Extracellular transmission of a DNA mycovirus and its use as a natural fungicide

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Mycoviruses are thought not to be infectious as free particles and to lack an extracellular phase in their life cycles, limiting the broad use of hypovirulence-associated mycoviruses in controlling fungal disease. Here, we demonstrate that purified particles of a DNA mycovirus, *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1), are infectious when applied extracellularly to its host *Sclerotinia sclerotiorum*. Virus particles isolated from an infected host can infect the hyphae of virus-free *S. sclerotiorum* directly when applied to hyphae grown on potato dextrose agar or sprayed on leaves of *Arabidopsis thaliana* and *Brassica napus*, regardless of vegetative compatibility affiliation. When applied to leaves, the virus can suppress the development of lesions. SsHADV-1 can also reduce disease severity and enhance rapeseed yield significantly under field conditions. SsHADV-1 has a narrow host range; it can infect *Sclerotinia minor* and *Sclerotinia nivalis*, sister species of *S. sclerotiorum*, and cause debilitation of these two fungi, but cannot infect or transfect other tested fungi, such as *Botrytis cinerea*, which shares the same family with *S. sclerotiorum*. Virus particles are likely to be very stable on the leaves of *A. thaliana* plants because viral DNA could be detected at 15 d postinoculation on unwounded leaves and at 10 d postinoculation on wounded leaves, respectively; however, this virus could not infect and move in plant cells. Our findings may prompt a reconsideration of the generalization that mycoviruses lack an extracellular phase in their life cycles and stimulate the search for other DNA mycoviruses with potential use as natural fungicides.

single-stranded DNA virus | biological control | crop fungal disease | rapeseed stem rot

Mycoviruses, also known as fungal viruses, are widespread throughout the major taxonomic groups of fungi (1). Most mycoviruses have RNA genomes, either double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA); one exception is the recently reported single-stranded circular DNA mycovirus Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) (2). Although many mycoviruses may not have any significant impact on their hosts, some may cause hypovirulence in plant pathogenic fungi (3–5). Examples of hypovirulence-associated fungal viruses that were used to control plant fungal diseases include the hypoviruses that were successfully used to control chestnut blight in Europe (2–7) and Rosellinia necatrix megabirnavirus 1 from the white root rot fungus *Rosellinia necatrix*, which showed significant potential for biological control (8).

Although the first report on mycoviruses was published in the 1960s (9), our knowledge about the biology of mycoviruses is still very limited compared with animal and plant viruses. Possible reasons for this situation are the paucity of information on the infection process and means of transmission of mycoviruses (4). Generally, mycoviruses are considered to lack infectivity as free particles, and they have been described as noninfectious or endogenous viruses (4, 10, 11). It is thought that mycoviruses lack an extracellular phase in their multiplication cycles, and viral transmission is generally limited to the intracellular routes, by hyphal anastomosis horizontally and via spores vertically (12). The rigid cell wall of fungi is thought to serve as a structural barrier against the uptake of virus particles from extracellular sources because protoplasts, competent cells, or cells during the sexual reproduction stage of *Saccharomyces cerevisiae* could be infected by purified virus particles (13). Recently, protoplasts of several fungi have been successfully transfected with purified virus particles mediated by PEG, and through such studies, it was possible to extend the host range of some mycoviruses (2, 8, 14–16). Nevertheless, there are no known examples of purified mycovirus particles that can infect intact fungal hyphae.

Sclerotinia sclerotiorum is a worldwide destructive fungal pathogen; it attacks more than 450 species and subspecies of plants, including many economically important crops, such as rapeseed, soybean, sunflower, and numerous vegetables (17). Previously, we isolated and characterized an ssDNA mycovirus, SsHADV-1, from the hypovirulent strain DT-8. We presented convincing evidence that SsHADV-1, which is phylogenetically related to geminiviruses, is the causal agent of hypovirulence in its host. In contrast to geminiviruses whose purified particles and viral ssDNA are not infectious (18), the purified virus particles and genomic DNA of SsHADV-1 are infectious when mixed with protoplasts of *S. sclerotiorum* in the presence of PEG (2). Also unlike geminiviruses, which could be transmitted by insect vectors, there are no known vectors for SsHADV-1. Furthermore, we found that this virus was surprisingly easy to transmit from strain DT-8 to strains belonging to other vegetative compatibility groups (VCGs), suggesting that this DNA mycovirus may have a different way to initiate infection of its host than that of known RNA mycoviruses and geminiviruses. In the present study, we explore the infectivity of SsHADV-1 particles toward *S. sclerotiorum* hyphae and the ability of SsHADV-1 to protect plants against *S. sclerotiorum* infection and damage. Here we demonstrated that SsHADV-1 can be transmitted extracellularly and holds the potential for use in biological control of *S. sclerotiorum* diseases.

Results

Extracellular Transmission of SsHADV-1. To determine the infectivity of cell-free purified virus particles, virus particles were mixed directly with either *S. sclerotiorum* protoplasts or hyphal

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fragments of strain Ep-1PNA367 in the absence of PEG. The results showed that most of the colonies from the regeneration medium (RM) plates were indeed infected with SsHADV-1 (Fig. S1). When virus particles were added to the potato dextrose agar (PDA) medium surrounding a young virus-free colony of *S. sclerotiorum*, and further incubated for 2–3 d, sectors from the virus-inoculated colony were observed (Fig. 1*A*). Mycelial agar plugs were taken from the sectored region and subcultured on fresh PDA plates, and the new colonies, although different in color, showed largely similar morphological features to those of the naturally infected strain DT-8 (Fig. 1*B*). These colonies were infected with SsHADV-1, as demonstrated by total DNA extraction and agarose gel electrophoresis analysis (Fig. 1*C*). Successful infection was further confirmed by viral transmission assays and virulence tests. Successful infection of intact hyphae on PDA plate occurred even when the concentration of the virus particles in the inoculum was 0.05 mg/mL. Viral DNA extracted from particles was used instead of whole particles to inoculate intact hyphae, hyphal fragments, or protoplasts of *S. sclerotiorum* under the same condition, but no infection was detected. Thus, virus particles of SsHADV-1 are highly infectious when applied extracellularly and can directly infect *S. sclerotiorum* hyphae, and whole virus particles are required for infection.

Early reports indicated that vegetative incompatibility of *S. sclerotiorum* in the field is very complicated because many VCGs may occur in the same field (19). Four strains belonging to different VCGs, which were isolated from the same rapeseed field, were confirmed by dual culturing on PDA (Fig. S2*A*) and then used instead of strain Ep-1PNA367 to test the infectivity of virus both on PDA plates and on leaves of *Arabidopsis thaliana* plants. The results showed that SsHADV-1 can infect all *S. sclerotiorum* isolates tested, and suppressed the extension of lesions on *A. thaliana* leaves (Fig. S2 *B* and *C*). All infected strains exhibited weakened pathogenicity (Fig. S3). Thus, SsHADV-1 can infect naturally occurring host isolates other than strains DT-8 and Ep-1PNA367, regardless of their VCG affiliation.

SsHADV-1 Inhibits Plant Infection by *S. sclerotiorum*. Virus particles have a protective activity against attack by *S. sclerotiorum*. When virus particles were spread on the leaves of *A. thaliana* followed by inoculation with *S. sclerotiorum*, only small or no lesions were observed on the virus particles-treated leaves. None of the virus-treated plants were killed in 6 d or longer, but all PBS buffer-treated plants were killed in 6 d (Fig. 2*A*; Fig. S4). When the virus preparation was adjusted to 100–500 μg/mL, disease suppression was still notable, although some infected leaves could be observed (Fig. 2*A*). Virus-infected cultures could be isolated from the atypical lesions induced on intact plants or detached leaves (Fig. S5), which indicated that conversion of *S. sclerotiorum* from virulent to hypovirulent by SsHADV-1 was the reason for inhibiting infection.

SsHADV-1 Suppresses Expansion of Lesions Induced by *S. sclerotiorum*. A crude preparation of virus particles has a therapeutic activity against the expansion of lesions by *S. sclerotiorum*. At 4 d post-inoculation (dpi; 2 d post-virus treatment), lesions on leaves of virus-treated plants expanded very slowly, or ceased to expand, and the lesion edge often turned yellow and black. Lesions on PBS buffer-treated plants, however, expanded quickly, and intense fungal hyphae could be observed. At 8 dpi, pathogen growth extended to the stem of PBS buffer-treated plants, and half of the plants collapsed. Although pathogen growth might rarely extend to the stem of virus-treated plants, only small lesions on the stem could be observed, and all plants remained standing and were not killed (Fig. 2*B*). Usually, the inoculated leaves of virus-treated plants were weakened (Fig. 2*B*) and most of them were detached from stem. At 15 dpi, only three of 16 virus-treated plants were killed by *S. sclerotiorum*, but all PBS-treated plants were killed. The therapeutic activity of SsHADV-1 is comparable to that of Carbendazim, a widely used fungicide. At 15 dpi, only two of eight Carbendazim (100 ppm)-treated plants were killed. Additionally, mycelia from some germinated sclerotia, collected from virus-treated plants, were found to contain virus.

Under field conditions, ascospores are released from apothecia during rapeseed's flowering stage, and infect petals to initiate the primary infection; the infected petals, when dropped on leaves and stems, lead to secondary infection. We found that if leaves of rapeseed were pretreated with virus particles, no lesions were induced through contact with the infected petals (Fig. S6). Thus, SsHADV-1 can protect plants by preventing the secondary infection. Field trials further revealed that SsHADV-1 could reduce both the incidence and severity of stem rot of rapeseed, and improve seed yield significantly (Table 1). The hyphal fragments suspension as a virus source, with or without amendment with UV protectors, showed strong activity against the attack by *S. sclerotiorum*, except when amended with isoflavone. When the hyphal

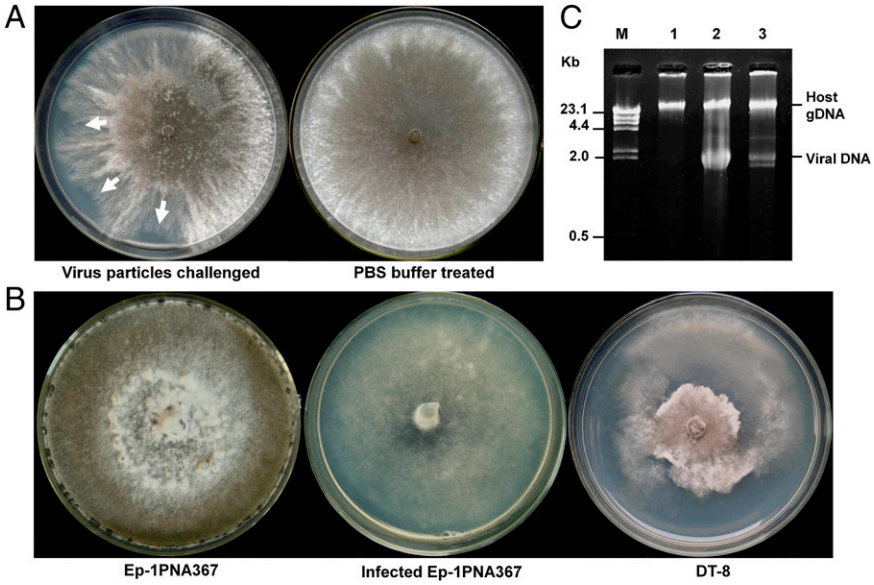

Fig. 1. Infection of *S. sclerotiorum* with purified particles of SsHADV-1 following extracellular application to intact hyphae on PDA plates. (*A*) Sectoring in a colony of strain Ep-1PNA367 could be observed at 2 dpi; sectors are indicated by arrowheads. Strain Ep-1PNA367 was grown on a PDA plate for 1 d, and then virus particles were added to the PDA medium around the margin of the young colony, but not to contact it. PBS buffer was used as a control. The photographs were taken at 4 dpi. (*B*) Colony morphology of newly infected strain Ep-1PNA367; colony morphology of the virus-free stain Ep-1PNA367 and the virus-infected strain DT-8 are also shown. All cultures were incubated on PDA medium for 7 d at 20 °C. (*C*) Electrophoretic profiles of genomic DNA extracted from the cultures shown in *B* are shown. DNA samples were fractionated on 1.0% agarose gel; the positions of host genomic DNA and viral DNA are indicated to the right. Lane M: λ-Hind III-digested DNA marker. Lanes 1–3, DNA samples of isolates Ep-1PNA367, virus infected Ep-PNA367, and strain DT-8, respectively.

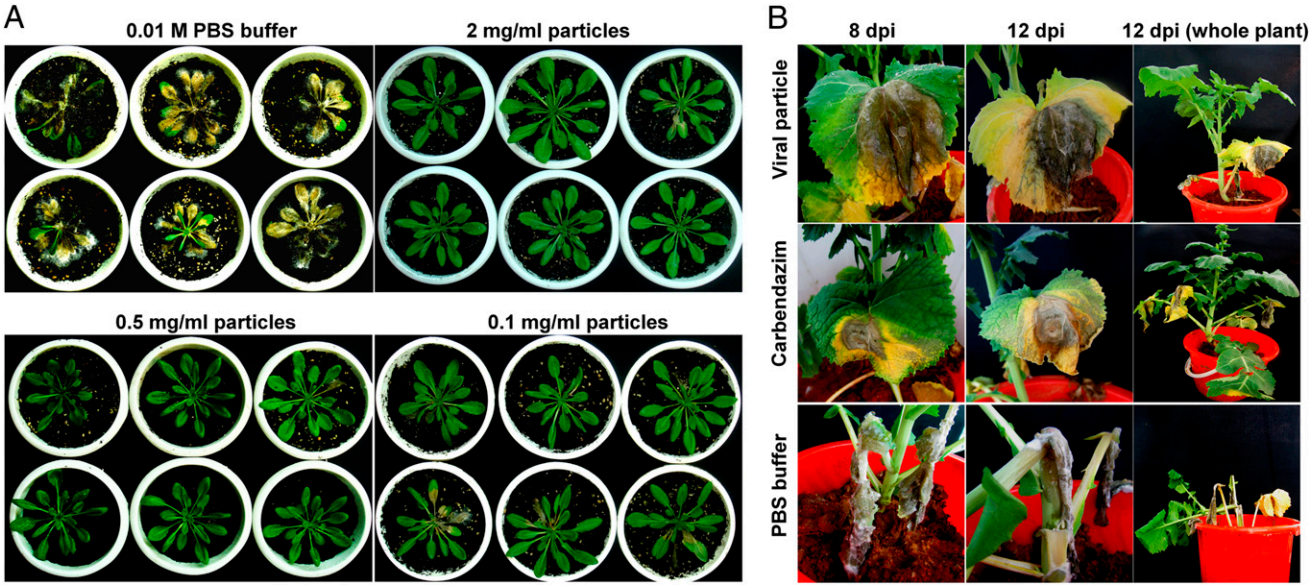

Fig. 2. Protection of plants against *S. sclerotiorum* using SsHADV-1 particles. (*A*) Virus particles prevented *S. sclerotiorum* from infecting and killing plants of *A. thaliana*. Viral particles at different concentrations were spread on leaves (only one leaf was sprayed for each plant), and mycelial agar plugs were placed on the treated leaves. The fungal pathogen-inoculated plants were kept in an incubator with high humidity (100%) at 20 °C, and photographs were taken at 6 dpi. (*B*) Therapeutic activity of SsHADV-1 against the attack of *S. sclerotiorum*. Crude preparation of virus particles inhibited the expansion of *S. sclerotiorum* on rapeseed plants and cured the lesions. *S. sclerotiorum* mycelial agar plugs were used to inoculate plant leaves, leaves were incubated for 2 d for lesion development, and then viral particles preparation, Carbendazim (100 ppm) or PBS buffer, was sprayed on plants at 2 dpi and 3 dpi, respectively. Three leaves were inoculated for each plant. Plants were maintained in an incubator with high humidity (100%) at 20 °C, and photographs were taken at 8 and 12 dpi.

fragments suspension was amended with isoflavone, no protective ability could be observed (Table 1). The biocontrol efficiency of SsHADV-1 is comparable to that of Prochloraz manganese (500 ppm; Table 1).

Extracellular Transmission of SsHADV-1 to Different *Sclerotinia* Species. Virus particles of SsHADV-1 were mixed directly with protoplasts of strain 3-4-1 of *Sclerotinia minor* and strain Let-19 of *Sclerotinia nivalis*, two sister species of *S. sclerotiorum*, respectively. Protoplast-regenerated cultures grew on PDA slowly and formed abnormal colony morphologies, and viral DNA was extracted from these abnormal cultures (Fig. 3). Thus, SsHADV-1 can infect these two fungi. However, virus particles of SsHADV-1 cannot infect other tested fungi, such as *Botrytis cinerea*—which, like *S. sclerotiorum*, is a member of the family *Sclerotiniaceae*—rice blast fungus *Magnaporthe oryzae*, and a sclerotial parasite *Coniothyrium minitans*, PEG-mediated transfection also failed. These results indicated that SsHADV-1 has a narrow host range and therefore should not present any biological safety concerns to other organisms when used as a biological control agent.

Stability and Biosafety of SsHADV-1. In another set of experiments, virus particles were spread on leaves of *A. thaliana* with either a brush only or with a brush and quartz sands. A PCR amplification assay was then used for detection of viral DNA in the inoculated and noninoculated leaves at different time points. Results showed that viral DNA could be detected at 15 dpi on unwounded leaves and at 10 dpi on wounded leaves, respectively (Fig. 4*A*). Thus, SsHADV-1 is apparently very stable on plant leaves. No viral DNA could be amplified from noninoculated leaves or newly emerging leaves on the virus-inoculated plants (Fig. 4*A*), suggesting that SsHADV-1 cannot infect *A. thaliana*. In situ hybridization tests using a fluorescent probe confirmed that SsHADV-1 did not replicate in plant cells (Fig. 4*B*).

Discussion

Although hypovirulence-associated mycoviruses are attractive as potential biological control agents against plant fungal diseases, the efficiency of virocontrol is not always satisfactory (4). The transmission of mycoviruses among host isolates is considerably limited by complicated vegetative incompatibility systems in a certain fungal population and also limited by field fitness of virus-infected strains (20, 21). Our present findings suggest that SsHADV-1 can be applied to plants at appropriate times as a protective treatment or therapeutic agent (i.e., a natural fungicide) against *Sclerotinia* diseases, thus avoiding the transmission and dissemination problems

Table 1. Efficacy of SsHADV-1 particles preparations for suppression of *Sclerotinia* stem rot of rapeseed under field conditions

Treatment	Disease incidence, %	Disease severity	Average yield, kg/12 M^2	Yield improvement, %
Virus particle suspension only	43.43 ± 5.51^{c}	22.57 ± 4.87^{c}	8.3 ± 0.28^{ab}	13.70
Virus particle suspension plus starch	44.91 ± 4.32^{bc}	21.43 ± 3.40^{c}	8.5 ± 0.27^{a}	16.44
Virus particle suspension plus isoflavone	55.26 ± 3.21^{ab}	30.23 ± 1.52^{ab}	7.38 ± 0.25^{c}	1.1
Virus particle suspension plus folacin	43.66 ± 6.67^{bc}	23.90 ± 4.55^{bc}	7.9 ± 0.22^{b}	8.2
Prochloraz manganese	39.81 ± 7.37^{c}	19.49 ± 3.25^{c}	8.5 ± 0.18^{a}	16.44
Control	65.37 ± 5.01^{a}	33.37 ± 3.50^{a}	7.3 ± 0.18^{c}	

Means followed by the same letters within each column are not significantly different at the $P < 0.05$ level of confidence according to Duncan's multiple range test.

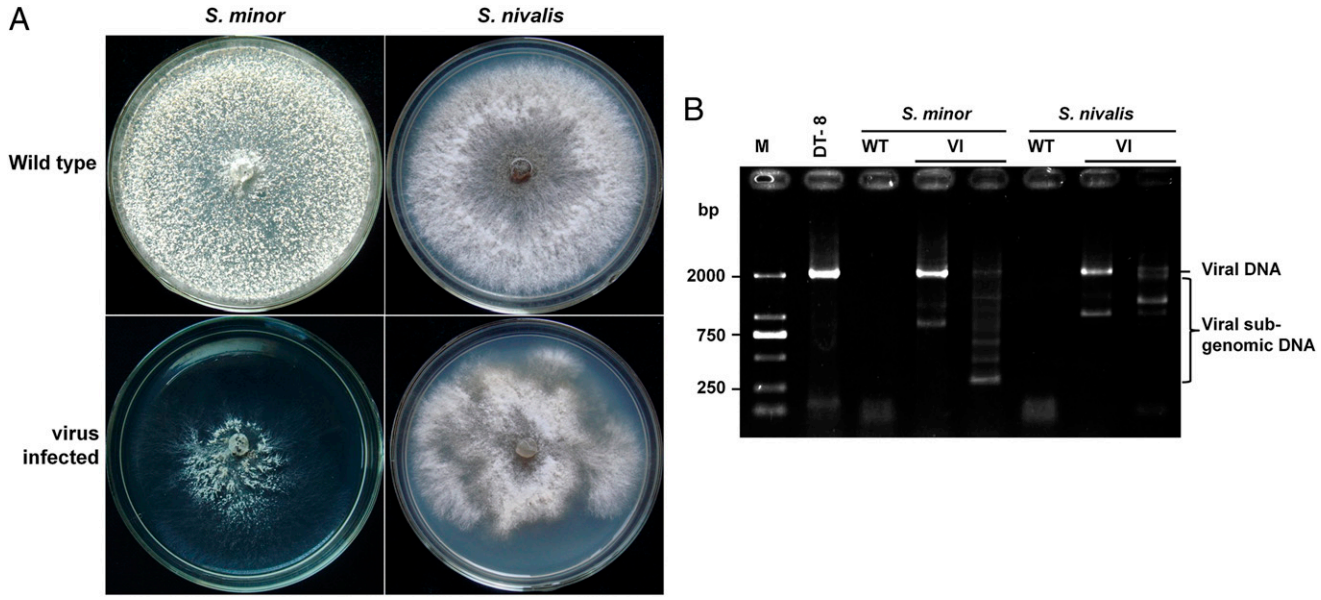

Fig. 3. Infection of protoplasts from two *Sclerotinia* species (*S. minor* and *S. nivalis*) closely related to *S. sclerotiorum* with purified SsHADV1 particles. (*A*) Colony morphology of virus-infected and wild-type strains of *S. minor* and *S. nivalis*. Strains were incubated at 20 °C for 5 d. (*B*) Viral genomic DNA was extracted from newly infected strain 3-4-1 of *S. minor* and strain Let-19 of *S. nivalis*. Viral genomic DNA is indicated. Lane M: DL2000 DNA marker.

caused by fungal host vegetative incompatibility and the fitness of virus-infected strains under field conditions.

SsHADV-1 can replicate and accumulate to high levels in its host, and virus concentrations of ∼1 mg/g of fresh mycelia can be reached using 10-d potato dextrose broth (PDB) cultures of strain DT-8. Thus, it is feasible to produce virus particles in quantities required for field application if an efficient method for virus production is developed. It should be noted that protective agents are required as amendments for commercial use to ensure particle infectivity is maintained for the desired duration. The narrow host range property of SsHADV-1 ensures the biosafety for field use. These features of SsHADV-1 point to a much-anticipated promise of application of mycoviruses for biological control of *Sclerotinia* diseases. However, there is much more work to be done before this virus could be used as a practical means to control plant diseases, such as improving virus stability during production, transportation, and application onto plant surfaces, and discerning the optimum time for virus delivery based on epidemiological studies of *Sclerotinia* diseases.

Our experimental results suggest that virus particles of SsHADV-1 are required for successful infection of intact hyphae or protoplasts of *S. sclerotiorum* because naked viral DNA is not infectious. Future work will explore the mechanism underlying SsHADV-1 entry into fungal cells. In this regard, detailed structural

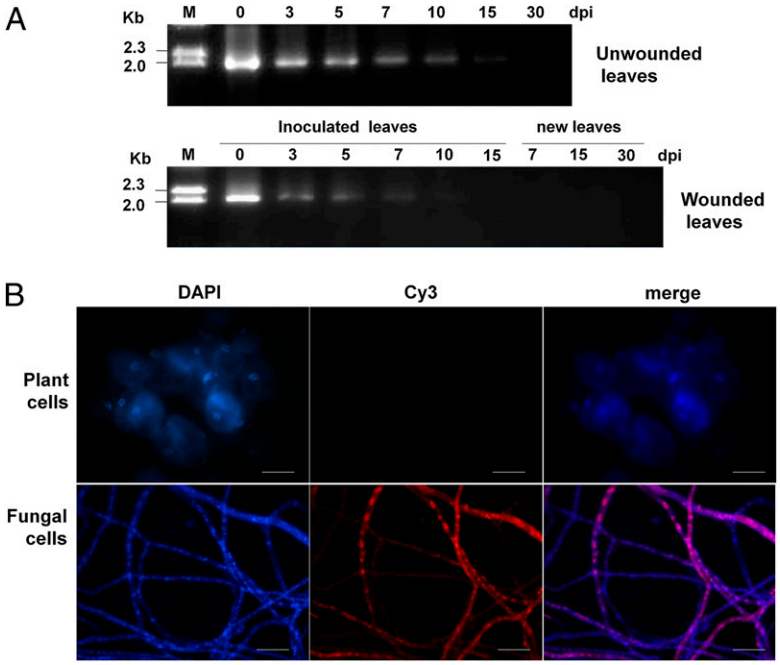

Fig. 4. Detection of SsHADV-1 on plant leaves and in plant cells. (*A*) Detection of SsHADV-1 on leaves of *A. thaliana* using specific primers designed based on viral sequence. Viral particles were spread on leaves with a brush or with a brush and quartz sand. After 0, 3, 5, 7, 10, 15, and 30 d, inoculated leaves, noninoculated leaves, and newly emerged leaves on inoculated plants were collected and subjected to total DNA extraction for PCR amplification of viral genomic DNA. (*B*) FISH analysis of SsHADV-1 replication in cells of inoculated leaves of *A. thaliana*. Fluorescence photomicrographs of plant cells and virus-infected fungal compartments of strain Ep-1PNA367 after FISH with biotinylated *CP* probes. Hybridization signals of the *CP* probe were detected using Cy3-labeled streptavidin. DAPI staining was used as a control. The fluorescence in plant cells or fungal compartments was examined with a fluorescence microscope (Nikon 80i); the blue signals were examined at 360 nm and the red signals excitation at 550 nm. (Scale bar: 20 μm.)

studies of virus particles, including electron cryomicroscopy and 3D image reconstruction at high resolution, may be necessary to reveal surface structures (fibers, attachment components) that may allow the particles to attach to the hyphae and eventually gain access to the inside of the hyphal compartments. Examination of the mechanisms used by viruses that breach the rigid cell walls of their host cells to gain entry (plant and bacterial viruses) reveals that entry in case of plant viruses is vector (insect) mediated or is due to mechanical damage to the cell wall that briefly breaches the plasma membrane of underlying cell. Bacterial viruses, however, have virion-associated attachment and penetration components, thus entry is receptor mediated. Therefore, it may be possible, but less likely, that SsHADV-1 DNA is brought into the fungal cell from externally attached particles. Mycoviruses are not infectious in the classic sense because infection cannot be initiated by exposing uninfected hyphae to purified particles (1, 11). This study demonstrates an exception and shows that SsHADV-1 particles can infect *S. sclerotiorum* via extracellular routes. This finding, when supported by discovery of additional infectious mycoviruses with demonstrated protective/therapeutic activities, may expand our understanding of the biological features of mycoviruses and contribute to progress in fungal virology.

Natural transmission of SsHADV-1 by insects or other vectors that feed on virus-infected fungi is conceivable considering the stability and the high virus concentration in infected fungal cells, but this has yet to be demonstrated for mycoviruses. It is of interest in this regard that SsHADV-1–like viruses were detected in different species of dragonflies, the best-known insect predators that accumulate viruses from their insect prey (22). Furthermore, many SsHADV-1–like viruses were recently found in other ecological niches (23–26). Surprisingly, abundant viruses with high similarity to SsHADV-1 were identified in the near-surface atmosphere and were proposed to be of fungal origin (27). Moreover, SsHADV-1 Rep gene-like sequences have been found integrated in the genomes of many fungi, including the rice blast fungus *Magnaporthe oryzae* and the mushrooms *Tuber melanosporum* and *Laccaria bicolor* (28). Taken together, these findings suggest that extracellular transmission of SsHADV-1–like DNA mycoviruses is plausible and that their host range may not be limited to *S. sclerotiorum* but include a wider range of other fungi. Thus, our results should also prompt the search for infectious DNA mycoviruses in other important plant pathogenic fungi.

Materials and Methods

Fungal Strains and Culture Media. The SsHADV-1–infected *S. sclerotiorum* strain DT-8, strain Ep-1PNA367, strain 3-4-1 of *S. minor*, and strain Let-19 of *S. nivalis* were previously described (2, 29, 30). Strains RL6, RL19, RL26, and RL29 are wild-type virus-free strains isolated from the same field in Hubei Province, China. All strains were grown on PDA plates at 20 °C, and stored on PDA slants at 4 °C; sclerotia produced on PDA plates were collected, air-dried, and stored at −20 °C. PDA amended with 75 μL/L of McCormick's red food coloring was used for vegetative compatibility tests (31). Strain HGYW-45, a virulent wild-type strain, was used to induce carpogenic germination of sclerotia and obtain ascospores according to the protocol described by Zhang et al. (32).

Preparation of Virus Particles. Virus particles were prepared using sucrose density gradient centrifugation as described previously (33). The purified virus particles were suspended in 0.01 M phosphate buffer (pH 7.0); concentration was adjusted to ∼2 mg/mL with a BioPhotometer (Eppendorf), filtrated through Millex-GP filter units (Millipore), and used in the infectivity assays. Virus particle concentration was ∼1 mg/g of 10-d-old hyphae.

Preparation of Fungal Protoplasts and Hyphal Fragments. Protoplasts of strain Ep-1PNA367 were prepared following the method described by Zhang et al. (32) with minor modification. Protoplasts were adjusted at a concentration of 1×10^7 protoplasts/mL. To obtain hyphal fragments, strains Ep-1PNA367, RL6, RL19, RL26, and RL29 were individually grown on cellophane membranes on top of PDA plates at 20 °C for 1 d. The mycelia were collected and ground with a mortar and pestle either with or without quartz sand with 1 mL sterile distilled water. Ground hyphae were washed twice with STC buffer (1.2 M sorbitol, 10 mM Tris·HCl, pH 7.5, 10 mM $CaCl_2$) and recovered using low-speed centrifugation.

Infectivity Assay of SsHADV-1. To determine the infectivity of virus particles, protoplasts suspension (100 μL) or hyphal fragments were gently mixed with purified virus particles (10 μL; 2 mg/mL). The mixture was incubated on ice for 30 min and then placed at room temperature for additional 30 min. The virus-treated protoplasts or hyphal fragments were regenerated at 20 °C for 3–4 d in regeneration medium to allow protoplasts to germinate and the generated hyphae to grow. Protoplasts of *S. minor*, *S. nivalis*, *B. cinerea*, sclerotial parasite *Coniothyrium minitans*, and rice blast fungus *M. oryzae* were also prepared and used in this infectivity assay.

To detect if virus particles can infect intact hyphae directly, mycelial agar plugs of *S. sclerotiorum* strain Ep-1PNA367 were grown on PDA at 20 °C for 1 d in 150 mm-diameter plates, and then virus particles were added at the periphery of 1-d-old colonies. Four days later, mycelial agar plugs were taken from sectors that developed on the inoculated colonies. These experiments were repeated three times, respectively.

Viral genomic DNA (2 mg/mL) was used to instead of virus particles to test if naked viral DNA can infect intact hyphae or protoplasts of *S. sclerotiorum*.

Viral Protection Ability Test. To explore the protection ability of virus particles against attack by *S. sclerotiorum*, purified virus particles were applied to plant leaves of 6-wk-old *A. thaliana* plants, Columbia ecotype, and then the treated leaves were inoculated with fungal pathogen. Briefly, 5 μL of a virus particle solution with different concentrations or 5 μL 0.01 M phosphate buffer (pH 7.0) were spread on *A. thaliana* leaves, and then mycelial agar plugs from actively growing colony margins of *S. sclerotiorum* strains were placed on virus-treated leaves with the mycelial side facing the leaf surface. The inoculated leaves were placed in an incubator at 20 °C and 100% relative humidity (RH) for further studies. Photographs were taken 48 h post-inoculation (hpi) for detached leaves and 6 dpi for intact plants. Both experiments were repeated at least three times, and at least three samples were used for each individual test.

To explore whether virus particles could inhibit a key step in the *S. sclerotiorum* infection process, another growth-chamber experiment was conducted. Detached leaves and petals were collected from rapeseed plants (cultivar FuYou 668) in an experimental field, leaves were placed on a moist paper towels in plastic trays, and then 10 μL (2 mg/mL) of virus particle suspension or 0.01 M phosphate buffer (pH 7.0) was added as droplets to leaf surfaces. Petals were washed twice in sterile distilled water and then placed on droplets of virus particle suspension. To inoculate with ascospores, an aliquot of 20-μL ascospores suspension at a concentration of 1×10^6 ascospores/mL from strain HGYW-45 was inoculated onto the center of the upside of petals. There were six replicates for each treatment. Trays were covered with plastic wrap and incubated in the growth chamber under fluorescent light at 20 °C with 100% RH. Photographs were taken 4 dpi. The experiment was repeated three times.

Crude preparations of virus particles were made to explore the therapeutic activity of SsHADV-1. SsHADV-1–infected strain Ep-1PNA367 was grown in PDB for 2 wk. The resulting mycelium was collected and freeze-dried, and ∼15-g samples of dried mycelia were obtained and ground to powder. The powdered homogenates were mixed with 120 mL of PBS buffer and shaken at 100 rpm for 2 h at 10 °C. After 3,500 × *g* centrifugation for 20 min, the supernatant was recentrifuged at 12,000 × *g* for 20 min, and the final resultant supernatant (∼80 mL) was collected and prepared for use. Rapeseed plants (cultivar FuYou 668) in late seedling stage were inoculated with mycelial agar discs of *S. sclerotiorum* for 2 d to allow lesion formation. Then, 40 mL of crude virus preparation or an equal volume of 100 ppm Carbendazim (Sigma) or PBS buffer only was sprayed onto the inoculated plants. The same number of sprays was repeated one additional time. There were 16 plants for crude virus particles treatment, eight plants for 100 ppm Carbendazim as positive controls, and eight plants for PBS buffer as negative controls. All treated plants were grown in a growth chamber under fluorescent light at 20 °C with 100% RH. Photographs were taken at 8 and 12 dpi, and the number of lodged rapeseed plants was calculated at 8, 10, 12, and 15 dpi. The experiment was performed at least three times, and repeated one time with tobacco plants.

Field Trials. Field trials were conducted at Huzhu, Qinghai Province, China, to determine the potential for controlling *Sclerotinia* stem rot of rapeseed. In fields, plants naturally infected by *S. sclerotiorum* were used. Two well-separated fields were chosen as repeats. Each field was divided into 24 plots,

and the size of each plot was 3 × 4 m (width × length). SsHADV-1–infected *S. sclerotiorum* strain Ep-1PNA367 was incubated in PDB in a 250-mL flask at 20 °C and shaken at 200 rpm for 7 d; the resulting mycelium was homogenized with a blender. The hyphal fragment suspension was diluted to 2.0 OD_{600} units (~1 × 10^5 cfu/mL) with distilled water. The hyphal fragment suspension was amended with UV protectors, such as 0.5% starch, 0.2% isoflavone, or 0.5% folacin, or without any amendments. Hyphal fragment suspensions were applied by spraying the suspensions with a hand sprayer onto aerial parts of rapeseed plants twice at 7-d intervals during the flowering stage. Water and a fungicide (Prochloraz manganese, 500 ppm) were used as negative and positive controls, respectively. Each treatment took up four plots and was distributed randomly in each field. At the maturity stage of rapeseed, the disease incidence, severity, and yield of all rapeseed plants in each plot were assessed. Disease incidence was defined as the percentage of infected plants, and disease severity of each plant was rated on a scale of 0 to 4 according to Li et al. (34).

Stability Test of SsHADV-1. A stability test of SsHADV-1 was carried out on leaves of 4-wk-old plants of *A. thaliana* Columbia ecotype. Virus particles solution (5 μL) was spread on leaf surfaces of living plants with a sterilized brush directly or with quartz sand and a sterilized brush. The virus-inoculated plants were placed in an incubator at 20 °C and 100% RH. The virus-inoculated leaves were collected at 0, 3, 5, 7, 10, and 15 dpi; the noninoculated leaves and newly emerging leaves were collected at 7, 15, and 30 dpi. All collected leaves were ground in liquid nitrogen immediately and subjected to a DNA extraction procedure. The intact viral genome DNA was detected with PCR amplification using a Large intergenic region forward primer (LIRFP) (5′ CCAGGGACGGGGACACTTC 3′) and a Large intergenic region reverse primer (LIRRP) (5′ GCCCAGAGACTCAA AGGGACAAC 3′).

Detection of SsHADV-1 in Plant Cells by Fluorescence in Situ Hybridization. FISH was conducted to assess whether SsHADV-1 could replicate in *A. thaliana* cells. Virus particles were spread on leaf surfaces of 6-wk-old *A. thaliana* with quartz sand and a brush. The treated leaves, collected at 7 dpi, were subjected to FISH analysis. A DNA fragment of viral *CP* was fluorescently labeled with Biotin-14-dCTP by the random primer method (BioPrime DNA Labeling System; Invitrogen) and used as a probe. In situ hybridization was carried out according to the protocol by Leitch et al. (35). Detection of virus in the hyphae of virus-infected strain Ep-1PNA367 was conducted as described by Baschien et al. (36), and used as a positive control. Hybridization signals of the *CP* probe were detected using Cy3-labeled streptavidin (Sigma). The fluorescence in plant cells or fungal cells was examined with a fluorescence microscope (Nikon 80i), and a DAPI or TRITC filter was used to select DAPI or Cy3 fluorescence, respectively. Images were captured with a Digital Sight DS-5 M digital camera using the soft NIS-Elements F Package Ver 3.0 (Nikon).

Nucleic Acid Extraction and PCR Confirmation. DNA extraction was conducted according to the procedures of Sambrook et al. (37). Viral DNA was confirmed by PCR amplification using a specific primer pair that was designed based on the *Rep* sequence (Rep-FP 5′ GTCACCACCCAAACATTACAAAG 3′ and Rep-RP 5′ AGCGTATTCCACGTCAGGTGC 3′) or the *CP* sequence (CP-FP: 5′ GGAGCATCCTCAACACGCATC 3′ and CP-RP: 5′ TACGAAGA AGGTCGGACGCC 3′).

Data Analysis. Statistical analyses of experimental data were performed using ANOVA and the SAS program. Treatment means were compared using least-significant difference at a probability of 5%.

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