CLONING AND CHARACTERIZATION OF CUCUMBER MOSAIC VIRUS COAT PROTEIN GENE FROM INFECTED BANANA PLANTS IN EGYPT

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Cucumber mosaic virus (CMV) is an important plant virus, affecting hundreds of plant species and causing numerous diseases (Palukaitis *et al*., 1992). CMV is the type member of the genus *Cucumovirus*, family *Bromoviridae*. It is one of the most economically important plant viruses in the world because it causes great losses in vegetables, ornamentals and fruits. The destructive effect is due to its rapid spread by more than 60 aphid species vectors in the field (Edwardson and Christie, 1991). The CMV genome is organized into three single-stranded messenger-sense genomic RNAs (RNAs 1, 2 and 3) and two major subgenomic RNAs (RNAs 4 and 4A, which serve for the expression of the 3′-proximal gene of RNAs 3 and 2, respectively). RNAs 1 and 2 codes for components of the replicase complex. RNA 2 codes for the 2b protein, which is expressed from subgenomic RNA 4A and is involved in the suppression of gene silencing. RNA 3 encodes the 3a protein, which is essential for virus movement (Palukaitis and Garcia-Arenal, 2003). The coat protein (CP) is expressed from RNA4 (Habili and Francki, 1974; Palukaitis and Garcia-Arenal, 2003). The CP is required for host range, encapsidation (Suzuki *et al*., 1991), systemic virus movement (Canto *et al*., 1997) and aphid transmission (Ng *et al*., 2000). CMV strains or isolates have been divided into subgroups I and II on the basis of serological data, peptide mapping of the CP, and nucleic acid hybridization (Edwards and Gonsalves, 1983; Owen and Palukaitis, 1988; Wahyuni *et al*., 1992). The use of test plant species to reliably distinguish CMV strains between subgroups was unsuccessful. Recent phylogenetic analysis of CMV by use of CP ORF and 5 non-translated region (NTR) sequences confirmed the grouping and also led to further subdivision of subgroup I into IA and IB (Roossinck *et al*., 1999). Also, recombination between subgroups IA and IB was reported (Chen *et al*., 2007). Banana (*Musa* spp.) is infected by several viral agents such as Banana bunchy top virus, Banana bract mosaic virus, Banana streak virus. However, cucumber mosaic virus is the most serious viral diseases and has devastating effect on commercial banana plantations (Niblett *et al*., 1994). In the present study, we report the CP gene sequence-based characterization of CMV infecting banana plants in Egypt and comparing the obtained se-

quence with other published sequences to get more reliable phylogenetic relationship of the different strains.

MATERIALS AND METHODS

Plant Materials

Banana plants (cv. Williams) with typical symptoms (leaf mosaic, yellow stripes in leaves, leaf distortion and stunting of plant) were collected from major banana growing areas of El-Behera governorate, Egypt.

DAS-ELISA

Virus detection in collected tissues was performed directly from infected banana plants without passage in other hosts. The double antibody sandwich-enzyme linked immunosorbant assay (DAS-ELISA), was performed as described by Clark and Adams (1977)**,** using polyclonal antibodies (PAbs) previously raised against CMV particles at the molecular plant pathogenesis laboratory, AGERI, Giza, Egypt. The ELISA values at 405 nm were determined after 20 and 60 minutes post incubation at room temperature. Sample with OD value two folds the negative control is considered as positive (Sutula *et al*., 1986).

Primer design

The primers for amplification of the coat protein region of CMV were designed using DNA sequences obtained from the National Center for Biotechnology Information (NCBI) database. The sequences were aligned using MegAlign, an alignment program in DNASTAR Lasergene 7 software (DNASTAR Inc., USA) and the general consensus regions were taken just within the coat protein. Primers were selected manually and *Bam*H1 site was added at the 5` end of each primer.

RNA extraction and RT-PCR

Extraction of total RNAs was performed using TriPure Isolation Reagent (Roche Diagnostics, USA) according to the manufacturer's instructions. Total RNA was extracted from naturally infected and healthy leaf samples of banana. The amplification of full sequence of CP gene was performed using two specific primers; CMVF *Bam*H1 (5`GGGAATTGGATCCATGGACAAAT CTGAATC`3) and CMVR *Bam*H1 (5` GATTGGATCCCGGAATCAGACTGG GAGCA`3). RT-PCR was carried out using a QIAGEN one step RT-PCR kit (QIAGEN, Germany) according to the manufacturer's instructions. RT-PCR reaction was performed in a reaction volume of 50 µl. The reaction contains 2 µg RNA, 10 µl of 5X RT-PCR buffer, 2 µl of dNTPs mix (10 mM), 2 µl RT-PCR enzyme mix, and 0.6 µM of each primer, then the total reaction volume was completed to 50 µl by RNase free dH_2O. The reaction was subjected to: one cycle at 50°C for 30 min, one cycle at 94°C for 15 min; 35 cycles, each consists of: 94°C for 1 min, 50°C for 45 sec, 72°C for 45 sec and the final cycle was extended for 10 min at 72°C. Amplified products (10 µl)

were separated on 2% (w/v) agarose gel in 1X TAE buffer by electrophoresis at 80V for 2 hrs. Gels were stained in ethidium bromide and photographed on a digital gel documentation system (BioRad, USA).

Cloning and sequencing of PCR amplicon

PCR product was cleaned directly with QIAquick PCR Purification Kit (Qiagen Inc., Germany) and cloned into pGEM®-T Easy Vector System I according to Manufacturer's instructions. After ligation, clones were transformed into competent cells of *Escherichia coli* Top 10 strain. The clones with recombinant plasmid were identified by blue/white colony screening on LB culture plate and restriction digestion. Plasmids from recombinants were isolated by alkaline lysis (Sambrook *et al*., 1989). Selected clones were screened by restriction digestion using *Eco*RI, followed by electrophoresis on 1.5% agarose. One of the positive clones was subjected to sequencing, using universal T7 and SP6 primers. DNA sequence was determined by fluorescent dideoxy chain terminator technology, BigDye Terminator Kit using Applied Biosystem 373A Sequencer (Applied Biosystems Crop., USA).

Sequence analysis and phylogenetic study

Putative CP sequence data were analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with existing sequences of CMV strains available in GenBank database. ORF were translated into amino acid residues and aligned with different strains using MegAlign and Expasy tools (www.expasy.org/tools/). Phylogenetic analyses were determined by Neighborhood Joining Bootstrap Method in Clustral X (1.81).

RESULTS AND DISCUSSION

DAS-ELISA

Since banana is an important consumable food crop grown commercially for its high nutritional value, identification of the virus infecting banana became essential so that effective diagnosis/control measures may be developed to minimize the losses and spread of the virus. A set of 18 Plant samples showing CMV like symptoms (Fig. 1), in addition to healthy samples were collected and subjected to virus detection by DAS-ELISA using PAbs specific to the viral particles of CMV. The test revealed three positive samples which represent 16.7%. The mean absorbance values at 405 for negative and positive controls were 0.20 and 2.01, respectively, while for positive samples; the values were 0.70, 0.88 and 0.99.

RT-PCR and cloning of CMV-CP gene

RT-PCR was successful in amplifying CMV-CP gene from one of the positive samples. A PCR product of the expected size (~683 bp) was obtained after amplification, while no amplification was obtained from healthy sample (Fig. 2). The amplified product was purified and cloned by direct ligation into pGEM®-T

Easy Vector System I, then transformed into *E. coli* Top 10 cells. Different white colonies were selected and tested by *Eco*R1 digestion to release the inserts. Figure (3) shows the released cloned fragments with the same expected size of (~683 bp), which confirming the association of CMV in the infected plants.

Analysis of sequence data

One of the confirmed clones was purified and sequenced using thermal cycle sequencing with dye terminators and ABI automatic sequencer. DNA sequence analysis revealed a fragment contains an uninterrupted open reading frame (ORF) of 657 nucleotides encoding a polypeptide of 219 amino acid residues (Fig. 4). DNA sequence was compared to those in public databases (Altschul *et al*., 1990; BLAST comparison with GenBank). The BLAST search revealed a high degree of similarity with other published CMV coat protein sequences. Deduced amino acid sequence was aligned with sixteen CMV coat protein sequences using Clustal W method of the MegAlign procedure supplement within the DNASTAR package. The result of alignment is summarized in Table (1) and Fig. (5). The alignment revealed that homology for the deduced amino acid sequence ranged between 98.2-100%. The analysis revealed a very high amino acid homology (100%) with the CMV-Fny strain isolated from *Cucurbita pepo* in USA and 99.5% with both CMV-Sny isolate from *Cucurbita pepo* in Israel and J isolate from *Cucumis sativus* L. in Poland. As shown in Fig. (6), a dendrogram was constructed using the Neighbour-Joining method. The phylogram illustrates the phylogenetic relationships between our isolate (CMV-Egy) and other published sequences based on the amino acids sequence. Visual comparison of the phylogenic CP tree showed that the CP of the Egyptian strain was closely grouped with CMV-Fny (USA) and CMV-Sny (Israeli) strains. However it showed divergence with other strains. Several reports indicated that CMV-Fny strain is related to subgroup IA (Canto *et al*., 1997; Verma *et al*., 2006; Balaji *et al*., 2008; Hellwald *et al*., 2001). These results have clearly established the genetic relatedness of CMV-Egy isolate with the members of IA subgroup. The strains of CMV have been divided into two subgroups (I and II). Further splitting of subgroup I into IA and IB has been proposed on the basis of sequence data, analysis of 5'-non-translated region of RNA3 of several strains and phylogenetic analysis of CP (Roossinck, 2002). An interesting finding that despite the different geographical origin of the selected strains, there is less variation in the CP gene of these strains. It has been reported that the CP interacts predominantly with itself or with the viral RNA and has little interaction with the host. CP interactions with the aphid vector are also probably minimal and mostly nonspecific, since more than 85 species of aphids can transmit CMV (Edwardson and Christie, 1991). Under natural conditions, CMV generally is transmitted by aphids, but the virus also has been transmitted by seed in some plant species (Neergard, 1977; Ali and Kobayashi, 2010). These findings

indicate that CMV can be transmitted from country to another through seeds. Therefore, this can explain the high similarity among different CMV isolates. High similarities and conserved regions were observed among the CMV subgroup members at CP level; therefore, this homology may be utilized for specific detection of different strains and for developing pathogen-derive resistance (PDR) in susceptible plants. It is clear that antibodies-based diagnosis will be efficient tools due to the reactivity of recognizing sharing epitopes which are present in both I and II subgroups which are highly conserved among these CMV strains. Therefore, using antisera against CP for the detection of CMV enabled reliable detection of all CMV isolates. Finally as noted above, Data presented here clearly indicate that CP region is sufficient to provide a simple and reliable method for detection and strain identification of banana CMV.

SUMMARY

Cucumber mosaic virus was detected in banana plants showing typical symptoms of CMV infection using DAS-ELISA. Coat protein cDNA was amplified, by RT-PCR using specific primers, and cloned into pGEM®-T Easy Vector, then transformed into *E. coli* Top 10 cells. The complete sequence of coat protein gene from CMV-Egy strain has been determined and compared to other known CMV-CP sequences. The alignment of the amino acid sequences revealed high homology (98-100%) between CMV-Egy and different CMV strains. Phylogenetic analysis also revealed that CMV-Egy is closely related with CMV-Fny isolated from USA and Sny isolated from Israel.

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Table (1): Coat protein gene sequences of various Cucumber mosaic virus strains used for comparison.

Accession Number	Origin	Strain	Isolation host	Identity (%)
AAA74483	Hawaii, USA	Hawaii	Musa	98.2
CAE51924	Australia	242	Silver beet	98.6
CAB43510	China	YN	tobacco	99.5
CAH17692	Spain	RT88	melon	98.6
AAM81372	California, USA	CK41	-	98.2
AEK69525	India	Ban-In	tobacco	98.2
AAM95241	India	-	Banana	98.2
AAY46237	Iran	FI3	squash	98.2
AAB07137	Israel	Sny	Cucurbita pepo	99.5
BAB11693	Indonesia	B2	Musa sapientum	98.2
CAC18661	Taiwan	LiTW	ornamental crops	98.2
ABY21418	Taiwan	25	Banana	98.2
AAY42625	Poland	J	Cucumis sativus L.	99.5
ACH48048	Serbia	650-07	Nicotiana tabacum	99.1
CAD42338	South Korea	LK	Lily	98.2
P69466	USA	Fny	Cucurbita pepo	100

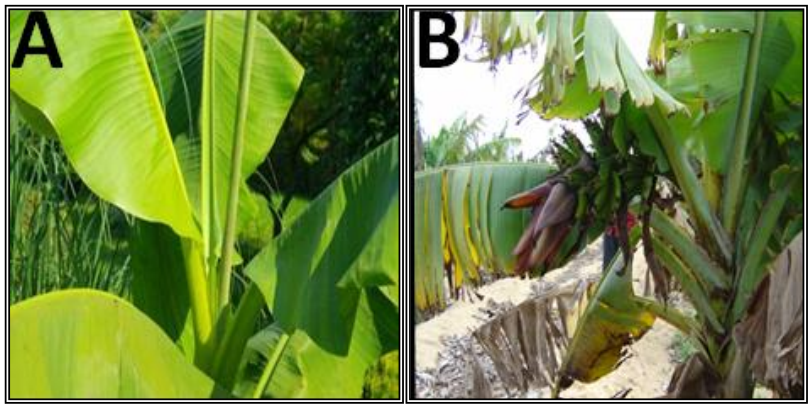

Fig. (1): Naturally infected Banana plants showing typical symptoms of CMV. A: healthy plant. B: infected plant with CMV.

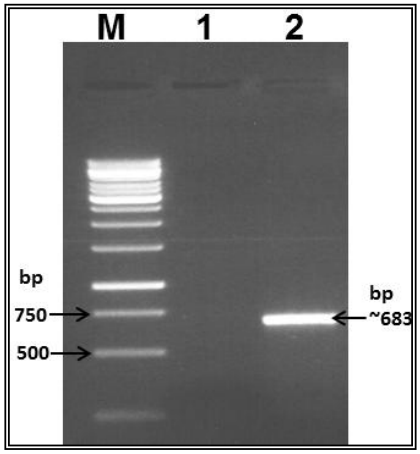

Fig. (2): Agorose gel electrophoresis showing ~683 bp amplicon after RT-PCR. M: 1 Kb DNA marker. Lanes1and 2: RT-PCR for healthy banana plant and infected banana plants, respectively.

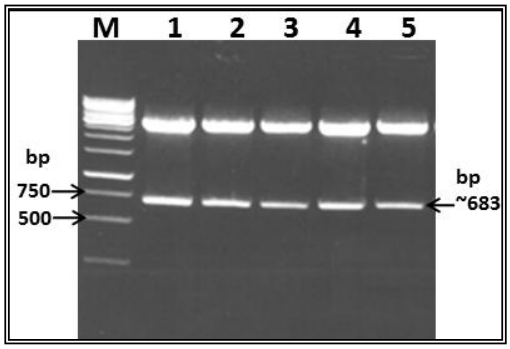

Fig. (3): Agarose gel electrophoresis of restriction digestion analysis of selected white colonies using *Eco*R1 enzyme. M: 1 Kb marker. Lanes 1-5: selected white colonies

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 1  ATGGACAAATCTGAATCAACCAGTGCTGGTCGTAACCGTCGACGTCGTCCGCGTCGTGGT
    M  D  K  S  E  S  T  S  A  G  R  N  R  R  R  R  P  R  R  G
61  TCCCGCTCCGCCCCTTCCTCTGCGGATGCTAACTTTAGAGTCTTGTCGCAGCAGCTTTCG
    S  R  S  A  P  S  S  A  D  A  N  F  R  V  L  S  Q  Q  L  S
121 CGACTTAATAAGACGTTAGCAGCTGGTCGTCCAACTATTAACCACCCAACCTTTGTAGGG
    R  L  N  K  T  L  A  A  G  R  P  T  I  N  H  P  T  F  V  G
181 AGTGAACGCTGTAGACCTGGGTACACGTTCACATCTATTACCCTAAAGCCACCAAAAATA
    S  E  R  C  R  P  G  Y  T  F  T  S  I  T  L  K  P  P  K  I
241 GACCGTGGGTCTTATTACGGTAAAAGGTTGTTACTACCTGATTCAGTCACGGAATATGAT
    D  R  G  S  Y  Y  G  K  R  L  L  L  P  D  S  V  T  E  Y  D
301 AAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCCGAAATTTGATTCTACC
    K  K  L  V  S  R  I  Q  I  R  V  N  P  L  P  K  F  D  S  T
361 GTGTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACTTATCCGTTGCCGCCATCTCT
    V  W  V  T  V  R  K  V  P  A  S  S  D  L  S  V  A  A  I  S
421 GCTATGTTCGCGGACGGAGCCTCACCGGTACTGGTTTATCAGTATGCCGCATCTGGAGTC
    A  M  F  A  D  G  A  S  P  V  L  V  Y  Q  Y  A  A  S  G  V
481 CAAGCCAACAACAAACTGTTGTATGATCTTTCGGCGATGCGCGCTGATATAGGTGACATG
    Q  A  N  N  K  L  L  Y  D  L  S  A  M  R  A  D  I  G  D  M
541 AGAAAGTACGCCGTCCTCGTGTATTCAAAAGACGATGCGCTCGAGACGGACGAGCTAGTA
    R  K  Y  A  V  L  V  Y  S  K  D  D  A  L  E  T  D  E  L  V
601 CTTCATGTTGACATCGAGCACCAACGCATTCCCACGTCTGGAGTGCTCCCAGTCTGA
    L  H  V  D  I  E  H  Q  R  I  P  T  S  G  V  L  P  V  stop
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Fig. (4): Nucleotide sequence of Egy-CMV CP gene. Total number of nucleotide bases is 657 and the amino acids residues is 219.

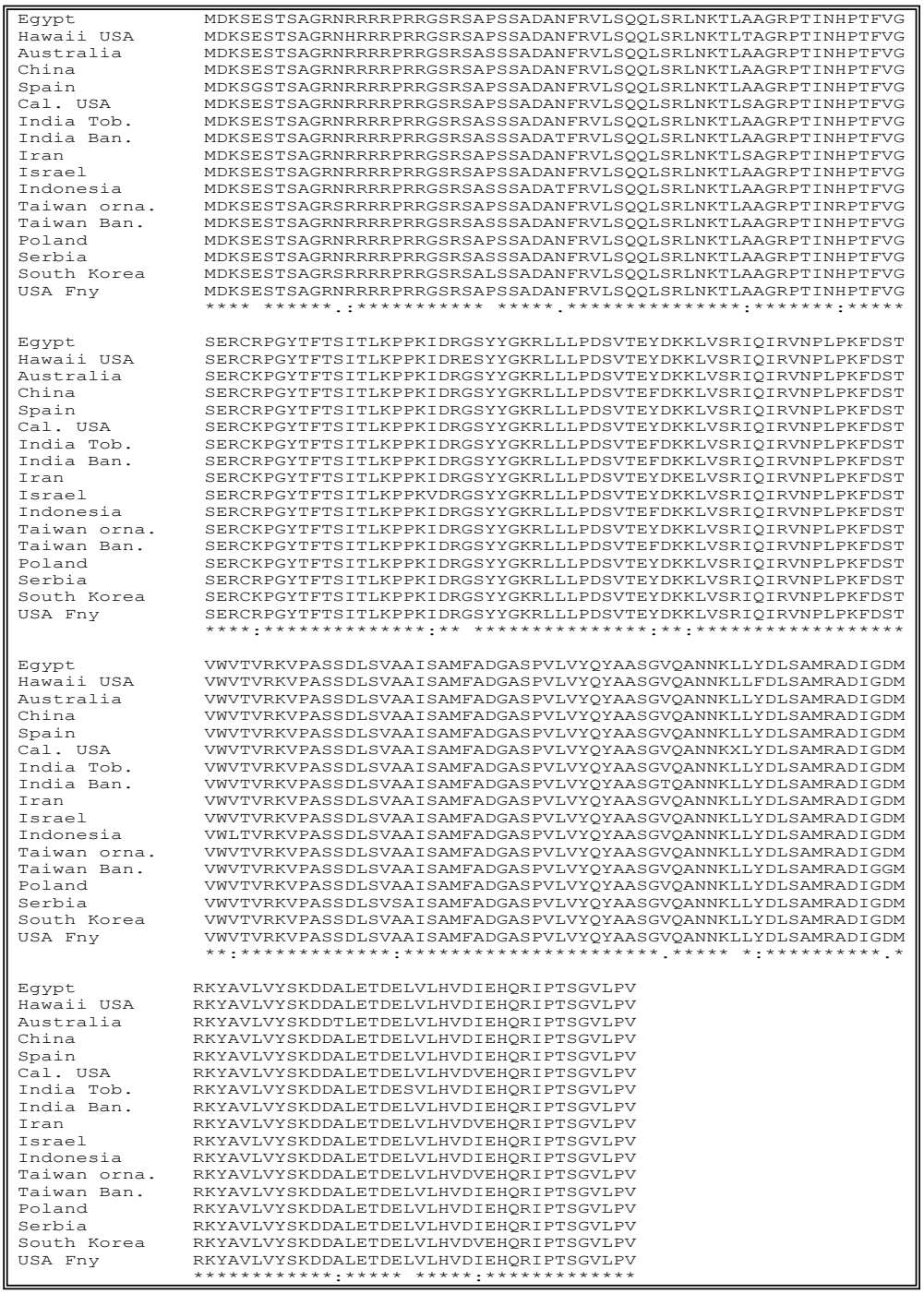

Fig. (5): Multiple sequence alignment of deduced amino acid of CMV-CP gene from different strains including Egyptian isolate (Egy-CMV).

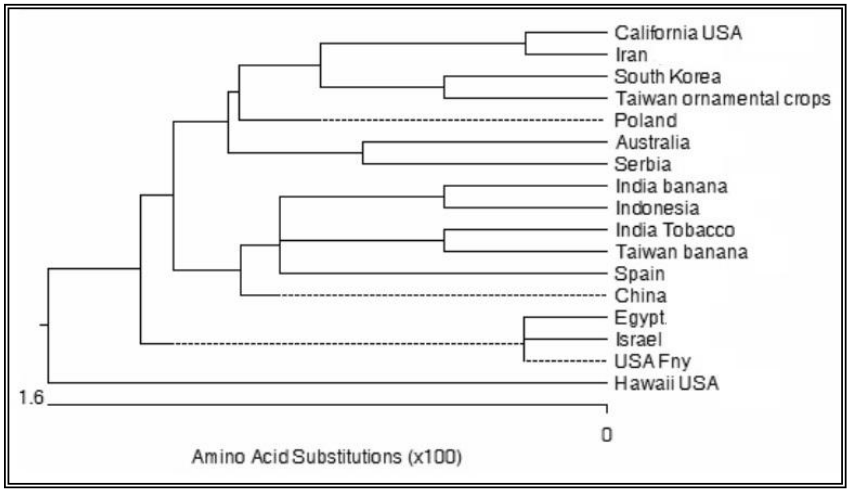

Fig. (6): Phylogenetic tree constructed from the alignment of amino acid sequences of coat protein gene of 17 CMV strains including Egy-CMV using Neighborhood Joining Bootstrap Method.