Molecular characterization of a novel single-stranded RNA virus, ChRV1, isolated from the plant-pathogenic fungus *Colletotrichum higginsianum*

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Abstract

In this study, a novel single-stranded RNA virus was isolated from the plant-pathogenic fungus *Colletotrichum higginsianum* strain HTC-5, and the virus was named “Colletotrichum higginsianum ssRNA virus 1” (ChRV1). The complete genome of ChRV1 is 3850 nucleotides in length with a GC content of 52% and contains two in-frame open reading frames (ORFs): ORF1 (smaller) and ORF2 (larger). ORF1 encodes a protein with the highest sequence similarity to proteins encoded by Phoma matteucciiola RNA virus 1 (PmRV1, 47.99% identity) and Periconia macrospinosa ambiguivirus 1 (PmAV1, 50.73% identity). ORF2 encodes a protein with a conserved RNA-dependent RNA polymerase (RdRp) domain with similarity to the RdRps of PmRV1 (61.41% identity) and PmAV1 (60.61% identity), which are recently reported unclassified (+) ssRNA mycoviruses. Phylogenetic analysis of the RdRp domain showed that ChRV1 grouped together with PmRV1, PmAV1, and other unclassified (+) ssRNA mycoviruses and had a distant relationship to invertebrate viruses and plant viruses of the family *Tombusviridae*. This is the first report of a novel (+) ssRNA virus infecting the phytopathogenic fungus *C. higginsianum*.

Mycoviruses (also called fungal virus) have been identified in all major fungal groups [1, 2]. The majority of mycoviruses belong to one of four groups: double-stranded RNA (dsRNA) viruses, positive-sense single-stranded RNA ((+) ssRNA) viruses, negative-sense single-stranded RNA ((-) ssRNA) viruses, and single-stranded DNA (ssDNA) viruses [3]. Due to the rapid development of high-throughput sequencing technologies, a growing number of (+) ssRNA mycoviruses have been discovered recently, many of which have not been assigned to formal taxa, such as Trichoderma harzianum ssRNA virus 1 (ThAV1), Setosphaeria turcica ssRNA virus 1 (StAV1), Verticillum longisporum ssRNA virus 1 (VIAV1), and Periconia macrospinosa ssRNA virus 1 (PmAV1) [4–6]. Gilbert et al. [6] recently proposed the establishment of the family “Ambiguiviridae” to include the above viruses and other recently described related viruses. Most mycoviruses are inconspicuous, causing few phenotypic effects in their fungal hosts. However, a few mycoviruses can induce phenotypic alterations causing hypovirulence of their fungal host and thus have potential as biological control agents for fungal diseases. The best example of this is the successful use of Cryphonectria hypovirus 1 (CHV1) to control chestnut blight disease in Europe [7].

*Colletotrichum higginsianum* is a hemibiotrophic pathogen that is responsible for anthracnose disease on cruciferous plants, such as *Brassica* and *Raphanus* [8], as well as *Arabidopsis*. The *Arabidopsis/C. higginsianum* pathosystem has emerged as an attractive model to study plant–pathogen interactions [9]. The discovery and identification of mycoviruses in *C. higginsianum* is an effective way to search for potential biological control agents against anthracnose disease. To date, only two mycoviruses from this fungus have been characterized: Colletotrichum higginsianum non-segmented dsRNA virus 1 (ChNRV1), identified in *C. higginsianum* strain IMI349063A in the USA [10], and Colletotrichum higginsianum mitovirus 1 (ChMV1), which we recently reported in *C. higginsianum* strain HTC-5 [11].

In this study, we characterized a (+) ssRNA mycovirus from *C. higginsianum* strain HTC-5, which we have...
provisionally named “Colletotrichum higginsianum ssRNA virus 1” (ChRV1).

Provenance of the virus material

*C. higginsianum* strain HTC-5 was isolated from a cabbage leaf with anthracnose disease in Changsha, Hunan Province, China, and identified as *C. higginsianum* in our previous study [11]. The fungus was cultured on potato dextrose broth at 28 °C in the dark. dsRNA was extracted as described by Morris and Dodds using the CF-11 cellulose chromatography method [12]. The extracted dsRNA was digested with DNase I and S1 nuclease (Takara, Dalian, China) to eliminate DNA and ssRNA prior to gel electrophoresis and visualization. A dsRNA segment ca. 3.9 kbp in size was visualized and purified (Fig. 1a) using a FastPure® Gel DNA Extraction Mini Kit (Vazyme Biotech Co., Ltd, Nanjing, China). A cDNA library was constructed using reverse transcriptase and random hexanucleotide primers (5’-CGA TCG ATC ATG ATG CAA TGNNNNNN-3’). Sequence gaps were filled by RT-PCR using sequence-specific primers based on the cDNA sequences obtained. The 5’- and 3’-terminal sequences were obtained by adapter ligation and PCR amplification as described previously [13]. All PCR amplicons were cloned into the pMD19-T vector (Takara, Dalian, China), and the resulting constructs were used to transform *Escherichia coli* DH5α cells. Recombinant clones were selected and sequenced, and each clone was sequenced independently at least three times. The complete, full-length cDNA sequence was assembled and submitted to the GenBank database with the accession number MW218984.

Sequence analysis, including ORF identification, homology searches, and conserved domain searches, were performed using the website of the National Center for Biotechnology Information (NCBI). Multiple alignments were conducted using Clustal X 2.0 [14] and annotated using GeneDoc [15] A phylogenetic tree was constructed using the neighbor-joining method in MEGA 7 [16]. Transmembrane domains were predicted using TMHMM Server v. 2.0.

Sequence properties

The complete genome sequence of ChRV1 is 3850 nt in length and contains two non-contiguous ORFs. The 5’ and 3’ untranslated regions (UTRs) of ChRV1 are 633 nt and 559 nt in length, respectively (Fig. 1b).

ORF1 encodes a 347-aa protein with a predicted molecular mass of 38.3 kDa. A BLASTp search showed that the aa sequence of the protein encoded by ChRV1 has the highest sequence similarity to proteins encoded by *Periconia macrospinosa ambiguivirus 1* (PmAV1) (50.73% identity; E-value, 7e-69; query coverage, 78%) and *Phoma matteucicicicola RNA virus 1* (PmRV1) (47.99% identity; E-value, 6e-83; query coverage, 86%), followed by other unclassified (+) ssRNA mycoviruses (Supplementary Table S1) whose functions are unknown. In addition, ORF1 and ORF2 were found in the same reading frame, and an amber stop codon (UAG) was found at the end of the ORF1, suggesting that the two ORFs are likely to produce a fusion protein with the downstream RdRp via a readthrough strategy, as described previously [6, 17]. Moreover, four transmembrane helices were predicted at the N-terminus of the ORF1 protein (Supplementary Fig. S1), suggesting that transmembrane domains are important for (+) ssRNA viruses and play a role in anchoring viral proteins to host membrane, as proposed for MoVA and VdRV [17, 18].

ORF2 was predicted to encode a 495-aa protein with a predicted molecular mass of 56.2 kDa. A BLASTp search showed that the aa sequence of the ORF2-encoded protein is most closely related to the RdRps of PmRV1 (identity, 61.41%; coverage, 98%; E-value, 0.0) and PmAV1 (identity, 60.61%; coverage, 92%; E-value, 0.0), followed by other unclassified (+) ssRNA mycoviruses, including soybean leaf-associated ssRNA virus 2 (SlaRV2) (identity, 58.04%; coverage, 91%; E-value, 1e-78), soybean leaf-associated ssRNA virus 3 (SlaRV3) (identity, 58.73%; coverage, 88%; E-value, 9e-173), MoVA (identity, 54.73%; coverage, 91%; E-value, 4e-161), and Setosphaeria turcica ambiguivirus 1 (StAV1) (identity, 51.14%; coverage, 95%; E-value, 1e-156) (Supplementary Table S1). The RdRp sequence of ChRV1 also shared sequence similarity with RdRps of Hubei tombus-like virus 12, Changjiang tombus-like virus 8, Changjiang tombus-like virus 15, and Wenling tombus-like virus 1, which are recently described invertebrate viruses [19]. It was also found to be similar to the RdRps of members of the plant virus family *Tombusviridae* (Supplementary Table S1). A conserved domain database (CDD) search and multiple protein alignment indicated that the 56.2-kDa protein encoded by ORF2 contained a conserved viral RdRp domain of the subfamily RdRP_3 (pfam00998) with five conserved motifs (Fig. 1c).

To further examine the relationship between ChRV1 and other mycoviruses, phylogenetic analysis was performed using the RdRp aa sequences of ChRV1 and other related RNA viruses. The results indicated that ChRV1 clusters together with PmRV1, PmAV1, and other unclassified (+) ssRNA mycoviruses but is distinct from the invertebrate and plant viruses (Fig. 2). All of these selected unclassified (+) ssRNA mycoviruses and ChRV1 contain two non-overlapping ORFs, and the RdRp domain is encoded by ORF2. Moreover, these unclassified (+) ssRNA mycoviruses are more similar in their genome organization to invertebrate viruses with two in-frame ORFs [18], and they
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1. **A single-stranded RNA virus from *Colletotrichum higginsianum***

   - Differ from members of the family *Tombusviridae*, suggesting that these unclassified (+) ssRNA mycoviruses are more closely related to invertebrate viruses than to plant viruses of the family *Tombusviridae*. In addition, the ChRV1 RdRp contains the amino acid triplet GDN in motif III, which differs from the GDD motif in (+) ssRNA viruses (Fig. 1c). The same triplet was found in previous studies including polymycoviruses [17, 20–22]. Taken together, we conclude that ChRV1 is a novel (+) ssRNA virus and should be considered a new member of the recently proposed family "Ambiguiviridae" [6].

2. **Supplementary Information**

   - The online version contains supplementary material available at https://doi.org/10.1007/s00705-021-05071-5.

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**Fig. 1** (a) Agarose gel electrophoresis showing the dsRNA banding pattern of ChRV1 isolated from *C. higginsianum* strain HTC-5. (b) Organization of the genome of ChRV1. The open reading frames (ORFs) and the untranslated regions (UTRs) are showed as open bars and single lines, respectively. (c) Multiple amino acid sequence alignment of RdRps of ChRV1 and other related mycoviruses. The conserved motifs are indicated by bold black lines and the Roman numerals I to V.
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Declarations

Conflict of interest All authors declare no conflicts of interest.

Ethical approval This article does not contain any experiments with human participants or animals performed by any of the authors.

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