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Molecular Cloning of Cowpea Golden Mosaic Geminivirus and its Relationship with Mungbean Yellow Mosaic Geminivirus

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ABSTRACT. Whitefly transmitted yellow mosaic and golden mosaic diseases are the major constraints in the productivity of cultivated pulses in India. Mungbean yellow mosaic geminivirus (MYMV) the whitefly transmitted geminivirus causing yellow mosaic disease in legumes, does not infect cowpea. A whitefly transmitted golden mosaic disease of cowpea was reported from India in 1980s. The causal virus referred to as cowpea golden mosaic geminivirus (CpGMV) has a very restricted host range within legumes and does not infect mungbean. Present investigation envisaged to characterize the virus causing golden mosaic disease in cowpea and to establish its relationship with MYMV. Results on vector transmission, and molecular cloning established that CpGMV is a Begomovirus belonging to family Geminiviridae. In CpGMV while DNA B population is normal, DNA A population is subgenomic and it is replication defective due to the lack of the ORF for the replication enhancer protein as revealed in PCR analysis. Relatedness of CpGMV and MYMV was confirmed by Southern hybridization. Nucleotide sequencing of a part of movement protein gene (BC1) and intergenic region established that though the causal virus differs from MYMV in its host range, it shares more than 95% sequence homology with blackgram isolate of MYMV. Phylogenetic analysis for intergenic region clearly revealed that the causal virus of golden mosaic disease of cowpea in Northern India is only a mutant of MYMV and it is totally different from the virus reported from Africa.

INTRODUCTION

Yellow mosaic diseases caused by whitefly-transmitted geminiviruses (WTGs) are the major constraints in improving the productivity of pulse crops in India. Among these, Mungbean yellow mosaic geminivirus (MYMV), the most virulent one that infects blackgram, mungbean and soybean was not known to infect cowpea till 1980. A whitefly (*Bemisia tabaci* Genn.) transmitted golden mosaic disease of cowpea was reported from India by Varma and Reddy in 1984. It has now spread in alarming proportion particularly in Northern India. The characteristic symptoms are conspicuous golden yellow mosaic, mottling or both which appear on young leaf lamina (Fig. 1). A similar disease has also been seen in Pakistan (Ahmad, 1978) and Nigeria (Singh and Allen, 1979). The causal virus, cowpea golden mosaic virus (CpGMV) (Varma and Reddy, 1984) in India has a very narrow host range and does not infect mungbean. Preliminary studies on viral morphology and transmission properties reveal that the virus is a whitefly-transmitted geminivirus like MYMV. However, epitope profile studies using panels of monoclonal antibodies to African and Indian cassava mosaic virus did not clearly differentiate it from MYMV (Swanson *et al.*, 1992). The present study was undertaken with the main objective of elucidating the

genome organization and defining the relationship between the two important WTGs *viz.*, cowpea golden mosaic geminivirus and mungbean yellow mosaic geminivirus.

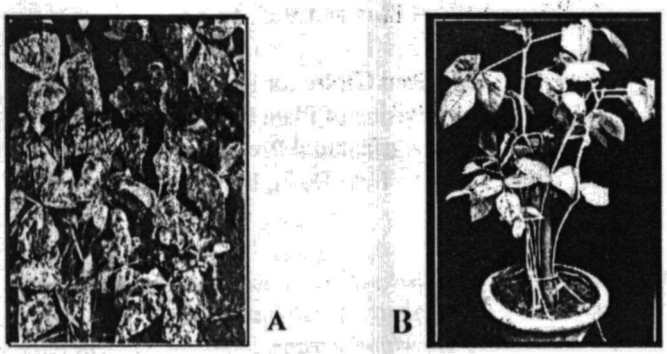

Fig. 1. A) Golden mosaic symptoms in cowpea in the field. B) Symptoms in cowpea cv. Pusa Komal inoculated through viruliferous whitefly.

MATERIALS AND METHODS

Establishment of virus

Cowpea plants naturally infected by CpGMV served as source of inoculum for this virus. The virus was established on young seedlings of *Vigna unguiculata* (L.) Walp. cv. Pusa Komal through whitefly inoculation with an acquisition access period (AAP) and inoculation access period (IAP) of 24 h each. Healthy plants were always inoculated at primary leaf stage using 10-12 whiteflies per plants.

Isolation and purification of replicative forms (RF) of viral DNA

Young leaves of cowpea cv. Pusa Komal showing golden mosaic symptom were harvested 10 days after inoculation. Total nucleic acid was isolated by the CTAB method (Dellaporta *et al.*, 1983). From the total nucleic acid, viral DNA was purified by cesium chloride-ethidium bromide (CsCl-EtBr.) density gradient centrifugation (Stanley and Townsend, 1985).

Cloning of viral genome

Molecular biological techniques followed were as given in Sambrook *et al.*, 1989. The purified viral DNA was cloned in pUC18 vector at *Pst* I site. *E. coli* strain DH5α was then transformed with this recombinant plasmid. Recombinant plasmid DNA was isolated from the clone by modified alkaline lysis method (Brinboim and Doly, 1979) and analysed by restriction with different enzymes.

Nucleic acid hybridization

Southern hybridization was carried out with radiolabelled probes (α^{-32}P, dCTP) prepared by random priming method (Feinberg and Vogelstein, 1984). The full length clones of DNA A and DNA B of blackgram isolate of MYMV (MYMIV-Bg) used in the present study were taken from Advanced Centre for Plant Virology, Division of Plant Pathology, Indian Agricultural Research Institute. For specific detection of whitefly transmitted germination (WTG) dot-blots were prepared with nucleic acid extracted from different plants and hybridization was done by using α^{-32}P dCTP labelled probe to full length DNA B and subgenomic DNA A of CpGMV.

Polymerase chain reaction (PCR)

To study the ORFs in DNA A clone, PCR was performed with a consensus Rep-CP primer designed based on conserved sequence of other WTGs (Briddon and Markham, 1994), MYMIV-Bg specific replication initiator protein (Rep), intergenic region (IR) and coat protein (CP) primers. The sequences of primers and region they amplify are given in, Table 1.

Table 1. Sequence of primers used in PCR analysis.

Primer	Sequence
MYMIV-Bg AC1 forward	5'ATGGATCCATGCCAAGGGAAGGTCGT3'
MYMIV-Bg AC1 reverse	5'TGAAAGCTTTCAATTCGAGATCGTCGA3'
MYMIV-Bg DNA A IR forward	5' TAGCAAAACGACCTTCCCTTGG 3'
MYMIV-Bg DNA A IR reverse	5' GCAGAAAAGTTAAAGTAACCCC 3'
MYMIV-Bg CP (UP1) forward	5'GCAGAATTCTGCCAAAGCGGACCTACG3'
MYMIV-Bg CP (UP3) forward	5'CATGAATTCATGTGGAGGAAACCTCGG3'
MYMIV-Bg CP reverse	5' TGAAAGCTTTCAATACAATCTTTATTA 3'
Consensus Rep-CP forward	5'GCATCTGCAGGCCCACATTGTCTTYNGT3'
Consensus Rep-CP reverse	5'GCATCTGCAGGCCCACATTGTCTTYNGT3'

The temperature profile and cycles performed were: 1 cycle for denaturation of DNA at 94°C for 2 min, 40 cycles which had 3 segments: denaturation at 94°C for 1 min, annealing at 55°C for 2 min and chain extension at 72°C for 3 min. After PCR, 5 µl of amplified product was subjected to electrophoresis in 1% agarose gel to observe the DNA fragments of predicted size. In all the PCR experiments, a reaction without any DNA but having all other reagents served as a negative control.

DNA sequencing by dideoxy chain termination method

DNA sequencing was carried out using *Taq* polymerase (Sequencing kit, MBI Fermentas) by dideoxy chain termination method. Sequencing was done using M13 forward and reverse primers. Sequencing was repeated twice for three clones. Data were analysed in BLAST (http://www.ncbi.nlm.nih.gov./blast/) and MacVector 6.3 programme. The analysis was done in Clustal W multiple alignments with open gap penalty of 10 and extent gap penalty of 5. Nucleotide sequences of other viruses compared were obtained from GenBank database. They are as given below: blackgram isolate of mungbean yellow mosaic Indian virus - MYMIV-Bg, AF126406; AF142440; Thailand isolate of mungbean yellow mosaic geminivirus - MYMV-Th, D14703, D14704; *Vigna mungo* isolate of mungbean yellow mosaic geminivirus - MYMV-Vi, AJ132574, AF262064; Nigeran isolate of cowpea golden mosaic geminivirus - CpGMV, AF029217. The nomenclature followed to describe different coding regions are as given by Hanley-Bowdoin *et al.* (1999).

RESULTS AND DISCUSSION

Host range and vector transmission

CpGMV isolate under study was transmissible only to cowpea and French bean through whitefly (Fig. 1). Attempts to transmit it to blackgram, mungbean and soybean did not succeed indicating the narrow host range of the virus isolate.

Investigation on the genome organization of cowpea golden mosaic geminivirus

Isolation of replicative forms of viral DNA

After fractionation and removal of CsCl and EtBr., all the fractions were subjected to 1% agarose gel electrophoresis. Two bands, one running on par with 3 kb and another with 1.6 kb band of the 1 kb molecular weight marker were visualised on staining the gel with EtBr. To confirm the viral nature of these DNA bands, Southern analysis was done.

Southern hybridization

Southern hybridization was done using radiolabelled probe to full length MYMIV-Bg DNA A. By analogy with viral replicative forms reported in other WTGs (Stanley and Townsend, 1985) three forms of viral DNA *viz.*, double stranded linear (LIN), supercoiled (SC) and single stranded (SS) were identified in the autoradiogram (Fig. 2.).

Supercoiled form (SC) gave stronger hybridization signal than other forms. It represented the active replication cycle of the viral genome inside cowpea. The fractions which gave stronger hybridization signal, were pooled, and an aliquot was taken for direct cloning.

Analysis of recombinant clones

Recombinant clones were restricted with *PstI*/*BglI* and fragments were separated in gel electrophoresis. Out of 11 clones 8 gave 2.7 kb band (Fig. 3A: lane 4-8, represent pCp7-pCp11 clone), an expected unit genome length of geminiviruses and 1.1 kb + 1.6 kb bands of the vector (pUC18). These full-length clones may represent population of DNA A or DNA B, the two component characteristics of Begomoviruses. By Southern hybridization using radiolabelled probe to MYMIV-Bg DNA A (Fig. 3 B), clone pCp7 was identified to represent DNA A of CpGMV.

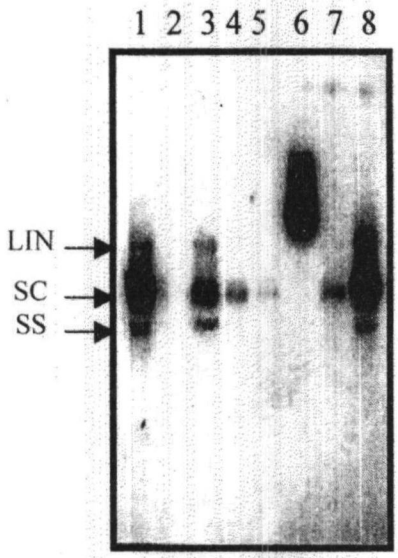

Fig. 2. Southern blot analysis of viral DNA purified by CsCl-Et Br. density gradient centrifugation. Blot hybridized with ^{32}P labelled probe to DNA A of MYMIV-Bg.

[Lane 1-5, 7, 8 - CsCl fractions, Lane 6 - Molecular weight marker. LIN, SC, SS denote double stranded linear, supercoiled and single stranded form of viral DNA respectively].

The other clones, which did not give any hybridization signal, were considered as DNA B clones of CpGMV and one of the 2.7 kb clones (pCp8) was selected as representative of DNA B clone.

Restriction analysis

In the case of clone pCp7, the viral insert got deleted in bacterial amplification. Several attempts to freshly clone DNA A from CsCl. EtBr. purified fraction, gave rise to 2.1 kb DNA A molecule while full length DNA B clones were obtained without any problem. Though the viral insert is only 2.1 kb it gave hybridization with MYMIV-Bg DNA A probe. In the absence of any other full length DNA A, clone pCp7 having 2.1 kb insert was taken for further work. Restriction analysis of clone pCp7 revealed that it had

an unique site for *Pst*I, *Bam*HI and *Hin*dIII while other enzymes used had no site(s). In the case of clone pCp8 (DNA B), the restriction analysis showed that it had a unique site for *Bam*HI, *Bgl*I, *Cla*I, *Hin*dIII, *Pst*I and two sites for *Xba*I and three sites for *Ssp*I.

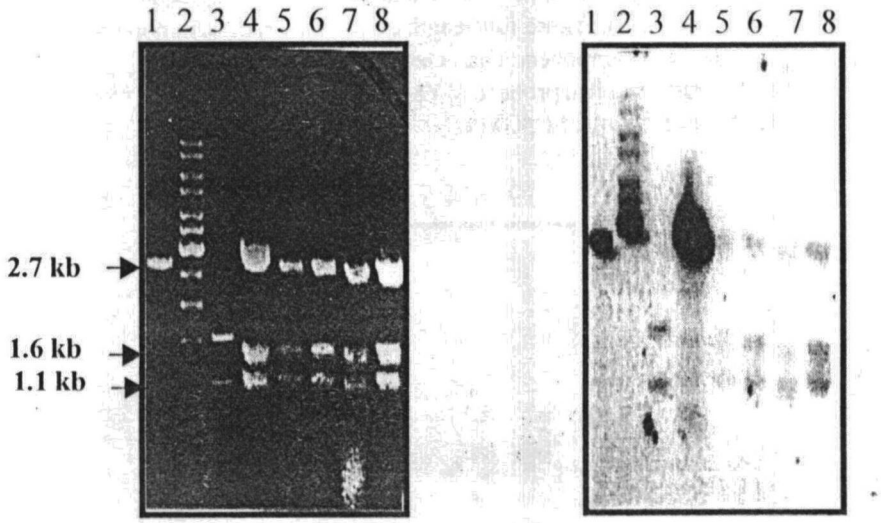

Fig. 3. A) Agarose gel electrophoresis of recombinant plasmid DNA restricted with *Pst*I/*Bgl*I. B) Southern blot analysis of recombinants DNA restricted with *Pst*I/*Bgl*I. Blot hybridized with ^{32}P labelled probe to DNA A of MYMIV-Bg. Only the strong signal (lane 4) is considered as positive hybridization.
[Lane 1 - pUC 18 vector restricted with *Pst*I, Lane 2 - Molecular weight marker, Lane 3 - pUC 18 vector restricted with *Bgl*, Lane 4-8 - DNA of recombinant plasmids (pCp7-pCp11) restricted with *Pst*I/*Bgl*I].

Recombinant clone analysis showed that the genome consists of two distinct population of DNA *viz*., DNA A and DNA B, confirming CpGMV as a member of typical bipartite geminivirus belonging to sub group III, Genus Begomovirus of the family *Geminiviridae*. DNA A clone pCp7 was 2.7 kb in length in the initial transformation but underwent deletion on subsequent subculturing. The deletion of the insert in plasmid vector in laboratory grown *E. coli* culture is a common phenomenon due to some adverse growth condition. Full length DNA A may be present in very low concentration and there may be problems in the replication of the full length molecule resulting in frequent occurrence of deleted 2.1 kb subgenomic DNA A. Occurrence of subgenomic DNA A, though rare, are not uncommon and seen in CLCuV (Harrison and Robinson, 1999).

Interrelationship between CpGMV and MYMV

Southern analysis of plant samples

Cross hybridization experiment with glasshouse-inoculated plants showed that CpGMV DNA B could detect MYMV infection in mungbean plants and *vice versa* (Fig. 4A-D). In dot-blot hybridization CpGMV DNA B probe detected MYMV infection in

blackgram, mungbean and soybean. Strong hybridization seen with DNA B probe point to the relatedness of the viruses.

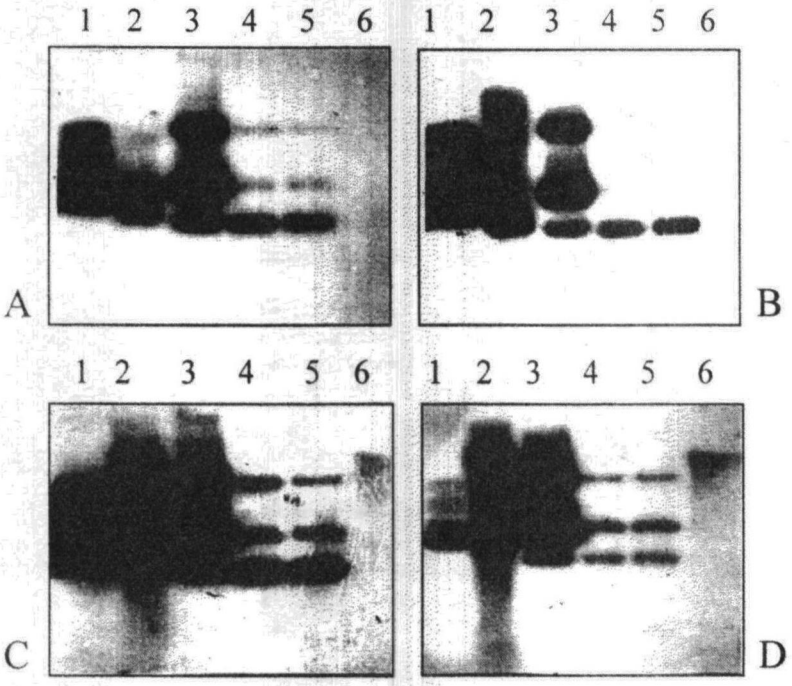

Fig. 4. Southern blot analysis of total DNA extracted from infected mungbean and cowpea plants using ^{32}P labelled probe to DNA A of CpGMV and MYMIV-Bg respectively (A and B) and ^{32}P labelled probe to DNA B of CpGMV and MYMIV-Bg respectively (C and D).

[Lane 1 - Molecular weight marker, Lane 2 - Total DNA extracted from MYMV infected mungbean plants, Lane 3-5 - Total DNA extracted from CpGMV infected cowpea plants, Lane 6 - Total DNA from healthy cowpea plant].

PCR analysis

Agarose gel electrophoresis of the amplified product clearly revealed that Rep, CP1, CP2 and IR specific primers of MYMIV-Bg amplified the CpGMV DNA A and products were also the same expected size as in MYMIV-Bg (Fig. 5A). But the Rep-CP primers could amplify only CpGMV DNA A and gave a fragment of expected 1.2 kb size. It did not give any product with MYMIV-Bg DNA A. From sequence analysis of MYMV, it was revealed that the variation in DNA A exists in the C terminal region of Rep (Fig. 5B). The total size of amplified fragments of Rep, CP and IR in the subgenomic clone was 2.1 kb which is equal to total size of this unstable clone. The deleted region represents ORF AC3/AC2, which codes for replication enhancer protein (REn) and transcription activator protein (TrAP). DNA A of CpGMV may not be stable and present in lower concentration due to instability in this region. It is possible that instability in REn region may hinder the multiplication of CpGMV genome within mungbean and other leguminous plants, resulting in the narrow host range of the virus.

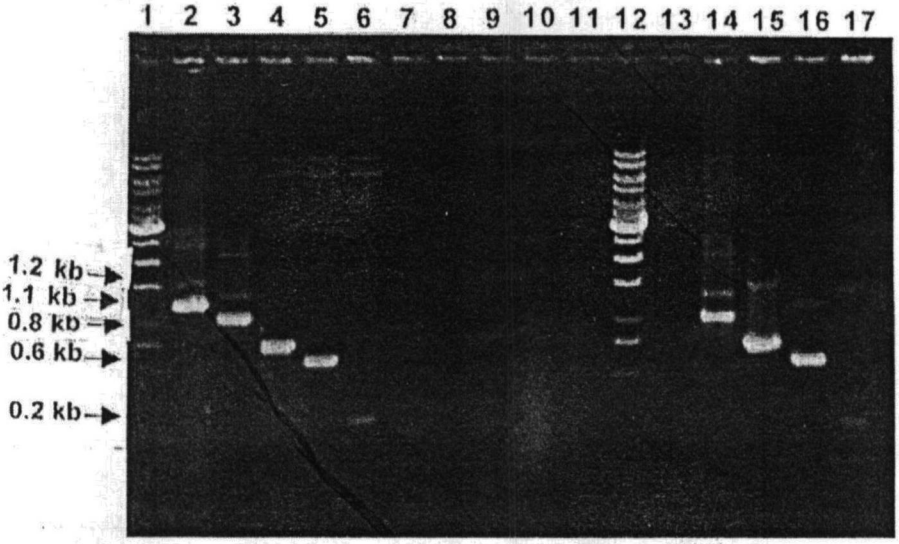

Fig. 5A. **Agarose gel electrophoresis of PCR amplified products.**
[Lane 1 and 12 - Molecular weight marker, Lane 2-6 - PCR amplification of DNA A of CpGMV using Rep-CP, Rep, CP1, CP2 and IR primers respectively, Lane 7-11 - reagent control of Rep-CP, Rep, CP1, CP2 and IR primers respectively, Lane 13-17 - PCR amplification of DNA A of MYMIV using Rep-CP, Rep, CP1, CP2 and IR primers respectively].

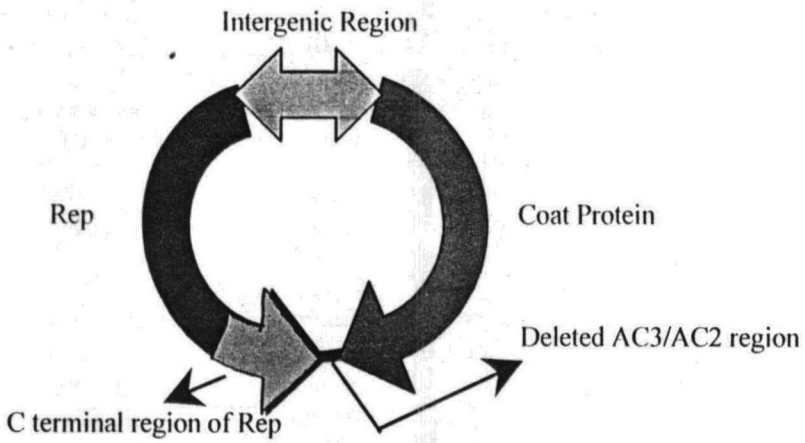

Fig. 5B. **PCR analysis indicates variation in DNA A exists in the C terminal region of Rep and the deleted region represents ORF AC3/AC2, which codes for replication enhancer protein (REn) and transcription activator protein (TrAP).**

In silico analysis of CpGMV DNA B

Sequencing was repeated twice for three clones (pCp 8, 9, 10). A total of 421-nucleotide sequence in viral sense (GenBank Accession No. AF289059) were analysed. The sequence data generated using M13 forward and reverse primers of the clone is partial and represent the core region of ORF BC1. Minor variation may be seen if entire genome is sequenced. However amongst Begomoviruses in DNA B high degree of homology is seen in ORF BC1 than in ORF BV1. Between CpGMV and other MYMV isolates too, it may be true. In BLAST analysis the sequence aligned maximum (97%) with ORF BC1 of DNA B of MYMIV-Bg (Table 2). There was also high level of homology shared with DNA B of MYMV-Vi (93%) and MYMV-Th (87%). The significant observation is the absence of homology with any Old World WTGs other than MYMV isolates. Comparison at nucleotide level between CpGMV and other MYMV isolates showed only few mismatches.

Table 2. Percentage identities in the nucleotide sequence of BC1 region between CpGMV and other WTGs.

Virus	Per cent identities
MYMIV-Bg	97
MYMV-Vi (isolate 1)	93
MYMV-Vi (isolate 2)	87
MYMV-Th	87

Comparison made in blast search (http://www.ncbi.nlm.nih.gov/blast/)

The putative protein product of ORF BC1 of CpGMV DNA B is compared with that of MYMIV-Bg and MYMV-Vi. Clustal W multiple alignment (Fig. 6) reveals that there is only one striking difference; an amino acid substitution of Lysine (K) at position 58th in the place of Arginine (R) between CpGMV and MYMIV-Bg. MYMV-Vi is distinct and shows differences from CpGMV and MYMIV-Bg.

In silico analysis of IR

The intergenic or common region sequence is highly specific for a virus and therefore helps in assessing the identity of the virus (Harrison and Robinson, 1999). From the sequence data of BC1, the location of the common or intergenic region was deduced near the *Hin*dIII site. Sequence was generated spanning this region and aligned with other MYMV isolates. 90 bp sequences upstream and downstream of the characteristic stem-loop structure seen in all geminiviruses (Hanley-Bowdoin *et al.*, 1999) were assumed to represent the common region. The putative intergenic region of CpGMV (Accession No. AF289058) is 193 nt long which includes the characteristic invariant nonanucleotide (TAATATTAC). IR of CpGMV DNA B has the features similar to organization of IR in other WTGs.

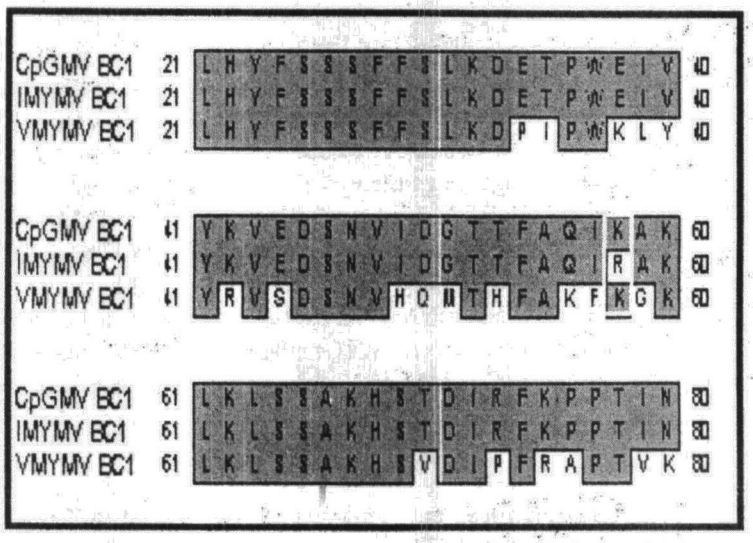

Fig. 6. Multiple alignment of predicted amino acid sequences of the BC1 protein (partial) of CpGMV, MYMIV-Bg and VMYMV. Shaded regions represent homology. Clustal W formatted alignment in MacVector 7.0 was used.

The nucleotide sequence of IR region of CpGMV DNA B was compared with that of different isolates of MYMV (MYMIV-Bg, MYMV-Vi and MYMV-Th) and CpGMV DNA A of Nigerian isolate. In all the cases, the regions compared were spanning between ~90 bp upstream and downstream of no namer sequences. CpGMV and the three MYMV isolates shared considerable degree of homology. It was highest with MYMIV-Bg (95%). The other two isolates of MYMV *i.e.*, MYMV-Vi and MYMV-Th differed to a greater extent from CpGMV (68 and 70% respectively) and MYMIV-Bg in the 5' region of IR.

The phylogenetic tree constructed (Fig. 7) based on nucleotide sequence of IR clearly reveals the relationship between MYMV isolates and CpGMV.

CpGMV Nigerian isolate is very different, well deviating and occupies a separate branch. Amongst the other four viruses, MYMV-Th and MYMV-Vi cluster together and form another branch, which is distinctly separated from the third branch in which CpGMV and MYMIV-Bg are clustered together. CpGMV differed from MYMIV-Bg in a stretch (50-55 nucleotide), a region presumed to play a role in specific recognition of DNA by the Rep protein. Hong and Harrison (1995) suggested that an isolate can be given the status of distinct virus, if they do not share homology in IR, and have less than 80% homology in ORFs other than CP. By that definition, it is concluded that CpGMV, present isolate from Northern India is not a distinct virus. It is proposed to rename the virus under study as MYMV-Cp (Cowpea isolate of MYMV) instead of CpGMV.

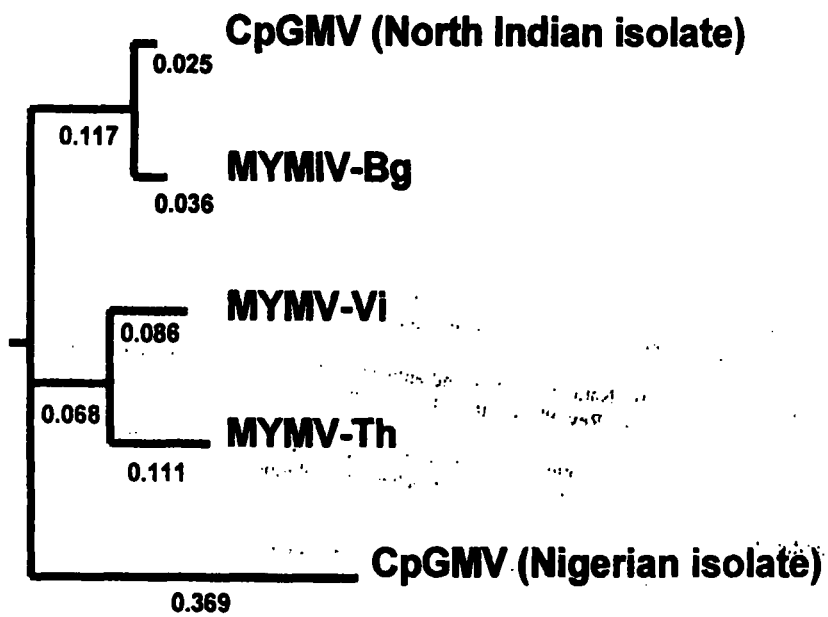

Fig. 7. Phylogenetic tree constructed based on alignments of nucleotide sequence of IR region. Number at nodes indicates percentage similarities. Clustal W formatted alignment in MacVector 7.0 was used.

CONCLUSIONS

The virus causing cowpea golden mosaic disease in Northern India is a whitefly transmitted bipartite geminivirus with a normal DNA B and a subgenomic, replication defective DNA A molecule and it is a strain of MYMV which might have originated due to mutation. The virus causing cowpea golden mosaic disease in India is distinctly different from that of the virus causing similar disease in Nigeria.

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