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MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *CITRUS TRISTEZA VIRUS* COAT PROTEIN FROM THE PUNJAB PROVINCE OF PAKISTAN

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ABSTRACT

Citrus is an important commercial crop around the globe including Pakistan that is being attacked by various pathogens including *Citrus tristeza virus* (CTV). The present study was carried out to investigate the molecular identification and characterization of the most lethal*Citrus tristeza virus* (CTV) from the major citrus producing region of Punjab (Pakistan). The total RNA from different varieties of sweet oranges, mandarins, grapefruits, lemons, limes, kumquats and important rootstock was extracted to be used as template for PCR using primers for the universal coat protein detection of CTV. Total RNA was extracted from the sweet oranges, mandarins, grapefruits, lemons and limes, kumquats and important rootstock varieties, which were then converted to cDNA to be used as a template in a conventional PCR using primers for the universal coat protein detection of CTV.A total of 250 samples were screened out of which 25 (10%) samples were found positive. The number of positive samples from each group includes20/100 sweet oranges, 2/70 mandarins, 1/32 lemon and limes and grapefruit 2/30 were observed. The results from the analysis of the PCR assay confirmed the presence of CTV as the gene fragment was amplified from by PCR at desired size of 672bp.The desired PCR amplified product was ubjected to Sanger sequencing and phylogenetic relationship using MEGA 7.0. showed 99% homology with the aligned American isolates.The amplified product was then sequenced and analyzed phylogenetically using MEGA 7.0 that showed 99% homology with American isolates.Hence the study proved to produce a significant preliminary data showing the current status of CTV that can be used for the creation of a pathogen tested citrus germplasm program for citrus growers in Pakistan. This study was conducted to evaluate the current CTV status of the citrus germplasm collection of a citrus research institute in Punjab, Pakistan, in order to produce preliminary data that can be used for the creation of a pathogen-tested citrus germplasm program for the citrus growers in Pakistan.

Keywords: *Citrus tristeza virus*, Coat protein, Molecular characterization, Phylogenetic analysis.

INTRODUCTION

Citrus fruit is one of the highest well-liked fruit crops. Over 140 countries worldwide are cultivating the citrus including many in the Asia Pacific and the fruit has gotten consideration due to its increased uses as nutritious and healthy drink during current years (Su, 2008). At present the world's citrus production is about 135.8 million tonnes on an area of about 96.8 million yielding about 140267.00 hg/ha in the year 2013. Islamic Republic of Pakistan occupies 14th position among the renowned citrus fruit producing countries for the year 2013. Pakistan's citrus production is about 2150000.00 tonnes on an area of about 195300.00 ha yielding about 110087.00 hg/ha in the year 2013 (FAO, 2017). Pakistan exported432061.494 metric tonnes of various citrus cultivars to many countries in the world and earned a colossal aggregate of foreign currency of about 15978.124 million rupees in the year 2012 (Ahmad *et al*. 2014).

Citrus crop is being affected by various pathogens including bacterial (Huanglongbing, Canker) (HLB, Canker), fungal (Scab), viral (Tristeza, Variegation) (CTV, CVV) and many others all over the world. These pathogens inhabit the citrus crop worldwide where the environment is humid and favourable for their growth and multiplication (Dalio *et al*. 2017).The areas around the world where citrus is grown, mostly the humid environment provides a perfect habitat to the above said pathogens causing significant damage to the citrus crop (Dalio *et al*. 2017).

There are above 30 viruses and virus like diseases in the world that attack the citrus fruit and among all the viral pathogens of citrus, *Citrus tristeza virus* (CTV) is possibly the most damaging virus of the industry (Atta *et al*. 2012). The spread of CTV around the globe is mainly through the movement and multiplication of already pathogen infected plant material or through aphid species.CTV was mainly dispersed through the movement and multiplication of the infected industrial plants or infected bud wood material and then locally it was spread by a few of aphid species (Cárdenas *et al*. 2002, Afechtal, 2013).*Citrus tristeza virus* (CTV) belongs to the genus *Closterovirus* that triggers one of the most deadliest pathogenic disease of citrus fruit (Djelouah and D'Onghia, 2000). CTV causes a significant damage to the citrus crop including the poor

fruit quality, yield and stem pits regardless of the rootstock used causing the complete deterioration of the citrus cultivars (Cambra *et al*. 2000, Zhou *et al*. 2011). *Toxoptera citricida* is the most effectual vector of CTV. The area around the globe where *T. citricida* is absent, another aphid specie (*Aphis gossypii*) is known to be the carrier vector for the spread of CTV. The regions of the world where *T. citricida* is non-existent, *Aphis gossypii* is the carrier of CTV (Gottwald *et al*. 2002, Batista *et al*. 2008).*Citrus tristeza virus* (CTV) causes a variety of symptoms on the citrus that are strain dependent, the type of citrus cultivar involved and the varietal combination of citrus. In Asia, various strains of CTV, generally referred to as seedling yellows (CTV-SY), tristeza (CTV-T), stem pitting (CTV-SP), and a mild type, have been widespread for many years. Any of these strains may exist in a citrus or they may come about together, as a complex (Bar-Joseph *et al*. 1989, Koizumi, 1995, Atta *et al*. 2012)

The causal agent of the tristeza disease is *Citrus tristeza closterovirus* (CTV), which is a flexuous rod shaped corpuscle the size of which is 2000nm and 12nm and the infectious particle of the virus contains a single stranded RNA (Xiao*et al*.2017)and consist of one single stranded positive-sense RNA molecule encapsulated withthe major capsid protein (CP) and minor CP (Raied *et al*. 2012).Therefore, the CP gene has been used as target for various methods developed for detection of CTV and strain identification including ELISA, western blot, peptide mapping, RT-PCR, hybridization and RT-PCR. In addition the CP genes of a large number of CTV isolates from different geographical regions and biological properties have been cloned and sequenced (Roy *et al*. 2003).

The presence and distribution of CTV in different citrus growing regions of Pakistan is largely known and their serological and biological properties are determined to some extent (Iftikhar *et al.* 2009; Atta *et al.*2012). However, there is no sequence information available for Pakistani CTV isolate and their molecular characteristics are completely unknown. In this study, molecular identification and characteristics of the T36 CTV isolate along with the sequencing of the major CP gene was done.The sequence was subjected to phylogenetic analysis with the other CP sequences submitted in GenBank from all over the world.

MATERIALS AND METHODS

Sample Collection: *Citrus tristeza virus* (CTV) symptomatic and asymptomatic leaf samples were collected from the citrus plants. The six major groups of citrus including Grapefruit, Mandarin, Kumquat, Lemon and Lime, Sweet Oranges and Rootstocks were obtained from orchards of Citrus Research Institute, Sargodha to check the prevalence of CTV in different cultivars of citrus. For each sample, at least 50 symptomatic and asymptomatic leaves were collected in labelled polythene bags and transferred to ice box until shifted to the laboratory at -20^{0}C.

Total RNA Isolation: Total RNA was extracted from the phloem containing midrib tissue of the leaves along with the petiole. For protocol 1, a total of 0.2gms of the plant tissues were homogenized in the equal volume of the extraction buffer I (100mM NaOAc, 10mM EDTA, 250mM NaCl, 1% triton 100X) and extraction buffer II (Acidic Phenol pH: 4.0) using a chilled pestle and mortar. The homogenized samples were kept at room temperature for 5min and then centrifuged at 12000rpm for 5min. The supernatant was transferred to the new tubes containing 3M KOAc, added three-column volume of chilled absolute ethanol, and centrifuged at 12000rpm for 10min. After this the supernatant was discarded and the RNA pellet was washed with 70% ethanol. The total RNA was suspended in nuclease free water (Thermo scientific). For protocol 2, total RNA extraction procedure was followed as described by Gambino *et al.* (2006) and for protocol 3, total RNA extraction procedure was followed as described byJordon-Thaden*et al.* (2015) Estimation of RNA yield and quality was done with a Nano Drop ND-1000 Spectrophotometer (OPTIZEN NanoQ) by measuring optical density at 260 nm and the ratio of the absorbance at 260 and 280 nm, respectively. The quality of RNA was also determined using 37% formaldehyde denaturing agarose gel electrophoresis. The total RNA after extraction was shifted to -80^{0}C immediately or cDNA was synthesized (Green and Smabrook, 2012).

Conventional RT-PCR Primers: The primers specific to the CP gene location based on GenBank accession number AY170468 were used to detect the CTV isolate in Pakistani citrus plants. The primers for the bidirectional PCR were previously reported (Saponari *et al*. 2008). The sequence and amplicon size for the primers is given in Table 1.

RT-PCR: The complimentary DNA (cDNA) synthesis was done using 3ul of the total RNA (500ng/ul concentration), 100uM antisense primer, 10mM dNTP's (Thermo scientific), 5X RT buffer (Thermo Scientific), 0.1M DDT and MMLV-RT (Thermo Scientific). The tubes were then incubated at 70^{0}C for 10 min, 37^{0}C for 5min, 42^{0}C for 60min and 70^{0}C for 7min in a thermal cycler in two steps. The cDNA was used as a template for the PCR and was stored at -20^{0}C prior to use.

Conventional PCR: The Dream Taq Green Master Mix (Thermo Scientific) was used for the conventional PCR assay. A 3ul of the cDNA template was used in a 25ul of the PCR reaction following the procedure described by Saponari *et al*. (2008). To determine the best annealing temperature, a gradient PCR was performed using the following conditions: one cycle at 94^{0}C for 5min, 35 cycles at 94^{0}C for 30 sec, 54^{0}C-62^{0}C for 60 sec and 72^{0}C

for 60 sec, followed by one cycle at 72^{0}C for 7min. The PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide. Expected amplicon size was produced at 672bp without any primer dimers.

Agarose Gel Purification and Sequence Verification: The Thermo Scientific Gene Jet PCR Gel Extraction Kit was used to purify the expected amplicon size on gel after electrophoresis. The expected amplicon from the PCR was cut out of the gel, purified, and used as a DNA template for further PCR amplification. Thermo Scientific Gene JET PCR Purification Kit purified the PCR productsand sequencing was done using primer pair in both orientations using the ABI Prism DNA sequencer using the sequencing protocols (Applied Biosystems, Perkin-Elmer Corp., Foster City, CA). The consensus coat protein sequence of the present study was compared and analysed by with those of the coat protein sequences obtained from Genbank databases.

RESULTS

Sampling and Field Scrutiny: The symptoms related to CTV such as stem pitting or quick decline were not observed clearly during the sampling from trees. Few of the samples that were collected on the basis of the symptoms showed vein clearing symptom and weak stem pitting. Other samples collected were asymptomatic for CTV but showing leaf curling, cupping, yellowing and protruding bark. All the samples collected were grafted on the Rough Lemon rootstock, which is known to be resistant to CTV. But the most infected group that showed positive results for CTV was Sweet Oranges group.

RNA extraction from Plants: Total RNA was extracted from both the symptomatic and asymptomatic citrus trees to check the presence of CTV in the Pakistani citrus trees. The three extraction procedures were used to isolate the total RNA from the petiole of the leaves. The procedure described above proved to yield a good quality of RNA that was proved by the NanoDrop analysis as well as 37% formaldehyde agarose gel electrophoresis (Figure 1). Also the freshly collected plant tissues yield a high quality of RNA as compare to the frozen plant tissue (Table 2).

Detection of CTV by RT-PCR: The presence of CTV T36 isolate was confirmed by RT-PCR. A 672bp DNA fragment corresponding to the major coat protein gene of the virus was amplified with 25 samples out of 250 samples (Figure 2). Both the symptomatic as well as the asymptomatic samples gave the positive results. The infection rate was recorded to about 10% in the Punjab region of Pakistan. The citrus group that showed positive results for CTV infection was Lemon & Lime, Sweet Oranges, Mandarins and Kumquats (Table 3). Among 30 Grapefruit plants tested, only 2 of them became positive for CTV and hence the infection rate was 6.6%. Similarly 12 Kumquat samples were tested for CTV and none of them were positive for CTV with 0 infection rate. Out of 32 Lemons and Limes plants, only 1 of them was positive. About 70 plants belonging to the Mandarin group were tested giving out only 2 positives for CTV. The major group that was found infected for CTV with 20 positives out of 100 was Sweet Orange with an infection rate of 20%. None of the rootstocks tested were found positive for CTV. Hence the total infection rate was found to be 10% for CTV T36 isolate coat protein unit.

CTV CP Gene Phylogenetic Analysis: Genbank (NCBI) has an abundant of coat protein sequences from all over the world. A neighbor-joining tree of the complete sequence of the major coat protein gene of the twenty CTV isolate from the Genbank along with sequence obtained from T36 CPU of the Pakistani germplasm was generated using MEGA7 (Kumar *et al*., 2016; Figure 3).

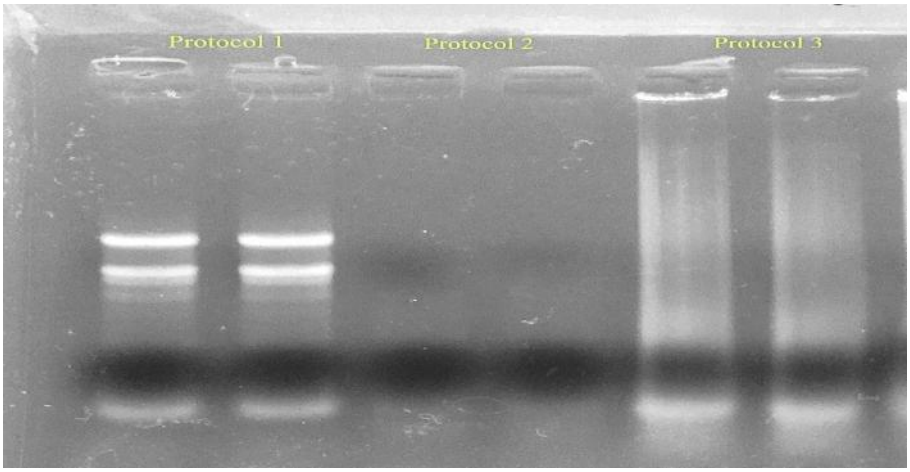

Fig. 1. Plant RNA on 37% formaldehyde agarose gel electrophoresis.

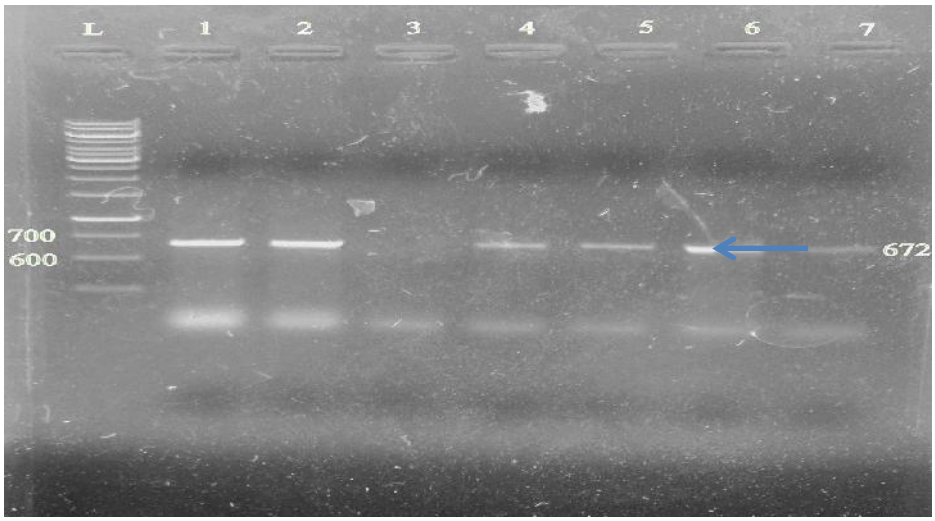

Figure 2. Amplification of CTV isolate from different samples of Citrus. L: 1kb Ladder; 1, 2, 4, 5, 6 showing positive results.

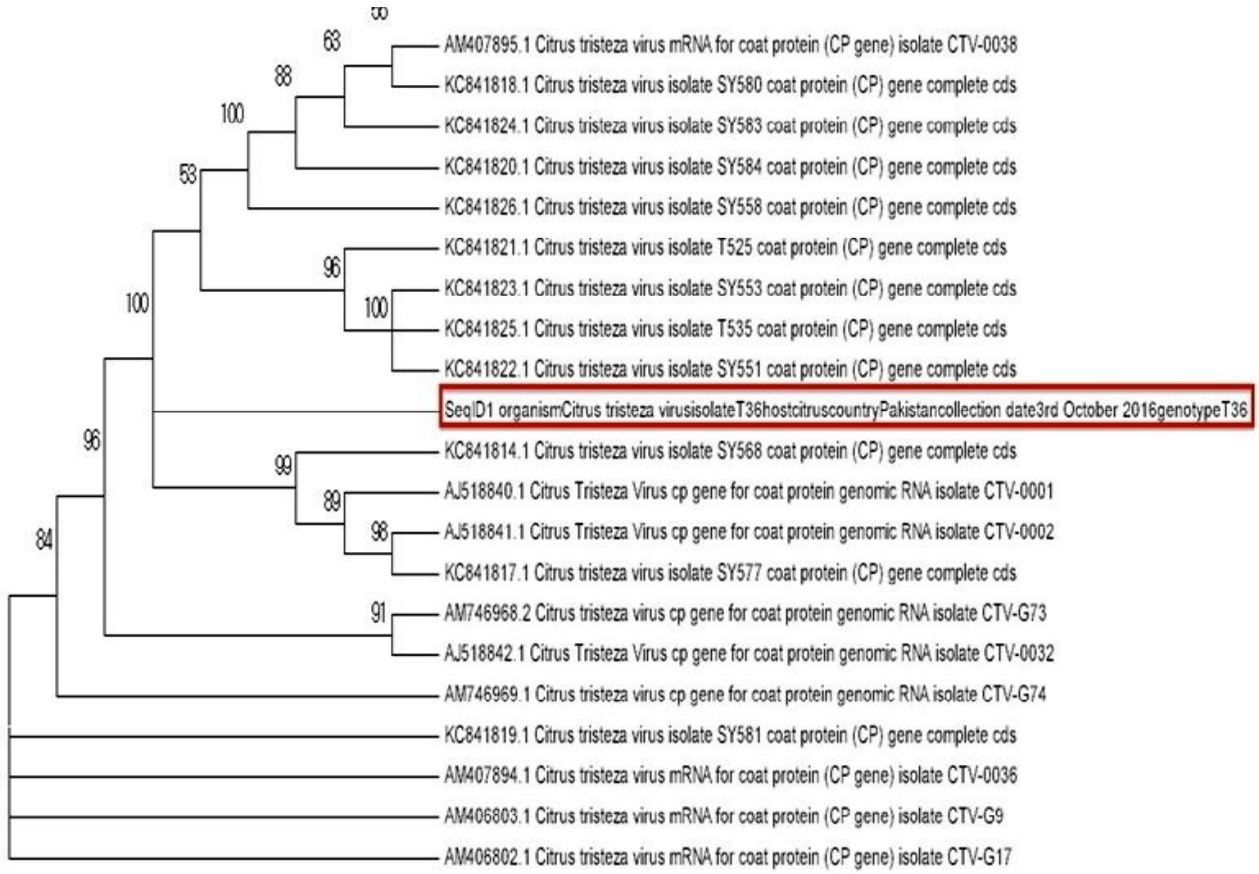

Figure 3. Evolutionary relationships of taxa: The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.29811344 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 672 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Table 1. Sequence of forward and reverse primer used for conventional RT-PCR.

Primer	Sequence 5'–3'	Expected amplicon size (bp)	Primer location
T36 CP-F (Forward)	ATGGACGACGAAACAAAGAAATTG	672	16,152–16,176
T36 CP-R (Reverse)	TCAACGTGTGTTGAATTTCCCA		16,802–16,823

Table 2. Comparison of the total RNA (ng/ul) from the Frozen and Fresh Plant tissues.

S. No.	Tissue Type	Average RNA concentration(ng/ul)
1	Fresh petiole	348.00 (Highest: 2013.2; Lowest: 12.35)
2	Frozen petiole	159.25 (Highest: 901.7; Lowest: 7.33)

Table 3. Detection of CTV via RT-PCR from various citrus species grown in Punjab.

Sr. No.	Citrus Group (Scion/Rootstock)	No. of samples tested	CTV positive	CTV negative	% Infection
1	Grapefruit/ Rough Lemon	30	2	28	6.6
2	Kumquat/ Rough Lemon	12	0	12	0
3	Lemon & Lime/ Rough Lemon	32	1	31	3.1
4	Mandarins/ Rough Lemon	70	2	68	2.9
5	Sweet Oranges/ Rough Lemon	100	20	80	20
6	Rootstocks	6	0	6	0
	Total	**250**	**25**	**225**	**10**

DISCUSSION

The present study conducted the first comprehensive national survey for the detection of CTV in Pakistan. The occurrence of the T36 isolate of CTV in the Punjab province that is the major citrus producing area of Pakistan was determined and confirmed with RT-PCR. The infection rate was 33% based on RT-PCR results, among which 20% infection rate was found in the Sweet Oranges varieties in the region. Previously studies were performed and reported using serological technique such as ELISA Preliminary survey conducted by a group of Italian and Pakistani experts in 1988, citrus in Pakistan was reported to be infected by a number of virus and virus-like diseases in KPK and Punjab province (Catara *et al*. 1988) and only CTV was confirmed by enzyme-linked immunosorbent assay (ELISA) and electron microscopy in a few locations in the country (Arif *et al*. 2005). But the studies later on suggested that the serological techniques i.e. ELISA are not enough to diagnose a disease. Hence a molecular technique such as PCR was introduced to detect and characterize a particular disease. Many researchers have showed comparison of ELISA and RT-PCR for CTV detection and they proved that more citrus trees were found to be infected using RT-PCR than by using ELISA. This was not surprising because RT-PCR is more sensitive than ELISA and the results were consistent with comparative studies of ELISA and RT-PCR for CTV detection (Cambra *et al*. 2002).

The total RNA recovered from the fresh and frozen tissues of citrus were also compared by Saponari *et al*. (2008) and reported the differences in amount of RNA recovered from fresh, frozen, and desiccated tissues. Desiccation and freezing resulted in low amounts of total RNA extracted.

The incidence of CTV previously reported in Pakistan with ELISA was lower to the incidence found during the present study because of the RT-PCR sensitivity and specificity (Arif *et al*. 2005,Hung *et al*. 2000). The infection of T36 isolate in the Sweet Oranges has been reported worldwide using ELISA, RT-PCR and qRT-PCR (Powell and Lin, 2005, Folimonova *et al*. 2008, Dawson *et al*. 2015, Bergua *et al*.2016). T36 isolate is renowned for quick decline symptom in citrus trees but the severity may vary from less severe decline, stunting, seedling yellow leading to chlorosis and reported previously.

Conclusions: This study presented the first national survey of CTV in Pakistan on the molecular identification and characterization of T36 isolate from major citrus growing region of Punjab. Sequencing the major CP gene of CTV isolates from this region of Pakistan will provide more information on the molecular properties of this isolate.Further work is required to identify and differentiate the isolates of CTV that are present in Pakistan through molecular characterization. Spread of disease in the orchards may be attributed to the presence of some aphid vectors that needs to be controlled by some means. A lot of work is required to be done in this aspect to save the citrus industry from this devastating virus.

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