Indian Journal of Biotechnology
Vol. 19, April 2020, pp 113-117

Identification of new strain of mungbean yellow mosaic virus infecting french bean (*Phaseolus vulgaris* L.) in Santiniketan

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Received 12 April 2018; revised & accepted 31 March 2020

Outbreak of yellow mosaic diseases emerged during 2017 in French bean (*Phaseolus vulgaris* L.) growing region of Santiniketan, Bolpur, West Bengal, India. Yellow mosaic disease was characterized by bright yellow mosaic spots on leaves. Amplification of an expected 598 bp DNA band has been done by polymerase chain reaction (PCR) using primers specific to Santiniketan coat protein (AV1) gene (HQ221570.1) of mungbean yellow mosaic India virus (MYMIV). Comparison of sequences obtained from PCR products with corresponding nucleotide sequences in GenBank, confirmed association of causative agent with mungbean yellow mosaic India virus. Nucleotide sequence analyses of MYMIV- Santiniketan isolates of French bean showed high sequence similarities (> 95%) with others isolates present world-wide. A close relationship of coat protein gene isolate of Santiniketan with DNA-A of Pant Nagar, Uttarakhand (DQ389152.1) of mungbean yellow mosaic India virus has been established on phylogenetic tree analysis. This report provides an evidence of MYMIV infections in French bean in Santiniketan.

Key words: French bean, MYMIV, Identification, Coat protein.

Introduction

Phaseolus vulgaris L. commonly known as French bean is an important legume crop which is grown widely in India. Beans are a rich source of protein, carbohydrates and vitamins especially of vitamin B complex which includes niacin, riboflavin, folic acid and thiamine[1-4]. The viral diseases in legume crop is common. Among viruses infecting the legumes, four distinct begomoviruses namely, mungbean yellow mosaic virus (MYMV), mungbean yellow mosaic India virus (MYMIV), horse gram yellow mosaic virus (HgYMV) and *Dolichos* yellow mosaic virus (DoYMV) identified across Southern Asia including India, Bangladesh, Pakistan, Sri Lanka and Thailand, are responsible for yellow mosaic disease (YMD)[5-6]. All these viruses are easily transmitted by whitefly, *Bemisia tabaci* while feeding on leaves[7-8]. MYMIV causes serious yellow mosaic disease that leads to high yield loss not only in beans but also in tomato, cassava and cotton[9-11]. An approximate monetary loss of $300 million has been documented for legume crops in India[12].

In the past, new strains or variants have been emerging by the epidemic outburst of the diseases, leading to recurrent loss of resistance[13]. Geminiviruses belong to the genus Begomovirus and are circular single-stranded DNA viruses characterized by twin icosahedral particles[14]. They have either bipartite genome (DNA-A and DNA-B) or a monopartite genome, homologous to the DNA-A of bipartite geminivirus[15-16]. DNA-A contains all the factors required for viral replication, encapsidation, transmission, and systemic spread and DNA-B for movement functions[17]. Among all genes present in the genome of the begomovirus, the coat protein gene AV1 is found to be the most conserved one[18-19].

During 2017, *P. vulgaris* plants in different fields in Santiniketan exhibited YMD symptoms like stunted growth, curling, yellowing and yellow mosaic symptoms on leaves and deformed fruits with small malformed fewer seeds in the pods (Fig. 1). To identify the suspected causative agent, total genomic DNA (gDNA) was extracted from nine cultivars of *P. vulgaris* with YMD symptoms and with no symptoms. Primers have been designed from Santiniketan West Bengal coat protein (AV1) gene (HQ221570.1) of MYMIV. Amplification of AV1 confirmed the association of a begomovirus with the disease.

Materials and Methods

Sample Source

Nine cultivars of *P. vulgaris* namely Anupam, Selection-9, Malgudi, Arjun, Falguni, Seville and

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Arkasuvidha were obtained from Bidhan Chandra Krishi Visvavidyalaya (West Bengal, India) and LAP 1, LAP 2 were obtained from Liebigs Agro Chem. Pvt. Ltd (Kolkata, India) and cultivated in the experimental field at the Department of Biotechnology, Visva-Bharati in Santiniketan, Bolpur (West Bengal, India). Leaf samples were collected based on typical symptoms of YMD (Fig. 1) as described by Qazi *et al.* (2007) and Naimuddin *et al.* (2011). Fresh tissue was directly subjected for virus detection and the remaining was stored in liquid N_2 at -80°C.

Genomic DNA Isolation

Genomic DNA was isolated from all the nine cultivars of *P. vulgaris* by revised cetyl trimethyl ammonium bromide (CTAB) method from uninfected leaves and infected leaves from the experimental field[20]. Approximately 100 mg of leaf sample was powdered in liquid N_2 and made a slurry with 1 ml of chilled CTAB buffer. In brief, the composition of CTAB buffer is 2% CTAB, 2% PVP-40, 100 mM Tris-HCl of pH 8.0, 1.4 M NaCl, 20 mM EDTA and 1% 2-mercaptoethanol. The homogenized sample was incubated for 10 min at 65°C. One ml of chilled chloroform: isoamyl alcohol in 24:1 ratio (vol/vol) was added and mixed thoroughly for a while. Ten min of spin was done thereafter at 10,000 g at 4°C. Again 1.5 ml of chilled isopropanol was added to 0.5 ml of soup and left for overnight incubation at -20°C. A 30 min of spin was performed at 10,000 g at 4°C. Soup was discarded and the pellet was washed twice in 70% ethanol. Next, pellet was allowed to dry for 10 min and dissolved into 20 mM Tris-HCl (pH 8.0). RNase treatment was done to remove RNA. The purity of isolated DNA was checked in micro volume spectrophotometer (JENWAY) and the integrity was checked on 0.8% agarose gel.

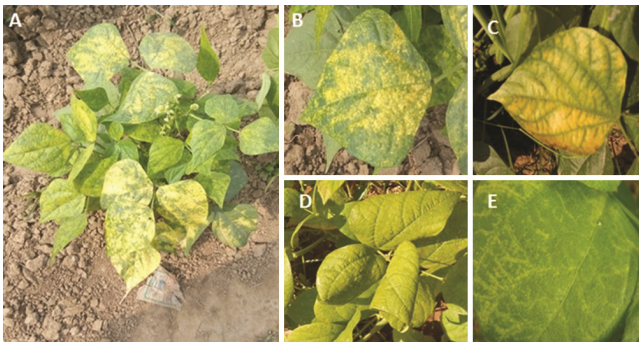

Fig. 1 —Disease symptoms of YMD by MYMIV infection in *P. vulgaris* in the field during 2012: A: Plant showing yellow mosaic patches on leaves B: Close-up of infected leaf with yellow mosaic patches. C: Typical yellowing of leaf. D: Curled deformed leaves. E: Yellowing and mosaic vein banding on leaf.

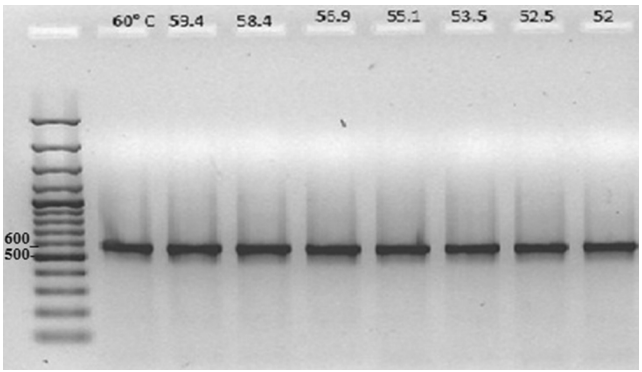

Fig. 2 — Amplified coat protein gene in a gradiant of annealing temperature of 52°C- 60°C.

Primer Designing

The partial cds of MYMIV (West Bengal isolate) coat protein (AV1) gene (HQ221570) was downloaded from the NCBI website (www.ncbi.nlm.nih.gov) and the primers were designed using Primer3 (Table 1).

Amplification of Coat Protein (AV1) Gene and Gel Elution

For the identification of viral strain in the collected samples, isolated genomic DNA samples were subjected to PCR using AV1 gene specific primers (Santiniketan Bolpur (West Bengal isolate) following an initial denaturation at 95ºC for 2 min and 40 cycles of denaturation at 95ºC for 10 sec, annealing at 52°C - 60°C for 20 sec and extension at 72ºC for 1 min. The reaction was completed with final extension at 72ºC for 7 min. Amplicons were run on 1.5% agarose gel to check amplification (Fig. 2).The amplified products were eluted with PrimePrep gel purification kit (GeNet Bio) and sent for sequencing.

Construction of Phylogenetic Tree

Partial sequences of the isolate were analyzed using database searches with other geminiviral sequences. The database searches were carried out by NCBI-BLAST program (http://blast.ncbi.nlm.nih.gov). The sequences of the isolate were aligned with the sequences of 21 MYMIV strains before the phylogenetic analysis using MEGA6 software[21] to see the homology or similarity pattern. A phylogenetic tree was constructed using maximum parsimony with 1000 bootstrap with 21 geminiviral coat protein gene reported world-wide.

Result and Discussion

Field Symptoms

Infected *P. vulgaris* nine cultivars showed leaf curl, yellowing, severe yellow mosaic, small leaf and

Table 1 — Primer for amplification of MYMIV AV1 gene.

Target	Forward primer	Reverse primer
AV1	5′GAAACCTCGGTTTTACC GACTGTATAG3′	5′TTGCATACACAGGA TTTGAGGCATGAG3′

dwarfism symptoms, a typical of the begomo viruses in the field that cause YMD in leguminous plants[22-23]. Initially only a few yellow spots appeared in the inter-veinal areas, but as day passed some patches appeared on the leaves and by the time the crop reached maturity more than 70% of the leaf area turned yellow.

DNA Isolation and Amplification of Coat Protein

RNase treated gDNA was run on 0.8% agarose gel to check the integrity of DNA (Fig. 2). AV1 gene was amplified by gene specific primers as mentioned in Table 1. From gradiant PCR, a temperature of 60°C has been selected as an ideal annealing temperature to amplify 598 bp of AV1 gene of MYMIV.

Sequence Similarity Assessment by BLAST

BLAST result showed that the nucleotide sequences of Santiniketan, Bolpur (WB, India) isolate has > 90% sequence similarities and identities with coat protein gene of the different strains of MYMIV (Table 2).

Table 2 — Percent identities of nucleotides of Santiniketan, Bolpur MYMIV coat protein gene isolates with coat protein gene of the selected geminiviruses reported worldwide.

Description	Max score	Total score	Query cover	E value	Identity	Accession
Mungbean yellow mosaic India virus segment DNA A, complete sequence_New Delhi	719	719	69%	0.0	98%	EU523045.1
Mungbean yellow mosaic India virus segment DNA A, complete sequence_Delhi	713	713	69%	0.0	98%	DQ389153.1
Mungbean yellow mosaic India virus [SoybeanTN] complete genome_Tamil Nadu	713	713	69%	0.0	98%	AJ416349.1
Mungbean yellow mosaic India virus segment A, complete sequence, isolate Palampur_Himachal Pradesh	708	708	69%	0.0	97%	FN794200.1
Mungbean yellow mosaic India virus precoat protein [AV2] and coat protein [AV1] genes, complete cds_Punjab	697	697	69%	0.0	97%	DQ389151.1
Mungbean yellow mosaic India virus, segment DNAA, complete sequence, clone MI9_Pakistan	682	682	69%	0.0	96%	FM208842.1
Mungbean yellow mosaic India virus, segment DNAA, complete sequence, clone MI2_Pakistan	680	680	69%	0.0	96%	FM208846.1
Mungbean yellow mosaic India virus, segment DNAA, complete sequence, clone MI13_Pakistan	680	680	69%	0.0	96%	FM208845.1
Mungbean yellow mosaic India virus, segment DNAA, complete sequence, clone MI12_Pakistan	680	680	69%	0.0	96%	FM208844.1
Mungbean yellow mosaic India virus segment DNA A, complete sequence, clone MI15_Pakistan	675	675	69%	0.0	96%	AM950268.1
Mungbean yellow mosaic India virus, segment DNAA, complete sequence, clone MI7_Pakistan	675	675	69%	0.0	96%	FM208843.1
Legume yellow mosaic virus complete genomic DNAA, isolate 14_Pakistan	675	675	69%	0.0	96%	AJ512495.1
Legume yellow mosaic virus complete genomic DNAA, isolate 130/12_Pakistan	669	669	69%	0.0	96%	AJ512497.1
Legume yellow mosaic virus complete genomic DNAA, isolate 130/7_Pakistan	669	669	69%	0.0	96%	AJ512496.1
Mungbean yellow mosaic India virus, segment DNAA, Vigna radiata av2 gene, av1 gene, ac5 gene, ac3 gene, ac2 gene, ac1 gene and ac4 gene, clone MI72, complete genome_Pakistan	664	664	69%	0.0	95%	FM955599.1
Mungbean yellow mosaic India virus precoat protein [AV2] and coat protein [AV1] genes, complete cds_Punjab	652	652	69%	0.0	95%	DQ389155.1
Mungbean yellow mosaic India virus segment DNA A, complete sequence_Punjab	652	652	69%	0.0	95%	DQ400847.1
Mungbean yellow mosaic India virus, segment DNAA, av2 gene, av1 gene, ac5 gene, ac3 gene, ac2 gene, ac1 gene and ac4 gene, clone MI76, complete genome_Pakistan	647	647	69%	0.0	95%	FM955600.1
Mungbean yellow mosaic India virus, segment DNAA, complete sequence, clone A14_Pakistan	647	647	69%	0.0	95%	FM208839.1
Mungbean yellow mosaic India virus, segment DNAA, complete sequence, clone A2_Pakistan	647	647	69%	0.0	95%	FM208835.1
Mungbean yellow mosaic India virus precoat protein [AV2] and coat protein [AV1] genes, complete cds_Pant Nagar, Uttarakhand	647	647	69%	0.0	95%	DQ389152.1

The sequence of the coat protein of new isolate was aligned with the published sequences of other 21 MYMIV strains present world-wide and phylogenetic relationship was assessed using MEGA6 software.

Phylogenetic Relationship of the New Coat Protein Isolate

The phylogenetic tree has been constructed with the other isolates of Geminiviruses present world-wide using maximum parsimony method. The phylogenetic tree was obtained from the alignment of nucleotide sequences of 21 published Geminiviruses using MEGA6 Software. This new Santiniketan isolate showed the maximum matching of 84% score with Pant Nagar DNA-A (DQ389152.1) of Uttarakhand (Fig. 3).

The development of complete YMD symptoms prominently on leaves along with malformed seeds in fruit, curved leaves and stunted growth after white flies seen on the leaves, confirmed the MYMIV as the causal agent which were transmitted by the white flies. Under molecular studies, the amplification of AV1 gene of MYMIV using primers developed from the Santiniketan, Bolpur (West Bengal) isolate further confirmed the association of this virus with the infection of MYMIV found at the experimental field in Santiniketan. For prediction of viral species and strains, a cut off of >90% sequence similarity has been used as a standard cut off[24]. In the present study, the > 90% sequence similarity of Santiniketan, Bolpur isolate with MYMIV reported world-wide (in BLAST analysis) suggested that this Santiniketan, Bolpur isolate is the MYMIV strain. A high sequence homology of Santiniketan, Bolpur isolate of 98-95% in BLAST analysis is seen with MYMIV isolates of Pakistan and different states of India namely Delhi, Tamil Nadu, Himachal Pradesh and Uttarakhand. A highest of 98% sequence homology was found with MYMIV isolates of Delhi (EU523045.1, DQ389153.1) and Tamil Nadu (AJ416349.1) and 95% with Pant Nagar (Uttarakhand) MYMIV isolate (DQ389152.1) (Table 2). Further phylogenetic analyses to study the relationship of Santiniketan, Bolpur isolate with other MYMIV strains present world-wide showed that all MYMIV and legume YMV infecting beans belong to one cluster (Fig. 3). The study showed that MYMIV from Santiniketan has very close genetical association to Pant Nagar DNA-A (DQ389152.1) of Uttarakhand. MYMIV infection on *P. vulgaris* has been reported in several studies but its strain identification has turned out to be the first report in this present study.

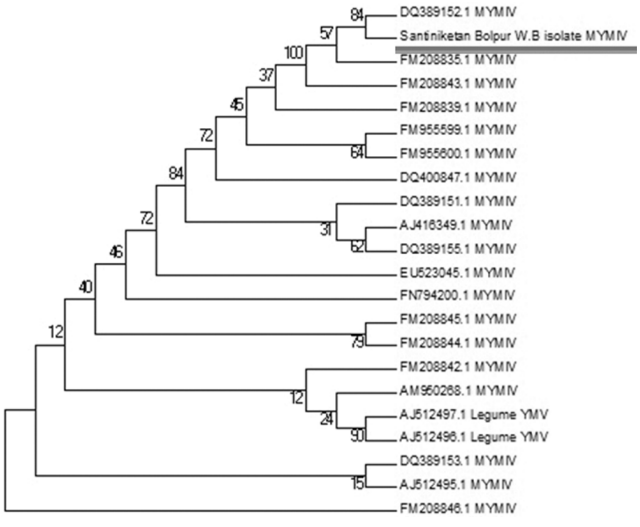

Fig. 3 — Phylogenetic tree generated by Maximum Parsimony obtained from alignment of nucleotide sequences of 21 gemini viruses using MEGA6 software. Values at the nodes indicate percentage of bootstrap support (out of 1000 bootstrap replicates). GenBank nucleotide accession numbers are indicated at the end of each branch.

Conclusion

The result presented here clearly indicated that the primer pair designed in this study can be used for molecular diagnosis of MYMIV infection. The region of the coat protein gene of Santiniketan, Bolpur (West Bengal, India) isolate in this study through PCR could be used for developing antiviral vector by cloning into a suitable plant transformation vector in an antisense orientation.

Acknowledgments

NP, VK and JB are grateful to the Department of Biotechnology Visva-Bharati for financial support.

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