Virus Research 159 (2011) 73–78

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Genomic characterization of a novel dsRNA virus detected in the phytopathogenic fungus *Verticillium dahliae* Kleb.☆

Yue-fen Cao[a], Xi-wu Zhu[a,b,*], Yu Xiang[b], De-qiang Li[a], Jia-rong Yang[c], Qian-zhuo Mao[a], Ji-shuang Chen[a]

[a] *Institute of Bioengineering, Zhejiang Sci-Tech University, Hangzhou, Zhejiang 310018, China*
[b] *Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, British Columbia V0H 1Z0, Canada*
[c] *College of Plant Protection, Northwest A F University, Yangling, Shaanxi 712100, China*

ARTICLE INFO

Article history:
Received 10 January 2011
Received in revised form 27 April 2011
Accepted 28 April 2011
Available online 6 May 2011

Keywords:
Verticillium dahliae chrysovirus 1
VdCV1
Mycovirus
dsRNA segments
Chrysovirus
Verticillium dahliae Kleb.

ABSTRACT

Four novel double-stranded RNA segments were detected in a *Verticillium dahliae* Kleb. strain (*V. dahliae* isolate 0-21), a causal fungal agent of Verticillium wilt disease of cotton. Each dsRNA genome segment contains a single large open reading frame (ORF) that encodes a distinctive protein with modest levels of sequence similarities to the corresponding putative proteins in the genus *Chrysovirus*. These include an RNA-dependent RNA polymerase (RdRp), a coat protein, an undefined replication-related protein and an ovarian tumor domain peptidase. Phylogenetic analysis of the four putative proteins unanimously indicated that they are evolutionarily related to viruses in *Chrysovirus*. The 5′- and 3′-untranslated regions of the four dsRNAs share highly similar internal sequence and contain conserved sequence stretches of UGAUAAAAAA(/U)UG(/U)AAAAA- (in the 5′-UTR) and -UUUACUACU (in the 3′-UTR), indicating that they have a common virus origin. Indeed, isometric virus-like particles (VLPs) with a diameter of approximately 34 nm were extracted from the fungal mycelia, and the four dsRNA segments were also detected in the virus-like particle (VLP) fraction. These results suggest that the mycovirus with four different dsRNA genome segments from the fungal isolate 0-21 is a new member of the genus *Chrysovirus*. We named the virus *Verticillium dahliae* chrysovirus 1 (VdCV1).

Mycoviruses may potentially be used as biological control agents of fungi based on reports that some mycoviruses have the ability to decrease virulence, growth rate and activities of the infected fungi (Ghabrial and Suzuki, 2009; McCabe et al., 1999; Nuss, 2000). For example, chestnut blight fungus, *Cryphonectria parasitica* infected with *C. hypovirus 1* stain EP713 (CHV1-EP713) or Euro7 (CHV1-Euro7) displayed severe reduction in fungal virulence, conidiation, pigmentation and fertility (Nuss, 2000). *Mycoreovirus 1* (MyRV1-Cp9B21) was shown to have little effect on sporulation and pigmentation of the *C. parasitica* but did attenuate the fungal virulence more dramatically than CHV1 (Sun et al., 2006). *Rosellinia necatrix mycoreovirus 3* (RnMYRV3-W370) was shown to be responsible for the hypovirulence of *R. necatrix*, a soil borne ascomycetous fungus that causes white root rot in fruit trees and other woody plants (Kanematsu et al., 2004). Recently, an unclassified novel double-stranded RNA (dsRNA) virus, *Alternaria alternata* virus 1 (AaV1) was found to impair the growth rate of the fungus *A. alternata* (Aoki et al., 2009). Most known phytopathogenic fungal viruses have dsRNA genome segments and are classified in the families *Totiviridae*, *Partitiviridae*, *Chrysoviridae* and *Reoviridae* (Ghabrial and Suzuki, 2009). Viruses in *Totiviridae* have a monopartite dsRNA genome segment of ~4.6–6.7 kbp in size. The genome segments of viruses *Partitiviridae* are composed of two dsRNAs ranging in size from 1.4 to 3.0 kbp. The *Chrysoviridae* consists of viruses containing four separately encapsidated dsRNA segments ranging in size from 2.4 to 3.6 kbp. The genome of viruses in the genus *Mycoreovirus* consists of 11–12 different dsRNA segments from 0.7 to 4.0 kbp in size (Ghabrial and Suzuki, 2009).

Verticillium dahliae Kleb. is the causal fungal agent of Cotton *Verticillium* wilt disease. The fungus was accidentally introduced into China from the United States in 1935 through commercial cotton import activity, and it has become the most devastating pathogen in cotton production. It also infects potato, soybean, tomato, beets and over 170 other agricultural corps (Tjamos et al., 2000). It is estimated that over 50% of the cotton fields in China are affected by the disease every year, causing annual reduction in yield of over 100,000 tons (Li et al., 2003). Strains of *V. dahliae* are classified into two pathotypes—defoliating (D) and nondefoliating (ND). D-strains are highly virulent and can completely defoliate the infected plant.

☆ The GenBank/ENBL/DDBJ accession numbers of the sequences reported in this paper are HM004067 through HM004070.

* Corresponding author at: Institute of Bioengineering, Zhejiang Sci-Tech University, Xiasha Road 2, Hangzhou, Zhejiang 310018, China. Fax: +86 571 8684 3196.
E-mail address: zhuxw9999@yahoo.com.cn (X.-w. Zhu).

0168-1702/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2011.04.029

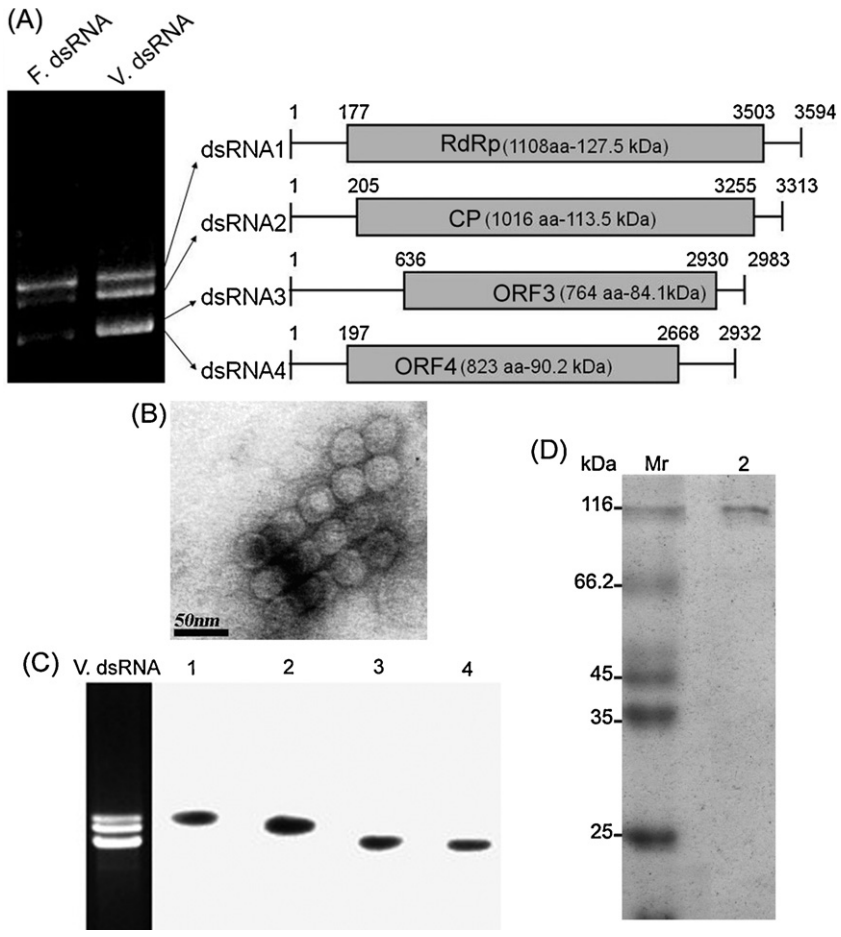

Fig. 1. Analysis of mycovirus in *V. dahliae* isolate 0-21. (A) 1.0% agarose gel electrophoresis of dsRNA segments (left) and diagrammatic representation of genome organization of the four dsRNAs (right). F. dsRNA, dsRNA segments extracted directly from fungal mycelia; V. dsRNA, dsRNA segments extracted from purified virus-like particle (VLP) fraction. (B) Isometric VLPs with buoyant densities of 1.32–1.36 g/cm^3 in CsCl gradients were extracted from *V. dahliae* isolate 0-21 and observed under transmission electron microscopy after negatively staining with 2% uranyl acetate. Bar = 50 nm. (C) Northern blot analysis of the dsRNA segments. RNA extracted from the VLPs using TRIzol reagent (Invitrogen, USA) was separated in a 0.8% agarose gel and transferred onto Hybond-N$^+$ membrane (Amerasham). Individual lanes were hybridized with ^{32}P-labeled probes specific for each of the four dsRNA segments (the putative open reading frame regions): 1, dsRNA1; 2, dsRNA2; 3, dsRNA3; 4, dsRNA4. (D) Coomassie brilliant blue stained 12% acrylamide gel (SDS-PAGE) with proteins extracted from VLP fraction (lane 2). Mr, protein molecular weight marker (Fermentas, SM0431).

ND-stains are relatively mild in virulence and cause wilt and partial or no defoliation (Schnathorst and Mathre, 1966). Currently, no fungicides are available to effectively control diseases caused by *V. dahliae*. No virus was previously reported to infect the fungus, either. With an aim to develop biological control agents of Cotton *Verticillium* wilt disease in the future, we investigated for the presence of viruses that may infect the fungus.

The first step in the search for viral infected fungus is to look for the presence of dsRNA in fungal cells since all RNA viruses have a dsRNA stage during their infection cycle. Plant and fungal cells do not normally contain detectable amounts of large molecules of dsRNA and the presence of dsRNA is considered to be an indicator of virus infection in plants or fungi (Marquez et al., 2007). Extraction of dsRNAs from mycelia was performed as reported (Chen et al., 2006). A total 11 *V. dahliae* isolates originally collected from cotton growing fields located in three district regions (Sanyuan, Ningwei and Weinan) with a radial distance of ~200 km in Shaanxi Province, China were screened for the presence of dsRNA. Only one isolate, *V. dahliae* 0-21 was discovered that had detectable levels of dsRNAs (approximately 2.0 μg/g mycelium powder) (Fig. 1A). DsRNAs were resolved by 1% agarose gel electrophoresis and consisted of four segments, termed dsRNA1, 2, 3 and 4, respectively, according to their decreasing size. DsRNA1 is near 3.6 kbp; dsRNA2 is approximately 3.3 kbp. DsRNAs 3 and 4 were both slightly less than 3.0 kbp and they were barely separable on 1% agarose gels. In subsequent experiments, the virus-like particle (VLP) fraction was isolated from the *V. dahliae* 0-21 isolate by an ultracentrifugation method in the presence of glycerol of two different densities (5% and 20%) as previously described (Chen et al., 2006). The RNAs extracted from the VLP fraction displayed the same pattern of migration in agarose gels as the dsRNAs extracted from the fungus. Four segments of dsRNA were detected, but more amount of dsRNA was evident (Fig. 1A). Moreover, the dsRNAs from the VLPs were used for cDNA synthesis and cloning. Full-length cDNA clones were obtained using a modified single-primer amplification technique (M-SPAT) (Potgieter et al., 2002; Chen et al., 2006). Sequence analysis revealed that the four dsRNAs have high similarities in their 5′- and 3′-untranslated regions (UTRs) (see analysis below) indicating a common viral origin. All four dsRNA segments showed sequence similarities to viruses in the genus *Chrysovirus* (further analysis below). In another independent experiment, the VLP fraction was subjected to an ultracentrifugation in a caesium chloride density gradient (1.10–1.40 g/cm^3) at 25,000 rpm (P70AT rotor, Hitachi) for 2 h. Intact VLPs with buoyant densities of 1.32–1.36 g/cm^3 in CsCl were observed under transmission electron microscopy after staining with 2% uranyl acetate. The VLPs were isometric vesicles with a diameter of approximately 34 nm (Fig. 1B). Northern blot analysis using ^{32}P-labeled cDNA probes specific for each of the four dsRNA segments (the putative open reading frame regions), which were made by random primer DNA labeling kit Ver.2.0 (Takara,