inoculated plants bombarded using the gene gun. Thus, this study was unable to confirm the pathogenicity of HgYMV infective clones using *N. benthamiana* as model plant.

Keywords: Horsegram, Begomovirus, *yellow mosaic viruses*, Particle bombardment, Gene gun

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Introduction

Begomoviruses are widely distributed in nature and are transmitted through the vector called *Bemisia tabaci*. They are classified as monopartite with a DNA-A-like component or bipartite containing DNA-A and DNA-B (Brown et al., 2012). DNA-A encodes six ORFs necessary for virus replication, transcription activation and encapsidation, while DNA-B component facilitate cell-cell and nucleocytoplasmic trafficking of the viral genome (Ha et al, 2008; Hanley-Bowdoin et al., 2013). Begomoviruses causes damage to economically important crop plants such as cowpea and horsegram, causing substantial yield losses every year in India (Muniyappa and Veeresh, 1984; Abarshi et al., 2017). Horsegram (*Macrotyloma uniflorum*) is an pulse crop (legume) that produces seeds, is an annual native to India. It is commonly cultivated in India and serves as good source of protein for rural inhabitants (Kadam and Salunkhe, 1985; Fuller and Harvey, 2006; Fuller and Murphy, 2018).

Several procedures have been developed for the introduction of DNA in to plant cells (Slater et al., 2003). These include the use of viral and bacterial vectors, microinjection, electroporation, transformation using silicon carbide whiskers and particle bombardment method (Negrutiu et al., 1987; Datta et al. 1990; Slater et al., 2003; Altpeter et al., 2005). The latter method of implanting (delivering) DNA into plant cell by bombarding cells with DNA-coated microparticles has rapidly become a popular technique because the process of DNA insertion is a simple mechanical operation (Altpeter et al., 2005). Particle bombardment is commonly employ technique for the delivery of transgenes

into growing tissues. This method remains the foremost of direct DNA transfer in plant biotechnology (James, 2003; Altpeter et al., 2005).

Particle bombardment method is also useful in biotechnological transformations involving plant viruses. This technique is employed in the transmission of poloeviruses (Hoffman et al., 2001). Particle bombardment is usually the method of choice for the inoculation of leaf tissues and whole plant with viruses that are hard to introduce through conventional mechanical infection. It also allow direct introduction of infectious viral DNA/RNA into a variety of plant species (Briddon et al., 1998; Altpeter et al., 2005). This study was initiated to develop an effective technique for precise infection and testing the pathogenicity of a begomovirus that infects horsegram. This is necessary as begomoviruses cannot be inoculated mechanically. The study involved PCR amplification of a begomovirus infecting horsegram, generating full-length infective clones of HgYMV, also testing the infectivity of HgYMV using particle bombardment method.

Materials and methods

Plant material and DNA extraction

The horsegram symptom bearing leaves were collected from infected plants in two areas of Bangalore, Karnataka State of India and stored at -80 °C freezer at NRI, University of Greenwich London. Leaves of horsegram plant showing symptoms typical of yellow mosaic disease were used in this study. Total DNA was extracted from the dried plant samples by using a CTAB extraction buffer according to the manufacturer's instruction (Maruthi et al., 2002).

Virus detection using Degenerate and specific primers

DNA template for PCR was prepared from Leaves of infected Horsegram plants. Amplification of begomovirus genome was proceeded using a pair of degenerate primers designed for the amplification of the DNA A and DNA B genomic components, Deng-A (5'TAATATTACCKGWKGVCCSC3') and Deng-B (5' TGGACYTTRCAWGGBCCTTCACA3') (Deng et al., 1994). To amplify DNA-A genomic component, two consensus outwardly extending primers were designed in the satellite conserved region (SCR) (HYMV-A 1500F, **5'CTGCAG**TGATGTTGTCCCCKG3'; HYMV-A 1500R, 5' **CTGCAG**CTCAACTCAGGARTGG3'). For DNA-B component, outwardly extending primers (D_HYMV-B2200F, 5'**GAATTC**ATGAATAAATGGCCG3'; D_HYMV-B2200R, 5'**GAATTC**ATAATTCCCTTGTCATG3') were designed from the conserved region of DNA. The PCR was run according to Abubakar et al. (2018). PCR products were then analysed by electrophoresis in 1 % agarose gels in Tris-buffer EDTA.

Elution of DNA from agarose gel

DNA elution from the gel, and purification were done using QIAGEN gel extraction kit protocol (Qiagen, UK).

Ligation and cloning

The pGEM^{R}-T vector and pGEM^{R}-T vector control tubes were centrifuged to collect contents at the bottom of the tube. A ligation reaction was set up and incubated for 2 hr at 37 ^{0}C. A 2 l of the ligated product was used for the transformation step.

Transformation of ligated PCR:pGEM^{R}-T vector

Two duplicate sets of LB/amp/X-gel plates were prepared for each ligation reaction. The plates were equilibrating to room temperature prior to plating. The tube containing the ligated PCR product:pGEM^{R}-T vector reaction was centrifuged to collect contents at the bottom of the tube. A 2 l of the ligated PCR product pGEM^{R}-T vector was added to sterile 1.5 ml microcentrifuge tube on ice. Then it was mixed by gently flicking the tube. 50 l of cells were carefully aliquot into each tube prepared in step 2. Note: Cells are highly fragile. The tubes were gently flicked to mix on ice for 20 min. The cells were heat shocked for 45- 50 seconds at exactly 42 ^{0}C. The tubes were returned to ice for 2 min. LB broth was added to the tubes (approximately 1,500 bp PCR products: 450 l LB; 500bp: 1.4ml LB). The tubes were gently inverted to mix and incubated for 1 hr at 37 ^{0}C.

Culturing of transformed cells

100 l of the transformation culture was spread

on one set of duplicate plates containing ampicillin (125 g/ ml) and X-gal (100 g/ ml) and on the other set, 100 l of the transformation culture was spread. It was incubated for at least 18 h at 37 °C and then at 4 °C for about 5-10 h to allowed clear development of blue colonies. Single white colonies were picked from the plates and inoculated into 3 ml LB broth containing ampicillin (250 g/ml). Colonies were grown overnight at 37 C on a shaker at 220 rpm.

Extraction of plasmid DNA from E. coli

The extraction of recombinant plasmid DNA from the bacterial cells also called "minipreparation" was performed by using QIAGEN plasmid miniprep kit according to the manufacturer's recommended procedure.

Screening of transformants for inserts

The amplified DNA-A and DNA-B of HgYMV1 and HgYMV2 were cloned into the plasmid vector pPGEMT-Easy (Promega, UK). The resulting clones were verified by restriction analysis, PCR and sequencing. The presence of inserts was checked by PCR using the primers HgYMV-A 1500F and HgYMV-A 1500R for DNA-A, and D_HgYMV-B2200F and D_HgYMV-B2200R for DNA-B.

Biolistic inoculation of cloned DNA-A and DNA-B genomic components

Test plants were grown in an insect proof glasshouse under ambient temperature of 26 C and a minimum 50 % relative humidity. In order to test the infectivity of HgYMV both DNA-A and DNA-B were inoculated onto 30 plants of the *N. benthamiana* species that were at the 3-5 leaf stage (for both biolistic and mechanical transmission procedure). DNA-A(pGEM^{R}-T) and DNA-B(pGEM^{R}-T) were inoculated together, linearized DNA-A and DNA-B construct were inoculated together, also undigested DNA-A and DNA-B construct were inoculated together.

Preparation of DNA and gold particles for use in gene gun

25 mg of gold particles (size 95) was weighed out in a 1.5ml eppendorf tube. 20 g of DNA was prepared in a 20 l volume TE buffer. DNA was added to the gold particles, and 25 l Xho buffer was added and Vortexed. 25 l of 0.1 M spermidine was added and Vortexed. 25 l of 25 % PEG M.W was added and centrifuged at 1300-1600 rpm. 25 l of 2.5 M $CaCl_2$ was added while vortexing. The mixture was incubated at room temperature for 10 min. Pulse in centrifuge – 20sec at max speed (13000 rpm). Supernatant was removed and discarded. The pellet was resuspended in 70 l of 100% ethanol (absolute ethanol) and sonicated briefly. The mixture was also centrifuged on pulse. 1 ml of 100% ethanol was added to a sterile scintillation vial. Gold pellet was resuspended in 100% ethanol from the final 10 ml volume and transferred gold to scintillation vial. Then it was sonicated briefly and stored at -20 ^{0}C until further use.

Shooting DNA into plants

The Tebzel plastic tube was laid (Natural Tebzel tubing 1/8"OD x 3/32" ID) on the back of a tray and secured with a tape. 1.5 cm tube was used per shot/ cartridge. The gold solution was sonicated briefly. The gold solution was injected into the Tebzel tubing using 2 ml syringe and needle. Gold particles coated with virus were allowed to settle for 30 min and it formed a visible precipitation line at the bottom. Ethanol was removed carefully by placing tissue at one end of the tube and drawing the ethanol out by absorption. The tube was left overnight for drying at room temperate and loaded into the cassette No. 1 and cut the excess tubing. Likewise the entire tube was loaded into several cassettes. The cassette was placed at the top of the gun and secured. The gun was plugged into the power supply and connected to the helium hose. The main tank valve was opened anticlockwise. The regulator valve was opened clockwise until the gauge reads 150 psi (Max 300 psi). The gun was placed at point blank range at underside of the youngest expanding leaf and shot. Finally, the main tank vale was closed clockwise and the regulator anticlockwise, the gun was shooting several times until the regulator gauge reads zero. *N. benthamiana* plants were incubated in the glasshouse for at least 15 days for symptom expression.

Results

Total DNA extraction from virus's infected leaves and PCR amplification

Total DNA was extracted from infected leaf tissue and the PCR was used to amplify various HgYMV DNA fragments using the

primer sequences shown in method section. The primer pair Deng A and Deng-B (degenerate primer) was used to amplify from the intergenic region (IR) to the coat protein gene, respectively. PCR failed to amplify expected bands from samples during initial DNA extractions (see Figure 1A). Reasons for this are many including the presence of inhibitors in the sample used for DNA extractions. The condition was optimised by modifying the CTAB-based DNA extraction of Lodhi et al. (1994). We circumvent the grinding in liquid nitrogen, the use of phenol and CsCl density gradient purification steps. The choice of the leaf tissue is important for DNA isolation. The use of matured leaves of horsegram has resulted in samples with inhibitors. Use of young leaves produced DNAs of sufficient quality to use in PCR.

Upon optimisation higher yields of clean DNA was obtained from horsegram leaves. The amplification in PCR using Deng et al (1994) primers indicated the successful DNA extraction and PCR amplification shown in Figure 1B. These results confirmed the infection of horsegram by a begomovirus.

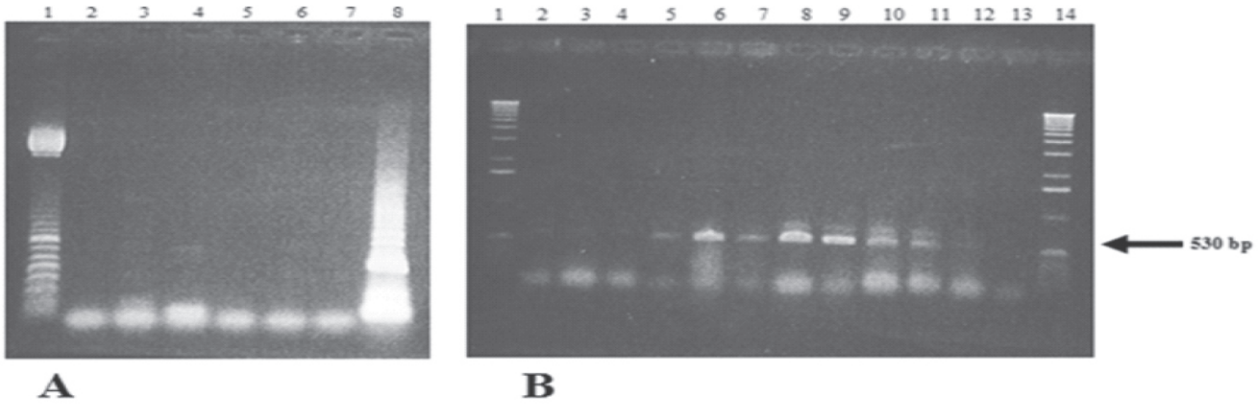

Fig. 1: (A) Gel photograph of PCR products generated using degenerate primer, Deng et al. (1994) primers showing the failed detection of begomovirus in different crop plants. The photograph indicated the absence of DNA in the samples analyzed. **(B)** Gel photograph of PCR products generated using degenerate primer, Deng et al. (1994) primers showing successful detection of begomovirus in different crop plants. Lane 1: ACMV (African cassava mosaic virus) +ve control, Lane 2: LYMV (diluted), Lane 3: FYMV (diluted), Lane 4: HgYMV (diluted), Lane 5: HgYMV (diluted), Lane 6: LYMV, Lane 7: FYMV, Lane 8-12: HgYMV, Lane 13: negative, Lane 1 and 14: are the sizes of marker as shown on the left and right of the figure.

Full-length DNA-A and DNA-B components

Expected PCR products of approximately 2.8 Kb were amplified, which further confirmed the presence of begomovirus in horsegram plants showing mosaic symptoms. In addition, these results indicated that the horsegram yellow mosaic disease was caused by a bipartite begomovirus which has both DNA-A and DNA-B components. Further confirmation of the presence of right size and type of inserts was confirmed following restriction digestion with PStI and EcoRI endonucleases for DNA-A and DNA-B clones, respectively. The products of restriction digestion separated on gel photographs are shown in Fig. 2.

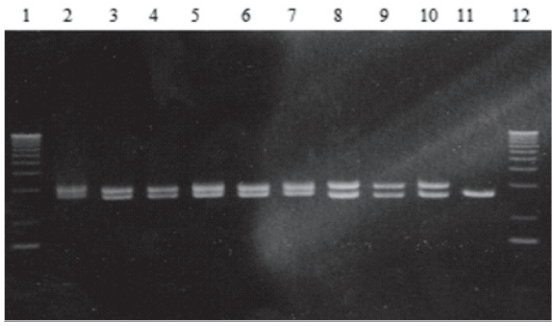

Fig. 2: Gel photograph clones digested with PstI endonuclease which confirmed the presence of full-length DNA-A insert in the clone. Lanes 2-11 represent bacterial colonies with insets of the DNA-A of HgYMV. Lanes 1 and 12 are the sizes 1-Kb molecular weight marker.

Sequencing the complete genome of HgYMV1 and HgYMV2 isolates

The complete genome of HgYMV isolate infecting horsegram was amplified by PCR. Phylogenetic of virus sequence of HgYMV begomovirus sequences were compared with those sequences of selected reference begomoviruses sequence from the gene bank. The sequences were aligned using the cluster method and the dendograms were reported in Abubakar et al. (2018).

Testing the infectivity of HgYMV in N. benthamiana

These experiments were conducted to verify whether the cloned constructs are capable of causing an infection in *N. benthamiana*, which would confirm their functional activity as a virus or whether heterologous components (DNA-A and DNA-B) of different virus types can effect infection in the plants.

The DNA-A and DNA-B of HgYMV genome were mixed and bombarded directly to *N. benthamiana*. Plasmid constructs were inoculated to *N. benthamiana* either as full-length clones excised from plasmid pGEM-T (PStI DNA-A and ECoRI for DNA-B), as linearized plasmid constructs and as undigested double stranded DNA. The results of this investigation are shown in Table1.0. Three different combinations of DNA-A and DNA-B of HgYMV strain inoculated by biolistic inoculation, resulted in having no symptom of infections of HgYMV in *N. benthamiana* 15 days post inoculation (Fig. 3). No symptoms were observed for mechanical innoculation; therefore the results were identical to those obtained with the particle bombardment procedure (data not shown).

Table 1: Biolistic inoculation of *N. benthamiana* with HgYMV linear, ligated and undigested DNA-A and DNA-B genomic components.

Inoculum	Amount of HgYMV DNA per plant (μg)	Number of plants infected/inoculated
Linearized DNA -A and DNA -B	20	0/10
Ligated DNA -A and DNA -B	20	0/10
Undigested DNA -A and DNA -B	20	0/10

Fig. 3: (A) *N. benthamiana* plants (bombarded using Helios gene gun, and mechanically inoculated) with DNA-A and DNA-B genomic components of HgYMV1 and HgYMV2 after 2 weeks of transplanting. **(B)** *N. benthamiana* plants showing no symptoms of HgYMV 15 day after bombardment with Helios gene gun.

Discussion

HgYMV were detected successfully using degenerate primers Deng A/B in the diseased samples. The inability to detect an HgYMV initially may be due to several reasons including the present of inhibitors such as polyphenols, polysaccharides, lipids and proteins. These inhibitors could have resulted in a negative PCR. There is also the possibility of single mismatch at the 3' end of the primers, which can result in negative PCR. The CTAB-based DNA extraction was optimization by modifying procedure from Lodhi et al. (1994). However, using Deng primers A/B indicated the presence of begomovirus DNA in horsegram plants. This indicated that PCR-screening of samples using Deng A/B primers alone would detect most of begomoviruses and was probably sufficient for the detection of begomoviruses. Very efficient methods for extracting DNA and RNA from roots and shoots of plants were reported in previous studies (Bekesiova et al., 1999; Rezadoost et al., 2016).

The genomes for two previously characterized bean-infecting begomoviruses from India, HgYMV1 and HgYMV2, have been cloned and completely characterized. Full-length infectious clones of DNA-A and DNA-B component for HgYMV1 and HgYMV2 were each cloned from symptomatic horsegram plant. Analysis of their genome sequences revealed that they are strains of begomovirus (see Abubakar et al., 2018).

The result of Helios gun delivery based transmission of HgYMV1 and HgYMV2 isolates as well as mechanical inoculation to *N. benthamiana* was relatively poor. None of the 50 *N. benthamiana* plants inoculated using both particle bombarded and mechanical methods produce HgYMV symptoms after two weeks of inoculation.

In all inoculated cases, no symptoms of HgYMV were visible in *N. benthamiana* that resembles infections of *N. benthamiana* with wild type viruses. This occurs as a result of additional restriction site in DNA-A clone, which may result to cleavage of bases in the DNA sequence. Restriction (cleavage) of bases will cause the conformational changes in DNA sequence. Therefore, since the confirmation of DNA-A in plants inoculated with cloned DNA was not pursued, it is likely that only DNA-B was present in inoculated plants replicating without DNA-A (which is responsible for symptoms development). The experiment conducted by Tuttle et al. (2012) shows that agro-inoculation resulted in efficiency and extent of silencing compared to biolistic inoculation.

The DNA-A of most *begomoviruses* has characteristics of monopartite and bipartite begomovirus species. In the absence of DNA-B, DNA-A can induce yellow mosaic on *N. benthamiana*. DNA-A alone is infectious and produces mild and delayed symptoms on *N. benthamiana*, the association of DNA-B increases symptom severity and also considerably shorten the incubation period. In the experiment conducted by Chakraborty et al. (2003) both ToLCGV-[Var] and *Tomato leaf curl Thailand virus*, DNA-A alone was shown to be infections, but DNA-B increased symptom severity. Therefore, the additional restriction in the DNA-A of HgYMV clones inoculated in this study, could not produce the symptom. Previous study shows that the *Agrobacterium* vectors were better than the biolistic VIGS vectors and hence contain less unique restriction sites for cloning in silencing fragments (Tuttle et al., 2012).

Conclusion

The most intriguing fact of this study is that, in most experiments such as PCR amplification, cloning and ligation, restriction digestion and sequence analyses we were able to detect the virus, but after particle bombarded and mechanically inoculated to *N. benthamiana* leaves, we did not found any symptoms. However, *N. benthamiana* plants inoculated gave inconsistent results, although amounts of HgYMV DNA-A and DNA-B used for each analysis were precisely quantified. These poor results could be explained by an additional restriction site in the DNA-A clone.

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