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Characterization of a novel *Sclerotinia sclerotiorum* RNA virus as the prototype of a new proposed family within the order *Tymovirales*

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ABSTRACT

Recent studies have shown that *Sclerotinia sclerotiorum*, an important plant pathogen fungus, harbors diverse mycoviruses. A new mycovirus, tentatively named as Sclerotinia sclerotiorum deltaflexivirus 1 (SsDFV1), was isolated from a *S. sclerotiorum* strain AX19 containing multiple dsRNA elements. The complete genome of SsDFV1 was shown to be 8178 nucleotides long excluding the poly (A) tail. SsDFV1 has a large putative open reading frame (ORF1) and three smaller ORFs (2–4). ORF1 encodes a putative methyltransferase-helicase-RdRp polyprotein of 2075 amino acids. ORFs (2–4) encode three putative small hypothetical proteins (<40 kDa) with unknown biological functions. No evidence for a coat protein encoded by SsDFV1 was obtained. Multiple alignment suggested that three conserved domains, RdRp, methyltransferase, and helicase, from SsDFV1 have lower identity (approximately 25%) with all the reported viruses of four approved families, *Alphaflexiviridae*, *Betaflexiviridae*, *Gammaflexiviridae* and *Tymoviridae* in the order *Tymovirales*. Moreover, a phylogenetic tree also suggested that the SsDFV1 could not be phylogenetically placed in any of the approved families, and forms a separated cluster distinct from other known viruses. Therefore, these combined results suggest that SsDFV1 could represent a new positive-sense single-stranded RNA virus with some unique molecular features, and we propose to create a tentative family Deltaflexiviridae that accommodates SsDFV1.

1. Introduction

Mycoviruses (fungal viruses) are prevalent in the major taxonomic groups of plant pathogenic fungi, and an increasing number of novel mycoviruses have been identified (Ghabrial and Suzuki, 2009; Xie and Jiang, 2014; Ghabrial et al., 2015). Those recently discovered mycoviruses not only enrich virus diversity and supply new evidences for viral evolution, but also broaden our new insights on basic principles of the virus life cycle (such as mycovirus transmission way). Two mycoviruses, Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) and Sclerotinia sclerotiorum negative-stranded RNA virus 1 (SsNsRV-1), were discovered in fungi, which firstly confirmed that fungi, like other organisms, could also be infected by single-stranded DNA virus and negative-stranded RNA virus (Yu et al., 2010; Liu et al., 2014). Aspergillus fumigatus tetramycovirus-1 (AfuTmV-1), isolated and characterized from the human pathogenic fungus, has unique features in viral genome and its infectious as dsRNA (Kanhayuwa et al., 2015). Sclerotinia sclerotiorum megabirnavirus 1 (SsMBV1) contains a conserved papain-like protease domain and further phylogenetic analysis revealed that horizontal gene transfer may have occurred from single-stranded RNA virus to double-stranded RNA virus (Wang et al., 2015). It is more important that some newly discovered and characterized mycoviruses are associated with hypovirulence and have the potential to be developed as biocontrol agents to combat plant fungal disease. Rosellinia necatrix megabirnavirus 1 (RnMBV1) and SsHADV-1 have been shown to effectively control white root rot disease under laboratory conditions and rapeseed rot disease under field conditions, respectively (Chiba et al., 2009; Yu et al., 2013).

Mycoviruses with positive-sense single-stranded RNA ((+)ssRNA) genomes usually accommodate a single molecule of linear RNA segment and are so far classified into seven families, including *Alphaflexiviridae*, *Barnaviridae*, *Endornaviridae*, *Gammaflexiviridae*, *Hypoviridae*, and *Narnaviridae*, as well as the newly proposed family Fusariviridae (Zhang et al., 2014;

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Ghabrial et al., 2015). With the exception of the members from *Alphaflexiviridae*, *Barnaviridae*, and *Gammaflexiviridae*, the other (+)ssRNA mycoviruses are unencapsidated and do not have true virions in their life cycles. The order *Tymovirales* is composed of four approved families (*Alpha-*, *Beta-*, *Gammaflexiviridae* and *Tymoviridae*), and all of the members contain a linear genome from 5.9 kb to 9.0 kb in length (Adams et al., 2011b). Members within the order *Tymovirales* have a diverse host range, which is also a characteristic of individual genera. Members of *Betaflexiviridae* and *Tymoviridae* have been shown to infect plants (Adams et al., 2011b), and a single member of *Gammaflexiviridae* only infects a filamentous fungus (Howitt et al., 2001; Adams et al., 2011b), whereas members of *Alphaflexiviridae* infect both plants and fungi in nature (Adams et al., 2011a).

Sclerotinia sclerotiorum (Lib.) de Bary, an ascomycete fungus, is a devastating necrotrophic fungal plant pathogen in agriculture and causes annual losses in a wide variety of crops throughout the world (Bolton et al., 2006). In China, *S. sclerotiorum* is a primary pathogenic factor for stem rot of rapeseed and is responsible for serious losses in rapeseed productivity every year. Screening mycoviruses associated with hypovirulence in the *S. sclerotiorum* population improves our understanding on the molecular pathogenicity mechanism of *S. sclerotiorum* and thus contributes to the development of virocontrol agents for rapeseed rot disease. *S. sclerotiorum* is increasingly recognized to harbor a wide range of viruses and some of the described mycoviruses confer hypovirulence (Jiang et al., 2013; Xie and Jiang, 2014; Liu et al., 2014). To date, at least 24 *S. sclerotiorum* mycovirus strains have been isolated, sequenced and characterized. Furthermore, 19 of 24 mycoviruses belong to (+)ssRNA viruses and distribute in five families including *Hypoviridae* (three hypoviruses, Xie et al., 2011; Hu et al., 2014; Khalifa and Pearson, 2014a; Marzano et al., 2015), *Alphaflexiviridae* (one sclerodarnavirus, Xie et al., 2006), *Endornaviridae* (one endornavirus, Khalifa and Pearson, 2014c), *Narnaviridae* (thirteen mitoviruses, Xie and Ghabrial, 2012; Khalifa and Pearson, 2013, 2014b; Xu et al., 2015) and the newly proposed family Fusariviridae (one fusarivirus, Liu et al., 2015).

In the present study, a (+)ssRNA mycovirus, SsDFV1 (Sclerotinia sclerotiorum deltaflexivirus 1), was isolated from a *S. sclerotiorum* strain AX19 harboring at least seven dsRNA elements. Genetic organization, multiple alignments and phylogenetic analysis suggested that SsDFV1 is similar to members of the order *Tymovirales*, but forms an independent phylogenetic branch that is distant from the approved members in this order. The taxonomic position of SsDFV1 was also discussed and proposed.

2. Materials and methods

2.1. Fungal strains and culturing

S. sclerotiorum strain AX19 was isolated from a sclerotial collected from diseased rapeseed in a rapeseed field of Anxiang county, Hunan province, PR China. Strain Ep-1PNA367 is a normal, virus-free strain that was obtained from a single-ascospore-isolation progeny of strain Ep-1PN (Xie et al., 2006). The two strains were grown on laboratory-produced PDA (200 g of peeled potato, 20 g of dextrose and 20 g of agar in 1000 ml distilled water) at 20–22 °C, and stored on PDA slants at 4–6 °C. The dried sclerotial were kept at −20 °C.

2.2. Analysis of the biological properties of strain AX19

Compared to virus-free strain Ep-1PNA367 (a referenced strain in this study), the biological properties (colony morphology, growth rate, and virulence) of the strain AX19 were assayed as previously described (Zhang et al., 2009). The colony morphology of the two strains on PDA medium was observed every day, and photographs were recorded at 9 days post-inoculation. The daily radial growth rates were calculated with a ruler four to six times at intervals of 24–48 h. For the virulence assay, actively growing mycelial plugs from individual strains were inoculated on detached rapeseed leaves. Inoculated leaves were maintained in a 20 °C incubator for 48 h. All of the biological experiments were done in five replicates and repeated at least twice. The data on biological properties data were subjected to analysis of variance (ANOVA) using the SAS® 8.0 program. The treatment means were compared with the least significant difference test at a $P = 0.05$ level.

2.3. dsRNA extraction and purification

Strain AX19 was cultured on PDA plate covered with cellophane membranes, and mycelium was harvested after a 2- to 3-day culture period with a sterile blade. The harvested mycelium was ground into a fine powder in liquid nitrogen with a mortar and pestle. The detailed protocol for dsRNA extraction in the presence of CF-11 cellulose (Sigma–Aldrich, Dorset, England) chromatography was conducted as previously described (Xie et al., 2006). The dsRNA sample was treated with DNase I and S1 nuclease (TaKaRa Dalian, China), and then the treated individual dsRNA segment was re-isolated and purified in 1% agarose-gel using a gel extraction kit (Axygene biosciences) and stored at −80 °C.

2.4. Virus particle purification

The virus particles were isolated from strain AX19 in accordance with the method previously described (Yu et al., 2010) and further purified with a sucrose gradient centrifugation. Each fraction from different sucrose gradients were collected with a sterile syringe, and the nucleic acids were released using phenol/chloroform extraction, which were then identified by RT-PCR with specific primers designed based on the cDNA sequences of dsRNA segments.

2.5. cDNA synthesis, molecular cloning and sequencing

Purified dsRNA segments were used as templates for the construction of cDNA libraries according to a protocol described previously by Xie et al. (2011) using a cDNA synthesis kit (Fermentas, Ontario, Canada) with tagged random dN6 primers (5′-CGATCGATCATGATGCAATGCNNNNNN-3′). Approximately 500 ng of purified dsRNA was mixed with 1.2 μM of random dN6 primers and 3 μl of dimethyl sulfoxide (DMSO), and diethylpyrocarbonate (DEPC)-treated double-distilled H_2O was added to a final volume of 12 μl. The mixture was heated to 95–98 °C for 15 min and chilled on ice for 5 min. cDNA synthesis was performed as described by the manufacturer's instructions (Fermentas, Ontario, Canada). After reverse transcription, random cDNA products were obtained using a single specific primer (5′-CGATCGATCATGATGCAATGC-3′) based on tagged random dN6 primers. To fill the gaps, overlapping clones were synthesized by RT-PCR using specific primers that were designed based on the obtained cDNA sequences with the above random primer. A series of reverse transcription PCRs was conducted to amplify the portions of the dsRNA genome that were not cloned by the initial random cDNA synthesis. All of the nucleic acid amplifications were performed using a C100™ Thermal Cycler (BIO-RAD). All of the expected PCR fragments were purified and cloned into the pMD18-T Vector (TaKaRa, Dalian, China) according to the manufacturer's instructions and then transformed into *Escherichia coli* JM109 competent cells. Positive clones were sequenced and analyzed with the DNAMAN program and the BLASTP program on the NCBI website.