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Molecular Detection and Characterization of Citrus Yellow Mosaic Virus Associated with Acid lime (*Citrus aurantifolia*)

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Citrus yellow mosaic disease is of high economic importance to the citrus industry in India. The mosaic disease is caused by Citrus yellow mosaic virus (CYMV) is a plant para-retrovirus, belongs to the Genus *Badnavirus* and family *Caulimoviridae*, with a DNA genome of 7.5 kb in size. The incidence of the disease ranged from 10-70% in citrus nurseries, and can cause reduction in fruit and juice yield up to 77 and 10%, respectively. The acid lime plants showing mosaic symptoms on the leaves as consisting of irregularly scattered light and dark green regions on the lamina were collected from Tirupati. Electron microscopy showed the presence of virus particles having bacilliform structure with size of ~30 x 130 nm. The CYMV genome was characterized using five sets of specific primers and a region of ORF III of CYMV associated with acid lime was cloned and sequenced. Upon sequencing of ORF III region produced a total of 899 bases. The homology search using NCBI revealed the highest sequence identity with other Indian isolate of CYMV infecting rangpur lime. Phylogenetic analysis of badnaviruses indicated that CYMV was most closely related to *Cacao swollen shoot virus*. Furthermore, high level of sequence conservation (89-99%) was noticed in the ORF III in three isolates infecting others species of citrus. The molecular tools developed in the present study could be used for large scale indexing of citrus plants to identify mosaic free plants and quick screening of planting material even at seedling stage without appearance of symptoms.

Key words: Molecular characterization, *Citrus yellow mosaic virus (CYMV)*, Acid lime, *Badnavirus*, Cloning.

Citrus (*Citrus species*) being the third most important fruit crop after mango and banana, occupies an important position in the horticultural wealth and Indian economy. However, Citrus industry faces severe loss to economical production as the production areas are being severely challenged by pest, disease, and environmental problems. The crop suffers from extensive damage caused by bacteria, viruses and virus like pathogens. Among them viruses are the major causes of diseases in citurs. Citrus yellow mosaic disease caused by Citrus yellow mosaic virus (CYMV) is the major one which has high economic importance to the citrus industry. In India, mosaic disease in citrus was reported in sathgudi sweet orange and khasi mandarins. The citrus yellow mosaic disease was first described by Dakshinamurti and Reddy (1975) in South India (Anantpur district of Andhra Pradesh) and was later detected in Northeast India in 1985 (Reddy and Murti, 1985; Ahlawat *et al*., 1985). Citrus yellow mosaic virus (CYMV) is a *Badnavirus* belongs to the family *Caulimoviridae*, (Pringle, 1999; Huang and Hartung, 2001 and Baranwal *et al*., 2005). It is one of the most serious citrus pathogen occurring in India, as it can infect nearly all citrus species and cultivars, especially sweet orange, mandarin, pummelo and grapefruit trees

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and ornamental citrus plants. (Alhawat *et al*., 1996a; Sai-Gopal *et al*., 1999). The incidence of the disease was observed from 10-70% in citrus nurseries in Andhra Pradesh (Ahlawat *et al*., 1996b). The disease can cause reduction in fruit yield up to 77% and juice up to 10% juice has 1.3% less ascorbic acid having more acidity in citrus orchards and citrus nurseries (Ahlawat, 2000). However, Sweet orange and pummelo orchards are reported as no longer productive due to CYMV infection (Ahlawat *et al*. 1996b; Ahlawat, 2000). The symptoms of mosaic disease appeared in leaves of infected field trees are bright yellow mottling to yellow flecking and vein banding and the infected trees are slightly stunted and leaf size is also smaller than in healthy trees. Trees affected by the mosaic disease not only produce a significant yield reduction, but also fruits with reduced quality juice (yellow depressed and green elevated areas symptoms), and ascorbic acid content (Ahlawat *et al*., 1996a; Ahlawat, 2000).

CYMV belongs to the genus *Badnavirus* and family *Caulimoviridae* (Pringle, 1998), have bacilliform virions measuring ~30 x 130 -150 nm in size and a circular covalently closed dsDNA genome (Lockhart, 1995). It has circular double stranded DNA genome of 7559 bp in length comprises of six putative open reading frames (ORFs) and an intergenic sequence lying on the plus strand of DNA with, each capable of encoding proteins with a molecular mass of greater than 10 kDa. ORF III, the largest ORF encodes a putative polyprotein for functions involved in virus movement, assembly and replication (Huang and Hartung, 2001). The genome also contains a plant tRNAmet binding site, which may serve as a primer for minus-strand DNA synthesis in its intergenic region. CYMV is serologically related to Banana streak virus (BSV), Cocoa swollen shoot virus (CSSV), Sugarcane bacilliform virus (SBV) and Commelina yellow mosaic virus (ComYMV) and belongs to the member of Badnavirus genus of the family *Caulimoviridae* (Ahlawat *et al*., 1966). Accurate information on the tools for indexing of virus infected trees is highly important for decisions to implement a virus eradication or suppression program (Sharma, *et al*., 2009). Studies have been conducted for serologically detection of CYMV in Citrus species using ELISA techniques in *Citrus sinensis* (Gaddam, *et al*., 2013). PCR based detection using degenerate primers (Ahlawat, *et al*., 1996b) and in rangpurlime (Baranwal, *et al*., 2005). However, Citrus yellow mosaic virus being poorly immunogenic could not be efficiently detected by immune-assay techniques like ELISA. However, its accurate detection can be done by using molecular approach, PCR based detection using primer of specific conserved sequences from the genome of the virus and other infectious agents. The present investigation was conducted to identify the citrus yellow mosaic virus using PCR based approaches and its characterization by cloning and sequencing of ORF III region. Furthermore, the PCR based (CYMV) specific primers have been validated which could be employed for quick detection of virus even at seedling stage before appearance of disease symptoms.

MATERIALS AND METHODS

Sample Collection and Electron Microscopy

The samples collected from the symptomatic plants of Acid lime showing Citrus yellow mosaic disease at Citrus orchard, Tirupati, India was used in the study**.** The mosaic like symptoms in leaves of acid lime trees was observed at the Horticultural Research Centre and Old campus of Sardar Vallabhbhai Patel University of Agriculture & Technology, Modipuram, Meerut, Uttar Pradesh. Leaves of acid lime plants suspected for mosaic disease was collected from both the locations were used for the detection presence of CYMV particles.

The symptomatic leaves of acid lime plants and leaves from healthy plant (control) were used for detection of virus particles using electron microscopy following the leaf dip method. The preparations were negatively stained using 2% aqueous uranyl acetate (UA) and the amount of virions present was also examined by EM at various stages of purification and in the final preparation. For ultrathin sections, pieces of leaf tissue (2.5 x 2 mm) were removed from healthy and mosaic disease of acid lime plants. Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 for 24 hr, rinsed and post-fixed in 0.2% osmium tetroxide in the same buffer for 24 hr. After dehydration through an alcohol series samples were passed through

propylene oxide for 10 rnin and then embedded in Emix resin (Emscope Laboratories Ltd, U.K.). Sections of 90 nm thickness were cut with a diamond knife on an LKB IV ultramicrotome. Sections were collected on formvar coated grids, doubly stained with 5% UA for 10 rnin and 5% lead citrate for 5 min, and examined in a JEOL l00 CX- I1 EM at the ACPV, IARI, New Delhi.

Extraction of Genomic DNA and PCR amplification

Total genomic DNA was isolated from 500 mg of electron microscopy positive symptomatic leaf tissues as well as leaves from healthy plants of acid lime using three different protocols, *viz.*, sodium sulphite method (Baranwal *et al*., 2003), Cetyltrimetyl ammonium bromide (CTAB) method and DNeasy plant mini Kit (Qiagen, Germany). The leaves grounded to fine powder in mortar and pestle using liquid nitrogen were used for DNA extraction. The qualitative and quantitative analysis of DNA was assessed by absorbance at 260/280 nm using Spectrophotometer (Bio-rad, USA) followed by running of DNA sample on 0.8% agarose gel along with uncut lambda DNA as standard (Fermentas, USA).

The sequences of CYMV specific primer pairs consisting of open reading frame (ORF) regions was kindly provided by Dr. V.K. Baranwal, IARI, New Delhi (Table 1) and synthesized from Operon, Germany were used to amplify the viral genome. PCR reaction for amplification of viral genome was performed in 50µl reaction consisted of 20 pmoles of specific primers, 200 µM each of dNTPs, 1.5 Units of *Taq* DNA polymerase, 1X reaction buffer, 1.5 mM $MgCl_2$ and 20 ng of template DNA. The PCR reaction conditions involved initial denaturation at 94˚C for 5 min followed by 35 cycles with denaturation at 94˚C for 30s, annealing at (specific for each primers pairs as mentioned in Table 1) for 1 min, and amplification at 72˚C for 2 min and final extension at 72 ˚C of 7 min. The PCR amplified products was resolved on 1% agarose gel along with 1kb DNA ladder (Fermentas, USA) in parallel for determination of the size of amplicon. The gel was visualized on UV transilluminator and documented using Gel Documentation System XR+ (Bio-Rad, USA).

Cloning and sequencing of PCR amplicon

The PCR reaction was performed with all five sets of specific primers and amplicon generated by primer combination, CYMV-4688F and CYMV-5587R derived from ORFIII region produced an expected size of fragment of about 900bp was excised and eluted from the agarose gel using QIAquick Gel extraction kit (Qiagen, Germany). The gel eluted and purified PCR product was quantified and ligated to pGEM-T Easy vector (Promega, USA) and then transformed in to *Escherichia coli* (*E. coli*) strain DH5α. Competent cells were prepared using $CaCl_2$ double suspension method while hot-shock method was adopted in *E. coli* transformation in terms of the protocol of Sambrook *et al*. (1989). The presence of positive clones was confirmed by blue/white colony screening and further recombinant clones were identified by restriction endonuclease digestion and colony PCR using the specific primer pair combinations. The selected recombinant clones were sequenced at the automated DNA sequencing facility at Department of Biochemistry, South Campus, University of Delhi, New Delhi, India.

Nucleotide sequence analysis and phylogenetic characterization

The nucleotide sequences obtained from the sequencing was used to make homology searches within GenBank's non- redundant database using the BLAST 2.2.8 (Basic Local Alignment Search Tool) algorithm available at http://www.ncbi.nlm. nih.gov/BLAST/ of the National Center for Biotechnology Information (NCBI), with the program BLASTn (Altschul *et al*., 1990). The sequences showed homology and the sequences of other badnaviruses known to infect different crops namely, ScBV, KTSV, CSSV, BSV, ComYMV, TaBV and CYMV were retrieved from NCBI (Table 2). The nucleotide sequences were aligned using Clustal W programme (http:// www.ebi.ac.uk/clustalw/) and comparison of aligned sequences was performed with BioEdit Sequence Alignment Editor (Hull, 1997) and phylogenetic tree was constructed using neighbour joining method. The robustness of the tree was determined by bootstrap sampling of multiple sequence alignment and a consensus tree was generated with treecon for Windows (version 1.3b) package. The restriction map of ORFIII of

CYMV was generated using NEB cutter software available at http://nc2.neb.com/NEBcutter2/.

RESULTS AND DISCUSSION

Electron Microscopy

The samples collected from the acid lime plants of different locations showed mosaic symptoms on the leaves, consisting of irregularly scattered light and dark green regions on the lamina. The leaves were collected at random taking into consideration either or all of the following symptoms including, stunting, chlorosis and uniformly distributed leaf mosaic, followed by leathery texture of matured leaves and mealy bug infection.

Electron microscopic study was performed for the detection of presence of CYMV in the samples. The leaf dip preparation from symptomatic leaf of acid lime plant collected from Tirupati showed typical bacilliform virus particles with particle length of 30 nm x 150 nm while, presences of other virus particles or mixed infection were not observed in the leaf sample (Fig.1). The results are in accordance of Ahlawat *et al*. (1996b) reported the size of CYMV, 30 x 130 nm approximately having all other *badnavirus* characteristics and non-enveloped bacilliform virus. Virus particles of similar morphology and size were found associated with yellow mosaic affected citrus plants reported by Pant and Ahlawat (1997). However, virus particles were not seen under electron microscopy in samples collected from horticultural research centre (HRC) and Old Campus of S.V. P. University of Agriculture & Technology, Meerut. The result suggests that the mosaic like symptoms observed on citrus plants at HRC and Old campus might be due to some abiotic

Table 1. List of Primers used for the characterization of Citrus yellow mosaic virus (CYMV) associated with acid lime

Primer pairs	Primer name and Sequences (5'-3')	Annealing Temperature	Amplicon size (bp)
1.	CYMV-997F- TGGTACCTTCTACGCTTACAA CYMV-2539R- AAGGACTGTTTGATTGACTC	55˚C	1500
2.	CYMV-2510F- GAGTCAATCAAACAGTCCTT CYMV-3440R-GAATCAGAAGTAAGCCTCTC	54˚C	930
3.	CYMV-3418F TTGGAGAGGCTTACTTGTG CYMV-4705R-CTCCTGTGTCAAGAATCGC	55˚C	1200
4.	CYMV-4688F-CGATTCTTGACACAGGAGC CYMV-5587R-CGCTACCTGCTGGAAAGCCAC	58˚C	899
5.	CYMV-6542F-TGGCTGATGCTCTGTCCAG CYMV-230R-AGATTAGATCACCTTTAGCG	54˚C	1200

Table 2. List of Badnaviruses known to infect other crops with accession numbers from GenBank database used for sequence analysis and phylogenetic study.

Sr. No.	Name of Viruses	Abbreviations	Accession No
1.	Banana streak virus	(BSV)	AJ 002234
2.	Cacao swollen shoot virus	(CSSV)	NC 001574
3.	Citrus yellow mosaic virus–H (Huang & Hartung)	(CYMV)	NC 003382
4.	Commelina yellow mosaic virus	(CoYMV)	NC 001343
5.	Kalanchoe top spotting virus	(KTSV)	NC 004540
6.	Sugarcane bacilliform virus	(ScBV)	AJ 277091
7.	Rice tungro bacilliform virus	(RTBV)	NC 001914
8	Citrus yellow mosaic virus –R (rangpur lime)	CYMV-R	DQ875213
9.	Citrus yellow mosaic virus –A (Acid lime)	CYMV-A	(Present study)
10.	Strawberry vein banding virus	(SVBV)	X97304

stress and nutritional deficiency. Since badnaviruses are week immunogenic and their detection by serological methods is not reliable hence, PCR was used for further detection and confirmation in the electron microscopy positive as well as negative samples.

PCR amplification of CYMV genomic regions

Total genomic DNA extracted from the leaves of electron microscopy (EM) positive and negative as well as leaves from healthy acid lime plants using three different methods. In comparison, sodium sulphite method (Baranwal *et al*., 2003) was found best and resulted in better yield and higher purity of DNA. However, CTAB method produced quality and quantity at par to sodium sulphite method. The qualitative analysis using spectrophotometer revealed the 260/280 ratio ranged from 1.7-1.9. However, DNeasy plant mini kit was found least effective and produced less quantity of genomic DNA. It may be due to the presence of higher level of polyphenolics and tannins in the citrus leaves which interfered in the precipitation of DNA. The higher yield of DNA by sodium sulphite method may be due to the sodium sulphite, effectively involved in the removal of PCR inhibitors from woody plants such as citrus resulting produced better yield and stability of DNA (Baranwal *et al*., 2003). Similarity Byrne *et al*. (2001) also reported that addition of sodium sulphite provides cleaner and more stable DNA possibly because it was effective to preventing the oxidation of nucleic acids upon the release of sap from cells and preventing degradation during storage and also agents for PCR inhibitors of woody plants.

In general all five primer sets used for amplification of viral genome found efficient in amplifying the CYMV genome in Tirupati samples produced the expected size of amplicons of 1500bp, 930bp, 1200bp, 899bp and 1200bp were amplified from respective primer sets (Table 1 and Fig.2).

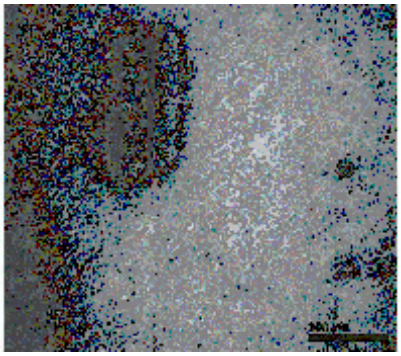

Fig. 1. Electron Micrograph of Citrus yellow mosaic virus (CYMV) associated with acid lime

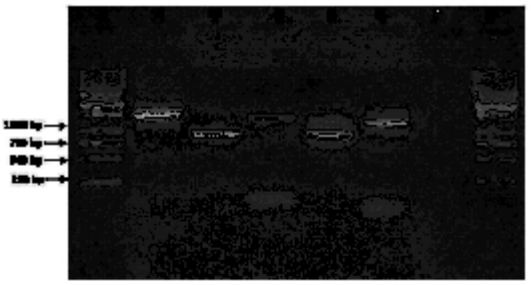

Fig. 2. PCR amplification of partial ORFIII region of CYMV associated with acid lime. Lane No: 1- 1 Kb ladder; 2- PCR amplified product of primer combination 997F-2539R; 3- 2510F-3440R; 4-3418F-4705 R; 5-4688 F-5587R; 6-6542F-230R; 7-Healthy sample; 8-1 Kb ladder

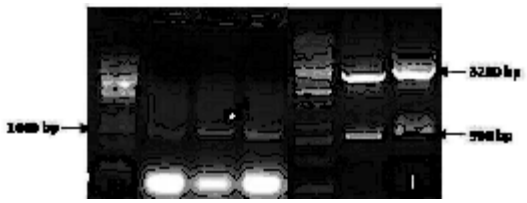

Fig. 3. Confirmation of recombinant clones using (a) Colony PCR with CYMV-4688F and 5587R (b) Restriction digestion analysis produced vector fragment 3.2 kb and cloned product of 899 bp

Fig. 4. Nucleotide sequence of the ORFIII region of Citrus yellow mosaic virus

However, no amplicons were found in the samples of collected from HRC and old campus of SVP UA&T, Modipuram it further confirmed the absence of CYMV in the symptomatic leaves. The size of CYMV genome reported by Huang and Hurtung, (2001) to 7559 bp in length and contains six putative open reading frames (ORFs) capable of encoding protein larger than 10 KDa. Among the ORFs present on the plus strand of DNA, ORF III is largest ORF encode putative polyprotien involved in the viral movement, capsid synthesis, and replication.

The PCR amplicon produced by CYMV-4688F and CYMV-5587R derived from ORFIII region produced an expected size of fragment of about 900bp from the leaves of microscopy positive samples. This amplicon region of ORFIII was cloned and presence of recombinant clones was identified by screening of blue/ white colonies. Among them only white colonies were used for further confirmation using colony PCR using sequence specific primer produced ~900bp fragment (Fig. 3) followed by restriction digestion analysis of recombinant plasmid using *Eco*RI yielded two fragments ~3.2 kb and ~900 bp from cloning vector and cloned fragment, respectively. The sequencing of cloned fragment produced a total of 899 bp nucleotides (Fig. 4). The restriction map of ORFIII of CYMV was generated using NEB cutter software showed restriction sites for different restriction enzymes namely, *Bam* HI, *Hind* III, *Sal* I, *Eco* RII, *Dpn I*, *Sal* I (Fig 5). The nucleotide sequences generated from ORFIII region were translated and produced 299 amino acids.

Phylogenetic analysis

Multiple sequence alignment of the nucleotides sequence revealed that it has high sequence identity of 99% indicating that the region

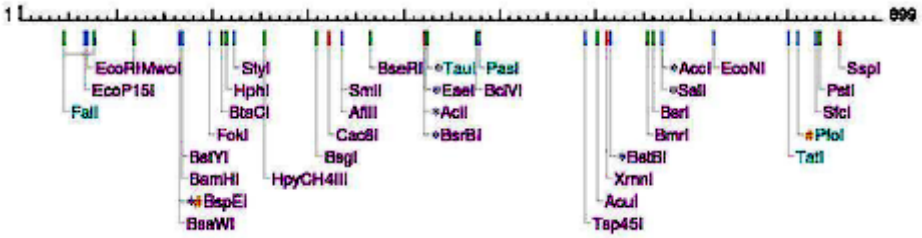

Fig. 5. Restriction map of ORFIII region of CYMV associated with acid lime

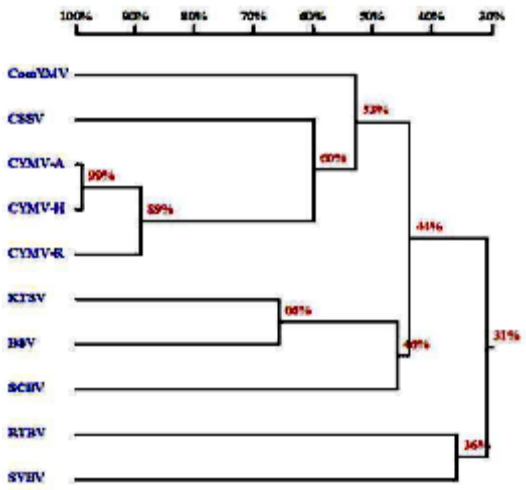

Fig. 6. Phylogenetic tree depicting relationship of various badnaviruses based on multiple alignments of ORF III region. (Virus names and their GenBank accession numbers are given in Table 2)

is conserved with other sequences of CYMV-H infecting sweet orange followed by 89% with the CYMV-R rangpur lime isolate (Fig. 6). On comparison of nucleotide sequences of CYMV with other badnaviruses, the isolates of CYMV were grouped and formed one cluster with CSSV. KTSV shared common homology with BSV and formed a separate group with SCBV. RTBV shared a common homology with SVBV. BSV did not group with either of these two clusters and formed a separate branch The result are in congruence with the study of (Huang and Hartung, 2001; Baranwal *et al.*, 2005) who conducted the phylogenetic analysis based on complete nucleotide sequences revealed that CYMV is more closely related to *Cacao swollen shoot virus*, CSSV and *Dioscorea bacilliform virus*-DBV.

In order to obtain a better understanding of the genetic variability of the virus with other

citrus mosaic disease affected plant. High sequence identity in amino acid of ORF III region of CYMV viral genome indicates lack of variability in ORF III. The greater identity of CYMV in ORF III with CSSV indicates that the virus might have moved from cacao to citrus or *vice versa*. Other badnaviruses reported from India are BSV (Cherian, *et al*., 2004) and ScBV (Viswanathan *et al*., 1996) both formed separate clusters with other badnaviruses, indicating their diverse origin. Variability in the intergenic region of the two isolates indicates that it can be used as a tool to distinguish different isolates of CYMV when sequences of intergenic region from CYMV infecting other citrus species become available. Ahlawat *et al.,* (1996b) reported that the CYMV was serologically related to CSSV, BSV, ComYMV and SCBV but the serologically closet is SCBV. In this study phylogenetic analysis based on ORFIII nucleotide sequences revealed that CYMV is most closely related to CSSV rather than SCBV. High similarity in nucleotide, as described earlier by Baranwal *et al.* (2005) was found in acid lime indicates that there is very less variability in ORFIII coding for polyproteins

The phylogenetic studied by Geering *et al.* (2000), based as conserved region of RNase H domain, also showed that the CSSV is most similar to DBV than to ScBV indirectly, supporting the result of present study. It was also found that the CYMV under investigation is more similar to other badnaviruses. However, RTBV was biologically distinct. Other badnaviruses reported from India *viz.,* Banana streak virus and Sugarcane bacilliform virus form separate clusters that indicate their diverse origin.

In most cases, mosaic disease affected seedlings do not show visible symptoms throughout the year. Hence it is essential to use sensitive techniques such as PCR. The protocol standardized for DNA isolation and PCR successfully detected the CYMV in infected citrus plants (with or without visible symptoms) in a wide range of DNA concentration ranging even at very less concentration. The method can be used for large scale indexing of citrus plants to identify mosaic free plants.

In present investigation, bacilliform particles of CYMV was detected in acid lime symptomatic leaves collected from Tirupati that indicates that amplification of viral genome was from the virus particles and not from the integrated host genome. However using the electron microscopy it was confirmed the CYMV infection was not present in the symptomatic samples collected from HRC and old campus of S.V..P. U.A &T, Modipuram, Meerut. The nucleotide sequences generated by cloning and sequencing of ORF III of CYMV associated with acid lime could be used to developed specific primers for PCR based detection of CYMV in acid lime and other citurs species.

CONCLUSION

Citrus yellow mosaic disease is widely distributed in India and is of great economic importance to the citrus industry. These domestic plants might be infected with viral disease, yet undetected due to negative or under expression of symptoms. Development of highly sensitive methods for detection of virus is essential as CYMV is poorly immunogenic, thus molecular diagnostic technique may be better and alternative. Electron microscopy showed the presence of virus particles having bacilliform structure with size of ~30 x 130 nm. The CYMV genome was characterized using five sets of specific primers and ORF III of CYMV associated with acid lime was cloned and sequenced. Upon sequencing of ORF III region produced a total of 899 bases. The homology search using NCBI revealed the highest sequence identity with other Indian isolate of CYMV infecting rangpur lime. The molecular tools generated in the present study may prove very helpful in quick detection of virus in planting material and can be used for production of virus free plants.

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