Construction of an infectious full-length and eGFP-tagged cDNA clone of a chilli ringspot virus isolate from Yunnan province, China

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Research Article

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Abstract

Chilli ringspot virus (ChiRSV; genus *Potyvirus*) was one of several viruses previously detected in pepper samples with severe yellowing and curling symptoms growing in Wenshan, Yunan province, China. We now report the full-length sequence of ChiRSV-YN/Wenshan (MZ269480) which has 88.5-98.9%. nucleotide identity to other published ChiRSV isolates. A full-length cDNA infectious clone was constructed. This cDNA and an eGFP-tagged clone were infectious leading to systemic symptoms in both *Nicotiana benthamiana* and *Capsicum* spp. Single infection by ChiRSV caused mild mosaic or leaf crinkling in *Capsicum frutescens L.* and *Capsicum annuum L.*

Main Text

Chilli ringspot virus (ChiRSV) was first reported in Vietnam in 2007 [6] and was classified as a distinct species in the genus *Potyvirus* in 2009 [3]. In 2011, the determination of the genomic sequence of ChiRSV-HN/14 isolate (from Hainan, China) provided further evidence that ChiRSV is a distinctive potyvirus [5]. The ChiRSV genome has 9,571 nucleotides (nt) of ssRNA and the virus has typical filamentous particles 780 nm in length [5, 8]. Recently, a further isolate of ChiRSV was reported from Yunnan province [8] which groups phylogenetically with the Hainan isolate. It has a broad host range among several economically important crops including chilli, tomato, tobacco, and cucurbitaceous vegetables [8, 11].

In a previous study using Next-generation RNA-Seq sequencing (NGS) and RT-PCR we showed that ChiRSV, pepper vein yellows virus, chilli veinal mottle virus, tomato zonate spot virus and cucumber mosaic virus were prevalent in samples of pepper (*Capsicum frutescens* L.) plants from Wenshan city Yunnan province, China which had severe yellowing and curling symptoms [12]. ChiRSV was detected by RT-PCR in 31 of the 89 symptomatic samples. In this study we first obtained the complete genomic sequence of ChiRSV from Wenshan city. Total RNA was isolated using RTIzol^{TM} Reagent (Invitrogen) and first strand cDNA was synthesized using ReverTra Ace-α-®(Toyobo) following the manufacturer's protocol. Reverse transcription (RT) was performed at 42°C for 60 min with M4T primers (Supplementary Table S1) followed by 72°C for 10 min using KOD-plus-Neo (Toyobo) as specified by the manufacturer. The subsequent PCR used primers DP-ChiRSV f/DP-ChiRSV r (Supplementary Table S1) and incubation at 98°C for 3min, followed by 35 cycles of 98°C for 30s, 55°C for 30s, 68°C for 1kb/min and a final incubation at 68°C for 10min. 5' and 3' RACE reactions were performed to obtain the complete 5'and 3' terminal sequences and three overlapping sections were amplified to verify the full-length sequence as described before [12]. The genome of ChiRSV-YN/Wenshan was 9653 nt long (Supplementary Figure S1A) and the sequence was deposited in GenBank with accession number: MZ269480.

ChiRSV-YN/Wenshan has 88.5-98.9% nucleotide identity to the other reported full-length genome sequence of ChiRSV (Supplementary Table S2) and with 93.0-99.3% amino acid identity in comparisons of their polyproteins. In comparisons among the predicted mature proteins, only the P1 protein was less than 80% identical (Supplementary Table S2). In a Maximum Likelihood (ML) phylogenetic analysis,

ChiRSV-YN/Wenshan clustered with other published ChiRSV isolates in a group distinct from other potyviruses (Supplementary Fig. S2).

To examine the biological characteristics of ChiRSV-YN/Wenshan and to provide a tool for future investigations of mixed virus infection in pepper plant, a full-length infectious clone (pChiRSV) was prepared. The recombination cloning method was used to insert the complete ChiRSV cDNA sequence between the duplicated 35S promoter and hepatitis delta virus ribozyme (HDV-Rz) as described previously [12]. Then, pChiRSV was transformed into *Agrobacterium tumefaciens* which was then delivered to *Nicotiana benthamiana* plantlets by infiltration. Symptoms of witches' broom and systemic mosaic were observed in systemic leaves at 6 dpi, and infected plants were significantly smaller at 10 dpi (Fig. 1A and Supplementary Table S4). RT-PCR and Western blot detected viral RNA and coat protein in the new non-inoculated leaves confirming that systemic infection had been established (Fig. 1A and 1B). Typical flexuous filamentous virions approximately 780 nm long were observed by transmission electron microscopy (TEM) in negatively-stained samples of the upper leaves (Fig. 1C). To investigate the symptoms of ChiRSV on peppers, seedlings of *Capsicum frutescens L.* and *Capsicum annuum L.* were mechanically inoculated using sap from infected *N. benthamiana* plants. At 15 dpi (day post inoculation), inoculated plants had mild mosaic (*C. annuum*) or leaf crinkling (*C. frutescens*) (Figure 2A and Supplementary Table S4), RT-PCR and Western blot confirmed that virus had spread systemically in the inoculated plants (Fig. 2B) and flexuous filamentous virions were observed in new non-inoculated leaves by TEM (Fig. 2C). These results show that the full-length cDNA clone of ChiRSV-YN/Wenshan could successfully infect both *N. benthamiana* and pepper plants.

To trace and observe infection by ChiRSV-YN/Wenshan more conveniently, an eGFP-tagged cDNA infectious clone was then constructed. The sequence encoding a NIa protease cleavage site (TTVYHQ/A) was introduced between the eGFP and CP coding sequences (Supplementary Fig. S1C). The infectious clone pChiRSV-GFP was transformed into *Agrobacterium tumefaciens* which was then delivered to *N. benthamiana* plantlets by infiltration. At 12 dpi, examination under a UV lamp showed eGFP fluorescence in the upper non-inoculated leaves of most plants (Fig. 3A; Supplementary Table S4). The symptoms on these leaves were similar to those produced by the untagged cDNA clone. Western blots showed that the coat protein and GFP could be detected in the new non-inoculated leaves (Fig. 3B) and typical flexuous filamentous virions were also observed in them by TEM (Fig. 3C).

In this study of ChiRSV-YN/Wenshan, only the P1 protein was significantly different to the mature proteins of other ChiRSV isolates. P1 is a multifunctional protein that participates in RNA binding, is associated with inclusion bodies, and plays roles in cell-to-cell and systemic movement [1, 2, 4, 9, 10]. The full-length cDNA clone of pChiRSV constructed in this study will provide a useful tool to investigate whether the diversity of P1 sequences among the isolates indicates that they have diverse roles. The ChiRSV-YN/Wenshan isolate was obtained in 2019 from plants with severe symptoms of PeVYD (pepper vein yellows disease). This disease is associated with co-infection by several viruses but especially the phloem-limited PoPeVYV (pod pepper vein yellows virus) together with PoPeVYVaRNA (pod pepper vein

yellows virus-associated RNA) [7, 12]. Our infectious clone will also be a useful tool for examining whether ChiRSV can assist infection by PoPeVYV in the absence of PoPeVYVaRNA.

Declarations

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

MH, SJ, SW and JP conceived and designed the experiments. EY, HZ, QW, YL and HC collected the samples. MH and SJ performed the experiments. FY and JC analyzed the data. MH, SJ, SW and JP wrote the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

Infectivity and symptoms of ChiRSV-YN/Wenshan following inoculation of *Nicotiana benthamiana* by pChiRSV. (A) Phenotype of *N. benthamiana* plants agroinfiltrated with viral infectious clone combinations or empty agrobacterium (CK) at 10 days post infiltration. (B) RT-PCR and Western blot confirming the presence of viral RNAs in systemic leaves of inoculated plants. (C) Typical potyvirus virions in negatively-stained samples of systemic leaves of plants inoculated with the ChiRSV infectious clone. Bars represent 100nm.

Figure 2

Symptoms caused by ChiRSV in peppers. (A) Symptoms on plants of *Capsicum annuum L* and *C. frutescens L*. inoculated with ChiRSV and photographed at 15 dpi (B) RT-PCR and Western blot confirming the presence of viral RNAs in systemic leaves of inoculated plants. (C) Virions observed by TEM in negatively-stained samples of systemic leaves. Bars represent 200nm.

Figure 3

Expression of eGFP by the infectious clone pChiRSV-GFP in *N. benthamiana*. (A) Symptoms in *N. benthamiana* plants inoculated with pCB301-CH (Mock) or pChiRSV-GFP under UV and natural light at 8 dpi. (B) RT-PCR and Western blot analysis confirming the presence of ChiRSV-GFP in the infected leaves of *N. benthamiana* plants. (C) Virions observed by TEM in negatively-stained samples of systemic leaves of plants inoculated with the pChiRSV-GFP infectious clone. Bars represent 200nm.

Supplementary Files

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