Complete genome sequences of a novel genotype of citrus tristeza virus infecting Chinese wild mandarin (Citrus daoxianensis S.W. He) in China

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Abstract

A moderate sequence heterogeneity isolate (JY-2) of CTV infecting Chinese wild mandarin (*C. daoxianensis* S.W. He) in were examined by RT-PCR and next-generation sequencing (NGS) of RNA sequencing (RNA-seq). Complete genomic sequences of JY-2 consisted of 19112 nucleotides (nts) were determined. Based on evidence of genomic characteristics, sequence analysis, and phylogenetic analysis of JY-2, one putative novel CTV genotypes JY should be considered. RT-PCR results showed that JY genotype was only in Chinese wild mandarin (*C. daoxianensis*) trees from Jiangyong (Hunan Province), China. These results will underpin further understanding of the molecular diversity of CTV and will provide a new insight into its evolution.

Full Text

Citrus tristeza virus, a member of the genus *Closterovirus*, family *Closteroviridae,* is one of the most economically destructive pathogens of citrus worldwide [1]. Although almost all citrus in China is grown on CTV-resistant *Poncirus trifoliata* or *P. trifoliata hybrid stock* rootstocks [2], the occurrence of tristeza disease with stem pitting phenotype in China is extremely common over the past decades [3,4].

The citrus tristeza virus (CTV) genome consists of a plus-sense single-stranded RNA of 19.3 kb organized in 12 open reading frames (ORFs), and 5’ and 3’ untranslated terminal regions [1]. CTV has high genetic diversity [5]. The complete genome average sequence identity between different CTV genotypes was 80.5-92.4% [5]. The sequence of the 3'-proximal region of the genome of CTV isolates is more conserved than that of the 5'-proximal region [5]. According to the sequence diversity and phylogenetic relationship of the CTV genome, CTV isolates can be divided into 11 genotypes: VT, T30, T36, T3, A18, B165 (T68), RB, HA16-5, S1, L1, M1 [4,5]. In the field, a single citrus plant generally is infected with more than one genotypes of CTV, complicating the genotypic and phenotypic associations since different combinations of genotypes affect symptom expression and disease severity [1,6].

China is the largest citrus-producing country in the world (FAOSTAT, 2019; http:// www.fao.org/faostat/) and has been suggested as the origin of CTV [7-9], with diverse citrus accessions providing rich sources to study citrus viruses. In the present study, a novel genotype isolate (JY-2) and a T3 isolate infecting Chinese wild mandarin (*Citrus daoxianensis* S.W. He) in were examined by next-generation sequencing (NGS) of RNA sequencing (RNA-seq), and the JY-2 genome sequence were generated. These data provide evidence that JY-2 is the type member of a new CTV genotype, called JY. In addition, phylogenetic analysis and specific detection were carried out for the JY genotype.

Leaf tissue from five non-symptomatic Chinese wild mandarin (C*. daoxianensis* S.W. He) trees (JY-1~5) was collected from Jiangyong (Hunan Province) for CTV and CTV genotypes detection. Sanger sequencing showed that the PCR products of T36-F/R primers [10] of JY-2 and JY-5 samples had moderate sequence heterogeneity. And NGS of RNA-seq was used to further characterize populations of the CTV in JY-2 sample [11]. Sequencing was performed on an Illumina Hi-seq 4000 platform done by

Novogene Tech, generating 150 bp paired-end reads. The obtained reads were assembled to produce contigs using Trinity [12]. BLAST analysis indicated that 68 contigs ranging in size from 250-4367 nt were related to CTV with nucleotide sequence similarity ranging from 77% to 100%. These contigs were used for viral genome of CTV assembly using SeqMan (DNASTAR Lasergene v7.1.0 software). Eventually, 9 assembled sequences covered 90% of the T3-KB isolate (MH051719) with nucleotide sequence similarity ranging from 92% to 99%, and 7 assembled sequences covered 98% of the CN-M1-ZT1 isolate (MH323442) with nucleotide sequence similarity ranged from 87% to 91%. These results revealed that a novel CTV variant (JY-2) and a T3 isolate co-existed in the JY-2 sample.

To determine the complete genomic RNA sequence of JY-2, RT-PCR was performed using the primer sets designed in this study (Fig. 1A, and Table S1). The 5’ and 3’ terminal sequences of genome were determined using a SMARTerÒ RACE 5’/3’ Kit (Clontech, Dalian, China). Sequencing these fragments, and assembling the contiguous sequence as described previously [11]. Sequence identities were obtained using ClustalW implemented in MegAlign (DNASTAR Lasergene v7.1.0 software package). Phylogenetic trees were constructed using the Maximum Likelihood (ML) method with 1000 bootstrap replicates in MEGA 7.0 [13]. Neighbor network (NN) construction was performed using SplitsTree 4.14.4 [14]. Putative recombination events were assessed using RDP5 software package [15].

The complete 19112 nucleotides (nts) genome of JY-2 isolate (ON094625) includes a 106 nts 5’-leader and a 119 nts 3’- trailer. The JY-2 isolate 3’ UTR is 156 nts shorter than that of CN-M1-ZT1 isolate (MH323442). Pairwise comparison of the genome sequences of JY-2 and other genotypes of CTV shows that the sequence variation between JY-2 and VT, T30, T3, T68, HA16-5, S1, and L1 (with nt identities ranging from 78.4 to 79.7%) is more than that of A18, M1, T36, and RB (with nt identities ranging from 82.1 to 89.4%) (Table 1). The nt and aa sequence of JY-2 show relatively lower identities in the 5' genomic region (the ORF1a, ORF1b, and *p33*) than regions further downstream (*p6* onwards) with other genotypes of CTV (Table 1 and Table S2). These results are consistent with previous reports suggesting that sequences at the 3' proximal region of the genomes of CTV isolates are more conserved than those at the 5' proximal region [5]. In contrast, a relatively high percentage nt and aa identity is shared between JY-2 and CN-M1-ZT1 at their ORF1a and ORF1b regions (Table 1). According to the genotypes or strains demarcation criteria for CTV (the complete genome sequence should differ by >7.5% at the nucleotide level and by >8% at both the nucleotide and amino acid levels in ORF1a) [5], the JY-2 isolate should be recognized as representative of a novel genotype.

To further reveal the relationship of JY-2 and other genotypes of CTV, maximum likelihood phylogenetic trees were constructed using 80 known completely sequenced isolates and JY-2 isolate. Phylogenetic analysis showed that JY-2 isolate fell into a separated clade clustered with CN-M1-ZT1 isolate (Fig. 1B). Moreover, the NN analysis (Fig. S1) suggested that the JY-2 isolate were separated into distinct sequence groups from those reported, which are temporarily named JY (JY-2). These results further support the proposal of classifying JY-2 isolate into a novel genotype. Putative recombination events for JY-2 isolate were assessed using RDP 5 software [15], but no reliable recombinant events were identified (Tables S3).

However, one recombination event in ORF1a and ORF1b of A18 isolate was identified (nt 9357–10694) with CTV isolates T3 and JY-2 suggested as putative parents (Tables S3).

To investigate the occurrence of JY genotype in China, a total of 278 cultivated citrus leaf samples from nine provinces or areas and 136 Chinese wild mandarin leaf samples from four citrus reserve areas were assayed by RT-PCR using CTV-specific primer sets CTV CP-F/R and CTV-JY-specific primer sets JY-7F/6R (Table S1). The results showed that the JY genotype was only detected in Chinese wild mandarin (*C. daoxianensis*) leaf samples from Jiangyong citrus reserve area (Hunan Province), but not in cultivated citrus leaf samples from Jiangyong. A total of 25 Chinese wild mandarin *(C. daoxianensis)* leaf samples from Jiangyong citrus reserve area, 56% (n=14) were positive for CTV and 48% (n=12) were positive for JY genotype of CTV (Fig. S2). These results suggest that JY genotype is not common in China.

In summary, based on evidence of genomic characteristics, sequence analysis, and phylogenetic analysis, one putative novel CTV genotypes isolated from *C. daoxianensis* were first described. These results will underpin further understanding of the molecular diversity of CTV and will provide a new insight into its evolution.

Declarations

Compliance with ethical standards

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Tables

Table 1
Averaged nucleotide (nt) and amino acid (aa) sequence identities for JY-2 isolate with other extant CTV genotype isolates

Genotype	Isolates[a]	Genome		ORF1a[b]		ORF1b[b]		p25[b]	
		nts[c]	nt%[d]	nt%[d]	aa%[d]	nt%[d]	aa%[d]	nt%[d]	aa%[d]
VT	T318A	19252	79.7	73.5	75	82	93	91.4	95.5
T30	T385	19259	79.1	73.1	74.2	79.6	92.3	90.6	94.6
T3	T3	19253	79.3	73.1	74.4	78.9	92.1	91.2	96.9
T68	T68-1	19246	78.8	71.9	74	79.2	90.5	91.7	96.4
HA16-5	Taiwan-Pum/M/T5	19236	78.6	72.3	73.5	78.9	89	91.2	93.7
S1	CA-S1-L	19248	78.4	71.6	72.9	78.3	90.1	91.1	94.2
L1	CN-L1-ZT1	19244	79.7	73	73.5	79.7	92.9	90.6	96.4
A18	A18	19302	82.1	76	74.9	90.9	96.3	93.6	97.3
M1	CN-M1-ZT1	19265	89.4	90.4	90.7	92.7	97.1	90.5	96.4
T36	T36	19296	87.4	87	82.9	91.9	96.3	91.2	95.1
RB	NZRB-TH30	19270	87.9	88.8	89.3	89.0	95.7	90.9	94.2
a: GenBank accession No. of CTV isolates: T318A, DQ151548; T385, Y18420; T3, KC525952; T68-1, JQ965169; Taiwan-Pum/M/T5, JX266713; CA-S1-L, KU589212; CN-L1-ZT1, MH323441; A18, JQ798289; CN-M1-ZT1, MH323442; T36, U16304; NZRB-TH30, FJ525434.									
b: ORF1a, Open Reading Frame 1a; ORF1b, Open Reading Frame 1b; p25, p25 gene.									
c: nts, nucleotides									
d: nt%, aa%, the nucleotide and amino acid identities between JY genotype isolate and other extant CTV genotype isolates.									

Figures

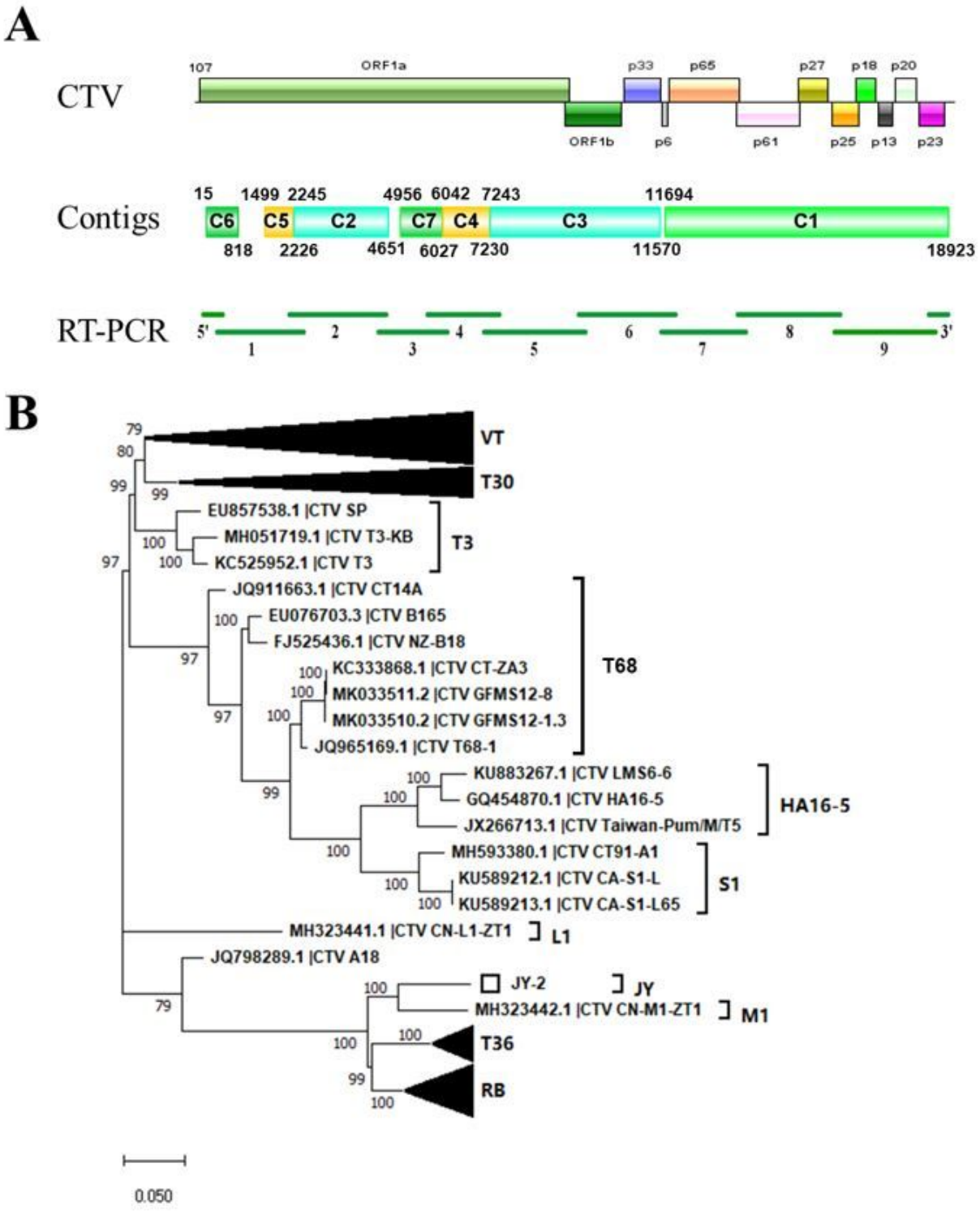

Figure 1

Amplification of overlapping fragments of the novel genotype and unrooted phylogenetic tree reconstructed of the citrus tristeza virus (CTV). A: Schematic representation of the CTV genome organization (drawn to scale) and the regions that were cloned and sequenced; B: Maximum Likelihood tree based on the complete genome nucleotide of representative isolates of the CTV. Bootstrap values

were obtained from 1,000 replications, and bootstrap values <70% were collapsed. The a isolate for which the complete genomes were sequenced in the present study are indicated by "□".

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