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Single-Strand Conformation Polymorphism (SSCP) and Nucleotide Sequence Analysis of *Citrus Tristeza Virus* in Egypt

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Abstract

Citrus Tristeza Virus (CTV) is the most economically important virus of citrus worldwide. CTV isolates differ by their biological characteristics, particular in the intensity of symptoms. The CTV genetic variability was analyzed using single-strand conformation polymorphism (SSCP) assay. The amplicon genes coding for CTV coat protein (CP) p23 and the non-structural p20 protein were analyzed using SSCP. As well as, sequence analysis were used to separate either distinct virus isolates for cloning the CP genes or variants (haplotypes) for sequencing. SSCP profiles of PCR amplified products from Ismailia Egyptian Isolate of CTV showed bands patterns corresponding to mild reference strains. While, Qalyubia Egyptian isolate of CTV showed band patterns corresponded to a CTV strain showing typical SSCP profiles with VT isolates and to California and Japan sever strains. The genes coding for p20 and p23 was amplified and the nucleotide sequences were determined for the isolate from Qalyubia in both directions. Blast analyses showed a nucleotide identity ranged from 95-99% with VT sever strains. SSCP analysis provided a rapid procedure to screen the genetic heterogeneity of the viral isolates reducing considerably the amount of nucleic acid sequenciation necessary to gain that knowledge. Results confirm that more than a single introduction of CTV could have occurred in Egypt over the years. The prevalence of severe isolates in the area of particular concern, and implications for the future of the CTV epidemics should be considered. PCR-SSCP gives reproducible results and permits to process a large number of samples at once. This method can improve its potentiality to simultaneously monitor and analyze the genetic diversity and structure of CTV population.

Keywords: Citrus, CTV, SSCP, Sequence analysis.

Introduction

Citrus tristeza virus (CTV) is still a major threat to the citrus industry in Egypt. CTV is the causal agent of the most economically important viral disease of citrus. It has been detected since 1958 in different Egyptian areas, but without relevance to the citrus industry due in part to monitoring and prompt eradication of infected plants (Nour-Eldin and Bishay 1958; Abdel-Salam, 1998; Fahmy *et. al*., 2009; Youssef *et. al*., 2016). CTV is a member of the genus *Closterovirus*, family *Closteroviridae*. Virions of CTV are flexuous filaments of about 2000×11 nm formed by two coat proteins, the major (CP, p25) covering most of the virion length, and the minor (CPm, p27) restricted to one of the termini (Febres *et al*. 1996). The genome of CTV is a positive-sense, single-stranded RNA of 19.3 kb with 12 open reading frames (ORFs) with three of them (p25, p20 and p23) acting as RNA-silencing suppressors (Ruiz-Ruiz *et al*. 2013). The sgRNAs for p20 and p23 are more abundant and probably produce more protein than other ORFs, with the possible exception of CP. However, among different CTV isolates, the ratios of these two sgRNAs vary in abundance in infected trees (Hilf *et al*.,1995). The p20 protein exhibited a high affinity for itself, suggesting

that it might aggregate in infected cells, and can be detected in CTV virion preparations, therefore p20 is presumed to form a structural component to the virus particle or to be bound to virions. The p20 protein is reported to be the major constituent of CTV amorphous inclusion bodies formed in infected protoplasts (Gowda *et al*., 2000). The p23 protein is unique to CTV in comparison with other closteroviruses. ORF 11 encodes the p23 protein and is adjacent to the 3'UTR of the CTV RNA genome. It is an RNA binding protein suspected to play a regulatory role in the expression of other CTV genes and may serve as an indicator of disease severity (Lopez *et al*., 2000). This has significance for finding markers for strain differentiation. It has also been predicted to have a ribosome binding capacity and was identified as the second CTV suppressor of post-transcriptional gene silencing (Lu *et al*., 2004). CTV isolates differing in biological, serological and molecular characteristics worldwide (Raied, *et al*., 2012). There are two major economically important syndromes caused by CTV that depend on the virus strains and host species or scion-rootstock combination: i) decline and death of citrus species grafted on sour orange or lemon rootstocks, and ii) stem-pitting, stunting, reduced yield and low fruit quality regardless of the rootstock used. The virus has been dispersed to new citrus areas mostly by propagation of infected budwood, followed by further local spread by several aphid species (Moreno *et al*. 2008). The virus has a host range restricted to most species of the family *Rutaceae* (Roistacher, 1991) and can be disseminated long distances by movement of virus-infected plant material and locally by several aphid species in semi-persistent mode (Lee and Bar-Joseph, 2000). Single Stranded Conformational Polymorphism (SSCP) analysis has been used to characterize populations of CTV and to identify haplotypes in these populations. SSCP can give a preliminary result regarding the potential symptom severity or population complexity (Sambade *et al*., 2002). Polymerase chain reaction analysis (PCR) with specific primers derived from the sequence of isolates previously characterized for their pathogenicity also allows screening many isolates in a short time (Ayllon *et al*., 2001). The aim of this work is to characterize CTV isolates present in Egypt by SSCP and sequence analysis for p20 and p23 genes, and to design management strategies for this disease in Egypt and to understand potential epidemic outbreaks.

Materials and Methods

Source of Isolates:

CTV isolates used in this experiment collected from citrus trees previously found to be infected by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) using a polyclonal CTV antibody (Agritest Srl) according to the manufacture's procedures and maintained in an insect-proof greenhouse as described before (Youssef *et. al*., 2016). Two CTV isolates were used in this study, one from Ismailia and one from Qalyubia Governorates in Egypt. Negative controls consisted of leaves taken from virus-free seedlings maintained in the greenhouse were used. The CTV reference isolates kindly provided as a nucleic acid from Dr. L.Ferretti (CRA-PVA, Roma, Italy). The isolates were California (SY568), Florida (T55), Sapin (T385), Japan (T388), Israel (VT), Italy (IT); their pathogenic characteristics have been described (Smbade *et al*., 2002). The reference isolates were classified into five biogroups according to the symptoms induced in a panel of indicator hosts (Garnsy *et al*., 2005). In short, isolates T55, IT and T385 mild isolates induce symptoms in Mexican lime (biogroup 1), VT and SY568 sever isolates cause decline of sweet orange grafted on sour orange rootstock , induce seedling yellows and cause stem pitting on grapefruit (biogroup 4) or on grapefruit and sweet orange T388 is a very sever isolate (biogroup5).

Nucleic acid Extraction and Single Strand Conformation Polymorphism Analysis (SSCP).

Total RNA was extracted from young shoots of phloem tissues, with RNeasy Plant Mini Kit (Qiagen Science, USA) according to manufacturer's instructions, and used as a templates for reverse transcription polymerase chain reaction (RT-PCR). RT-PCR were performed using the primers P20A and P20B amplifying a 561 bp encompassing the CTV p20gene (Guerri *et al*., 1991) and PM50 and PM51 amplifying 697 bp encompassing CTV p23gene (Sambade *et al*., 2002). One-step RT-PCR reactions were set up using 2 µL of tRNA in 25µl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mMKCl, 3 mM MgCl2, 0.4 mM dNTPs, 1µM of each primer, 4 U of RNaseOut (Invitrogen, Carlsbad, CA, USA), 20U of SuperScript II reverse transcriptase-RNaseH (Invitrogen,Carlsbad, CA, USA) and 2 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was performed at 47° C for 30 min, followed by denaturation at 94°C for 2 min.

The following PCR conditions were used: 30 cycles each of 94°C for 30s, 50°C for 30s, and 72°C 40s; and final extension 72°C for 5 min. The resulting RT-PCR products were separated by electrophoresis in a 2% agarose gel using GeneRuler™ 100bp DNA Ladder (Fermentas), and detected by ethidium bromide staining.

Table (1).Primers used for the RT-PCR amplification of sequence variants of the p20 and p23 genes of CTV.

Target	primer	Primer sequence (5'to3')	Binding site	Authers
CTV p20 gene	fP20A	ACAATATGCGAGCTTACTTTA	17691–17710	(Guerri *et al*.,1991)
CTV p20 gene	rP20B	AACCTAACAGCAAGATGGA	18229–18246	
CTV p23 gene	fPM50	ACTAACTTTAATTCGAACA	18347-18365	(Sambade *et al* 2002)
CTV p23 gene	rPM51	AACTTATTCCGTCCACTTC	19026-19044	

For SSCP analysis, 2µl of the RT-PCR product was mixed with 18 µl of the denaturing solution (95% formamide and 0.05% bromophenol blue), heated for 10min at 95°C, and chilled on ice.). Electrophoresis was carried in a non-denatured 8% polyacrylamide gel using 1xTBE buffer, at 200 volts for 4h at 4°C (Sambrook *et al*., 1989). Gels were stained with silver nitrate (Beidler *et al*., 1982).

Molecular cloning and nucleotide sequence analysis.

The variables detected with SSCP were cloned in a pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufactories' instruction manual. Two clones from each SSCP variant were sequenced in both directions using ABI PRISM DNA 377sequncer (Perkin-Elmer). The obtained sequences were aligned using the National Center for Biotechnology Information (NCBI) BLAST server (http://blast.ncbi.nlm.nih.gov/Blast) with the following reference sequences of the CP genes p20 and p23 from the GenBank: VT, Florida-USA (EU937519); T68, Florida-USA (JQ965169); VT, Israel (U56902); NZ-SP, New Zealand (EU857538); CTV-B5, India (HQ912023); QS4, Syria (FN662711); Pakistan (HQ329228): (HQ329236); C315, Argentina (AY962343); NUagA, Japan (AB046398); SY568, California-USA (AF001623); T318, Spain (DQ151548) and TAR-SwO, Sicily-Italy(JQ422280). Phylogenetic tree were constructed using MEGA 4 software package (Tamura *et al*., 2007).

Results

SSCP Analysis

RT-PCR amplification using primers P20A and p20B (Guerri *et al*.,1991) to amplify *p20* gene and primer PM50 and PM51 (Sambade *et al*., 2002) to amplify *p23* gene, were used as afirst step screening PCR to amplify sequnces conserved in VT, IT, SY568, T388, T385, T55 (reference isolates), and to identify the two Egyptian isolates. The two Egyptian isolates yielded the expected DNA fragment for each primer. In every PCR electrophoretic profiles a segment of 561bp for *p20* gene (Fig. 1) and 679bp for *p23* gene (Fig.2). No amplification was obtained from non infected plants grown in the greenhouse.

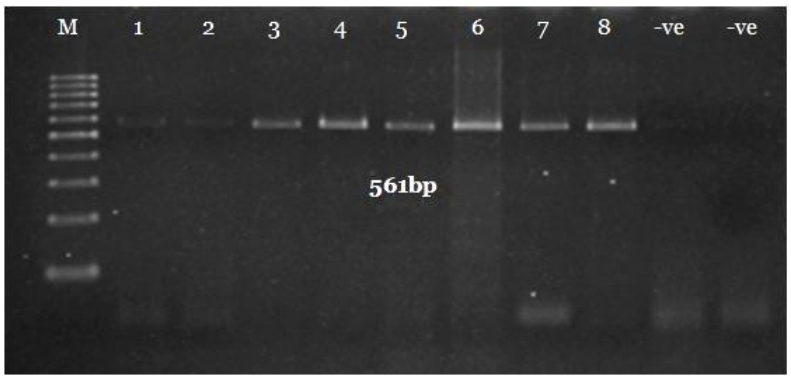

Fig.(1).1% Agarose gel electrophoresis of PCR amplification for *p20* gene fragments generated for SSCP analysis. Lane M, GeneRuler™ 100bp DNA Ladder (Fermentas);(1) sample from Ismailia; (2) sample from Qalyubia;(3) VT; (4) IT; (5) SY568; (6) T388;(7) T385; (8) T55 and (–ve) negative control.

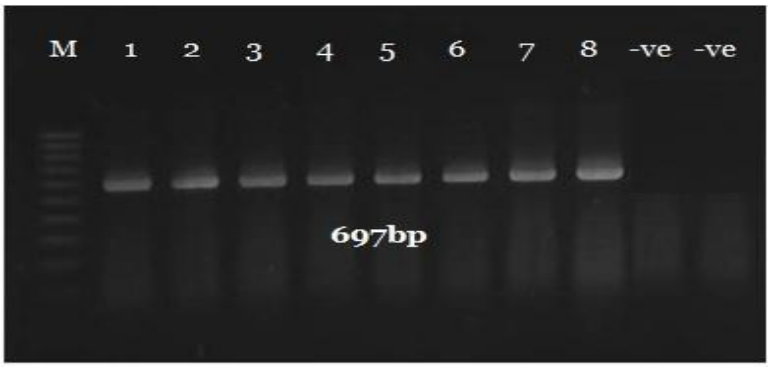

Fig. (2). 1% Agarose gel electrophoresis of the conserved PCR of *p23* gene from 8CTV isolates using primers PM50 and PM51. The expected product is 697bp. Lanes: (M) GeneRuler™ 100bp DNA Ladder (Fermentas), (1) Ismailia; (2) Qalyubia; (3) VT; (4) IT; (5) SY568; (6)T388; (7) T385; (8) T55 and (–ve) negative control.

RT-PCR and SSCP analysis were performed for the *p20 and a p23* gene of the CTV isolates (Fig.3 A &B). Variation analysis of the CTV population was preliminarily assessed for the two Egyptian isolates by comparing their viral RNA diversities revealed by SSCP analysis. The electrophoretic profiles of SSCP analysis were conducted by observing the number and position of the bands. Comparisons were established amongst CTV isolates obtained from different CTV samples with their respective controls and with the severe isolates used as controls. Approximately two to three bands were observed, suggesting the presence of two strains (Fig.3A&B). Citrus Tristeza isolates, like other RNA viruses, contain a population of genomic variants, the composition of which may affect their biological characteristics. Analysis of SSCP is a simple technique that allows detection of minor variations in the nucleotide sequence of DNA fragments without sequencing. Data obtained by studying of SSCP analysis revealed that: the eight isolates (Six identified isolates in addition two isolates from Egypt) were already electrophoresed and gave clear fragments; the fragments were in deferent distance according to the position of the fragments; Egyptian isolate from Ismailia is closely related to IT, T385 and T55 which identified as mild strains belong to biogroub (1). While, the isolate from Qalyubia is closely related to VT isolate which identified as severe strain belong to biogroub (4) and related also to SY568 and T388 which identified as severe strains. Accordingly, Egyptian isolates is related to two groups of mild and sever isolates.

Results obtained from the SSCP technique confirmed the presence of a discrepancy between the two isolates under study, where as the Ismailia isolate was mild and Qalyubia was sever. The subsequently studies confirmed the molecular characterization of mild strain in Egypt (Amin *et al*., 2006). Nucleotide sequence analysis was applied on the severe strain from Qalyubia governorate.

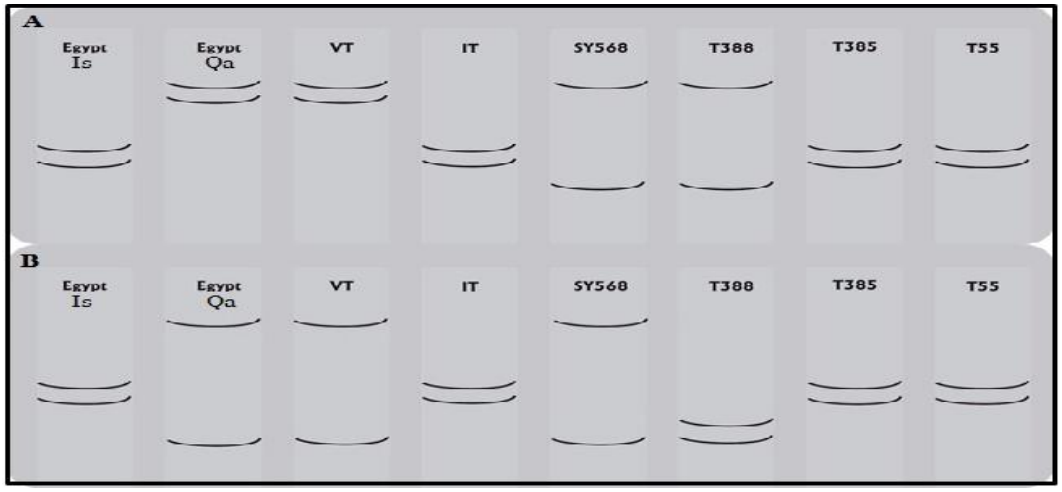

Fig.(3). Representative diagram for Single-Strand Conformation Polymorphism (SSCP) patterns of *p20 gene* (A) and p23 gene (B) amplified from different CTV isolates (Egypt isolates from Ismailia (IS) and Qalyubia (Qa) respectively) in comparison with the obtained reference isolates(VT, IT, SY568, T388, T385 and T55). SSCP analysis was performed by electrophoresis under non-denaturing conditions in 8% acrylamide gel, at 4C, and 200 V for 4 h.

Nucleotide sequence analysis

Searches for recombination events among the sequences were applied on the isolate from Qalyubia – Egypt. Multiple sequence alignments were produced for the genes coding for p20 and p23 using BLAST and phylogenetic trees generated using MEGA 4 software. Comparison with sequences from GenBank showed that p20 gene amplified from Qalyubia isolate was closely related to Florida VT isolate and Syria QS4 isolate (nucleotide identity 99%), to Israel VT isolate and Pakistan isolate (98% identity), to South Africa CT-ZA3 isolate; New Zeland SP isolate; India B5 isolate; Argentina C315 isolate; Sicily-Italy TAR-SwO isolate (97% identity) and to Florida-USA T68 isolate; Japan NUagA isolate; California-USA SY568 isolate and Spain T318 isolate (96% identity). P23 gene amplified form Qalyubia isolate was closely related to Florida VT isolate and Israel VT isolate (99% identity), to South Africa CT-ZA3 isolate and New Zealand SP isolate (98% identity); to India B5 isolate (97%); to Pakistan isolate (96% identity) and to Florida-USA T68 isolate; Japan NUagA isolate; California- USA SY568 isolate and Spain T318 isolate (95%identity).

Phylogenetic analysis

Phylogenetic analysis of the nucleotide sequences of the p20 gene grouped this isolate in the same clad with several sever reference strains sharing identities ranged from 96% with (T68, NUagA, SY568,and T318); to 97% with(CT-ZA3, SP, B5, C315 and TAR-SwO); to 98% with (VT and Pakistan) and to 99% with(VT and QS4) Fig.(4A). Phylogenetic analysis based on the nucleotide sequences of p23 gene confirmed that this isolate from Qalyubia on the same main clad of the sever reference isolate sharing identities ranged from 95% with (T68, NUagA, SY568, T318); to 96% with (Pakistan); to 97% with (B5); to 98% with (CT-ZA3 and SP) and to 99% with (VT Florida and VT Israel) Fig.(4B). Results collected from sequence analysis and phylogenetic tree indicated that, there was a high degree of homology between the Egyptian isolate from Qalyubia and the sever reference strains (VT, SY568, NUag A and SP) retrieved from GenBank by BLAST.

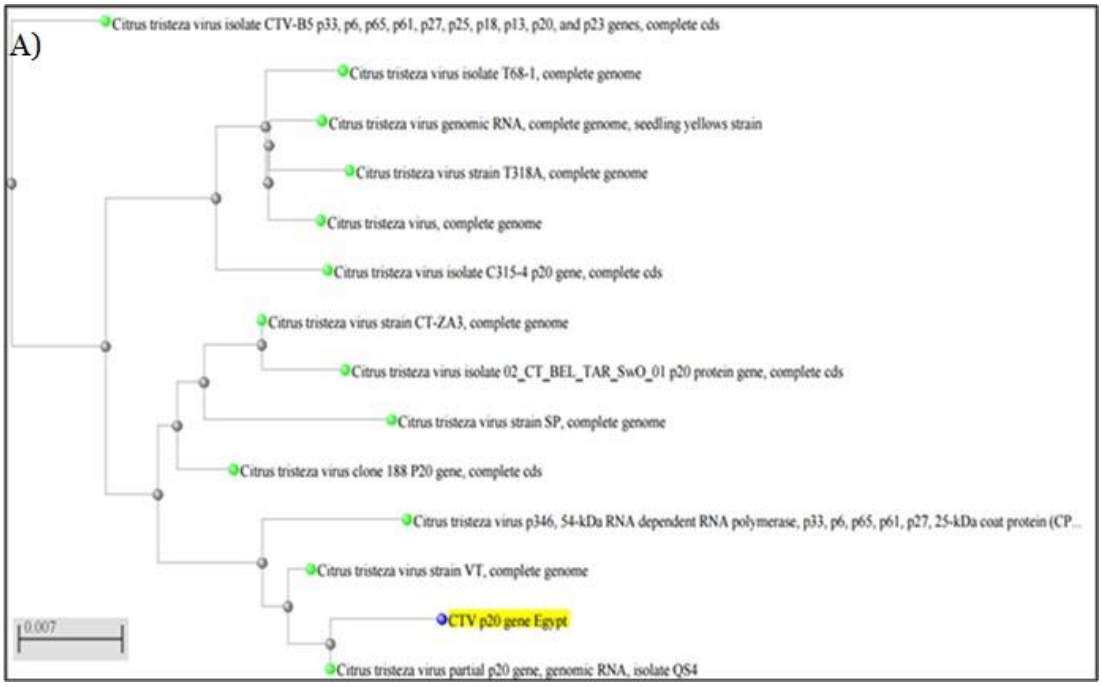

Fig. (4A).Phylogenetic trees generated by the neighbour-joining method from the alignment of the nucleotide sequences of the major CP (A), *P20* gene of selected *Citrus tristeza virus* isolates using MEGA (Version 4.1). Scale bar indicates changes per nucleotide.

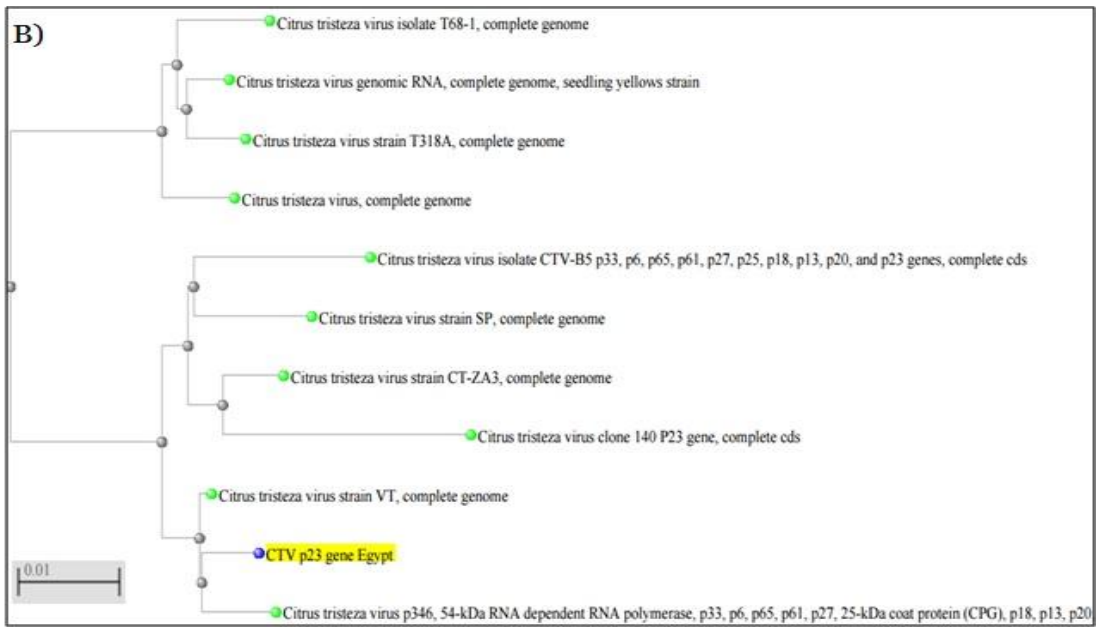

Fig. (4B). Phylogenetic trees generated by the neighbour-joining method from the alignment of the nucleotide sequences of the major CP (B), *P23* gene of selected *Citrus tristeza virus* isolates using MEGA (Version 4.1). Scale bar indicates changes per nucleotide.

Discussion

In the last 10 years CTV became wide spread in some areas of the Mediterranean basin, and infected citrus trees harbouring severe strains of the virus have been reported in different countries (Saponari *et al*., 2009; Malandraki *et al*., 2011). Field plants are exposed to continuous environment changes and successive infections by aphids. The coexistence of variants with a high nucleotide diversity within-CTV population of Egypt isolates might probably reflect changing environment conditions and/or the high frequency of infections by aphids in the field. The adaptation of CTV populations to a new host could be an important factor to select the haplotypes present in the viral isolates. This SSCP analysis was originally developed to analyze viral genome diversity because the procedure combines simplicity, low cost, and the potential for use with many samples (Sambade *et al*., 2002). Additionally, it has enough sensitivity to detect a single nuceotide difference in relatively large DNA fragments (up to 700 nucleotides) (Rubio *et al*.,1996); these differences among profiles are consistent if the experimental conditions are carefully maintained. Guerri *et al*. (1991) have compared the dsRNA patterns from 125 randomly selected CTV-infected citrus trees, and have found up to 16 different profiles, which is indirect evidence of genetic variation based on the population of D-RNAs. SSCP analysis by Orita *et al*. (1989) of complementary DNA from two CTV genes has also been used to characterize the population of sequence variants for several CTV isolates (Ayllon *et al*., 1999). This method can also detect variations within these populations after aphid transmission or host passage (Rubio *et al*., 2000). Kong *et al*. (2000) have characterized the population structure and genetic diversity of five California CTV isolates. Rubio *et al*. (2001) have reported no correlation between geographical origin and nucleotide distance in their comparison of two groups of CTV isolates from Spain and California (USA). The objective of this work was to find a potential molecular marker that could be used for quick identification and differentiation of CTV isolates. SSCP analysis was useful for screening many samples very quickly to establish the degree of homogeneity in the virus population. Because SSCP analysis of the *p20* and *p23* genes gave similar results, either of these two regions can be used to differentiate isolates. The SSCP exploratory analysis also allowed us to determine which isolates to further characterize with differential PCR, which corroborated the SSCP results. Molecular characterization by single-strand conformation polymorphism (SSCP) of RT-PCR amplified products of the genes coding for p20 and p23 from selected CTV Egyptian isolates tested in this study showed different SSCP profiles for both genes. RT-PCR analyses using a panel of multiple molecular markers and sequencing of these two genes provided molecular evidence for the presence of CTV genotypes genetically related to the reference (VT, SY568, NUagA and SP) sever strains in addition

the common mild strain. . The sequence analysis and phylogenetic tree strongly support such conclusion showing a 99% of nucleotide identities between Qalyubia isolate and several sever reference strains (VT, SY568, NUagA and SP). This was unexpected since these strains were did not propagate in Egypt. According to these results we can conclude that CTV populations in Egypt contain new genetic variants, these variants could produce a severe phenotype in inoculated plants, under certain conditions, involving environmental ,presence of the vector and host factors. That could contribute to spread the severe variants of the virus. Mandatory program are therefore required to prevent or suppress further introduction and spread of the virus (D'Onghia, 2009). Effective quarantine, eradication and budwood certification programmes are the best measures to avoid CTV introduction or dispersal in CTV-free areas (Ferretti *et al*., 2014) . Rapid identification of strain severity is critical for disease control because CTV is readily spread by propagation and aphid vector (Yokomi *et al*., 2010). Finally, PCR-SSCP gives reproducible results and permits to process a large number of samples at once. This method can improve its potentiality to simultaneously monitor and analyze the genetic diversity and structure of CTV population.

Acknowledgments

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