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Full Length Article

Variation in the Coat Protein Gene Among *Citrus Tristeza Virus* Isolates from Khyber Pakhtunkhwa and Punjab Provinces, and Islamabad Capital Territory of Pakistan

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Abstract

Citrus tristeza virus (CTV) is a plant pathogenic virus belonging to the genus *Closterovirus* and family *Closteroviridae*. It is transmitted by vegetative propagation and by several aphid species. It has been reported that CTV has killed millions of citrus trees worldwide. CTV has previously been reported in Pakistan. Symptom based sampling was carried out from the orchards of citrus rich districts of Punjab and Khyber Pakhtunkhwa (KPK) provinces of Pakistan and screened by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) and 48 samples were positive. The major coat protein-coding gene of the positive samples were amplified, sequenced and phylogenetic analysis was carried out. Nucleotide sequence analysis revealed 90–100% similarity within indigenous 48 isolates, 91.1 to 100% similarity with six isolates previously reported from Pakistan and 97.7 to 99.7% similarity with T3 USA, VT USA, VT Israel, RB New Zealand, VT India and an Indian isolate of unknown genotype. A maximum likelihood phylogenetic tree indicated that the CTV population is diverse in Pakistan with different isolates consisting of one major isolate of T3, and three minor isolates of VT, RB and recombinant VT IND genotypes. The most common group (T3 like) is comprised of 36 of samples and is dispersed all over the country irrespective of the region and province. The remaining three groups are related to VT Israel, VT India and RB New Zealand confined to specific regions. These results indicated that a citrus virus free certification program could reduce the risk of spreading these isolates. © 2019 Friends Science Publishers

Keywords: Citrus; DAS-ELISA; PCR; Phylogenetic analysis; Genotype

Introduction

Citrus tristeza virus (CTV) is member of the genus *Closterovirus* and family *Closteroviridae*. It is a phloem associated plant virus with the largest RNA plant virus genome and the second largest among all other viruses (Bar-Joseph *et al*., 1979; Febres *et al*., 1996) and is widely spread in all citrus growing areas of the world, especially where sour orange was used as a rootstock. The virus is spread by vegetative propagation through grafting (Dawson *et al*., 2015) and is spread by aphids in a semi-persistent manner (Bar-Joseph *et al*., 1989). The prominent aphid species known to transmit CTV are *Aphis gossypii*, *A. spiraecola* and *Toxoptera citricida*. Out of these species *T. citricida* is reported to be more efficient in viral transmission (Roistacher and Bar-Joseph, 1987) and considered to be majorly responsible for the spread of CTV in citrus rich regions including South America, Australia, Asia and South Africa (Albiach-Marti *et al*., 2000). In the past CTV has caused the death of millions of citrus trees worldwide with more than 30 million trees reported in Brazil and Argentina during the 1930s, 10 million trees in Spain during the 1960s and 6.6 million trees in Venezuela during the 1980s (Rocha-Pena *et al*., 1995). It causes a range of symptoms on the host plant including vein clearing, stem pitting, seedling yellowing and rapid decline of trees. Mild isolates produce vein-clearing symptoms (Bar-Joseph *et al*., 1989). Stem pitting and quick decline are believed to be caused by severe isolates.

The CTV virion is filamentous with particles size of 2000 nm long and 12 nm wide (Kitajima *et al*., 1964). The viral genome consists of positive sense single stranded RNA which is non-segmented and vary from 19,226 to 19,302 nucleotides. The 3' half of the genome is relatively conserved with 90% sequence identity, on the other hand the 5' half of the genome has less than 70% sequence similarity (Ayllon *et al*., 2001). The genome contains 12 open reading frames (ORFs) encoding 19

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proteins and untranslated regions (UTRs) at both 5' and 3' ends (Karasev *et al*., 1995; Vives *et al*., 1999). The two 5' end open reading frames (ORFs) encode replication machinery encoding helicase (HEL), RNA dependent RNA polymerase (RdRp) and Methyl transferase (MT) domains and are directly translated from genomic RNA (Karasev *et al*., 1995) Ten 3' open reading frames (ORFs) are expressed from sub genomic RNAs (Hilf *et al*., 1995). The genome contains two different coat proteins encoded by two open reading frames (ORFs). A major coat protein encapsidates about 95% of the genome and a minor coat protein which encapsidates only 5% of the genome (Febres *et al*., 1996). Many phylogenetic studies using the coat protein gene sequences have been performed to assess the variability among CTV isolates.

In Pakistan, prevalence of CTV has long been suspected. In 1987 it was confirmed through enzyme linked immunosorbent assay (ELISA) and electron microscopy from the citrus rich provinces *i.e.,* Punjab and KPK (Grimaldi and Catara, 1989). The presence of virus and virus-like diseases have been reported in ten major citrus growing districts of KPK and most of the orchards are declining in both KPK and Punjab provinces (Arif *et al*., 2005; Iftikhar *et al*., 2009). Incidence of CTV infection increased in KPK from 16 to 48% from 2002 to 2010 and one of the main reasons for the decline of citrus orchards is the use of virus infected sour orange and rough lemon root stock (Arif *et al*., 2015). Despite the extensive presence of CTV, there was a lack of knowledge about the genetic diversity of this virus from the two major citrus rich provinces in Pakistan. This study was, therefore, conducted to study the variability in the local populations of citrus tristeza virus (CTV) present in the regions of two major citrus producing provinces of Pakistan.

Materials and Methods

Sampling and Serological Detection

The leaf and bark samples of 70 adult plants showing decline and stem pitting symptoms were collected from orchards in Islamabad Capital Territory (ICT), near six cities of Punjab including Sargodha, Bhalwal, Kot Momin, Faisalabad, Toba Tek Singh, Fateh Jang and six cities of KPK including Mardan, Nowshera, Sawabi, Khanpur, Haripur and Peshawar (Fig. 1). Samples were collected during March 2015 to March 2016. Samples were taken from four citrus species including: 25 sweet orange (Mosambi), 10 hybrid mandarin (Kinnow), 10 lemon (Kaghzi Lemon) and 25 Mandarin (Feuterell's Early). Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was performed (Clark and Adams, 1977) using the CTV DAS-ELISA kit (BIOREBA). Absorbance readings were recorded by microplate reader (Multiskan EX, V.2.3, Thermo Fisher Scientific) at 405 nanometers (nm) after one hour incubation.

Total RNA Extraction and cDNA Synthesis

Total RNA was purified by the Tri-Reagent method from positive samples (Chomczynski, 1993). Isolated RNA from each sample was used to synthesize the cDNA with coat protein (CP) gene specific reverse primer (5-′TCAACGTGTGTTGAATTTCC-3′ Ammar *et al*., 2013) by using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA). The cDNA was purified (PureLink PCR Purification Kit, Thermo Fisher).

Amplification, Cloning and Sequencing of CTV CP Gene

The CTV major CP gene specific primers (FORWARD:5′-ATGGACGACGAAACAAAGA-3′) and (REVERSE:5′-TCAACGTGTGTTGAATTTCC-3′) (Ammar *et al*., 2013) were used in a PCR reaction (GoTaq, Promega, Madison, WI, USA) using 10 μM of forward and reverse primer. The PCR cycle consisted of 94ºC for 5 min, followed by 30 cycles of 94ºC for 30 sec, 52ºC for 30 sec and 72ºC for 1 min followed by a final extension of 72ºC for 10 min. The products were verified by agarose gel electrophoresis. Amplified PCR products were purified (PureLink Quick PCR Purification Kit, Invitrogen) ligated and cloned (pGEM-T Easy Vector Systems, Promega). Single individual clones per PCR product were sequenced in each direction (MCLAB, South San Francisco, CA USA). The sequences were trimmed and assembled using Sequencher version 5.1 (Gene Codes Corp., Ann Arbor, MI, USA) and the consensus sequences were submitted into Gen Bank.

Phylogenetic Analysis

Multiple sequence alignment of the sequences were carried out by ClustalW (Larkin *et al*., 2007) using 19 reference sequences including 5 from USA, 2 from New Zealand, 3 from India, 1 from Spain, 1 from China, 1 from Israel and 6 previously reported from Pakistan. The genetic relationship of these isolates with our 48 isolates was examined using the maximum-likelihood algorithm using the Molecular Evolutionary Genetic Analysis v. 7.0 (Mega7) software (Kumar *et al*., 2016). Bootstrap values generated using 1000 puzzling steps calculated by the JTT matrix-based model (Jones *et al*., 1992). Nucleotide sequences were translated by the web-based program ExPASy (http://web.expasy.org/translate/). Amino acid sequences were also aligned and analyzed as above.

Results

CTV Specific Symptoms and Serological Detection

CTV related symptoms including decline and stem pitting (Fig. 2) were observed in orchards of cities of KPK and Punjab provinces and 70 samples were collected. Forty-eight samples were found positive by DAS-ELISA including 5 from Sargodha, 1 from Bhalwal, 1 from Kot

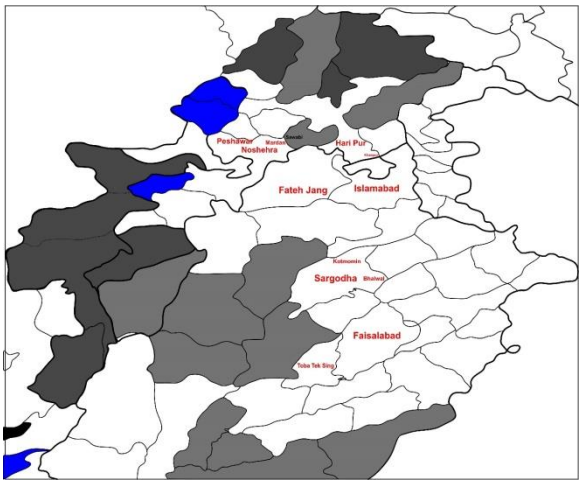

Fig. 1: A map of KPK and Punjab provinces of Pakistan showing the regions from where the samples were collected

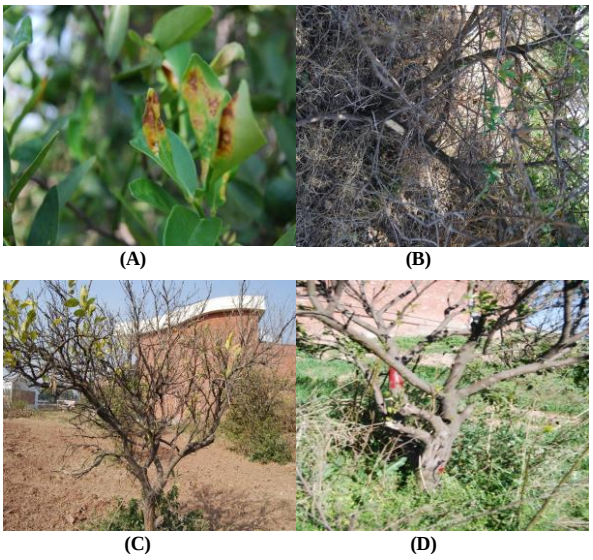

Fig. 2: Symptoms observed during the survey to collect samples for CTV screening. These plants were positive for CTV infection by DAS-ELISA. **(A)** Necrotic symptoms on lemon plant observed in Sawabi. **(B)** Declining of lemon plant observed in Khanpur. **(C)** Declining of feuterell's early plant observed in ICT. **(D)** Stem Pitting on sweet orange (Mosambi) plant observed in ICT. Depressions can be seen by focusing on lower part of the stem

Momin, 2 from Faisalabad, 2 from Toba Tek Singh, 13 from ICT, 1 from Fateh Jang, 2 from Mardan, 5 from Nowshera, 5 from Sawabi, 7 from Khanpur, 3 from Haripur and 1 from Peshawar.

There were 18 infected sweet orange (Mosambi), 13 Mandarin (Feuterell's early), 9 hybrid mandarin (Kinnow) and 8 lemon (Kaghzi lemon). Spectrophotometric values varied from 0.4 to 1.9 and all infected samples had an absorbance no less than 0.4 and minimally two times larger than the negative control.

Amplification and Sequence Analysis of CTV CP Gene

The major CP gene of CTV was amplified from 48 samples found positive by DAS-ELISA. A full-length fragment of 672 bp was revealed. Products were cloned and sequenced. Sequences were submitted in GenBank and accession numbers were obtained (Table 1). Pairwise percentage nucleotide identity within isolates of this study revealed similarity of 90 to 100% and 91.1 to 100% with six previously reported Pakistani isolates (Atta *et al*., 2017). Whereas when nucleotide sequences from isolates of the present study were compared with thirteen isolates from other countries of the world, it revealed that 36 isolates irrespective of any specific region of two provinces showing 97.7 to 99.1% nucleotide identity with T3 USA and 98 to 99% identity with an Indian isolate of unknown genotype. Two isolates from Khanpur city of KPK showed identity of 98.3% with the RB New Zealand. Four isolates from Nowshera and Sawabi cities of KPK showed 98.3 to 98.9% identity with VT USA and 98 to 98.6% identity with VT Israel. Six isolates including five from ICT and one from Sargodha cities of Punjab showed 98.8 to 98.9% identity with VT India.

Nucleotide sequence alignment of the 48 isolates revealed 127 variable positions when compared with the consensus sequence possessed by most of the isolates. Alignment of protein sequences showed 36 variable positions out of which 22 are conservative replacements and 14 are radical replacements. Seventeen isolates showed radical replacement of amino acids including Sawabi10 which showed six radical replacement followed by four in Nowshera11, Nowshera27 and Nowshera43, three in Khanpur64 and Khanpur85, two in Islamabad14, Islamabad25, Islamabad33, Islamabad36, Islamabad37 and Sargodha86. Islamabad41, Khanpur45. Fatehjang6, Tobateksingh12 and Haripur6 only showed one such replacement. Protein sequence of all isolates contained phenylalanine at position 124 that is the only amino acid which determines the reactivity of severe isolate with MCA13 monoclonal antibody (Pappu *et al*., 1993).

Phylogenetic Analysis

The phylogenetic tree constructed by using nucleotide sequences of the isolates from present study and 19 representative reference isolates (including six previously reported from Pakistan) indicated that Pakistani isolates are closely related to the worldwide isolates (Fig 3). A divergence pattern was observed in the phylogenetic tree. Two clear distinct lineages appeared. Pakistani isolates appeared in four different groups with the genotypes T3 USA, RB New Zealand, VT Israel and VT India. Forty-two Pakistani isolates,

Table 1: Name, genotype/isolate and accession numbers of the isolates used in this study. First nineteen are the reference sequences from GenBanK

Region	Isolate name/Genotype	Accession number	Region	Isolate name	Accession number
India	ARP1	LN997804.1	ICT	Islamabad12	MF498908
USA (Florida)	T30	EU937520.1	Nowshera (KPK)	Nowshera27	MF498909
USA (Florida)	T36	KC517485.1	Peshawar (KPK)	Peshawar08	MF498910
USA (Florida)	T3	KC525952.1	ICT	Islamabad14	MF498911
USA (Florida)	T68	JQ965169.1	ICT	Islamabad25	MF498912
New Zealand	T68	FJ525436.1	ICT	Islamabad33	MF498913
China	T68	JQ911663.1	ICT	Islamabad36	MF498914
India	T68	EU076703.3	ICT	Islamabad37	MF498915
New Zealand	RB	FJ525435.1	Nowshera (KPK)	Nowshera34	MF498916
Spain	T30	Y18420.1	Sargodha (Punjab)	Sargodha71	MF498917
India	VT	HM573451.1	Sargodha (Punjab)	Sargodha92	MF498918
USA (Florida)	VT	KC517494.1	Khanpur (KPK)	Khanpur32	MF498919
Israel	VT	U56902.1	Khanpur (KPK)	Khanpur43	MF498920
Pakistan	108	HQ329188	Khanpur (KPK)	Khanpur64	MF498921
Pakistan	109	HQ329189	Khanpur (KPK)	Khanpur85	MF498922
Pakistan	131	HQ329190	Nowshera (KPK)	Nowshera51	MF498923
Pakistan	143	HQ329193	Sawabi (KPK)	Sawabi29	MF498924
Pakistan	159	HQ329195	Sawabi (KPK)	Sawabi43	MF498925
Pakistan	179	HQ329197	Khanpur (KPK)	Khanpur40	MF850323
ICT	Islamabad91	MF461395	Khanpur (KPK)	Khanpur45	MF850324
Mardan (KPK)	Mardan101	MF327273	Haripur (KPK)	Haripur6	MF850325
Khanpur (KPK)	Khanpur20	MF461396	Sargodha (Punjab)	Sargodha86	MF850326
Mardan (KPK)	Mardan120	MF461397	Toba Tek Singh (Punjab)	Tobateksingh3	MF850327
ICT	Islamabad200	MF461398	Haripur (KPK)	Haripur9	MF850328
Bhalwal (Punjab)	Bhalowal54	MF461399	Sawabi (KPK)	Sawabi21	MF850329
Sawabi (KPK)	Sawabi19	MF461400	ICT	Islamabad41	MF850330
Kot Momin (Punjab)	Kot Momin26	MF461401	Faislabad (Punjab)	Faislabad44	MF850331
Sargodha (Punjab)	Sargodha22	MF461402	Sawabi (KPK)	Sawabi10	MF850332
ICT	Islamabad40	MF461403	Nowshera (KPK)	Nowshera43	MF850333
Faislabad (Punjab)	Faislabad65	MF461404	ICT	Islamabad100	MF850334
ICT	Islamabad46	MF461405	Fateh Jang (Punjab)	Fatehjang6	MF850335
Sargodha (Punjab)	Sargodha16	MF498904	Haripur (KPK)	Haripur4	MF850336
Toba Tek Singh (Punjab)	Tobateksingh12	MF498905			
ICT	Islamabad10	MF498906			
Nowshera (KPK)	Nowshera11	MF498907			

including 36 of the present study and six previously reported appeared with an Indian isolate of unknown genotype in the same group with T3 USA. Four isolates are closely related with VT Israel. Two isolates showed relatedness with RB New Zealand. Six isolates appeared with the VT India. Isolates including T36 genotype from USA, T68 from four different countries and T30 from two countries are not closely related to any isolate from Pakistan.

Discussion

Citrus tristeza virus (CTV) coevolved with the host plant and humankind has widely distributed this virus through the onset of commercial citrus industries requiring the production and distribution of superior citrus varieties (Bar-Joseph *et al*., 1989). In the beginning of citrus cultivation, the transport of citrus plants was not easy and it was done primarily through seeds. CTV cannot spread through seed but later when whole plants or cutting of plants were transferred between different regions of the world it caused the distribution of genotypes of this virus throughout citrus producing regions of the world. CTV has been detected in Pakistan by transmission electron microscopy (Grimaldi and Catara, 1989), serology (Arif *et al*., 2005; Iftikhar *et al*., 2009; Ammar *et al*., 2013) and sequence analysis (Atta *et al*., 2017). However, these diagnostic methods do not indicate the genetic diversity of CTV in Pakistan.

This study reveals the wider relationship of isolates of CTV from citrus growing areas in Punjab and KPK provinces of Pakistan with genotypes from around the world. The coat protein gene sequences of 48 isolates were used for the phylogenetic analysis. Slight differences in the CP gene sequences are known to correlate with the biological activity and geographical origins of the isolates of CTV (Herrera-Isidron *et al*., 2009). The nucleotide sequence information is the most authentic procedure for the estimation of genetic variation (Rubio *et al*., 2001) and CP gene sequences has previously been used for differentiation of mild and severe genotypes (Pappu *et al*., 1993; Niblett *et al*., 2000; Cevik *et al*., 2013). CTV classification has been grouped previously into six genotypes along with one putative genotype HA16-5 (Harper, 2013), so the coat protein (CP) gene sequences of the representative isolates of six

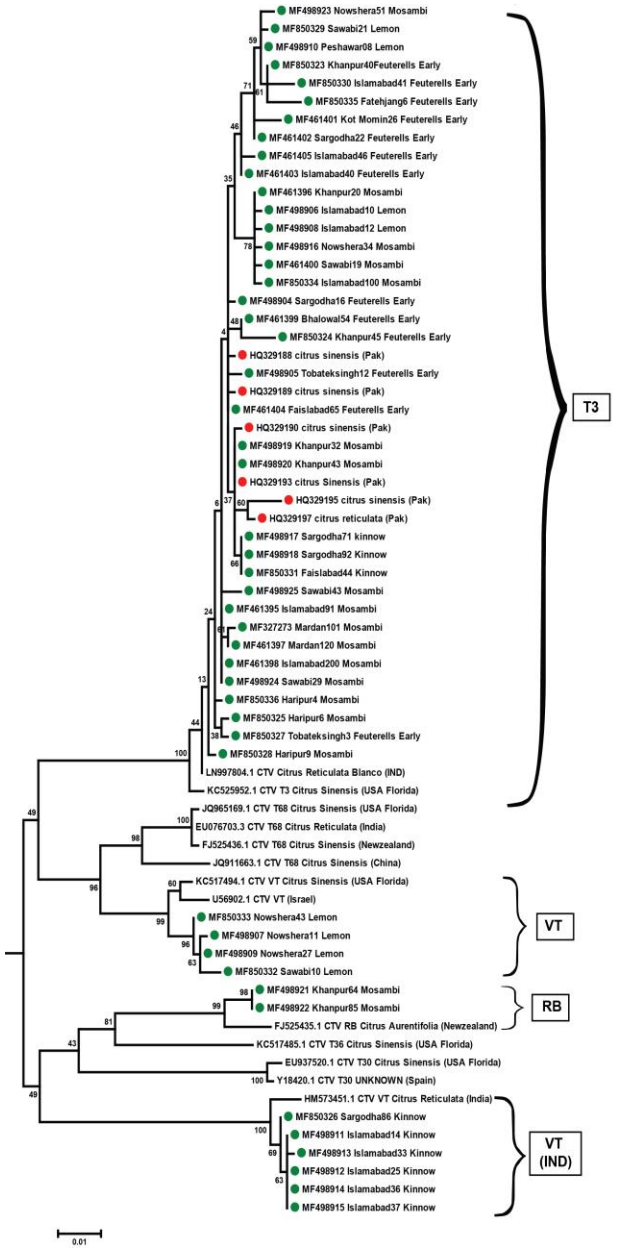

Fig. 3: A maximum likelihood phylogenetic tree generated by MEGA version 7.0. Bootstrap value from 1000 replicates is mentioned at each node. Indigenous Isolates representing accession numbers, Isolate name and host variety. Country of origin is also mentioned with reference isolates. Isolates of present study are labeled with green circles whereas previously reported isolates from Pakistan are labeled with red circles

genotypes from different countries was used to identify the prevalent genotypes in Pakistan's citrus producing regions.

Isolates of CTV can be differentiated based on reactivity with specific monoclonal antibodies (MABs) and mutagenic analysis revealed that a particular replacement of amino acid in specific epitopic domain of coat protein could bring a change in the reactivity of particular MAB (Wu *et al*., 2014). Most of the severe isolates are reactive to MAB MCA13 whereas mild isolates generally do not react with this particular antibody (Permar *et al*., 1990). The worldwide isolates of CTV can easily react with monoclonal antibody 3DF1 and their reactivity is determined by three residues *i.e.,* aspartic acid, lysine and phenylalanine at position 2, 4 and 13 of the major coat proteins (Pappu *et al*., 1995). It was observed that 46 out of 48 isolates of the present study contain these specific residues but we have found an exception in two isolates including Sawabi21 and Haripur4. These two isolates contain glutamic acid at position 2 instead of aspartic acid. This replacement in these two isolates does not seem to bring a major structural change in the coat protein because the two alternating residues belong to the same group of negatively charged amino acids. The only difference between the two is an extra carbon in the R chain of glutamic acid. There is only one amino acid difference at position 124 of the coat protein between MCA13 reactive and non-reactive isolates, the reactive isolates should contain phenylalanine at position 124 (Pappu *et al*., 1993). All of our 48 isolates contain phenylalanine at this particular position, which indicated that these isolates are severe and can easily react with MAB MCA13. The epitope for MAB 4H6 has been reported between nine C-terminal amino acids from 215 to 223 (Nikolaeva *et al*., 1996). In our study, we have examined the replacement of amino acids with different properties in positions 215 of isolate Islamabad41, 222 of isolate Khanpur45 and 223 of isolate Tobateksingh12. Single radical replacement in an epitope consist of only nine amino acids increase a chance of causing a change in the epitopic structure and difference in reactivity of these three isolates and all other isolates with MAB 4H6.

The nucleotide identity within isolates of this study was 90–100%, which may be due to transfer of bud wood between the nearby cities by local farmers. Average nucleotide identity of major CP gene between all known genotypes of CTV was calculated and maximum identity of 96.4% was observed between isolates of VT and T68 genotypes (Harper, 2013). About 97.7 to 99.7% nucleotide similarity was observed between our 48 isolates with T3 USA, V.T., USA, VT Israel, VT India, RB New Zealand and an Indian isolate of unknown genotype. This indicates that Pakistani isolates are related to three world known genotypes *i.e*., T3, VT and RB.

The phylogenetic tree also revealed close relatedness of Pakistani isolates with these three genotypes. Previously it was observed that based on major CP gene six Pakistani isolates from three cities of Punjab are related with VT Israel (Atta *et al*., 2017). However, our phylogenetic tree showed the most prevalent genotype is T3 (consisting of 36 of our isolates and six previously reported Pakistani isolates) while VT Israel is in a separate sub-clade related to only four of our isolates and one USA isolate. T3 clade consisting of a total of 44 isolates was supported with a

bootstrap value of 49%. Interestingly the T3 clade divided into two sub-clades consisted of a single USA isolate and an Indian isolate with 42 Pakistani isolates. This grouping was strongly supported with a bootstrap value of 100%. These results indicated that the T3 is the major genotype of CTV in Pakistan and is present in all major citrus producing regions of the country. The transfer of citrus germplasm from the USA to the sub-continent before the division of India and Pakistan has likely resulted in the presence of both the T3 genotype reported from the USA along with an Indian isolate of unknown genotype that share a high similarity with many Pakistani isolates obtained in this study. Four isolates from KPK are also closely related with VT Israel and VT USA suggesting an introduction of that genotype from one of those countries. In addition, two isolates from the same province are closely related with RB New Zealand so it is likely these genotypes are confined to those cities in KPK. Interestingly isolates most closely related to VT India isolates are confined to samples obtained from Punjab, which shares a border with India.

A recombinant Indian Isolate KPG3 was sequenced recently and was a member of VT genotype (Biswas *et al*., 2012), while phylogenetic analysis based on full genome placed this isolate in the same group with VT Israel and VT USA (Harper, 2013). However, based on major CP gene we have found that this isolate appeared with six of our isolates in a separate clade. This clade was supported with a bootstrap value of 49% and supported the hypothesis that recombinant isolates are also present in Pakistan, but a full genome study is required to verify this hypothesis.

From this phylogenetic study of Pakistani CTV coat protein p25 gene we have identified four major clades of CTV related to isolates from other major citrus producing countries including the USA, India, New Zealand and Israel suggesting the virus has been introduced on separate occasions from various regions of the world.

Conclusion

CTV isolates from the major citrus producing provinces of Pakistan *i.e*., Punjab and KPK are most closely related to three genotypes from the USA, India, New Zealand and Israel. Majority of isolates sequenced in this study (36) plus six previously sequenced Pakistani CTV isolates grouped into a single sub-clade. This indicated the distribution of CTV through the exchange of infected buds and tree cuttings between farmers in this region and transmission by the aphid vectors. Certified virus free planting bud wood and rootstock and virus screening of trees provoke the farmers wish to take cuttings that may help reduce the incidence of this virus in Pakistan. Certification schemes would reduce the risk of introducing further CTV isolates from other citrus producing regions of the world and reduce the spread of isolates between citrus growing regions of Pakistan.

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