British Microbiology Research Journal
9(3): 1-9, 2015, Article no.BMRJ.18939
ISSN: 2231-0886

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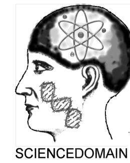

Molecular and Serological Studies for Detection and Identification of Cotton Leaf Curl Virus in Cotton Plant (*G. barbadense* L.) in Egypt

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2015/18939
Editor(s):
(1) Laleh Naraghi, Plant Disease Research Department, Iranian Research Institute of Plant Protection, Tehran, Iran.
Reviewers:
(1) Jeffrey Lim Seng Heng, Malaysia Agriculture Research and Development Institute, Malaysia.
(2) M. Angels Calvo Torras, Universdad Autonoma De Bacrelona, Spain.
Complete Peer review History: http://sciencedomain.org/review-history/9944

Original Research Article

Received 18th May 2015
Accepted 8th June 2015
Published 29th June 2015

ABSTRACT

Aims: Cotton production contributes in economic security in Egypt. Cotton leaf curl virus (CLCuV) belongs to the *Begomovirus g*enus and *Geminiviridae* family that is transmitted by the whitefly *Bemisia tabaci*. It is the greatest current threat where cotton is grown. In Egypt, a distinct *Begomovirus* causing leaf curl has been identified in okra, tomato, hollyhock and other *malvaceous* species suggesting that it could possibly infect Egyptian cotton. The aim of this study was the detection and isolation of this Virus from Egyptian cotton fields.
Methodology: Diseased leaves of cotton, okra and tomato plants were collected from different localities during 2009; samples were subjected to DAS-ELISA for viral detection. Coat protein (CP) gene of the virus was amplified by PCR, sequenced and aligned with the others *Begomovirus* available in the GenBank. Cloning and sub cloning of CP gene for cotton virus were performed and its recombinant protein was separated on SDS-PAGE. DBIA was also used to identify protein of the virus.
Results: The amplified fragment of the CP gene was about 280bp in all samples. The CP gene of CLCUV in our study shared 97% identity with CLCuV isolate from Pakistan while, CP gene

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amplicon of okra leaf curl virus (OLCUV) showed identity of 84% with three isolates of tomato leaf curl virus (TYLCV) from Chian. The recombinant protein of CLCUV was about 24kDa and positive relatedness was shown between this recombinant protein and TYLCuV antiserum.

Conclusion: our study showed the existence of such virus in *malvaceous* species as okra plant may be causes a serious problem for cotton production in Egypt. Also, the CP gene is a good tool for examination the existence of the virus in the infected fields. The purified protein of CLCUV can use as an antigen to produce its antibodies.

Keywords: CP gene; CLCUV; OLCUV; TLCUV; DAS- ELISA; DBIA.

1. INTRODUCTION

Malvaceae plants are widely cultivated in Egypt. It includes cotton, okra and several *Hibiscus sp*. The most important one is Egyptian cotton which is known all over the world's for its superiority & quality and plays a major role in economic income. Egypt produces 50% of the world's produce of long and extra long staple cotton [1].

Cotton leaf curl disease (CLCuD) is a serious viral disorder of several plant species in the family *Malvaceae,* the most Important of which is cotton (Genus: *Gossypium* L.). The disease occurs across Africa and southern Asia [2]. Plants affected by this disease exhibit very unusual symptom, consisting of vein swelling, upward or downward cupping of the leaves, and the formation of enations on the main Veins on the undersides of leaves [3]. CLCuV belongs to the *Begomovirus g*enus and *Geminiviridae* family. This family has small ssDNA genomes. Their genome is approximately 2.5–3.0 kb in size which is encapsidated in characteristic twinned particles. They are classified into four genera ((*Mastrevirus, Curtovirus, Topucovirus* and *Begomovirus*) depending on their genomes arrangement, insect mode of transmission and sequence relatedness [4,5].

Begomovirus is the largest genus among the *Geminivirdae* family. They infect dicotyledonous plants especially *Malvaceae* plants and are transmitted by the whitefly *Bemisia tabaci. Begomovirus* are bipartite with two molecules of circular single stranded DNA (A and B), *DNA-A* encodes proteins that are essential for encapsidation and replication, *DNA-B* encodes nuclear shuttle protein (NSP or BV1) and movement protein (BC1 or MP) required for systemic spread [6]. The viral DNA replicates via double stranded intermediate in the nuclei of infected plants [7] and NSP is essential for the transport of viral DNA across the nuclear envelope while MP is required for cell to cell movement through the plasmodesmata (PD) [8] However, the coat protein (CP) is shown to complement the function of NSP when disabled by mutation [9].

In Egypt, a distinct *Begomovirus* has been identified in the *Malvaceous* host, hollyhock and other species as a result of that the virus will be predominate and may be able to cause disease in cotton plant [10]. To date, few data have been collected on *Begomoviruses* affecting *Malvaceae* plants in Egypt, rarely on cotton, occasionally on okra and frequently on hollyhock (*Athea rosea*) [11]. On the other hand, typical *Geminivirus* associated symptoms of leaf enations and curling were observed on other species as tomato cultivars, hybrids and lines that were susceptible to the tomato yellow leaf curl virus (TYLCV). The disease was epidemic in fall season at Giza, Fayoum, Ismailia provinces, while it had low incidence at Nubaria (Beheria) and Sabaheia (Alexandria) districts [12]. Thus, Potential risk of the spread of *Begomoviruses* in natural conditions to some important plants needs to be further assessed. Therefore, the present study aimed to detect and identify the Leaf Curl Virus in Cotton fields in Egypt. To achieve this purpose serological study such as enzyme linked immunosorbent assay (ELISA) and dot blotting immuno-binding assay (DBIA) were performed as well as Polymerase chain reaction (PCR) in presence of specific primers was carried out to amplify the coat protein (CP) gene of the virus. Cloning and sub cloning were executed for CP gene of cotton virus. DNA sequencing for a fragment of CP gene of cotton and okra virus were also implemented.

2. MATERIALS AND METHODS

2.1 Plant Material

Okra plant had always been cultivated around the cotton fields in Egypt. For this reason, diseased young leaves of cotton and Okra plants showing the symptoms of leaf curl virus were collected during season 2009 from six localities (El-Beheira, El-Gharbia, El-Dakahlya, El-Monofeya, Banysuef and Alexandria) of cotton

fields. Moreover, infected leaves of tomato plants with TYLCuV was also collected and studied as a positive control while, healthy leaves of cotton plant was taken as a negative control in the present study. The excised leaf samples were washed with sterile water, dried then sealed in plastic bags and stored at -20ºC until usage.

2.2 Elisa Detection

The double antibody sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) was performed in presence of specific antiserum of TYLCV for detection and screening the CLCuV and OLCuV in the collected samples as described [13] using ELISA kit (SRA 19500/1000, Agdia, USA).

2.3 Viral Replicative form (RF) Extraction from Plant Tissues

The DNA replicative form of virus (RF) was extracted from one gram of the infected leaf tissues by the method of [14]. RF was visualized on 1% agarose gel and photographed using gel documentation system (Alpha-chem. Imager, USA).

2.4 Polymerase Chain Reaction (PCR)

A pair of degenerate oligonucleotide primers [15] were corresponding to (forward; 5`-GTCGCAGGATTATTCACCG-`3 and reverse; 5`-GTATGTAGATCAATATTCAGAAA-`3) was used to amplify the DNA fragments of CP gene of leaf curl virus for cotton, okra and tomato plants. PCR reaction mixture was carried out in a final volume of 25 µl, consisting of 1 µl DNA template, 5 µl of 5x buffer, 2.5 µl MgCl2, 3 µl dNTPs, 20 pmol primer, 1U Taq DNA polymerase and nuclease-free water. Amplification conditions consisted of denaturation at 94°C for 5 min and 34 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extention at 72°C for 1 min with a final extension at 72°C for 10 min. PCR products were analyzed on 1% agarose gel and photographed using gel documentation system (Alpha-chem Imager, USA).

2.5 Cloning of CP Gene

The amplified PCR products of CP gene of the virus was purified using Extraction Kit (Promega, USA) and cloned into the plasmid vector pCR 2.1-TOPO using the TA Cloning Kit (Invitrogen, USA) according to the manufacturer's instructions.

2.6 Protein Purification Using Affinity Chromatography and SDS –Page

The insert from the recombinant was sub cloned into the prokaryotic expression vector pPROEX-HTa using pPROEX-HTa Prokaryotic Expression System (Life Tecchnologies, USA). Bacterial protein was purified using affinity chromatography. The bound protein was eluted with Buffer (20MmTris –HCL, pH 8.5, 5 mM 2-mercaptoethanol, 100 KCL, 10% glycerol and 100 Mm imidazole). 15 µl of each sample (Bacterial protein) was separated through 12% SDS-PAGE gel as described [16]. The gel was stained in Coomassie brilliant blue R-250 (1%) and visualized for protein bands on a white light transilluminator. molecular weight of bands was determined by protein marker (Fermentas, USA).

2.7 Dot Blotting Immuno-Binding Assay (DBIA)

Dot blot technique was performed in presence of TYLCV antiserum [17] where, 2µl of the purified recombinant protein were spotted onto the PVP membrane at the center of the blot region (grid) and left to dry. Then, the membrane was incubated in10 cm blocking Buffer (5% BSA in TBS-T) for 30 minutes at room temperature. After that, the membrane was incubated with the primary antibody (1: 1000 in BSAL/TBS-T) for 30 min at room temperature and washed three times with TBS-T then, incubated with the secondary antibody (alkaline phosphatase–conjugated dilution in Blocking Buffer) for 30 min. following washing three times with TBS-T. Membrane was incubated with ECL reagent for 1 min and agitated until color was observed and kept it.

2.8 Sequence Analysis and Phylogenetic Construction

DNA sequencing was carried out for the amplified CP fragments of CLCuV and OLCuV using automated DNA sequencer ABI (Macrogene Company, Korea). The fragment sequences in the present study were submitted into NCBI Genbank under accession numbers EU515240 and EU515241 for cotton and okra plants, respectively and aligned with the other begomovirus sequences available in the GenBank database using clustal w (1.8) program (http://ww2.ebi.ac.uk/clustalw). Phylogenetic tree were also constructed using Mega 4 software program.

3. RESULTS AND DISCUSSION

A method integrating the serological assay as well as PCR amplification offers the most efficient system for rapid detection of virus [18]. In the current study, Leaf samples showing different gradients of typical curling symptom of *Begomovirus* like upward or downward leaf , leaf curling, thick dark green veins and vein swelling were collected (Fig. 1). The described symptom of this disease in the infected samples in the present investigation was typical to the symptoms induced by Cotton leaf curl Gezira virus which were observed in cotton in Sudan [19]. Moreover, the last symptoms which were detected in cotton in Sudan were also similar to those described in cotton and okra in Pakistan during an epidemic in 1990 [20]. The presence of virus in diseased plant samples was firstly checked by DAS-ELISA assay in the present study where, Polyclonal antiserum of Tomato yellow leaf curl virus (TYLCV) succeeded to detect the presence of leaf curl virus in diseased tissue of cotton and okra plants. Positive results were observed in both infected tissues since, virus concentration was 0.38 OD and 0.49 OD in cotton and okra plants respectively, compared with 0.65 OD in a positive control (Fig. 2). polyclonal antisera of the Tomato leaf curl Bangalore virus (ToLCBV) that were produced in rabbit by injecting native virus and recombinant coat protein of ToLCBV successfully detected *Begomovirus* infections in tomato plant fields using DAC–ELSIA [21] .

Gemini viral genome is more suitable for PCR amplification because it replicates via a double-stranded circular DNA intermediate (replicate form), which can serve as a template for DNA amplification. In recent past, PCR-based rapid detection methods have been developed for several viruses [22]. *Geminiviruses* are a family of plant viruses which is characterized by possessing small circular single stranded DNA genome of about 2.6 to 2.8 kb [23]. [24] found that the *Geminiviridae* family contains one or two circular ssDNA molecules of size 2.5 to 3 kb as their genome. The previous investigations confirmed our results which showed the viral DNA replicative form (RF) of leaf curl virus was approximately 2.8 kb in size in each of infected cotton, okra and tomato plants.

In the present study, the replicative form of viral DNA was subjected to PCR in presence of specific primer pairs to amplify coat protein (CP) gene of the virus. The results indicated that the CP gene band was detected at approximately 280 bp in all infected isolates Fig. (3). Our results agreed with [25] who used the same primer to amplify a fragment of CP gene for six isolates of CLCuV from infected cotton and an isolate of OLCuV from okra plant in Pakistan [26]. Used degenerate primers for amplification of CP gene to confirm the presence of *Begomoviruses* in the leaves of diseased bean plants in green house.

Fig. 1. A, B and C (on the left) represent healthy leaf of cotton, okra and tomato plants, respectively. D, E and F (on the right) represent naturally infected leaf of cotton, okra and tomato plants with leaf curl virus respectively showing up ward or down ward leaf curling, thick dark, green veins and vein swelling

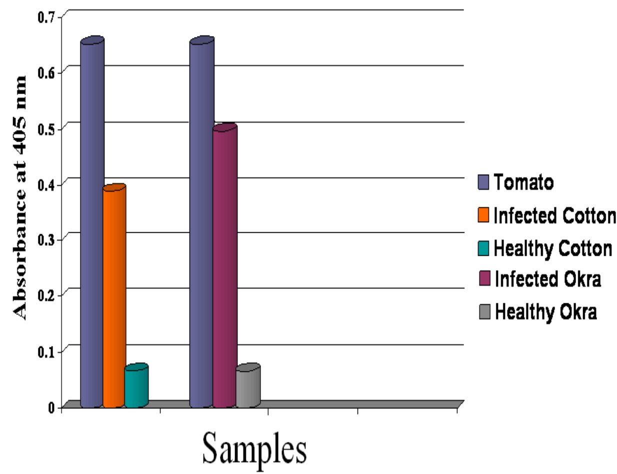

Fig. 2. Detection of leaf curl virus in infected cotton and okra plants using 1/1000 of TYLCV polyclonal antiserum

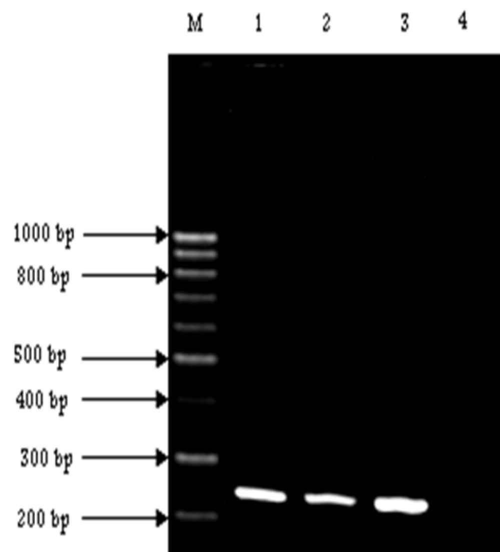

Fig. 3. 2% Agarose gel electrophoresis of PCR product of CP gene. M, 1000bp DNA marker; Lane 1→ CP gene in Cotton, Lane 2→ CP gene in Okra, Lane 3→ CP gene in Tomato (positive control) and Lane 4→healthy cotton plant (negative control), respectively

Coat protein (CP) gene of cotton leaf curl virus (CLCUV) was cloned into the plasmid vector (pCR2.1-TOPO). The universal primer M13 was used in the current study to confirm the presence of the insert fragment (CP gene) inside the vector. Subcloning was performed for the recombinant plasmid (pCR2.1-TOPO-CP gene) using expression vector (pPROEX.HTa). Protein of CP gene was induced in *E. coli* with isopropyl-β-D-thiogalactopyranoside (IPTG) synthetic inducer. After purification of the deduced mature protein, a unique protein with molecular weight 24kDa was shown through SDS-PAGE as in Fig. (4). [27] reported that the Clone with the amplified product specific to coat protein gene of Pepper leaf curl virus (PepLCV) through expression vector (pET161) showed presence of unique protein mobility with molecular weight 28 kDa.

Dot blot immunobinding assay (DBIA) is efficient for detection of *Geminiviruses* as well as other viruses in Egypt [28]. In the present investigation, Dot blot hybridization technique succeeded to

detect protein of CLCUV from the recombinant expression vector (pPROEX.HTa). Positive reaction (purple color) was appeared as a result of the hybridization between the extracted protein of the virus and the ECL reagent specific that refers to the presence of the virus in the diseased leaves of cotton plant as shown in Fig. (5). Our results confirmed by the studies of [29,30] found that Dot blot hybridization technique is useful to detect SqLCV DNA in blotted squash leaves that were collected from qaluobiya Governorate in Egypt and detect OLCV in the field of okra cultivars and green house plants in Saudi Arabia.

The DNA sequence of a target gene is one of the most promising methods for detection and identification of strains. Comparative studies of the nucleotide sequences of genes provide a powerful mean for studying phylogentic relationships over a wide range of taxonomic levels [31]. Comparative nucleotide sequence alignment in our study showed that the CP sequence of cotton leaf curl virus (CLCUV) which was under accession number EU51540 in Egypt shared 97% sequence identity with AJ002454 which represents CLCuV from cotton in Pakistan. The high percentage nucleotide (nt) sequence identities that was observed between cotton virus in Egypt and cotton virus from Pakistan suggesting that Egypt isolate may be a recent introduction from Pakistan. Data sequencing for the CP gene amplicon of okra leaf curl virus (EU515241) in the present study showed high nucleotide sequence identity of 84% with TYLCV isolates from tomato in China under accession numbers AJ784152, FN392884 and AM698121 in GenBank. The results of phylogenetic tree in the present investigation reflected that there were clearly distinct variations in nucleotide sequences between cotton and okra isolates as each isolate was aligned separately with the coding sequences of other isolates published in the NCBI GenBank and placed in a separated sub cluster as shown in Fig. (6). It should be emphasized that partial sequences are not enough to distinguish among isolates. A comprehensive characterization on host range, virus transmission and complete genome sequence needs to be conducted in order to classify virus isolates [32].

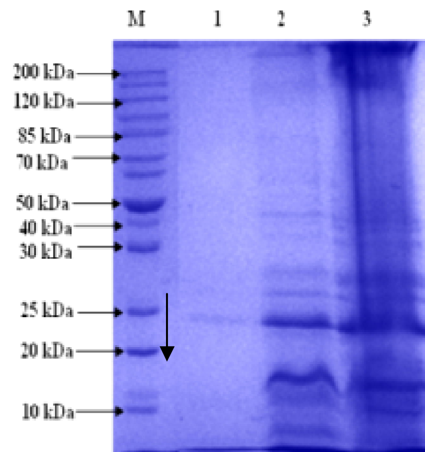

Fig. 4. 12% SDS-PAGE electrophoresis of protein purification of CP gene cotton leaf curl virus where, M→ 200 kDa protein marker; Lane 1→ eluted protein fraction approximately at 24 kDa, Lane 2→ unbound protein fraction and Lane 3→crude protein, respectively

Fig. 5. DBIA for detection of cotton leaf curl virus in infected cotton plant where, Lane 1 represents crude protein; Lane 2 represents purified protein

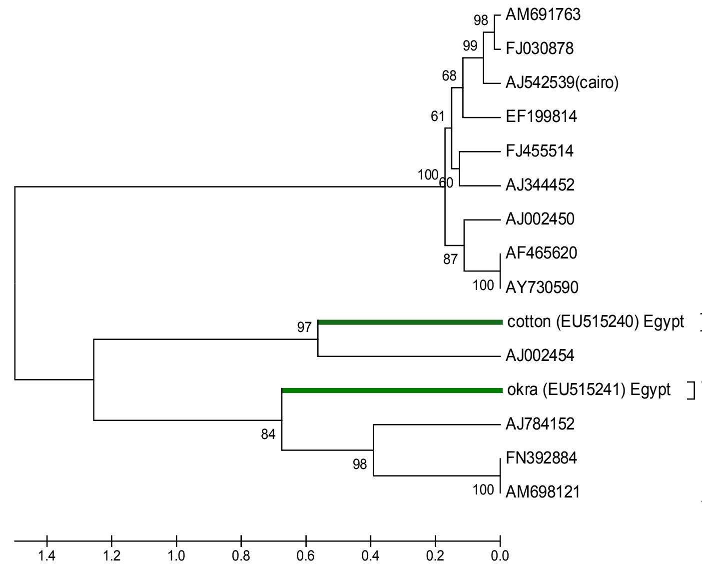

Fig. 6. Phylogenetic tree for CP gene of cotton and okra leaf curl viruses based on the DNA nucleotide sequence compared with the other coat protein (CP) genes of the *Begomoviruse* listed into GenBank

4. CONCLUSION

The results of our study indicated to the existence of such virus in *Malvaceous* species as cotton, okra plants and the others such as tomato plants. The spread of these *Begomoviruses* in natural conditions may cause a serious problem for the cotton production in Egypt. The CP gene of cotton leaf curl virus in our study was successfully cloned; expressed in *E.coli* and the protein of the CP gene was produced .This result is useful because the purified protein can be used as an antigen to produce antibodies to CLCuV and the availability of antiserum to CLCuV will allow the development of a practical serological method for CLCuV detection. Also, it can conclude that the coat protein gene is considered as a good tool for viral detection and classification.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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