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Prevalence of *Citrus tristeza virus* in North Eastern region of India and molecular characterization of its isolates

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ABSTRACT

Incidence of *Citrus tristeza virus* (CTV) in commercially important citrus species; Khasi mandarin (*Citrus reticulata*), Assam lemon (*Citrus limon*) and rough lemon (*Citrus jambhiri*) in Assam, Meghalaya, Arunachal Pradesh and Nagaland of Northeast (NE) regions of India was studied using double antibody sandwich ELISA (DAS-ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR). Incidence of CTV disease varied with regions and species of citrus. Incidence was more in Assam lemon than in Khasi mandarin and rough lemon. The viral inoculum was found to be more in Assam and rough lemon than in Khasi mandarin as indicated by higher titre of infected samples. Eight CTV isolates from this region were characterized based on sequencing of coat protein (CP) gene. Sequence analysis showed that CP gene of the present CTV isolates shared 95-99% identity among them. Seven CTV isolates AP3, AP5, JRT1, JRT5, N1, N15 and TK1 were found to be phylogenetically related to each other forming one genogroup and isolate TK 5 was different from other isolates forming another group. Although, recombination events are weak, recombination-detecting program RDP3 showed that majorities of the present isolates are putative recombinants.

Key words: *Citrus tristeza virus*, CP gene, diversity, incidence.

INTRODUCTION

Citrus is one of the most widely grown and economically important fruits crops of the world. *Citrus tristeza virus* (CTV), a member of the genus Closterovirus, is one of the most important plant viruses challenging citrus cultivation around the globe (Bar-Joseph *et al*., 2). CTV cause major loss in all most all the citrus growing regions of India, including its north eastern regions, where it is a major problem (Ahlawat, 1). CTV is a phloem limited aphid transmitted virus and characterized by their very long and flexuous particles of 2000 × 11 nm in dimension. It is one of the longest plant viruses and its genome consists of positive sense single stranded RNA (Karasev *et al*., 8) with twelve open reading frames (ORFs), expressing up to nineteen protein products. It contains two major coat proteins, p25 (coat protein, CP) and p27 (CP minor, CPm) of molecular weight 25 and 27 kDa encoded by ORF7 and ORF6, respectively.

North Eastern region of India is a treasure house of citrus germplasms and considered to be one of the important centres of origin of citrus. The citrus orchards of NE regions are prone to decline due to CTV and its aphid vectors are well established in the region resulting in efficient dispersal of this disease (Bhagabati *et al*., 3; Tarafdar *et al*., 11). Kashyap *et al*. (13) reported CTV in different citrus fruit species of north east India through serological and nucleic acid based detection. Previously, Tarafdar *et al*. (11) studied genetic diversity of CTV using seven isolates from Assam and five isolates from Meghalaya based on CP gene and a fragment of 5'ORF1a region. However, CTV isolates in Arunachal Pradesh and Nagaland were not characterized at molecular level and incidence of the disease was not studied. Therefore, in the present study an effort has been made to study the distribution of CTV in Northeast region covering.

MATERIALS AND METHODS

The citrus growing areas of eight districts; Jorhat, Tinsukia and Kamrup in Assam; Lohit, Papum Pare and West Siang in Arunachal Pradesh; Ri-Bhoi in Meghalaya and Mokokchung in Nagaland was surveyed to determine the incidence of CTV as well as genetic variability in isolates. The orchards of three important commercial species, Khasi mandarin (*Citrus reticulata*), Assam lemon (*Citrus lemon*) and rough lemon (*Citrus jambhiri*) were surveyed during the period of October, 2011 to February, 2012. Citrus trees from each of the species were selected randomly and twigs were collected from two age groups, (i) below 10 years, and (ii) above 10 years plantations.

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The ELISA kit with polyclonal antibody (IgG) was procured from Bioreba AG and detection done following manufacture's recommendation. CTV IgG was added to each wells and incubated at 30°C for 4 h followed by three washings with phosphate buffer saline supplemented with 1% Tween 20® (PBS-T). Leaf midrib tissue was homogenized in extraction buffer and added to each well and incubated, washed as earlier and alkaline phosphatase labelled enzyme conjugated antibody was added to each well and incubated. The plate was again washed and substrate, p-nitrophenyl-phosphate was added to each well. ELISA plate was incubated at room temperature (25°C) and the reaction was recorded at 405 nm within 30-60 min. The incidence percentage of CTV in a representative species was worked out on the basis of number of positive trees.

Molecular detection of CTV was done using RT-PCR. Total RNA from the leaf tissues of infected samples was isolated using SV Total RNA extraction kit (Promega) following the manufacturer's recommendations. Specific primers, forward primer KLM543 (5' CTCTAGATCTTTTGAATTATGGACGAC 3') and the reverse primer KLM544 (5'CGCGAATTCAACAGATCAACGTGTGT3') targeting CP gene, 672 nt of CTV genome were used for PCR using the method suggested by Biswas (4). The first strand cDNA was synthesized from the total RNA using the reverse primer. For each PCR, a reaction mixture was prepared containing, 2.5 µl of cDNA as a template, 2.5 µl of 10 X *Taq* buffer, 1.25 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.25 µl of each forward and reverse primers (10 pmoles), 0.25 µl of *Taq* DNA polymerase (1 U/ µl) and 17.5 µl of nuclease free water. Constituents were mixed well by vortexing and the PCR was run in a thermocycler (Bio-Rad) at 94°C for 3 min., followed by 30 cycles of denaturation (94°C for 30 sec.), annealing (60°C for 1 min.) and extension (72°C for 1 min.) and then finally, one cycle at 72°C for 10 min. for final extension and 4°C for infinity. The PCR products were analysed in agarose gel (1%) electrophoresis in 1X TAE buffer containing 200 ng of ethidium bromide/ml.

Eight isolates from three NE states was taken randomly, of which four were from Assam (JRT1, JRT15, TK1 and TK5), two from Arunachal Pradesh (AP3 and AP5) and two from Nagaland (N1 and N15). The PCR products were purified and cloned into pGEM-T vector (Promega, Madison, USA) and positive clones were selected and sent for sequencing using the vector derived primers M13F and M13R in AB13130 Genetic Analyzer (Chromous Biotech, Bangalore).The consensus sequences were used for further analysis. The sequences were aligned through multiple alignments with Clustal X version 1.81. (Thompson *et al*., 12), nucleotide identity matrix was carried out using Gene-Doc version 2.6.002. The maximum likelihood phylogenetic trees were constructed using the program MEGA4 (Biswas *et al*., 5).

Sequence comparison was done with CTV isolates, T36 (Florida, severe, U16304), T30 (Florida, mild, AF260651), VT (Israel, severe, U56902), B165 (India, stem pitting, EU076703), HA16-5 (Hawai, GQ454870) and NZRB-G-90 (New Zealand, resistance breaking, FJ5254232) representing the six recognised CTV genotypes identified worldwide (Biswas *et al*., 5). The CP gene of Indian isolates K5, K10, K27, Kpg2, Kpg3, P14 and TP6 representing seven CP gene-based genogroups recognized in India (Biswas *et al*., 5&6). Further, CP gene of isolates AG28, MB3, AR1and AR13 of representing four genogroups in Northeast India, under the genogroups K10, K27, K5, and Kpg3 (Tarafdar *et al*., 11) were taken for analysis. The presence of putative recombination events were identified using recombination detecting program V 3.44 (RDP3) implementing seven algorithms, RDP, GenConv, Bootscan, MaxChi, Chimera, SiScan and 3SEQ (Martin, 9) using default parameter values for the different detection programmes. When the same recombination events were detected by three or more algorithms, they were considered to be putative recombination events.

RESULTS AND DISCUSSION

DAS-ELISA result showed the current scenario of CTV infection in North-eastern states (Table 1). Viral inoculum was found to be more in Assam lemon and rough lemon than in Khasi mandarin as indicated by higher titre of infected samples. Incidence of CTV disease varied with regions and across all the four states covering three citrus species. The older orchard showed higher disease incidence compared to the new orchards acting as major reservoir of viral inoculum. The ELISA results were confirmed by RT-PCR yielding a 672 nt desired product (Fig. 1). The pair-wise sequence analysis of CP genes of CTV isolates were carried out (Table 3). The present CTV isolates shared 95-100% sequence identity among them out of which the isolate TK5 was found to be distinct, sharing 95-96% sequence identity with remaining seven present isolates. In overall sequence analysis, the present isolates showed a sequence identity in the range of 91-100% with other Indian and international isolates.

The phylogenetic analysis using CP gene showed that the present eight CTV isolates fell into two genogroups. Isolates JRT1, JRT5, TK1, AP3, AP5, N1 and N15 fell in one along with previously reported K5 type of CTV from Darjeeling hills; the K5 type includes CTV isolates AG18, AR1, AR2 and AR16 from Assam,

Table 1. Incidence of CTV in Northeast India based on DAS-ELISA.

State	District	No of tree infected/ No. of tree tested (% infection)								
		Khasi mandarin			Assam lemon			Rough lemon		
		Incidence	Range OD_{405}	RT-PCR	Incidence	Range OD_{405}	RT-PCR	Incidence	Range OD_{405}	RT-PCR
Assam	Jorhat	12/26 (46.15)	0.67 - 2.3	+ve	21/46 (45.62)	0.92 - 3.79	+ve	16/29 (55.17)	0.69 - 3.06	+ve
	Tinsukia	14/31 (45.16)		+ve	17/30 (56.67)		+ve	14/23 (60.86)		+ve
	Kamrup	9/23 (39.13)		+ve	21/34 (57.14)		+ve	16/29 (55.17)		+ve
Arunachal Pradesh	Lohit	16/35 (45.71)	0.79 - 2.21	+ve	14/27 (51.85)	0.80 - 3.01	+ve	12/29 (41.37)	0.69 - 3.02	+ve
	Papaum Pare	16/33 (48.48)		+ve	16/29 (55.17)		+ve	13/31 (41.93)		+ve
	West Siang	17/33 (51.51)		+ve	16/29 (55.17)		+ve	12/26 (46.15)		+ve
Meghalaya	Ri bhoi	28/54 (51.85)	0.7 - 1.98	+ve	26/50 (52)	0.99 - 3.32	+ve	26/51 (59.05)	0.81 - 2.73	+ve
Nagaland	Mokokchung	33/56 (58.93)	0.78 - 2.01	+ve	24/38 (63.15)		+ve	23/43 (53.48)	0.81 - 2.93	+ve

Av.OD_{405} value of healthy, buffer, positive control is 0.196, 0.181, and 0.343, respectively.

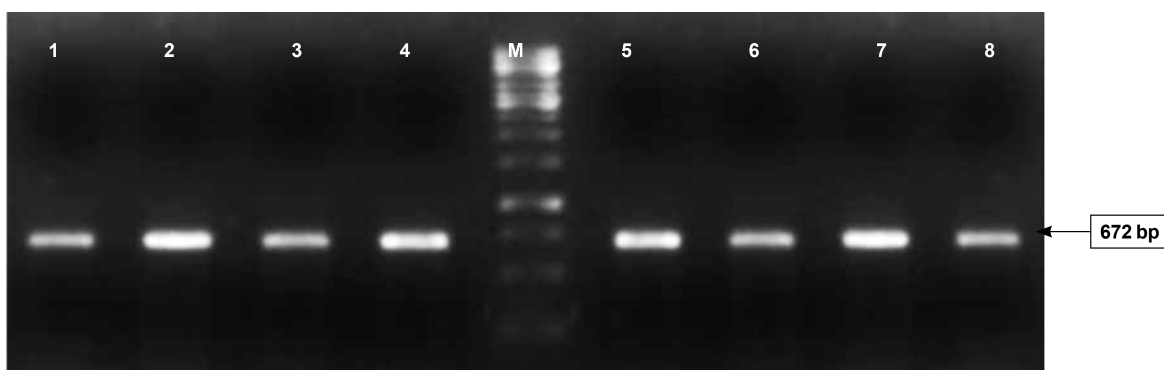

Fig. 1. Detection of CTV infection in different citrus species of Northeastern states through RT-PCR; Agarose gel electrophoresis showing amplification of 672 nt CP gene, with lane M: 1 kb ladder, lane 1-8: eight CTV infected citrus samples.

and MU6 of Meghlaya, Akt1 of Central India and many CTV isolates of the Darjeeling hills (Biswas *et al.*, 5; Tarafdar *et al.*, 11). The present isolate TK5 fell in another group along with previously reported AR13 type isolate which include isolate AG24 of Assam, TPU of Tirupati, South India and Kpg3 type isolates, which include isolates of Northeast including the Darjeeling hills, North, Central and South India (Biswas *et al.*, 5; Tarafdar *et al.*, 11) and Hawaii isolate HA16-5 (Fig. 2). Genetic diversity in CTV based on sequence analysis has been reported worldwide (Biswas *et al.*, 6). Occurrence of divergent CTV isolates in all the citrus growing geographical region of India have been reported (Biswas, 4; Sharma *et al.*, 10; Biswas *et al.*, 5; Tarafdar *et al.*, 11). Recently, genetic diversity of CTV in Assam and Meghalaya of Northeast was found to be common and existence of CP gene-based four genotypes has been reported (Tarafdar *et al.*, 11).

Although, the present study analysing eight CTV isolates could not identify new CTV entity, however, it confirmed presence of atleast four genogroup in Assam, Meghalaya, Arunachal Pradesh and Nagaland

Table 2. Origin of identified CTV isolates under study.

Isolate	Origin	Accession No.	Symptom on	
			Assam lemon	Kagzi lime
AP3	Lohit, Arunachal Pradesh	KC986380	Vcl, Chl, St	Vcl
AP5	Papum pare, Arunachal Pradesh	KC986381	Vcl, Chl	Vcl
JRT1	Jorhat, Assam	KC986382	Vcl, Chl	Vcl, Chl
JRT5	Jorhat, Assam	KC986383	St, Chl	Chl
N1	Mokokchung, Nagaland	KC986384	Chl, Vcl	Vcl
N15	Mokokchung, Nagaland	KC986385	St, Chl	Vcl
TK1	Tinsukia, Assam	KC986386	Vcl, Chl	Vcl,Chl
TK5	Tinsukia, Assam	KC986387	St, Chl	Vcl, Chl

Vcl = Vein clearing, Chl = Chlorosis, St = Stunting

Table 3. Nucleotide identity (%) of CP gene of present NE CTV isolates with other Indian and international isolates.

Seq->	AP3	AP5	JRT1	JRT5	N1	N15	TK1	TK5	AR1	K5	AR13	Kpg3	MB3	K27	AG28	TP6	Kpg2	K10	P14	VT	T30	T36	B165	HA16-5	NZRB-G90
AP3	ID	100	99	99	99	100	100	96	100	98	96	95	91	90	91	93	92	92	92	92	92	92	91	96	92
AP5		ID	99	99	100	99	100	96	100	98	96	96	92	91	91	93	92	92	92	92	92	92	92	96	92
JRT1			ID	98	98	98	99	96	99	97	96	95	91	90	91	93	91	92	91	91	91	91	91	96	92
JRT5				ID	99	99	99	95	99	98	96	95	91	90	90	93	91	92	91	91	91	91	91	95	92
N1					ID	99	100	96	99	98	96	95	91	90	91	93	91	92	92	92	92	92	91	96	92
N15						ID	99	96	99	98	96	95	91	90	91	93	92	92	92	92	92	92	91	96	92
TK1							ID	96	100	98	96	95	92	91	91	93	92	92	92	92	92	92	92	96	92
TK5								ID	96	97	97	97	92	91	91	93	93	92	93	92	92	93	92	98	93
AR1									ID	98	96	96	91	90	91	93	91	92	92	92	92	92	91	96	92
K5										ID	97	96	92	90	91	93	92	92	92	92	92	92	92	97	93
AR13											ID	97	92	90	90	93	92	92	92	92	92	92	92	98	93
Kpg3												ID	92	90	91	95	92	92	92	92	92	92	91	98	92
MB3													ID	96	92	91	93	94	93	98	93	92	96	91	93
K27														ID	91	90	92	93	91	96	91	91	94	90	91
AG28															ID	93	91	97	91	93	91	91	93	90	91
TP6																ID	90	96	91	91	92	91	92	93	91
Kpg2																	ID	92	94	94	92	94	93	92	97
K10																		ID	92	94	93	92	94	92	92
P14																			ID	93	93	98	93	92	94
VT																				ID	92	93	96	92	93
T30																					ID	93	93	92	92
T36																						ID	93	92	93
B165																							ID	91	93
HA16-5																								ID	93
RB-G90																									ID

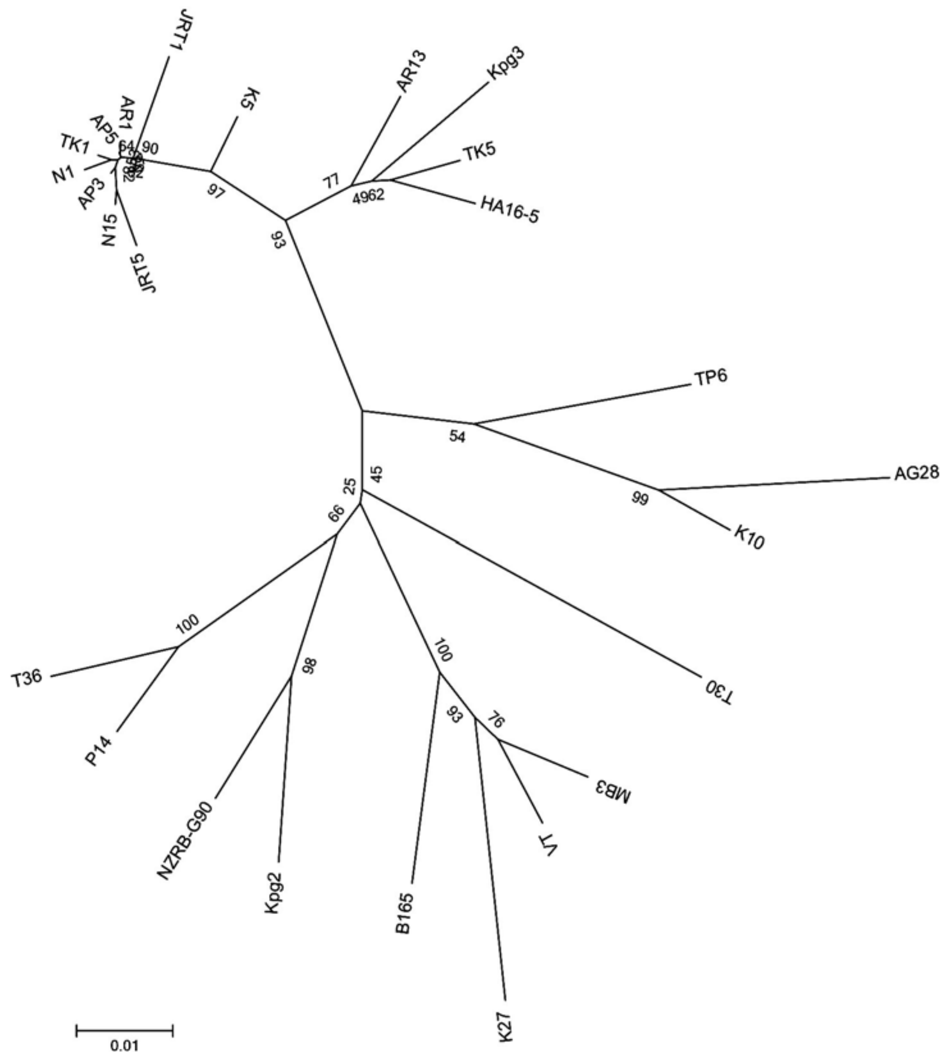

Fig. 2. Maximum likelihood phylogenetic tree showing relationships among NE CTV isolates with other Indian and international CTV isolates using CP gene. Scale bar represent the genetic distance, proportional to the number of nucleotide differences between branch nodes. Number represents the bootstrap values.

of Northeast (Table 2). The previous studies of Biswas *et al*. (5) and Tarafdar *et al*. (11) and that of present study concluded that in the Northeastern citrus growing regions with its adjoining Darjeeling hills there occurs at least five CTV genogroups. However, more number of CTV isolates are needed to be studied to get a clear picture of genetic diversity in CTV of Northeast, as this region is believed to be one of important centre of origin of citrus.

Of seven algorithms implemented in RDP3, only algorithm SiScan detected recombination event in CP gene of the present CTV isolates. One recombination event in same position at 565-222 nt of six isolates, AP3, AP5, JRT1, N1, N15 and TK1 and one event at 18-687 of isolate JRT5. No recombination event was identified in isolate TK5. Interestingly, all the recombinant isolates involved sequence of MB3 as a major and Kpg2 as a minor donor sequences. Although, the recombination events in the present isolates are weak, the result revealed that recombination phenomenon is one of the major criteria for development of CTV variants. Recombination also concluded that present recombinant isolates may be evolutionary related to each other as they involved same donor sequences. The exchange of genetic material between divergent sequence is one of the main forces in driving genetic diversity in CTV isolates in India reported earlier (Biswas *et al*., 5 & 6; Sharma *et al*., 10; Tarafdar *et al*., 11).

Present study along with previous studies concluded that the CTV is widely distributed in

Northeast region of India with high degrees of disease incidence. Extensive genetic variability and occurrence of at least four CTV genogroup in Northeast citrus growing regions was determined. The distinct CTV groups identified will lead to development of group-specific primers for accurate and quick detection of the predominant virus group. The distribution map of CTV and its variants in India will be useful in understanding disease epidemiology and designing suitable management program of this virus in India. As the disease incidence is high and CTV infected citrus planting materials are randomly distributed to the farmers in this region, sanitation and replanting with virus-free propagative materials will be an effective method to reduce the economic losses of citrus.

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