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A novel mycovirus closely related to hypoviruses that infects the plant pathogenic fungus *Sclerotinia sclerotiorum*

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ABSTRACT

Three dsRNA segments, two similarly sized at 9.5 kbp and a third one of approximately 3.6 kbp, were extracted from a hypovirulent strain SZ-150 of *Sclerotinia sclerotiorum*. The complete cDNA sequence of one of the two large dsRNA segment (10398 bp, excluding the poly (A) tail) reveals a single ORF that encodes a polyprotein with conserved domains of putative papain-like protease, UDP glucose/sterol glycosyltransferase, RNA-dependent RNA polymerase and viral RNA Helicase. This virus is closely related to *Cryphonectria hypovirus* (CHV) 3/GH2 and CHV4/SR2 in the family *Hypoviridae* and designated as Sclerotinia sclerotiorum hypovirus 1 (SsHV1/SZ-150). The satellite-like 3.6 kbp dsRNA segment (S-dsRNA) shares high sequence identity with the 5′-UTR of SsHV1/SZ-150. SsHV1/SZ-150 alone is not the primary causal agent for hypovirulence of strain SZ-150 since strains without the S-dsRNA show normal phenotype. This is the first report of a naturally occurring hypovirus that infects a fungus other than *Cryphonectria parasitica*.

Introduction

Mycoviruses (or fungal viruses) are of common occurrence in all major fungal groups and typically contain dsRNA genomes (Ghabrial and Suzuki, 2009). An increasing number of novel RNA and DNA mycoviruses have recently been characterized at the genomic level (Aoki et al., 2009; Chiba et al., 2009; Lima et al., 2010; Liu et al., 2009; Tuomivirta et al., 2009; Urayama et al., 2010; Vainio et al., 2010; Wu et al., 2010; Yu et al., 2010). Although most of known dsRNA mycoviruses cause little or no obvious symptoms to their hosts (Ghabrial and Suzuki, 2009), many of the novel mycoviruses with ssRNA and ssDNA genomes are associated with debilitation/hypovirulence phenotypes in their plant pathogenic fungal hosts (Ghabrial and Suzuki, 2009; Pearson et al., 2009; Yu et al., 2010). Mycoviruses that confer hypovirulence to their hosts are potential biocontrol agents of plant pathogenic fungi as well as for use in fundamental studies to probe the molecular basis of fungal pathogenesis (Ghabrial and Suzuki, 2009; Nuss, 2005; Pearson et al., 2009). Cryphonectria hypoviruses, especially *Cryphonectria hypovirus* 1 (CHV1), represent the classic and successful examples for biological control of chestnut blight in Europe (Nuss, 2005). Likewise *Rosellinia necatrix megabirnavirus* 1 that infects *Rosellinia necatrix* was demonstrated to be of potential significance for biological control of the white root rot (Chiba et al., 2009). The hypovirus/*Cryphonectria parasitica* system also has become the model for exploring in depth the molecular interactions between mycoviruses and their hosts including the mechanisms underlying fungal pathogenesis (Nuss, 2005).

The fungus *Sclerotinia sclerotiorum* is a cosmopolitan, economically important, and necrotrophic fungal pathogen that infects over 450 plant species and is responsible for significant annual yield losses in many crops (Boland and Hall, 1994; Bolton et al., 2006). Stem rot of rapeseed caused by this fungal pathogen is the most important disease of rapeseed in China, especially in the area of Yangtze River. Because of lack of resistant cultivars, the control of stem rot of rapeseed is dependent on chemical fungicides, such as carbendazim and dimethachlon. However, a carbendazim-resistant strain of *S. sclerotiorum* was detected in 1997 (Pan et al., 1997), and dimethachlon-resistant strains were reported in 2009 (Ma et al., 2009). More importantly, the risk to the environment and food supply from the use of chemical fungicides is of considerable concern.

Hypovirulence-associated mycoviruses that infect *S. sclerotiorum* have the potential to be developed as an alternative means to control stem rot of rapeseed, however selection of appropriate viruses is the key step for virocontrol. Some mycoviruses with either RNA genome or DNA genome have been found in *S. sclerotiorum* (Liu et al., 2009,

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2010; Xie et al., 2006; Yu et al., 2010; Zhang et al., 2009), suggesting that *S. sclerotiorum* is suitable for many mycoviruses. In this study, we identified a mycovirus, designated Sclerotinia sclerotiorum hypovirus 1 (SsHV1/SZ-150), in a hypovirulent strain of *S. sclerotiorum* and showed that it is closely related to the hypoviruses CHV3 and CHV4. We also show that this novel putative hypovirus from *S. sclerotiorum*, like CHV4, has limited impact on host virulence. The genomic organization and potential biological function of a 3.6 kbp dsRNA segment (S-dsRNA) that is associated with infection by SsHV1/SZ-150 are also discussed.

Results

DsRNA segments in S. sclerotiorum SZ-150

DsRNA isolated from the mycelial mass of strain SZ-150 was subjected to agarose gel electrophoresis. In addition to genomic DNA, two distinct bands could be observed under UV light, one sized at approximately 9.5 kbp, and the other was sized at 3.6 kbp. The two segments were confirmed to be dsRNA in nature because both of them were resistant to digestion with DNase I and S1 nuclease (Fig. 1A).

Nucleotide sequence and genome organization of mycovirus SsHV1/SZ-150

The 9.5-kbp dsRNA segment was gel purified and subjected to cDNA cloning with random RT-PCR amplification. Thirty-five cDNA clones were obtained and completely sequenced. Although all clones were shown to be of viral origin, the clones could be divided into two groups based on BLASTP results. One group showed sequence similarity to the putative RNA-dependent RNA polymerase of cucurbit yellows-associated virus (family *Potyviridae*) (Accession number: X92203), and the dsRNA was presumed to represent a putative fungal virus, which we tentatively named Sclerotinia sclerotiorum RNA virus 1 (SsRV1/SZ-150). The second group of clones showed sequence similarity to mycoviruses in the family *Hypoviridae*. Thus, there are two dsRNA segments of similar size that could not be separated by agarose gel electrophoresis, and thus represent two different mycoviruses.

A full-length cDNA clone of the hypovirus-like dsRNA segment was generated based on clones obtained by random RT-PCR and a combination of RACE. The genetic organization of the hypovirus-like dsRNA segment is shown in Fig. 1B. The complete genomic sequence is 10398 nucleotides (nts) long excluding the 3′-terminal poly (A) tail. Computer analyses revealed that the full-length cDNA sequence has a single putative open reading frame (ORF) beginning at AUG (nt positions 542–544) and terminating at UAG (nt positions 9386–9388). It encodes a polyprotein of 2948 amino acids with an approximate molecular mass of 337 kDa. The deduced polyprotein amino acid sequence contains conserved domains of papain-like protease (Prot), UDP glucose/sterol glucosyltransferase (UGT), RNA-dependent RNA polymerase (Pol) and viral RNA Helicase (Hel) (Fig. 1B). These four conserved regions were each separately aligned with sequences from selected viruses and subjected to phylogenetic analysis. No other integral ORFs were detected in the genome of the hypovirus-like dsRNA segment with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The 5′- and 3′- untranslated regions (UTRs) of the mycoviral RNA were determined as 541 and 1010 nts, respectively. The hypovirus-like dsRNA segment has a similar genomic organization to viruses in the genus *Hypovirus*. Thus, we presumed that the hypovirus-like dsRNA segment is a replicative form of a ssRNA mycovirus, and this mycovirus was named Sclerotinia sclerotiorum hypovirus 1 (SsHV1/SZ-150). The sequence has been deposited in GenBank under accession number JF781304.

Phylogenetic analysis of SsHV1/SZ-150 and relationships to other mycoviruses

To understand the phylogenetic relationship between SsHV1/SZ-150 and other selected mycoviruses including CHV1/EP713, CHV2/NB58, CHV3/GH2, CHV4/SR2 and Fusarium graminarium virus 1 (FgV1/DK21), phylogenetic analyses were performed based on the complete amino acid sequences of these viruses. The results showed that SsHV1/SZ-150 is most closely related to CHV3/GH2 and CHV4/SR2 (Fig. 1C). Multiple alignment analysis showed that the amino acid sequence of the putative polyprotein of SsHV1/SZ-150 shares high sequence identity with those of CHV3/GH2 (58.1%) and CHV4/SR2 (48.0%).

Alignment of the first 100 nt sequences of the 5′-UTR of SsHV1/SZ-150 and those of CHV3/GH2 and CHV4/SR2 showed that this region is highly conserved among SsHV1/SZ-150 and the two hypoviruses;

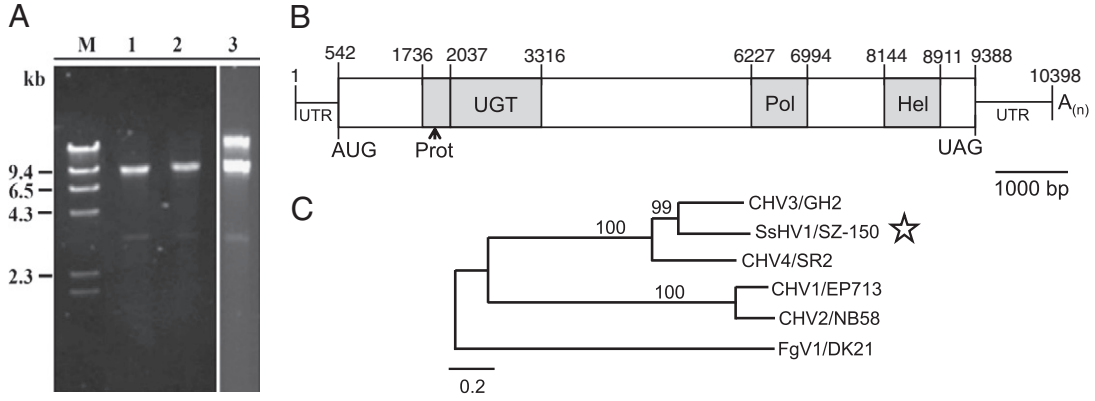

Fig. 1. (A). dsRNA profile of samples extracted from strain SZ-150 of *S. sclerotiorum*. Lane 1, dsRNA sample was treated with DNaseI; Lane 2, dsRNA sample was treated with both DNase I and S1 nuclease; Lane 3, nontreated dsRNA sample showing both host genomic DNA (top) and viral dsRNA. Size of dsRNA (right) and DNA ladder standards (left, M) are indicated as kilobase pairs (kb). (B). Schematic representation of the genomic organization of mycovirus SsHV1/SZ-150. Open reading frame (542–9388 nt) encodes a putative viral protein with 2948 amino acids that contains four conserved domains highlighted by gray color shading: papain-like proteinase (Prot, 1736–2050 nt), UDP glucose/sterol glucosyltransferase (UGT, 2037–3316 nt), RNA-dependent RNA polymerase (Pol, 6227–6994 nt) and viral RNA Helicase (Hel, 8144–8911 nt). 5′-Untranslated region (UTR, 1–541 nt) and 3′-UTR (9389–10398 nt) were indicated as line. AUG (542–544 nt) denotes the initiation codons and UAG (9386–9388) denotes the stop codons. Scale bar corresponds to a nucleotide length of 1000 bp. (C). Phylogenetic analysis of SsHV1/SZ-150 and selected mycoviruses based on full-length amino acid sequences of the viral polyproteins. A neighbor joining unrooted tree is shown. Bootstrap values (%) obtained with 1000 replicates are indicated on branches and branch lengths correspond to genetic distance; scale bar at lower left corresponds to a genetic distance of 0.2. Mycovirus (acronym/strain; GenBank accession) included Cryphonectria parasitica hypovirus 1 (CHV1/EP713; NP_041091.1), C. parasitica hypovirus 2 (CHV2/NB58; NP_613266.1), C. parasitica hypovirus 3 (CHV3/GH2; NP_051710.1) C. parasitica hypovirus 4 (CHV4/SR2; YP_138519.1) and Fusarium graminearum virus 1 (FgV1/DK21; AAT07067.2).

SsHV1/SZ-150 shared a higher sequence identity to CHV3/GH2 (70.0%) than to CHV4/SR2 (61.0%) in this 5′ UTR region. Moreover, one absolutely conserved region (100% identity in a 21 nts stretch from nt position 78 to 98) was shared by SsHV1/SZ-150 and the other two hypoviruses CHV3/GH2 and CHV4/SR2 (Fig. 2A). The 3′ -UTR of SsHV1/SZ-150 is 1010 nts in length. Direct comparison of the 3′ terminal 100 nt sequence, excluding the ploy A tail, of the 3′-UTR of SsHV1/SZ-150 showed that it also shared high sequence identity with that of CHV3/GH2 (68.0%) and CHV4/SR2 (55.0%), particularly in a stretch of 32 nts located at nt positions 10311 to 10398 (Fig. 2B). The remainder of 3′-UTR sequence of SsHV1/SZ-150, on the other hand, showed little or no sequence similarity to CHV3/GH2 and CHV4/SR2.

Multiple alignments of the N-terminal amino acid sequence of the putative polyprotein of SsHV1/SZ-150 and that of other selected viruses revealed little similarity between the putative protein of SsHV1/SZ-150 and that of other hypoviruses. This region comprises a putative papain-like protease motif with conserved predicted autoproteolytic catalytic site at amino acid position containing Cys^{406} and His^{453} residues (Fig. 3A). Furthermore, a putative polyprotein cleavage site (at position Gly^{297}) was found downstream of the deduced papain-like proteinase motif based on sequence alignment with other hypoviruses. This polyprotein cleavage site in CHV3/GH2 (Gly^{297}) has been confirmed using in vitro translational analysis (Yuan and Hillman, 2001), and in protease p48 of CHV1, Gly^{418} was found to be essential for autoptoteolysis and Ala substitutions for their flanking amino acids are frequently tolerable for the cleavage (Shapira and Nuss, 1991). Phylogenetic analysis based on papain-like proteases motif of different hypoviruses and plum pox virus was conducted and the results showed that the papain-like motif of SsHV1/SZ-150 is more closely related to that of CHV4/SR2 than to that of CHV3/GH2 (Fig. 3A).

Multiple alignments further revealed that SsHV1/SZ-150 contains a conserved domain of the UGT gene, which was consistent with the previous report on CHV4 (Linder-Basso et al., 2005). The homologous regions of UGT in the putative polyprotein of SsHV1/SZ-150 showed high sequence identity to that in CHV3 (60.6%) and CHV4 (51.5%). Like hypoviruses CHV3/GH2 and CHV4/SR2, the region (aa 500–925) of the putative polyprotein of SsHV1/SZ-150 showed significant sequence similarity to the UDP-glucose/sterol glucosyltransferase (UGT) genes from fungi (*S. sclerotiorum* and *Neurospora crassa*), higher plants (*Arabidopsis thaliana* and *Populus trichocarpa*) and even bacteria (*Amycolatopsis mediterranei*). A phylogenetic tree derived from the alignment of the UGT motif also suggested that UGT gene sequence of SsHV1/SZ-150 clusters with those of CHV3/GH2 and CHV4/SR2 (Fig. 3B).

A BLASTP analysis using NCBI database revealed the presence of RdRp and helicase domains in the polyprotein of SsHV1/SZ-150 (Fig. 2C) and that they shared high sequence identity with the RdRp and helicase domains of mycoviruses belonging to the family *Hypoviridae* (Fig. 3C). The RdRp domain of SsHV1/SZ-150 is 80.9% and 71.5% identical to that of CHV3/GH2 and CHV4/SR2, but significantly less conserved in comparison with that of CHV1/EP713 (15.8%) and CHV2/NB58 (18.4%). The phylogenetic tree based on the RdRp domain further supported that SsHV1/SZ-150 is more closely related to CHV3/GH2 and CHV4/SR2 than to CHV1/EP713 and CHV2/NB58. The helicase domain of SsHV1/SZ-150 and other related viruses contained the conserved motifs GKST box, DExH box and QRxGR box that are characteristic motifs in the superfamily 2 of helicases (Hall and Matson, 1999) (Fig. 3D). Multiple alignments of amino acid sequences of the helicase domain from SsHV1/SZ-150 and other hypoviruses revealed that the helicase domain in SsHV1 has 69.5% identity to that in CHV3/GH2 and 56.9% identity to that in CHV4/SR2. The phylogenetic tree of the helicase domain showed comparable results to those of the RdRp domain alignment analysis, except that the helicase domain of SsHV1/SZ-150 is more closely related to FgV1 than to that of CHV1/EP713 and CHV2/NB58. In summary, comparison of the full-length amino acid sequence and conserved domains of SsHV1/SZ-150 suggested that SsHV1/SZ-150 was most closely related to the hypoviruses CHV3/GH2 and CHV4/SR2.

Nucleotide sequence and genome organization of S-dsRNA

The full-length cDNA sequence of S-dsRNA was determined and the sequence was deposited in the GenBank under accession number JN084008. Sequence analysis indicated that it is 3643 nts in length excluding the Poly (A) tail. A single large putative ORF (nt positions 318 to 2237) was identified on the positive strand of the S-dsRNA cDNA. The 5′- and 3′-UTRs were determined to be 317 and 1046 nts in length, respectively (Fig. 4A). Alignment of the 5′-UTR sequences of SsHV1/SZ-150 and the S-dsRNA showed that the first 104 nts were 100% identical. The sequence from nt position 105 to 174 of the S-dsRNA segment, on the other hand, shares only 51.5% identity with the corresponding sequence of SsHV1/SZ-150. Meanwhile the sequence from nt position 175 to 317 of S-dsRNA shares 81.1% identity with the nucleotide stretch from position 399 to 540 of SsHV1/SZ-150 (Fig. 4B). No other region of sequence identity between the SsHV1/SZ-150 and S-dsRNA was detected at the nucleotides level. The ORF encodes a protein of 639 amino acids with an approximate

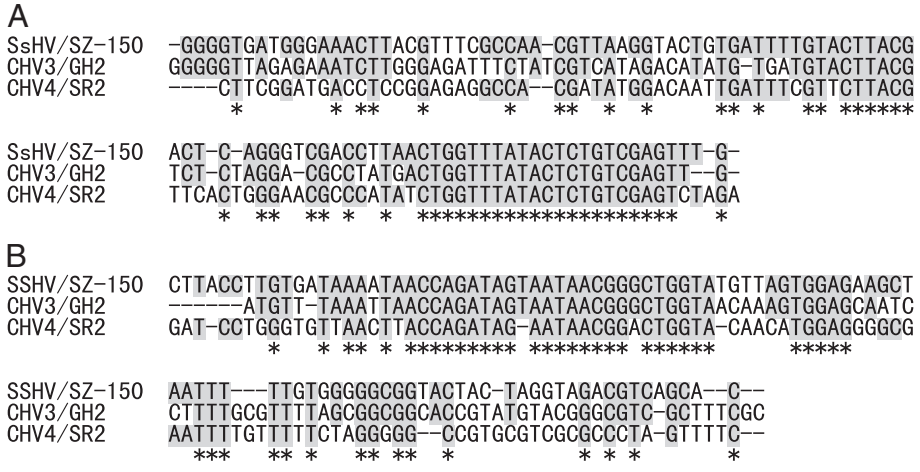

Fig. 2. Multiple alignment of the first 100 nucleotides of the 5′ -UTR of SsHV1/SZ-150 (A) and the last 100 nucleotides, excluding the poly (A) tail, of the 3′-UTR (B) with that of CHV3/GH2 and CHV4/SR2. Identical nucleotides in SsHV1/SZ-150 and the other two related mycoviruses were shown by asterisks. Identical nucleotides in SsHV1/SZ-150 and one or the other of the two mycoviruses were marked by gray-boxes. Abbreviations of mycovirus names and GenBank accession numbers are as shown in Fig. 1.

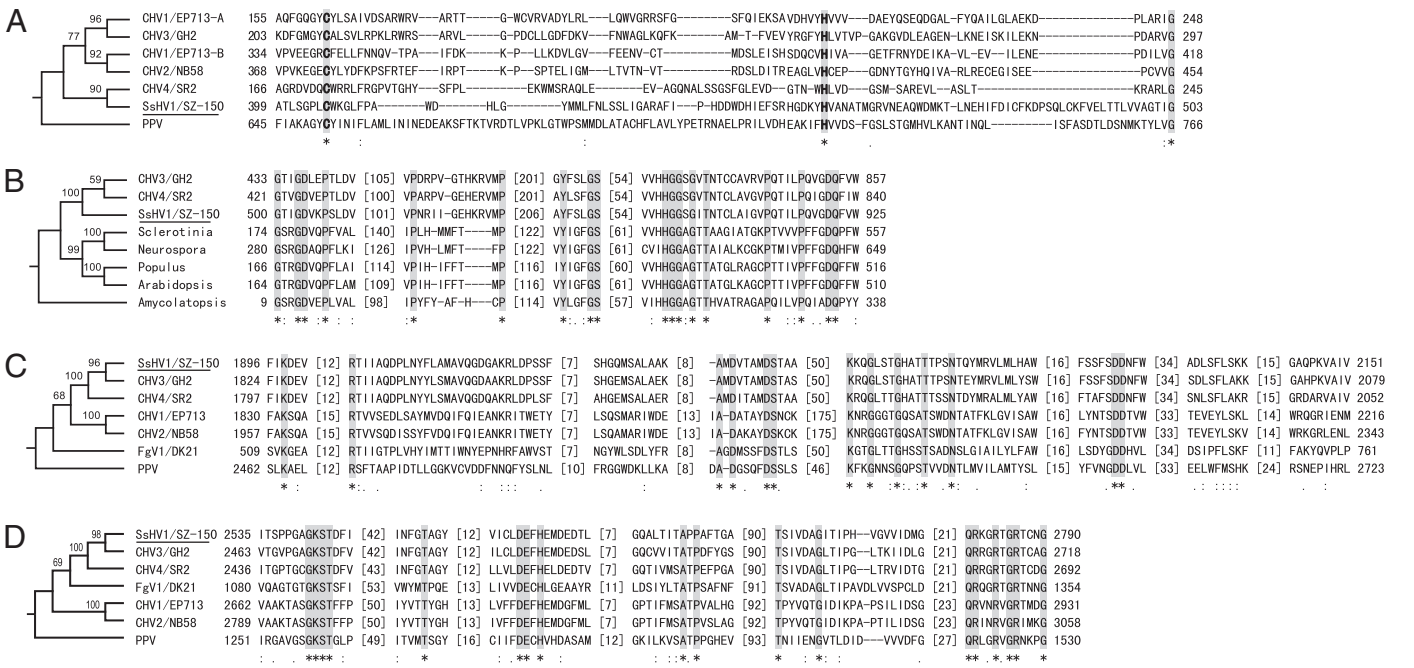

Fig. 3. Phylogenetic analysis (left) and amino acid sequence alignment (right) of the papain-like protease domain, UDP glucose/sterol glucosyltransferase domain, RNA-dependent RNA polymerase domain and RNA Helicase domain. (A). Analysis of the papain-like protease domain. The positions of catalytic Cys^{406} and His^{453} residues in SsHV1/SZ-150 correspond to those previously described in CHV1/EP713, CHV2/NB58, CHV3/GH2 and CHV4/SR2 (Linder-Basso et al., 2005). The C-terminal boundaries of the aligned segments correspond to the cleavage sites. (B). Analysis of UDP glucose/sterol glucosyltransferase (UGT) domain in SsHV1/SZ-150 with that in selected mycoviruses, fungi and plants. (C). Analysis of the RNA-dependent RNA polymerase (RdRp) domain in SsHV1/SZ-150 with that in selected viruses. (D). Analysis of the RNA helicase domain in SsHV1/SZ-150 with that in selected viruses. Identical residues are shaded, asterisks, colons and dots indicate conserved amino acid residues and semi-conserved amino acid residues, respectively. Numbers in square brackets correspond to the number of amino acid residues separating the motifs. The unrooted phylogenetic tree was constructed using the neighbor joining method based on amino acid sequences alignment. Bootstrap values (%) obtained with 1000 replicates are indicated on branches. Abbreviations of mycovirus names and mycoviral sequence accession numbers were as shown in Fig. 1, with the addition of *C. parasitica hypovirus* 1-ORF A (CHV1/EP713-A; NP_041090), *C. parasitica hypovirus* 1-ORF B (CHV1/EP713-B; NP_041091), Plum pox virus (PPV; NP_040807), *Sclerotinia sclerotiorum* (Sclerotinia, XP_001592979.1), *Neurospora crassa* (Neurospora, XP_957752.1), *Populus trichocarpa* (Populus, XP_002302172.1), *Arabidopsis thaliana* (Arabidopsis, AAD39269.1) and *Amycolatopsis orientalis* (Amycolatopsis, AAK31353.1).

molecular mass of 71 kDa (Fig. 4A). A BLASTP and conserved motif search using the deduced amino acid sequence of the putative protein revealed that no sequence similarity to any known protein in the protein database. Furthermore, no sequence motif was observed using PROSITE motif database except for phosphorylation and glycosylation sites. Thus, the protein encoded by S-dsRNA segment is a hypothetical protein based on the present study.

Hypovirulence-associated traits of S. sclerotiorum strain SZ-150

Strain SZ-150 differs significantly in its biological properties compared to its protoplast derivative isolates SZ-150/R59 and SZ-150/R6, the latter two also differed in dsRNA profile from strain SZ-150 (see below). Whereas isolates SZ-150/R59 and SZ-150/R6 induced typical lesions on detached rapeseed leaves that were kept at 20 °C for 72 h following inoculation, strain SZ-150 failed to infect the leaves (Fig. 5A), thus suggesting that strain SZ-150 is hypovirulent. Furthermore, strain SZ-150 grew slowly on PDA plates and developed abnormal colony morphology with many sectors at the colony margin and black and brown pigment in colony on the PDA plates (Fig. 5A and Table 1).

SsHV1/SZ-150 alone is not the primary causal agent of hypovirulence in strain SZ-150 of S. sclerotiorum

To determine the causal role of SsHV1/SZ-150, SsRV1/SZ-150 and the S-dsRNA in the hypovirulence phenotype of strain SZ-150, ninety-nine colonies derived from protoplast regenerants of strain SZ-150 were selected for further studies. The phenotypes of these regenerants were diverse and could be divided into three types (Type I, II, III) based on growth rates, colony morphology and pathogenicity. Type I (7 regenerants as represented by isolate SZ-150/R32) showed similar phenotype to strain SZ-150 in colony morphology and virulence to detached leaves of rapeseed. Type II (85 regenerants as represented by isolate SZ-150/R6) grew much faster on PDA and exhibited stronger virulence to the detached leaves of rapeseed than strain SZ-150, but the pigment produced by these regenerants was black, which was similar to that produced by strain SZ-150. Type III (7 regenerants as represented by isolate SZ-150/R59) showed normal phenotype in growth rates, colony morphology and pathogenicity (Fig. 5A).

To further determine whether SsHV1/SZ-150 is the determinant of hypovirulence in strain SZ-150, transmission assays of hypovirulence traits were conducted using dual culture methods. Pursuant to contact between hyphae of strain SZ-150 and SZ-150/R59^{hyg}, or of strain SZ-150 and SZ-150/R6^{hyg} on PDA plate, isolates SZ-150/R59^{hyg} and SZ-150/R6^{hyg} were converted to hypovirulent phenotypes. We designated the newly produced hypovirulent isolates as SZ-150/R59T3 and SZ-150/R6T1, respectively. There were no significant differences among SZ-150/R59T3, SZ-150/R6T1 and strain SZ-150 in colony morphology, growth rate and virulence (Fig. 5A and Table 1). The results revealed that the hypovirulence-associated traits of strain SZ-150 could be transmitted to virulent isolates via hyphal anastomosis.

Based on dsRNA profiles and RT-PCR amplification assays, regenerant isolate SZ-150/R59 was found to harbor only SsRV1/SZ-150, and isolate SZ-150/R6 to harbor both SsHV1/SZ-150 and SsRV1/SZ-150, but lacks the S-dsRNA segment. Furthermore, newly generated hypovirulent isolates (SZ-150/R59T3 and SZ-150/R6T) were found to harbor SsHV1/SZ-150 and SsRV1/SZ-150 as well as the S-dsRNA segment (Fig. 5B). Thus, we conclude that SsRV1/SZ-150 alone is not the primary causal agent for hypovirulence in strain SZ-150, and that S-dsRNA segment is likely required for conferring full-scale hypovirulence in strain SZ-150. Furthermore, no protoplast regenerants that harbor only

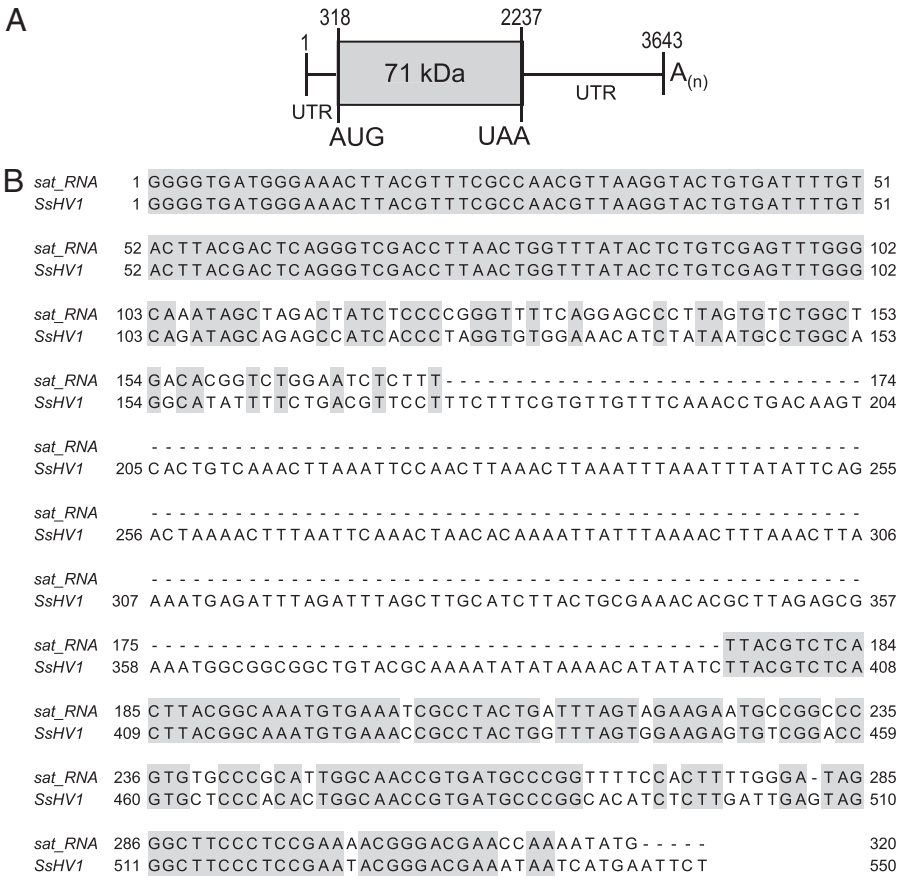

Fig. 4. Molecular characterization of the S-dsRNA segment. (A) Schematic representation of the genetic organization. The ORF is indicated by a gray rectangular. (B) Alignment of 5′-UTR of S-dsRNA segment (S-dsRNA) and the SsHV1/SZ-150. Identical nucleotides are shaded.

S-dsRNA segment were isolated and S-dsRNA segment does not encode RdRp, suggesting that the S-dsRNA could be a satellite-like RNA that is dependent on SsHV1/SZ-150 for replication.

Discussion

In this study, we described the properties of a novel mycovirus SsHV1/SZ-150 and an associated 3.6 kbp dsRNA (S-dsRNA) segment that infect the plant pathogenic fungus *S. sclerotiorum*. The nucleotide sequence and genome organization of SsHV1/SZ-150 were determined and characterized. The genome of SsHV1/SZ-150, excluding the poly (A) tail, comprises an ssRNA of 10398 nts that contains a single putative ORF encoding a large polyprotein with four conserved domains including a putative protease, UGT, RdRp and helicase. Sequence and phylogenetic analyses of the putative polyprotein strongly support the conclusion that SsHV1/SZ-150 is most closely related to the hypoviruses CHV3/GH2 and CHV4/SR2, in the family *Hypoviridae*. Therefore, SsHV1/SZ-150 is proposed to constitute a new species in the genus *Hypovirus*, family *Hypoviridae*. Members of the family *Hypoviridae* are phylogenetically related to positive-strand RNA viruses in the family *Potyviridae* (Hillman and Suzuki, 2004; Koonin et al., 1991). The prototypes of four species presently recognized in the genus *Hypovirus* are CHV1/EP713 (Shapira et al., 1991), CHV2/NB58 (Hillman et al., 1994), CHV3/GH2 (Smart et al., 1999) and CHV4/SR2 (Linder-Basso et al., 2005). These have all been characterized for genome structure, phenotype changes and their impact on virulence of their hosts. Furthermore, the impact of CHV1 on virulence, mycelial growth and colony morphology of fungi that are phylogenetically related to *C. parasitica* was observed following artificial transfection (Chen et al., 1996; Sasaki et al., 2002). However, mycoviruses belonging to the family *Hypoviridae* have not been found in other fungi. To our knowledge, this is first report of a naturally occurring hypovirus that infects a fungus other than *C. parasitica*. Moreover, CHV1 and CHV2 were both detected in North America and Asia (Peever et al., 1997, 1998), but CHV3 and CHV4 were only found predominantly in North America (Linder-Basso et al., 2005; Smart et al., 1999). Thus, our finding may expand our knowledge of the ecology and evolution of mycoviruses in the family *Hypoviridae*.

Although the SsHV1/SZ-150 justifiably belongs to the family *Hypoviridae*, it differs in genome organization. Direct comparison in genome size between SsHV1/SZ-150 (10.4 kbp) and other hypoviruses reveals that it is larger than CHV3/GH2 (9.8 kbp) (Smart et al., 1999) and CHV4/SR2 (9.1 kbp) (Linder-Basso et al., 2005), but smaller than CHV1/EP713 (12.7 kbp) (Shapira et al., 1991) and CHV2/NB58 (12.5 kbp) (Hillman et al., 1994). Furthermore, the most significant difference in genome organization between SsHV1/SZ-150 and other hypoviruses reported previously is the length of its 5′ and 3′-UTRs. The 5′-UTR of SsHV1 is 541 nts in length (Fig. 1B) and is longer than those of the other four hypoviruses, which contain less 500 nts. The 3`-UTR in the genomic RNA of SsHV1/SZ-150 was shown to be 1010 nts in length (Fig. 1B), which is significantly longer than those of the other hypoviruses with lengths of 409 to 851 nts (Linder-Basso et al., 2005). Based on the high degree of sequence conservation among certain regions of the 5′ and 3′ terminal of SsHV1/SZ-150, CHV3/GH2 and CHV4/SR2 (Fig. 2), it is likely that these 5′ and 3′ conserved terminal sequences, are important for replication and genome expression of hypoviruses.

In the present study, we demonstrated that the hypovirulent strain SZ-150 harbors two phylogenetically unrelated mycoviruses and a satellite-like S-dsRNA segment. Our experimental results suggest that the satellite-like RNA segment is an important player in conferring hypovirulence and debilitation on strain SZ-150, since SsHV1/SZ-150 in the absence of the S-dsRNA segment has only a limited effect on the

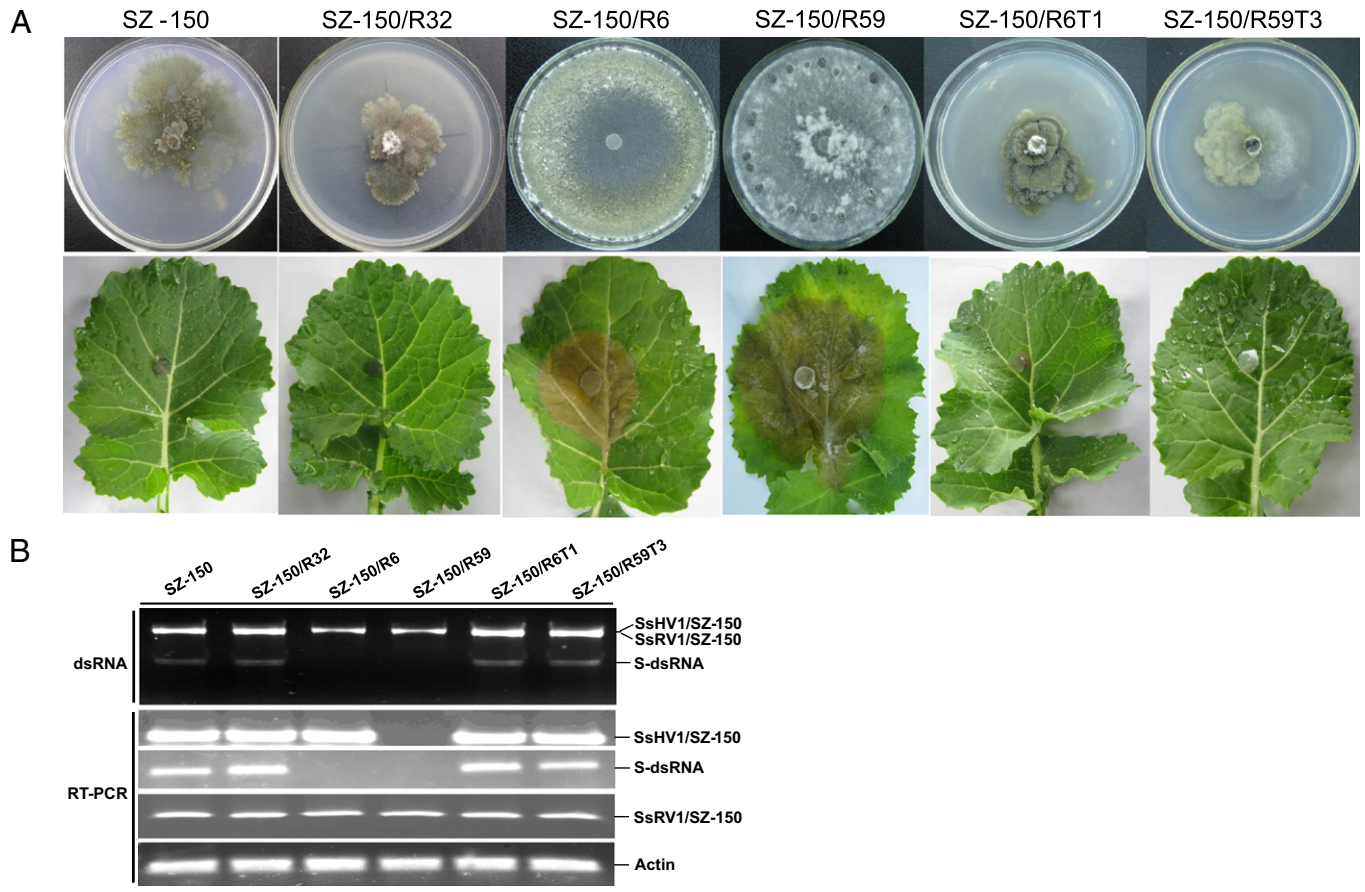

Fig. 5. Colony morphology, virulence and mycovirus content. (A). The colony morphology (upper panels) and virulence (lower panels) of hypovirulent *S. sclerotiorum* strain SZ-150 and its protoplast derivatives. (B). Detection of mycoviruses SsHV1/SZ-150 and SsRV1/SZ-150 and the satellite-like RNA (S-dsRNA) in individual isolates with dsRNA extraction and RT-PCR method. The actin gene was used as an internal control. The predicted lengths of the RT-PCR products for SsHV1/SZ-150, SsRV1/SZ-150 and S-dsRNA are 252, 462 and 250 nts, respectively.

biological properties of its host, and SsRV1/SZ-150 has no obvious effect on virulence and biological properties if infecting the host alone (Fig. 5A and Table 1). Whereas the hypovirus CHV3/GH2 could reduce the virulence of its host *C. parasitica*, it has no obvious effects on other phenotypes including pigmentation and conidiation (Smart et al., 1999). Likewise CHV4/SR2 has only a limited effect on virulence and colony morphology (Linder-Basso et al., 2005). The current study thus indicates SsHV1/SZ-150, like the hypoviruses CHV3/GH2 and CHV4/SR2, may only have mild effect on the virulence of their hosts.

In this study, we proposed that the S-dsRNA segment might be a satellite-like RNA of SsHV1/SZ-150 based on sequence analysis and biological data presented (apparent co-infection with SsHV1/SZ-150). Satellite RNAs have been described in association with several mycovirus systems, but not those involving hypovirulence in fungi (Deng and Boland, 2004; Romanos et al., 1981; Wickner, 1996). The hypovirus CHV3/GH2 has two satellite RNAs (dsRNA-3 and dsRNA-4), of which dsRNA-4 was confirmed to encode a small polypeptide of 9.4 kDa, as shown in an in vitro translation experiment (Hillman et al., 2000; Yuan and Hillman, 2001). However, the biological functions of these satellite RNAs remain unknown. Our results suggest that the S-dsRNA segment is most likely involved in the hypovirulence phenotype of *S. sclerotiorum* strain SZ-150 and its associated traits, suggesting that it, directly or indirectly, enhances the hypovirulence traits of *S. sclerotiorum*. It is of interest to note that the size of S-dsRNA

Table 1
Biological properties of *S. sclerotiorum* strains used in this paper.

Strains	Growth rate (cm/d)[a]	Lesion diameter (cm)[b]	Mycoviruses detected	Pigmentation[c]
SZ-150	0.77 ± 0.39	0	SsHV1/SZ-150, SsRV1/SZ-150 and S-dsRNA segment	Black, brown
SZ-150/R59	2.35 ± 0.04	4.38 ± 1.13	SsRV1/SZ-150	White
SZ-150/R6	2.30 ± 0.00	3.15 ± 0.15	SsHV1/SZ-150, SsRV1/SZ-150	Black, brown
SZ-150/R59T3	0.33 ± 0.07	0	SsHV1/SZ-150, SsRV1/SZ-150 and S-dsRNA segment	Black, brown
SZ-150/R6T1	0.47 ± 0.06	0	SsHV1/SZ-150, SsRV1/SZ-150 and S-dsRNA segment	Black, brown
LSD (0.01)	0.52	0.41	–	–

[a] Values are means of six replicate PDA plates. Radial hyphal extension (cm/d) was measured on the PDA plates at 20 °C. Significant differences between means were determined by the LSD (least significant difference) at P = 0.01.
[b] Values are means of six replicates of virulence test on detached rapeseed leaves. Lesion diameter was measured 72 h after inoculation at 20 °C. Significant differences between means were determined by the LSD at P = 0.01.
[c] Pigmentation type was recorded following 10 days of growth at 20 °C.

segment is larger than any known satellite RNAs and that it shares high sequence identity with the 5′-UTR of SsHV1/SZ-150. The latter property is unusual for satellite RNAs which generally share very little or no sequence similarity with helper viruses (Bruening, 2002). Future research using full-length infectious transcripts of S-dsRNA and SsHV1/SZ-150 is needed to confirm the satellite nature of S-dsRNA and its role in hypovirulence.

Material and methods

Fungal strains and culture condition

Sclerotinia sclerotiorum strain SZ-150 was isolated from a sclerotium obtained from a diseased rapeseed (*Brassica napus*) plant in Suizhou county, Hubei province, PR China. Isolates SZ-150/R32, SZ-150/R59 and SZ-150/R6 were derived from strain SZ-150 via protoplast isolation and regeneration. The protoplast-derived isolates were labeled with a hygromycin-resistance gene (*hph*) using the *Agrobactirium tumfacience* mediated transformation method and the hygromycin-resistant isolates showed no significant differences from their parent isolates in biological properties. All *S. sclerotiorum* isolates were maintained on potato dextrose agar (PDA) at 20–22 °C and stored on PDA slants at 4 °C.

DsRNA isolation and purification

To isolate dsRNA, strain SZ-150 was cultured on cellophane membranes overlaying a PDA plate for 10 days, and then the mycelial mass was collected and ground in liquid nitrogen with a mortar and pestle to fine powder. DsRNA was isolated with CF-11 cellulose (Sigma-Aldrich, Dorset, England) chromatography, as previously described (Xie et al., 2006). The dsRNA nature of the samples was further confirmed based on resistance to DNase I and S1 nuclease (TaKaRa, Dalian, China). The isolated dsRNA was agarose gel purified with a gel extraction kit (Axygene biosciences) and stored at −80 °C before use.

Molecular cloning, sequencing and sequence analysis

cDNA cloning and sequencing of dsRNA isolated from strain SZ-150 were performed following the method of Liu et al. (2009) and Darissa et al. (2010) using a cDNA synthesis kit (Fermentas) with tagged random primers-dN6 (5′-CGATCGATCATGATGCAATGCNNNNNN-3′). About 200 ng dsRNA was mixed with 1.2 μM random primers-dN6 and 3 μl 100% dimethyl sulfoxide, and diethyl pyrocarbonate-treated double-distilled H_2O was added to a final volume of 12 μl. The mixture was heated to 95 to 98 °C for 15 min and chilled on ice for 5 min. The dsRNA was reverse transcribed in a reaction mixture containing 200 units of RevertAid™ Reverse Transcriptase (Fermentas, USA), 50 mM Tris-HCl (pH8.0 at 25 °C), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, and 20 units of RiboLock™ RNase Inhibitor (Fermentas, USA) and distilled water to a final volume of 25 μl. The mixture was incubated at 42 °C for 1 h. After Reverse Transcription (RT), the mixture was treated with NaOH (Final concentration 0.1 M at 68 °C for 60 min) for denaturation, and then 2.5 μl 1 M HCl and 2.5 μl 1 M Tris–HCl (pH 8.0) were added. The reaction mixtures was incubated at 68 °C for 1–2 h to neutralization and anneal, and form a mixture of partial double-stranded cDNA library finally, which was then purified with a gel extraction kit and eluted in 42 μl of distilled water. The purified cDNA library was used for synthesis of completely double-stranded cDNAs as follows: Ten units of the Klenow Fragment (TaKaRa), 5 μl 10 × Klenow buffer, 2 μl 10 mM dNTPs were added to reaction mixture, and distilled water was added to a final volume of 50 μl. The reaction was incubated at 37 °C for 60 min. The random cDNA products were then amplified using a single specific primer (5′-CGATCGATCATGATGCAATGC-3′) based on tagged random primers-dN6. DNA amplifications were carried out using a C1000™ Thermal Cycler (BIO-RAD). The amplified PCR products were cloned into pMD18-T Vector (TaKaRa) according to the manufacturer's instructions and then transformed into competent cells of *Escherichia coli* JM109. Positive clones were sequenced and analyzed with the DNAMAN program and BLASTP program on NCBI website. Gaps in sequences between different clones were determined using RT-PCR amplification.

To obtain the 5′ and 3′ terminal sequence of dsRNA, a method described by Xie et al. (2006) was used. Briefly, the 3′ terminus of each strand of dsRNA was ligated to the phosphorylated 5′-end oligonucleotide 5′-GCATTGCATCATGATCGATCGAATTCTTTAGTGAGGGTTAATTGCC-(NH2)-3′ using T4 RNA ligase at 4–8 °C for 18 h. The oligonucleotide-ligated dsRNA was then denatured and used for the reverse transcription reaction with reverse transcriptase and 3 pmol of a primer with sequence complementary to the oligonucleotide used for RNA ligation (oligoREV: 5′-GGCAATTAACCCTCACTAAAG-3′). The reaction product was treated with RNase H as described earlier and the cDNA was amplified with another primer complementary to the RNA ligation oligonucleotide (5′-TCACTAAAGAATTCGATCGATC-3′) and the sequence-specific primer corresponding to the 5′-and 3′-terminal sequences of the dsRNA, respectively. The expected PCR products from cDNA sequence of dsRNA were fractionated by electrophoresis on an agarose gel and purified using a gel extraction kit. The PCR product was cloned into the pMD18-T Vector and sequenced.

Sequencing was carried out by the dideoxynucleotide termination method using a Big Dye Terminator Sequencing kit (BigDye terminator v. 2.0; ABI) and an ABI PRISM 377–96 automated sequencer (BGI). M13 universal primers or sequence-specific primers were used for sequencing and every base was determined by sequencing at least three independent overlapping clones in both orientations.

Sequence analysis, alignments and phylogenetic analysis were carried out by using DNAMAN, CLUSTAL_W and BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of previously reported mycoviruses and other sequences referenced to in this paper were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genomes) and used for comparative analyses. On the basis of the aligned sequences, phylogenetic trees were constructed by the neighbor-joining method using the MEGA version 4.0 programs (Kumar et al., 2008).

Mycovirus detection in different isolates

Three dsRNA segments were extracted from strain SZ-150, among them, two segments were similarly sized that could not be separated on agarose gel. cDNA cloning and sequence analysis showed the presence of two distinct RNA genomes of mycoviruses. To determine whether the two mycoviruses could replicate independently, or if strain SZ-150 could be cured by protoplast regeneration, specific primers were designed based on the two viruses genome sequences. Total RNA was prepared using the TRIzol reagent (Invitrogen) and used for RT-PCR amplification. The specific primers (pSsHVF: 5′-CCAAAACAACCCTGTCGCC-3′ and pSsHVR: 5′-CAACAAACCTGTCAGCACCC-3′) were used for the mycovirus SsHV/SZ-150, specific primers (pSsRVF: 5′-GGGTGACGACGGATGGGTA-3′ and pSsRVR: 5′-CACAGGCAGCTTCGCTACTCT-3′) were used for mycovirus SsRV/SZ-150 and specific primers (pSsSatF: 5′-GAGTTGGCTCTTCTGGATACGA-3′ and pSsSatR: 5′-AAGTGGTTGTTGAGTTGGGTTC-3′) were used for S-dsRNA segment. At the same time, specific primers (actin-qF2: 5′-GAGCTGTTTTCCCTTCCATTGTC-3′ and actin-qR4: 5′-GACGACACCGTGCTCGATTGG-3′) were used for actin gene as control (Sexton et al., 2009).

Biological properties of S. sclerotiorum strain SZ-150 and its derivatives

The methods of Zhang et al. (2009) were followed to assess the mycelial growth, colony morphology and virulence on detached leaves of rapeseed (*Brassica napus*) of strain SZ-150 and its

derivatives SZ-150/R32, SZ-150/R59 and SZ-150/R6, SZ-150/R59T3 and SZ-150/R6T1.

Date analysis

Experimental data were subjected to analysis of variance (ANOVA) using SAS®8.0 program. Treatment means were compared with least significant difference (LSD) test at $P = 0.01$ level.

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