First Report of *Citrus leaf blotch virus* in Lemon in China

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ABSTRACT

Citrus is one of most important economic fruit crops in China. *Citrus leaf blotch virus* (CLBV) is a virus species of genus *Citrivirus* in family *Betaflexiviridae*. This virus was first reported from Nagami kumquat in association with bud union disorder (Navarro et al. 1984). It is transmitted in citrus by grafting and seed (Guerri et al. 2004). CLBV was found recently in sweet cherry (Wang et al. 2016) and kiwifruit (Zhu et al. 2016) in China. In May 2015, one Eureka lemon sample (*Citrus limon* (L.) Burm.f.) displaying yellow vein clearing symptoms was found in a citrus orchard in Wanzhou in Chongqing municipality during a survey for virus-like symptoms. Leaf tissues were collected and sent to the Beijing Genomics Institute for total RNA preparation, small RNA (sRNA) library construction, and sequencing. De novo assembly of sRNA reads was performed using CLC Genomics

Workbench (CLC bio v10.0, Denmark), and 623 contigs >50 nucleotides (nt) were obtained after removal of host sRNAs. BLASTn and BLASTx searches against nonredundant nucleotide and protein databases revealed eight contigs similar to *Citrus yellow vein clearing virus* (CYVCV) and five contigs to CLBV. The largest CLBV contig of 4,683 nt was 98% identical to CLBV isolate NZ_G78 (EU857540). From this sequence, two primer pairs, F2 (5'-AGAGGGAAAGGTCTTGGAGTG-3')/R2 (5'-GCAATCTATCAGCAGTCAGGA-3') and F3 (5'-AAGTCAATGGCAAAGAAGCG-3')/R3 (5'-TCGTGGTGAGATTTCGTCTG-3'), were designed from this contig and which in RT-PCR successfully amplified 412 nt (partial replicase polyprotein and movement protein) and 597 nt (partial movement protein) fragments from the symptomatic lemon tissue. These fragments were cloned, sequenced, and found to be the appropriate CLBV genomic regions characterized from the contig. Bark grafting was performed from the lemon plant to 'Dweet' tangor plants and characteristic chlorotic mottle symptoms developed on leaves by 6 months after grafting; cloning and sequencing from 'Dweet' tissues confirmed the presence of CLBV. To further investigate the distribution of the virus, 20 citrus samples collected from different regions of China were tested by RT-PCR. Three infected tangor (*C. reticulata* × *C. sinensis*) samples were identified, one each from trees of varieties Harumi, Reikou, and Shiranuhi from Hunan, Sichuan, and Zhejiang provinces, respectively. The aforementioned 412 and 597 nt genomic regions were amplified, cloned, and sequenced from these samples. BLASTn searches of the clones from Harumi (KX924465 and KX924464), Reikou (KX924466 and KX924467), and Shiranuhi (KX924468 and KX924469) and Wanzhou (KX924470 and KX924471) revealed all sequences had at least 98% identity with CLBV isolate NZ_G78. To the best of our knowledge, this is the first report of CLBV infection on citrus in China. As CLBV has been reported to be capable of invading the apical meristem, results indicate the necessity for detection and therapy in citrus germplasm and nursery programs in China.

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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. 2002a; 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 (Navarro et al. 1984), and later associated with bud union disorder on Troyer citrange (Citrus sinensis x 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 recently 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, N. benthamiana, N. cavicola, N. glutinosa and N. clevelandii included (Vives et al. 2008; Guardo et al. 2009; Chavan et al. 2013). In China, CLBV was initially found in kiwi, and then in sweet cherry (Wang et al. 2016; Zhu et al. 2016;).

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. 2002b). 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 provinces 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′-AGCCATAGTTGAACCATTCCTC-3′) and CLBV-R (5′-GCAGATCATTCACCACATGC-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 PreimeScriptTM one-step RT-PCR Kit Ver.2 (TaKaRa, China). PCR amplification was performed in a total reaction volume of 50 ul with 1.25 ul each primer in pair (10 umol/L) according to the reagent manuals. The thermal cycling conditions for RT-PCR were one cycle of 45 ℃ for 30 min and 94 ℃ for 3 min, 40 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 150-180 s (adapt for amplicon length), and a final elongation at 72 ℃ 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 GeneBank by CLC Main Workbench 7.9 (Qiagen, German). The phylogenetic tree was constructed by the Clustal-W and Neighbor-joining method with 1000 bootstrap replications using the MEGA version 7.0 program.

3. Results

15 citrus samples were detected to be CLBV positives by RT-PCR and were divided into four classes by sequence analysis, which were collected from four different cultivars Reikou in Sichuan (LH), Yura Wase in Zhejiang (YL), Bingtangcheng in Hunan (BT) and Fengjie 72-1 navel orange in Chongqing (FJ), respectively. Complete genome sequence of a typical isolate for each class was deposited in GenBank database under accession number MF784853 (CLBV-LH),

MF784854 (CLBV-YL), MF784855 (CLBV-BT) and MF784856 (CLBV-FJ). The complete genome of all the four CLBV isolates were 8747 nucleotides (nt) long, excluding the poly (A) tail. The isolates were 73.7%-98.1% identical to other available CLBV isolates from GeneBank. Analysis of the genomic structures showed that all the four isolates were similar to previously reported CLBV citrus isolate (AJ318061) (Vives et al. 2001), and CLBV-LH, CLBV-YL, CLBV-BT, CLBV-FJ genomes shared 97.9%, 97.8%, 98.0% and 98.0% identity, respectively, with AJ318061 isolate. These four isolates all contained two untranslated regions (UTR) of 73 and 541 nt at the 5' and 3' end, respectively. The ORF1 began at nucleotide position 74 and ended at position 5962, encoded a polypeptide of 1,962 aa. ORF2 (nt 5962-7050) encoded a MP that was 362 aa. ORF3 (nt 7115-8206) coded a 363 aa CP. The identities for ORF1, ORF2 and ORF3 of the four isolates comparing with the NCBI's were 71.7%-98% nt (78.3%-98.7% aa), 77.4%-97.8% nt (95.9%-99.5% aa) and 77.5%-98.6% nt (88.2%-99.7% aa), respectively. Sequence alignments demonstrated that CP and MP region of CLBV isolates were both highly conserved in either citrus or kiwi in respects of alignments of whatever nucleotide or amino acid.

To reveal phylogenetic relationships, a tree was carried out based on the genomic sequences of the four CLBV isolates and nine others in the NCBI database. CLBV isolates from citrus in China were clustered in the same clade within the phylogenetic tree, and were closely related to the New Zealand isolate (EU857540) (Fig 1).

4. Discussion

CLBV was first detected in kiwi fruit plants with a rate of 13.3% which grown in China (Zhu et al. 2016). Subsequently, CLBV was found in sweet cherry and citrus with a rate of 15% in China (Wang et al. 2016; Cao et al. 2017). In contrast, our result showed a lower infected rate of 5.2% in citrus samples likely due to random sampling, while the statistics covers still limited for only parts of regions were investigated and only four cultivars were found CLBV-infected. Generally, complete genome sequence of CLBV for Chinese isolates were only reported in sweet cherry and kiwifruit. In our study, four genome sequences of CLBV isolated from citrus were obtained, of which CLBV-LH and CLBV-YL, CLBV-BT and CLBV-FJ were identified as Citrus reticulata (L.) and Citrus sinensis (L.), respectively. The four isolates shared high resemblance to each other and homologous with those from other countries, indicating low genetic variation within citrus groups of CLBV isolates from whatever geographical origins, as previously reported (Vives et al. 2002a).

5. Conclusion

To our knowledge, this study provides the first complete nucleotide sequences of CLBV isolates from citrus in China, therefore enriched bioinformatics database. Surveys for CLBV with scope of six citrus producing areas, could locate its spreading and severity to some extent. These mentioned details would form the basis for development of molecular diagnostic and hence a more effective disease control strategy.

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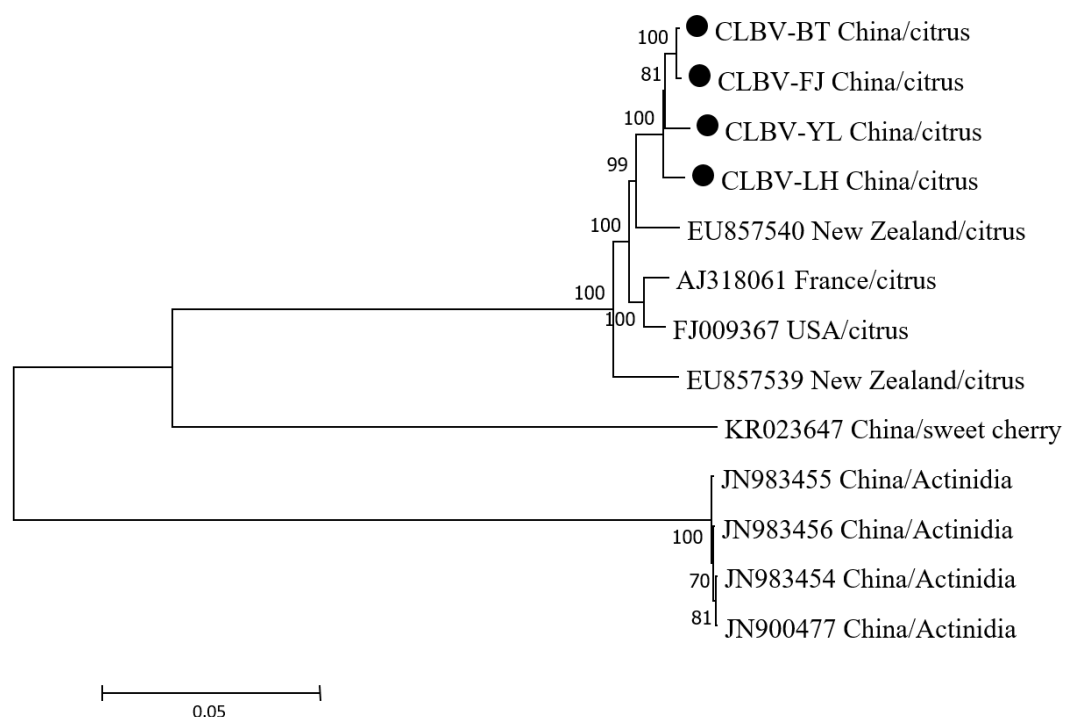
Fig. 1 Phylogenetic tree based on the genome sequences of the four CLBV isolates under study and other available CLBV isolates from GeneBank. The tree was constructed by the Neighbor-joining method with 1000 bootstrap replicates. The accession number, country and host of each isolate were indicated.