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Evidence for a novel negative-stranded RNA mycovirus isolated from the plant pathogenic fungus *Fusarium graminearum*

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ABSTRACT

Here we describe a novel (−)ssRNA mycovirus, Fusarium graminearum negative-stranded RNA virus 1 (FgNSRV-1), isolated from *Fusarium graminearum* strain HN1. The genome of FgNSRV-1 is 9072 nucleotides in length, with five discontinuous but linear ORFs (ORF I-V). Phylogenetic analysis based on entire L polymerase sequences indicated that FgNSRV-1 is related to the (−)ssRNA mycovirus Sclerotinia sclerotiorum negative-stranded RNA virus 1 (SsNSRV-1), and other mycoviruses. Our data suggest that FgNSRV-1 can be classified into the family *Mymonaviridae*, order *Mononegavirales*. Putative enveloped virion-like structures with filamentous morphology similar to SsNSRV-1 were observed in virion preparation samples. The L proteins of FgNSRV-1, and other fungal mononegaviruses, were found to be related to L protein-like sequences in some fungal genome, supporting the hypothesis that there is coevolution occurring between mycoviruses and fungi. Besides, clearing the virus from the infected host fungus resulted in no discernable phenotypic change.

1. Introduction

Mycoviruses infect most of the major taxonomic fungi families, including plant-pathogenic fungi, yeasts, and mushrooms (Ghabrial and Suzuki, 2009; Pearson et al., 2009). Most mycoviruses contain either positive (+) single-stranded (ss) or double-stranded (ds) RNA genomes. There are also some exceptions, such as the mycovirus Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1), that possesses a ssDNA genomes (Yu et al., 2010). Negative-stranded (−) ssRNA viruses were first demonstrated infecting fungi by Kondo et al. (Kondo et al., 2013) based on an exhaustive search in fungal genomes and transcriptome libraries using extant (−)ssRNA virus sequences as queries. The strong evidence was presented for the existence of (−) ssRNA mycovirus, Sclerotinia sclerotiorum negative-stranded RNA virus 1 (SsNSRV-1), and some other related viruses (Gale et al., 2002; Liu et al., 2014). SsNSRV-1 is closely related to members of the *Nyamiviridae* and *Bornaviridae* families (Liu et al., 2014) and has recently been taxonomically classified into a novel family, *Mymonaviridae* (genus *Sclerotimonavirus*) in the order *Mononegavirales*. Most members of this clade have non-segmented, single-stranded negative-sense RNA (8.9–19 kb) genomes, although there are some exceptions (Hou et al., 2002). Interestingly, while there are many mononegaviruses found in vertebrates and invertebrates, only a limited number appears to infect plants. Additionally, very few (−)ssRNA viruses have been shown to infect fungi (Dietzgen et al., 2017; Kondo et al., 2013; Seong et al., 2006; Walker et al., 2015).

Most mycoviruses infection do not cause any morphological changes in their hosts (Ghabrial and Suzuki, 2009), such as Fusarium graminearum hypovirus 1 (FgHV1, family *Hypoviridae*) (Wang et al., 2013). However, some mycoviruses including Fusarium graminearum virus 1 (FgV1 in the proposed family *Fusariviridae*) (Chu et al., 2002) can lead to debilitating symptoms in their pathogenic fungal hosts. These include reduced mycelial growth, and decreased production of spores and/or sclerotia, and suppressed biosynthesis of secondary metabolites, and attenuated aggressiveness or virulence (Nuss, 2005; Pearson et al., 2009). This suggests mycoviruses could be a promising biocontrol agent for combating fungal disease. For example, the (+)ssRNA mycovirus Cryphonectria hypovirus 1 (CHV1) in the family *Hypoviridae* has been successfully used for the biological control of chestnut blight in Europe (Anagnostakis, 1982; Heiniger and Rigling, 1994). Most of these researches have provided valuable insights into the molecular pathogenesis of plant-pathogenic fungi (Nuss, 2005).

Fusarium graminearum is a devastating filamentous fungus with a worldwide distribution. The organism causes fusarium head blight

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(FHB) disease in several economically important crops, such as wheat, maize, and barley (Goswami and Kistler, 2004). *F. graminearum* has also been reported as soybean pathogen in South America numerous times, where it leads to pod discoloration, seed decay, and root rot (Barros et al., 2014; Martinelli et al., 2004; Pioli et al., 2004). Many mycoviruses have been identified from *F. graminearum* (Chu et al., 2002, 2004; Darissa et al., 2011; Kwon et al., 2007; Li et al., 2016, 2015; Wang et al., 2013; Yu et al., 2009, 2011) and related fungi, such as Fusarium graminearum virus 1 (FgV1), FgV2, FgV3, FgV4, FgV5 (Chu et al., 2002, 2004; Yu et al., 2009; Wang et al., 2017), FgV-ch9, Fusarium graminearum hypovirus 1 (FgHV1/HN10), FgHV2/JS16, Fusarium graminearum deltaflexivirus 1 (FgDFV1) and Fusarium graminearum mycotymovirus 1 (FgMTV1/SX64) (Darissa et al., 2011; Wang et al., 2013; Li et al., 2015, 2016; Chen et al., 2016b), of which FgV1, FgV-ch9 and FgHV2/JS16 were associated with the hypovirulence of *F. graminearum*. Nevertheless, to date, no (−)ssRNA virus has been characterized from *F. graminearum*. In this study, we describe the sequence, molecular and biological characteristics of a novel (−)ssRNA mycovirus isolated from *F. graminearum* strain HN1. We have proposed naming this virus, Fusarium graminearum negative-stranded RNA virus 1 (FgNSRV-1). Phylogenetic analysis of the viral L polymerase sequences indicates that FgNSRV-1 is related to SsNSRV-1 and other fungal mononegaviruses. This is evidence for a naturally occurring novel negative-stranded mycovirus infect the plant pathogen fungus *F. graminearum*.

2. Results and discussion

2.1. Detection and particle properties of a (−)ssRNA virus in F. graminearum strain HN1

The HN1 isolate was identified as *F. graminearum* by PCR amplification of the EF-1α fragment (O'Donnell et al., 2000) (data not shown). A previously characterized dsRNA mycovirus, Fusarium graminearum dsRNA virus 5 (FgV5), was also identified and characterized from strain HN1 (Fig. 1A) (Wang et al., 2017). The novel virus FgNSRV-1 we identified was not found via agarose gel electrophoresis (Fig. 1A) but rather after a random-primed reverse transcription polymerase chain reaction (RT-PCR). Firstly, dsRNA samples were mixed with tagged random primers-dN6 (5′-GACGTCCAGATCGCGAATTCNNNNNN-3′) to perform the reverse transcription reaction. Secondly, the resulting random cDNAs were amplified with a single specific primer (5′-GACG TCCAGATCGCGAATTC-3′). The complete nucleotide sequence of FgNSRV-1 was determined by sequencing of random-primed cDNA, specific-primed RT-PCR and a standard 3′RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE). The viral genome was found to be 9072 nt in length and lacked a poly (A) tail at its 3′-terminus.

To reconfirm the presence of FgNSRV-1 in dsRNA of HN1, specific primer pair 9F (the starting nucleotide position was 96 nt) and 9R (the starting nucleotide position was 8998 nt) were selected based on the already determined full-length genome sequence of FgNSRV-1, to amplify the nearly full-length of FgNSRV-1 by RT-PCR amplification. Firstly, the first strand cDNA of the genomic and antigenomic RNA were synthesized by reverse transcriptase (RT) with primer 9F and primer 9R, respectively, using purified dsRNA as templates. Secondly, the first strand cDNA of the genomic and antigenomic RNA and the host genomic DNA (gDNA) were used as templates, respectively, to conduct RCR amplifications with primer pair 9F and 9R. Agarose gel electrophoresis of these PCR amplification products were showed in Fig. 1B. These results showed that no fragments were amplified directly using HN1 gDNA as a template. However, amplified fragments were present when the first strand cDNA of the genomic and antigenomic RNA were used as templates. It is indicated that FgNSRV-1 is present in dsRNA of HN1, but also independent of HN1 genome. Nevertheless, FgNSRV-1 was not detected by northern blot in HN1 dsRNA (data not shown), which is similar to the result that no dsRNA signals were detected for negative-strand RNA viruses using a immunofluorescence analysis of dsRNA-specific antibody reported by Weber et al. (2006). This is the first negative-stranded mycovirus identified in *F. graminearum*. The complete genomic sequence of FgNSRV-1 has been deposited in GenBank with accession no. MF276904.

The virions of FgNSRV-1 purified from mycelia of strain HN1 are filamentous, 35–50 nm in diameter, ~1200 nm in length (Fig. 1C), and similar to SsNSRV-1 (25–50 nm in diameter, ~1000 nm in length) (Liu et al., 2014). Some short rod-like particles were also observed in the purified preparations (Fig. 1D and E), which are similar to the condensed-nucleocapsid structures of SsNSRV-1 and other mononegaviruses such as Measles virus and orchid fleck virus (Liu et al., 2014; Ruigrok et al., 2011; Kondo et al., 2009). Compared with SsNSRV-1 nucleocapsids, some loose nucleocapsid-like structures were also observed in the FgNSRV-1 preparations, having single, left-handed, helical structures (Fig. 1D and E). Considering the similarities in the particle structures between FgNSRV-1 and SsNSRV-1, it is suggested that the FgNSRV-1 virions may be enveloped by a membrane, and called "enveloped virion-like structures" (EVLSs)", as described in SsNSRV-1 (Liu et al., 2014).

2.2. Organization of the FgNSRV1 genome

The full-length genome of FgNSRV-1 was predicted to possess five considerable open reading frames (ORFs I–V), with some small overlapping ORFs (~0.3 kb). These five discontinuous ORFs were laid out linearly in the antiviral genome (Fig. 1F). The ORFs of FgNSRV-1 putatively encode five proteins (termed P I to P V) with predicted molecular masses ranging from 6 kDa (P III) to 221 kDa (P IV) (Table 1). The 3′-untranslated region (UTR) and 5′-UTR of FgNSRV-1 RNA were 126 nt and 220 nt long, respectively (Fig. 1B). The 3′- and 5′-ends of FgNSRV-1 RNA had perfect complementarity for the first six residues (3′-UCCUGC——GCAGGA-5′) (Fig. 1G). This terminal complementarity is a common feature among mononegaviruses (Chen et al., 2016). Furthermore, a conserved non-coding sequence (3′-…AUAAU/AUUAUUU UGAAUCCU… − 5′) was identified downstream of each ORF (Fig. 1H). Similar sequences were also found up and downstream of ORF I and ORF V, respectively (Fig. 1H). These regions are most likely the gene-junction regions in FgNSRV-1 genome and are broadly similar to sequences found in other mononegaviruses (Pringle and Easton, 1997). Interestingly, the putative gene-junction sequence we identified in FgNSRV-1 is very similar to that of SsNSRV-1. Each of the six genes SsNSRV-1 can be transcribed independently with the 3′-UCCU/A… − 5′ gene start and 3′-…AUAA(U/A)UU(A/C)UUUU-5′ stop sequences (Liu et al., 2014), suggesting that the transcription termination and initiation strategies in the two fungal mononegaviruses may be highly conserved.

Our further BLAST analysis of FgNSRV-1 revealed that there was 96% nucleotide identity with soybean leaf-associated negative-stranded RNA virus 1 (SaNSRV-1) that was a previously identified virus in a soybean leaf metatranscriptomic study. However, sequences identified in the soybean leaf metatranscriptomic study have not characterized in detail (Marzano and Domier, 2016). FgNSRV-1(9072 nt) is only 31 nt longer than SaNSRV-1 (9041 nt), and a pairwise comparison of the nucleotide and amino acid sequences between FgNSRV-1 and SaNSRV-1 showed highly sequence similarity ranging from 93.9% (nucleotide of ORF I) to 98.8% (nucleotide of ORF III), and 97.3% (amino acid of P I) to 100% (amino acid of P III) (supplementary Table S1). These results suggest that FgNSRV-1 is a negative-stranded RNA mycovirus that is closely related to SaNSRV-1 and that both viruses likely belong to the same viral species. Therefore, SaNSRV-1 appears to have been derived from a fungus (probably *F. graminearum* or a related species) that is associated with soybean leaves (Broders et al., 2007).

Further BLAST analysis using the predicted amino acid sequences of FgNSRV-1 P I to V showed moderate or low identity (23–55%) with corresponding proteins found in other fungal mononegaviruses (e.g., SsNSRV-1 to −4) (Liu et al., 2014; Marzano and Domier, 2016) or