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Research Article

INCIDENCE AND MOLECULAR DIAGNOSIS OF *CITRUS TRISTEZA VIRUS* IN MANDARIN (*CITRUS RETICULATA*) ORCHARDS OF SIKKIM

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ABSTRACT
Citrus tristeza virus (CTV), an aphid (*Toxoptera citricidus*) transmitted *closterovirus*, is the causal agents of decline disease in citrus worldwide. CTV is a phloem-limited, flexuous filamentous plant virus with particle size of 2000 X 11 nm. It contains positive sense, ssRNA genome, ~19.3kb in length comprising 12 ORFs (ORF1a-b and ORFs 2-11) potentially encoding at least 19 putative proteins and two UTRs. Sikkim which falls under Northeastern Himalayan region of India is known to produce quality mandarin (*C. reticulata*) commercially. CTV is a major problem in this region causing decline of mandarin orchards which are being wiping out. Occurrence of CTV in mandarin orchards in Sikkim State has also been reported earlier but till to date CTV isolates of this State has not been characterized genetically. The different orchards of East and West Sikkim were surveyed. CTV incidence of 40-60% in East and 60-100% in West were estimated based on the testing with Direct antigen coated-ELISA (DAC-ELISA) and polymerase chain reaction (PCR). Seven CTV isolates; three from East and four from West were characterized based on cloning and sequencing of 5'ORF1a gene fragment (404nt) from L-ProI domain. The sequence analysis of 5'ORF1a gene fragment showed that the present isolates shared 98-100% nt identity among them and grouped together. Phylogenetic analysis showed that all of the present Sikkim CTV isolates are related with decline inducing Indian isolate Kpg3 and Israel severe isolate VT. CTV isolates of Sikkim were characterized for the first time and found to be genetically related with decline inducing Indian CTV isolate Kpg3; indicating decline inducing CTV isolates are common in Northeastern Himalayan region of India.

Keywords: *Citrus Tristeza Virus, Sikkim Mandarin, ELISA, RT-PCR*

INTRODUCTION
Citrus tristeza virus (CTV) is a member of mono-partite single-stranded RNA virus of 20 kb nucleotide length with flexuous thread-like particles measuring 10-12 nm x 2000 nm with 12 ORFs encoding 19 putative proteins (Joseph and Lee, 2000; Joseph *et al.,* 2002). It is considered as the very destructive disease in most of the citrus growing countries of the world causing decline and death of significant population of citrus trees (Ahlawat, 1997; Joseph *et al.,* 2002; Pena *et al.,* 1995). CTV is predominantly transmitted through grafting and insect vector, brown citrus aphid (BrCA), *Toxoptera citiricidus,* in semi-persistent manner (Joseph *et al.,* 1989; Pena *et al*., 1995). The symptoms of CTV in different species of citrus include decline of fruits, stem pitting, seedling yellowing, vein clearing and flecking, vein corking depending on the scion/rootstock combinations. Symptoms also depends on CTV strain, time of infection, environ-mental conditions etc (Lee and Garnsey, 1996; Lee and Joseph, 2000). *Citrus tristeza virus* is a century old problem of citrus production in all the citrus growing states of India (Ahlawat and Raychaudhuri, 1998; Ahlawat, 1997). This virus has killed more than one million citrus trees in India till to date (Ahlawat, 1997). The virus has infected nearly all the citrus species and their relatives along with their hybrids in India (Ahlawat *et al.,* 1992; Ahlawat and Pant, 2003; Biswas *et al*., 2004). Sikkim mandarin (*C. reticulata*) is one of the major cash crop of Sikkim with high export value. Now-a-days this crop has drastically reduced. Several orchards of Sikkim mandarin are being wiped out gradually due to severe attack of CTV with incidence of 46.32% has been reported earlier based on visual observation of decline symptoms, inconsistent biological indexing and limited serological tests of field samples (Kishore

et al., 2010). However, extensive surveys on occurrence and distribution of CTV in this areas and employment of recently advanced molecular diagnostic methods were not followed. In the present study different advanced and molecular diagnostic tools based on enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR) have been standardized.

MATERIALS AND METHODS

Survey of Citrus Orchards

Surveys were conducted in the Sikkim mandarin (*C. reticulata*) orchards of citrus growing areas in East and West Sikkim including Rumtek, Nazitam, Sang, Bhir kuney and Chakung, Paki gaon, Lingchom, Salley, Tikjyak respectively during 2016-17 to determine the occurrence of the virus, to evaluate its incidence, to identify and characterize the CTV isolate.

Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect antibody coated ELISA test for detection of CTV from infected samples were performed by the method described by Clark and Bar-Joseph (1984). Optimum concentration of antigen (dilution 1: 20), conjugate (antirabbit IgG labelled with alkaline phosphatase, Sigma) (dilution 1:10000), antisera (CTV antisera, Unit of Plant Virology, Indian Agricultural Research Institute, New Delhi) (dilution 1:1000) and substrate (pNPP, Sigma) (dilution 0.6 mg/ml) were used. The colour reaction was measured at 405 nm by Sunrise Tecan ELISA reader.

Reverse Transcriptase-PCR, Cloning and Sequencing of Genomic Regions of CTV Isolates

Four CTV isolates designated as SML(b), SMT(c), SMS(b), SMC(b) from five orchards of West and three designated as SMR4, SMN5 and SMS1 from three orchards of East were taken for molecular characterization based on sequencing of 404 nt fragment of ORF1a (L ProI domain) (5'ORF1a) and complete CP (ORF7, 672nt) genes. Total plant RNA was isolated from tender bark tissues using SV total RNA isolation system (Promega, Madison, USA). The first strand cDNA was synthesized using M-MLV-Reverse transcriptase (Promega, Madison, USA) following the method used earlier (Biswas, 2010). The 5'ORF1a fragment and CP gene of the present CTV isolates were amplified by polymerase chain reaction (PCR) using protocol and primers used previously (Biswas, 2010). The amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Maryland), cloned into the T&A cloning vector system (RBC, UK) and grown in *E. coli* strain DH5α using standard method. The clones of viral DNA were sequenced by vector derived M13 forward and M13 reverse primers in an automatic sequencer (ABI 3011, Chromous Biotech Pvt. Ltd., Bangalore, India). Two clones of each isolate were sequenced and consensus sequences were taken for further analysis. Sequences of two independent clones of each genomic fragment shared an identity of more than 99.5% among them, which indicated that they were amplified from same genotype.

Results and Discussion

Results

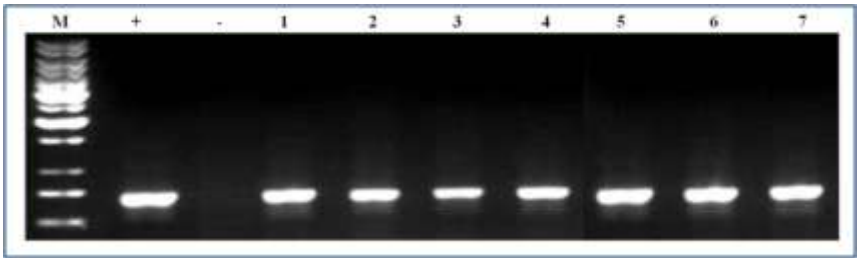

Figure 1: Polymerase Chain Reaction of Nucleotide Sequence of Citrus Tristeza Virus of Sikkim Based on 5′ORF 1a Fragment (404nt), Lanes 1-3: Three CTV Isolates of East Sikkim, Lane 4-7: Four Isolates of West Sikkim and Lane M:1 kb Ladder and +: CTV Infected Kagzi Lime Maintained in Greenhouse; –: Pooled Healthy Samples of Kagzi Lime, Sweet Orange and Sikkim Mandarin

Table 1: Disease Incidence of *Citrus Tristeza* Virus in Sikkim

District	Location	No.pl Infected/No. pl Tested (%pl Infection)	OD Value at 405 (X Fold)	Symptoms	Isolate Taken for Molecular Study	PCR Conformation
East Sikkim	Rumtek	6/15(40%)	0.78-1.18 (~3)	Chl.pg	Skm1	+ve
	Nazitam	12/20(60%)	0.64-1.27 (~3)	Chl,pg,st	Skm2	+ve
	Sang	4/40(40%)	0.64-0.80 (~2)	Chl,pg	Skm3	+ve
	Bhir Kuney	0/15(0%)	2.23-4.19 (~6.10)	Chl	-	+ve
West Sikkim	Chakung	15/15(100%)	2.31-4.19 (~6.10)	chl.st,pg	Skm4	+ve
	Pakigaon	12/25(80%)	2.31-2.96 (~5-7)	Chl,st,pg	Skm5	+ve
	Lingchom	12/20(60%)	1.08-2.73 (~3-7)	Chl,pg	Skm6	+ve
	Tikjyak	10/10(100%)	1.65-2.58(~4-6)	Chl,pg	Skm7	+ve
	Salley	15/15(100%)	2.16-2.30(~5)	chl	-	+ve

OD values of positive, healthy and buffer control are 1.89, 0.42 and 0.29, respectively; X fold titer values of infected samples were calculated compared with the OD values of healthy control; Chl=Chlorosis, Pg=Poor growth, St=Stunting.

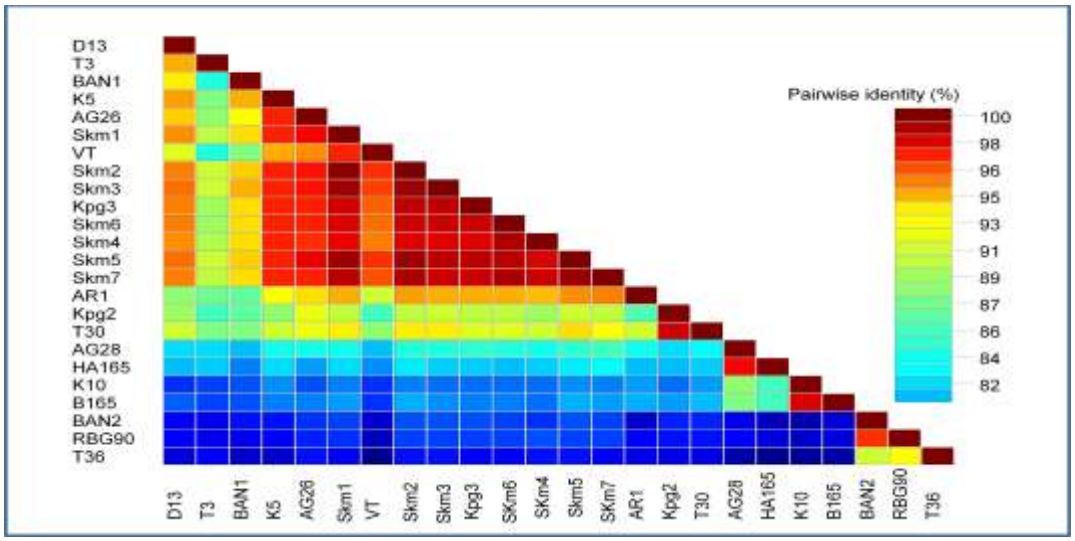

Figure 2: Colour-Coded Pair-Wise Nucleotide Identity (%) Matrix of CTV Isolates Based on 5'ORF1a Fragment of CTV Genome, each Colour Cell Represents a Percent Identity Score between Two CTV Isolates (One Indicated Horizontally to the Left and the other Vertically at the Bottom); A Coloured Key Indicates the Correspondence between Pair Wise Identities and Colours Displayed in the Matrix

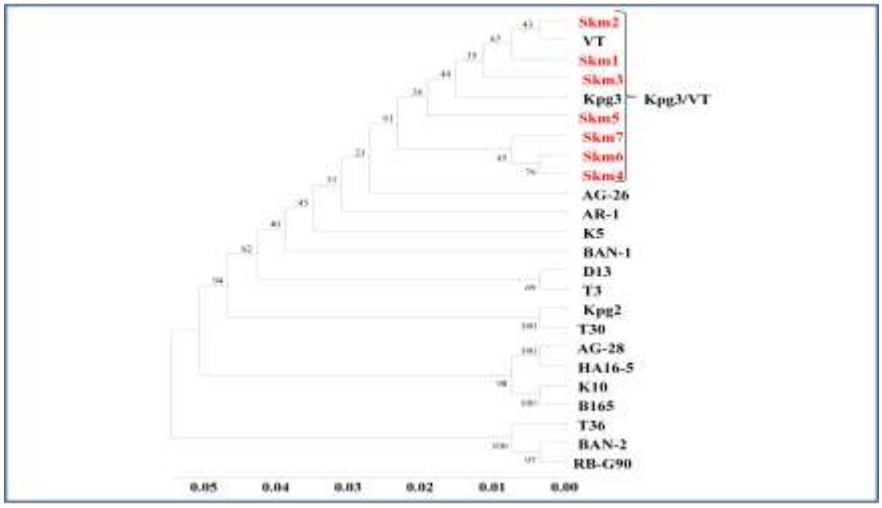

Figure 3: Phylogenetic Relationships among CTV Isolates Using Maximum Likelihood Parameter (1,000 Bootstrap) Based on 5'ORF1a Fragment

Three designated as SMR4, SMN5 and SMS1 from three orchards of East Sikkim were taken for molecular characterization based on sequencing of 404 nt fragment of ORF1a (L ProI domain) (5'ORF1a). Total plant RNA was isolated from tender bark tissues using SV total RNA isolation system (Promega, Madison, USA). The first cDNA strand was synthesized using M-MLV-Reverse transcriptase (Promega, Madison, USA) following the method used earlier (Biswas, 2010). The 5'ORF1a fragment and CP gene of the present CTV isolates were amplified by polymerase chain reaction (PCR) using protocol and primers used previously (Biswas, 2010). The amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Maryland), cloned into the T&A cloning vector system (RBC, UK) and grown in E. coli strain DH5α using standard method. The clones of viral DNA were sequenced by vector derived M13 forward and M13 reverse primers in an automatic sequencer (ABI 3011, Chromous Biotech Pvt. Ltd., Bangalore, India). Two clones of each isolate were sequenced and consensus sequences were taken for further analysis. Sequences of two independent clones of each genomic fragment shared an identity of more than 99.5% among them, which indicated that they were amplified from same genotype.

Sequence Analysis

The corresponding sequences of CTV isolates, VT (U56902), T36 (U16304), T30 (AF260651), T3 (KC525952), B165 (EU076703), HA16-5 (GQ454870), and NZRB-G90 (FJ525432) representing the seven recognized CTV genotypes which is identified worldwide (Melzer *et al.*, 2010; Biswas *et al.*, 2012a; Harper, 2013) were used for sequence comparison.

Randomly, 6 samples from the West and three samples of East districts showing positive ELISA reaction were subjected to PCR using specific primer targeting CP gene of CTV genome. All the citrus samples were found to be PCR positive. Based on ELISA results, CTV incidence of 40-60% in East and 60-100% in West were estimated (Table 1).

Cloning of Gene Sequence of CTV Isolates

The CP genes and 5'ORF1a fragments (L ProI domain) of four CTV isolates, designated as SML (b), SMT (c), SMS (b) and SMC (b) of West and three designated as SMR4, SMN5 and SMS1 of East Sikkim were amplified by specific primer pairs and the amplicons were purified, cloned, analyzed and compared with other isolate (Table 1 and Figure 1).

Sequence Analysis and Genetic Variation in CTV Isolates

Pair wise sequence analysis for 5' ORF1a showed that the present CTV isolates shared 98-100% nt identities among them. Similar of 80-100% nt identities were found among Indian and among all the CTV isolates (Figure 2). In the phylogenetic analysis, all the present isolates Skm1, Skm2, Skm3, Skm4, Skm5,

Skm6 and Skm7 segregated into one geno-group and grouped together along with previously reported decline inducing Indian CTV isolate Kpg3 and Israel severe isolate VT (Figure 3).

Discussion

The present study determined occurrence of several variants of CTV in Sikkim hills. In the present study when 7 CTV isolates of East and West Sikkim were characterized which revealed that *Citrus tristeza virus* (CTV) are widely distributed in mandarin orchards in Sikkim hills of India. To detect the CTV accurately and quickly in this region, the need of an advanced molecular diagnostic approach is the most urgent need. Diagnosis based on visible symptoms is generally unreliable because factors like climatic conditions (particularly temperature) citrus species and occurrence of different strains of CTV influence the induction of symptoms in the plants.

Earlier, diagnosis of CTV in mandarin tree was reported to be very difficult as no visible symptoms were developed on infected mandarin trees (Ahlawat 1997), but ELISA test detected CTV to be present in these symptomless plants.

These results show that symptomless infection in mandarin plants in the Sikkim hills are very common. Some isolates of CTV produce severe symptoms like stem pitting in various citrus species in many citrus growing countries (Pena *et al*., 1995; Joseph *et al*., 1989) but in the present studies, no such symptoms were observed in this region, ELISA is a widely used method for rapid detection of CTV in infected plants in all the citrus growing countries (Gransey and Camba, 1991; Ahlawat *et al*., 1992; Hilf and Garnsey, 2002).

It is a quick, reliable and effective detection method of CTV and can be used for many samples at a time. In the present studies DAC-ELISA effectively detected CTV infection in the citrus plant samples. The RT-PCR using specific primers has been reported to be a sensitive, reliable, specific, and quick method to detect CTV in citrus trees (Hilf and Garnsey, 2002; Biswas *et al*., 2004).

In this report RT-PCR was used and detected CTV by amplifying the CTV genomes from the infected samples. For the sake of precision, rapidity and convenience in accurately detecting CTV in a large number of samples rapidly, this method would be obviously the best among the methods tested and used for mapping *Tristeza* in Sikkim.

Conclusion

The present study revealed that incidence of CTV is very high in Sikkim state of Northeastern Himalayan region of India. CTV isolates in mandarin growing areas of Sikkim has been characterized genetically for the first time. The CTV isolates of Sikkim are genetically related to CTV isolates Kpg3, (mandarin decline inducing isolate of Darjeeling hills). This indicates the decline inducing isolates are common in Sikkim and Darjeeling hills of India.

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