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Molecular Detection and Partial Characterization of Begomovirus Associated Yellow Mosaic virus Disease of Soyabean (*Glycine max* L.)

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ABSTRACT: The study's primary aim was to explore the molecular connection between the virus responsible for soybean yellow mosaic disease and related viruses. Symptoms of the disease involve yellowing leaves, leaf rolling and stunted growth in infected plants. Specific degenerate primers for begomoviruses were utilized to detect the causative virus. Gene fragments, measuring 1000bp for the coat protein and 900bp for the movement protein, were amplified from both naturally infected and whitefly-inoculated plants. Comparative analysis of the virus's CP gene sequence with other begomoviruses showcased clustering with horse gram yellow mosaic virus (HgYMV) segments from a horse gram isolate and an HgYMV isolate from India, Bangalore, infecting French beans, sharing a 98 per cent similarity, respectively. Similarly, when assessing the virus's MP gene sequence against other begomoviruses, it clustered with Horsegram yellow mosaic virus segments from a horse gram isolate and the HgYMV isolate from India, Bangalore, which infects French beans, showing a 99 per cent similarity, respectively. Consequently, the findings distinctly reveal that the yellow mosaic virus infecting soybeans is closely related to the one infecting horse gram and French beans, leading to its recognition as a strain of horse gram yellow mosaic virus (HgYMV).

Key words: Soyabean**,** Begomovirus, PCR, coat protein, movement protein, Molecular Characterization.

INTRODUCTION

Soybean, scientifically known as *Glycine max* (L.) Merrill, originally hails from East Asia and is classified by the UN Food and Agricultural Organization (FAO) as an oilseed rather than a pulse. Often dubbed the 'Golden bean' or the 'Super legume' of the twentieth century, soybean seeds stand out as an exceptional source of unsaturated fatty acids, high-quality proteins and dietary fibers. Notably, soybean oil is abundant in polyunsaturated fatty acids, such as linoleic acid and alphalinolenic acid, which offer significant health benefits. Collectively, soybean oil and protein make up 60 percent of the soybean's dry weight, with protein constituting 40 percent and oil 20 percent (Anon., 2012).

Soybeans, a significant oilseed crop on a global scale, are cultivated extensively in India. However, they face susceptibility to various diseases, including Rust (*Phakopsora pachyrhizi*), Alternaria leaf spot (*Alternaria* spp.), Downy mildew (*Peronospora manshurica*), Bacterial leaf spot (*Xanthomonas campestris* pv. *glycines*), Anthracnose (*Colletotrichum spp*), Charcoal rot (*Macrophomina phaseolina*), Purple seed stain (*Cercospora kikuchii*), Soybean cyst nematode (*Heterodera glycines*) and viral infections. In India, soybeans are notably affected by several prominent viral diseases, including Yellow mosaic virus (YMV), soybean mosaic virus (SMV), groundnut bud necrosis virus (GBNV), bean pea mottle virus, soybean crinkle leaf geminivirus, and cowpea mild mottle carla virus (CMMV) (Lal *et al.*, 2005). Among these, Yellow mosaic virus (YMV) stands out for its economic impact, significantly hampering crop growth and yield. This disease severely affects soybean cultivation in Northern India, parts of South India, Sri Lanka, Bangladesh, Pakistan, and Thailand (Bhattacharyya *et al*., 1999). The emergence of Yellow mosaic disease in soybeans was first recorded in North India in the early 1970s. Nearly all soybean varieties grown in central India are vulnerable to YMV. The virus accountable for yellow mosaic disease belongs to the Geminiviridae family, specifically the Begomovirus genus, a group of viruses that infect dicot plants. These viruses are exclusively spread by the whitefly *Bemisia tabaci* in a persistent manner. Begomoviruses can possess a monopartite genome, comprising a single circular single-stranded DNA component around 2.7 kb, known as DNA-A, or bipartite genomes containing two similarly sized components designated as DNA-A and DNA-B.

Within the DNA-A genome are four key genes responsible for encoding the coat protein (CP), replication initiator protein (Rep), transcriptional transactivator protein and replication enhancer protein. The DNA-B component harbors two genes encoding

proteins crucial for virus movement, host range, and pathogenicity. The coat protein (CP) gene in begomoviruses displays significant conservation, and the N-terminal sequence identity of the CP has been an effective means of identifying begomovirus isolates (Borah and Dasgupta 2012). Both in plants and insects, begomoviruses manifest as nuclear inclusion bodies, detectable through diverse methods such as light microscopy, ultrastructural electron microscopy, serological assays, DNA hybridization assays, polymerase chain reaction (PCR), and immuno-capture PCR. Molecular cloning and sequencing of viral genomes play a vital role in identifying viruses and facilitating comparisons of their relationships with other viral isolates. Begomoviruses universally encode a protective coat protein crucial in determining their transmissibility by the whitefly vector *Bemisia tabaci*, forming a protective layer for the virus particle. Consequently, the CP (coat protein) gene displays significant conservation among begomoviruses originating from the same geographical region, adapted to the local vector population for transmission. Due to its highly conserved nature, the coat protein sequence has been widely employed for provisional species identification. CP primers have proven effective in amplifying a fragment of most, if not all, begomoviruses, irrespective of their origin in the Old World or the New World. This rapid detection, coupled with comparing the sequence with reference begomovirus CP sequences, has been an efficient identification method. While previous studies have detailed symptoms and management strategies for soybean yellow mosaic disease, the etiology and phylogenetic relationships with other begomoviruses have remained elusive. This study aims to delineate the symptoms, detection methods and establish the phylogenetic ties with other significant begomoviruses.

MATERIALS AND METHOD

A. Collecting samples and extracting DNA

Virus cultures derived from infected soybean plants grown in MRS, Hebbal (Bengaluru) were utilized. The process of detecting the virus and cloning its DNA was undertaken at the Department of Plant Pathology, UAS, GKVK, Bengaluru, India. Initially, total genomic DNA was extracted from soybean plants exhibiting symptoms of yellow mosaic, using a modified CTAB Method (Rouhibakhsh *et al.,* 2008). This extracted DNA underwent PCR amplification, employing Gemini virus-specific primers capable of amplifying both the coat protein (CP) and movement protein (MP) regions found in numerous begomoviruses.

B. The impact of DNA extract dilution on PCR amplification of viral genomic DNA

PCR was used to evaluate the effect of diluting the template DNA for yellow mosaic. The DNA extracts from soybean yellow mosaic were then diluted with distilled water (SDW), varying from 1:10 to 1:60, before being employed in PCR amplifications.

C. Amplification of viral DNA through PCR

PCR was conducted in a 25 µl reaction mixture, comprising 12.5 µl of Fermentas 2x master mix, 0.5 µl of Taq DNA polymerase (3 U/µl), 2 µl of each specific primer for the Begomovirus genes (as detailed in Table 1), 4 µl of DNA template, and the remaining volume adjusted with sterile distilled water. The amplification procedure involved an initial cycle at 94°C for 2 minutes, followed by 35 cycles at 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes, with a final cycle at 72°C for 10 minutes, as outlined in the protocol by Naimuddin and Akram (2010). The resulting PCR products were separated on a 1% agarose gel containing ethidium bromide in 1x TBE buffer, and the banding pattern was visualized using a gel documentation unit.

D. Extracting, sequencing, and conducting phylogenetic analysis

The DNA from the agarose gel in TBE buffer underwent extraction and purification using the QIA quick Gel Extraction Kit (Cat. No. 28704; Qiagen, Germany) following the manufacturer's guidelines. Subsequently, the eluted product was forwarded to the National Centre for Biological Sciences, Bengaluru, for sequencing, using the primer walking method. The acquired sequences were aligned and combined to form a full-length sequence, utilizing the "nucleotide blast" tool within the Basic Blast Programs and the "align two (or more) sequences" function available in specialized blast programs. These tools were accessible through the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). For constructing neighbor-joining phylogenetic trees and performing evolutionary analysis, the MEGA 6.06 software package, described by Tamura *et al.* (2013), was employed. The robustness of the resultant trees was evaluated through bootstrap sampling of multiple sequence alignments, carried out with 1000 replications.

Table 1: Oligonucleotide primers utilized for amplifying the genomic components of soybean yellow mosaic virus.

Primers	Oligonucleotides	References
HYMV-CP-F	5’-ATGCTTGCAATTAAGTACTTGCA-3’(23 nt)	Naimuddin and Akram (2010)
HYMV-CP-R	5’-TAGGCGTCATTAGCATA GCA-3’ (20 nt)	
HYMV-MP-F	5’-ATGGAGCATATTCCGGTGCA-3’ (20 nt)	
HYMV-MP-R	5’-TTACA(G/A)GGTTTTGTTTACAGT-3’ (22 nt)	

RESULTS AND DISCUSSION

A. Extraction of complete DNA and conducting PCR amplification

The total viral DNA was extracted using a modified CTAB protocol. PCR tests were performed with a set of degenerate primers, successfully amplifying the expected DNA fragments of the coat protein gene (1000bp) and the movement protein gene (900bp) from both naturally infected and whitefly-inoculated soybean plants. These primers have been widely used for identifying begomoviruses in various crops and their vector, *Bemisia tabaci*, in prior studies. However, some adjustments were needed in the PCR protocol as the viral DNA didn't amplify following the standard procedure recommended by Wyatt and Brown (1996). Modifications involved changes in the annealing temperature and duration. An annealing temperature of 55°C for 1 minute proved suitable for amplifying soybean yellow mosaic viral DNA, contrasting the standard protocols typically using an annealing temperature of 55°C for 2 minutes in Gemini virus detection. This adjusted PCR protocol effectively amplified the Gemini virus-specific coat protein gene (1000bp) (Plate 2) and movement protein gene (900bp) (Plate 3) from samples of infected soybean plants.

B. Sequence Analysis

The coat protein (CP) gene sequence serves as a valuable tool in begomovirus classification. Comparing the CP and MP sequences of SYMV with selected begomoviruses revealed a 98% nucleotide identity with Horse gram yellow mosaic virus (HgYMV) DNA-A horse gram isolate (AM932427) and a 99% nucleotide identity with various isolates including HgYMV-Bangalore-Frenchbean (KC019306) and HgYMV-DNA-A-Limabean (AM932429) (Table 2 and 3). Symptom observation, *Bemisia tabaci* transmission, and PCR detection of begomovirus-specific DNA products from infected plants supported the conclusion that the disease is caused by a begomovirus.

Plate 1. Cultivation and management of soybean yellow mosaic virus on soybean plants within a greenhouse condition.

Sequences representing diverse begomovirus groups were downloaded for phylogenetic analysis, resulting in three major clusters: MYMIV, HgYMV, and MYMV (Plate 1 & 2). SYMV formed a distinct cluster within the HgYMV group, known for inducing yellow mosaic disease symptoms in Horsegram and French bean.

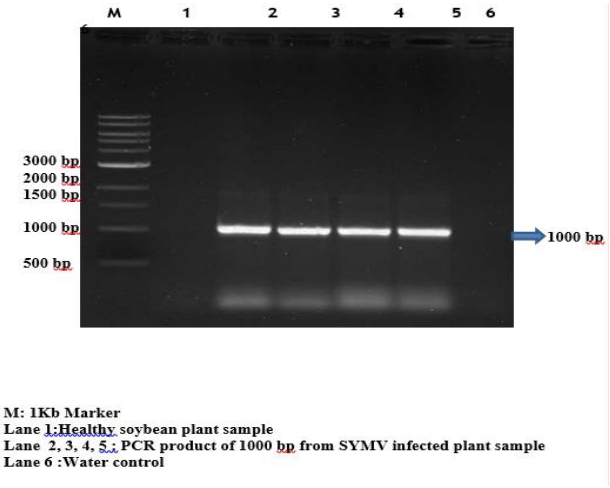

Plate 2. Gel image depicting the PCR amplification of the coat protein gene of SYMV in soybeans, utilizing the HYMV-CP-F/HYMV-CP-R primer pair.

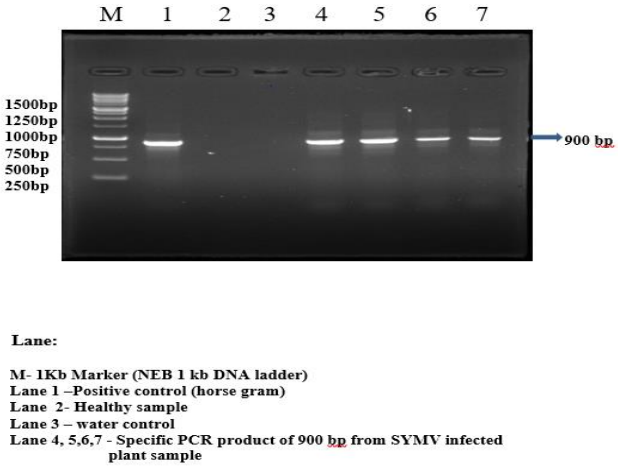

Plate 3. Gel image displaying the PCR amplification of the movement protein gene of SYMV in soybeans, employing the HYMV-MP-F/HYMV-MP-R primer pair.

Comparative analysis of the CP and MP genes in the soybean yellow mosaic virus isolate GKVK, in contrast to other yellow mosaic viruses affecting legume crops, distinctly separates it from MYMV and MYMIV. It forms a cluster with HgYMV isolates from horsegram and French bean found in Tamil Nadu, Bangalore, and Sri Lanka, showcasing significant divergence from other sequences. The diversity and emergence of new geminiviruses are likely due to recurrent recombination among them (Fauquet *et al.*, 2003). Decryption sequencing of the CP gene revealed similarities between YMV-infected samples of blackgram, cowpea, and greengram with the MYMV Tamil Nadu isolates. However, the YMV-infected horsegram sample exhibited similarities to HgYMV (Maheshwari *et al.*, 2014).

Table 2: Nucleotide sequence identity of the coat protein gene in soybean yellow mosaic virus compared to other begomoviruses.

Sr. No.	Homology with begomoviruses and their genomi components	Accession number	Percent homology
1.	Horse gram yellow mosaic virus segment DNA-A horse gram isolate	AM932427	98
2.	Horse gram yellow mosaic virus clone PBdBq04 segment DNA-A	KP752088	98
3.	Horse gram yellow mosaic virus isolate India: Bangalore: French bean	KC019306	98
4.	Horse gram yellow mosaic virus: Full genome	AJ627904	98
5.	Horse gram yellow mosaic virus DNA-A Chittorisolate	KR053204	98
6.	Mungbean yellow mosaic virus isolate VN5 segment DNA-A complete sequence	JX244173	88
7.	Mungbean yellow mosaic virus isolate VN1 segment DNA-A complete sequence	JX244172	88
8.	Mungbean yellow mosaic virus clone VA1 segment DNA-A complete sequence	KC911722	88
9.	Mungbean yellow mosaic virus DNA-A Complete sequence	AY271892	87
10.	Mungbean yellow mosaic virus-Vigna [Maharashtra]	AF314530	87
11.	Soybean yellow mosaic virus AV1gene CP	AJ315963	86
12.	Mungbean yellow mosaic India virus DNA-A	AY27893	83
13.	Mungbean yellow mosaic India virus-[cowpea] DNA-A complete genome	DQ389154	83
14.	Mungbean yellow mosaic India virus-[Akola]DNA-A	AY271893	82
15.	Mungbean yellow mosaic India virus[Bangladesh] complete genome	AF314145	82

* The per cent nucleotide identities were obtained from NCBI nucleotide BLAST

Table 3: Nucleotide sequence identity of the movement protein gene in soybean yellow mosaic virus compared to other begomo viruses.

Sr. No.	Homology with begomoviruses and their genomic components	Accession number	Percent homology
1.	Horsegram yellow mosaic virus segment DNA-A horse gram isolate	AM932427	99
2.	Horsegram yellow mosaic virus clone PBdBq04 segment DNA-A	KP752088	99
3.	Horsegram yellow mosaic virus isolate India: Bangalore :Frenchbean	KC019306	99
4.	Horsegram yellow mosaic virus: Full genome	AJ627904	99
5.	Horsegram yellow mosaic virus segment DNA-A-Lima bean isolate	AM932429	99
6.	Mungbean yellow mosaic virus DNA-A complete sequence	AY271892	88
7.	Mungbean yellow mosaic virus isolate VN1 segment DNA-A complete sequence	JX244172	88
8.	Mungbean yellow mosaic virus clone VA1 segment DNA-A complete sequence	KC911722	88
9.	Mungbean yellow mosaic virus DNA-A Complete sequence	AY271892	87
10.	Mungbean yellow mosaic virus-soybean [Pakistan]	AY26999	86
11.	Mungbean yellow mosaic India virus clone PBds DNA-A	KP779633	85
12.	Mungbean yellow mosaic India virus [soybean] DNA-A	AY049772	84
13.	Mungbean yellow mosaic India virus[soybean] complete genome	AY049773	84
14.	Mungbean yellow mosaic India virusDNA-AcloneM13	KM208845	83
15.	Mungbean yellow mosaic India virus [Mungbean Pakistan] complete genome	AY269992	83

* The per cent nucleotide identities were obtained from NCBI nucleotide BLAST

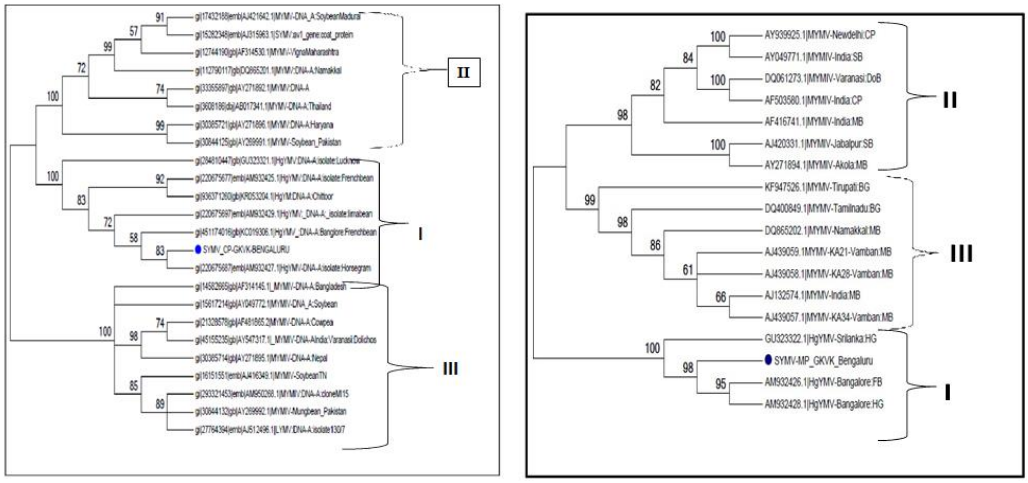

Fig. 2. Phylogenetic tree derived by comparing the nucleotide sequence of the movement protein gene of SYMV. with other begomoviruses from database.

Additionally, Qazi *et al.* (2006) reported the first association of MYMIV with yellow mosaic disease in mothbean in Pakistan. To confirm the virus's identity, PCR employed primers specific to the DNA-B encoded nuclear shuttle protein (NSP) gene of MYMIV (Hussain *et al.*, 2004). This led to the amplification of an approximately 800 bp fragment, subsequently cloned and sequenced. The complete 771 bp sequence of the NSP gene (Accession no AM 233490) displayed a 95-92 percent nucleotide sequence identity (94-92 percent amino acid similarity) with the NSP gene of other MYMIV isolates.

CONCLUSIONS

The scrutiny of soybean yellow mosaic virus (SYMV) and its relation to other begomoviruses offers crucial insights into their genetic diversity and classification. The study highlighted the close genetic kinship between SYMV isolates from Bengaluru and Horse gram yellow mosaic virus (HgYMV), especially evident in the coat protein (CP) and movement protein (MP) gene sequences. This distinction underscores SYMV's unique nature and its divergence from other legume-affecting yellow mosaic viruses. Additionally, the presence of diverse begomovirus groups with their unique genetic profiles emphasizes the role of recombination in generating their variability. These revelations deepen our comprehension of begomovirus genetic diversity and their impact on soybean farming, notably in areas afflicted by yellow mosaic disease.

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Conflict of Interest. None.

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