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Nucleotide sequence of ORF 11-p23 suppressor gene of *Citrus tristeza virus* isolate of different citrus growing states of India and determination of extensive genetic diversity

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Citrus tristeza virus (CTV), a member of the genus *Closterovirus* is responsible to cause tremendous losses of citrus industries in India and worldwide. The virus particles are flexuous long filamentous, contain positive sense long monopartite single stranded RNA and transmitted by citrus aphid particle (*Toxoptera citricida*). In present study, twelve Indian CTV isolates, AP3, AP5, D5, D7, JRT1, JRT12, M1, N1, N15, RS1, TK1 and TK5 from different citrus growing region of India were characterized based on full length (630 nt) ORF11 suppressor gene (sup gene). The sup genes were sequenced and analyzed with other Indian and international isolates. The present Indian CTV isolates shared 88-100% nucleotide identity and 87-100% amino acid identity among them. The present isolates were distributed in to three genogroups and all the isolates studied in this study fell into six genogroups. Recombination analysis using a recombination detecting program, RDP3 could not identify recombination events in ORF11 of any of the Indian isolates. However, present study suggests that ORF11 suppressor gene of some international isolates (T30 and RB-G90) showed recombination site. The present study revealed the extensive genetic diversity in suppressor gene of CTV in India.

Key words: Citrus, *Citrus tristeza virus*, ORF11, suppressor gene, genetic variability

INTRODUCTION

Citrus tristeza virus (CTV), a member of the genus Closterovirus, family Closteroviridae, causes huge losses to citrus trees worldwide. The virions are flexuous filamentous particle, measuring 10–12×2000 nm in size and transmitted by brown citrus aphid (*Toxoptera citricida*) in semi persistent manner (Bar-Joseph and Dawson, 2008). CTV infects most of the cultivated citrus species, hybrids and citrus relatives and produces various kinds of symptoms like vein clearing, vein corking, seedling yellowing, stem pitting and decline of plants, depending on scion/rootstock combinations, virus strain and environment (Lee and Bar-Joseph, 2000).

Citrus is cultivated in all the geographical zones of India and CTV occurs in all the areas wherever citrus is grown. The overall CTV incidence was reported to be 26.3% in Vidarbha region, 47.1-56.0% in Northeast, 36-50% in South and 16-60% in North-Northwest India (Biswas *et al.* 2014). All

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the commercial citrus species and its hybrids grown in different areas of India are infected by CTV and this disease has killed more than 1 million citrus trees in India (Ahlawat, 1997; Biswas, 2008).Genetically and in biological host reaction, CTV is diverse in nature thus occurring several divergent of CTV genotypes in the world including India. Extensive genetic variation of CTV in Indian has been reported. Six to eight CTV genotypes analyzing several isolates using sequence of CP gene and 5' ORF1a gene fragment have been identified (Biswas *et al.*, 2012a; Sharma *et al.*, 2012).

CTV genome contains positive sense, monopartite ssRNA of 19.3 kb length containing 12 ORFs (ORF1a and 1b and ORF 2-11) potentially encoding at least 19 putative proteins (Bar-Joseph and Dawson, 2008). The genome contains at least three suppressor genes, importantly 32 terminal ORF11 encoding p23, ORF8 encoding p25 (coat protein) by ORF10 encoding p20 (Lu *et al.*, 2004; Soler *et al.*, 2012; Flores *et al.*, 2013). The protein p23, encoded by ORF11, is a unique protein mediating key virus-host interaction, works in intracellular level to overcome host antiviral defense and has no significant similarity between p23 and other amino acid sequences deposited in the database (Flores *et al.*, 2013). The p23 suppressor gene construct has been used for *Agrobacterium* transformation of several citrus species (Ghorbel *et al.*, 2000; Soler *et al.*, 2012) confer resistance against CTV.

In India, molecular characterization and genetic variation of ORF11 gene encoding p23 protein has not been reported so far. Therefore, in the present study CTV isolates of different citrus growing regions of India are taken, characterized them based on sequencing of full length ORF11 suppressor gene of 630 (sup gene) and genetic variations among them.

MATERIALS AND METHODS

CTV isolate

A total number of twelve CTV isolates originated from different citrus growing regions of India were maintained in different citrus hosts in insect-proof greenhouse at Advanced Centre of Plant Virology (ACPV), Indian Agricultural Research Institute (IARI), New Delhi (Table 1). CTV infection was confirmed using direct antigen coated-indirect enzyme linked immuno-sorbent assay (DAC-ELISA) protocol described earlier (Biswas, 2008).

RNA extraction, cDNA synthesis, cloning and sequencing

Total plant RNA was isolated from tender bark tissues of citrus samples using SV total RNA isolation system (Promega, Madison, USA). The first strand cDNA was synthesized using the procedure used earlier (Biswas, 2010). The complete sup genes of all the 12 CTV isolates were amplified by PCR using specific primer pairs, forward KK707 (F) 52-GTCGACATG GGAYDATACTAGCGGAC-32 and reverse KK708 (R) 52-GGATCCTCAGATGAAGTG GTGTTC-32, following the protocol described earlier (Biswas *et al.*, 2012a). The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Maryland) and cloned into T&A cloning vector system I (RBC, Taipei, Taiwan) using the standard method. Two clones of each sample isolate were sequenced using vector derived primers M13 Forward and M13 Reverse in an automatic sequencer (ABI 3011, Chromous Biotech, Bangalore). The consensus sequences were taken for further analysis.

Sequence analysis

The sup gene sequences of international CTV isolates, T36 (Florida severe, U16304), T30 (Florida mild, AF260651), VT (Israel severe, U56902), B165 (Indian severe, EU076703), HA16-5 (Hawaii isolate, GQ454870) and NZRB-G90 (New Zealand resistant breaking isolate, FJ525432) all are representative of six CTV genotypes recognized worldwide (Melzer *et al.*, 2010; Biswas *et al.*, 2012b) were used for sequence analysis. The sup gene sequence of Indian decline inducing CTV isolate Kpg3 (HM573451) reported earlier (Biswas *et al.*, 2012b) was also used for analysis. Multiple sequence alignments were carried out using the program Clustal X (version 1.81) and pair-wise homology matrix of the CTV isolates were carried out compared using Gene Doc version 2.6.002. The maximum likelihood phylogenetic relationships were determined using the program MEGA4 (Tamura *et al.*, 2007). The presence of putative recombination events were identified using recombination detection program (RDP3) implementing seven algorithms, RDP, GeneConv, Bootscan,

MaxChi, Chimera, SiScan and 3SEQ (Martin *et al.*, 2009) using default parameter values for the different detection programs. When the same recombination events were detected by two or more algorithms, they were considered as evidence of putative recombination.

RESULTS AND DISCUSSION

The complete sup genes of the present CTV isolates were amplified in PCR using specific primer pair. The amplicons of 12 isolates (Fig, 1) were purified, cloned and sequenced. The sequence of the present sup genes were analyzed and compared with other Indian and International CTV isolates. The length of the complete sup gene of all the present isolates was estimated to be 630 nt which was similar to the sup gene of other CTV isolates. The pair wise sequence analysis showed that present isolates shared 88-100% nt identity among them. A range of 87-100% nt was estimated overall including Indian and International CTV isolates. The present isolates, TK1, RS1, TK5, N1 and N15 shared 98-100% nt identity, whereas, isolates AP3, AP5, JRT1, JRT12 and M1 shared 97-99% nt identity among them (Fig, 2). The isolates D5 and D7 were found to be identical showing 100% nt identity between them. The deduced amino acid of sup gene sequences of CTV isolates was analyzed and there were 87-100% amino acid identity among the present Indian isolates and overall 87-100% amino acid identity. The pattern in sequence variation in nucleotide and deduced amino acid of suppressor genes of CTV isolates was similar (Fig, 2). Phylogenetic analysis of deduced amino acids of sup gene showed that the present isolates were distributed into three genogroups (Fig, 3). The isolates, AP3, AP5, JRT11, M1 and TK 12 fell into first group along with Israel isolate VT, isolates N1, N15, RS1, TK1 and TK5 into distinct second group, and isolates D5 and D7 into third group along with Indian isolate B165 reported earlier. The phylogenetic analysis using nucleotide sequence showed similar pattern of relationships which was obtained using deduced amino acid sequence. The present study showed four genogroups among Indian iso-

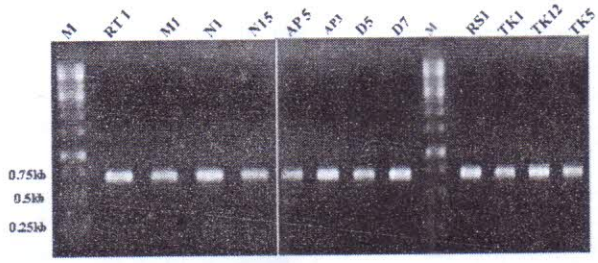

Fig. 1 : Agarose gel electrophoresis showing PCR amplification of full length of ORF 11 suppressor gene (630 nucleotide) of 12 CTV isolates using specific primers; Lane M denotes 1kb marker and other lanes denote 12 isolates of CTV

Table 1 : Characterization of ORF11 suppressor gene of CTV isolates from different citrus growing areas of India

Citrus growing states	Area	Source citrus plant	Isolate designated as	Maintained in citrus host	OD value in ELISA at 405nm (X fold)*	Accession number
Arunachal Pradesh	Lohit	Assam lemon (*Citrus lemon*)	AP3	Kagzi Lime	2.2(4.5)	KC562143
	Papum pare	Assam lemon	AP5	Kagzi Lime	1.82(4.0)	KC562142
Delhi	IARI Farm	Sweet orange (*C. sinensis*)	D5	Kagzi Lime	1.87(4.0)	KC577594
	IARI Farm	Sweet orange	D7	Sweet orange	2.32(4.0)	KF913716
Assam	Jorhat	Assam lemon	JRT1	Sweet orange	2.39(5.0)	KC562144
	Tinsukia, Citrus Research Station	Kagzi Lime (*C. aurantifolia*)	RS1	Assam lemon	2.20(4.5)	KF913715
	Tinsukia	Assam lemon	TK1	Kagzi Lime	1.21(3.0)	KC562146
	Tinsukia	Assam lemon	TK5	Kagzi Lime	1.89(4.0)	KJ094313
	Tinsukia	Sweet orange	TK12	Kagzi Lime	2.28(5.0)	KF913717
Nagaland	Mokokchung	Kagzi Lime	N1	Kagzi Lime	1.91(4.0)	KC562145
	Mokokchung	Khasi Mandarin (*C. reticulate*)	N15	Sweet orange	1.93(4.0)	KC562141
Maharashtra	Katol	Sweet orange	M1	Kagzi Lime	2.34(5.0)	KF913718

*OD value in positive control and healthy control are 2.39 and 0.48 respectively. X fold of titter compared to healthy control

lates as sup gene of Kpg3 was distinct. Overall six genogroups of CTV isolates were identified worldwide. This is for the first time to determine the sequence variability in ORF 11 suppressor gene in Indian CTV isolates. Earlier, it has been reported

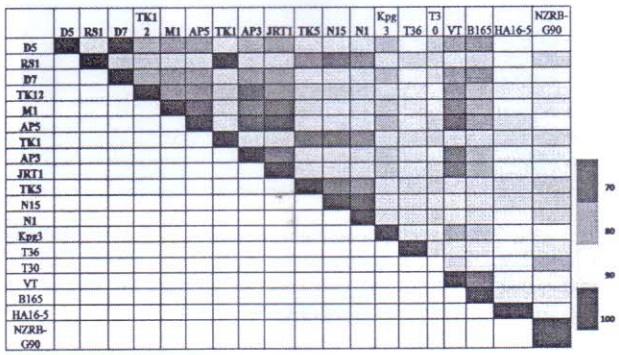

Fig. 2 : Percent deduced amino acid sequences identity of ORF 11 suppressor gene of Indian and other CTV isolates

that sequence variation is 10% or less in the 32 half among the CTV isolates and considered to be more conserved (Lopez *et al.*, 2000). However,

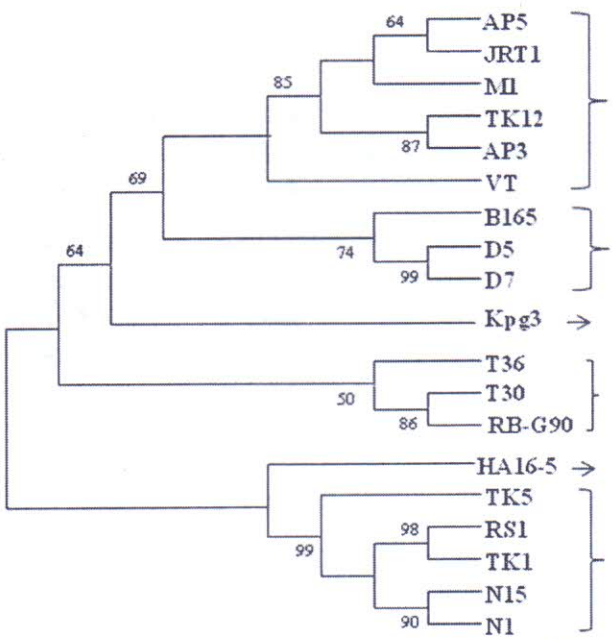

Fig. 3 : Dendrogarm showing the genetic relationship of ORF 11 suppressor gene of Indian and other CTV isolate based on deduced amino acid sequences

present study showed 87-100% nt identity sharing among the sup gene sequences of CTV of 3' half genome, suggesting sequence in 32 terminal ORF 11 sup gene of the CTV genome is not conserved showing more than 10% nt identity. These findings suggested that genetic variations in suppressor gene are responsible in changing pathogenicity in different isolates of CTV.

The present result indicated the extensive variability in sup gene in CTV isolates including Indian isolates. Several factors could contribute to such complex populations of CTV: the perennial nature of citrus plant, chronic infection for many years with 'continuous crop-host availability' with multiple infestations by aphid vectors and geneteic recombination by exchanging genetic materials among divergent sequences (Martin *et al.*, 2009; Biswas *et al.*, 2012a, 2012b). Analyzing CP gene, ORF1a fragments and complete genome, genetic recombination as a cause of evolution divergent CTV isolates in India has been reported earlier (Sharma *et al.*, 2012; Biswas *et al.*, 2012a, b; Singh *et al.*, 2013).

A recombination detecting program, RDP3 could not identify recombination events in ORF11 sup gene in the present Indian isolates, however, international isolates T30 and RB-G90 showed recombination events. The isolate RB-G90 shows strong recombination in the nucleotide position of 1-6 and 420-672nt of the ORF11 gene, that qualified by three algorithms. But recombination event in isolate T30 is very week. The ORF11 encodes p23 protein which is a unique protein and has no homologue with other amino acid sequences of the proteins deposited in the database (Flores *et al.*, 2013). As the p23 protein is unique, thus it may be one of the reason that recombination phenomenon in this gene sequences is uncommon.

Development of a molecular-based management strategy for plant virus requires a clear understanding of the genetic variability of the virus isolates. In the present study, genetic diversity in CTV populations in India based on ORF11 suppressor gene was examined, and suppressor gene-based distinct CTV groups were identified, that led to develop group specific suppressor gene construct, that may be used for transformation of citrus plant to develop citrus plant resistant to CTV.

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