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SHORT COMMUNICATION

Complete genome sequences of four isolates of *Citrus leaf blotch virus* from citrus in China

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Abstract

Citrus leaf blotch virus (CLBV) is a member of the genus *Citrivirus*, in the family *Betaflexiviridae*. It has been reported CLBV could infect kiwi, citrus and sweet cherry in China. Of 289 citrus samples from six regions of China, 15 were detected to be infected with CLBV in this study. The complete genome of four isolates of CLBV was obtained from Reikou in Sichuan (CLBV-LH), Yura Wase in Zhejiang (CLBV-YL), Bingtangcheng in Hunan (CLBV-BT), Fengjie 72-1 in Chongqing (CLBV-FJ), respectively. While they all represented 8 747 nucleotides in monopartite size, excluding the poly(A) tail, each of the isolates coded three open reading frames (ORFs). Identity of the four isolates ranged from 98.9 to 99.8% to each other and from 96.8 to 98.1% to the citrus references in GenBank by multiple alignment of genomes. A phylogenetic tree based on the genome sequences of available CLBV isolates indicated that the four isolates were clustered together, suggesting that CLBV isolates from citrus in China did not have obvious variation. This is the first report of the complete nucleotide sequences of CLBV isolates infecting citrus in China.

Keywords: *Citrus leaf blotch virus* (CLBV), genome sequence, *Citrivirus*

1. Introduction

Citrus leaf blotch virus (CLBV) is a member of the genus *Citrivirus* in the family *Betaflexiviridae (*Adams *et al*. 2012). It has been found in Spain, Japan, USA, France, Austrialia, Italy, New Zealand, Cuba, and China (Vives *et al*. 2002b; Guardo *et al*. 2007; Harper *et al*. 2008; Hernández-Rodríguez *et al*. 2016; Cao *et al*. 2017). CLBV was first detected in Nagami Kumquat (*Fortunella margarita* Lour. Swingle) from Corsica, France (Navarro *et al*. 1984) and later was found to associate with bud union disorder on Troyer citrange (*Citrus sinensis*×*Poncirus trifoliata*) (Galipienso *et al*. 2001). It is a graft- and seed-transmissible pathogens, albeit at low percentages in citrus (Guerri *et al*. 2004). This virus is able to infect a wide range of hosts including numerous fruits such as citrus, kiwi, sweet cherry and an ornamental plant peony (Galipienso *et al*. 2001; Chavan *et al*. 2013; Wang *et al*. 2016; Gress *et al*. 2017), as well as several herbaceous hosts, such as *Nicotiana occidentails*, *Nicotiana benthamiana*, *Nicotiana cavicola*, *Nicotiana glutinosa*, and *Nicotiana clevelandii* included (Vives *et al*. 2008; Guardo *et al*. 2009; Chavan *et al*. 2013). In China, CLBV was initially found in kiwi,

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and then in sweet cherry and citrus (Wang *et al*. 2016; Zhu *et al*. 2016; Cao *et al*. 2017).

The virus particle of CLBV is filamentous and flexuous, 960 nm long and 14 nm in diameter. The genome of CLBV is a single-stranded positive-sense RNA with full length of approximately 8.7 kb, excluding a 3′-terminal poly(A) tail, and comprises three overlapping open reading frames (ORFs). ORF1 encodes a polyprotein with a molecular mass of about 227.4 kDa which contains the viral replication components with methyl-transferase, AlkB-like, OTu-like peptidase, papain-like protease, helicase and RNA-dependent RNA polymerase (RdRp) motifs. ORF2 encodes a 40.2-kDa putative cell-to-cell movement protein (MP). ORF3 codes an approximately 40.7-kDa coat protein (CP) (Vives *et al*. 2001). In previous studies, CLBV produced two 3′-coterminal and two 5′-coterminal subgenomic RNAs which were essential for the viral infection (Vives *et al*. 2002a). In this study, the complete nucleotide sequences of CLBV isolates from different citrus cultivars and regions were first obtained in China. This study would provide the basis for further studies on molecular evolution of CLBV.

2. Materials and methods

2.1. Virus isolates and RNA extraction

For this research, a total of 289 citrus samples were collected amongst different cultivars from several major citrus-growing regions of China including Zhejiang, Hunan, Sichuan, Jiangxi, Yunnan, and Chongqing. Total RNA extracts were obtained from the leaf tissues using TRIzol® Reagent (Thermo-Fisher, USA) according to the manufacturer's instructions, and then one-step RT-PCR detection with primer pair CLBV-F (5′-AGC CATAGTTGAACCATTCCTC-3′) and CLBV-R (5′-GC AGATCATTCACCACATGC-3′) (Harper *et al*. 2008) was used. The four CLBV-infected samples featured with different cultivars and geographical locations were selected for amplification of the complete virus genome.

2.2. RT-PCR and genome cloning

In order to obtain full genomic sequence, specific primers were initially designed into overlapping fragments based on the sequences of a CLBV isolate from GenBank (accession no. FJ009367) (Table 1). For the determination of the 5′- and 3′-terminal sequences of the genomic RNA, a commercial RACE Kit (Invitrogen, USA) was used. All fragments were amplified using the PreimeScript™ One-Step RT-PCR Kit version 2 (TaKaRa, China). PCR amplification was performed in a total reaction volume of 50 μL with 1.25 μL each primer in pair (10 μmol L^{-1}) according to the reagent manuals. The thermal cycling conditions for RT-PCR were one cycle of 45°C for 30 min and 94°C for 3 min, 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 150–180 s (adapt for amplicon length), and a final elongation at 72°C for 10 min.

PCR products of given size were verified and purified using a gel DNA extraction kit (OMEGA Bio-Tek, USA). These final amplicons were cloned into pEASY-T1 vector (TransGen Biotech, China), and five clones per fragment were custom sequenced.

2.3. Sequence analysis

The clones derived from six overlapping fragments were assembled with classic option and the outcome sequences were modified and decided (including 5′- and 3′-RACE) by DNASTAR 7 (DNASTAR Inc., USA). The genomic sequence and both nucleotides and amino acids (aa) sequence of ORF1, ORF2 and ORFE3 were separately compared with other available CLBV isolates in GenBank by CLC Main Workbench 7.9 (Qiagen, German). The phylogenetic tree was constructed by the Clustal-W and neighbor-joining method with 1 000 bootstrap replications using the MEGA version 7.0 Program.

Table 1 Primers designed for PCR amplification of *Citrus leaf blotch virus* (CLBV) genomic RNA

Primer name	Primer sequence (5′→3′)	Position (nt)	Product size (bp)
CL-1F	GCCTCAAAGGTGGGTATT	239–256	2874
CL-1R	GTATCTACTCCAGAAAGGGCT	3092–3112	
CL-2F	CCATAGAGATAATGAACGGG	2746–2765	2757
CL-2R	GCCAGTGTGTTGAATAGGAA	5483–5502	
CL-3F	ATAGGGAGGAAAGGTTGG	4470–4487	2359
CL-3R	TGAAGCCGTCTCTGACTTA	6810–6828	
CL-4F	ACTAACCATCAACGCTGC	6378–6395	2293
CL-4R	AACACACCTTCACCCTCTAC	8651–8670	
CL-5′-RACE-GSP1	CTGAAACCTGTCTTCTGGAT	421–440	440
CL-3′-RACE-GSP1	AGCCTGAAGAACAGAACACT	8368–8387	380