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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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, Yunnan province, China. We now report the full-length sequence of ChiRSV-YN/Wenshan (MZ269480), which has 88.5-98.9% nucleotide sequence 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. Recombinant clones containing the P1 protein coding region of other ChiRSV isolates differed in their pathogenicity. Single infection by ChiRSV caused mild mosaic or leaf crinkling in Capsicum frutescens L. and Capsicum annuum L.

Chilli ringspot virus (ChiRSV) was first reported in Vietnam in 2007 [5], and in 2009, it was classified as a member of a distinct species in the genus Potyvirus [3]. It has a broad host range that includes several economically important crops such as chilli, tomato, tobacco, and cucurbitaceous vegetables [8, 11].

In a previous study using next-generation 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 the city of Wenshan in Yunnan province, China, that exhibited severe yellowing and curling symptoms [12]. ChiRSV was detected by RT-PCR in 31 of the 89 symptomatic samples. In this study, we determined the complete genome sequence of a ChiRSV isolate from Wenshan. Total RNA was isolated using TRizol 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 was performed using the primers DP-ChiRSV f and DP-ChiRSV r (Supplementary Table S1), with incubation at 98°C for 3 min, followed by 35 cycles of 98°C for 30 s, 55°C for 30 s, and 68°C for 1 min and a final incubation at 68°C for 10 min. 5’ and 3’ RACE reactions were performed to obtain the complete 5’- and 3’-terminal sequences, and three overlapping regions were amplified to verify the full-length sequence as described previously [12]. The genome of ChiRSV-YN/Wenshan was found to be 9653 nt long (Supplementary Fig. S1A), and the sequence was deposited in the GenBank database with accession number MZ269480.

ChiRSV-YN/Wenshan has 88.5-98.9% nucleotide sequence identity to the other reported full-length genome
sequences of ChiRSV (Supplementary Table S2) and 93.0-99.3% amino acid sequence identity to their polyproteins. Its sequence is very similar throughout its genome to that of another isolate from Yunnan (KX258620) [9], but the P1 protein has less than 80% sequence identity to those of isolates from Hainan and Hunan (Supplementary Table S2). The full genome sequences of ChiRSV isolates and closely related potyviruses were aligned using MUSCLE and analyzed for recombination using a variety of methods on the RDP4 recombinant platform. No recombination events were predicted (Supplementary Material).

To examine the biological characteristics of ChiRSV-YN/Wenshan and to provide a tool for future investigations of mixed virus infection in pepper plants, a full-length infectious clone (pChiRSV) was generated by homologous recombination, using a CloneExpress MultiS One Step Cloning Kit (Vazyme). The recombination cloning method was used to insert the complete ChiRSV cDNA sequence into the previously reported pCB301-MD vector [12]. The resulting construct, pChiRSV, was then introduced into Agrobacterium tumefaciens by transformation and delivered to young Nicotiana benthamiana plants 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 S3). Using RT-PCR and Western blot, viral RNA and the coat protein were detected in new non-inoculated leaves, confirming that systemic infection had been established (Fig. 1A and B). 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 days post-inoculation (dpi), inoculated plants exhibited mild mosaic (C. annuum) or leaf crinkling (C. frutescens) (Fig. 2A and Supplementary Table S3), RT-PCR and Western blot confirmed that the 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 examine the biological significance of the differences in their P1 proteins, recombinant clones of ChiRSV-YN/Wenshan were constructed by homologous recombination in which the P1 sequence was substituted for that of ChiRSV-HaiN (JQ234922) or ChiRSV-HuN (KX379001) (Supplementary Fig. 1C and Supplementary Table S1). Following inoculation of N. benthamiana plants as described above (Supplementary Table S3), similar viral symptoms appeared on the systemic leaves of those inoculated with pChiRSV-HaiN-P1 and pChiRSV, but only mild symptoms were observed on those inoculated with pChiRSV-HuN-P1 (Fig. 3A). Quantitative RT-PCR and Western blot indicated that there were significant differences in the levels of accumulation of viral RNA or proteins in the order pChiRSV > pChiRSV-HaiN-P1 > pChiRSV-HuN-P1 (Fig. 3B and C). The P1 amino acid sequences of the HaiN and HuN isolates

Fig. 1 Infectivity and symptoms of ChiRSV-YN/Wenshan following inoculation of Nicotiana benthamiana with 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 RNA in systemic leaves of inoculated plants. A 225-bp fragment of the UBC2 gene (GenBank no. KU726872) was amplified and used as an internal control. Equal protein loading was demonstrated using Ponceau S staining of the membranes. (C) Typical potyvirus virions in negatively stained samples of systemic leaves of plants inoculated with the ChiRSV infectious clone. Bars represent 100 nm.
are about 80% identical. These results indicate that differences in the P1 proteins affect viral RNA accumulation. P1 is a multifunctional protein that participates in RNA binding and suppression of posttranscriptional gene silencing. It is associated with inclusion bodies and plays roles in cell-to-cell and systemic movement [1, 2, 4, 6, 9, 10]. Differences in any or all of these functions may affect symptom severity.

To trace and observe infection by ChiRSV-YN/Wenshan more conveniently, an eGFP-tagged cDNA infectious clone was then constructed. A sequence encoding an N1a protease cleavage site (TTVYHQ/A) was introduced between the eGFP and CP coding sequences (Supplementary Fig. S1C). The infectious clone pChiRSV-GFP was introduced 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 (Supplementary Fig. S2A; Supplementary Table S3). 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 (Supplementary Fig. S2B), and typical flexuous filamentous virions were also observed in them by TEM (Supplementary Fig. S2C).

The ChiRSV-YN/Wenshan isolate was obtained in 2019 from plants with severe symptoms of pepper vein yellows disease (PeVYD). This disease is associated with coinfection by several viruses, but especially with the phloem-limited pod pepper vein yellows virus (PoPeVYV) together with pod pepper vein yellows virus-associated RNA (PoPeVYVaRNA) [7, 12]. Our infectious clone will be a useful tool for examining whether ChiRSV can assist infection by PoPeVYV in the absence of PoPeVYVaRNA.
Supplementary Information  The online version contains supplementary material available at https://doi.org/10.1007/s00705-022-05457-z.

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

Fig. 3  Symptoms and virus accumulation in Nicotiana benthamiana plants inoculated with wild-type and recombinant ChiRSV clones. (A) Phenotypes of N. benthamiana plants agroinfiltrated with the infectious viral clones pChiRSV, pChiRSV-HuN-P1, and pChiRSV-HaiN-P1 or empty agrobacterium (CK) 14 days after infiltration. (B) RT-qPCR confirming the accumulation of viral RNAs in systemic leaves of inoculated plants. For relative quantification of each RNA, the UBC2 gene of N. benthamiana was selected as an internal control. (C) Western blot confirming the accumulation of viral coat protein (CP) in systemic leaves of inoculated plants. The average grayscale values from membranes were quantified using ImageJ software and are shown below the image.

Declarations

Conflict of interest  The authors declare that they have no conflict of interest.

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