International Journal of Plant & Soil Science

34(22): 638-644, 2022; Article no.IJPSS.90809
ISSN: 2320-7035

Coat Protein Characterization of *Mungbean yellow mosaic virus* Infecting Blackgram (*Vigna mungo* L. Hepper) in Tamil Nadu

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2022/v34i2231418

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/90809

Original Research Article

Received 05 June 2022
Accepted 10 August 2022
Published 12 August 2022

ABSTRACT

The Yellow Mosaic Disease is the main constraint for decreased yield in blackgram production. Coat protein gene of *Mungbean yellow mosaic virus* (MYMV) has been characterized and phylogenetic tree was constructed. The genomic DNA from diseased plants was collected and it was tested for it's quality and quantity using a Nanodrop. The isolated DNA was subjected to polymerase chain reaction using begomovirus universal primers for preliminary confirmation of the virus. The samples amplified for universal primers were subjected for gene specific amplification using DNA A primers. The DNA A primers resulted in the amplification of the expected size of 1000 bp amplicon. The DNA fragments were sequenced and the sequence results of DNA A were compared with other sequences available in NCBI and it showed higher similarity with *Mungbean yellow mosaic virus* banglore coat protein gene (accession no: MK409376). The sequence was submitted in NCBI database and accession numbers were obtained. The phylogenetic tree was constructed using mega 11 software by comparing the selected strains with our isolate.

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Keywords: Blackgram; mosaic; coat protein; characterization; sequence; phylogeny.

1. INTRODUCTION

Blackgram (*vigna mungo* l. Hepper) is an important pulse used in Indian diet, because the protein content of blackgram is three times more than that of the cereals. The blackgram is used as a supplement of the cereals [1]. Blackgram is widely cultivated in countries like India, Bangladesh and Nepal [2]. In India, the area under blackgram is 4.6 million hectares. The annual production of blackgram in India is 24.5lakh tons which contributes to the 23% of total pulse production in India. In India Madhya Pradesh is the largest producer of blackgram followed by Uttar Pradesh, Rajasthan and Maharashtra. In southern India, Tamil Nadu is one of the leading producers of black gram with an area of 886.9 hectares, producing 365.3 tons annually with the productivity of 412 kg/ha. The blackgram crop is grown during kharif and rabi. Due to the ability of the crop to withstand adverse environmental conditions, it can be grown as rice fallow crop with minimal care, which is a most common practice by farmers. But there are several biotic factors that are involved in hindering the production and yield of blackgram which are pests and diseases, among which diseases like Yellow Mosaic Disease [3], Leaf Crinkle Virus [4], Cercospora leafspot [5], Bacterial leaf spot and Powdery mildew [6] play a major role. Yellow mosaic disease is detected as the major constraint for the blackgram and greengram production in India [7].

In India, yellow mosaic disease is first reported by Nariani in 1960 in Indian Agricultural Research Institute, later the causal organism is diagnosed as a virus i.e. *Mungbean yellow mosaic virus* [8]. In 1968, Williams *et al* has reported the occurrence of MYMV in blackgram [9]. Yellow Mosaic Disease caused by begomovirus belonging to family geminiviridae. The begomovirus are the ssDNA viruses that are transmitted in a non-propagative persistent and circulative manner by the white flies (*Bemisia tabaci*) [10,11,8,12]. This disease is mainly occurred by four distinct begomoviruses they are, *Mungbean yellow mosaic virus*(MYMV), *Horsegram yellow mosaic virus* (HGYMV), *Dolichos yellow mosaic virus* (DOYMV) and *Mungbean yellow mosaic india virus* (MYMIV). But MYMV and MYMIV are of most importance as they cause severe yield losses in grain pulses especially in greengram and blackgram [13]. The symptoms of MYMV appears mostly on leaves and also in pods and seeds. The visible symptoms on leaves will start as yellow spots which later turn into the notable yellow and green alteration in mosaic pattern in later stages it leads to necrosis and death of the leaves. The pods produced by the diseased plant are misshapen and small compared to healthy pods. The disease will usually spread from pods to seeds. The diseased seeds can be visually separated from the healthy seeds as they are ill filled, not in a regular shape and in some cases yellow discoloration is also seen on infected seeds.

MYMV is a begomovirus containing bipartite genome implying that it contains DNA A and DNA B as it's genome. The DNA A containing the coat protein gene is responsible for symptom expression and vector transmission [14,15].

2. MATERIALS AND METHODS

2.1 Collection of Diseased Samples

The samples were collected from the plants showing typical and prominent symptoms of Yellow Mosaic Disease which include yellow color patches alternating with green color showing mosaic pattern. Though the mature leaves are showing prominent and noticeable symptoms, the samples are collected from young leaves i.e., emerging and expanding leaves because the concentration of virus will be more in the developing meristematic tissues. The diseased samples were collected from the experimental fields of Department of Pulses, Tamil Nadu Agricultural University, Coimbatore.

2.2 Plant Genomic DNA Isolation

As the virus undergoes its replication in the nucleus of the plant cell, the total genomic DNA of the plant was isolated using the Gem- CTAB method with slight modifications. Gem-CTAB method is a DNA isolation method specifically modified for the detection of begomoviruses in legumes [16]. The buffer containing 100mm Tris-HCl, 10mm EDTA, 2.0m NaCl, and 2% CTAB is prepared and autoclaved after adjusting the $\boldsymbol{p^H}$ to 8.0. β-mercaptoethanol is added prior to the isolation procedure. To 100mg of leaf sample taken in pre chilled pestle and mortar, 1ml of the buffer was added, transferred into 1.5ml eppendorf tube and kept for incubation at @65°c for one hour. During incubation the tubes were

intermittently mixed to keep the buffer and plant sample homogenous. After incubation, 0.8 volumes of chloroform: iso- amyl alcohol was added to the tubes and mixed gently by inversion until the formation of emulsion. The tubes were centrifuged at 10,000rpm for 10 min at 4°c in a refrigerated centrifuge. The chloroform: iso- amyl alcohol step was repeated when the phenol content in the sample was more. The supernatant/ the upper aqueous layer was collected into a new tube. 0.6 volume of ice cold isopropanol was added to the tubes and incubated at -20°C overnight. After overnight incubation, the tubes were centrifuged at 10,000 rpm for 10 min to obtain the nucleic acid pellet. The obtained pellet was washed twice with 70% ethanol. After ethanol wash the tubes are kept open at room temperature for the ethanol to evaporate completely from the DNA. 50µl of double sterile distilled water was added to dissolve the pellet. The isolated DNAs were stored at -20°c for further use.

2.2.1 Quality and quantity check of isolated DNA

The isolated plant DNA were checked using gel electrophoresis by making them to run on 0.8% agarose gel after staining with ethidium bromide. 6X DNA loading dye was added to the DNA to make them visualized under UV light.

The concentration of the DNA was measured by using Nanodrop. The absorbance of the sample DNA placed in nanometer at 220 nm of wavelength has given the concentration of the DNA and also the level of protein contamination in DNA.

2.3 PCR Based Detection Using Universal Primers

For the preliminary confirmation of the isolated DNA for the presence of begomovirus, the DNA was subjected to Polymerase Chain Reaction using universal degenerative primers of begomovirus i.e., PALr772 and PALc1960 [17]. The sequence of the primer set is PALr772-5'GGNAARATHTGGGATGGA3' and PALc1960-5'ACNGGNAARACNATGTGGGC3'.The reaction mixture was added to sterile PCR tube containing 10µl of taq DNA polymerase, optimized buffer, and DNTP mixture in a ready-to-use master mix (2X Takara EmeralAmp GT PCR Mastermix), 1µl of each forward primer reverse primers and template DNA, the final volume was adjusted to 20µl using double sterile distilled water. The temperature profile followed was initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes and elongation at 72°C for 3 minutes, the final extension step was carried out at 72°C for 10 minutes. The PCR product was kept in hold at 4°C. The Polymerase Chain Reaction was carried out in the Mastercycler® nexus gradient thermal cycler. The primer set is expected to amplify the DNA fragment of ~1.5 Kb.

After the PCR reaction the samples were made to run on 1.2% agarose gel using 1x TAE buffer after staining the gel with ethidium bromide. The gel was imaged under an alpha imager to know the amplification of the sample to the desired size to confirm the presence of the virus.

2.4 Amplification Using Gene Specific Primers

By the confirmation of the presence of begomovirus in the sample, we used the MYMV specific primers to amplify DNA A of MYMV. The forward primer of 5'ATG GG (T/G) TCC GTT GTA TGC TTG3' sequence and the reverse primer of the sequence 5'GGC GTC ATT AGC ATA GGC AAT3' were used to amplify the coat protein region of MYMV [3]. The reaction mixture was prepared by adding 25µl of master mix, 2 µl of forward primer, 2 µl of reverse primer and 2 µl of diluted template DNA. The final volume was adjusted to 50 µl using nuclease free water. The Polymerase Chain Reaction was carried out in Mastercycler® nexus gradient thermal cycler using a temperature profile of initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 54°C for 2 minutes and elongation at 72°C for 3 minutes, the final extension step was carried out at 72°C for 10 minutes. The amplified PCR products were analyzed and the size of amplicons was interpreted utilizing electrophoresis on 1 per cent agarose gel stained with ethidium bromide using 1x TAE buffer. In a UV -gel documentation unit (Alpha Imager, USA), the gel was observed, and the findings were recorded [18-20].

2.5 Sequencing, Sequence Analysis and Construction of Phylogenetic Tree

The samples amplified for Coat Protein region of MYMV using gene specific primers were sequenced by sanger sequencing. The sequence obtained was searched in NCBI- BLAST to know

Table 1. Details of primers used and anticipated amplicon sizes

S.No	Name of the Primer	Sequence 5' To 3'	Annealing Temperature (°C)	Anticipated Amplicon Size	Reference
1	PALR772	5'GGNAARATHTGGGATGGA3'	55	~1.5KB	[17]
2	PALC1960	5'ACNGGNAARACNATGTGGGC3'			
3	MYMV-CP-F	5'ATGGG(T/G) TCCGTTGTATGCTTG3'	54	1000BP	[3]
4	MYMV-CP-R	5'GGCGTCATTAGCATAGGCAAT3'			

Table 2. List of accession numbers provided by ncbi to the mymv isolate and coat protein of this study

S. No	Location of Sample collection	Isolate name	Primers used	Accession number
1	National pulse research station, Vamban	Mungbean yellow mosaic virus Tamil Nadu isolate in blackgram	PALr772 and PALc1960	OP121618
2	Department of Pulses, TNAU	Coat protein gene of Mungbean yellow mosaic virus Tamil Nadu isolate	MYMV-CP-F and MYMV-CP-R	OP121617

the sequence similarity with other begomoviruses. Sequence identity matrix tool, Bio Edit software was used to assemble and analyze the data. Multiple alignments were made and amino acid sequence was obtained by ClustralW programme. The sequence was deposited in NCBI BLAST and the accession numbers were obtained (Table 2). The phylogenetic tree was constructed based with the available sequences using MEGA 11 Software.

3. RESULTS AND DISCUSSION

The isolated DNA from diseased plants has sown a clear non smeared band on 0.8% agarose gel. The quantity of DNA using nanodrop revealed that all the DNAs are at good quantity of 1000-2000 ng/µl.

The diluted DNA were subjected for amplification using Roja's primer, they showed an amplicon at ~1.5 kb which is the expected size (Fig. 2). Then the samples amplified using gene specific primers that were DNA a (coat protein) showed the amplification at 1000bp (Fig. 3). For further confirmation the samples of DNA A amplification were given for sequencing. The nucleotide sequence has blasted in NCBI and showed similarity of 98% query coverage with coat protein genes of MYMV existing in the database. The sequence was deposited in NCBI database and accession number was obtained. The accessions numbers obtained were op121618 for *Mungbean yellow mosaic virus* Tamil Nadu Isolate in blackgram and op121617 for Coat Protein gene of *Mungbean yellow mosaic virus* Tamil Nadu isolate. Phylogenetic tree was

Table 3. The nanodrop readings of isolated plant genomic DNA representing it's concentration

S.No	A260/230	A260/280	Conc. (ng/µl)
1	1.539	1.516	2175.4
2	1.306	1.344	2119.2
3	0.946	1.096	2156.0
4	0.926	1.073	2225.6
5	1.097	1.300	2043.3
6	1.515	1.513	2238.3
7	1.295	1.337	2272.0
8	1.573	1.634	2156.9
9	1.853	1.790	2070.8
10	1.629	1.789	2041.0

Fig.1. Blackgram Leaves showing the green and yellow patches alternating to give mosaic symptoms

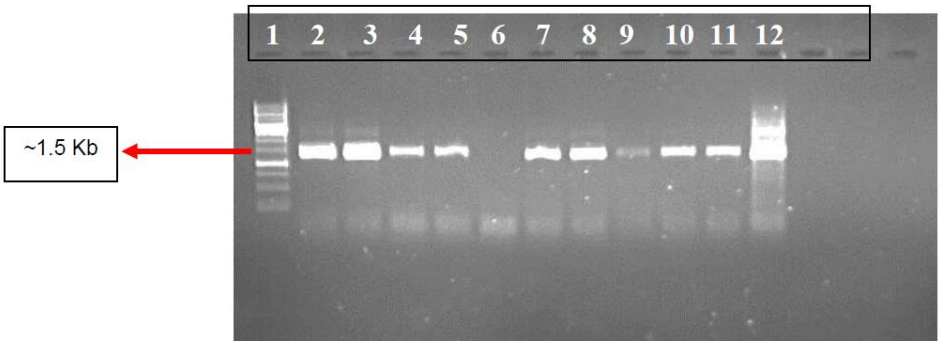

Fig. 2. Agarose gel electrophoresis image of infected plant genomic DNA amplified with Universal primers

Lane1- 1Kb Ladder, Lane2- Sample1, Lane3- Sample2, Lane4- Sample3, Lane5- Sample4, Lane6- sample5, Lane7- Sample6, Lane8- Sample7, Lane9- Sample8, Lane10- Sample9, Lane11- Sample10 Lane12- Positive control

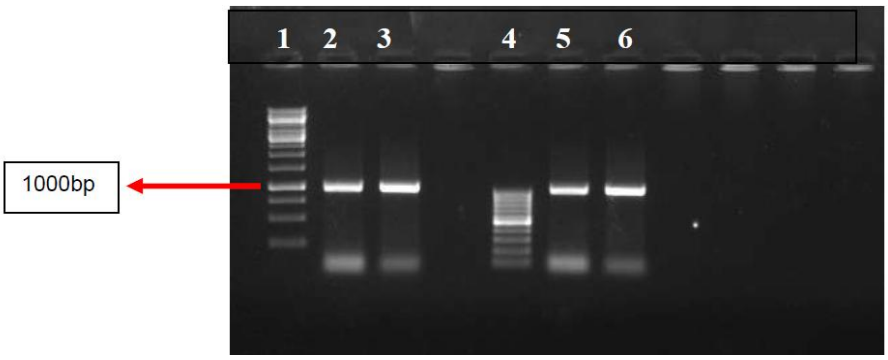

Fig. 3. Agarose gel electrophoresis image of Gene specific amplification of MYMV

Lane1- 1Kb Ladder, Lane2- Sample1, Lane3- Sample2, Lane4- nil
Lane4- 100bp ladder, Lane5- sample1, Lane6- Sample2

constructed using Mega 11 software using Bootstrap sampling with 1000 replications in which our isolate has showed sequence similarity with other deposited MYMV DNA a segment sequences (Fig. 4). Our isolate has showed close similarity with *Mungbean yellow mosaic virus* banglore coat protein gene (accession no: MK409376).

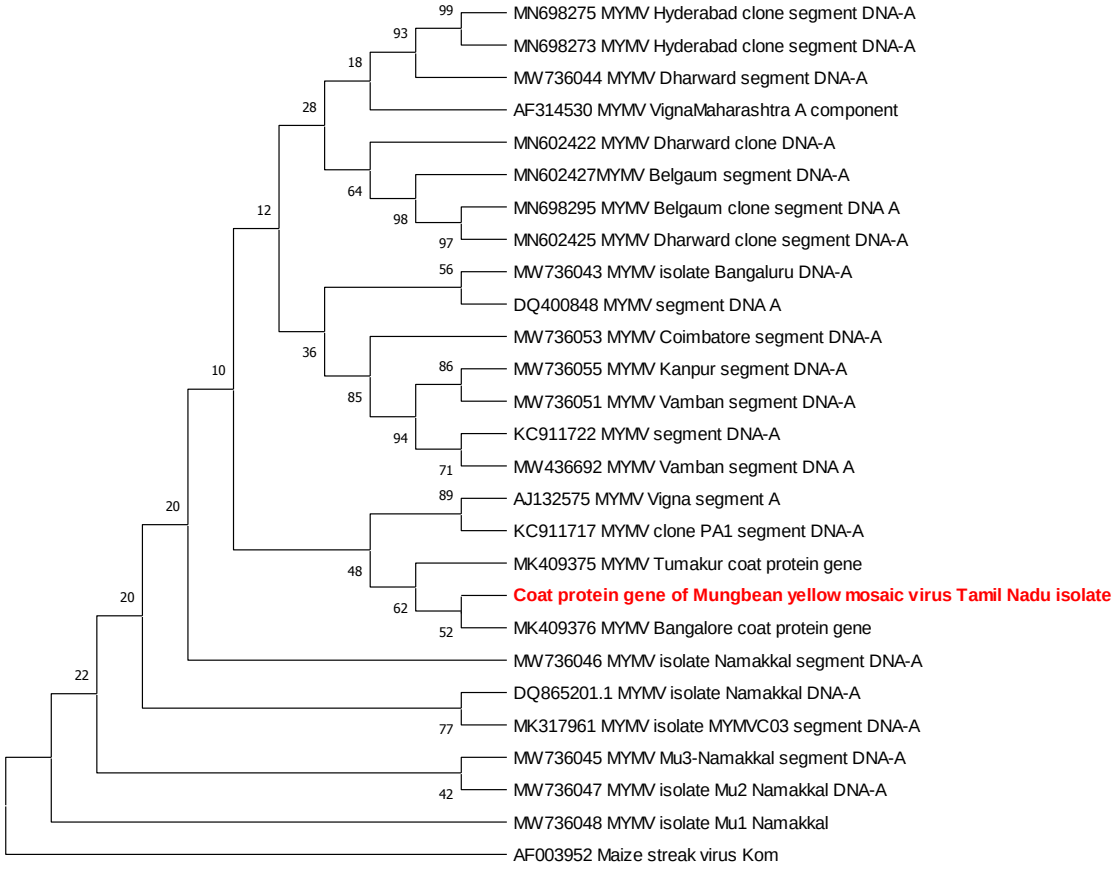

Fig. 4. Phylogenetic tree of coat protein gene of Mungbean yellow mosaic virus Tamil Nadu isolate

Phylogenetic tree was built for coat protein gene of Coat protein gene of Mungbean yellow mosaic virus Tamil Nadu isolate with coat protein genes of other MYMVs by using Neighbor joining method in Mega 11 software programme. Maize streak virus was taken as outgroup

4. CONCLUSION

The diseased samples collected from fields of Department of Pulses has showed the typical symptoms of MYMV. The DNA isolated from diseased samples using GEM- CTAB method was of good quality as well as the quantity. The expected amplicon size was showed with Roja's primer and gene specific primers. The coat protein of MYMV is sequenced and submitted in NCBI database. NCBI has allotted accession numbers to the submitted sequences. The phylogenetic tree has shown similarity with *Mungbean yellow mosaic virus* Banglore coat protein gene (accession no: MK409376).

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Plant Pathology, Department of Nanoscience and Technology and Department of Plant Biotechnology Tamil Nadu Agricultural University, Coimbatore for providing facilities required to carryout this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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