Molecular Characterization of Coat Protein Gene of Horse Gram Yellow Mosaic Virus (HgYMV) from Southern India

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Abstract

Horse gram leaf samples showing yellow mosaic symptoms gave positive results with HgYMV specific primer pairs (HgYMV-CP-F/HgYMV-CP-R) and yielded amplicons of ~1000 bp whereas no PCR product was obtained from DNA extracted from healthy samples in PCR. The 1000 bp PCR products were cloned, sequenced, assembled and its length was determined as 885 bp including 111 bp pre-coat protein at 5’ end and 774 core coat protein. The length of the predicted amino acid sequence was found to be 257. Phylogenetic tree based on full length coat protein gene sequences of HgYMV with 23 other geminivirus sequences downloaded from NCBI Genbank formed three major clusters of MYMIV, HgYMV and MYMV. The present isolate formed unique cluster with HgYMV group that cause yellow mosaic disease symptoms in horsegram (AJ627904.1) and frenchbean (GU323321.1). Comparison of complete nucleotide sequence of coat protein gene of HgYMV indicated that coat protein gene of HgYMV-GKVK-Bangalore isolate had maximum homology with HgYMV-Tamil Nadu:HG [AJ627904.1] (99.2%), followed by HgYMV-Srilanka:FB [GU323321.1] (95.2%). HgYMV-GKVK-Bangalore isolate had 85.2-86.9 per cent identity and 80.2-81.9 per cent identity with MYMV and MYMIV isolates, respectively. Comparison of predicted amino acid sequence of coat protein gene of HgYMV revealed that HgYMV-GKVK-Bangalore isolate shared maximum amino acid identity with HgYMV-Tamil Nadu:HG [AJ627904.1] (99.2%), followed by HgYMV-Srilanka:FB [GU323321.1] (97.2%). HgYMV-GKVK-Bangalore isolate had 91-92.2 per cent identity and 82.8-84.8 per cent identity with MYMV and MYMIV isolates, respectively. From the above results, it is clear that HgYMV-GKVK-Bangalore is closely related to HgYMV isolates rather than MYMV and MYMIV.

Keywords
Coat protein gene, Horsegram, Yellow mosaic virus, Whitefly, Begomovirus, Phylogenetic analysis, Sequence comparison

Introduction
Horsegram (Macrotyloma uniflorum (Lam.) Verdc.) commonly known as kulthi, is one of the hardiest and drought tolerant crops, grown extensively in peninsular India as poor man’s pulse crop. Horsegram is an indigenous plant cultivated in India, other Asian countries and Africa (Jayan and Maya, 2001). Among viral diseases, yellow mosaic virus is one of the major constraints for its cultivation in peninsular India and was first observed in...
southern districts 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; Williams et al., 1968 and Prema, 2013). 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., 1976). The disease caused yellow discoloration on the leaves that led to irregular, small, greenish yellow mosaic symptoms. Severe infection led to stunted growth of the plant and reduction in the leaf size (Muniyappa et al., 1987; Prema and Rangaswamy, 2017).

Geminiviruses which belong to family Geminiviridae are small plant viruses characterized by a 16-18 nm × 30 nm geminate particles consisting of two joined incomplete icosahedral encapsidating either monopartite or bipartite circular single stranded (ss) DNA genome molecules of about 2700 nucleotides (Harrison et al., 1977; Francki et al., 1980; Harrison and Robinson, 2002). Geminiviruses have been grouped into four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus* depending on their vector, host range and genome characteristics (Fauquet and Stanley, 2003; Fauquet et al., 2003).

The viruses causing yellow mosaic diseases of legumes across Southern Asia are identified as four distinct bipartite Begomoviruses namely Mungbean yellow mosaic India virus, Mung bean yellow mosaic virus, Horsegram yellow mosaic virus and Dolichos yellow mosaic virus (Qazi et al., 2007). Most of the yellow mosaic viruses infecting legumes in India were transmitted by the whitefly (*B. tabaci* Genn.) and not seed or soil borne or sap transmissible, share a very narrow host range within legumes and cause biologically indistinguishable symptoms, making specific identification of the viruses difficult (Nene, 1972).

At present literature is not available at genomic level for HgYMV from Karnataka. The information on phylogenetic relationship with that of other YMV isolates associated with other legumes is not available. So, in this context, the present study was undertaken to characterize the HgYMV by sequencing the core region of the CP and to establish its genetic relationship with other legume yellow mosaic viruses.

**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 1). 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 eppendorf 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 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 T10E0.1 buffer (10 mM Tris-Cl 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 coat protein of horsegram yellow mosaic virus, specific primers available in the literature were tried to amplify coat protein region of yellow mosaic viruses of nearly 1000 bp. Primers specific to HgYMV (HgYMV-CP-F- ATG CTT GCA ATT AAG TAC TTG CA/HgYMV-CP-R- TAG GCG TCA TTA GCA TAG GCA) were used for amplification of coat protein gene of horsegram yellow mosaic virus (HgYMV). Primers were designed to get the complete coat protein gene of various yellow mosaic viruses of legume hosts by taking 100 extra nucleotides on both the sides of the gene (Naimuddin and Akram, 2012).

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 ºC for 2 min for primers HgYMV-CP/-HgYMV-CPR and extension at 72 ºC for 3 min, followed by one step of final extension at 72 ºC 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 coat protein gene 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 CP specific primers. The resultant positive clones were fully sequenced in both directions using universal M13 forward and reverse primers. Full length sequence of coat protein 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 coat protein gene of HgYMV with other geminiviruses**

Pairwise and multiple sequence alignment of the full length of coat protein sequence 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 coat protein gene sequences of HgYMV with 23 other geminivirus sequences downloaded from NCBI Genbank (Table 1). 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).

Results and Discussion

Molecular cloning and sequencing of coat protein gene of yellow mosaic virus infecting horsegram (HgYMV)

The PCR results indicated that the virus could be detected from 1:20 to 1:40 dilutions. In PCR, horsegram samples showing yellow mosaic symptoms gave positive results with HgYMV specific primer pairs (HgYMV-CP-F/HgYMV-CP-R for DNA A) and yielded amplicons of ~1000 bp (Plate 2) which clearly indicated that the yellow mosaic disease of horsegram at Bangalore is caused by Horsegram yellow mosaic virus whereas no PCR product was obtained from DNA extracted from healthy samples. It revealed that yellow mosaic disease of horsegram cultivated in and around Bangalore was caused by HgYMV.

The 1000 bp PCR product was eluted from agarose gel and cloned into pTZ57R/T vector and transformed into competent E.coli cells (DH5α strain). The recombinant clones containing CP gene were sequenced, assembled and its length was determined as 885 bp including 111 bp pre-coat protein at 5’ end and 774 core coat protein. The length of the predicted amino acid sequence was found to be 257.

Phylogenetic analysis, nucleotide sequence and amino acid sequence comparison of coat protein gene of yellow mosaic virus infecting horsegram

Phylogenetic tree based on full length coat protein gene sequences of HgYMV with 23 other geminivirus sequences downloaded from NCBI Genbank formed three major clusters of MYMIV, HgYMV and MYMV (Fig. 1). The present isolate formed unique cluster with HgYMV group that cause yellow mosaic disease symptoms in horsegram (AJ627904.1) and frenchbean (GU323321.1). The less horizontal spread of branches showed less sequence variation among these isolates. The branching out of different MYMIV and MYMV isolates suggested that these were entirely different viruses separated early in the evolution. From the above results, it is evident that yellow mosaic disease of horsegram in Bangalore is more closely related to other HgYMV isolates from different regions rather than MYMIV and MYMV.

Comparison of complete nucleotide sequence of coat protein gene of HgYMV with 23 other geminivirus sequences available in the NCBI Genbank (Table 2) indicated that coat protein gene of HgYMV-GKVK-Bangalore isolate had maximum homology with HgYMV-Tamil Nadu:HG [AJ627904.1] (99.2%), followed by HgYMV-Srilanka:FB [GU323321.1] (95.2%). HgYMV-GKVK-Bangalore isolate had 85.2-86.9 per cent identity and 80.2-81.9 per cent identity with MYMV and MYMIV isolates, respectively. HgYMV-GKVK-Bangalore CP nucleotide sequence showed 86.9 per cent identity with MYMV: Cambodia:MB [AY271892.1], followed by 86.8 per cent with MYMV-Maharashtra:SB [AF314530.1] and 85.2 per cent with MYMV-Pakistan:SB [AY269991.1]. It had 8.19 per cent, 81.7 per cent and 80.2 per cent homology with
MYMIV-India:SB [AY049772.1], MYMIV-Pakistan:MB [AY269992.1] and MYMIV-Akola:MB [AY271893.1] isolates, respectively. From the above results, it is clear that HgYMV-GKVK-Bangalore is closely related to HgYMV-Tamil Nadu:HG and HgYMV-Srilanka:FB rather than MYMV and MYMIV. The full description of various isolates with their Genbank accession numbers used for sequence comparison were furnished in Table 8.

**Plate 1** Horsegram plants showing typical symptoms of yellow mosaic virus

**Plate 2** Amplification of coat protein gene of YMV infecting horsegram using HYMV-CP-F/HYMV-CP-R primer pair

Lane:
M- 1Kb Marker (Fermentas 1 kb DNA ruler)
Lane 1, 2, 3, 4 - Specific PCR product of 1000 bp from HgYMV infected sample
Lane 5 - Healthy horsegram plant DNA
Lane 6 - Water control
Figure 1: Phylogenetic tree obtained from comparison of complete nucleotide sequence of coat protein gene 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)
**Table 1** List of geminiviruses used for comparison of coat protein gene sequences, 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-Haryana:MB    | Haryana              | Mungbean (MB)  | AY271896.1      |
| 4       | Mungbean yellow mosaic virus   | MYMV-Namakkal:MoB  | Namakkal             | Mothbean (MoB) | DQ865201.1     |
| 5       | Mungbean yellow mosaic virus   | MYMV:Combodia:MB   | Combodia             | Mungbean (MB)  | AY271892.1      |
| 6       | Mungbean yellow mosaic virus   | MYMV-Madurai:SB    | Madurai              | Soybean (SB)   | AJ421642.1      |
| 7       | Mungbean yellow mosaic virus   | MYMV-Pakistan:SB   | Pakistan             | Soybean (SB)   | AY269991.1      |
| 8       | Mungbean yellow mosaic virus   | MYMV-Maharashtra:SB| Maharashtra          | Soybean (SB)   | AF314530.1      |
| 9       | Mungbean yellow mosaic virus   | MYMV-India:Tamil Nadu:MB | Tamil Nadu       | Mungbean (MB)  | AB017341.1      |
| 10      | Mungbean yellow mosaic virus   | MYMV-India:MB      | India               | Soybean (SB)   | JN368438.1      |
| 11      | Mungbean yellow mosaic India virus | MYMIV-Indonesia:SB | Indonesia           | Soybean (SB)   | JN368437.1      |
| 12      | Mungbean yellow mosaic India virus | MYMIV-Akola:MB    | Akola                | Mungbean (MB)  | AY271893.1      |
| 13      | Mungbean yellow mosaic India virus | MYMIV-India:SB    | India                | Soybean (SB)   | AY049772.1      |
| 14      | Mungbean yellow mosaic India virus | MYMIV-Indonesia:YL B | Indonesia           | Yard long bean (YL B) | JN368437.1|
| 15      | Mungbean yellow mosaic India virus | MYMIV-Pakistan:BG | Pakistan             | Blackgram (BG) | FM208845.1      |
| 16      | Mungbean yellow mosaic India virus | MYMIV-Indonesia:LB | Indonesia           | Mungbean (MB)  | AY269992.1      |
| 17      | Mungbean yellow mosaic India virus | MYMIV-Indonesia:LB | Indonesia           | Yard long bean (YL B) | JN368434.1|
| 18      | Mungbean yellow mosaic India virus | MYMIV-Indonesia:LB | Indonesia           | Yard long bean (YL B) | JN368432.1|
| 19      | Mungbean yellow mosaic India virus | MYMIV-Indonesia:LB | Indonesia           | Mungbean (MB)  | AY271895.1      |
| 20      | Mungbean yellow mosaic India virus | MYMIV-Varanasi:Do | Varanasi             | Fieldbean (Do) | AY547317.1      |
| 21      | Mungbean yellow mosaic India virus | MYMIV-Indonesia:LB | Bangladesh           | Mungbean (MB)  | AY416349.1      |
| 22      | Mungbean yellow mosaic India virus | MYMIV-Jabalpur:SB | Jabalpur             | Soybean (SB)   | AY416349.1      |
| 23      | Mungbean yellow mosaic India virus | MYMIV-Palampur:FB | Palampur             | Frenchbean (FB) | FN794200.1      |
Table 2 Nucleotide and amino acid sequence identities of coat protein gene of yellow mosaic virus infecting horsegram with other geminiviruses

| Sl. No. | Accession number | Sequences | Nucleotide sequence identity | Amino acid sequence identity |
|---------|------------------|-----------|------------------------------|----------------------------|
| 1.      | AJ627904.1       | HgYMV-Tamilnadu:HG | 99.2                         | 99.2                       |
| 2.      | GU323321.1       | HgYMV-Srilanka:FB  | 95.2                         | 97.2                       |
| 3.      | AY271896.1       | MYMV-Haryana:MB    | 85.6                         | 92.2                       |
| 4.      | DQ865201.1       | MYMV-Namakkal:MoB  | 86.4                         | 91.4                       |
| 5.      | AY271892.1       | MYMV-Combodia:MB   | 86.9                         | 91.8                       |
| 6.      | AJ421642.1       | MYMV-Madurai:SB    | 86.4                         | 91.0                       |
| 7.      | AY269991.1       | MYMV-Pakistan:SB   | 85.2                         | 91.4                       |
| 8.      | AF314530.1       | MYMV-Maharashtra:SB| 86.8                         | 91.8                       |
| 9.      | AB017341.1       | MYMV-India:SB      | 86.5                         | 91.0                       |
| 10.     | AJ132575.1       | MYMV-Thailand:MB   | 86.4                         | 91.8                       |
| 11.     | JN368438.1       | MYMIV-Indonesia:SB | 80.7                         | 83.6                       |
| 12.     | AY271893.1       | MYMIV-Akola:MB     | 80.2                         | 83.2                       |
| 13.     | AY049772.1       | MYMIV-India:SB     | 81.9                         | 84.4                       |
| 14.     | JN368437.1       | MYMIV-Indonesia:YLB| 80.8                         | 84.0                       |
| 15.     | FM208845.1       | MYMIV-Pakistan:BG  | 81.9                         | 84.4                       |
| 16.     | AY269992.1       | MYMIV-Pakistan:MB  | 81.7                         | 84.8                       |
| 17.     | JN368434.1       | MYMIV-Indonesia:YLB| 80.7                         | 83.2                       |
| 18.     | JN368432.1       | MYMIV-Indonesia:YLB| 80.7                         | 84.0                       |
| 19.     | AY271895.1       | MYMIV-Tamilnadu:MB | 80.7                         | 84.0                       |
| 20.     | AY547317.1       | MYMIV-Varanasi:Do  | 80.8                         | 84.4                       |
| 21.     | AF314145.1       | MYMIV-Bangladesh:MB| 80.7                         | 82.8                       |
| 22.     | AJ416349.1       | MYMIV-Jabalpur:SB  | 81.6                         | 84.8                       |
| 23.     | FN794200.1       | MYMIV-Palampur:FB  | 81.3                         | 84.8                       |
Comparison of predicted amino acid sequence of coat protein gene of HgYMV with 23 other geminivirus amino acid sequences available in the NCBI Genbank (Table 2) indicated that HgYMV-GKVK-Bangalore isolate shared maximum amino acid identity with HgYMV-Tamil Nadu:HG [AJ627904.1] (99.2%), followed by HgYMV-Srilanka:FB [GU323321.1] (97.2%). HgYMV-GKVK-Bangalore isolate had 91-92.2 per cent identity and 82.8-84.8 per cent identity with MYMV and MYMIV isolates, respectively. It shared 92.2 per cent, 91.8 per cent, 91.8 per cent, 91.8 per cent and 91 per cent nucleotide identity with MYMV-Haryana:MB [AY271896.1], MYMV-Maharashtra:SB [AF314530.1], MYMV-Tamil Nadu:MB [AI132575.1], MYMV:Combodia:MB [AY271892.1] and MYMV-Madurai:SB [AJ421642.1] isolates. It had 84.8 per cent, 84.8 per cent, 84.8 per cent and 82.8 per cent identity with MYMIV-Pakistan:MB [AY269992.1], MYMIV-Jabalpur:SB [AJ416349.1], MYMIV-Palampur:FB [FN794200.1] and MYMIV-Bangladesh:MB [AF314145.1]. From the above amino acid sequence alignment data, it is clear that HgYMV-GKVK-Bangalore isolate is more closely related to HgYMV-Tamil Nadu:HG and HgYMV-Srilanka:FB rather than MYMV and MYMIV.

This was also validated during earlier investigations carried out by Naimuddin and Mohd. Akram (2010), Kamaal Naimuddin et al. (2011), Naimuddin et al. (2011), Obaiah (2011), Mohammad Nurul Islam et al. (2012), Naimuddin and Akram (2012), Prema and Rangaswamy (2018a), Prema and Rangaswamy (2018b), Prema and Rangaswamy (2018c) and Prema and Rangaswamy (2018d).

Coat protein genes have traditionally proven useful for plant virus identification and classification (Mayo and Pringle, 1998). Because of its high degree of conservation, the coat protein ORF (CP or AV1) is the only begomovirus sequence approved by the International Committee on Taxonomy of Viruses for ascertaining the identity of a begomovirus (Mayo and Pringle, 1998), and the sequence comparison has been used to identify and classify geminiviruses (Padidam et al., 1995; Brown et al., 2001).

As per the latest guidelines if nucleotide identity at coat protein or 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 International Committee on Taxonomy of Viruses (ICTV) accepts the classification of begomoviruses based on CP gene sequences, when full length sequences are not available (Rybicki et al., 1998). Member of the genus Begomovirus are known to form clusters according to geographical origin with distinct branches for viruses from America, Africa and Asia. According to these guidelines, the yellow mosaic virus isolates from horsegram is a variant of horsegram yellow mosaic virus because of 99.2-95.2 per cent homology at nucleotide level.

Phylogenetic analysis based on coat protein gene sequences of HgYMV with 23 other geminivirus sequences formed three major subgroups consisting of of MYMIV, HgYMV and MYMIV. HgYMV-GKVK-Bengalore isolate fell within the HgYMV subgroup and deviated from MYMV and MYMIV isolates indicating that virus specificity depends on host and geographical location. The present results from nucleotide and amino acid sequence comparison using CP gene sequences had clearly revealed that the yellow mosaic virus infecting horsegram was entirely distinct from yellow mosaic virus infecting greengram and blackgram.
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