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ANNOTATED SEQUENCE RECORD

Complete nucleotide sequence of a new carlavirus in chrysanthemums in China

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Abstract

A new virus causing a serious stunt disease of chrysanthemum was identified in China by high-throughput sequencing (HTS) and named chrysanthemum virus R (CVR). The complete sequence of CVR was determined by reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The genomic RNA of CVR consists of 8,874 nucleotides (nt), excluding the poly(A) tail, contains six putative open reading frames (ORFs), and has a genomic organization typical of members of the genus *Carlavirus*. BLAST analysis of the full genome sequence showed low similarity (38%–56% sequence identity) to other members of the genus *Carlavirus*. BLAST analysis and phylogenetic analysis based on the amino acid (aa) sequences of the CVR replicase and coat protein (CP) confirmed that CVR is a distinct member of the genus *Carlavirus*.

Chrysanthemum (*Chrysanthemum morifolium* Ramat, family Asteraceae), is a perennial herbaceous plant and one of the most popular cut flowers. Some cultivars of chrysanthemum, such as "Hangju", "Huaiju", and "Boju", are also used as important traditional Chinese medicines. Chrysanthemum production is severely affected by viruses and viroid diseases; approximately 20 viruses and viroids have been reported to infect chrysanthemums [7]. Nine viruses and viroids, including CVB, tomato aspermy virus (TAV), cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), potato virus Y (PVY), potato virus X (PVX), zucchini yellow mosaic virus (ZYMV), chrysanthemum stunt viroid (CSVd), and chrysanthemum chlorotic mottle viroid (CChMVd), have been identified in chrysanthemum in China [3, 4, 9].

In February 2017, chrysanthemum plants displaying stunted growth (Fig. 1a) were found in the Yanqing District of Beijing, China. To identify the causal agent(s) of this stunting, total RNA was extracted from symptomatic leaves using TRNzol Reagent (Tiangen, Beijing, China) according to the manufacturer's instructions and used in RT-PCR to detect specifically CVB, TAV, CSVd, CMV, and members of the genera *Potyvirus* and *Tobamovirus*. None of the expected PCR products were obtained. Therefore, the total RNA samples were further analyzed by high-throughput sequencing (HTS) on an Illumina HiSeq 2500 platform. The sequence reads were processed to remove low-quality and adaptor sequences, after which they were assembled using the *de novo* assembly algorithms of Velvet [8] with a *k*-mer of 17. The assembled contigs were annotated against the GenBank Virus Reference Database (ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral/) using the local BLASTn program. Based on the BLASTn search results, 26 contigs ranging from 46 to 183 bp were found to share a high level of sequence identity (74%–89%) with several carlaviruses. To confirm the HTS results, genomic sequencing was performed using RT-PCR and RACE PCR with the cloning strategy shown in Fig. 1b. Most of the genomic sequence of the target virus was obtained by amplifying six overlapping

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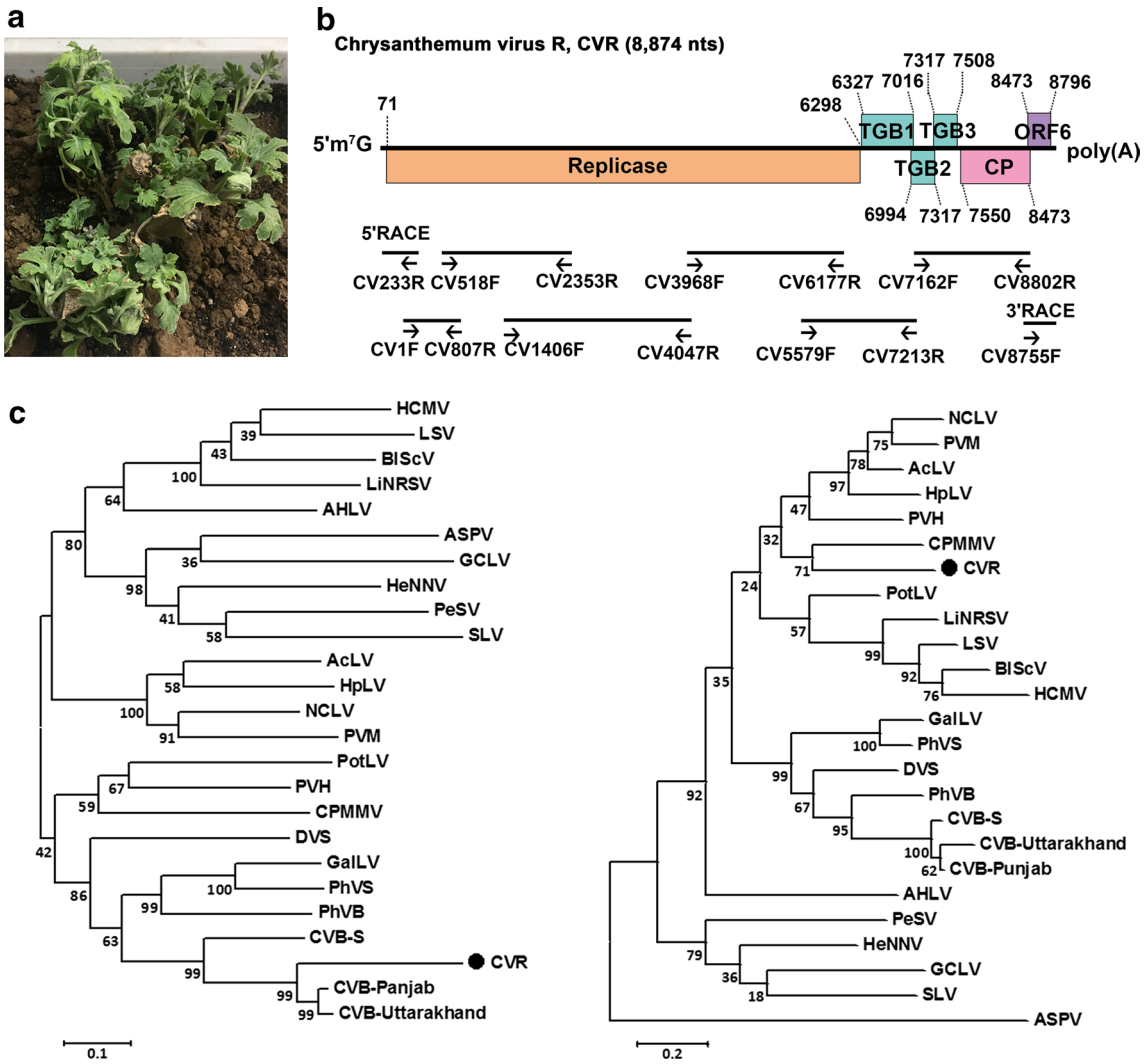

Fig. 1 Characterization of a novel carlavirus, chrysanthemum virus R (CVR), isolated from chrysanthemums in China. (**a**) Symptomatic chrysanthemum plant from which CVR was isolated. (**b**) Genomic organization of CVR and the genome cloning strategy used in this study. (**c**) Phylogenetic relationships of CVR and 23 reported carlaviruses based on the amino acid sequences of their replicases (left) and coat proteins (right). Phylogenetic trees were constructed using the maximum-likelihood method in MEGA 5.2.1 software. Bootstrap values (1,000 replicates) are shown below the branches. The following viruses were included in the analysis: aconitum latent virus (AcLV, AB051848), American hop latent virus (AHLV, KR185345), blueberry scorch virus (BlScV, AY941198), *cowpea mild mottle virus* (CPMMV, KC884246), CVB isolate Punjab (CVB-Panjab, AM493895), isolate Uttarakhand (CVB-Uttarakhand, AM765838), isolate S (CVB-S, AB245142), daphne virus S (DVS, AJ620300), gaillardia latent virus (GalLV, KJ415259), garlic common latent virus (GCLV, JF320810), hydrangea chlorotic mottle virus (HCMV, EU754720), helleborus net necrosis virus (HeNNV, FJ196836), hop latent virus (HpLV, KP861891), ligustrum necrotic ringspot virus (LiNRSV, EU074853), lily symptomless virus (LSV, LC004126), narcissus common latent virus (NCLV, AM158439), pea streak virus (PeSV, KP828803), phlox virus B (PhVB, EU162589), phlox virus S (PhVS, EF492068), potato latent virus (PotLV, EU433397), potato virus H (PVH, JQ904630), potato virus M (PVM, D14449), and shallot latent virus (SLV, HQ258896). Apple stem pitting virus (ASPV, D21829) was used as an out-group

PCR fragments with specific primers designed according to the HTS contig sequences (Supplementary Table S1). The sequences of the 5′ and 3′ untranslated regions (UTRs) were determined by 5′- and 3′-RACE PCR with primers CV233R and CV8755F, respectively (Supplementary Table S1). The genomic sequence of the target virus was found to be 8,874 nt in length, excluding the poly(A) tail. BLASTn analysis of the complete sequence showed that the virus shares low sequence identity (38%–56%) with known members of genus *Carlavirus*. Therefore, we propose that the identified virus, which we have named "chrysanthemum virus R" (CVR) is a new member of genus *Carlavirus*. The genomic sequence of CVR was deposited in the GenBank database with accession number MG432107.

The CVR genome contains six ORFs and has the typical features of a carlavirus (Fig. 1b). ORF1 (nt 71–6,298)

follows a short 5′-UTR and encodes a viral replicase of 2,075 aa residues. Five domains were identified in the CVR replicase using the Conserved Domain Database on the NCBI website (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi): viral methyltransferase (MTR), 2OG-Fe (II) oxygenase superfamily, carlavirus endopeptidase, superfamily 1 viral RNA helicase, and RNA-dependent RNA polymerase (RdRp). A conserved motif, ^{1,916}SGX$_3$TX$_3$NTX$_{22}$GDD^{1,951} (X, any aa residue), was found near the C-terminus of the RdRp domain. An ovarian tumor (OTU) family domain, which is present in some carlaviruses, was not found in the replicase of CVR. Three overlapping ORFs, ORF2 (nt 6,327–7,016), ORF3 (nt 6,994–7,317), and ORF4 (nt 7,317–7,508), are immediately downstream of the CVR replicase and potentially form a triple gene block (TGB) involved in viral movement. ORF5 (nt 7,550–8,473) encodes a CP of 307 aa residues. ORF6 (nt 8,473–8,796) encodes a cysteine-rich protein (CRP) of 144 aa residues. Similar to CRPs in other carlaviruses, the CVR CRP has a nuclear localization signal (NLS) at aa 47–50 and a zinc finger-like motif (ZF) at aa ^{57}CX$_2$CX$_{12}$CX$_4$C^{78}, adjacent to the NLS. It was recently reported that the N-terminal region, NLS and ZF motifs in CRPs are critical factors that determine viral pathogenicity [2].

BLAST analysis with the sequences of the CVR replicase and CP indicated that the CVR replicase shares the highest sequence identity with the replicase of CVB isolate Uttarakhand (CVB-Uttarakhand, GenBank accession no. AM765838), with 64% nt sequence identity and 67% aa sequence identity, whereas the CVR CP has the highest sequence identity with the CP of narcissus common latent virus (NCLV, AM158439), with 58% nt sequence identity and 51% aa sequence identity. These values are all well below the species demarcation threshold (72% nt sequence identity and 80% aa sequence identity) for the genus *Carlavirus* [1], so CVR is proposed to be a new carlavirus. To determine the taxonomic position of CVR, phylogenetic trees were constructed using the maximum-likelihood method (1,000 replicates) in MEGA 5.2.1 software using the aa sequences of the replicases and CPs of CVR and 23 reported carlaviruses. Apple stem pitting virus (ASPV, D21819) was used as an out-group. The phylogenetic tree based on replicase aa sequences revealed that CVR clusters closely with CVB-Uttarakhand and CVB-Punjab (AM493895), while the phylogenetic tree based on CP aa sequences revealed that CVR clusters closely with cowpea mild mottle virus (CPMMV, KC884246). As recombination within the full-genome sequences of CVB has been reported previously [6], we tested for the presence of recombination within the full genome sequences of CVR and of representatives of other carlavirus species using Recombination Detection Program version 4 (RDP4) [5], which revealed no evidence of significant recombination within the CVR genome.

We also determined the incidence of the virus and compared the incidence of CVR with that of CVB, one of the most commonly occurring chrysanthemum viruses [9]. Seventeen chrysanthemum seedling samples were collected randomly from a chrysanthemum-growing region in Beijing, China. The total RNA of the seedlings was extracted and subjected to RT-PCR to detect CVR and CVB with the primer pairs CV7162F/CV8802R and CVB-U/CVB-L, respectively (Supplementary Table S1). The RT-PCR results showed that eight plants were infected by CVR (47% incidence), whereas seven plants were infected by CVB (41% incidence) (Supplementary Fig. S1). These results suggest that the incidence of CVR in chrysanthemums is likely to be at least as high as that of CVB.

In this work, we determined the complete genome sequence of CVR, a new carlavirus infecting chrysanthemums in Beijing, China. CVR merits significant research because it is associated with severe stunting in chrysanthemums and is one of the most common chrysanthemum viruses in China.

Compliance with ethical standards

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

Research involving human participants or animals This study did not include experiments with human participants or animals performed by any of the authors.

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