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Molecular Characterization of DNA-A Component of Horse Gram Yellow Mosaic Virus (HgYMV) from Southern India

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A B S T R A C T

PCR was employed to establish association of begomovirus through amplification of geminivirus specific PCR product. In order to determine the complete nucleotide sequence of DNA-A component of HgYMV, several universal primers/ abutting primers and specific primers available in the literature were tried to amplify full length DNA-A of 2.7 kb. The amplification of full length of DNA-A component of HgYMV was achieved with all the primers such as AC-abut and AV-abut, HgYMVAF and HgYMVAR and HYMV-A1500F and HYMV-A1500R. Virus specific DNA fragments of approximately 2700 bp were obtained from DNA of infected horsegram samples. No PCR product was obtained from DNA extracted from healthy samples and water control. An annealing temperature of 55º C for 2 min was found suitable for amplification of full length of DNA-A component of HgYMV. The PCR product (approx. 2700 bp) of DNA-A component of HgYMV amplified with HgYMVAF and HgYMVAR primers was cloned, sequenced and assembled and its length was determined as 2654 nucleotides. The nucleotide sequences of DNA-A component of horsegram yellow mosaic virus were compared with those of selected begomoviruses obtained from the NCBI database. Comparison of the complete DNA-A sequence of HgYMV-GKVK-Bangalore with other bipartite begomoviruses revealed 82-84 per cent identity with isolates of Mungbean yellow mosaic virus (MYMV), 78-81 per cent with Mungbean yellow mosaic India virus (MYMIV), 98 and 95 per cent identity with the accessions of horsegram and frenchbean isolates of HgYMV from Tamil Nadu and Srilanka, respectively. The phylogenetic tree built using the DNA-A of HgYMV-GKVK-Bangalore showed three clusters, with HgYMV-GKVK-Bangalore falling in cluster II, Mungbean yellow mosaic virus (MYMV) isolates in cluster I and Mungbean yellow mosaic India virus (MYMIV isolates) in cluster III. Analyses of HgYMV-DNA-A-GKVK-Bangalore isolate sequence showed typical features of bipartite begomoviruses characterized by six conserved open reading frames in DNA-A.

Introduction

Horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.) popularly known as poor man's pulse crop, is a hardy legume which belongs to family Leguminosae and sub-family Papilionaceae. Horsegram is an indigenous plant cultivated in India, extensively grown in dry areas of Karnataka, Tamil Nadu, Maharashtra, Andhra Pradesh, Madhya Pradesh, Orissa, Chattisgarh, Bihar, Himachal Pradesh, West Bengal, Jharkhand, Rajasthan and Gujarat (Khedar *et al.,* 2008). Horsegram is a popular pulse crop of Karnataka, grown in districts like Mysore, Mandya, Chamarajnagar, Tumkur, Hassan, Bijapur, Kolar, Chitradurga and Koppal districts.

Important diseases affecting horsegram are Powdery mildew (*Erysiphe polygoni*), Dry root rot (*Macrophomina phaseolina*), Anthracnose (*Colletotrichum lindemuthianum*), Rust (*Uromyces phaseoli typica*), Leaf spot (*Cercospora cruenta*) and Cottony stem rot (*Sclerotinia sclerotiorum*). Among viral diseases, yellow mosaic virus is one of the major constraints for its cultivation in peninsular India and was first observed in southern districs of Karnataka. Horsegram yellow mosaic disease transmitted by white fly, *Bemisia tabaci* (Gennadius) was prevalent in most parts of South India (Muniyappa and Reddy, 1976; Muniyappa *et al.,* 1975 and Williams *et al.,* 1968). The disease incidence ranged from 50 to 100 per cent in both summer and early rainy season crops causing substantial loss in grain yield (Muniyappa *et al.,* 1975). The increasing spread of the horsegram yellow mosaic disease due to increase in *Bemisia tabaci* population resulted in almost complete loss of the crop during summer (Muniyappa *et al.,* 1978).

Horsegram yellow mosaic disease is shown to be caused by the genus Begomovirus belonging to family Geminiviridae. Geminiviruses are small plant viruses characterized by 16-18 nm x 30 nm geminate particles consisting of two joined incomplete icosahedra encapsidating either monopartite or bipartite circular single stranded (ss) DNA genome molecules of about 2700 nucleotides (Harrison *et al.,* 1977). They form the second largest family, Geminiviridae of plant viruses. Geminiviruses have been grouped into four genera- Mastrevirus, Curtovirus, Topocuvirus and Begomovirus depending on their vector, host range and genome characteristics.

Viruses of the genus begomovirus typically have bipartite, circular, covalently closed ss DNA molecules (DNA-A and DNA-B) each of about 2.6-2.8 kb, single stranded DNA (ssDNA) genomes. All the functions required for replication, control of gene expression and encapsidation coded on DNA-A and genes involved in intra and intercellular movement are coded on DNA-B (Harrison *et al.,* 1977).

The symptoms appeared as faint yellow discoloration on the young leaves in the beginning. As the disease progressed, the leaves showed mosaic mottling. The mottles were irregular, small, greenish yellow in colour and intermixed with normal green patches. Later on, the mottles become enlarged and turn bright yellow and eventually become completely bleached. Severe infection led to stunted growth of the plant and reduction in the leaf size (Muniyappa *et al.,* 1976 and Prema, 2013).

Horsegram which is grown extensively throughout the year in Karnataka, is more susceptible to yellow mosaic disease transmitted by whitefly vector, *Bemisia tabaci*. The first report of the yellow mosaic disease of horsegram was by Williams *et al.,* (1968). Later preliminary electron microscopic studies revealed geminate particles (Muniyappa *et al.,* 1987) from the infected leaves. Swanson *et al.,* (1992)

showed that monoclonal antibodies raised against African cassava mosaic virus reacted with extracts from yellow mosaic diseased horsegram leaves, indicating the geminivirus origin of the disease. Literature is also available on epidemiology (Muniyappa *et al.,* 1978), virus-vector relationships (Rajkumar, 2006) and sources of resistance to HgYMV (Rajkumar *et al.,* 2009; Parimala *et al.,* 2011; Prema *et al.,* 2013; Prema and Rangaswamy, 2017). At present, information is not available on HgYMV at genomic level from Karnataka and also its phylogenetic relationship with other YMV isolates associated with other legumes. So, the present work describes the molecular characterization of the DNA-A component of HgYMV by cloning and sequencing of full genome.

Materials and Methods

Sample collection

Horsegram plants showing severe yellow mosaic and mottling symptoms were collected from field at the Zonal Agricultural Research Station, GKVK, University of Agricultural Sciences, Bengaluru, Karnataka (Plate 1a-1e). Samples from healthy plants were collected as controls.

Nucleic acid extraction

The total genomic DNA was extracted from leaf tissues of healthy horsegram plants and YMV infected horsegram plants based on the method of Rouhibakhsh *et al.,* (2008). One hundred and fifty milligrams of fresh YMV infected leaf tissues were ground with liquid nitrogen using sterile pestle and mortar. The whole ground sample was transferred into a fresh 1.5-ml eppendorf tube. 1500 µl of prewarmed (65° C) DNA extraction buffer was added to ground sample taken in 1.5-ml eppendorf tube (added in situ just before DNA extraction). The whole crude sap was incubated for 30 min at 60° C in a water bath with occasional mixing. The supernatant (750 µl) was transferred into a fresh 1.5-ml effendorf tube and mixed with equal amount (750 µl) of Phenol: chloroform: isoamyl alcohol (25: 24:1) by vertexing. The samples were then centrifuged at 13,000 rpm for 10 min using micro centrifuge. The aqueous supernatant was collected in to a fresh 1.5-ml eppendorf tube. The DNA was precipitated by mixing with 300 µl of chilled isopropanol + 30 µl of 7.5 M Ammonium acetate by inversion. The tubes were centrifuged at 13,000 rpm for 10 min. The resulted pellet was washed with 70 per cent ethanol, dried in a vacuum drier for 10 min and re-suspended with 40 µl of $T_{10}E_{0.1}$ buffer (10 mM Tris-HCl of pH 8.0 and 0.1 mM EDTA of pH 8.0) and stored at -20° C. All the DNA extracts were further diluted from 1:10 to 1:40 in single distilled water (SDW) before using for PCR amplifications. The quality and quantity of DNA was assessed at 260 nm and 280 nm using UV spectrophotometer.

Primers used, PCR amplification and gel electrophoresis

In order to determine the nucleotide sequence of DNA-A component of horsegram yellow mosaic virus, specific primers available in the literature were tried to amplify DNA-A component of yellow mosaic viruses of nearly 2700 bp (Table 1). Amplification was performed in Thermocycler (Eppendorf Mastercycler gradient, Hamburg, Germany) programmed for one step of initial denaturation at 94º for 2 min and 35 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºCfor 2 min for primers and extension at 72 ºC for 3 min, followed by one step of final extension at 72 °C for 10 min. PCR was conducted with Dream Taq Master mix (Fermentas) in total reaction mixture volume of 25 µl that contained Dream Taq Master mix-13 µl; dH2O-4 µl; forward and reverse

primers (20 pmole/µl)-2 µl each; DNA template (total nucleic acid-100ng/µl)- 4 µl and PCR products were subjected to electrophoresis in 1% agarose at 50 V for 45 minutes in Electrophoresis system - SCOTLAB (Anachem Ltd.) in Tris-acetate-EDTA buffer containing ethidium bromide @ 0.1%. The gel was observed under Gel Documentation System (IMAGO Compact Imaging System, B & L Systems, Isogen Lifescience, The Netherlands).

Cloning and sequencing of DNA-A component of YMV infecting horsegram

The PCR products were purified from agarose gel using Qiagen Gel Extraction kit (Qiagen, Hilder, Germany). All amplicons were cloned into the plasmid vector pTZ57R/T using InsTAcloneTM PCR Cloning Kit following the manufacturer's instructions. Transformed colonies were screened and selected on LB agar medium amended with ampicillin, X-gal and IPTG. Isolated plasmids from transformed positive clones were confirmed for the presence of insert using the respective DNA-A component specific primers. The resultant positive clones were fully sequenced in both directions using universal M13 forward and reverse primers. Full length sequence of DNA-A component of HgYMV was obtained by aligning of forward and reverse reaction sequences (Sambrook and Russel, 2001).

Phylogenetic analysis, nucleotide and amino acid sequence comparison of DNA-A component of HgYMV with other geminiviruses

Pairwise and multiple sequence alignment of the full length sequence of DNA-A component of HgYMV was done using MEGA 5.1 multiple alignment tool (Tamura *et al.,* 2007). The phylogenetic neighbor-joining trees and evolutionary analysis were conducted using MEGA 5.1 software package based on DNA-A component sequence of HgYMV with 23 other geminivirus sequences downloaded from NCBI Genbank (Table 2). Robustness of trees was determined by bootstrap sampling of multiple sequence alignment with 1000 replications. Comparison of the nucleotide and amino acid sequences of YMV was analysed by using sequence identity matrix tool of Bio-Edit software (Version 7.9.1).

Finding genes and ORFs

Locations and lengths of genes/ ORFs/ CDS were detected and analyzed using online tool "ORF Finder" at www.ncbi.nlm.nih.gov and further confirmed by online tool GENSCAN. The "ORF Finder" was a graphical analysis tool which finds all ORFs of a selectable minimum size in a sequence. This tool identified all ORFs using the standard or alternative genetic codes.

Results and Discussion

Standardization of viral DNA isolation method from horsegram infected by yellow mosaic virus

In the present study, PCR technique for detection of yellow mosaic virus infecting different legumes was standardized by modifying the DNA extraction method as no viral DNA was detected with the standard CTAB DNA isolation protocol suggested by Lodhi *et al.,* (1994) and Maruthi *et al.,* (2002). The modified method involved extraction with a modified CTAB buffer containing 0.65% Sodium sulphite, 2% PVP-40 and 1% SDS. Using this method, PCR amplifiable DNA could be extracted from mature leaves of legume hosts rich in polyphenols, tannins and polysaccharides. It was very interesting to observe that DNA obtained by CTAB method which has been

routinely used for isolating DNA for PCR detection of geminivirus did not support the amplification of yellow mosaic viral DNA. Phenolic compound and glycoprotein present in the plant appears to inhibit amplification of viral DNA or affect the quality of DNA suitable for PCR. The yellow mosaic viral DNA amplification was possible and reproducible with this modified method. This protocol has been used by many workers to isolate replicative forms of geminivirus for direct cloning of gemini viral DNAs. The geminivirus specific product was not detected with template DNA isolated by normal CTAB method. This showed that quality of DNA extracted by modified method was superior for PCR detection of virus causing yellow mosaic as compared to other two common plant DNA extraction protocols. The results of the present study revealed that the protocol described here is thus useful for obtaining good quality viral DNA from legumes for PCR detection of the virus.

Detection and differentiation of begomoviruses by serological methods using polyclonal and monoclonal antibodies were often met with problems in tropical legumes (Swanson *et al.,* 1992). Begomoviruses were well suited for PCR based detection and identification as they replicate via a double stranded DNA intermediate, which can readily be a template for amplification. Isolation of begomoviral DNA by CTAB method with minor modifications has been recommended by several workers (Briddon and Markham, 1994; Deng *et al.,* 1994; Mansoor *et al.,* 1999; Jose and Usha, 2003; Rothenstein *et al.,* 2005). However, these methods were not applicable to legume hosts as jelly like insoluble precipitate bind to DNA pellet.

A relatively inexpensive protocol for the detection of genomic components of whitefly-transmitted begomoviruses in symptomatic legumes in the field was described by Rouhibakhsh *et al.,* (2008). The method involved extraction with a modified CTAB buffer containing mercaptoethanol upto 5 per cent and sodium chloride concentration from 1.4 to 2.0 M. Using this method PCR amplifiable DNA could be extracted from mature leaves of legume hosts rich in polyphenols, tannins and polysaccharides. The non-coding region and full-length DNA-A, DNA-B components of yellow mosaic viruses were consistently amplifiable from 97 samples, out of 136 tested in PCR reaction, employing primers specific for intergenic regions and full-length genome. The system was robust and the protocol would be useful for the detection and identification of begomoviruses infecting grain legumes.

Standardization of PCR protocol for detection of DNA-A component of horsegram yellow mosaic virus

The total DNA isolated from infected horsegram samples was used for polymerase chain reaction. PCR was employed to establish association of begomovirus through amplification of geminivirus specific PCR product. Various dilutions i. e., 1:10 to 1:40 were prepared and subjected to PCR. The PCR results indicated that the virus could be detected from 1:20 to 1:40 dilutions. In order to determine the complete nucleotide sequence of DNA-A component of HgYMV, several universal primers/ abutting primers and specific primers available in the literature were tried to amplify full length DNA-A of 2.7 kb. The amplification of full length of DNA-A component of HgYMV was achieved with all the primers such as AC-abut and AV-abut, HgYMVAF and HgYMVAR and HYMV-A1500F and HYMV-A1500R. Virus specific DNA fragments of approximately 2700 bp were obtained from DNA of infected horsegram samples. No PCR product was obtained from DNA extracted from healthy

samples and water control (Plate 2 and 3). An annealing temperature of 55º C for 2 min was found suitable for amplification of full length of DNA-A component of HgYMV. This PCR protocol amplified HgYMV specific fragment of 2700 bp from infected horsegram samples but not from healthy sample and water control.

PCR detection of horsegram yellow mosaic virus was standardized by using the PCR conditions suggested by Rouhibakhsh *et al.*, (2008). The repeated and reproducible viral DNA amplification (2700 bp) was obtained only with the annealing temperature of 55º C for 2 min by using horsegram yellow mosaic virus specific oligonucleotide primers as well as universal primers. This indicated that the causal agent of horsegram yellow mosaic virus is a begomovirus. Different workers viz., Rouhibaksh *et al.,* (2008), Anburaj *et al.*, (2010) and Maruthi *et al.,* (2006) have designed different set of primers to amplify full genome of DNA-A from different legumes.

Full-length DNA-A component of dolichos yellow mosaic virus by polymerase chain reaction was amplified by PCR using a set of abutting primers designed to anneal around nucleotide position 1500. Expected PCR products of size-2.8 kb were obtained from the primers [DYMV-A&B70F and DYMV-A&B70R], (Maruthi *et al.,* 2006). Full-length amplification of DNA-A of HgYMV-[IN:Coi] was achieved by a pair of abutting primers (HgYMVAF and HgYMVAR) by PCR amplification (Anburaj *et al.,* 2010). Begomovirus degenerate primers UPV1/UPC2 specific for DNA-A were used in PCR (Shahid *et al.*, 2012). A PCR product of the expected size (approximately 2.6 kb) was amplified from all symptomatic plants and no amplification products of the expected size were obtained from healthy or asymptomatic plants, confirming the association of a begomovirus with the disease.

The whitefly transmitted geminiviruses of the genus begomoviruses are important pathogens of vegetables and fibre crops in subtropical and tropical agro-ecosystems. Because of the dramatic increase in population densities of *B. tabaci* in 1970s (Bird and Maramorosch, 1978) and later establishment of B-biotype of *B. tabaci* in USA and elsewhere (Brown and Bird, 1992), begomoviruses have become recognized as emerging pathogens (Brown, 1994). The plethora of new and uncharacterized begomoviruses isolated from diverse locations worldwide necessitates the development of an accurate and simple method for their rapid and accurate identification. Serology is not suitable for begomovirus characterization because high titre antisera are difficult to prepare and lack sufficient specificity. Consequently, the DNA based approaches including PCR has supplemented serology for detection, identification and classification of begomoviruses. So, a PCR method that permits sensitive and accurate detection of YMV infecting horsegram was optimized in this study.

Cloning, sequencing and phylogenetic analysis of DNA-A component of HgYMV

The PCR product (approx. 2700 bp) of DNA-A component of HgYMV amplified with HgYMVAF and HgYMVAR primers was cloned into plasmid vector pTZ57R/T and transformed using *E. coli* (Figure 1 and 2). The insert in the vector was confirmed by colony PCR (Plate 4a and 4b). Plasmids were isolated and sequenced bi-directionally to ensure sequence identity and reliability and the sequences. The DNA-A sequences were assembled and its length was determined as 2654 nucleotides.

Currently, there are at least two sequences available that showed a close identity of 98-95 per cent with HgYMV-GKVK-Bangalore isolate. Phylogenetic analyses clearly

indicated that DNA-A component of HgYMV-GKVK-Bangalore, HgYMV-Tamil Nadu-HG and HgYMV-Srilanka:FB have close relationships. The phylogenetic analysis of the DNA-A component of HgYMV-GKVK-Bangalore showed three clusters, with HgYMV-GKVK-Bangalore falling in cluster II, Mungbean yellow mosaic virus (MYMV) isolates in cluster I and Mungbean yellow mosaic India virus (MYMIV isolates) in cluster III (Figure 3). This showed that these sequences were highly different from remaining sequences. The frequent recombination among geminiviruses could be the reason behind diversity and emergence of new geminiviruses (Fauquet *et al.,* 2003).

The virus causing horsegram (*Macrotyloma uniflorum*) yellow mosaic disease has been shown to be a typical Old World bipartite begomovirus (Anburaj *et al.,* 2010). Full-length amplification of DNA-A of HgYMV-[IN:Coi] was achieved by a pair of abutting primers (HgYMVAF and HgYMVAR). A pair of abutting primers (HgYMVBF and HgYMVBR) was designed and the full-length DNA-B of HgYMV-[IN: Coi] was amplified. The amplicon was cloned into pTZ57R (MBI Fermentas) and the clone was sequenced.

The complete viral genome was obtained from Horsegram yellow mosaic virus (HgYMV:FB) sample using specific PCR primers. Component-A contained 2735 nucleotides and component-B contained 2669 nucleotides. Component A shared between 95.1 and 96.6 per cent, and component-B between 93.5 and 96.3 per cent sequence identity with the four available genomes in GenBank (Monger *et al.,* 2010). HgYMV has been identified in India where it was found to infect various legumes: bambara groundnut, french bean, groundnut, lima bean, mungbean, pigeon pea, soybean and horsegram (Muniyappa *et al.,* 2008).

Complete DNA-A components of DoYMV isolates from Mysore and Bangalore, South India, were sequenced, but several attempts to identify DNA-B were unsuccessful. DoYMV isolates shared DNA-A nucleotide identities of 92.5-95.3 per cent with previously described isolates from North India and Bangladesh. They were most similar to mungbean-infecting begomoviruses at 61.6–64.4 per cent of DNA-A nucleotide identities. Phylogenetic analyses of DNA-A sequences grouped the dolichos-infecting and mungbean infecting begomoviruses into a distinct cluster away from begomoviruses infecting nonleguminous plants in the Indian subcontinent (Maruthi *et al.,* 2006).

The components of a begomovirus causing cowpea golden mosaic disease (CGMD) in western India were isolated, cloned and sequenced (John *et al.,* 2008). Aliquots of replicative form DNA were digested with a range of restriction endonucleases to identify single-cutting enzymes giving linear products of approximately 2,700 nucleotides in length, which were cloned into suitably digested pUC18. Two clones, one each of the potentially full-length DNA-A and DNA-B (produced using restriction enzymes KpnI and BamHI, respectively) were selected for further analysis. A phylogenetic dendrogram, based on an alignment of the complete DNA-A sequences of the majority of legume-infecting begomoviruses showed that DNA-A of Anand 25 to segregate with the MYMIV sequences. However, together with another isolate originating from cowpea (MYMIV-[IN:Var:Cp]) and a clone originating from Bangladesh (MYMIV-[BD:98] form a distinct cluster basal to the remaining MYMIV DNA-A sequences indicating that Anand 25 is an isolate of MYMIV.

A begomovirus associated with yellow mosaic disease in lima bean (*Phaseolus lunatus*) was cloned, sequenced by Shahid *et*

al., (2012). The virus has a bipartite genome of which DNA-A had 2745 nucleotides and DNA-B had 2669 nucleotides and had a typical bipartite begomovirus genomic organization from the Old World. The sequences showed the highest levels of nucleotide identity (99.2 % for DNA-A and 98.9 % for the DNA-B) to the 'Indian' strain of Mungbean yellow mosaic India virus (MYMIV). Complete sequences of 44 components (23 DNA-A, 19 DNA-B and 2 betasatellites) were determined by Muhammad Ilyas *et al.,* (2010). Sequencing of the three DNA-A and two DNA-B clones thus obtained confirmed infection by three distinct begomoviruses: bean golden mosaic virus, *Sida micrantha* mosaic virus and okra mottle virus, the last of which was reported recently to be a novel virus infecting okra plants in Brazil (Fernanda *et al.,* 2009).

The frequent recombination among geminiviruses could be one of the reasons behind the diversity and emergence of new geminiviruses. Full-length DNA-A sequence analyses of more than 200 viruses have led to a guideline to identify a begomovirus species (Fauquet *et al.,* 2003). An analysis established that legume yellow mosaic viruses are genetically isolated begomoviruses (Javaria Qazi *et al.,* 2007).

Grain legumes and pulses were affected by yellow mosaic virus disease caused by whitefly transmitted begomoviruses. The affected host plants in India were horsegram, frenchbean, soybean, limabean, mungbean, blackgram, cowpea, fieldbean and pigeonpea (Varma *et al.,* 1992). It is not yet clear that the diseases in different grain legumes are caused by different strain of same virus species or by different virus species. Molecular data on nucleotide and predicted amino acid sequences of various protein products clearly revealed the involvement of two virus species i.e., MYMV and MYMIV (Usharani *et al.,* 2004).

The begomovirus genomes are typically 2.8 kb nucleotides in length and encode genes both in complementary and virion sense from a non-coding intergenic region that contains promoter sequences and the origin (ori) of virion strand DNA replication. The ori consists of a predicted hairpin structure that contains the absolutely conserved (for geminiviruses) nonanucleotide (TAATATTAC) loop sequence and repeated motifs upstream known as iterons. The two components of bipartite begomoviruses are referred to as DNA-A and DNA-B (2700-2800 nt). The DNA-A encompasses all virus encoded functions required for DNA replication, control of gene expression, overcoming host defenses and encapsidation, whereas DNA-B encodes two proteins involved in intra and intercellular movement (Briddon *et al.,* 2010). The two components share little identity with exception of conserved region (CR) of 200 nt. The CR maintains the integrity of the genome, ensuring that replication of both components can be initiated by DNA-A encoded the replication-associated protein (Rep).

The virion-sense strand encodes the genes required for insect transmission and movement in plants, coat protein (CP) and V2 protein. The complementary-sense strand encodes the following proteins: the replication-associated protein (Rep) required for viral DNA replication, which is a rolling circle replication initiator protein that recognizes the iterons and nicks within the nonanucleotide sequence to initiate replication (Hanley-Bowdoin *et al.,* 2004); the transcriptional up-regulates the late (virion-sense) genes, is a suppressor of post-transcriptional gene silencing (PTGS) and overcomes virus-induced hypersensitive cell death (Hussain *et al.,* 2007); the replication enhancer protein (REn), which is involved in creation of an environment favorable for virus replication (Settlage *et al.,* 2005); and the C4

protein, for which the function remains unclear but for some viruses is a pathogenicity determinant and a suppressor of PTGS (Saeed *et al.*, 2008), activator protein (TrAP), which for bipartite begomoviruses. The full length DNA-A sequences are widely used for taxonomic purpose for identification of strains, variants and classification of distinct species. Since, all the functions required for replication, control of gene expression and encapsidation coded on DNA-A, an attempt has been made in this investigation to characterize DNA-A component of viral genome of horsegram yellow mosaic virus by cloning and sequencing.

Analyses of HgYMV-DNA-A-GKVK-Bangalore sequences showed typical features of bipartite begomoviruses characterized by six conserved open reading frames in DNA-A (Table 3 and Figure 4). The total length of DNA-A sequence was determined as 2654 nucleotide in length and encodes 2 ORFs (AV1 and AV2) on sense strand and 4 ORFs on anti-sense strand (AC1, AC2, AC3 and AC4). The DNA-A encodes AV1 (position 315-1089 nt) and AV2 (156-497 nt) on virus sense strand and remaining four genes (AC1, AC2, AC3 and AC4) were encoded on virion complementary strand. The relative nucleotide position of remaining four ORFs on DNA-A were AC1 (1501-2600 nt), AC2 (1208-1633 nt), AC3 (1103-1460 nt) and AC4 (2177-2499 nt). The ORFs were similar with respect to size and location of yellow mosaic viruses.

Table.1 List of oligonucleotide primers used for amplification of DNA-A component of yellow mosaic virus infecting horsegram

Primer Name	Nucleotide sequence (5'→3')	Target molecule	Product size	Annealing temperature	Reference
HgYMVAF	ATCATACTGAGAACGCTTTG	DNA-A	2.7 kb	55	Anburaj *et al.*, (2010)
HgYMVAR	TGTCATACTTCGCAGCTTC				
AC-abut	GTAAAGCTTTACGCATAATG	DNA-A	2.7 kb	55	Rouhibaksh *et al.*, (2008)
AV-abut	AAAGCTTACATCCTCCAC				
HYMV-A1500F	CTGCAGTGATGTTGTCCCCKG	DNA-A	2.7 kb	55	Maruthi *et al.*, (2006)
HYMV-A1500R	CTGCAGCTCAACTCAGGARTGG				

Table.3 Six open reading frames (ORFs) of DNA-A component of HgYMV with nucleotide position

Sl. No.	ORFs	Nucleotide position
1	AV1	315-1089 nt
2	AV2	156-497 nt
3	AC1	1501-2600 nt
4	AC2	1633-1208 nt
5	AC3	1460-1103 nt
6	AC4	2177-2499 nt

Table.2 List of geminiviruses used for comparison of DNA-A sequence, their origin, host species and NCBI accession numbers

Sl. No.	Virus species	Abbreviation	Geographical origin	Host species	Accession number
1.	Horsegram yellow mosaic virus	HgYMV-Tamil Nadu:HG	Tamil Nadu	Horsegram (HG)	AJ627904.1
2.	Horsegram yellow mosaic virus	HgYMV-Srilanka:FB	Srilanka	Frenchbean (FB)	GU323321.1
3.	Mungbean yellow mosaic virus	MYMV-Madurai:SB	Madurai	Soybean (SB)	AJ421642.1
4.	Mungbean yellow mosaic virus	MYMV-Vietnam:MB	Vietnam	Mungbean (MB)	JX244175.1
5.	Mungbean yellow mosaic virus	MYMV-Maharashtra:SB	Maharashtra	Soybean (SB)	AF314530.1
6.	Mungbean yellow mosaic virus	MYMV-Namakkal:MoB	Namakkal	Mothbean (MoB)	DQ865201.1
7.	Mungbean yellow mosaic virus	MYMV-Pakistan:SB	Pakistan	Soybean (SB)	AY269991.1
8.	Mungbean yellow mosaic virus	MYMV-Japan:MB	Japan	Mungbean (MB)	D14703.1
9.	Mungbean yellow mosaic virus	MYMV-Navasari:SB	Navasari	Soybean (SB)	DQ389144.1
10.	Mungbean yellow mosaic virus	MYMV-Combodia:MB	Combodia	Mungbean (MB)	AY271892.1
11.	Mungbean yellow mosaic virus	MYMV-Tamil Nadu:BG	Tamil Nadu	Blackgram (BG)	DQ400848.1
12.	Mungbean yellow mosaic virus	MYMV-Haryana:MB	Haryana	Mungbean (MB)	AY271896.1
13.	Mungbean yellow mosaic India virus	MYMIV-Palampur:FB	Palampur	Frenchbean (FB)	FN794200.1
14.	Mungbean yellow mosaic India virus	MYMIV-Nepal:KB	Nepal	Kidneybean (KB)	JN543395.1
15.	Mungbean yellow mosaic India virus	MYMIV-Kanpur:CP	Kanpur	Cowpea (CP)	DQ389154.1
16.	Mungbean yellow mosaic India virus	MYMIV-Pantnagar:SB	Pantnagar	Soybean (SB)	DQ389152.1
17.	Mungbean yellow mosaic India virus	MYMIV-Bareily:CP	Bareily	Cowpea (CP)	DQ389145.1
18.	Mungbean yellow mosaic India virus	MYMIV-Akola:MB	Akola	Mungbean (MB)	AY271893.1
19.	Mungbean yellow mosaic India virus	MYMIV-Meerut:CP	Meerut	Cowpea (CP)	DQ389147.1
20.	Mungbean yellow mosaic India virus	MYMIV-Ludhiana:SB	Ludhiana	Soybean (SB)	DQ389151.1
21.	Mungbean yellow mosaic India virus	MYMIV-Nepal:MB	Nepal	Mungbean (MB)	AY271895.1
22.	Mungbean yellow mosaic India virus	MYMIV-India:CP	India	Cowpea (CP)	AF481865.2
23.	Mungbean yellow mosaic India virus	MYMIV-Pakistan:CP	Pakistan	Cowpea (CP)	AY269990.1
24.	Mungbean yellow mosaic India virus	MYMIV-Tirupati:BG	Tirupati	Blackgram (BG)	JX110618.1
25.	Mungbean yellow mosaic India virus	MYMIV-India:SB	India	Soybean (SB)	AY049772.1
26.	Mungbean yellow mosaic India virus	MYMIV-India:BG	India	Blackgram (BG)	AF126406.1
27.	Mungbean yellow mosaic India virus	MYMIV-Ludhiana:BG	Ludhiana	Blackgram (BG)	DQ400847.1
28.	Mungbean yellow mosaic India virus	MYMIV-Indonesia:SB	Indonesia	Soybean (SB)	JN368438.1
29.	Mungbean yellow mosaic India virus	MYMIV-Indonesia:YLB	Indonesia	Yard long bean (YLB)	JN368437.1
30.	Mungbean yellow mosaic India virus	MYMIV-Varanasi:Do	Varanasi	Fieldbean (Do)	AY547317.1

Table.4 Percentage identity of DNA-A component of HgYMV-GKVK-Bangalore with other begomoviruses

Sl. No.	Virus species	Abbreviation	Accession number	Percentage identity
1.	Horsegram yellow mosaic virus	HgYMV-Tamil Nadu:HG	AJ627904.1	98
2.	Horsegram yellow mosaic virus	HgYMV-Srilanka:FB	GU323321.1	95
3.	Mungbean yellow mosaic virus	MYMV-Madurai:SB	AJ421642.1	84
4.	Mungbean yellow mosaic virus	MYMV-Vietnam:MB	JX244175.1	84
5.	Mungbean yellow mosaic virus	MYMV-Maharashtra:SB	AF314530.1	84
6.	Mungbean yellow mosaic virus	MYMV-Namakkal:MoB	DQ865201.1	84
7.	Mungbean yellow mosaic virus	MYMV-Pakistan:SB	AY269991.1	83
8.	Mungbean yellow mosaic virus	MYMV-Japan:MB	D14703.1	82
9.	Mungbean yellow mosaic virus	MYMV-Navasari:SB	DQ389144.1	83
10.	Mungbean yellow mosaic virus	MYMV-Combodia:MB	AY271892.1	84
11.	Mungbean yellow mosaic virus	MYMV-Tamil Nadu:BG	DQ400848.1	84
12.	Mungbean yellow mosaic virus	MYMV-Haryana:MB	AY271896.1	83
13.	Mungbean yellow mosaic India virus	MYMIV-Palampur:FB	FN794200.1	81
14.	Mungbean yellow mosaic India virus	MYMIV-Nepal:KB	JN543395.1	81
15.	Mungbean yellow mosaic India virus	MYMIV-Kanpur:CP	DQ389154.1	81
16.	Mungbean yellow mosaic India virus	MYMIV-Pantnagar:SB	DQ389152.1	81
17.	Mungbean yellow mosaic India virus	MYMIV-Bareily:CP	DQ389145.1	81
18.	Mungbean yellow mosaic India virus	MYMIV-Akola:MB	AY271893.1	80
19.	Mungbean yellow mosaic India virus	MYMIV-Meerut:CP	DQ389147.1	81
20.	Mungbean yellow mosaic India virus	MYMIV-Ludhiana:SB	DQ389151.1	81
21.	Mungbean yellow mosaic India virus	MYMIV-Nepal:MB	AY271895.1	81
22.	Mungbean yellow mosaic India virus	MYMIV-India:CP	AF481865.2	79
23.	Mungbean yellow mosaic India virus	MYMIV-Pakistan:CP	AY269990.1	81
24.	Mungbean yellow mosaic India virus	MYMIV-Tirupati:BG	JX110618.1	79
25.	Mungbean yellow mosaic India virus	MYMIV-India:SB	AY049772.1	80
26.	Mungbean yellow mosaic India virus	MYMIV-India:BG	AF126406.1	78
27.	Mungbean yellow mosaic India virus	MYMIV-Ludhiana:BG	DQ400847.1	80
28.	Mungbean yellow mosaic India virus	MYMIV-Indonesia:SB	JN368438.1	81
29.	Mungbean yellow mosaic India virus	MYMIV-Indonesia:YLB	JN368437.1	80
30.	Mungbean yellow mosaic India virus	MYMIV-Varanasi:Do	AY547317.1	79

Plate.1a Horsegram plant infected with Horsegram yellow mosaic virus (HgYMV)

Plate.1b Horsegram leaves showing typical mild to severe yellow mosaic symptoms

Plate.1c Complete yellowing of leaves caused by HgYMV

Plate.1d Reduction in leaf size caused by HgYMV

Plate.1e Stunted growth of horsegram plants due to HgYMV

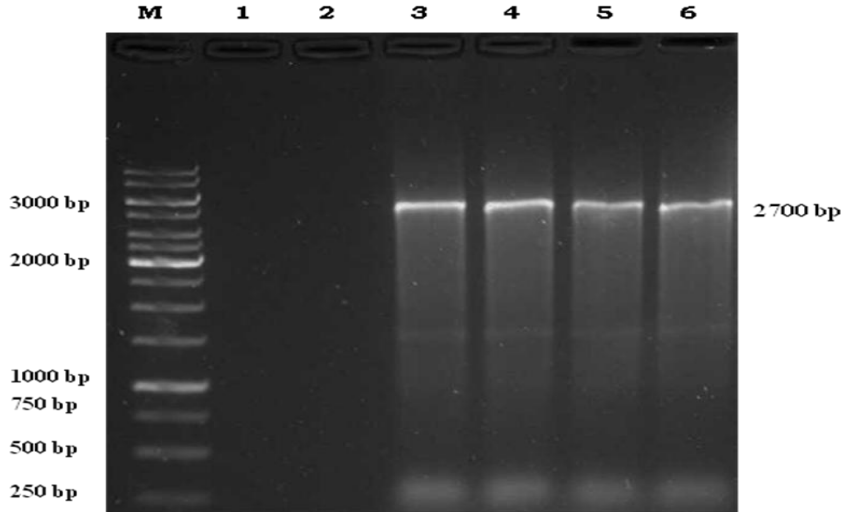

Plate.2 Amplification of full length DNA-A fragment of HgYMV using AC-abut/ AV-abut and HYMV-A1500F/ HYMV-A1500R primer pair

Lane:
M- 1Kb Marker (abm 1 Kb DNA marker)
Lane 1 –Healthy horsegram plant DNA
Lane 2 – Water control
Lane 3, 4- Specific PCR product of 2700 bp from HgYMV infected sample using AC-abut/ AV-abut primer pair
Lane 5, 6 - Specific PCR product of 2700 bp from HgYMV infected sample using HYMV-A1500F/ HYMV-A1500R primer pair

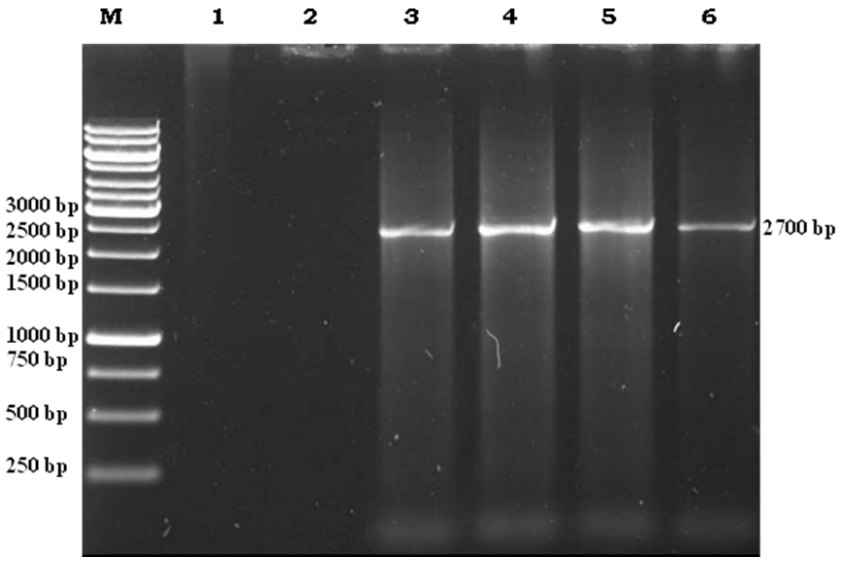

Plate.3 Amplification of full length DNA-A fragment of HgYMV using HgYMVAF/HgYMVAR primer pair

Lane:
M- 1Kb Marker (Fermentas 1 kb DNA ruler)
Lane 1 –Healthy horsegram plant DNA
Lane 2 – Water control
Lane 3, 4, 5, 6 - Specific PCR product of 2700 bp from HgYMV infected sample using HgYMVAF/HgYMVAR primer pair

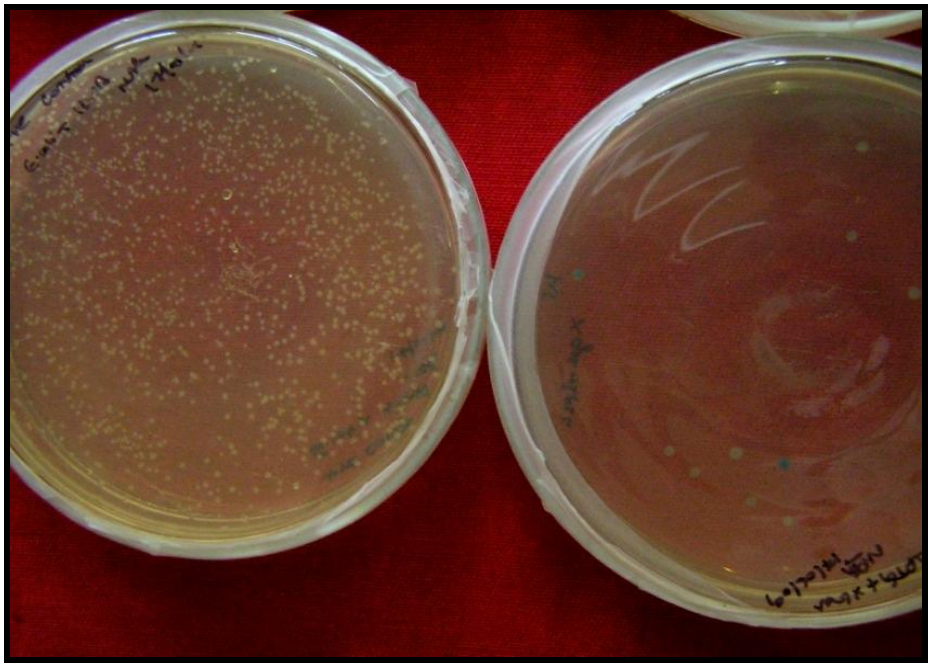

Plate.4a Colony of transformants on LB media

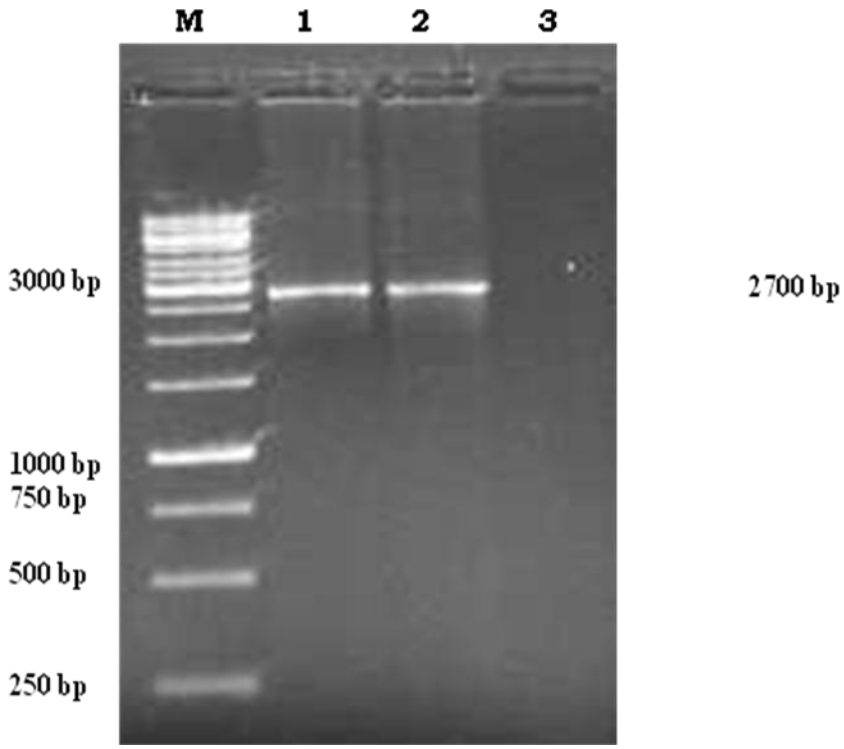

Plate.4b Screening of transformants for DNA-A component of HgYMV inserts by colony PCR

Lane:
M- 1Kb Marker (Fermentas 1 kb DNA ruler)
Lane 1, 2 -HgYMV DNA clone
Lane 3 - Non transformed plasmid

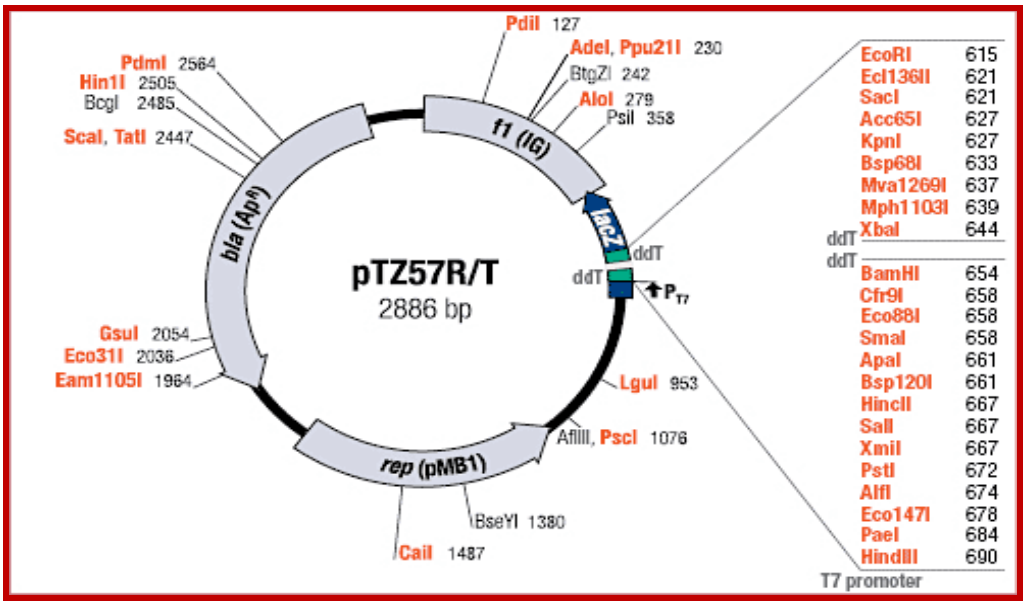

Figure.1 Restriction map, multiple cloning site and sequence of vector pTZ57R/T vector (Fermentas, Germany)

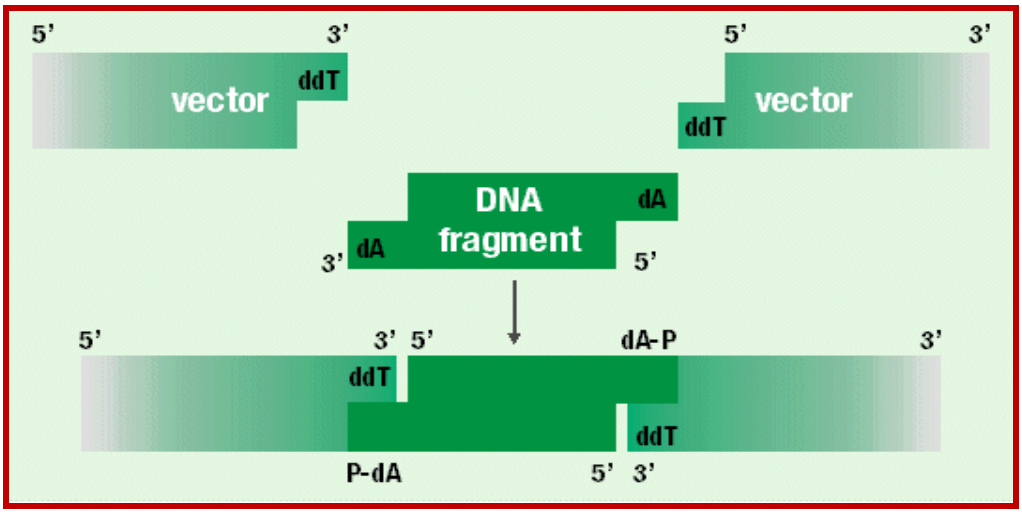

Figure.2 Ligation of a PCR fragment into the pTZ57R/T vector

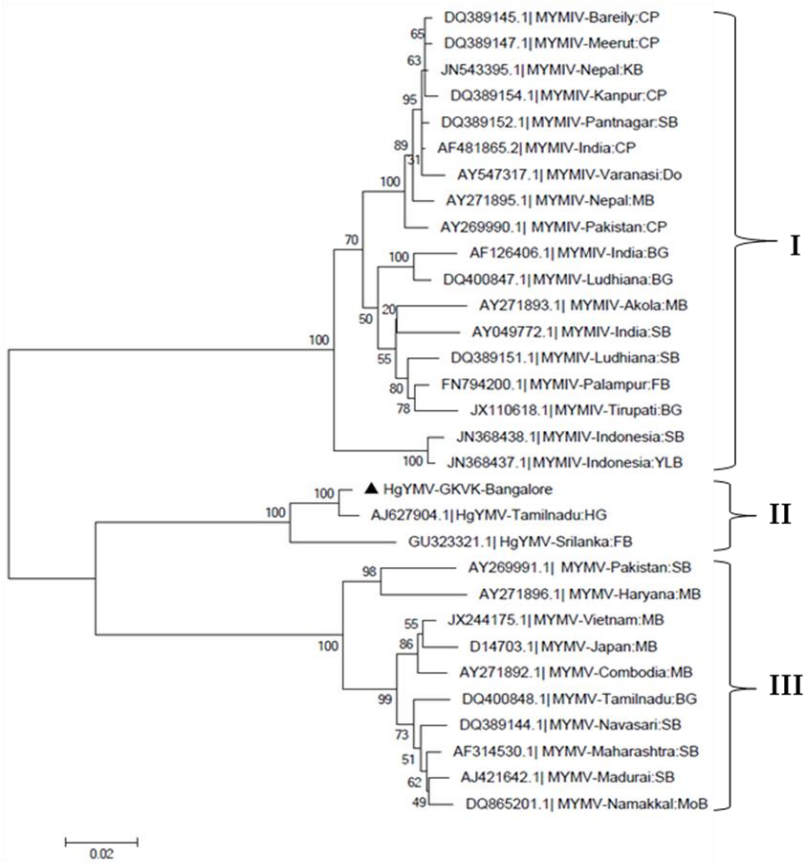

Figure.3 Phylogenetic tree obtained from comparison of complete nucleotide sequence of DNA-A component of HgYMV with other geminiviruses from database. The dendrograms are calculated using neighbor-joining algorithm of MEGA 5.1 version. Numbers at nodes indicate percentage bootstrap confidence scores (1,000 replications)

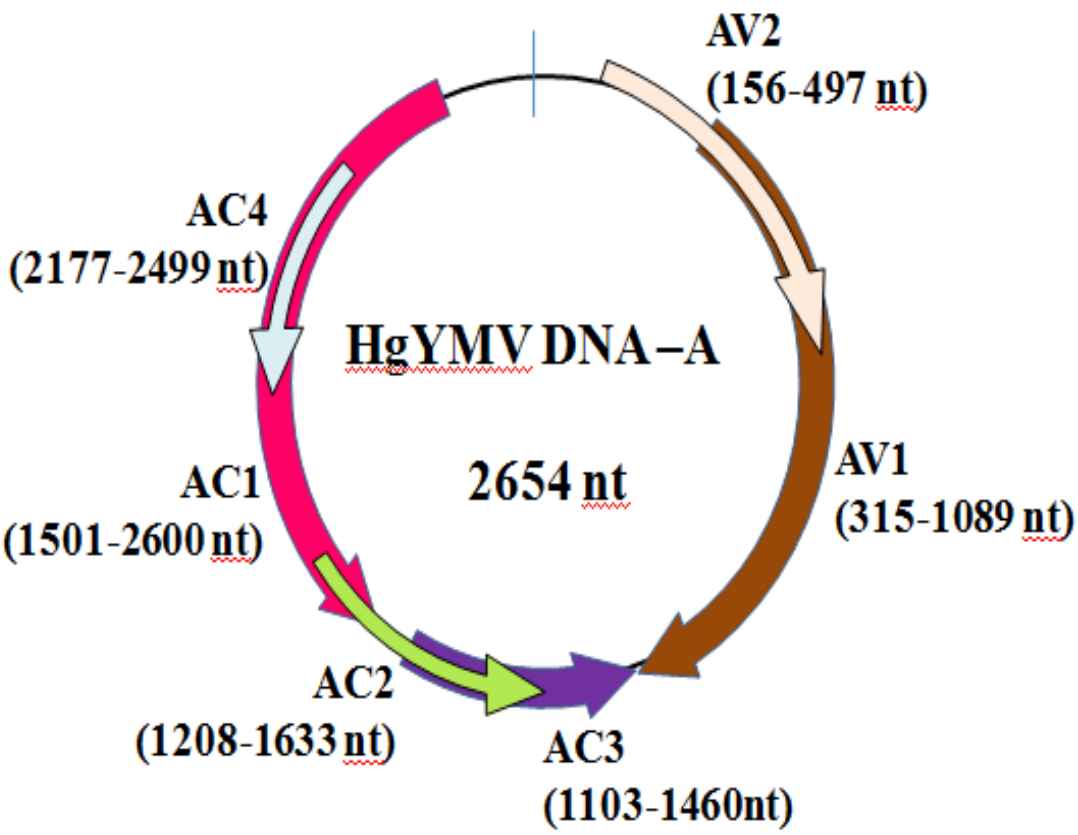

Figure.4 Schematic representation of DNA-A component of HgYMV showing 6 open reading frames with nucleotide position

Complete analysis of DNA-A sequence of Horsegram yellow mosaic virus-GKVK-, Bangalore isolate showed presence of 6 open reading frames (ORFs) similar to that of DNA-A component of other begomoviruses. The ORFs were named by analogy with other known sequences of begomoviruses (Briddon *et al.,* 2010). Analysis of ORFs of DNA-A of present HgYMV-GKVK-Bangalore isolate revealed that it had genomic organization similar to that of DNA-A component of many other begomoviruses like ICMV (Hameed and Robinson, 2004), MYMIV-Sb (Usharani *et al.,* 2004), Cotton leaf curl virus (Kirthi *et al.,* 2004), Dolichos yellow mosaic virus (Sudheer Kumar Singh *et al.,* 2006), Cowpea golden mosaic virus (John *et al.,* 2008) and HgYMV-Coimbatore-TN:Hg (Anburaj *et al.,* 2010). The genomes of CoGMV, KuMV and ClGMV were bipartite. All DNA-A molecules contained four complementary-sense ORFs (AC1, AC2, AC3 and AC4) and two virion-sense ORFs (AV1 and AV2), with the exception of CoGMV, which did not contain an AV2 ORF (Cuong Ha *et al.,* 2008). Complete sequences of 44 components (23 DNA-A, 19 DNA-B and 2 betasatellites) were determined by Muhammad Ilyas *et al.,* (2010). Analysis of sequences for potential open reading frames (ORFs) using ORF Finder showed 23 clones to have an arrangement of genes typical of monopartite (or DNA-A components of bipartite) begomoviruses originating from the OW, whereas 19 clones had an arrangement typical of the DNA-B components of bipartite begomoviruses.

Viral sequences were aligned, ORFs were deciphered and the genome organization was found to have typical features of begomoviruses. There are two virion sense genes, ORF AV2 and ORF AV1 (coat protein, CP) in DNA-A and one ORF BV1 (nuclear shuttle protein, NSP) in DNA-B. The complementary sense genes are ORF AC1 (replication initiation protein, Rep), AC2 (transcription activator protein, TrAP), AC3 (replication enhancer protein, REn), AC4 and AC5 in DNA A and ORF BC1 (movement protein, MP) in DNA-B (Usharani *et al.*, 2004). Complete nucleotide sequence analysis of DNA A showed > 89 per cent identity with MYMIV and hence this isolate was designated as MYMIV-[Sb].

Nucleotide sequence comparison of DNA-A component of yellow mosaic virus infecting horsegram with other begomoviruses

Comparison of the complete DNA-A sequence of HgYMV-GKVK-Bangalore with other bipartite begomoviruses revealed 82-84 per cent identity (Table 4) with isolates of Mungbean yellow mosaic virus (MYMV), 78-81 per cent with Mungbean yellow mosaic India virus ; 98 and 95 per cent identity with the accessions of horsegram and frenchbean isolates of HgYMV from Tamil Nadu and Srilanka, respectively.

This was also validated during earlier investigations carried out by Anburaj *et al.*, (2010), Obaiah (2011), Monger *et al.*, (2010), Maruthi *et al.*, (2006), Shahid *et al.*, (2012), Usharani *et al.*, (2004) and Prema (2013). As per the latest guidelines if nucleotide identity of complete DNA-A sequence is >90%, it will be considered as variant, strain or isolate of the same virus and <90% will be considered as distinct species in begomovirus classification (Fauquet *et al.*, 2008). The present results from ORFs sequence homology, phylogenetic relationship and complete DNA sequence similarity at nucleotide level clearly established that yellow mosaic virus of horsegram in Karnataka is HgYMV.

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