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Cloning, Expression and Protein Purification of AV1 Gene of *Okra Leaf Curl Virus* Egyptian Isolate and Genetic Diversity between Whitefly and Different Plant Hosts

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Abstract*—Begomoviruses*** are economically important plant viruses that infect dicotyledonous plants and exclusively transmitted by the whitefly *Bemisia tabaci*. Here, replicative form was isolated from Okra, Cotton, Tomato plants and whitefly infected with *Begomoviruses*. Using coat protein specific primers (AV1), the viral infection was verified with amplicon at 450 bp. The sequence of OLCuV-AV1 gene was recorded and received an accession number (FJ441605) from Genebank. The phylogenetic tree of OLCuV was closely related to *Okra leaf curl virus* previously isolated from Cameroon and USA with nucleotide sequence identity of 92%. The protein purification was carried out using His-Tag methodology by using Affinity Chromatography. The purified protein was separated on SDS-PAGE analysis and an enriched expected size of band at 30 kDa was observed. Furthermore, RAPD and SDS-PAGE were used to detect genetic variability between different hosts of *okra leaf curl virus* (OLCuV), *cotton leaf curl virus* (CLCuV), *tomato yellow leaf curl virus* (TYLCuV) and the whitefly vector. Finally, the present study would help to understand the relationship between the whitefly and different economical crops in Egypt.

Keywords—*Begomovirus*, AV1 gene, sequence, cloning, whitefly, okra, cotton, tomato, RAPD, phylogenetic tree and SDS-PAGE.

I. Introduction

GEMINIVIRIDAE is a large family of plant viruses, including some members as *Geminiviruses.* Genomes is circular single-stranded DNA genomes encapsidated in twin particles. Based on their genome arrangement, insect vector and host range. *Geminiviruses* are classified into seven genera: *Becurtovirus* (2 Species), *Begomovirus* (192 Species), *Curtovirus* (3 Species), Eragrovirus (1 Species), *Mastrevirus* (29 Species), *Topocuvirus* (1 Species), and *Turncurtovirus* (1 Species) [1], [2].

Begomoviruses which contains more than 180 species, so this genus had been a large number of important diseases [3] *Begomoviruses* are transmitted by whitefly *Bemisia tabaci* predominantly in the tropical and subtropical regions of the world and cause severe disease in dicot plants including tomato, pepper, cassava, beans, cotton and cucurbits [4], [5].

Begomoviruses can be subdivided into two major groups: bipartite viruses, those with a genome composed of two ~2.6 kb DNA components (referred to as DNA-A and DNA-B), which are prevalent in the New World and monopartite viruses, those with a genome composed of a single ~2.9 kb genomic DNA, which are prevalent in the old world many monopartite *begomoviruses* are associated with satellite DNAs (alpha satellites and beta satellites), some of which enhance pathogenicity [6]-[8]. The DNA-A component codes the coat protein "CP" (AV1), replication-associated protein "Rep" (AC1), transactivation protein "TrAP" (AC2) and replication enhancer protein "REn" (AC3), and has an intergenic region between CP and Rep genes described by [9]. The Coat protein (CP; V1), required for encapsidation, insect transmission and movement in plants [10], [11]. At some point during RCR, CP interferes with nicking of DNA thus limiting the viral DNA copy number [12], [13].

Okra (*Abelmoschuse sculentus*, family *Malvaceae*) is a widely grown vegetable crop in West Africa, where it originated. In West Africa, *Okra leaf curl* disease (OLCD) has become increasingly important. OLCD symptoms include stunted growth, leaf curling, distortion, mosaic, mottling and yellowing. A new *begomovirus* species like *okra yellow crinkle virus;* (OYCrV) displays a range of symptoms depending on the severity of the infection typically thickening and yellowing of small veins on the lower surface of young leaves [14].

First viruses were recorded and isolated from species in the family *Malvaceae* and other dicotyledon families. Capsid protein (CP) open reading frame was analysed and grouped into three geographical clusters, corresponding to isolates collected in Punjab, Sindh, or both provinces. Random amplified polymorphic DNA analysis of the *B. tabaci* population showed that the intra population diversity was high at both the local and regional scales studied by [15].

This study aims to characterize *begomovirus* in Egypt. Define the genetic diversity and the phylogenetic relationship between *Begomovirus* infecting some economical crops with whitefly vector on DNA and protein levels. The set of isolates considered here has not been jointly analyzed before, nor has the role of the host plant and insect vector in their evolution been investigated.

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II. Materials and Methods

A. Virus Sources and Associated Symptoms

Begomovirus and the premature instars of the Whitefly (Bemisia tabaci) were collected from infected okra, cotton and tomato plants. Vein thickening, yellowing and leaf curling visually identified infected leaves. Leaves with stunting symptoms were washed by sterilized distilled water and finally preserved in plastic bags and storage in-20°C until used.

B. Detection of the Coat Protein Gene (AV1).

Total DNAs (Replicative Form; RF) was extracted from collected symptomatic okra, cotton, tomato leaves and from whiteflies which infested these plants as well [16]. Polymerase Chain Reaction (PCR) was performed for the detection of OLCuV in tissues and Whitefly using coat protein gene specific primers AV1-sense 5'TAGAGCTCAATTCGTTACA GAGTC3' and AV1-antisense 5'GTCGCAGGATTATTCAC CG 3' [17].

C. DNA Sequencing and Phylogenetic Tree of the OLCuV-AV1

DNA sequence of the coat protein gene (AV1 gene) was obtained by Macrogene Company (Korea). The obtained DNA nucleotide sequence was analyzed using NCBI-BLAST [18] to confirm the identity of the sequence. The CP gene sequence for OLCuV was aligned in comparison to the other *Begomoviruses* available in the GenBank database. Sequences were used for comparison using MEGA 4 [19], and the phylogeny was tested with bootstrap method. The phylogenetic tree was analyzed and generated based on UPGMA statistic method.

D. Cloning, Expression, Protein Purification of OLCuV-AV1 Partial Coat Protein Gene and SDS-PAGE

This is done by using the procedure described by [20].

E. Random Amplified Polymorphism DNA-PCR and the Phylogenetic Relationships

PCR analysis was performed using the extracted viral DNA (Replicative Form) from infected Okra, cotton, tomato plants and a whitefly sample. Five random primers (Table I) were used to compare the genetic diversity between samples. PCR reaction components were performed in 25µL containing 1µL of DNA (30ng); 5µL10x buffer; 2µL of dNTPs (10mM of each); 2.5µL $MgCl_2$ (25mM) 5µL primer (10pmol); 0.2µL of 5U Taq DNA Polymerase (Promega, USA) and 9.3µL of sterile water. The applied PCR program was as follows: initial denaturation at 95°C for 5 min.; 40 cycles at 95°C for 1 min.; annealing at 30-34°C for 1 min and extension at 72°C for 1min. A final extension step at 72°C for 10 min. PCR products were separated on agarose gel electrophoresis using 2% (w/v) agarose in 0.5x TBE buffer. The size of each band was estimated by using DNA molecular weight marker. Finally, the gel was photographed by using gel documentation system. Bands of viral DNA (RF) pattern were scored manually for all the samples studied as 0.0 and 1.0. A Similarity dendrogram for each sample was produced using software program Jaccard 'coefficient (Jc).

TABLE I
SEQUENCE OF RAPD PRIMERS, NUMBERS AND FRAGMENT, NUMBER OF MONOMORPHIC BANDS NUMBER OF POLYMORPHIC FRAGMENTS, POLYMORPHISM PERCENTAGE; POLYMORPHIC INFORMATION CONTENT OBTAINED FROM WHITEFLY, OKRA, COTTON AND TOMATO USING 5 RANDOM PRIMERS

No.	Primers	Sequences 5`-----3`	Total number of bands	Number of monomorphic bands	Number of polymorphic bands	% polymorphic
1	RAPD3	ACCGCCGAAG	26	1	25	96.2
2	RAPD5	AAAGCTGCGG	18	0	18	100
3	RAPD6	AGCCACCGAA	23	3	20	86.9
4	RAPD7	ACCTGAACGG	14	6	8	57.1
5	RAPD8	TGCCGAGCTG	22	0	22	100
Total	--------	--------	103	10	93	---

F. Viral Protein and SDS-PAGE

Total soluble protein was extracted from plant tissues with *leaf curl virus* and from whitefly as well [21].

III. Results and Discussion

Okra, cotton and tomato plants infected with *begomoviruses* were collected from natural fields in three different Egyptian governorates (Bahera, Dakahlia and Menoufia). Symptoms observed were vein darkening, severe vein thickening and downward leaf curling on the top third part of the leaf, Figs. 1 (A) and (B). Our data agree with those of [22]-[24] they reported that the typical symptoms of *begomoviruse* including of leaf curling, mosaic, vein yellowing and more generalized leaf yellowing, often accompanied by stunting of plant growth.

A. DNA Extraction of the Virus Replicative form (RF) in Different Plant Host and Whitefly

The viral DNA (replicative form) was extracted from the infected okra, cotton and tomato leaves and whitefly. As showed in Fig. 1 (C), the DNA yield and density were higher in the insect than in other plants (okra, cotton and tomato) supporting the hypothesis that *begomovirus* was firstly propagated in the insect then transmitted to the host plant. Similar results have been reported by [25], on the basis of the genome structure of *geminiviruses* transmitted by whitefly, it was assumed that a bipartite genome would also be found for TYLCV. However, the viral RF for all the clones was obtained from one DNA A-like component. In addition, [26] reported that the direct role of V2 protein plays in regulating a switch from RF-RF synthesis to RF-SS synthesis.

B. Amplification of the Coat Protein Gene (AV1) and Phylogenetic Tree

In the current study, PCR amplification was carried out on DNAs isolated from the infected okra, and whitefly using specific primers of the Coat Protein (AV1) gene. As expected, the size of the (AV1) Part of DNA-A amplicon was about ~450 bp Fig. 1 (D). Similarly, [27] reported that the predicted length of TYLCV-DNA gene that was amplified from tomatoes infected with viruliferous whiteflies as well as from the insect progeny (eggs, crawlers, and adults) was about 410 bp. In addition, our data agreed with previous studies conducted by [28] confirming positive results of *begomovirus* prepared from infected *Lantana camara* plants and the PCR product of the coat protein gene fragment was about ~550 bp [29] found that DNA-A amplified from all samples infected by *begomoviruses* was 580 bp. Finally, the molecular size of the viral coat protein genes of *Tomato Yellow Leaf Curl Virus* (TYLCV) and (TYLCSV) were 462 and 135 bp respectively, reported by [30].

The partial sequence of AV1 gene for *Okra Leaf Curl virus-DA1* isolate was aligned and compared with the other *begomoviruses* available in the GenBank databases that showed considerable homology. The sequence of AV1 gene for *Okra Leaf Curl virus-DA1* was recorded and received accession number (**FJ441605**).

Phylogenetic analysis was performed based on multiple alignments of coat protein (AV1) sequences available on Genebank. *Okra Leaf Curl virus-DA1* (**FJ441605**) is closely related to the *okra leaf curl virus* from Cameroon and USA (**NC013017 and EU024119**, respectively) with nucleotide sequence identity of 92% Fig. 1 (E). Previous studies showed that the complete or the partial DNA- A sequences of nine Pakistani *geminivirus* isolates from cotton (CLCuV-PK) or from okra (OLCuV-PK) were compared with different types of virus isolates. The resulted sequences revealed that isolates from leaf curl-affected okra had virtually the same sequences as those from cotton [31]. Moreover, the phylogenetic tree of the amino acid sequence of the putative (Coat Protein) CP compared with some other mono and bipartite *geminiviruses* revealed a highest identity of 97.3% with Pakistan *cotton leaf curl virus* (CLCuV-62) [32], [33] reported that the nucleotide sequence similarity ranged from 90% to 98.7% in (CLCuV), 55.2–55.5% with *Bhendi yellow vein mosaic virus*, 55.8% with *Okra leaf curl virus* and 51.70% with *Tomato leaf curl virus* isolates. Meanwhile, the lowest similarity was 47.8% with CLCuV Sudan isolate. In addition, the nucleotides sequences of the viral genomes from some crops such as okra, cotton, tomato and pepper were aligned with two *begomovirus* species (*Cotton leaf curl Gezira virus* and *Okra yellow crinkle virus*) as well as with new recombinants of *begomovirus* species such as *Okra leaf curl Cameroon virus*, *Cotton leaf curl Gezira betasatellite* and a new *alphasatellites*. Tomato and pepper leaf curl isolates were shown to be a new type of *begomoviruses*, referred to as West African tomato-infecting new alpha satellites and beta satellites *begomoviruses* [23].

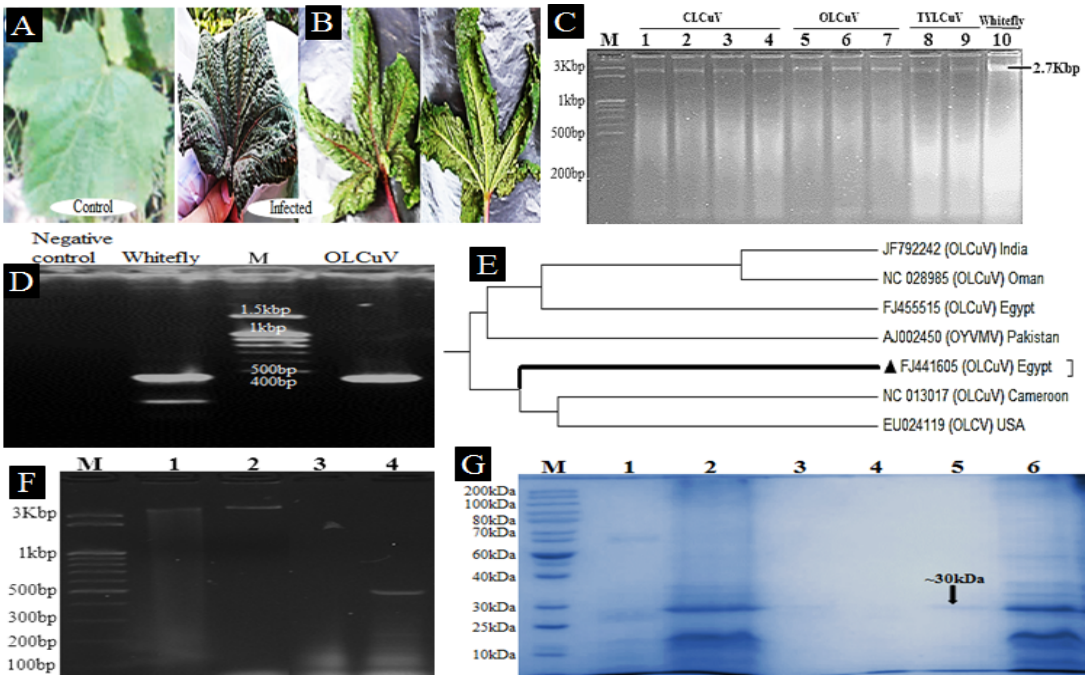

Fig. 1 (A) Healthy okra leaves; (B) Symptoms on okra leaves infected with *Leaf curl virus*; (C) DNA replicative form about 2.7 Kbp isolated from different hosts (cotton; okra; tomato) and the whitefly; (D) PCR amplification of AV1 gene, negative control; whitefly; M, 1.5kbpDNA marker; OLCuV; respectively on 2% agarose gel electrophoresis; (E) Dendrogram illustrating the phylogenetic relationships of AV1gene for OLCuV based on the DNA nucleotide sequences and compared to the other genes of *Begomoviruses* listed in the GenBank; (F) 2% agarose gel observed the recombinant plasmid: M, 3 Kbp DNA marker; Lane 1, pCR 2.1-TOPO plasmid (3.9kbp); Lane 2, recombinant plasmid (OLCuV-AV1 gene) with approximately 4.350kbp; Lane 5, confirmation by PCR using AV1 primers with empty pCR 2.1-TOPO plasmid; 6,

confirmation by PCR with recombinant OLCuV-AV1 gene; (G) SDS-PAGE analysis showing the purified protein of OLCuV-AV1 (~30KDa) as a result of the recombinant OLCuV-AV1 gene expression. Where M, 200kDa protein marker; Lane 1, empty pCR 2.1-TOPO plasmid; Lane 2, recombinant plasmid (OLCuV-AV1 gene); 3-5, eluted protein fraction approximately at 30kDa, Lane 6, crud protein

C. Expression of AV1 (CP) Gene and Protein Purification

In order to induce the partial coat protein of the OLCuV. The OLCuV-AV1 gene was inserted in the pCR2.1-TOPO plasmid and the recombinant clones were confirmed through restriction digestion analysis Fig. 1 (F). The positive fragments were purified and cloned into the expression vector (pPROEX-HTa) then transformed into *E. coli* cells. The expressed protein was purified and a band of ~30kDa was detected on SDS-PAGE Fig. 1 (G). In the *Sweet potato leaf curl virus* (SLCV) the partial coat protein (CP) sequence was cloned into the pJET/blunt cloning vector. The BgIII restriction enzyme digestion of the recombinant plasmid showed a single band at the expected size of the insert band 550 bp as well as another band at the expected size of the digested vector ~ 3000 bp [34]. Furthermore, the SPLCV-CP gene isolated from *sweet potato leaf curl virus* was cloned into the expression vector pMAL-c2E as a fusion protein with maltose-binding protein, and transformed into *E. coli* strain XL1-Blue. After gene induction, a fusion protein of 72 kDa was purified by amylose affinity chromatography. Digested with enterokinase that cleaved the fusion protein into a 42.5 kDa maltose-binding protein and a 29.4 kDa protein [35].

D. Genetic Diversity between the Whitefly Vector and Different Host Plants

The Random Amplified Polymorphism DNA (RAPD) was used to identify the different DNA profiles between *okra leaf curl virus, cotton leaf curl virus*, *tomato yellow leaf curl virus* and the whitefly vector. Here, three isolates of cotton, and three isolates of okra from (Bahera, Mansoura and Menofia governorates) as well as two isolates of tomato from (Bahera and Alexandria governorates) and one whitefly sample were analyzed using five random primers. Results reveal that the RAPDs analyses showed different levels of polymorphism between the genetic profiles of Whitefly, CLCuV, OLCuV and TYLCuV as shown in Fig. 2 All the isolates gave from 9-13 bands patterns ranged from 1500 bp to less than 100 bp. The primer named as RAPD7 produced three monomorphic bands of 220 bp, 200 bp and 100 bp between whitefly and all CLCuV, OLCuV and TYLCuV isolates. Similarly, [36] used RAPD-PCR technique with three primers to differentiate between 17 samples of *B. tabaci* that were collected from five locations and different hosts in Jordan.Furthermore, the genetic structure of *begomoviruses* and the whitefly vector from cotton-growing areas in Pakistan, including the provinces of the Punjab, and Sindh where CLCuD is rampant, has been reported in the northern part of the province [37]-[39]. The extent of genetic diversity and the evolution of recombination role were analyzed in the CLCuV like *begomoviruses* [31], [39], [40].

E. Phylogenetic Relationship Based on RAPD-PCR Analysis

Genetic variability occurred due to the host plants in whitefly (*B. tabaci*) populations that were collected from fields of different crops. The whitefly types hold specificity for different host plants under study that have evolved as three distinct genetic groups; the first one includes cotton, sida and soyabean, the second group had potato and bringal and the last one includes tomato [41]. In the present study, genetic similarity between all the previously mentioned samples were estimated from the data scored in the RAPD PCR reaction using the Jaccard coefficient (Jc). This coefficient is based on the presence or the absence of unique and shared fragment produced by RAPD to compute the similarity matrices. These similarity matrices were used to generate a dendograme using the UPGMA method.

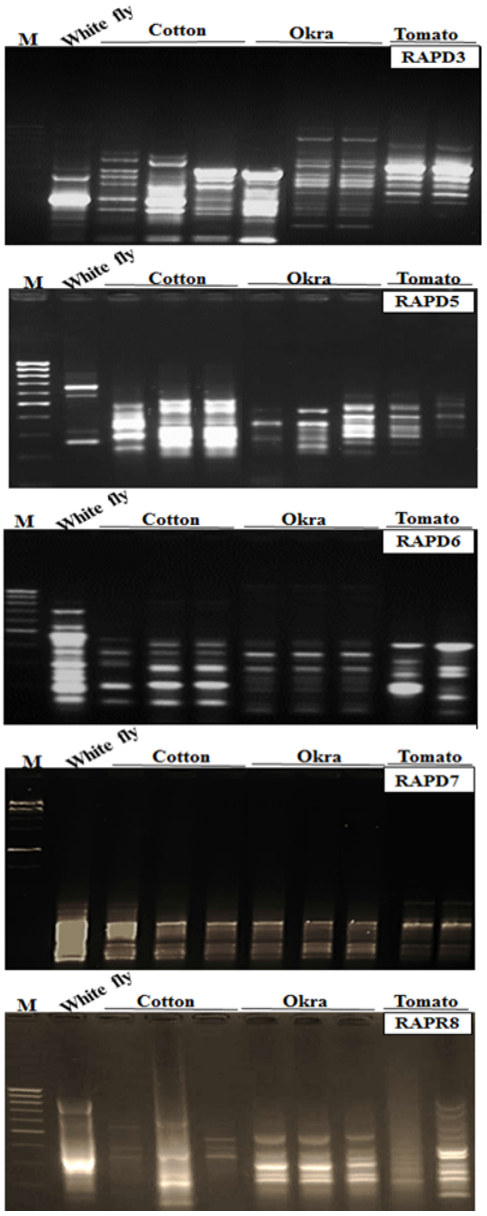

Fig. 2 A representative RAPD-PCR reaction using five different arbitrary primer's for the differentiation of whitefly (Insect), cotton,

okra and tomato (Plants). M: 100bp DNA ladder; Lane1, whitefly; Lane 2-4, CLCuV; Lane 5-7, OLCuV; Lane 8-9, TYLCuV, respectively

Our results reveal that, the highest similarity was observed within the isolates of (OLCuV) from Mansoura and Menofia governorates, with a score of (87%). Meanwhile, the lowest similarity was between the whitefly (vector) and (OLCuV) from Menofia governorate, with a score of (42.8%) Fig. 3. A dendrogram was constructed using banding patterns obtained from the 5 RAPD-PCR primers, Two major groups (groupA and group B) were generated. The major group A comprises only one isolate of whitefly vector. Group B consists of cluster A1 includes two subcluster (AI and AII). The sub-cluster AI includes CLCuV isolates from Bahira, Mansoura and Menofia, while subcluster AII contains isolates of TYLCuV from Bahira and Alexandria Moreover, cluster A2 contain OLCuV isolates from Bahira, Mansoura and Menofia.

F. Detection of Viral Proteins Using SDS-PAGE

Proteins have multiple function during the virus life such as viral replication; host gene regulation and silencing suppression; virus assembly; and vector transmission [42], [43]. In order to investigate the relationship between the *leaf curl virus* and the whitefly (*Bemisia tabaci*), total protein isolation was performed using the (SDS-PAGE) analysis. Protein was extracted from different infected leaves and the insect vector. Results showed that, some samples had different patterns and the protein profiles obtained were C1 protein (Mw ~41 kDa), AV1 protein (Mw~30kDa), C3 protein (Mw ~15.9 kDa) and C4 protein (Mw ~10.5kDa) in the whitefly vector, CLCuV, OLCuV and TYLCuV, respectively Fig. 4. Previous studies reported that the monopartite *begomovirus*, encodes genes in both orientations. C1 protein is the (Rep) protein so it is involved in viral replication. AV1 protein is the Coat protein (CP), C3 protein is the (Ren) protein, it enhances the viral DNA accumulation, infectivity and symptom expression .C4 protein is involved in the development of disease symptoms during viral infection [44]-[46].

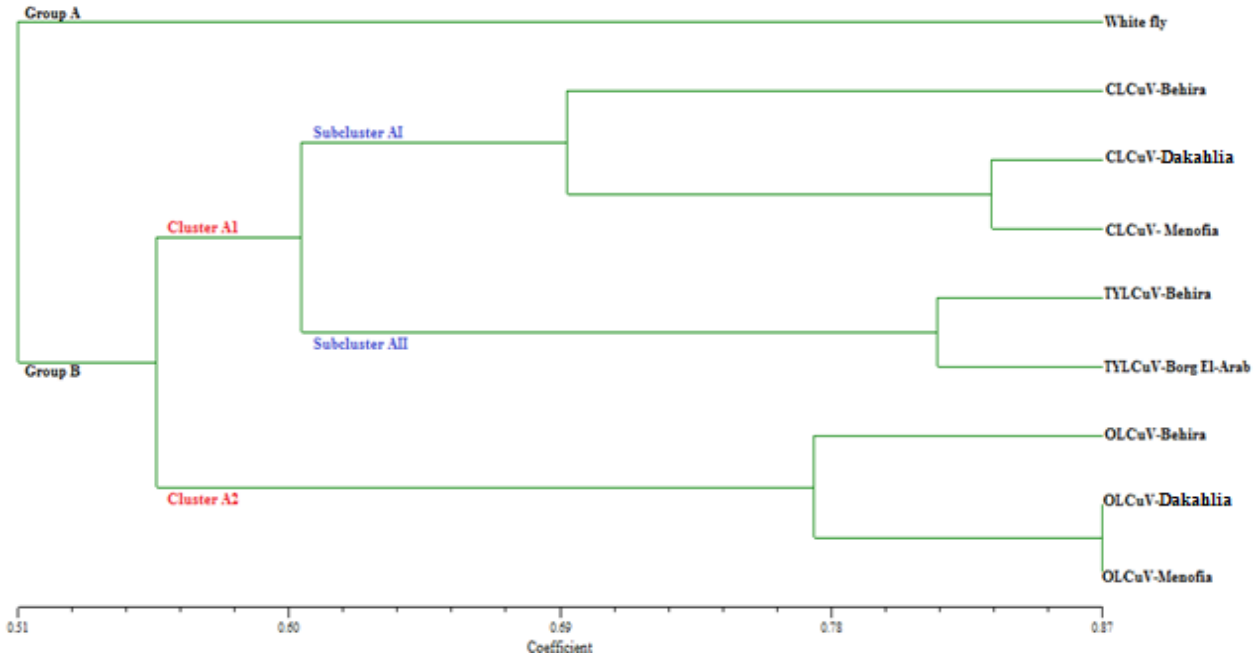

Fig. 3 The Phylogenetic tree illustrating RAPD-PCR for all isolates of whitefly and different hosts (cotton, okra, tomato) from different governorates (Bahira, Dakahlia, Menofia and Alexandria).

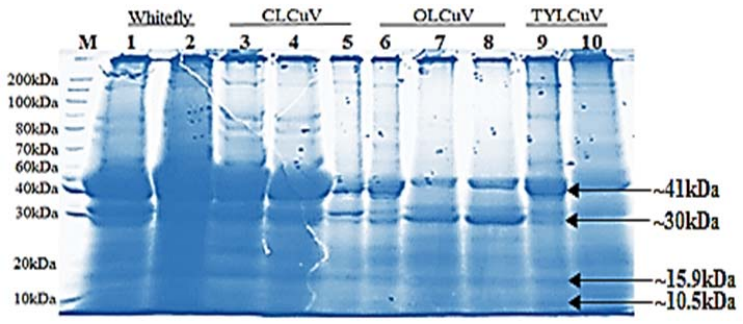

Fig. 4 12% SDS-PAGE analysis protein was isolated from different plants infected by *begomovirus* and the whitefly vector., where M: is protein marker Lane 1-2, whitefly; Lane 3-5, CLCuV; Lane 6-8, OLCuV; Lane 9-10, TYLCuV, respectively

IV. Conclusion

In this study, the *begomoviruses* that effect on different economic crops in Egypt (such as: Okra, Tomato and Cotton) through by feeding of whitefly on this crops. In addition, the study of genetic diversity using RAPD-PCR and SDS-PAGE techniques observed that the effect of whitefly on the viral structure and function according to the type of plant hosts.

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