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REGULAR ARTICLE

# COAT PROTEIN GENE BASED CHARACTERIZATION OF *CUCUMBER MOSAIC VIRUS* ISOLATES INFECTING BANANA IN INDIA

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## SUMMARY

Banana plants showing typical yellow stripes on leaves as symptoms, in addition to leaf distortion and stunting of plant were collected from Karnataka (KAR), Maharashtra (MH) and Uttar Pradesh (UP) in India. The causal agent was identified as *Cucumber mosaic virus* (CMV) on the basis of transmission electron microscopy and reverse transcription polymerase chain reaction (RT-PCR). Complete coat protein (CP) gene of all isolates were amplified using gene specific primers for coat protein (CP), followed by cloning into desired cloning vector for sequencing. Sequenced region were found containing complete single open reading frame of 657 nucleotides, potentially coding 219 amino acids. Sequence analysis of CP gene showed 93%-98% (at nucleotide) and 94%-99% (at amino acid) sequence identity between all three Indian isolates. On comparing CP gene sequences of CMV KAR, CMV MH and CMV UP with CMV P isolate (*Physalis minima*); we got 94%, 99% and 96% identity respectively. High degree identity at nucleotide level between these isolates of banana and *Physalis minim*a (a weed) suggest that *Physalis minima* could be an alternate host of CMV banana. Phylogenetic analysis of nucleotide along with amino acid sequence of coat protein gene revealed that all our isolates belong to IB subgroup. In short, it appears that there occurs a high incidence of CMV infecting banana belonging to IB subgroup in most parts of Indian subcontinent.

**Key words:** Banana, CMV, CP gene, RT-PCR

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## 1. Introduction

Banana is the largest fruit crop of India accounting 33% of the total fruit production and has great socio-economic significance. Banana plantation is subjected to various natural calamities among which diseases constitute a major setback to the production of this crop. Diseases, particularly virus borne are considered as major threat due to abundance of insect vectors and easily available alternate hosts. *Cucumber mosaic virus* causing yellow mosaic and stripes on leaves, besides causing leaf distortion along with stunting of banana plant, contribute as a major serious threat for banana cultivation (Niblett *et al*., 1994). *Cucumber mosaic virus* (CMV) causing banana mosaic disease is an important virus, belonging to genus *Cucucmovirus* and family *Bromoviridae* (Roossinck *et al*., 1999; Yot-Dauthy and Boove, 1966). The main culprit behind high disease incidence rate and lesser productivity as cucumber mosaic virus was first described in Australia by Magee in 1930's (Magee, 1940), previously by various names including infectious cholorosis, heart rot, virus sheath rot, cucumber mosaic and banana mosaic (Stover, 1972). CMV has the broadest host range of any known virus, infecting more than 1,000 species of plants and has been found in all parts of the world (Edwardson and Christie, 1991).

CMV is multicomponent single stranded virus, with three positive-sense RNA's (RNA 1, RNA 2 and RNA 3) and an additional subgenomic RNA (RNA 4) derived from RNA 3 (Hubili and Francki, 1974; Paden and Symons, 1973). RNA 1, RNA 2 encode 1a, 2a proteins involved in virus replication (Hayes

and Buck, 1990), while as RNA 3a encode 3a movement protein (MP) (Suzuki *et al*., 1991) and 3b expressed from RNA 4 coat protein (CP) (Hubili and Francki, 1974, Schwinghamer and Symons, 1975). A large number of strains of CMV have been described previously and classified into two subgroups I and II on the basis of biological, serological properties and nucleotide sequence homology (Anderson *et al*., 1995; Palukaitis *et al*., 1992; Quedma *et al*., 1989). Phylogenetic analysis of a number of CMV isolates has led to further subdivision of subgroup I into IA and IB (Palukaitis *et al*., 1992). RNA viruses have potential for high genetic variation due to the absence of proofreading ability of the RNA replicase.

Although banana is grown widely in Karnataka, Maharashtra and Uttar Pradesh region of India, surveys of plants for CMV infection and differentiation of viral isolates have been yet not been reported up to large extent from these major banana growing states of India. Therefore, for acquiring knowledge of virus diversity at genetic level, we carried out study regarding characterization of these banana infecting CMV isolates using coat protein (CP) gene for establishing their genetic relatedness with other previously reported sequences of CMV, classified on the basis of biological and serological properties in to subgroup I and II respectively.

## 2. Materials and Methods

### Maintenance of virus culture and electron microscopic study

Banana plants showing leaf mosaic, yellow stripes in leaves, leaf distortion and stunting of plant were collected from major banana growing areas of India (Karnataka, Maharashtra, and Uttar Pradesh). Virus cultures were maintained under insect proof glass house condition. Transmission electron microscopic studies of the infected leaf samples were performed at Electron Microscopic Facility, All India Institute of Medical Sciences, India. For this, samples were fixed in modified karnovsky fluid's (David *et al*., 1973) buffered with 0.1 molar Sodium Phosphate Buffer (pH 7.4). Fixation was done for 2hrs in 1% Osmium tetraoxide in the same buffer at $4^0$C. After several washes in 0.1 M Sodium Phosphate Buffer, the specimens were dehydrated in graded acetone solution and embedded in CY212 araldite. Ultra thin section of 60-80 nm thickness were cut using an ultra cut E (Reichert Jung) ultramicrotome and the sections were stained in alcoholic uracil acetate (10 min) and lead citrate (10min), before examining corresponding grids under transmission electron microscope (Philips, CM-10) operated at 60-80 Kv.

### RNA isolation and RT-PCR of coat protein (CP) gene

RNA was isolated from both infected as well as from healthy tissue culture based leaf tissue (Negative control) of banana samples using RNeasy® Plant Mini Kit (Qiagen, Germany) as per instructions of the manufacturer. Isolated RNA were checked by electrophoresis on the 2% agarose gel and quantified spectrophotometrically. Extracted RNA was used for cDNA synthesis using RevertAid H minus cDNA synthesis kit (Fermentas, Canada). PCR reactions of CP gene along with plasmid DNA (Cloned CP) of *Physalis minima* (positive control) and tissue culture based healthy banana leaf (negative control) were performed using gene specific primers (Forward-5'-ATGGACAAATCTGAATCAACC-3'and Reverse-5'-TCAAACTGGGAGCACCCC-3') in TC-312 thermal cycler (TECHNE, UK). The 25 $\mu$l reaction volume include 2mM $MgCl_2$, 200 $\mu$M of each dNTP (Cinnagen, Iran), 0.75 U Taq DNA Polymerase (Biotools, Spain), 0.2 $\mu$M of Forward and Reverse primer for CP and 15 ng of cDNA and 10ng of plasmid DNA (Clone CP) of *Physalis minima* (Positive control). Amplification reactions were carried out using the following thermal profile: $95^0$C for 5min (1 cycle) followed by 30 cycles each with $95^0$C 1 min (Denaturation), 53 $^0$C 1 min (Annealing), 72 $^0$C 1 min (Extension) along with a final extension at $72^0$C for 7min (1 cycle). Amplified product (10µl) were separated on 2% (w/v) agarose gel along with 100bp DNA ladder (Fermentas, Canada) in 1X TAE buffer by electrophoresis at 80V for 2hrs. Gels were stained in ethidium bromide and

photographed on a digital gel documentation system (Alpha InfoTech, USA).

**Cloning and sequencing for CP gene**

Eluted PCR products of CP gene after purification were cloned into pGEM-T® Easy vector (Promega, WI, U.S.A.). The ligated products were transformed in competent cells of *Escherichia coli*, XL1-Blue strain showing resistance to tetracycline. The transformed clones were selected on LA plates containing 100µg/ml ampicillin and 12.5 µg/ml tetracycline (Sambrook and Russel, 2001). Selected clones were for the desired gene were cross checked by performing colony PCR as well as restriction digestion using *Eco*RI, followed by electrophoresis on 1.5% agarose. Clones were designated as CMV KAR, CMV MH, and CMV UP for Karnataka, Maharashtra and Uttar Pradesh samples respectively. Positive clones with inserts of the required size were subjected to sequencing, using universal T7 and SP6 primers. The sequencing was done in DNA Sequencing Facility available at University of Delhi (South Campus), India. Amino acid sequences for coat were generated using Expasy translational tools (http://expasy.org/tools).

**Sequence analysis and phylogenetic study**

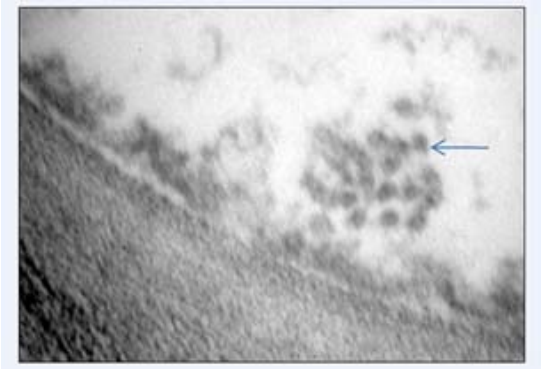
Coat protein (CP) gene sequence of three isolate were submitted to EMBL Database (Accession No's: AM055602, DQ640743 and AM158321 respectively). Furthermore, these sequences were compared with previously reported sequences of CMV belonging to different subgroups [I (A and B) and II], from both Indian as well as those reported from different parts of the world. Nucleotide Blast program was used to identify related sequences available in Genbank database. CP gene nucleotide and amino acid sequences of CMV isolates used for comparison are listed in Table 1. Percent sequence identity of these sequences were determined using Bio-Edit program (Version 7.0.0). Multiple alignments of nucleotide and deduced amino acid were carried out using CLUSTAL W (Version 1.82) and Bio-Edit (Version 7.0.0). The alignment files created by ClustalW were bootstrapped 1000 times for generating phylogenetic tree by neighbour-joining using Clustal X and TREEVIEW Win 32 (version 1.6.6) software.

## 3. Results

**Maintenance of virus culture and electron microscope studies**

Virus cultures on banana plant were maintained under insect proof condition for electron microscopy and other studies. Transmission electron microscopy of infected banana leaves revealed presence of a polyhedral virus particle of ~28-29 nm diameter (Fig. 1). The shape and size of the virus particle were identical with those of CMV particle obtained from other sources.

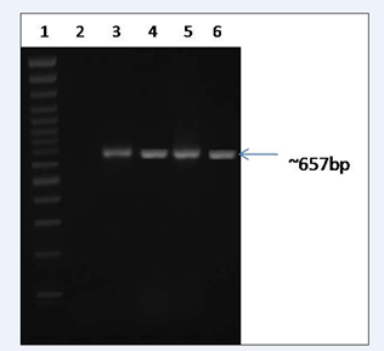
Fig 1: Electron micrograph of a thin section of infected leaf sample of banana showing viral particle (~29nm in diameter)



**RT-PCR and cloning of coat protein (CP) gene**

PCR amplification of the CP gene using gene specific primers resulted in the amplification of ~657 bp fragment from all three samples (KAR, MH and UP) along with positive control, but no amplification was observed in tissue culture based healthy sample (negative control) (Fig 2). After purification PCR products were successfully cloned into pGEM-T® Easy vector. Colony PCR along with restriction digestion of plasmid DNA isolated from selected clones were carried out for the conformation of insertion product of desired gene.

Fig 2: Gel photograph of RT-PCR amplicons




Lane 1: 100 base pairs DNA ladder
Lane 2: Negative control
Lane 3: Positive control (CMV P)
Lane 4–6: Amplified CMV CP- Karnataka, Maharastra and Uttar Pradesh

**Sequence analysis and phylogenetic study**

Sequence analysis of CP gene revealed that the sequenced region contain a single open reading frame, comprised of 657 nucleotides with initiation and termination codon. Sequences of CMV CP infecting banana of all our isolates on comparing with CP gene sequences of available CMV isolates from India as well as few representative isolates from different part of the world, revealed that all our isolates share 93% to 98% at nucleotide and 94% to 99% homology at amino acid level respectively. However, the sequence identity between banana isolates from India and rest of the world was lower at both nucleotide (75-94%) and amino acid (75-97%) levels (Table 1). CMV isolates from KAR (AM055602) and UP (AM158321) showed highest 98% and 99% identity in their nucleotide and amino acid sequences respectively (Fig 3). Interestingly, banana isolate of CMV from Maharashtra (DQ 640743) shared 99% and 100% identity with *Physalis minima* (X89652) isolate of CMV.

Multiple alignment studies for nucleotide and amino acid sequences of CMV KAR with other reported isolates of CMV from India as well as different parts of the world revealed 90-93% and 92-96% identity with CMV IA Subgroup strains and 75-76% and 76-79% identity in nucleotide and amino acid sequences with the members of subgroup II respectively. CMV KAR showed a high identity with the strain of CMV subgroup IB (91-95% in nucleotide and 93-99% amino acid) (Table 2). Thus, on the basis of phylogenetic studies, CMV KAR isolate appears to be an active member of CMV IB subgroup.

In a similar fashion, CMV MH revealed 89-91% and 91-95% identity at nucleotide and amino acid sequences with CMV IB subgroup strains and 74-76% nucleotide and 75-79% amino acid at nucleotide and amino acid level with members of subgroup IB. CMV MH showed high identity with the strain of CMV subgroup IB (89-99% in nucleotide and 92-100% amino acid) (Table 2). Phylogenetic analysis here again reveals that CMV MH isolate belongs to CMV subgroup IB. Similarly, sequence analysis of CMV UP isolate revealed 89-92% nucleotide and 93-96% amino acid with the strains of CMV Subgroup IA and 74-76% nucleotide and 75-79% amino acid with members of CMV subgroup II. Our UP isolate showed high sequence identity with the strain of CMV subgroup IB (89-96% in nucleotide and 93-99% amino acid) (Table 2). Based on above finding regarding phylogenetic analysis of CMV UP, it appears as being the member of CMV subgroup IB. Multiple sequence alignment of CMV CP based on deduced amino acid sequences of subgroup IA, IB and II Isolates have revealed that CMV P and CMV KAR differs at four positions, CMV P and CMV UP at two position, but at the same time CMV MAH showing absolute identity with CMV P.

A phylogram was constructed using cp nucleotide alignment of various strains of CMV (IA, IB and II) favors the results of sequence similarity between CMV P and CMV KAR, CMV MH and CMV UP with CMV subgroup IB. On the basis of phylogram, it is cleared that all Indian CMV banana isolates belongs to subgroup IB. However Hawaii isolate (U31219) of CMV banana belong to subgroup IA and China isolate (AF268598) of banana belonging to subgroup II. Clear clusters of CMV subgroups IA, IB and II formed in the phylogram. (Fig. 4) These analyses showed that both I and II (A & B) subgroup of CMV

isolates infecting banana occur in different part of the world.

Fig 3: Coat protein amino acid sequence alignment of banana isolates with that of subgroup IA, IB and II isolates of CMV (from India and other parts of the world) using Bio-Edit (Version 7.0.0). Dots (.) indicates identity at a given position. For abbreviations of virus, country of origin and accession number, see Table 1 Asterisk (*) indicate banana isolates

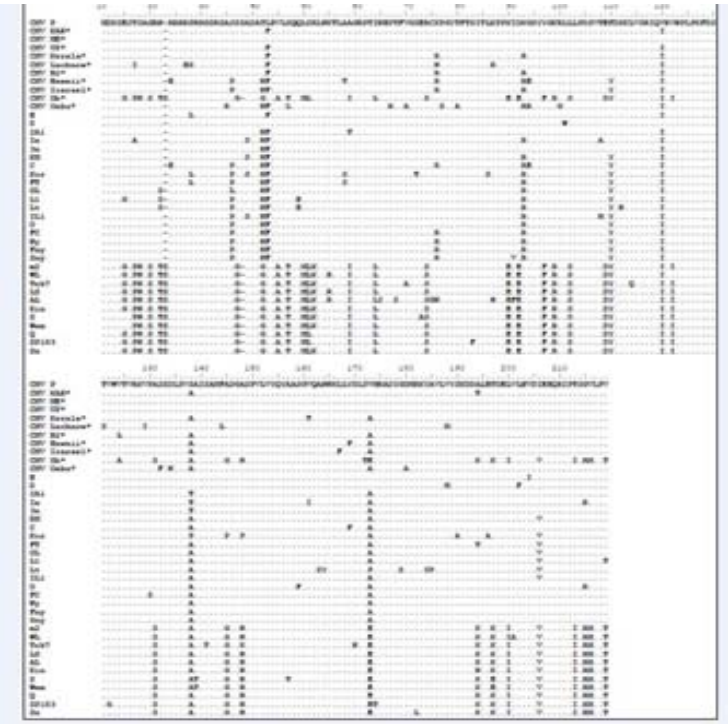
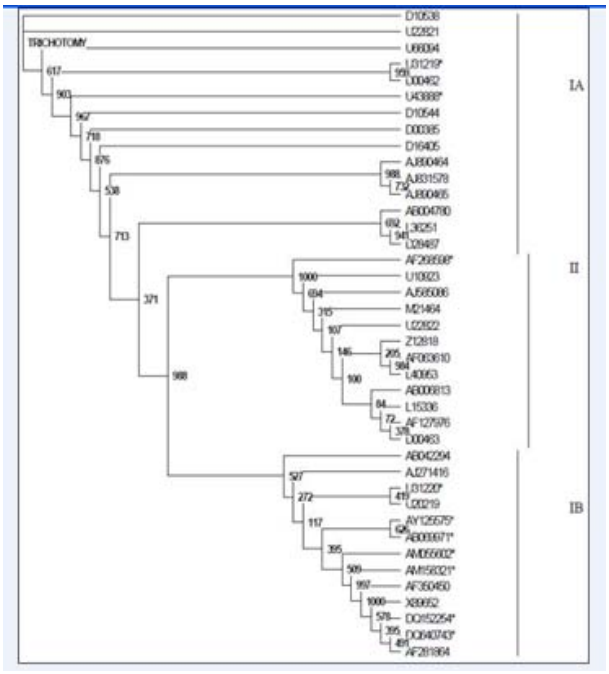


Fig 4: Phylogram, drawn by Neighborhood Joining Bootstrap method (bootstrap analysis with 1000 replicates) in Clustal X (version 1.83) and rooted tree were generated using TREEVIEW. Phylogenetic relationship illustrated by the multiple alignments of the coat protein nucleotide sequences (30 distinct and 10 banana isolates) of CMV CP. Sequences for comparison between banana and IA, IB & II isolates were obtained from GenBank. Accession number shown to each of the isolates and their country name and designation are given in table 1. Asterisk (*) indicates banana isolates



| Subgroup | Strain/Isolate | Accession No. | Country of Origin |
|---|---|---|---|
| - | CMV KAR* | AM055602 | India |
| - | CMV MH* | DQ640743 | India |
| - | CMV UP* | AM158321 | India |
| - | Kerala* | AY125575 | India |
| - | Lucknow* | DQ152254 | India |
| - | B2* | AB069971 | Indonesia |
| - | Hawaii* | U31219 | Hawaii |
| - | Israel* | U43888 | Israel |
| - | Xb* | AF268598 | China |
| - | Oahu* | U31220 | Hawaii |
| IB | CMV P | X89652 | India |
| IB | H | AF350450 | India |
| IB | D | AF281864 | India |
| IB | 2A1 | AJ271416 | USA |
| IB | Ix | U20219 | USA |
| IB | 3a | AB042294 | Japan |
| IA | KM | AB004780 | Japan |
| IA | C | D00462 | USA (NY) |
| IA | Korea | L36251 | Korea |
| IA | FT | D28487 | Japan |
| IA | OL | AJ890464 | India |
| IA | Ll | AJ831578 | India |
| IA | Lt | AJ890465 | India |
| IA | IL1 | D16405- | Japan |
| IA | O | D00385 | Japan |
| IA | FC | D10544 | USA |
| IA | Ny | U22821 | Australia |
| IA | Fny | D10538 | USA- NY |
| IA | Sny | U66094 | Israel |
| II | M2 | AB006813 | Japan |
| II | WL | D00463 | Unkwon |
| II | Trk7 | L15336 | Hungary |
| II | LS | AF127976 | USA |
| II | AL | AJ585086 | India |
| II | Kin | Z12818 | UK |
| II | S | AF063610 | USA |
| II | Wem | L40953 | Unknown |
| II | Q | M21464- | Australia |
| II | SP103 | U10923 | USA |
| II | Sn | U22822 | Australia |

Table 1: Source of CMV coat protein gene sequences used for comparison of banana isolates and subgroup IA, IB and II. Asterisk (*) indicates banana isolates

Table 2: Comparison among coat protein gene of banana isolates of CMV (India and other parts of the world) and Physalis minima isolate (% nucleotide identity top right and % amino acid identity bottom left)

| | AMO55602 | AM158321 | DQ640743 | AY125575 | DQ152254 | AB069971 | U31220 | U31219 | U43888 | AF268598 | X89652 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AMO55602 | * | 95 | 94 | 95 | 93 | 94 | 91 | 92 | 93 | 76 | 94 |
| AM158321 | 99 | * | 96 | 94 | 95 | 93 | 89 | 91 | 92 | 76 | 96 |
| DQ640743 | 98 | 99 | * | 94 | 98 | 93 | 89 | 91 | 92 | 76 | 99 |
| AY125575 | 97 | 97 | 96 | * | 93 | 95 | 92 | 92 | 93 | 77 | 94 |
| DQ152254 | 94 | 95 | 94 | 94 | * | 91 | 87 | 89 | 91 | 75 | 97 |
| AB069971 | 97 | 97 | 96 | 99 | 94 | * | 91 | 92 | 92 | 77 | 92 |
| U31220 | 93 | 93 | 92 | 93 | 88 | 93 | * | 90 | 90 | 75 | 89 |
| U31219 | 94 | 94 | 94 | 96 | 91 | 96 | 92 | * | 98 | 76 | 91 |
| U43888 | 96 | 96 | 95 | 97 | 92 | 97 | 93 | 97 | * | 77 | 92 |
| AF268598 | 78 | 78 | 78 | 77 | 75 | 77 | 74 | 77 | 77 | * | 76 |
| X89652 | 98 | 99 | 100 | 96 | 94 | 96 | 92 | 94 | 95 | 78 | * |

## 4. Discussion

Cucumber mosaic disease caused by CMV has attained a serious status in most of the banana growing states of India. Importance of the disease stems from the fact that it is responsible for losses to the tune of 40%. Realizing the potential threats of cucumber mosaic disease of banana, it is feared that in future Indian banana growing areas might be highly affected by this disease. Aim of our study illustrates detection and cloning of CP gene of field isolates to correctly identify the disease and to assess similarity or variability among isolates of CMV infecting banana in India as well as other CMV isolates from rest of the world. For this, coat protein gene of three CMV isolates collected from three different states of India were cloned and sequenced. High degree of nucleotide sequence identity (98-99%) between isolates of banana and *Physalis minim*a (a common weed), suggest that *Physalis minima* could be an alternate host of CMV banana. Phylogenetic analysis suggested that all three CMV isolates of banana viz. CMV KAR, CMV MH and CMV UP belong to subgroup IB. These results has clearly established the genetic relatedness of our isolates with members of IB subgroup, in accordance with those reported earlier for isolate of CMV infecting banana in India.

CMV known to infect various species of plant has been adapted successfully to new host and new environment. Recombination and reassortment play important role in CMV evolution. Recombinants of RNA3 were significantly more frequent than recombinants for RNA1 and RNA2. Coat protein being the main structural protein of CMV, has been found to play a determinative role in the transmission and symptom modulation of this virus. It has been found that single amino acid change in CP of CMV determine symptoms in *N. glutinosa* and specific sites in the viral CP of CMV affect its systemic movement in squash. Since banana is an important fruit and vegetable crop, it becomes necessary to characterize the viruses infecting the crop so that effective control measures or quarantine step can be develop to minimize the losses caused by viruses. RT-PCR based detection systems significantly improves monitoring and forecasting of banana mosaic epidemics. Data presented in this communication clearly indicate that CP region is sufficient to provide a simple and reliable method for detection and strain identification of banana CMV.

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