# Molecular and serological studies on a plant virus affecting strawberry

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## ABSTRACT

*Viruses form a major threat to the strawberry industry in Egypt causing severe economic losses. Rapid and simple methods for the detection of the major strawberry viruses are absent due to the lack of sensitive diagnostic tools. Plants showing virus-like symptoms (VLS) were collected from the field and subjected to indirect enzyme-linked immunosorbent assay (I-ELISA) tests using the polypeptide CP3 antiserum specific for whitefly transmitting geminivirus (WTG) in addition to Tomato yellow leaf curl geminivirus (TYLCV) polyclonal antiserum. In addition, plants were subjected to PCR as a molecular diagnosis test for further confirmation. Experiments proved that the virus could be transmitted mechanically, by viruliferous whiteflies and by grafting. Inoculated strawberry plants with viruliferous whiteflies showed curling and upward cup shape of the leaves. Primers specific for whitefly transmitted geminivirus were used in PCR diagnosis of the inoculated plants. Based on the positive molecular and serological diagnosis results, we concluded that the virus belongs to WTGs. PCR was also carried out for the inoculated plants using primers specific for TYLCV, however negative amplification was obtained indicating that the virus under this study is not a TYLCV. Electron microscopy of purified virus preparation showed the presence of geminate virus particles about 18x20 nm. Antiserum was raised against the purified virus and used for indirect-ELISA to measure the antigenicity of the raised antibodies. Western blot analysis was also used for confirmation of the specificity of the raised antiserum. The isolated virus was given the name strawberry leaf curl geminivirus (StLCV) and it represents the first record of WTG that infect strawberry plants in Egypt.*

***Key words:*** *Strawberry, virus, molecular and serological techniques.*

## INTRODUCTION

Strawberry (*Fragaria* spp.) is one of the most economically important floral crops. According to the Egyptian Agricultural Statistics (2004); the total cultivated area of strawberry is 8677 Faddens, giving a yield of 99091 tons. It is an important commercial fruit being grown in different parts of the country, with a great potential for export if raised from desirable virus free planting material to increase production and maintain quality. Many viruses form a major threat to the strawberry industry in Egypt, causing severe economic losses. There are four main aphid-borne viruses infecting strawberry: Strawberry crinkle rhabdovirus (SCV), Strawberry mild yellow edge Potyvirus (SMYEV), Strawberry mottle virus (SMoV) and Strawberry vein banding Caulimovirus

(SVBV) worldwide (Thompson and Jelkmann, 2003).

Geminiviruses are characterized by having a genome of a single stranded DNA contained in geminate particles. Within this group of viruses, two main subgroups can be recognized; one is transmitted by leafhoppers and the other by whiteflies (Goodman, 1977). Members of the family *Geminiviridae* can be divided into three subgroups based on genome organization, host range and type of insect vector (Hamilton *et al.*, 1983 and Rojas *et al.*, 2000). Subgroup I (genus *Masterovirus*); comprises viruses that have a single component genome, transmitted by leafhopper, and usually infect monocotyledonous plants. It includes maize streak virus (MSV) and wheat dwarf virus (WDV). Subgroup II (genus *Curtovirus*); members of subgroup II have a monopartite genome and are transmitted by leafhopper vector. They infect only dicotyledonous plants like beat curly top virus (BCTV). Subgroup III (genus *Begomovirus*); members of this subgroup have monopartite genomes (2.5 Kb) as well as bipartite ones. For bipartite viruses; the two genomic DNAs have similar size (2.6 Kb) and are designated A and B. However, they differ in sequence except for a common region of 200–250 bp, nearly identical in the genome components of any given virus but differs between different viruses. It is reported that both genome components are required for infectivity.

Diseases caused by geminiviruses have long been recognized as limitations to the cultivation of several important crops including; maize, cassava, bean, cucurbits and tomato in tropical and sub tropical regions of the world (Brown *et al.*, 1995). Symptoms of geminivirus include the following in various combinations: a bright yellow mosaic, chlorotic mottle, chlorotic leaf margins, leaf rolling, leaf distortion, puckering of leaves, reduction in leaf size, stunting of the infected plant, and flower abscission (Polston and Anderson, 1997). Most purified preparations of Geminiviruses contained geminate particles of about 18x30 nm (Francki *et al.*, 1979). Aref *et al.* (1995) reported that the electron micrographs of the purified TYLCV showed isometric particles in pairs (geminate) with a dimension of 20x30 nm.

Polymerase chain reaction (PCR) is an extremely sensitive and specific technique for the detection and identification of plant pathogens (Innis *et al.*, 1990 and Saiki *et al.*, 1988). Idris *et al.* (2003) used degenerate primers for PCR to confirm the presence of Begomoviruses in the leaves of naturally infected symptomatic bean plants maintained in the greenhouse using primers that direct the amplification of the *cp* gene. Morris *et al.* (2002) stated that the detection of TYLCV in tomato plants was achieved two weeks after whitefly fumigation with improved frequency of detection at four weeks. They also found that PCR was a more sensitive method than triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) for the detection of TYLCV isolates in all tested hosts. Specific primers for whitefly transmitting geminivirus (WTG) were used for viral diagnosis in different plants (Abdallah *et al.*, 2002 and Abou-Jawdah *et al.*, 2006). Rabbits, rats and mice are commonly used for the production of mono-specific antiserum used in diagnosis and identification of plant viruses as well as in the evaluation of the function of a specific viral genome (Akad *et al.*, 2004). The present study aimed to characterizing strawberry infecting virus (es) which cause thread to strawberry orchards in Egypt and developing sensitive methods for their detection.

## MATERIALS AND METHODS

### Plant Materials

Strawberry plants with virus-like symptoms (VLS) were collected from orchards in Giza, Behaira and Ismailia Governorates during the years 2004-2005.

### ELISA detection

The indirect ELISA was used for virus detection as described by Banttari and Goodwin (1985).

### PCR detection

Primers used in this study were designed from the nucleotide sequences for the consensus sequence of the genome of WTGs and from TYLCV Egyptian isolate (TYLCV-Eg) (Abdallah *et al.*, 2000). The oligonucleotide primers were synthesized at the Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt, on an ABI 392 DNA/RNA Synthesizer (Applied BioSystem, Lincoln Center Drive, Foster City, CA, USA). The position of the primers, direction and sequence are listed in Table 1. The PCR was carried out as described by Essam *et al.* (2004) with an annealing temperature of 55°C for 35 cycles.

***Table (1): Nucleotide sequences, position of the primers and the expected amplified PCR products***

| Primers name | Nucleotide sequences (5' ----------3') | Additional code (underline) | Position of the amplified region | Length of the amplified fragments |
|---|---|---|---|---|
| *cp*-F | CGGAATTCACATGTCGAAGCGACCAGG | *Bam* HI | 467-1253 | 787 |
| *cp*-R1 | CGGGATCCTTAATTTGATAATGAATC | *Eco* RI | | |
| HD-1 | CGGAATTCGCCCACCAATAACTGTAGC | *Eco* RI | 1855-2528 | 674 |
| HD-2 | CGGGATCCGCAGTCCGTTGAGGAAACTTAC | *Bam* HI | | |
| HD-3 | CGGGATCCAAACAGGTCAGCACATTTCC | *Bam* HI | 391-1049 | 659 |
| HD-4 | GGGGTACCTATATGAGGAGGTAGGTCC | *Kpn* I | | |

### Virus isolation

Groups of 100-200 virus-free whitefly, previously reared on sweat potato and tested with PCR, were given an acquisition access period of 48 hr on infected strawberry plants. Viruliferous whiteflies were transferred to healthy strawberry plants in an insect wooden proof cage at two leaf stage for an additional 48 hr to insure of plants inoculation. Plants were tested with PCR (Abdallah *et al.*, 2002) for the virus presence after 17 days of inoculation using WTG specific primers (HD-1&HD-2 and HD-3&HD-4) and TYLCV specific primers (Cp-F & Cp-R).

### Mechanical and grafting transmission of the virus

Infected strawberry plants produced from viruliferous whiteflies inoculation were used for mechanical transmission of healthy tomato plants (*Lycopersicon esculentum* Mill). The buffer used is composed of 0.1 M phosphate buffer, pH 7.0. Crude sap was obtained by grinding the infected young leaves of strawberry plants with the buffer. Mechanical inoculation was conducted by rubbing sap using a cotton swab on the dusted leaves with Carborundum (400 meshes) of the test plants. Inoculated plants were caged in an insect proof greenhouse at 25°C. A healthy plant was used as a negative control and inoculated with the phosphate buffer only. In grafting, healthy tomato plants were inoculated by wedge grafting as described by Mansour and Al-muss (1992) using pieces from the runners of virus infected strawberry plants. Plants were tested using PCR after 15-20 days of grafting with

WTGs specific primers and TYLCV specific primers and symptoms were recorded weekly.

### Virus purification

Virus was purified from inoculated tomato plants after symptoms appearance according to Hammond *et al.* (1983) with some modifications as described by Essam *et al.* (2004). The purified virus particles were stained with 2% (w/v) uranyl acetate and examined under the electron microscope.

### Antiserum production

Raising antiserum against the purified virus was carried out according to the method described by Ball *et al.* (1990) using white mice ' Balb- C' (6-8 weeks- old). A volume of 0.1 mg/ml was used as a primary response; antigen is administered in about 0.1 ml intravenous injection, followed by five intraperitoneal injections of 0.2 mg/ml containing 0.1 ml incomplete Freund's adjuvant, and injection were given every 7 days. Finally, blood was collected weekly after 3 weeks from the last injection. Indirect ELISA technique (Banttari and Goodwin (1985) was applied with different concentrations (1/1000, 1/2000 and 1/10000) of the obtained antiserum for measuring the sensitivity of the raised antiserum against the virus.

### Western blot analysis

This test was carried out using two identical SDS-polyacrylamide gels (15%) for the purified virus and negative control (healthy plant) (Laemmli, 1970). After electrophoresis the first gel was stained using Coomassie Brilliant Blue stain. The second gel was subjected to western blot analysis as described by Towbin *et al.* (1979). The substrate buffer was added to the membrane, agitated until color was observed and the reaction was stopped by adding $H_2O$.

## RESULTS AND DISCUSSION

### Collecting plant materials from strawberry orchards

Strawberry fields from different Governorates were visited for collecting plants with VLS. Field observations showed that the major virus symptoms were leaflets with cupped, curling shapes, bright yellow edge, reduction in leaf size, leaf rolling and vein banding. In addition heavy infestations of whiteflies were observed and associated with VLS that resemble geminivirus symptoms (Fig. 1). To confirm the presence of WTG in these plants, they were subjected to ELISA and PCR detection.


***Fig. (1):Strawberry plants collected from open fields associated with whiteflies infestation showing virus like symptoms such as leaf curling (A) and cupped shape leaves (B)compared to healthy symptomless plants (C).***

**ELISA detection**

ELISA was found to be indecisive in distinguishing between Begomovirus, especially those present in the same area due to the presence of common epitops in the coat protein region of WTG (Harrison *et al.*, 1977, Padidam *et al.*, 1995 and Abdel Salam, 1999). In this study, plants were diagnosed with I-ELISA using the polypeptide CP3 antiserum specific for WTG and TYLCV polyclonal antiserum. Both antisera were diluted 1/1000 before use. TYLCV-infected tomato plants were used as a positive control. Results showed that both antisera gave positive reactions with the majority of the examined plants (Figure 2). These results were in agreement with that obtained by Akad *et al.* (2004) when using TYLCV polyclonal antibodies against infected tomato plants.

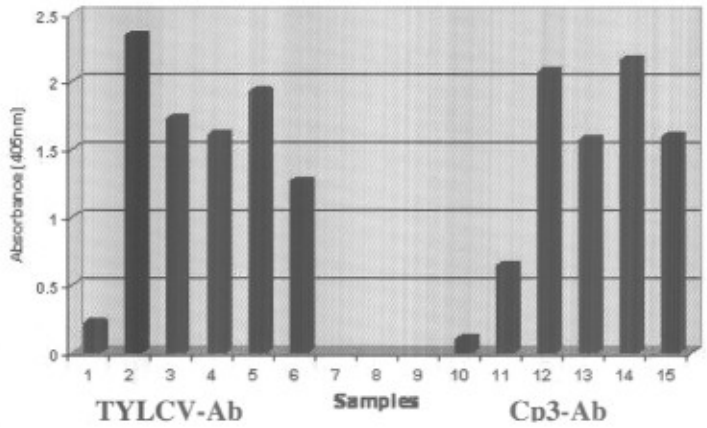


***Fig. (2): Detection of WTG in VLS strawberry plants using 1/1000 of TYLCV polyclonal (lanes 1-6) and CP3 polypeptide antiserum lanes (10-15) by means of indirect ELISA. Columns 1 and 10 represent the negative control while 2 and 11 represent the positive control***

**PCR analysis**

DNA extracted from VLS plants was used in PCR analysis with WTG specific primers (HD-1 and HD-2). The cloned TYLCV genome was used as a positive control in PCR experiments. Five samples out of ten gave positive results as the positive control at a molecular weight of 674 bp (Fig. 3). Results indicated that the virus which infected the examined strawberry plants belongs to WTGs. These results agreed with those obtained by Rojas *et al.* (1993) and Wyatt and Brown (1996) using specific or degenerate oligonucleotide primers. They reported that the PCR primers, which were designed at highly conserved sequence within the genome of WTG, could be applied for broad-spectrum PCR-based virus detection.

**Virus isolation**

Plants were tested for the presence of the viral genome after 15-17 days of inoculation using WTGs specific primers (HD-1&HD-2 and HD-3&HD-4). Inoculated plants were able to amplify the expected size fragment with the two sets of primers (Fig. 4). In addition, the TYLCV *cp* specific primers were also used in PCR tests with DNA extracted from inoculated plants to avoid any plants with mixed infection.

The symptoms appeared on strawberry plants after 30-45 days of inoculation with viruliferous whiteflies included leaf curling

the leaves (Fig. 6). Virus transmission was confirmed by PCR technique (Fig. 6).

PCR results indicated that the samples gave positive results 15 days post mechanical transmission using WTGs specific primers and negative amplification with TYLCV-*cp* primers confirming that this virus is a WTG but not TYLCV. It has been reported before by Aref *et al.* (1995) and Abdallah *et al.* (2000) that TYLCV is not transmitted mechanically.


***Fig. (5): Symptoms appeared on strawberry plants after 30-45 days of inoculation with viruliferous whitefly including curling and upward cup shape of the leaves (A), compared to normal healthy strawberry plants (B).***


***Fig.(6): Symptoms appeared on mechanically transmitted tomato plants 30-45 days post inoculation showing leaf curling and downward cup shape are illustrated in (A&B) while (C) represents a grafted tomato plant and (D) healthy tomato plant.***

In the case of grafting inoculation symptoms were also recorded 30-45 days post grafting (Fig. 6 D), which included downward cup shape and curling of the leaves as those obtained from mechanical transmission. The results of infection were confirmed by PCR 15-17 days post grafting. Grafted plants produced the expected size fragments with WTGs specific primers to detect the genome of the virus but not with TYLCV specific primers. Previous reports stated that WTGs can easily transmitted by wedge grafting (Abdel-Salam, 1990; Allam *et al.*, 1994; Brunt *et al.*, 1996; Aref *et al.*, 1995 and Abdallah *et al.*, 2000).

**Virus purification**

StLCV was purified from the mechanically inoculated tomato plants after symptoms appearance. The yield of the virus was measured as 0.9 mg obtained from 100 g infected tomato plant tissues with a purity of

1.3 at $A_{260/280}$. The method used for virus purification was able to produce a good yield and immunogenic virus particles for raising antibodies against StLCV. The examination showed geminated, icosahedral particles typical to Begomoviruses particles (20x 30 nm) as indicated in Fig. 6. Essam *et al.* (2004) reported that the purified TYLCV yield was 3.09 mg/Kg of infected tomato tissues, while Aref *et al.* (1995) obtained a yield of 1.52-2.8 mg/100 g of infected tomato tissues.

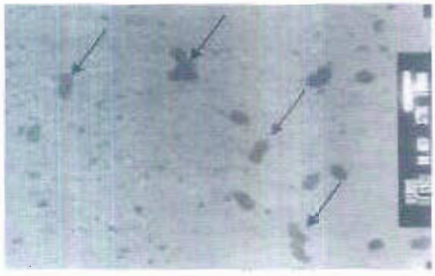

***Fig. (7): Electron micrograph of purified virus particles stained with 2% uranyl acetate.***

**Antiserum production**

Abdel-Salam *et al.* (1998) induced an antiserum against Hollyhock leaf crumple virus by injecting a New Zealand white rabbit. They mentioned that the rabbit were bled one week after the last injection. In this study, antiserum was raised against the purified virus preparation by injecting white mice 'Balb-C' (6-8 weeks-old) with 0.1 mg/ml purified virus as a primary response in the tail followed by five interaperitoneal injection of 0.2 mg/ml. Blood samples were collected weekly after 3 weeks from injection. Indirect ELISA was used to measure the antigenicity of the raised antibodies. Infected and healthy strawberry plants were investigated using 1/1000, 1/2000 and 1/10000 dilutions of the antiserum against 1/10 of tested plants (Fig. 8).

**Western blot analysis**

Results showed that a sharp band produced with the purified virus (lanes 1&2) at a molecular weight of 32 KD corresponding to the band appeared with the stained gel, but this band was absent with a healthy plant sample (Fig. 9). This test was carried out to confirm that the antiserum produced is specific for the viral Cp. Aref *et al.* (1995) reported that TYLCV preparation has a major protein of 30 KD which was detected in the purified preparation of TYLCV particles from *Nicotiana benthemiana*.

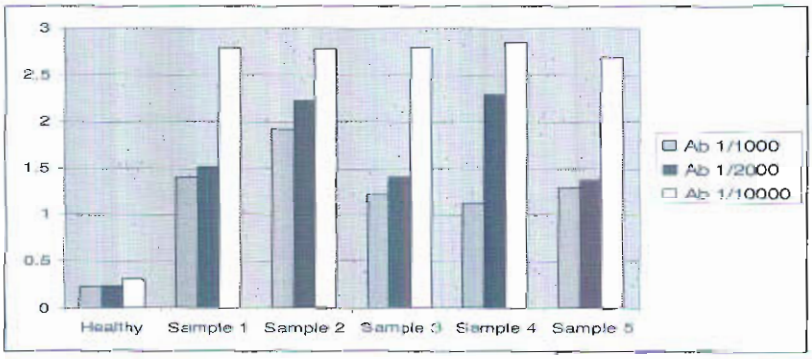


***Fig. (8):Detection of StLCV in infected strawberry plants using dilution of 1/1000, 1/2000 and 1/10000 antiserum and 1/10 of samples by means of indirect- ELISA.***

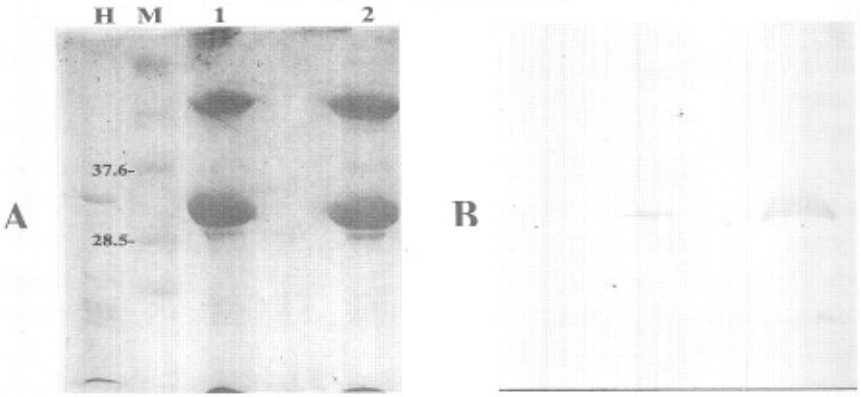


***Fig. (9): SDS-PAGE of the purified virus (A) stained by Coomassie Brilliant Blue and (B) western blot analysis using antiserum of StLCV. Purified virus gave sharp band (lane 1 and 2) of 32 KDa which was absent with healthy plant (H). Lane M represents the Mobi Tec. marker.***

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## الملخص العربي

### دراسات جزيئية و سيرولوجية على أحد فيروسات النبات التي تصيب الفراولة

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تُشكل الفيروساتُ تهديدا رئيسي لزراعةِ الفراولة في مصر مما يُسببُ خسائرَ إقتصادية كبيرة.لا تتوفر الطرق السريعة والبسيطة لكشفِ فيروساتِ الفراولة الرئيسيةِ وذلك بسبب قلةِ الوسائل التشخيصيةِ الحسّاسةِ. تم تجُميع النباتات التي تظهر عليها أعراضَ اصابة شبة فيروسَة مِنْ الحقل وأخضعَت إلى اختبار الاليزا غير المباشر وذلك باستعمال الاجسام المضادة عديدة الكونات المتخصصة لفيروسات geminivirus التى تنتقل بالذبابة البيضاء وكذلك باستعمال الاجسام المضادة عديدة النسل المتخصصة لفيروس التفاف الاوراق الاصفر فى الطماطم(TYLCV) . بالإضافة الى ذلك أُخضعتْ النباتات إلى إختبار التشخيص الجزيئي باستخدام تفاعل البلمرة المتسلسل(PCR) للتأكيد. أثبتتْ التجاربَ بأنّ الفيروسَ يُمْكِنُ أنْ ينتقل ميكانيكياً بواسطة الذبابة البيضاء و كذلك بالتَطعيم.اظهرت نباتات الفروله الملقحة باستعمال الذبابه البيضاء تجعد والتفاف الاوراق لاعلى . كذلك باستعمال الاجسام المتخصصة لفيروسات الجيمنى التى تنتقل بالذبابة البيضاء فى الكشف باستخدام تفاعل البلمرة المتسلسل للنباتات المختبرة كانت النتائج ايجابية. بالاعتماد على النتائج الموجبة المتحصل عليها بالوسائل السيرولوجية والجزيئية فى التشخيص يمكن القول بان هذا الفيروس يتبع WTGs وكذلك تم استخدام تفاعل البلمرة المتسلسل للكشف على النباتات المختبرة باستخدام بادئات متخصصة للكشف عن فيروس التفاف الاوراق الاصفر فى الطماطم اعطت نتائج سالبة مما يوكد ان هذا الفيروس تحت الدراسة ليس فيروس التفاف الاوراق الاصفر فى الطماطم . وباستعمال الميكروسكوب الالكترونى لتصوير تحضير نقى من الفيروس اظهرت لنتائج وجود جسيمات فيروسية بمقياس ١٨ x ٢٠نانوميتر. تم انتاج اجسام مضادة متخصصة لهذا الفيروس واستعمالها فى الكشف باختبار الاليز غيرا لمباشر وكذلك استعمال تحليلات western blotting لقياس فاعليته وللتاكد من تخصص الاجسام المضادة المتحصل عليها. تم اعطاء اسم لهذا الفيروس المعزول هو تجعد الاوراق الفيروسى فى الفراولة StLCV ويعتبر هذا هو التسجيل الاول لفيروس تابع للـ WTGs يصيب نباتات الفراولة فى مصر.