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Molecular characterization of two positive-strand RNA viruses co-infecting a hypovirulent strain of *Sclerotinia sclerotiorum*

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ABSTRACT

Two dsRNA segments, the replicative forms of two ssRNA viruses of SsHV2/SX247 (Sclerotinia sclerotiorum hypovirus 2) and SsDRV/SX247 (*Sclerotinia sclerotiorum debilitation-associated RNA virus*), were isolated from the hypovirulent strain SX247 of *Sclerotinia sclerotiorum*. SsDRV/SX247 has the highest similarities (81% aa identity) with the previously reported virus SsDRV/Ep-1PN. The genome of SsHV2/SX247 is 15,219 bp in length with a poly-A tail, and it has only one large putative open reading frame (ORF) that encodes a polyprotein containing RNA-dependent RNA polymerase (RdRp) and viral RNA helicase domains. The RdRp domain shares amino acid similarity with that of CHV1 (23%). However, the genome organization of SsHV2/SX247 is significantly different from that of CHV1 on genomic size and ORFs. We conclude that SsDRV/SX247 is a novel strain in species SsDRV of genus *Sclerodarnavirus*, whereas SsHV2/SX247 is a representative member of new proposed lineage *Gammahypovirus* in the family *Hypoviridae* and confers hypovirulence in its host.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is an ascomycetous plant pathogenic fungus with worldwide distribution. This pathogen attacks more than 400 species of plant hosts including rapeseed (*Brassica napus*), soybean, sunflower and numerous vegetable crops (Boland and Hall, 1994), and is responsible for a significant yield loss of these corps (Bolton et al., 2006). Because it is difficult to control diseases caused by this pathogen via cultural practices, the control of diseases caused by *S. sclerotiorum* mainly relies on the application of fungicides. However, fungicide sprays often exhibit an associated risk of developing fungicide-resistant fungal isolates (Ma et al., 2009; Gossen et al. 2001; Kuang et al., 2011) and may pose potential negative impacts on both environmental safety and food safety. The hypovirulence-associated mycoviruses have potential for exploitation as alternative biological control agents. Furthermore, the mycovirus/*S. sclerotiorum* system may be exploited to identify new virulence factors of *S. sclerotiorum* (Li et al. 2008; Zhu et al., 2013). Because different types of viruses vary implications strategy, host range, stability, impact on host virulence, selection of appropriate viruses is essential for virocontrol. Furthermore, identifications of new viruses from *S. sclerotiorum* will offer new insights into implementation of virocontrol of *S. sclerotiorum* (Xie and Jiang, 2014). Since hypovirulence and dsRNA elements in *S. sclerotiorum* were firstly reported in 1990s (Boland, 1992), eleven viruses (seven viruses associated hypovirulence from unclassified ssDNA virus, *Sclerodarnavirus*, *Hypovirus*, and *Mitovirus*), including ssRNA viruses, dsRNA viruses, and ssDNA viruses in *S. sclerotiorum*, have been detected and characterized at molecular level (Yu et al., 2010; Jiang et al., 2013; Khalifa and Pearson, 2013; Xiao et al., 2014; Xie and Jiang, 2014). Thus, the host fungus, *S. sclerotiorum*, provides an excellent system for the exploration of virus-host interactions.

The family *Hypoviridae* contains a single genus of *Hypovirus* and four recognized virus species isolated from *Cryphonectria parasitica* have been identified as belonging to the genus *Hypovirus*: CHV1 (*Cryphonectria hypovirus 1*) (Hillman et al., 1990, 1995), CHV2 (Hillman et al., 1992, 1994), CHV3 (Smart et al., 1999), and CHV4 (Linder-Basso et al., 2005). However, the impact of these four virus species on the biological features of *C. parasitica* and their genome features are varied. CHV1/EP713 or CHV2/NB58-infected strains of *C. parasitica* exhibit markedly reduced virulence to chestnut trees, and the infections results in female sterility of the fungus (Hillman et al., 1990). Furthermore, the impact of CHV1/EP713 on *C. parasitica* was directly confirmed following artificial transfection (Chen et al., 1994; Sasaki et al., 2002). The hypoviruses CHV3/GH2 and CHV4/SR2 have a limited effect on their host (Smart et al., 1999; Linder-Basso et al., 2005). CHV1, which has a positive strand RNA genome and two ORFs, is the typical

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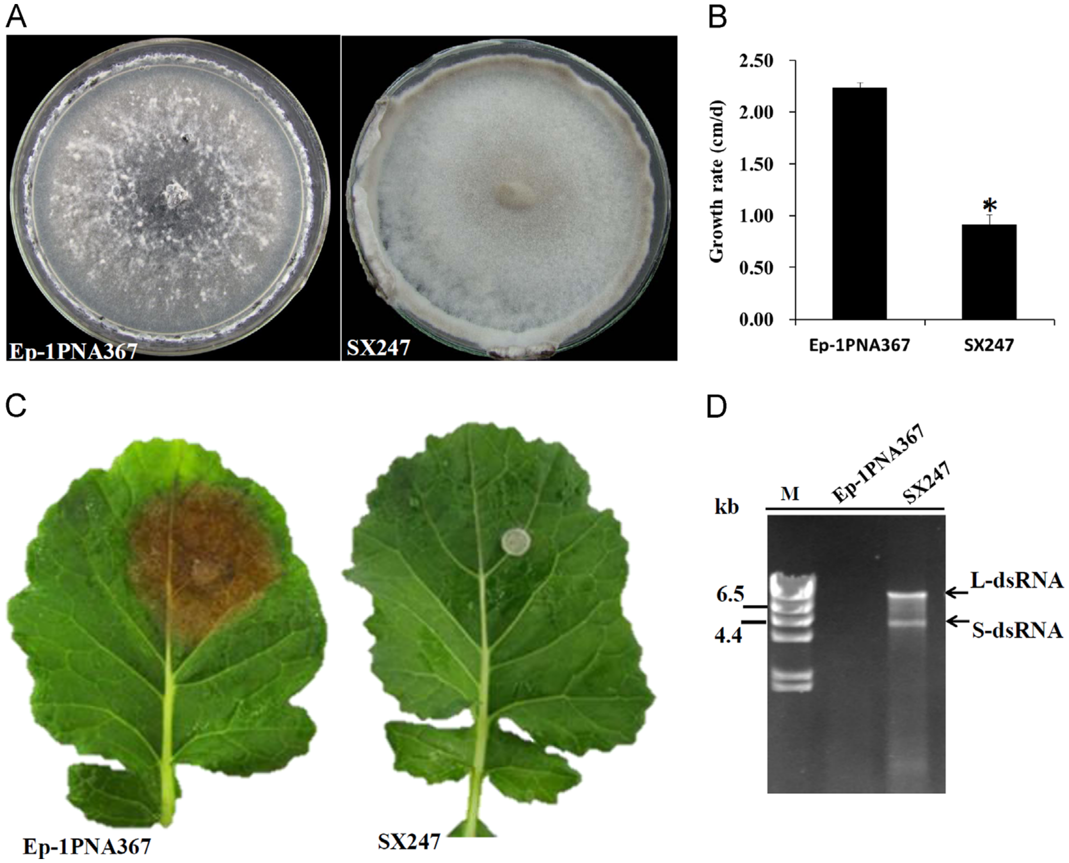

Fig. 1. Hypovirulence-associated traits of strain SX247 of *S. sclerotiorum* and dsRNA extraction. (A) Abnormal colony morphology of strain SX247 grown on a PDA plate at 20 °C for 15 days and normal colony morphology of the virus-free strain Ep-1PNA367; and (B) compared to the virus-free strain Ep-1PNA367, strain SX247 grew slowly on a PDA plate. The bars represent the standard deviations from the mean ($n=6$). The asterisk on top of the bars in C indicates that the differences are statistically significant ($P < 0.05$). (C) Hypovirulence phenotype of strain SX247 as demonstrated by infected leaves of rapeseed plants, which were maintained at 20 °C for 3 days post-inoculation; and (D) dsRNA profile of samples extracted from strain SX247 and Ep-1PNA367 of *S. sclerotiorum*. All samples were treated with DNase I and S1 nuclease. DNA ladder standards (left, M) are indicated as kilobase pairs (kb).

species of the current genus and is the first species of the genus *Hypovirus* for which full-length genome sequence of a representative strain were reported. CHV1/EP713 has become the subject of the most intensive research of any filamentous fungal virus (Hillman and Suzuki, 2004; Dawe and Nuss, 2013). To date, hypoviruses have been isolated and characterized from four other phytopathogenic fungi: *S. sclerotiorum* (Sclerotinia sclerotiorum hypovirus 1, SsHV1) (Xie et al., 2011), *Valsa ceratosperma* (Valsa ceratosperma hypovirus 1, VcHV1) (Yaegashi et al., 2012), *Fusarium graminearum* (Fusarium graminearum hypovirus 1, FgHV1) (Wang et al., 2013), and *Phomopsis longicolla* (Phomopsis longicolla hypovirus 1, PlHV1) (Koloniuk et al., 2014). It is noteworthy that these four novel hypoviruses are associated with latent infections in their natural hosts. Recently, Yaegashi et al. (2012) proposed that the family *Hypoviridae* contains two lineages "*Alphahypovirus*" and "*Betahypovirus*". A phylogenetic analysis and a review of their genome features revealed that CHV3, CHV4, VcHV1, SsHV1, and PlHV1 may be assigned to "*Betahypovirus*" lineage, whereas CHV1, CHV2, and FgHV1 belong to the "*Alphahypovirus*" lineage.

We previously reported two positive strand RNA viruses SsHV1 and SsDRV (*Sclerotinia sclerotiorum debilitation-associated RNA virus*) in *S. sclerotiorum*. SsHV1 is closely related to hypoviruses 3 and 4 in family *Hypoviridae* and confers strong hypovirulence that correlated with co-infection with sat-like RNA of SsHV1. In the absence of sat-like RNA, the SsHV1-infected strain displayed dark brown colony coloration, but had a mild effect on growth and virulence of *S. sclerotiorum* (Xie et al. 2011). SsDRV was the first well-characterized virus at the molecular level in *S. sclerotiorum*. Genomic RNA of SsDRV/Ep-1PN contains only a single ORF that codes a putative RNA replicase that contains methyltransferase, helicase and RNA-dependent RNA polymerase (RdRp) domains (Xie et al., 2006). A new genus *Sclerodarnavirus* was established in family *Alphaflexividae* based on the characteristics of SsDRV (Adams et al., 2011). SsDRV is associated with hypovirulence of strain Ep-1PN (Li et al., 1999; Xie et al., 2006).

In this study, the hypovirulent strain SX247 was co-infected with SsDRV/SX247 (Sclerotinia sclerotiorum debilitation-associated RNA virus) and SsHV2/SX247 (Sclerotinia sclerotiorum hypovirus 2). SsDRV/SX247 has high sequence identity (81%) with the previously reported positive strand RNA virus SsDRV. SsHV2/SX247 shares the highest sequence identity with CHV1. We also found that the genome organization and molecular features of SsHV2/SX247 are unique and have significant difference from those of the previously reported members of the family *Hypoviridae*. It is more important that SsHV2/SX247 confers hypovirulence to *S. sclerotiorum*. Thus, the characterization of SsHV2/SX247 will be valuable for understanding its role in pathogenesis, for determining its potential as a virocontrol agent and exploiting the ecological diversity, and for the analysis of the evolution of viruses from family *Hypoviridae*.

Results

Two dsRNA segments isolated from hypovirulent strain SX247

Compared to the virus-free strain Ep-1PNA367, strain SX247 showed abnormal colony morphology (Fig. 1A). Strain SX247 grew on potato dextrose agar (PDA) at a rate of 0.9 cm/day, which is 60% slower than the growth of strain Ep-1PNA367 (2.35 cm/day)