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Short communication

Characterization of a novel single-stranded RNA virus, closely related to fusariviruses, infecting the plant pathogenic fungus *Alternaria brassicicola*

Jie Zhong[a,1], Hong Hong Shang[a,1], Chuan Xia Zhu[b], Jun Zi Zhu[a,b], Hong Jian Zhu[a,c], Yan Hu[a], Bi Da Gao[a,*]

[a] Hunan Provincial Key Laboratory for Biology and Control of Plant Diseases and Insect Pests, Hunan Agricultural University, Nongda Road 1, Furong District, Changsha City, Hunan Province 410128, PR China
[b] Changde Agriculture & Science Research Institute, Changde City, Hunan Province 415000, PR China
[c] Southern Regional Collaborative Innovation Center for Grain and Oil Crops in China, Hunan Agricultural University, PR China

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ABSTRACT

The alternaria blackspot of rapeseed is one of the most prominent diseases of rapeseed. It is caused by three species of the genus *Alternaria*: *Alternaria brassicicola*, *Alternaria brassicae*, and *Alternaria raphanin*. Here we report a novel positive-sense RNA virus from an *A. brassicicola* strain 817-14. The virus has a 6639 nucleotide (nt) long genome, excluding a poly (A)-tail, and was predicted to contain three putative open reading frames (ORF1, ORF2, and ORF3). The large ORF1 encoded a 174-kDa polyprotein (composed of 1522 amino acid residues) containing a conserved RNA-dependent RNA polymerase (RdRp) domain and a helicase domain. The other two smaller ORFs encoded polypeptides with unknown function. Homology search and phylogenetic analysis, based on the RdRp and helicase domains, suggest that this virus is related to and grouped with Sclerotinia sclerotiorum fusarivirus 1 (SsFV1), Rosellinia necatrix fusarivirus 1 (RnFV1), Fusarium graminearum virus-DK21 (FgV1), and Penicillium roqueforti RNA mycovirus 1 (PrRV1), all of which belong to a newly proposed family Fusariviridae. For this study, we designed the virus as "Alternaria brassicicola fusarivirus 1" (AbFV1). Virus elimination revealed that AbFV1 has no conspicuous impact on the biological properties of its host.

Mycoviruses or fungal viruses are prevalent among the major taxonomic groups of fungi, including yeasts, oomycete, and filamentous fungus, most of which belong to plant-pathogenic fungi (Ghabrial and Suzuki, 2009; Pearson et al., 2009; Xie and Jiang, 2014). Mycoviruses, with an RNA genome, were classified into three groups: double-stranded RNA (dsRNA), positive-sense single-stranded RNA (+ssRNA), and negative-sense single-stranded RNA (−ssRNA) (recently discovered) (Liu et al., 2014; Ghabrial et al., 2015). The classification of novel mycoviruses is continually updated and refined to capture the ever-increasing number of them that are reported, some of which remain to be unassigned. Mycoviruses consisting of an +ssRNA genome have been classified into seven families: Alphaflexiviridae, Barnaviride, Endornaviridae, Gammaflexiviridae, Hypoviridae, Narnaviridae, and a newly proposed family Fusariviridae. The family Fusariviridae was named from the prototype Fusarium graminearum virus-DK21 (FgV1) that infecting the *Fusarium graminearum* fungus. Up to now, the sequences of some viruses in NCBI database (i.e., Sclerotinia sclerotiorum fusarivirus 1 [SsFV1], Penicillium roqueforti SsRNA mycovirus 1 [PrRV1] and Rosellinia necatrix fusarivirus 1 [RnFV1], Macrophomina phaseolina single-stranded RNA virus 1 [MpRV1]) were found to closely related to FgV1 and might be accommodated to this family.

Many mycoviruses may cause latent infection in their fungal hosts (Ghabrial and Suzuki, 2009). Some mycoviruses, however, can produce serious phenotypic alterations in their fungal hosts, including hypovirulence and debilitation. This suggests that a few mycoviruses can be employed for biological control of the fungal disease, as has been adequately proven with Cryphonectria hypovirus 1 (CHV1), to control chest blight disease in Europe (Nuss, 2005). Moreover, the coevolution of some mycoviruses with their hosts has contributed to the establishment of some host-mycovirus systems, which is beneficial for

* Corresponding author. Fax: +86 731 84635136.
E-mail addresses: bdgao@aliyun.com, 1601389330@qq.com (B.D. Gao).
[1] These authors contributed equally to this work.

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studying virus-host interactions (Wang et al., 2015; Xie and Jiang, 2014) (i.e., Cryphonectria parasitica-hypovirus, Helmintosporium victoriae-HvV190S, Sclerotinia sclerotiorum-mycovirus, Rosellinia necatrix-mycovirus, and Fusarium graminearum-mycovirus). This occurrence may enhance the understanding of fungal and viral pathogenesis at a molecular level. Mycoviruses, which are prevalent throughout most fungi taxonomic groups, are divergent in biological properties and molecular characteristics. The discovery of more mycoviruses is advantageous for greater insight into viral evolution and may be critical to progress in mycovirology.

The genus *Alternaria* encompasses a complex group of filamentous fungi – some of which are major pathogens on many kinds of plant species – and is responsible for major losses on a wide range of crops (Woudenberg et al., 2013). The alternaria blackspot of rapeseed, caused by *Alternaria brassicicola*, *Alternaria brassicae*, and *Alternaria raphanin*, is one of the most important diseases generating massive economic losses in yearly rapeseed production in China. Currently, screening for mycoviruses, that have a potential for biocontrol, is regarded as an initial step for virocontrol, an alternative disease control measure (deemed environmentally friendly) for fungal disease management. *Alternaria* species have been reported to harbor several viruses. Virus-like dsRNAs were detected in *Alternaria alternate* pathotypes isolated from cotton seeds and Japanese pear trees (Aoki et al., 2009; Shepherd, 1988; Hayashi et al., 1988). Importantly, a new mycovirus (Alternaria alternata virus 1 [AaV-1]), whose genome composed of four dsRNA segments, was identified and showed potential for biocontrol agent. AaV-1 is distinct from most common mycoviruses (i.e., totiviruses, chrysoviruses, partitiviruses, reoviruses, and hypoviruses) but related to the Aspergillus mycovirus 341 (AsV341) (Aoki et al., 2009; Hammond et al., 2008). Furthermore, dsRNA elements in the *A. alternate* Japanese pear pathotype are associated with morphological changes in the host fungus through some negative effects, such as apoptosis-like cell death (Fuke et al., 2011). Two not-sequenced dsRNAs (molecular weight of 8.3 kbp and 5.5 kbp) were detected from *A. alternate* and found to be non-encapsidated and associated with spherical membrane vesicles in infected hosts (Zabalgogeazcoa et al., 1997). For *Alternaria longipes*, a mycovirus with a non-segmented dsRNA genome has recently been reported and was related to the unassigned Curvularia thermal tolerance virus (Lin et al., 2014). A novel member (designated "Alternaria brassicicola endornavirus 1" [AbEV1]) of the family Endornaviridae was characterized by an *A. brassicicola* strain of 817-14 (Shang et al., 2015). Here we report on the molecular characterization of a novel putative (+ssRNA) virus from the *A. brassicicola* strain 817-14 and termed it "Alternaria brassicicola fusarivirus 1" (AbFV1). This fungus strain has been confirmed to harbor AbEV1 and AbFV1 (two unrelated mycoviruses). We determined the complete sequence and analyzed the genome organization of AbFV1. The biological effects of this virus on its host were also evaluated.

The *A. brassicicola* strains used in this study were collected from rapeseed pods displaying alternaria blackspot symptoms (in May 2013, from the Hunan province of China). For dsRNA extraction, mycelial plugs were inoculated in potato dextrose (PD) broth in an orbital shaker at 110 rpm for 4–7 days at 27 °C. DsRNAs were extracted from fungal mycelia by the CF-11 cellulose chromatography method described by Morris and Dodds (1979). The dsRNA samples were digested with DNase I (RNase-free) and S1 nuclease (TaKaRa, Dalian, China) to eliminate contaminating single-stranded RNA (ssRNA) and DNA and to confirm their dsRNA nature. The dsRNA banding pattern and molecular size were estimated by agarose electrophoresis and visualization. We tested 25 strains for the presence of dsRNAs. As showed in Fig. 1a, five strains were detected to be dsRNA positive, with the size ranging from 2.5 kbp to 10 kbp. We selected the *A. brassicicola* strain 817-14 containing two dsRNA banding (10 kbp and 6 kbp, respectively) for further analysis (Fig. 1c). In this study, we determined the full-length cDNA sequence of the 6 kbp dsRNA that represents the genome component of a virus tentatively named AbFV1. The 6 kbp dsRNA segment was purified from strain 817-14 by a gel extraction kit (TaKaRa, Dalian, China) and used for cDNA cloning. Full-length cDNA clones were obtained by cDNA library synthesis using random hexadeoxynucleotide primers (TaKaRa) and RT-PCR to fill the gaps in the initial round of random cDNA synthesis. The 5′- and 3′-terminals of the dsRNA sequences were obtained by adaptor ligation to the 3′ end of each strand with T4 RNA ligase (Fermentas) and PCR amplification (Zhong et al., 2014). Sequence similarity searches were conducted using BLASTp program in the NCBI database (Altschul et al., 1997). ORFs and conserved domains were found by the ORF finder and conserved domains in the NCBI database as well. Multiple sequence alignments and phylogenetic analysis were carried out using the CLUSTALX (Thompson et al., 1997) and MEGA 6 programs (Tamura et al., 2013). Phylogenetic trees were generated using the neighbor-joining (NJ) method, with a bootstrap test of 1000 replicates. The full-length cDNA sequences were assembled and deposited in GenBank with the accession number of KT581960. Considering the genome characteristics and sequences similarities to other viruses in family Fusariviridae, we tentatively suggested the 6 Kbp sequenced dsRNA represents the genome component of a virus tentatively named AbFV1.

The genomic organization of AbFV1 was indicated by a schematic representation shown in Fig. 1d. The complete genome of AbFV1 was 6639 nt long, excluding the poly (A)-tail, with a GC content of 47.3%. The polyA tail was composed of 17 continuous A in the 3′-end of the genome. The coding strand of AbFV1 contained three ORFs (ORF1, ORF2, and ORF3) on different frames of the genomic plus strand. The 5′- and 3′-untranslated regions (UTRs) of AbFV1 were 83 and 219 nt long, respectively.

ORF1 of AbFV1 was predicted to encode a putative 174-kDa polyprotein with 1522 aa residues. A CDD search against the NCBI database found two conserved sequence domains: an RdRp (RdRp_1, pfam00680) and a helicase (Hel; Helicase_C, pfam00271). A BLASTp search of the NCBI protein database, using the putative protein sequences encoded by ORF1, showed a significant sequence identity (33–49%) to viruses in family Fusariviridae (including SsFV1, PrRV1, FgV1, RnFV1, and MpRV1), as well as lower levels of identity to the RdRp domain of viruses in family *Hypoviridae* and other related viruses (Table 1), such as Cryphonectria hypovirus 4 (CHV4), Valsa ceratosperma hypovirus 1 (VcHV1), Phomopsis longicolla hypovirus 1 (PlHV1), Sclerotinia sclerotiorum hypovirus 1 (SsHV1), and Cryphonectria hypovirus 3 (CHV3). Moreover, some bacterial RNA helicases in *Pseudoalteromonas* sp. were found to be homologous with a relatively low level of sequence identity (from 25% to 28%) to the Hel domain of AbFV1. According to our homology search results, AbFV1 was proposed to be a (+ssRNA) mycovirus, exhibiting similarities between the RdRp and Hel domains of the AbFV1 ORF1-encoded protein and the corresponding regions of other known (+ssRNA) viruses, which could be further confirmed via phylogenetic analysis.

ORF2 (nt 4649–4861) potentially encoded a small 70 aa protein with an approximate molecular mass of 7.8-kDa. This 7.8-kDa protein showed no significant sequence identity to any known viral protein in the NCBI database.

ORF3 (nt 4948–6420) encoded a putative 54-kDa protein (495aa). A homology search based on the deduced amino acid sequences of ORF3 showed no sequence identity to any known viral protein as well, with the exception of a hypothetical protein encoded by the ORF2 of SsFV1. No putative conserved domain was detected from the ORF2 and ORF3 encoded proteins. However, as has been described in virus RnFV1 (Zhang et al., 2014), a transmembrane (TM) domain was predicted at the C-terminal regions of the ORF3 encoded protein (Fig. S1), using the TMHMM