A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus

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Mycoviruses are viruses that infect fungi and have the potential to control fungal diseases of crops when associated with hypovirulence. Typically, mycoviruses have double-stranded (ds) or single-stranded (ss) RNA genomes. No mycoviruses with DNA genomes have previously been reported. Here, we describe a hypovirulence-associated circular ssDNA mycovirus from the plant pathogenic fungus *Sclerotinia sclerotiorum*. The genome of this ssDNA virus, named Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1), is 2166 nt, coding for a replication initiation protein (Rep) and a coat protein (CP). Although phylogenetic analysis of Rep showed that SsHADV-1 is related to geminiviruses, it is notably distinct from geminiviruses both in genome organization and particle morphology. Polyethylene glycol-mediated transfection of fungal protoplasts was successful with either purified SsHADV-1 particles or viral DNA isolated directly from infected mycelium. The discovery of an ssDNA mycovirus enhances the potential of exploring fungal viruses as valuable tools for molecular manipulation of fungi and for plant disease control and expands our knowledge of global virus ecology and evolution.

mycovirus | ssDNA virus | geminivirus | *Sclerotinia sclerotiorum* | hypovirulence

Fungi cause catastrophic diseases in all major crops with considerable impact on human lives. For example, the recent re-emergence of the deadly wheat black stem rust fungus is likely to threaten the world's breadbaskets (1). Application of chemical fungicides is the major method used for control of fungal diseases of economically important crops, especially when resistant cultivars are lacking. To reduce the dependence on fungicides, highly efficient and environmentally friendly alternative methods to control diseases are desirable. Mycovirus-mediated hypovirulence is a phenomenon in which the virulence of fungal pathogens is reduced or even completely lost as a consequence of virus infection (2). Hypovirulence is thought to play a role in counterbalancing plant diseases in nature (3), and it was used successfully to control chestnut blight (caused by the fungus *Cryphonectria parasitica*) in Europe (4, 5). The successful utilization of hypoviruses for biological control of the chestnut blight fungus has attracted much interest and led to the discovery of hypovirulent strains in other fungi.

Mycoviruses are known to infect and multiply in all major taxa belonging to the kingdom Fungi. Typically, mycoviruses have either double-stranded (ds) or single-stranded (ss) RNA genomes; viruses with DNA genomes have not been reported to infect fungi. Although Rhizidiomyces virus, which infects *Rhiziodiomyces* sp., was previously reported as a dsDNA fungal virus (6), the fungal-like host *Rhiziodiomyces* sp. is in fact a water mold belonging to the kingdom Protista (7). Thus, it is not known whether DNA viruses could naturally multiply in fungi.

Geminiviruses, which only infect plants, have circular ssDNA genome and twinned quasi-icosahedral particles (8). Plant diseases caused by geminiviruses have threatened agricultural productivity in most tropical and subtropical areas in the world (9). Nowadays, due to changes in agricultural practices, as well as the increase in global trade in agricultural products, these diseases have spread to more regions (10, 11). Several possible scenarios for the evolution of geminiviruses have been developed, however, there still are many questions to be answered, and it is very difficult to ascertain how ancient geminiviruses or their ancestors are (12–14).

Sclerotinia sclerotiorum is a notorious plant fungal pathogen that inhabits the soil worldwide. It has a wide host range including more than 450 species and subspecies among 64 genera of plants (15). Diseases caused by *S. sclerotiorum* cannot be controlled efficiently because no resistant cultivars are available and fungicides are difficult to deliver into canopies and soil. Hypovirulent strains of *S. sclerotiorum* have been isolated (16, 17), and two other RNA viruses infecting the hypovirulent strain Ep-1PN were identified (18–20). In the present study, we report on a unique geminivirus-related ssDNA mycovirus that confers hypovirulence to *S. sclerotiorum* and thus holds the potential for use in studies on the interactions between a DNA virus and fungi, as well as for possible use in biological control of fungal diseases of important crops.

Results

Hypovirulence and Associated Traits of Strain DT-8 of *S. Sclerotiorum*. Strain DT-8 grew slowly on potato dextrose agar (PDA) and developed colony morphology similar to that of the hypovirulent strain Ep-1PN (Fig. 1*A* and D). The hypovirulence phenotype of strain DT-8 is even more pronounced than that of strain Ep-1PN when inoculated onto *Arabidopsis thaliana* (Fig. 1*B*) or detached leaves of *Brassica napus* (Fig. S1). Although strain DT-8 was able to produce sclerotia, the time required for sclerotial initiation was about 3–5 days longer than that for the normal strain and the sclerotia were randomly distributed on the colony surface. Furthermore, the size of the sclerotia produced by strain DT-8 was significantly smaller than that of the normal strain (Fig. 1*C*).

Strain DT-8 was cured of its hypovirulent phenotype either by hyphal-tip culturing or protoplast regeneration, and the cured isolates of DT-8, such as DT-8VF, regained virulence on plants

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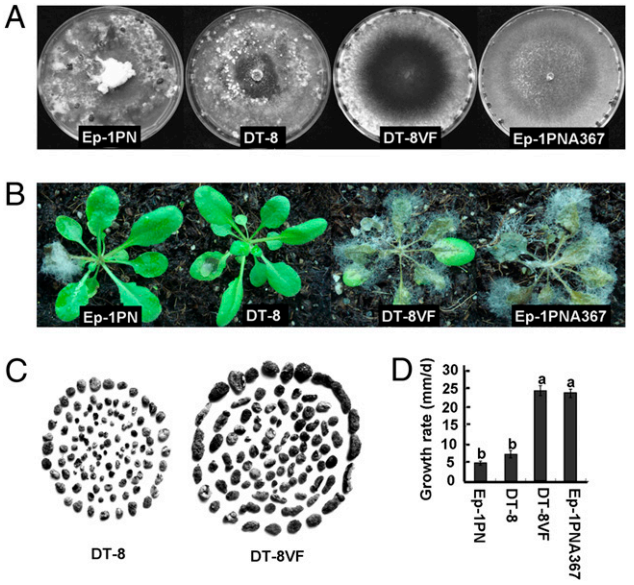

Fig. 1. Hypovirulence-associated traits of strain DT-8 of *S. sclerotiorum*. (*A*) Abnormal colony morphology of strain DT-8 grown on a PDA plate at 20 °C for 15 days; (*B*) hypovirulent phenotype of strain DT-8 as exhibited by infected *Arabidopsis thaliana* plants, which were maintained at 20 °C for 4 days postinoculation; (*C*) small sclerotia produced by strain DT-8 on a PDA plate at 20 °C for 30 days; and (*D*) growth rate of strain DT-8 relative to other strains, bars represent standard deviation from the mean (n = 8). The small letters on top of the bars in *D* indicate whether the differences are statistically significant ($P < 0.05$). The RNA virus-infected hypovirulent strain Ep-1PN and its sexual progeny Ep-1PNA367 (virus-free) were used as controls; DT-8VF is a virus-free culture derived by hyphal tipping of strain DT-8, and showed a normal phenotype of *S. sclerotiorum*.

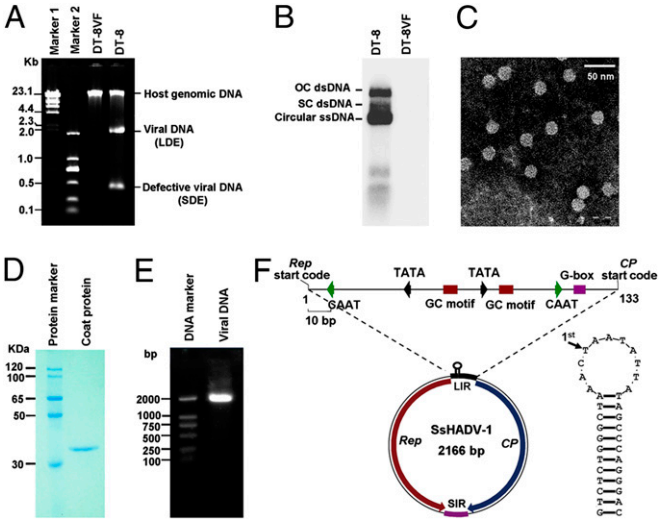

Fig. 2. Genomic characteristics and particle morphology of *Sclerotinia sclerotiorum* hypovirulence associated DNA virus 1 (SsHADV-1). (*A*) Total DNA extracted from mycelia of strains DT-8 and DT-8VF. DNA samples were fractionated on 1.0% agarose gel. The positions of host genomic DNA, viral DNA, or the large DNA element (LDE) and defective viral DNA or small DNA element (SDE) of strain DT-8 are indicated. Lane M1, λ-*Hin*d III-digested DNA Marker; lane M2, DL2000 DNA ladder marker (TaKaRa). (*B*) Southern blot analysis of total nucleic acid extracted from mycelia of strains DT-8 and DT-8VF. The forms of viral DNA are indicated as OC dsDNA [open circular double-stranded (ds) DNA], SC dsDNA (supercoiled dsDNA), and circular single-stranded (ss) DNA. A 379-bp DNA fragment of *Rep* was PCR amplified and labeled with α-^{32}P dCTP and used as a probe. (*C*) Viral particles observed under transmission electron microscopy. Particles were purified from mycelia of strain DT-8 and negatively stained with 1% uranyl acetate. (Scale bars, 50 nm.) (*D*) SDS/PAGE analysis of SsHADV-1 coat protein. Samples collected from fractions corresponding to 25% sucrose of sucrose gradients were subjected to SDS/PAGE. The size of the Coomassie blue-stained protein was estimated by comparison with protein markers. (*E*) Agarose gel electrophoresis on 1% agarose of DNA extracted from sucrose gradient fractions containing virus particles. (*F*) Genome organization of SsHADV-1. Functional ORFs (coding for CP and Rep) were displayed as thick arrows. The positions of the potential stem–loop structure, large intergenic region (LIR) and small short intergenic region (SIR) were marked; the stem-loop structure was shown on the right. LIR is also shown in an expanded form to indicate the elements of the bidirectional promoter.

comparable to that of the normal strains (e.g., strain Ep-1PNA367) and developed normal colony morphology (Fig. 1 *A* and *D*). Furthermore, the traits of these cured isolates can be reverted to the hypovirulence phenotype following contact with the original hypovirulent strain DT-8 (Fig. S2). Thus, strain DT-8 shows typical characteristics of virus-mediated debilitation/hypovirulence.

DNA Elements Associated with Strain DT-8 of *S. Sclerotiorum*. Virus-mediated hypovirulence in pathogenic fungi is usually caused by either double-stranded (ds) or single-stranded (ss) RNA viruses. However, several attempts to extract dsRNA from strain DT-8 were unsuccessful. The genomic DNA of strain DT-8 was extracted and resuspended in RNase A-containing TE buffer, and the DNA sample was electrophoresed on 1% agrose gel and stained with ethidium bromide. Surprisingly, besides the fungal genomic DNA, two additional DNA bands with sizes of about 2 kb [large DNA element (LDE)] and 0.3 kb [small DNA element (SDE)], respectively, were clearly observed in a stained gel (Fig. 2*A*). These two DNA elements could be digested with DNase I (active against ssDNA and dsDNA) and S1 nuclease (active against ssDNA or ssRNA) but could not be digested with either Exonuclease III (active against linear dsDNA) or Exonuclease I (active against linear ssDNA), suggesting that these elements are circular ssDNA molecules or linear ssDNA with modified 5′ and 3′ ends (Fig. S3*A*).

Interestingly, strain DT-8VF, which was derived from strain DT-8, lacked the additional ssDNA elements characteristic of DT-8 (Fig. 2 *A* and *B*). However, after contacting the colony of strain DT-8, newly obtained isolates of strain DT-8VF contained the two ssDNA elements, and their colony morphology and virulence were as debilitated as the original strain DT-8 (Fig. S2). Thus, the ssDNA elements are likely to be associated with hypovirulence of strain DT-8.

Cloning and Sequencing of the Full-Length DNA Elements of Strain DT-8. Full-length DNA clones of LDE were obtained by PCR amplification, confirmed by Southern blot analysis (Fig. 2*B*) and subjected to sequence analysis. Sequencing results showed that the full-length DNA of LDE is 2,166 nt in length and contains two large ORFs, whose expressions were confirmed by northern hybridization and RT-PCR analysis (Fig. S4 *A* and *B*). One ORF, whose expression codes for a putative coat protein, is located on the sense strand and the other ORF is situated on the complementary-sense strand and codes for a putative replication initiation protein (Rep) (Fig. 2*F*). The putative Rep has two conserved domains, namely geminivirus Rep catalytic domain (Gemini_AL1) and geminivirus Rep protein central domain (Gemini_AL1_M) with conserved motifs for rolling-circle replication (21) (Fig. 3*A*). Two intergenic regions separate the two ORFs, the large intergenic region (LIR) contains 133 nt and the small intergenic region (SIR) contains 119 nt (Fig. 2*F*). Both LIR and SIR also have similar characteristics to those of viruses in the genus *Mastrevirus* in the family Geminiviridae (22). LIR has an unusual nonanucleotide TAATATT↓AT at the apex of a potential stem-loop structure, which is recognized at

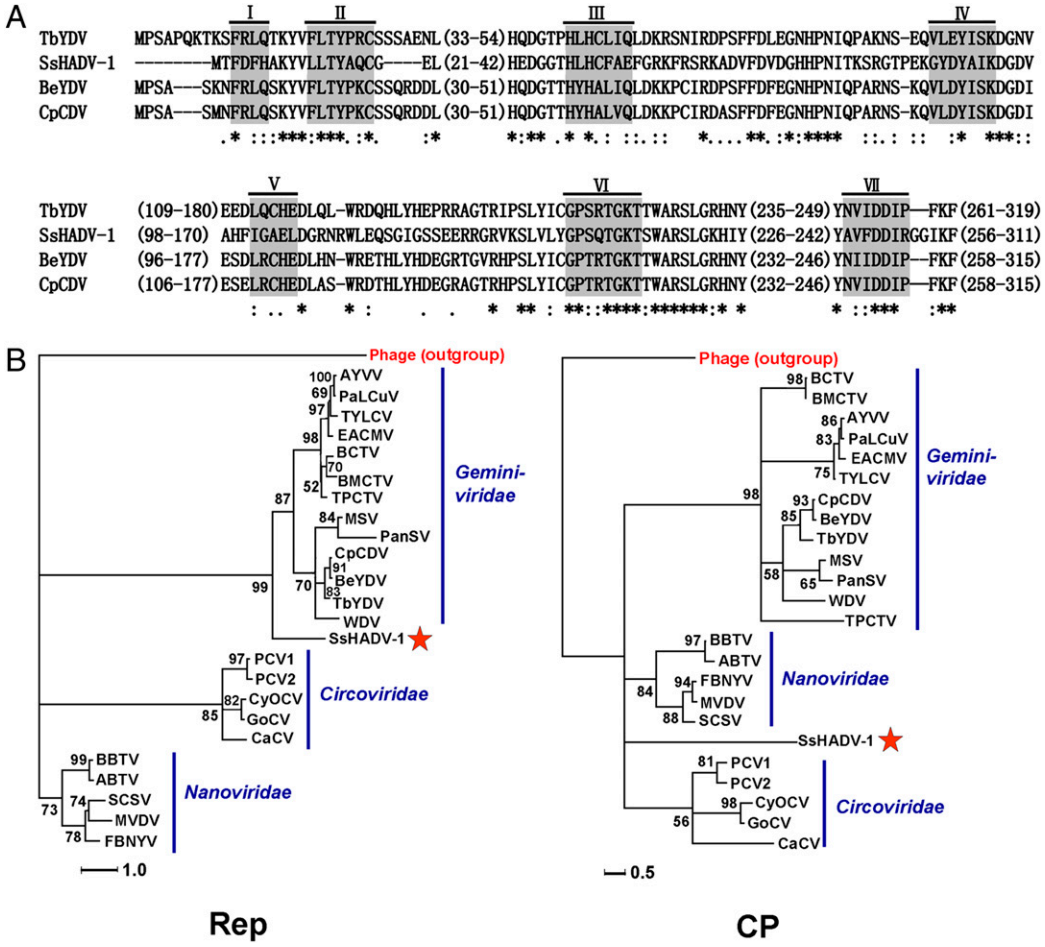

Fig. 3. Phylogenetic analysis of SsHADV-1. (*A*) Amino acid sequence alignment of Rep of SsHADV-1 and selected viruses from the genus *Mastrevirus* in the family Geminiviridae. The conserved motifs (I to VII) were shaded with light gray color; Asterisks indicate identical amino acid residues, and colons indicate similar residues. Conserved motif Ito VII are IRD, RCR-I, RCR-II, RCR-III, RBR, NTP Binding-A, and NTP binding-B, respectively. The Alignment was generated by using CLUSTALX (2.0). Numbers in brackets are the positions of amino acid residues that are not listed. (*B*) Phylograms of the Rep and CP of SsHADV-1 and selected circular ssDNA viruses in the families Geminiviridae, Nanoviridae and Circoviridae. Multiple alignments of amino acids and tentative phylogenetic trees generation were referred to the method described by Liu et al. (20). The position of SsHADV-1 is indicated by a red star. See Table S1 for abbreviations of virus names and viral protein accession numbers used for phylogenetic analysis.

a specific position (↓) by the Rep during the initiation of virion DNA replication (23). Therefore, the large DNA element most likely represents a DNA viral genome.

Sequence analysis showed that the SDE comprises a mixture of ssDNA molecules, 236–284 nt in length, representing defective DNA derived from LDE. They lack the two ORFs but contain both LIR and SIR (Fig. S5). The short direct repeat sequences flanking LIR and SIR may represent signal nucleotides for deletion (24).

Viral Particles in Strain DT-8 of *S. Sclerotiorum*. To confirm that LDE represents a viral genomic DNA, mycelial mass of strain DT-8 grown on a PDA plate was collected and subjected to a virus purification protocol that includes sucrose gradient centrifugation as the final step. The major band containing viral particles, which was present in the gradient region corresponding to 25–30% sucrose, was collected and the viral particles were recovered by centrifugation. Negatively stained viral particles observed with an electron microscope were nontwinned isometric particles, 20–22 nm in diameter (Fig. 2*C*). The viral particles were examined for coat protein (CP) and viral genomic DNA contents. A DNA band, similar in size to the LDE extracted directly from mycelia of strain DT-8, was extracted from the viral particles (Fig. 2*D*). Like the ssDNA form of M13 phage genomic DNA, the DNA extracted from viral particles can be digested by ssDNA nuclease S1, but not by Exonuclease I (Fig. S3*B*). Only one major protein with a molecular mass of about 35 kDa was resolved by SDS/PAGE analysis (Fig. 2*E*). The size of this isolated protein is similar to that predicted for coat protein based on DNA sequence analysis. Furthermore, N-terminal sequencing analysis further confirmed that the isolated protein was in fact the coat protein. Thus, we verified that the large DNA element in strain DT-8 represents a circular ssDNA viral genome, which we named Sclerotinia sclerotiorum hypovirulence associated DNA virus 1 (SsHADV-1).

Sequence and Phylogenetic Analyses of SsHADV-1. Sequence analysis of the full-length amino acid sequence of the putative Rep of SsHADV-1 showed that it shares the highest sequence identity with the Reps of chickpea chlorotic dwarf virus (25), bean yellow dwarf virus (26), and tobacco yellow dwarf virus (27) (Table S2). These three viruses that infect dicotyledonous plants belong to the genus *Mastrevirus* (Fig. 3*A*). Phylogenetic analysis of SsHADV-1 and selected ssDNA viruses showed that the putative Rep of SsHADV-1 clusters with members of the family Geminiviridae, but it is distinct from ssDNA viruses in the families

Nanoviridae and Circoviridae (Fig. 3*B*). Although SsHADV-1 appears to be related to geminiviruses, it is clearly different from all documented geminiviruses. In addition to the difference in particle morphology, the genome of this fungal DNA virus has an unusual nonanucleotide and is smaller than those of plant geminiviruses in the genus *Mastrevirus* (24). The genome of SsHADV-1 only codes for two proteins (CP and Rep), but lacks a gene for movement protein (MP), which is a key protein of plant geminiviruses for cell-to-cell movement. Furthermore, the Rep gene on the complementary strand lacks an intron. Moreover, phylogenetic analysis of the CP of SsHADV-1 and that of viruses in the families Geminivirdae, Nanoviridae, and Circovirdae shows that SsHADV-1 CP is phylogenetically divergent because it does not cluster with any of the viruses included in this analysis (Fig. 3*B*). Thus, SsHADV-1 most likely belongs to a family that is phylogenetically related to the family Geminiviridae. Interestingly, we found that SsHADV-1 CP shares the highest sequence similarity with a marine putative protein (Marine_PP) encoded by a whole genome shotgun sequence (GenBank accession no.: AACY024124290) from a metagenomic data of a microbial community from Sargasso Sea (28). The percent identity and similarity of these two proteins are 37.8% and 52.6%, respectively (Fig. S6). Considering that a short gene sequence possibly coding for a viral Rep is present on the same fragment as this Marine_PP gene, this putative protein is likely of a viral origin. It is thus possible that SsHADV-1 is more closely related to the putative marine virus (most likely infecting algae) than to plant geminiviruses.

Infectivity of Purified SsHADV-1 Particles and Viral DNA Extracted Directly from Infected Mycelium. Mycelial agar discs were taken randomly from regeneration plates of transfected *S. sclerotiorum* protoplasts and transferred into fresh PDA plates. Isolates Ep-1PNA367-NT3 and Ep-1PNA367-NT7 were derived from colonies where viral DNA-transfected protoplasts were regenerated; and isolates Ep-1PNA367-PT2 and Ep-1PNA367-PT6 were derived from colonies where viral particle-infected protoplasts were regenerated. Colony morphology and hyphal growth of these four isolates were similar to those of strain DT-8 but different from the virus-free strain Ep-1PNA367 (Fig. 4*A*). Viral DNA also was extracted successfully from these cultures and confirmed with PCR amplification using the specific primers, which were designed based on the *Rep* sequence (Fig. 4 *B* and *C*). Thus, both purified viral particles and viral DNA-enriched fractions isolated directly from infected mycelium were able to transfect *S. sclerotiorum* protoplasts.

Transmission of SsHADV-1 from Strain DT-8 to a Vegetatively Incompatible Strain of *S. sclerotiorum*. Vegetative incompatibility between two fungal individuals often limits the transmission of mycoviruses, and the most likely reasons are compartmentation and cell death of the fusing hyphal cells (29, 30). However, transmission of hypoviruses between two vegetatively incompatible strains has been observed previously (31). In our experiment, successful transmission of SsHADV-1 between vegetatively incompatible strains of *S. sclerotiorum* was confirmed by dual-culture of DT-8 and the vegetatively incompatible virulent strain Ep-1PNA367, which was labeled with hygromycin-resistance gene. Virus-infected isolates of strain Ep-1PNA367 were frequently obtained from dual culture plates (7 from 21 dual-culture plates), and all newly infected isolates of strain Ep-1PNA367 were converted to hypovirulence and showed a similar phenotype to that of strain DT-8 (Fig. S7).

Discussion

Previously, DNA viruses were known to infect animals, plants, protists, and prokaryotes but not fungi (32). Here, we demonstrated that a DNA virus could replicate in a fungus in nature and reported an ssDNA virus infecting fungi. The combination of

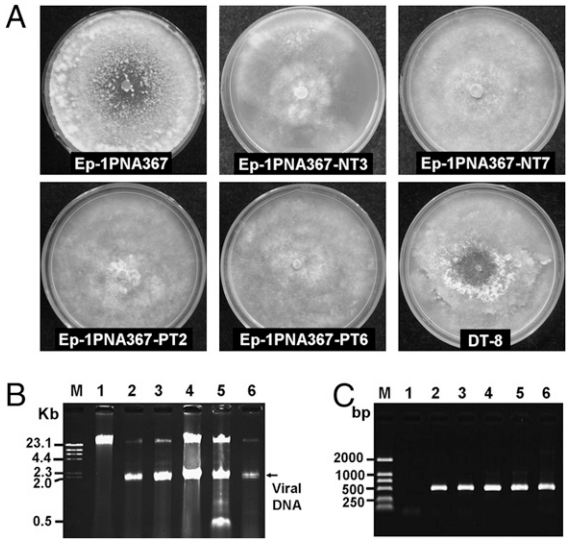

Fig. 4. PEG-mediated protoplast transfection with purified SsHDV-1 particles and viral DNA extracted directly from infected mycelium. Colony morphology of the virus-free strain Ep-1PNA367 and its newly transfected isolates. Ep-1PNA367-NT3 and Ep-1PNA367-NT7 were isolated from transfectants with purified viral DNA, whereas Ep-1PNA367-PT2 and Ep-1PNA367-PT6 were isolated from transfectants with viral particles. Colonies were grown on PDA plate at 20 °C for 7 days. (*B*) Viral DNA extracted from transfected cultures of strain Ep-1PNA367. Lane 1, strain Ep-1PNA367; lane 2, Ep-1PNA367-NT3; lane 3, p-1PNA367-NT7; lane 4, Ep-1PNA367-PT2; lane 5, Ep-1PNA367-PT6; lane 6, strain DT-8; and lane M, λ-*Hin*d III-digested DNA Marker. (*C*) Viral DNA of newly transfected cultures were confirmed by PCR amplification with primer pair designed from the *Rep* sequence of SsHADV-1. Lane M, DL2000 DNA ladder marker; lane 1 to lane 6 are as the same as in *B*.

S. sclerotiorum–SsHADV-1 also provides an excellent system for examining fungal factors involved in DNA virus replication and maintenance. Furthermore, the discovery of ssDNA mycoviruses may expand our knowledge of global virus ecology and evolution.

Based on phylogenetic analysis of Rep of SsHADV-1 and selected viruses, SsHADV-1 is related to plant geminiviruses. Although geminiviruses represent a serious threat to crops (10, 11), the origin of geminiviruses is presently unknown (12–14). It has been hypothesized that the progenitor of geminiviruses originated from an ssDNA replicon (plasmid molecules) that acquired new genes and captured other molecules for adaptation to plants. It was also hypothesized that there once existed a premastrevirus before the appearance of geminiviruses (13). Because the Rep and genome structure of SsHADV-1 are very similar to those of mastreviruses, but the CP is distinct from that of all tested geminiviruses (Table S2), it is possible that SsHADV-1 and geminiviruses share a common ancestor but acquired coat proteins independently. On the other hand, because the genome of SsHADV-1 is so small and basic, we assume that it is more likely to resemble a possible precursor of a premastrevirus, as portrayed by Nawaz-ul-Rehman and Fauquet (13). The discovery of SsHADV-1 may provide another avenue for studying the evolution of geminiviruses and their potential ancestors.

Mycoviruses perhaps have a unique path to evolution. Compared with typical plant geminiviruses, SsHADV-1 does not have an *MP* and its *Rep* lacks an intron. We do not know at present how or why SsHADV-1 or its ancestor lacks the *MP* and the intron. However, deletions or lack of *CP*, *MP*, and other genes are often found in many fungal RNA viruses (2, 19, 20). Thus, it is possible that there exists a common evolutionary mechanism for both RNA and DNA viruses in fungi.

As plant geminivirus-based vectors have proven to be very efficient in plant functional genomics studies (33–35), SsHADV-1

and its associated defective genomic DNA have the potential to be developed as attractive tools for gene expression and functional studies in fungi. Unlike geminiviruses, whose purified particles and viral ssDNA are not infectious (36), purified SsDAV-1 particles or DNA preparations enriched in viral DNA (dsDNA and ssDNA) that are isolated directly from infected mycelium are infectious. The genome of SsHADV-1 is very small, the titer in hyphae of *S. sclerotiorum* is quite high, and viral DNA can be easily extracted from infected strains. Furthermore, the PEG-mediated protoplast transfection assays are convenient for manipulation. All of these attributes make SsHADV-1 a promising means for providing genetic tools for studies on fungal functional genomics.

Furthermore, our finding that SsHADV-1 confers hypovirulence to its plant pathogenic fungal host has implication for biological control of important fungal pathogens. Fungal virus-mediated hypovirulence is believed to have an important role in counterbalancing the effects of plant diseases in nature. In this regard, it should be noted that biological control of chestnut blight using hypovirulence-associated hypoviruses was the only successful example in a limited area (37). Our experiments also demonstrated that SsHADV-1 can be transmitted from strain DT-8 to vegetatively incompatible *S. sclerotiorum* strains with relatively high frequency. SsHADV-1 isolates have been found in other *S. sclerotiorum* hypovirulent strains, which were isolated in 2008 from the same rapeseed field, but were vegetatively incompatible with strain DT-8. Hypovirulent strains were also isolated from other fields in 2009. Thus, SsHADV-1 can spread naturally under field conditions. Moreover, we expect that the host range of SsHADV-1 could be expanded to include other important plant pathogens considering that yeast was demonstrated to support replication of the DNA-A component of a plant geminivirus (38). Thus, the potential of SsHDAV-1 as a biological control agent to control plant fungal diseases needs to be explored.

Materials and Methods

Fungal Strains and Culture Media. *S. sclerotiorum* strain DT-8 was isolated from a sclerotium produced on a diseased rapeseed (*B. napus*) from Hunan Province, China. Hypovirulent strain Ep-1PN was shown to be infected with two RNA viruses (19, 20, 39). Ep-1PNA367 is a normal virus-free strain obtained by single-ascospore-isolation progeny of strain Ep-1PN, as verified by RT-PCR. Strain DT-8VF is a virus-free strain derived by hyphal tipping of strain DT-8 and was confirmed by Southern hybridization analysis. All strains were grown on PDA (Difco) plates at 20–22 °C, and stored on PDA slants at 4–6 °C; sclerotia produced on PDA plates were collected, air-dried, and stored at −20 °C.

Growth and Virulence Assays. Mycelial growth, colony morphology, and virulence test on detached leaves of rapeseed were evaluated according to the procedures described by Zhang et al. (40). The number of sclerotia produced in each colony was counted, and the size of sclerotia was measured. The virulence of fungal strains on six-week-old plants of *Arabidopsis thaliana* Columbia ecotype was evaluated as described for rapeseed. The inoculated Arabidopsis plants were checked for survival at 4 dpi.

Nucleic Acid Extraction and Molecular Hybridization Analyses. To obtain mycelial samples of strain DT-8 and other strains, mycelia were grown on cellophane membranes on PDA at 20–22 °C. DNA was extracted in CTAB following the procedures of Sambrook et al. (41). Total RNA samples were prepared using a TRIzol kit (Invitrogen). RNA samples were treated with DNase I (RNase Free) before use. Southern and Northern blot analyses were conducted as described previously (19, 42).

Molecular Cloning and Sequencing. To obtain a partial DNA sequence of the large DNA element in strain DT-8, the large DNA element was collected and purified with a DNA gel kit (AXYGEN). The purified DNA was used as a template for PCR amplification using 9-mer random primers, and a DNA fragment about 400 bp was amplified with one random primer (5′ CGTCGAATA 3′). The DNA fragment was confirmed with Southern blot hybridization analysis.

A full-length clone of the large DNA segment was amplified by Inverse-PCR, using the forward primer FP163 (5′CTTGACGGGAGAAATCGGTG 3′) and the reverse primer RP139 (5′CCGCTGGTGACTCATACTCG 3′), which were designed based on available sequences. The cloning strategy of Dry et al. (43) was followed for cloning the small DNA element. A pair of abutting primers, SgRP65 (5 CCAGGGA CGGGGACACTTC 3′) and SgFP83 (5′GCCCAGAGACTCAAAGGGACAAC 3′), were used to reclone the same DNA fragment.

Sequence and Phylogenetic Analyses. DNA sequence analysis and assembly were made with the DNAMAN version 5.2.9 (Lynnon Biosoft) software package including nucleotide statistics and ORF searching. Sequence similarity and protein motifs searches of GenBank databases were conducted using the BLAST program in National Center for Biotechnology Information website (44). Two sequence comparisons were made using the Needle program of the European Molecular Biology Open Software Suite (EMBOSS). Multiple alignments of amino acids and tentative phylogenetic trees generation were refereed to the method described by Liu et al. (20).

Purification of Virus Particles and Characterization of Coat Protein and Viral DNA. Virus particles were purified by sucrose density gradient centrifugation as described by Pinner et al. (45). Viral samples were negatively stained with 1% uranyl acetate on carbon-coated 400-mesh copper grids and observed with an FEI Tecnai G^2 20 TWIN transmission electron microscope.

Samples corresponding to gradient fractions containing 25% sucrose were subjected to SDS/PAGE. N-terminal end sequencing was performed by Shanghai Applied Protein Technology Company. Viral DNA was isolated from purified particles by SDS/phenol extraction, and confirmed with PCR amplification using primers SgRP and SgFP.

For transfection assays, a viral DNA-enriched fraction containing ssDNA and dsDNA [replicative form (RF)] was isolated from total DNA of infected mycelium by a method as described by Palmer et al. (46).

Protoplast Preparation and Transfection Assays. Protoplasts of *S. sclerotiorum* were prepared following the method described by Zhang et al. (40) with minor modifications. Protoplasts were resuspended at a concentration of 1×10^8 protoplasts per mL in STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$). The protoplast stock (containing 5% dimethyl sulfoxide) was routinely stored at −80 °C. Purified SsHADV-1 particles and viral DNA extracted directly from infected mycelium were filtered with Millex^R GP filter units (Millipore) and used to transfect fungal protoplasts. PEG-mediated transfection tests were conducted according to previous reports (47–49) with minor modifications. Protoplasts were regenerated in regeneration medium at 20 °C for 3–4 days. Mycelial plugs were cut at random from the regenerated colonies and transferred to fresh PDA plates. To verify that the transferred cultures were transfected by SsHADV-1, colony morphology of newly transfected isolates of strain Ep-1PNA367 were compared with that of strains Ep-1PNA367 and DT-8, and further confirmed by PCR amplification of viral DNA using a gene specific primer pair that was designed based on the *Rep* sequence (pRep-F 5′ GTCACCACCCAAACATTACAAAG 3′ and pRep-R 5′ AGCGTATTCCACGTCAGGTGC 3′).

Transmission Test. To test the possible transmission of hypovirulence and SsHADV-1 from strain DT-8 to a vegetatively incompatible virulent strain (Ep-1PNA367), strain Ep-1PNA367 was labeled with the hygromycin B resistance gene (*hph*) using *Agrobacterium*-mediated transformation as described by Li et al. (42). The transmission test between DT-8 and labeled Ep-1PNA367 (named strain Ep-1PNA367^R) was conducted using the method described by Zhang et al. (40).

Data Analyses. Statistical analysis of experimental data were performed using ANOVA and the SAS program. Treatment means were compared using least significant difference (LSD) at a probability of 5%.

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