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Identification and Molecular Characterization of Mung Bean Yellow Mosaic Virus in French Bean through Coat Protein Gene

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ABSTRACT

Background: French bean (*Phaseolus vulgaris* L.), is one of the important legume vegetables grown in India. The disease mung bean yellow mosaic virus (MYMV) limits successful production of beans. MYMV has become an epidemic in bean growing regions, particularly in the tropical and subtropical countries. In summer, the disease is more serious and widely distributed and reported in various countries. The current study is aimed at identification of MYMV and molecular characterization of mung bean yellow mosaic virus in French bean. This study will be helpful for early detection of the virus, so that better preventive measures can be taken to control MYMV.

Methods: The investigations were conducted during 2016-2017, all the agro climatic zones of Karnataka province were surveyed. The coat protein (CP) gene of MYMV was amplified using gene specific primer with DNA isolated from MYMV infected leaves samples in french bean. Polymerase chain reaction products were Sequenced and analysed using a bioinformatic tool.

Result: The CP gene decrypt sequences analysis revealed that the identity and similarity in global alignment for all the obtained sequences ranging from 80.8 to 95.3% with reference to MYMV, whereas for horse gram yellow mosaic virus (HYMV) it ranges from 90.4 to 99.1%. Two distinct yellow mosaic viruses infecting french bean (MYMV and HYMV) were identified and it was observed that there exists considerable genetic variation among these species. Present work showed that the CP region is efficient enough to provide a simple, rapid and reliable method for early detection of MYMV infections in french bean, which would help to develop proper management strategies to control these viruses.

Key words: Gemini virus, HYMV, MYMV, Mung bean, Whitefly, Yellow mosaic disease.

INTRODUCTION

French bean is one of the most important legume vegetables grown for its green tender pods, processing as canned, frozen or freeze dried products. The successful productions of beans is limited by the disease mung bean yellow mosaic virus (MYMV) and has become epidemic in bean growing region, particularly in tropical and subtropical countries. The disease is found during all the three seasons in bean growing areas, but it is more severe during summer, because of high whitefly population.

The disease is caused by mung bean yellow mosaic virus, which is easily transmitted by whiteflies. The virus belongs to family *Geminiviridae*, genus *Begomovirus* (Mayo and Pringle 1988, Jindal *et al*., 1998) and is isometric paired, 18 × 30 nm in size, have single stranded DNA. These particles are limited to phloem-associated elements in infected plants (Srivastava, 1979). Initial symptoms appear in the form of irregular yellow patches of various sizes, which coalesce to form larger patches of bright yellow colour and this is accompanied with general stunting of plants. In severe cases, entire leaves may turn yellow and plant bears few flowers and small curved pods (Chenulu *et al*., 1979). The production loss due to MYMV in french bean has not been systematically studied. However, the yield loss due to this virus in french bean is accounted from10 to 100 per cent (Marimuthu *et al*., 1981).

Based on nucleotide sequence data of the genomic components of YMV (Yellow mosaic virus), two distinct *begomoviruses*, Mungbean yellow mosaic India virus and MYMV (Morinaga *et al*., 1990) were suggested to be associated with the etiology of YMD in legumes in south Asia including India. This has probably already been suggested that MYMV causes severe YMD in french bean in southern parts of India (Karthikeyan *et al*., 2004). More often the identification of a particular strain of YMV affecting these crops creates a challenge by simple morphological observations. Hence the suitable genomic approach should be devised to detect the early infections in these crops. Therefore, the present study was designed to identify different isolates of MYMV affecting french bean in all the regions of Karnataka, so that better preventive measures could be taken for the management of this virus. In this study coat protein gene was taken for the identification of the presence of different strains of MYMV as it is well

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characterized and widely used for the virus identification, classification and characterization (Mahajan *et al*., 2011).

MATERIALS AND METHODS

The mung bean yellow mosaic virus infected french bean leaves were tested for the presence of MYMV in the infected plants samples, total genomic DNA was isolated from the both infected as well as non-infected leaf samples. Isolated DNA samples were subjected to PCR amplification using MYMV gene specific coat protein (CP) primers. The MYMV CP gene specific markers, RHA, MYMV, CP, IGRA, IGRB, YFP1 and YFP2 were screened for gene specific amplification.

Details of experiment

Research was conducted during 2016-2017 in the Department of Plant Pathology in collaboration with the Department of Plant Biotechnology at College of Horticulture Bengaluru, University of Horticultural Sciences, Bagalkot.

Sampling of MYMV

The MYMV infected french bean samples were collected from all the agro-climatic zones of the Karnataka. Total five infected leaves samplesfrom each agro-climatic zones were collected. The samples (infected plant leaves showing distinct disease symptoms) from each location were collected and immediately placed in liquid nitrogen (Fig 1). The samples were stored at -80°C for further analysis. Healthy plants samples were also collectedin the field at College of Horticulture, Bengaluru as a control.

DNA extraction

The total genomic DNA was extracted from leaf tissues of healthy and MYMV infected frenchbean leaves. Genomic DNA was prepared by the following methods described by Krishna and Jawali (1997) with a few minor modifications. Frozen tissue sample of two grams collected from pool of 4-5 leaves from each plant was ground into fine powder in liquid nitrogen, using autoclaved mortar and pestle. Thegrounded sample was transferred into a 1.5 ml eppendorf tube. 1500 µl of pre-warmed (65°C) CTAB DNA extraction buffer was added to grounded 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 to fresh 1.5 ml eppendorf tube. The DNA was precipitatedovernight 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 $T_{10}E_1$ 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.

PCR amplification and gel electrophoresis

In order to determine the nucleotide sequence of coat protein in mungbean yellow mosaic virus, specific primers available in the literature were tried to amplify coat protein region of yellow mosaic viruses. Details of seven MYMV gene specific coat proteins (Cp) marker sequence information are presented in Table 1.PCR was performed in Thermocycler, programmed for one step of initial denaturation at 95°C for 4 min and 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min for the listed primers and extension at 72°C for 3 min, followed by one step of final extension at 72°C for 10 min. PCR was conducted with Dream Taq Master mix in total reaction mixture volume of 25 l that contained Dream Taq Master mix- 13 µl; dH_2O - 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 in Tris-acetate- EDTA buffer containing ethidium bromide @ 0.1%. The gel was observed under Gel Documentation System.

Data analysis

Only clear and unambiguous amplicons of MYMV CP markers were scored. The sizes of the amplified fragments were estimated with the help of Alpha image software by

Fig 1: Infected plant leaves showing distinct disease symptoms.

Table 1: List of MYMV CP gene specific markers used for molecular variation in MYMV infected french bean.

Marker	Forward sequence (5'-3')	Reverse sequence (5'-3')	Amplicon Size in (bp)	Reference
RHA	TCAAGCTCCCGGTGCATGTTGCA	GTAAAGCTTTACGCATAATG	750	Obaiah *et al.*, 2013
MYMV	ACACGAGCTCCTCTACCCCGATATCGAT	ACACGGATCCGTTGATACACAGGAT TG	750	Mohammad *et al.*, 2012
CPF	GGTGGATCCCCATGTAAAGTTCAATCA	ATGAAGCTTCATGGCTTGTTCCTTACAAGC	2.7K	Haq *et al.*, 2011
IGRA	TCA AGC TTT GCGTTT ATA GCA AA	GAG AAT TCT TGCCAA CAT GCA CCG GA	2.7K	Haq *et al.*, 2011
IGRB	GAC AGT CTTCTG TTC CAG AT	ACG AAC GAC ATTGTG CGT AT	2.7K	Haq *et al.*, 2011
YFP1	GCGGAATTACGATACCGCC	GATGCATGAGTAC A T G C C	704	Richa *et al.*,2014
YFP2	GCCAAAGCGGAATTACGA	GCCTCTTGGTGGTTGTAAAC	648	Richa *et al.*, 2014

gel documentation system using 100 bp DNA ladder (NEB) as size standard. Markers were scored for the presence or absence of the corresponding amplicons among the different samples. Further, PCR amplified sample from each zone was purified and confirmed by DNA sequencing. Sequence analysis done through different *in silico* algorithms and NCBI Database. The DNA A and DNA B of bipartite Begomoviral particles, the specific loci / region corresponding to DNA A or DNA B has been targeted and PCR amplified using specific primer pairs. Standard PCR reaction conditions and thermal profiles were adapted and optimized to this viral DNA amplification to amplify from test samples. The PCR products was sequenced from service providers and analyzed for curing the sequences using bioinformatics tools. Statistical tools such as MEGA 4, BioEdit, PHYLIP and Darwin were used to generate phylogenetic trees to elucidate the genetic structure and population genetic parameters of this virus group.

RESULTS AND DISCUSSION

The mung bean yellow mosaic virus infected french bean leaves were tested for the presence of MYMV in the infected plants samples, The result of PCR analysis was found to amplify a ~750 bp fragment of the CP gene.The markers RHA, MYMV, CP, IGRA, IGRB and YFP2 resulted in no amplification (Fig 2). In the present study, amplification of a bright ~704 bp amplicon was observed in collected samples. The marker YFP1 CP gene showed specific amplification from different samples of the entire agro-climatic zones studied in Karnataka (Fig 3). Further, one PCR amplified sample from each zone was purified and confirmed by DNA sequencing. Sequence analysis through NCBI BLAST showed that the genes were significantly similar with the MYMV, MYMIV, TMYMV and HYMV CP gene sequences

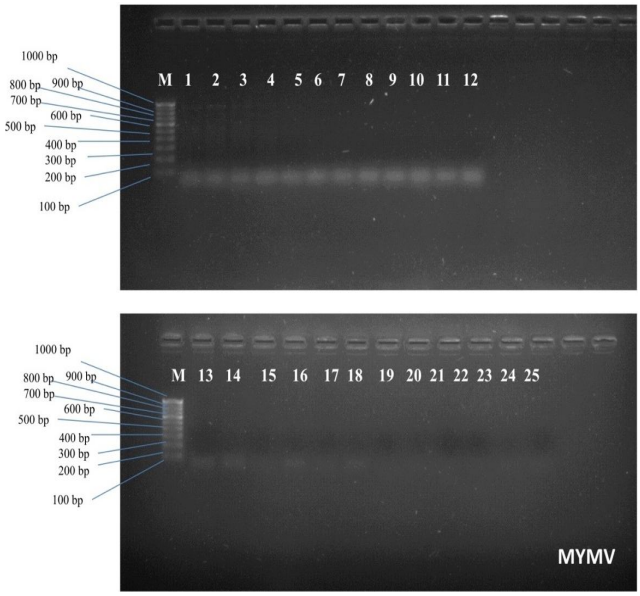

Fig 2: MYMV CP gene specific amplification profile of different French bean sample.

deposited in the NCBI database. The DNA sequences of the entire MYMV CP gene were subjected to pair wise alignment using Bioedit pair wise alignment tool for the obtained sequence. Global as well as the local alignment was done for all the sequences and the identity and similarity percentages of the sixteen sequenced genes were analysed in NCBI database. Identity and similarity in global alignment for all the obtained sequences ranged from 80.8 to 95.3% with reference to MYMV, whereas the with respect to HYMV ranges from 90.4 to 99.1%. Dendrogram was generated using the 16 sequences from different agro-climatic zones. The UPGMA clustering method clearly separated isolates into two major clusters labelled, 1 and 2 respectively (Fig 4). Phylogenetic tree was constructed by using MEGA6 program version 6.1. The observation of dendrogram indicated that cluster 1 was subdivided two sub-clusters, sub- cluster 1 (1.1) and Sub-cluster 2 (1.2). The sub-cluster 1.1 and 1.2 comprised of 11 and 12 samples which belongs to northern dry zone. The cluster 2 was subdivided two sub- clusters, sub-cluster 1 (2.1) and Sub-cluster 2 (2.2). The sub-cluster 2:1 divided into two groups, (2.1.1) and (2.1.2). The sub-cluster 2.1.2 consists of only sample 4 which found in northern transition, while sub-cluster 2.1.1 was subdivided two sub-clusters, sub- cluster 1 (2.1.1.1) and Sub-cluster 2 (2.1.1.2). Sub cluster again sub divided into two sub-clusters sub-cluster 1 (2.1.1.1.1) consist of samples 19 and 26 having central dry zone and sub-cluster 2 (2.1.1.1.2) consist of sample 18 which having northern transition zone. The sub-cluster (2.1.1.2) consists of sample 21 and 32 having southern transition zone (Fig 4). The sub-cluster (2.2) divided into two sub-clusters sub-cluster 1 (2.2.1) and Sub-cluster 2 (2.2.2). The sub cluster 2.2.1 consist of sample no 22 from hilli zone. The sub- cluster 2.2.2 again sub divided into 2 sub cluster 2.2.2.1 and 2.22.2. The sub-cluster 2.2.2.1 consist of line no 10 from southern transition zone. The 2.2.2.2 sub divided into 2 sub cluster. Sub cluster 1 (2.2.2.2.1) and sub cluster 2 (2.2.2.2.2), cluster 2.2.2.2.1 showed line no 14 and 31 from southern dry zone. Similarly, sub cluster 2.2.2.2.2 subdivided into two sub cluster 2.2.2.2.2.1 and 2.2.2.2.2.2 having line no 27, 29, 9 and 25 from eastern dry zone (Fig 4).

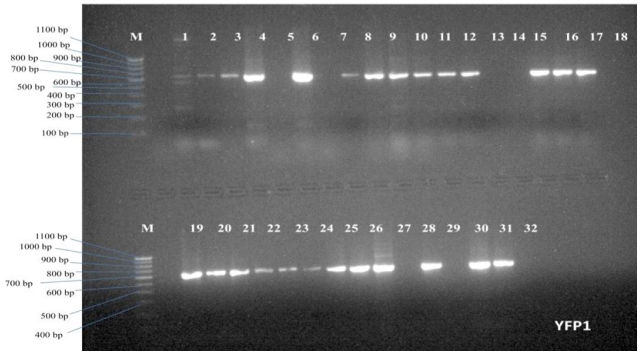

Fig 3: MYMV CP gene specific YFP1 amplification profile of different French bean sample

Frenchbean production is seriously affected by MYMV in all the agro-climatic zones of Karnataka. MYMV being an important viral disease limits the beans production in India (Varma *et al*., 1992). Initially, DNA was isolated from infected leaf samples collected from different growing areas of Karnataka. Amplification of CP gene primers specific to the geminiviruses has already been reported (Deng *et al*., 1994). Hence, CP gene sequence specific primers were designed to determine the variability present in the MYMV isolates. One set of gene specific primers YFP1/YRP1 were found to be successful in the amplification of YMV CP gene of the expected size (~704 bp). CP gene sequence analysis revealed that the identified virus is homologous to MYMV

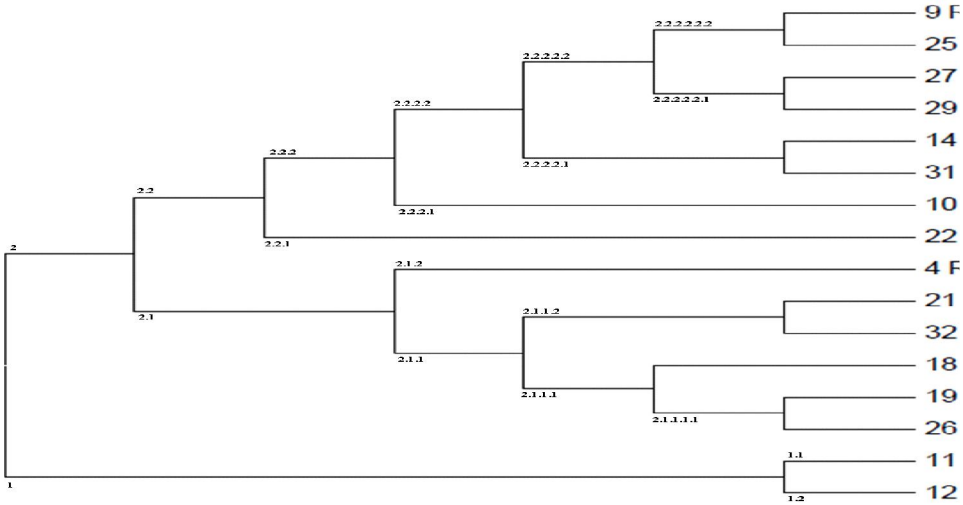

Fig 4: UPGMA phenogram of 16 MYMV isolates of French bean sequence based on average distance of MYMV CP gene specific fragment frequencies.

9: Eastern dry zone, 25: Eastern dry zone, 27:Eastern dry zone, 29: Eastern dry zone, 14: Southern dry zone, 31: Southern dry zone, 10: Southern transition zone, 22: Hilli zone, 4: Northen transition zone, 21: Southern transition zone, 32: Southern transition zone, 18: Northen transition zone, 19: Central dry zone, 26: Central dry zone, 11: Northen dry zone, 12: Northen dry zone.

and HYMV. Phylogenetic tree and pairwise alignment showed that the MYMV isolated from French bean showed the similarity index of significantly similar with the MYMV, MYMIV, TMYMV and HYMV whereas the Hg-MYMV CP1 was found to be 90.4 to 99.1% similar to HYMV. These data indicate the presence of two distinct MYMVs in Karnataka region (MYMV and HYMV species) and there is considerable genetic variation among these species. Further, these virus species are classified as a separate species in the seventh report of International Committee on Taxonomy of Viruses (ICTV) (Fauquet and Stanley, 2005). In addition, HYMV which causes infection specific to Horsegram could also infect other crops as evident from the present study. This finding is in concordance with one of the earlier study (Fauquet and Stanley, 2005). CP genes are the highly conserved genes in the family Geminiviridae (Wyatt and Brown, 1996). This gene sequence, which effectively predicts discrete strains, species and taxonomic lineages of begomoviruses, was accepted by the ICTV as a desirable marker for virus identity when a full-length genomic sequence is not available (Rybicki, 1994). Pairwise alignment of MYMV and HYMV showed nearly 85 % similarity among the two species, which indicate that both the species are similar but nevertheless, could be identified as different.

CONCLUSION

The present study indicated that the CP gene is efficient enough to provide a simple, rapid and reliable method for begomovirus detection and identification. Furthermore, two distinct YMVs (MYMV and HYMV species) were identified, having considerable genetic variation occurring in french bean across different parts of Karnataka. Results obtained in this study will be helpful for early detection of this virus, so that better preventive measures can be taken to control MYMV.

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