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MOLECULAR CHARACTERIZATION OF GEMINIVIRUS CAUSING YELLOW VEIN MOSAIC IN PUMPKIN

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Abstract: Molecular properties of the virus causing pumpkin yellow vein mosaic (PYVM) disease in Kerala were characterized. The complete coat protein (CP) gene of 771bp and movement protein (MP) gene of 846bp were amplified through Polymerase Chain Reaction (PCR) using specific primers. The nucleotide sequences of CP and MP genes exhibited maximum homology with *Squash leaf curl China virus*- [Pumpkin Coimbatore] segment A and *Squash leaf curl China virus* [Pumpkin: Coimbatore] DNA B respectively. Phylogenetic analysis of the sequences revealed that, genome organization of the isolate was typical of bipartite begomovirus and showed close relationship to geminiviruses infecting vegetables, especially cucurbits and tomato. PCR amplification of yellow vein mosaic infected weed species (*Ageratum conizoides, Emilia sonchifolia, Synedrella nodiflora, Hibiscus surattensis*), ash gourd, okra and bitter gourd showing distortion mosaic symptoms failed to yield CP and MP genes, revealing that, the virus infecting these plants may be different from PYVM virus.
Keywords: pumpkin yellow vein mosaic virus, coat protein, movement protein.

Introduction

Pumpkin (*Cucurbita moschata* Duch. Ex Poir) is an important vegetable grown extensively in India throughout the year for its mature and immature fruits. The crop is infected with several virus diseases like pumpkin mosaic (PM), pumpkin yellow vein mosaic (PYVM), squash leaf curl mosaic, pumpkin enation mosaic and pumpkin mild mosaic (Ghosh and Mukhopadhyay, 1979). All these diseases were reported from time to time primarily based on symptoms, physical properties, transmission and host range. The cultivation of pumpkin has often suffered serious setback due to severe outbreak of mosaic diseases, particularly yellow vein mosaic and pumpkin mosaic (Latha, 1992).

PYVM was reported in northern India during early 1940s (Vasudeva and Lal, 1943). Later, the disease was reported from different parts of the country and the causal agent is a

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geminivirus, belonging to genus *Begomovirus.* The virus is transmitted by whitefly, *Bemisia tabaci* and is not sap transmissible (Capoor and Ahmed, 1975; Jayashree *et al.,* 1999). Incidence of PYVM and associated yield losses were reported to be high during February to May, when the vector population is at its peak (Muniyappa *et al.*, 2003). A yield loss of up to 90 per cent has been recorded in pumpkin due to this disease (Singh *et al.,* 2009).

Muniyappa *et al.* (2003) reported that, initial symptom appears as faint vein clearing in tertiary veins of younger leaves. This extends gradually to secondary and primary veins as prominent vein yellowing, coalescing to yellow mosaic. Vein yellowing on older leaves causes early senescence, retarding the plant growth. The leaf size gets reduced, flowers senesce prematurely and fruits become unfit for marketing (Maruthi *et al.,* 2007; Singh *et al.,* 2009).

Two species of geminiviruses, with bipartite genome, causing PYVM have been reported from India. Tomato leaf curl New Delhi virus-India (Maruthi *et al.,* 2007) has been reported from North India and squash leaf curl China virus-India, from South India. Later, bipartite *Squash leaf curl Palampur virus* was also reported to be associated with PYVM (Jaiswal *et al.,* 2011). Most viruses causing yellow vein mosaic in crop pants belong to genus *Begomovirus*. Although attempts have been made to characterize the causal agent based on its biological characteristics, information on molecular biology of the causal organism is scanty.

Geminiviruses possess characteristic geminate particles (18 nm× 30 nm) encapsidating single stranded circular DNA (Harrison and Robinson, 1999; Rojas *et al*., 2005). They may be monopartite with single DNA molecules (DNA-A) or bipartite with two DNA molecules (DNA-A and DNA-B). DNA-A (2.5 kb) encodes proteins required for encapsidation and viral replication and DNA-B (2.6 kb) for movement functions. DNA-A generally possesses 6 ORFs with 2 ORFs [AV1 (CP) and AV2] in the virion sense DNA and 4 ORFs (AC_1 and AC_4) in complementary sense DNA (Lazarowitz, 1992). DNA-A encodes replication-associated protein (Rep), replication enhanced protein (REn), coat protein (CP), and transcriptional activator protein (TrAp). DNA-B encodes nuclear shuttle protein (NSP) and movement protein (MP), which are involved in virus movement within the host plant. Here we report molecular characterization of geminivirus causing yellow vein mosaic in pumpkin in Kerala.

MATERIALS AND METHODS

Extraction of total genomic DNA

Total DNA was extracted from young pumpkin leaves with characteristic yellow vein symptoms (Fig. 1), collected from the research field of College of Horticulture, Thrissur, using CTAB method (Rogers and Benedich, 1994). The quality and quantity of DNA extracted was tested by agarose gel electrophoresis and spectrophotometry at 260 and 280nm.

Designing of primers based on conserved genomic sequence

Degenerate primers were designed from conserved boxes obtained through multiple sequence alignment (ClustalW) of CP and MP sequences of Gemini viruses infecting vegetables. Primers were targeted for amplification of 900bp region of coat protein gene on DNA-A and 800bp region within movement protein gene (Table 1).

PCR amplification of coat protein and movement protein genes

PCR amplification parameters for primers CPF and CPR designed for CP gene included 30 cycles of denaturation for 2 min at 94ºC, primer annealing for 1 min at 55.8ºC and primer extension for 2 min at 72ºC, with a denaturation temperature of 94 ºC for 45 sec and a final extension for 10 min at 72ºC. All amplifications were performed in volumes of 25µl containing 2.5 µl Taq buffer, dNTPs 10 mM each, primers 1 µl (10 pM) each, Taq DNA Polymerase (0.3 U) and template DNA 1µl (50 ng). PCR products were electrophoresed (100 V) on 1% agarose gel stained with ethidium bromide.

For amplification of movement protein gene, primers MFP and MRP were used with the same PCR conditions as followed for CP gene except for annealing which was performed at 58.5ºC for 1 min.

Cloning of PCR products

Amplification products were purified from gel using DNA gel extraction kit (Axygen Biosciences, USA). The purified PCR products were ligated in pGEMT Easy Vector System (Promega Corporation, USA) using heat shock method and cloned in competent cells of *E. coli* (JM109). Putative recombinants obtained through blue-white screening were confirmed for presence of insert by colony PCR using T7 and SP6 universal primers. Amplicons of expected size were sequenced using automated sequencer (ABI–31100 Genetic Analyzer, USA) that uses fluorescent labeled dye terminators and primers.

Amplification of full length MP gene

Full length MP gene (1363bp) was amplified from PYVMV using primers MFP1 and MRP1 (Table 1).

Sequence analysis

Sequence analysis of CP and MP gene sequences was carried out using standard bioinformatics tools. Nucleotide sequence were compared by BLAST to sequence available at NCBI (http://www.ncbi.gov/. blast). The ORFs present in the sequences were determined by ORF finder (www.ncbi.nlm.gov/orf). Homology search of ORFs was performed using the tool BLASTP.

Phylogenetic trees were obtained by importing the aligned sequences into the MEGA programme version 5.05. (http://www. megasoftware.net/features.html) with neighbor joining algorithm (Tamura *et al.,* 2007). Bootstrapping for 1000 replications was performed to determine the reliability of the tree. The details of accessions used in phylogenetic analysis are given in Table 2 and 3.

Validation of primers

Samples of yellow vein infected pumpkin leaves were collected from farmers' fields in three districts of Kerala (Thrissur, Palakkad and Malappuaram). Five infected leaf samples collected from each location were used for DNA extraction followed by PCR amplification. Four healthy samples served as negative control. Apparently healthy (symptomless) leaves from diseased plants were also included in the study.

Detection of PYVMV in weeds and other hosts

Weed plants (*Ageratum conyzoides, Emilia sonchifolia, Hibiscus surattensis* and *Synedrella nodiflora*) and vegetable crops (okra and ash gourd) exhibiting yellow vein symptoms (Fig. 2) and hence, suspected to be collateral hosts of the virus were used for PCR assay. Bitter gourd affected with distortion mosaic was also tested for presence of PYVMV. DNA was extracted from leaves by CTAB method. For okra NaCl concentration was increased from 1.4M to 2M in 2X extraction buffer and from 0.7M to 1M in CTAB solution. PCR amplifications with CP and MP specific primers were then carried out.

RESULTS

Total plant DNA extraction

Total plant DNA of pumpkin leaves showing typical yellow vein symptoms extracted using CTAB method yielded good quality DNA, indicated by a crisp, intact band on agarose gel and the concentration of DNA was 4.2µg /ml.

Amplification of CP and MP genes

PCR with geminivirus group specific primers yielded amplicons of ~900 and ~700bp for CP and MP genes respectively (Fig.3).

Amplification of full length MP gene

PCR amplification with the primer pair MFP1 and MFP2 yielded amplicon of ~1300bp) (Fig 4).

Sequence analysis of PYVMV

CP gene: In BLAST search, CP gene showed 98% nucleotide sequence identity with *Squash leaf curl China virus*- [Pumpkin Coimbatore] segment A (AY184487.3) and 97% similarity with *Squash leaf curl China virus*- [Pumpkin: Lucknow] segment A (DQ026296.2). The sequence also showed more than 90% of similarity to Tomato leaf curl New Delhi virus. The coding sequence contained six open reading frames, the longest having 771bp. Blastp analysis, showed maximum homology with coat protein of *Squash leaf curl China virus*– [Pumpkin:Varanasi] and *Tomato leaf curl New Delhi virus.* Partial ORFs of AC_3 and AC_5 proteins of begomiviruses were also observed in the sequence. The coat protein gene is observed to be the most conserved gene among the begomoviruses.

MP gene: MP gene sequence showed maximum similarity of 98 % to *Squash leaf curl China virus* [Pumpkin: Coimbatore] DNA B (AY184488.1). ORF search followed by the blastp analysis revealed that the longest ORF (846bp) belonged to BL1 (movement) protein of leaf curl china virus-[pumpkin Lucknow] begomovirus. It showed maximum similarity to movement protein [Squash (AAQ91817.1). Phylogenetic analysis also revealed that the virus is closely related to SLCCV (Fig. 5).

Phylogenetic analyses were performed with MEGA version 5.05 using the neighbor-joining method. Branch supports were evaluated by constructing 1,000 trees by bootstrap analysis. Bootstrap values are shown above or under the horizontal line. Horizontal lines are proportional in length to the number of nucleotide differences between branch nodes.

Validation of primers

PCR amplification using CP and MP specific gene specific primers yielded amplicons of expected size in all fifteen infected samples and none in healthy samples (Fig. 6). One among the four symptomless leaf samples of infected plant also showed amplification (Fig. 7). Therefore these primers could also be used to detect latent infection where no external symptoms are expressed.

Detection of PYVMV in weeds and other crop plants

PCR amplification was carried out with MP and CP specific primers using DNA samples of weed plants (*Emilia sonchifolia, Ageratum conyzoides, Synedrella nodiflora* and *Hibiscus surattensis*) and from vegetable crops (okra, ash gourd and bitter gourd). DNA from

infected pumpkin leaves were kept as positive control (Fig.8). No amplification was obtained in any of the samples except positive control which indicated that none of these plants can act as collateral host of the virus.

DISCUSSION

Yellow vein mosaic disease on *Cucurbita moschata* caused by a begomovirus has become severe during recent years in India, resulting in losses up to 90%. The disease is transmitted by whiteflies (*B. tabaci* biotype B). In this study we made an attempt to characterize the virus based on the nucleotide sequence of coat protein and movement protein genes.

PCR amplification with CP and MP specific primers yielded amplicons of expected size (900 and 700 bp respectively) from the infected samples. Both the primer sets yielded single intact bands. There was no amplification in the healthy samples, Hence these primers could be used in developing molecular diagnostic kit for rapid detection of the pathogen.

The ability of both CP and MP specific primers to amplify products from PYVMV affected samples indicates that the virus associated with this disease is probably bipartite as CP and MP genes, encoded by DNA A and B respectively. Moreover, earlier reports on geminivirus genome organization suggest that monopartite virus do not posses DNA B or MP and both the encapsidation and movement functions are controlled by CP (Harrison *et al.*, 2002; Fauquet and Stanley, 2003).

Based on nucleotide sequence and phylogenetic analysis, both the CP and MP gene sequences showed maximum similarity to Squash leaf curl China virus [Pumpkin: Coimbatore] which agrees with the observations made by Singh *et al*. (2009). Muniyappa *et al*. (2003) reported PYVM disease in Karnataka state is caused by a strain of tomato leaf curl New Delhi virus.

The coat protein may probably be the highly conserved protein as the blastp analysis of the sequence showed highest similarity to both SLCCV and ToLCNDV with the same score. The MP showed more similarity to SLCCV than ToLCNV on blast p analysis.

Numerous common weeds were found infected with geminiviruses (Brown and Bird, 1992), but little is known of their relationship with crop infecting geminiviruses. These plants may act as collateral hosts for the virus and they must be identified and eradicated from the premises of the field. In an attempt to identify the collateral host of PYVMV, PCR amplification was also performed in weed plants, (*Ageratum conyzoides*, *Emilia sonchifolia*, *Synedrella nodiflora*, and *Hibiscus surattensis*), crop plants (Okra and ash gourd) with yellow

vein symptom and bitter gourd infected with distortion mosaic. None of these samples produced amplification. Reports on *Ageratum* and okra revealed that the yellow vein mosaic in these plants are caused by monopartite begomovirus with only DNA A and a satellite DNA molecule while, PYVM is caused by bipartite virus (Swanson *et al*., 1998; Muniyappa *et al*., 2003; Xiong *et al*., 2006; Singh *et al*., 2009; Shih *et al*., 2009).

The absence of amplicons in okra, bittergourd and ash gourd could be due to the fact that the virus could be of different strain. This further confirmed the narrow host range of the virus. However, there may be some plants which may be acting as source of the virus inocula without showing any external symptom or the symptom may not be expressed as in the case of PYVMV infected bottle gourd, exhibited crinkling of leaves (Muniyappa *et al*., 2003). Hence detection and elimination of such hosts is important for the disease management. Amplification in symptomless sample shows that these primers could be effectively utilized to detect latent infection and symptomless carriers.

Further investigation based on electron microscopic study is required to confirm the bipartite nature of the virus. The primers designed in the study could be used for molecular detection of PYVMV.

The sequence information of coat protein and movement protein genes obtained in the study could be further exploited in crop improvement programmes for developing PYVMV resistant varieties. The sequence information of different genes could be used to serve the purpose. Multiple approaches for engineering resistance to geminiviruses have been employed in plants, in most cases in the model plant system. Such technologies can also be applied to crop plants like pumpkin and they would bring significant benefit to crop breeding and production. Geminivirus genes that could confer pathogen derived resistance include CPs, replicases and MPs. defective interfering RNAs, DNAs and non translated RNAs also play a major role in disease resistance.

Significant advances in understanding the mechanism of resistance and the nature of genes involved will lead to increased and sustainable resistance (Beachy, 1997). CP and MP mediated resistance can confer resistance to a broader range of viruses. CP-MR is the first reported and most widely studied mechanism. It has been used to confer resistance to a number of viruses in a variety of plant species. The CP produced by the transgene interferes with the disassembly of the virus thus inhibiting the infection. Furthermore specific mutations to the known sequences can confer much greater levels of resistance than the wild type. Similarly movement protein mediated resistance inhibits the virus by interfering with the

virus movement within the plant. Hence, the knowledge in structure of the virus gene could aid in design of mutant genes that possess increased efficacy and breadth in protection. So, there is an immediate need to characterize such hitherto unknown viruses in these areas and to develop a clearer picture of the geographical distribution of these viruses.

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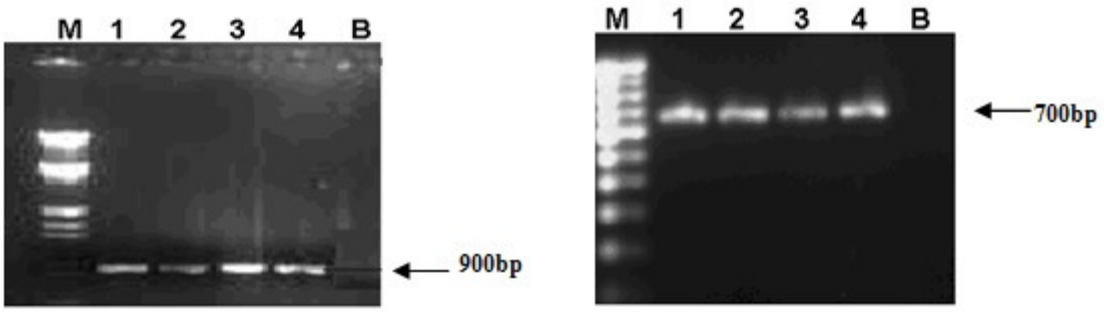
Fig. 1. Pumpkin leaves exhibiting yellow vein mosaic symptom

(a)

(b) (c)

Fig. 2. *Agertum conyzoides* (a) Okra (b) and ashgourd (c) leaves exhibiting yellow vein mosaic symptom



(a) (b)

Fig. 3. (a) Coat protein gene of PYVMV amplified by PCR Lane M: λDNA EcoR1/HindIll double digest. Lane 1-4: Infected pumpkin; Lane B: Blank (b) Movement protein gene of PYVMV amplified by PCR. Lane M:100bp DNA ladder; Lane 1-4: Infected pumpkin; Lane B: Blank

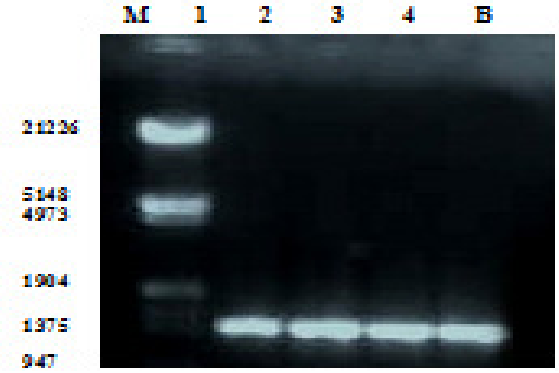
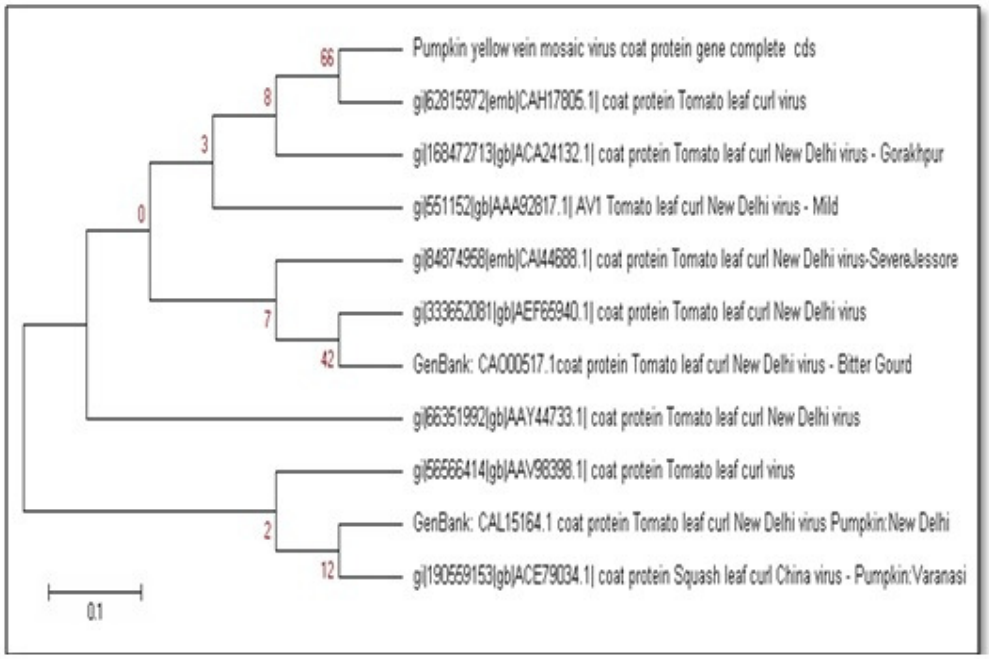
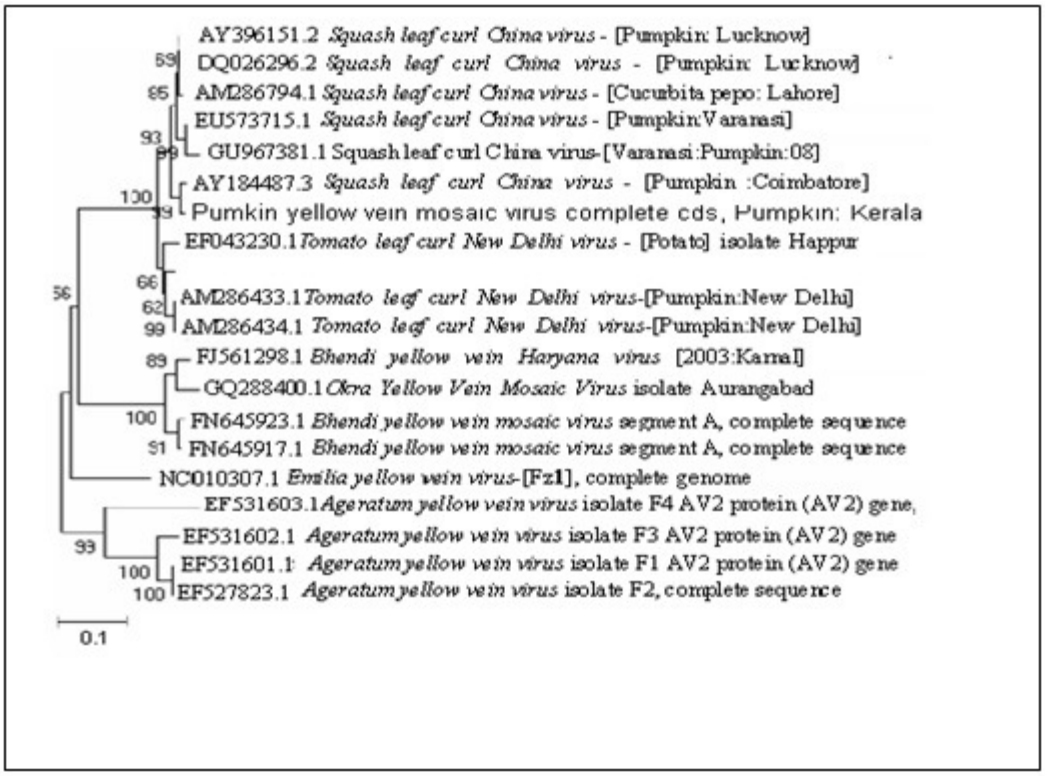


Fig. 4. Amplification of full length MP gene ORF of PYVMV on PCR Lane M: λDNA EcoR1/Hind lll double digest; Lane 1-4: infected pumpkin, Lane B: Blank

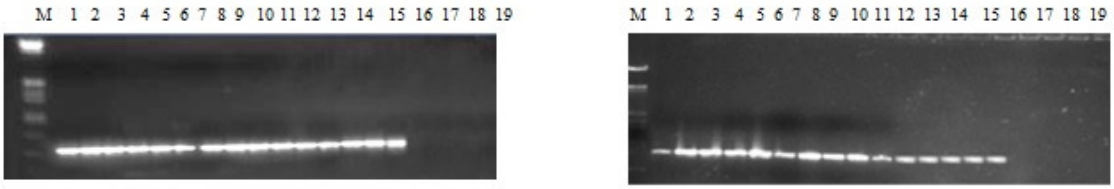


(a) Phylogenetic tree of PYVMV CP gene inferred using Neighbor-Joining method



(b) Phylogenetic tree of MP gene inferred using Neighbor-Joining method

Fig. 5. Phylogenetic consensus tree showing the relationship of pumpkin yellow vein mosaic virus [Kerala:Pumpkin] with other begomoviruses based on an alignment of nucleotide sequences.

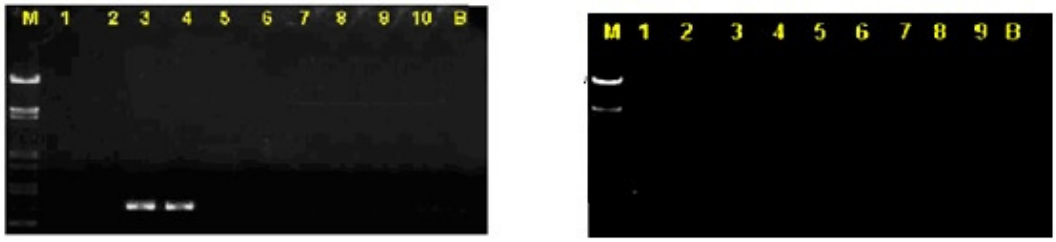


(a) Amplification of CP gene (b) Amplification of MP gene

Fig. 6. Detection of PYVMV by amplification of CP and MP gene in infected and healthy pumpkin Lane M: λDNA *Eco*R1/*Hind*lll double digest; Lanes 1-15: Infected ; Lane16-19: Healthy

(a) PCR amplification of CP gene (b) PCR amplification of MP gene

Fig. 7. Validation of primers for PYVMV detection in apparently healthy pumpkin samples M: λDNA *Eco*R1/*Hind*lll double digest ; 1-4: Apparently healthy samples; 5: Infected pumpkin B: Blank



(a) (b)

Fig. 8. (a) PCR amplification of CP gene. Lane M: λDNA *Eco*RI/*Hind*lll double digest; 1- *Emilia*; 2- *Synedrella*; 3 & 4- Infected pumpkin; 5- *Ageratum*; 6- *Hibiscus*; 7-Okra; 8- Ash gourd; 9- Bitter gourd; 10- Healthy pumpkin; B- Blank. (b) PCR amplification of MP gene. Lane M: λDNA *Eco*RI/*Hind*lll double digest; 1- Infected pumpkin; 2-*Emilia*; 3- *Synedrella*; 4- *Ageratum*; 5- *Hibiscus*, 6- Okra; 7- Ash gourd; 8- Bitter gourd; 9-Healthy pumpkin; B- Blank. PCR amplification of PYVMV in weeds and vegetable crops showing yellow vein symptoms.

Table 1. Details of primers for amplification of CP and MP genes of PYVMV

Primer	Nucleotide Sequence	Length (bp)	Tm (ºC)	Expected amplicon size (bp)
CFP	5'GCAAACAACTATGGCGAAGC3'	20	60.0	896
CRP	5'TGTTGGGGGTGATTGGTATT3'	20	58.0	
MFP	5'GATGCATGAAAATTGATCACG3'	21	58.0	678
MRP	5'GGA/GGCGTTTTCATTA/C/TGATTTC3'	21	58.0	
MFP1	5'CAATGTAATGC/TTAAATTACATTG3'	23	59.0	1363
MRP1	5'CCATACCCCAATATACCATAG3'	21	57.0	

Table 2. NCBI accessions of geminivirus sequences used for phylogenetic analysis of CP gene from PVVMV from Kerala

Accession	Description
AY184487.3	*Squash leaf curl China virus* - [Pumpkin :Coimbatore] segment DNA-A, complete sequence
DQ026296.2	*Squash leaf curl China virus* - [Pumpkin: Lucknow] segment DNA A, complete sequence
AM286794.1	*Squash leaf curl China virus* - [Cucurbita pepo: Lahore] AV2 gene, CP gene, AC1 gene, TrAP gene, ReN gene, AC4 gene and AC5 gene, clone CPoAL4(3)
EU573715.1	*Squash leaf curl China virus* - [Pumpkin:Varanasi] segment DNA-A, complete sequence
GU967381.1	Squash leaf curl China virus-[Varanasi:Pumpkin:08] segment DNA-A, complete sequence
AY396151.2	*Squash leaf curl China virus* - [Pumpkin: Lucknow] coat protein (AV1) gene, complete cds
AM286434.1	*Tomato leaf curl New Delhi virus*-[Pumpkin:New Delhi] segment DNA-A, complete sequence, isolate 2
AM286433.1	*Tomato leaf curl New Delhi virus*-[Pumpkin:New Delhi] segment DNA-A, complete sequence, isolate 1
EF043230.1	*Tomato leaf curl New Delhi virus* - [Potato] isolate Happur segment DNA A, complete sequence
AM850115.1	*Tomato leaf curl New Delhi virus* DNA-A complete genome, isolate Himachal
NC010307.1	*Emilia yellow vein virus*-[Fz1], complete genome
FN645923.1	*Bhendi yellow vein mosaic virus* segment A, complete sequence, clone 10c-RCA-AII-F
FN645917.1	*Bhendi yellow vein mosaic virus* segment A, complete sequence, clone 10a1-RCA-AI-F
GQ288400.1	*Okra Yellow Vein Mosaic Virus* isolate Aurangabad coat protein (AV1) gene, complete cds
FJ561298.1	*Bhendi yellow vein Haryana virus* [2003:Karnal] isolate OY76 segment DNA-A, complete sequence
EF531603.1	*Ageratum yellow vein virus* isolate F4 AV2 protein (AV2) gene, complete cds; and coat protein (AV1) gene, partial cds
EF531602.1	*Ageratum yellow vein virus* isolate F3 AV2 protein (AV2) gene
EF531601.1	*Ageratum yellow vein virus* isolate F1 AV2 protein (AV2) gene
EF527823.1	*Ageratum yellow vein virus* isolate F2, complete sequence

Table 3. NCBI accessions of geminivirus sequences used for phylogenetic analysis of MP gene in PYVMV from Kerala

Accession	Description
HM566113.1	*Squash leaf curl China virus* DNA B, complete sequence
GU967382.1	*Squash leaf curl China virus* [Varanasi: pumpkin: 08], segment B, complete sequence
AF509742.1	*Squash leaf curl China virus* [B] segment DNA B, complete sequence
AM260208.1	*Squash leaf curl China virus* DNA B genome, isolate Hn6
AM260208.2	*Squash leaf curl China virus* DNA B genome, isolate G25
AB085794.1	*Squash leaf curl Philippine virus* DNA B genome, complete sequence
AM286435.1	*Tomato leaf curl New Delhi virus* [Pumpkin: New Delhi], segment DNA B complete sequence, isolate 2
DQ873412.1	*Tomato leaf curl New Delhi virus* [chilli: Bahrain], movement protein gene complete cds
AY184488.1	*Squash leaf curl China virus*- [Pumpkin: Coimbatore], segment DNA- B, complete sequence
AM778959.1	*Squash leaf curl China virus* DNA B, complete sequence
AM709505.1	*Squash leaf curl China virus* DNA B, complete sequence
FJ85988101	*Squash leaf curl China virus* [India: Varanasi: Pumpkin] segment DNA-B, complete sequence