Fungal DNA virus infects a mycophagous insect and utilizes it as a transmission vector

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Mycoviruses are usually transmitted horizontally via hyphal anastomosis and vertically via sexual/asexual spores. Previously, we reported that a gemycircularvirus, *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1), could infect its fungal host extracellularly. Here, we discovered that SsHADV-1 could infect a mycophagous insect, *Lycoriella ingenua*, and use it as a transmission vector. Virus acquired by larvae feeding on colonies of a virus-infected strain of *S. sclerotiorum* was replicated and retained in larvae, pupae, adults, and eggs. Virus could be transmitted to insect offspring when larvae were injected with virus particles and allowed to feed on a non-host fungus. Virus replication in insect cells was further confirmed by inoculating *Spodoptera frugiperda* cells with virus particles and analyzing with RT-PCR, Northern blot, immunofluorescence, and flow cytometry assays. Larvae could transmit virus once they acquired virus by feeding on virus-infected fungal colony. Offspring larvae hatched from viruliferous eggs were virus carriers and could also successfully transmit virus. Virus transmission between insect and fungus also occurred on rapeseed plants. Virus-infected isolates produced less repellent volatile substances to attract adults of *L. ingenua*. Furthermore, *L. ingenua* was easily observed on *Sclerotinia* lesions in rapeseed fields, and viruliferous adults were captured from fields either sprayed with a virus-infected fungal strain or nonsprayed. Our findings may facilitate the exploration of mycoviruses for control of fungal diseases and enhance our understanding of the ecology of SsHADV-1 and other newly emerging SsHADV-1–like viruses, which were recently found to be widespread in various niches including human HIV-infected blood, human and animal feces, insects, plants, and even sewage.

mycovirus | biological control | mycophagous insect | mutualism | gemycircularvirus

Mycoviruses infect fungi and replicate in fungal cells that are widespread in all major fungal groups (1). As a part of the virus world, mycoviruses may play an ecologically important role in nature (2). Furthermore, hypovirulence-associated mycoviruses that infect fungal plant pathogens may have potential to control plant diseases (1, 3–5). Moreover, mycoviruses could potentially be explored to control human fungal diseases (6). Mycoviruses are also very important toward an understanding of the diversity, evolution, and ecology of viruses; they are often found to be phylogenetically related to plant and animal viruses (7, 8), and some newly discovered mycoviruses showed linkages between dsRNA and ssRNA viruses (9–11). Chiba and Suzuki found that one mycovirus could interfere with the replication of an unrelated mycovirus (12). More recently, Zhang et al. discovered that a capsid protein encoded by a dsRNA mycovirus could package a capsidless single-stranded (+) RNA virus genome (13). Mycoviruses are thought to lack an extracellular phase in their lifecycles, and some mycoviruses are associated with latent infections (1) and are only transmitted horizontally via hyphal anastomosis between vegetatively compatible individuals or transmitted vertically through asexual and/or sexual spores (1). Several studies implied the presence of vector organisms for mycoviruses in nature (14–16); *Thyreophagus corticalis* (Acari, Acaridae) was found to be involved in the spread of Cryphonectria parasitica hypovirus 1 by carrying undigested virus-infected hyphae (17). However, possible transmission of mycoviruses via vectors still needs to be carefully investigated.

Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) is a circular single-stranded DNA virus with a genome of 2,166 nt, and its particles are isometric with a diameter of 20–22 nm (18). This virus was originally isolated from a hypovirulent strain of *S. sclerotiorum*, a fungal plant pathogen. After similar ssDNA viruses were detected in dragonflies, SsHADV-1 and SsHADV-1–like viruses were grouped together in a new genus designated *Gemycircularvirus* (19). Surprisingly, gemycircularviruses are exceptionally widespread in nature. The DNA of SsHADV-1–like viruses was detected in a microbial community from the Sargasso Sea (18) and was found in fecal samples of unexplained human diarrhea (20) and in various animal feces (21–23), insects (such as mosquitoes, dragonflies, and damselflies) (19, 24–26), and plants (27). SsHADV-1–like viral DNA was found in human brain and serum samples from multiple sclerosis patients (28), in human HIV-positive blood (29), in the cerebrospinal fluids of Sri Lankan patients with unexplained encephalitis (20), in blood samples of experimental rats (30), and in the liver and spleen of a horse with fatal idiopathic hepatopathy (31). These discoveries suggest that gemycircularviruses represent a group of emerging viruses, which are widespread in nature, and some of them may impact human and animal health.

Significance

***Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1) was originally isolated from a fungal pathogen; recent research revealed that SsHADV-1–like viruses are widespread in various niches including human tissues. Here, we discovered that SsHADV-1 could infect a mycophagous insect, *Lycoriella ingenua*, when larvae fed on virus-infected fungus; and viruliferous adults could transmit SsHADV-1 transovarially. We further found that SsHADV-1–infected fungus could suppress the production of repellent volatile substances to attract adults to lay eggs on its colony and that virus infection could stimulate female adults to produce more eggs. Our findings may facilitate the exploration of mycoviruses to control fungal diseases and suggest that insects may play an important role in the transmission and distribution of these newly emerging viruses.**

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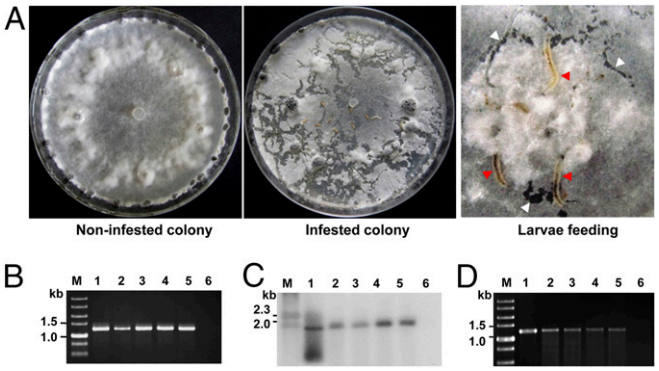

Fig. 1. *L. ingenua* feeding and virus detection. (*A*) Larvae feeding on a fungal colony in a PDA plate. Larvae (indicated by red arrowheads) feeding on hyphae and generating galleries (indicated by white arrowheads) on a colony. (*B*) PCR detection of virus in *L. ingenua*. A virus-specific DNA product could be amplified from *L. ingenua* reared on strain DT-8. The diameter of dish was 90 mm, and the width of left photograph was about 13 mm. (*C*) Southern blot analysis of virus in *L. ingenua*. (Lanes for *B* and *C*) Lane M, DNA size markers; lane 1, DNA from strain DT-8 (positive control); lanes 2–5, DNA samples from eggs, larvae, pupae, and adults reared on strain DT-8, respectively; lane 6, DNA from larvae reared on strain DT-8VF (negative control). A DIG-labeled probe comprising a DNA fragment (814 bp) that corresponds to viral genomic DNA was prepared and used to probe the Southern blots. (*D*) Virus retention time in the body of larvae, as assayed by PCR. Lane M, DNA marker; lane 1, DNA sample from strain DT-8; lanes 2–5, DNA samples from larvae starved for 1, 3, 5, and 7 d, respectively; lane 6, DNA sample from larvae reared on strain DT-8VF.

SsHADV-1 has a narrow fungal host range. It could not replicate in *Botrytis cinerea*, a closely related fungus that shares the same family with *S. sclerotiorum* (32). However, the fact that viral DNA of SsHADV-1 could be detected in urban river sediments (33) and in damselfly (26) suggests that SsHADV-1 may have hosts belonging to different kingdoms, as *S. sclerotiorum* usually does not occur in river sewage and does not naturally infect damselfly. Insects in the genus *Lycoriella* feed on fungi, and some of them, such as *Lycoriella ingenua*, are notorious pests of cultivated mushrooms (34). We observed that *L. ingenua* could also feed on mycelia of *S. sclerotiorum* in the laboratory (Fig. 1*A* and Fig. S1) and suspected that this mycophagous insect might acquire and transmit fungal viruses when fed on colonies of virus-infected fungi.

Results

Acquisition of SsHADV-1 by *L. ingenua* from a Virus-Infected Fungal Isolate. SsHADV-1–free larvae were reared on a colony of virus-free strain DT-8VF, and the emerging pupae were collected and placed on a colony of virus-infected strain DT-8. When adults emerged, the female adults laid eggs, and the newly hatched larvae, which consumed hyphae, were allowed to develop on the virus-infected colony of strain DT-8. Larvae, pupae, adults, and eggs were taken from these colonies and subjected to virus detection assays. Viral genomic DNA was PCR-amplified from larvae, pupae, adults, and eggs (Fig. 1*B*), and the sequences of the PCR products were identical to that of the SsHADV-1 genomic sequence. Southern blot analysis further confirmed that virus is present on and/or in larvae, pupae, adults, and eggs (Fig. 1*C*). Immunofluorescence detection with an antibody to viral coat protein showed that virus existed in cells of larvae, pupae, adults, and eggs (Fig. 2). Larvae, when fed on virus-infected colony, could acquire virus in a short period of acquisition feeding, as virus was detected in 5 larvae out of 10 pursuant to feeding on a colony of strain DT-8 for 30 min (Fig. S2*A*). We could not judge, however, whether the virus had already entered into insect tissue or was still retained in fungal hyphae in the insect digestive tract. Nevertheless, SsHADV-1 viral genomic DNA was detected in larvae that were starved for as long as 7 d (Fig. 1*D*).

To reduce the probability that virus detected from insect was potentially derived from contaminating virus-infected hyphal debris, either viruliferous pupae developed on a colony of strain DT-8 or virus-free larvae that were injected with virus particles suspension were placed on colonies of the nonhost fungus *B. cinerea* to generate offspring, and then the newly emerging larvae on *B. cinerea* colonies were sampled for virus detection individually using PCR amplification. Thirty nine out of 46 and 41 out of 46 of the sampled two groups of larvae (those derived from viruliferous pupae and virus-injected larvae), respectively, were found to be virus-infected (Fig. S3). Thus, *L. ingenua* could pass SsHADV-1 to its offspring.

Replication of SsHADV-1 in Insect Cells. Because SsHADV-1 *Rep* and *CP* gene transcripts in insects reared on strain DT-8 were successfully detected with RT-PCR amplification (Fig. S4*A*), we suspected that SsHADV-1 could replicate in insect cells. To further confirm this finding, purified SsHADV-1 particles (300 ng/μL) either nondiluted or serially diluted were used to inoculate *Spodoptera frugiperda* cells (Sf9), which were grown in Grace's medium, and then the cells were subjected to virus detection with RT-PCR amplification, Northern blot, immunofluorescence, and flow cytometry assays. Again, virus-specific DNA could be amplified with virus-specific primers (Fig. 3*A*). Moreover, RT-PCR amplification (Fig. 3*B*) and Northern blot analysis (Fig. 3*C*) established that viral *Rep* and *CP* genes were expressed in virus-inoculated Sf9 cells. Furthermore, viral fluorescence signals were observed in virus-inoculated Sf9 cells using immunofluorescence detection assays (Fig. 3*D*). The infection rates of insect cells were measured with flow cytometry, and the results showed that infection of insect cells inoculated with virus particle suspension (30 ng/μL) could be detected at 12 h post-inoculation (hpi); then the infection rate increased up to 60% at 24 hpi, and this infection level was maintained for 72 hpi (Fig. 3*E*). Virus infection could be still detected when the original virus particle suspension (300 ng/μL) was diluted 1,000-fold and assayed at 72 hpi (Fig. 3*E*). The results of passage experiments showed that SsHADV-1 replicated in second-passage Sf9 cells, as viral DNA and transcripts of *Rep* and *CP* were successfully detected with qRT-PCR (Fig. S4 *B* and *C*). Taken together, these results suggest that SsHADV-1 could replicate in cultured cells of an experimental insect host, *S. frugiperda*, as well as in cells of its presumptive natural host, *L. ingenua*.

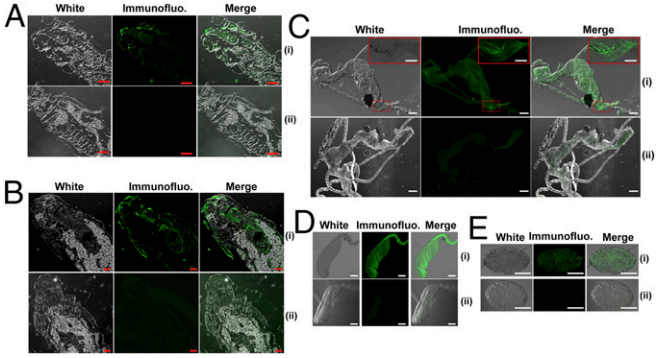

Fig. 2. Immunofluorescence detection of SsHADV-1 in *L. ingenua*. Insects developed either on strain DT-8 (*i*) or DT-8VF (*ii*) (negative control) were used for detection. (*A*) Detection in larvae. Longitudinal sections of larvae (head parts) were shown. (*B*) Detection in pupae. Longitudinal sections of pupae (head parts) were shown. (*C*) Detection in midgut of female adults. Photographs shown at the upper corner were magnified from a part of the midgut (indicated by red boxes). (*D*) Detection in ovarian duct of female adults. (*E*) Detection in eggs dissected from female adults. (Scale bars at the bottom right, 200 μm for *A* and *B*, 100 μm for *C*–*E*, and 50 μm for the upper corner in *C*.) Samples were stained with secondary antibody FITC conjugated to viral particle-specific monoclonal antibody prepared with viral CP protein. The immunofluorescence reaction was observed under a confocal microscope (fv1000mp, Olympus).

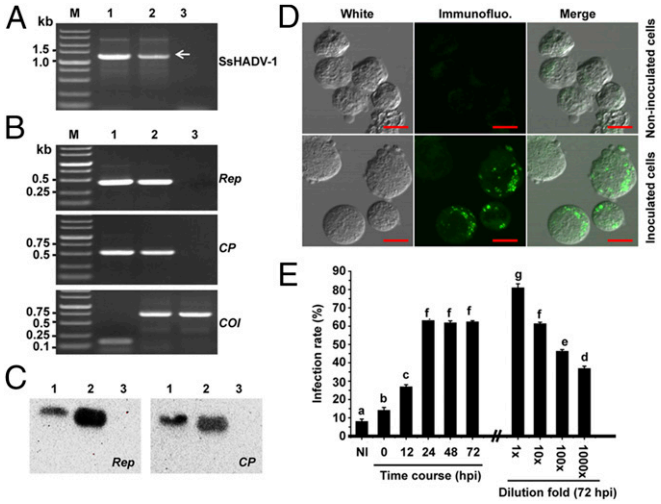

Fig. 3. Replication of SsHADV-1 in *S. frugiperda* cells (Sf9). (*A*) PCR amplification of SsHADV-1 in virus-inoculated Sf9 cells. Viral DNA was amplified from virus-inoculated Sf9 cells (indicated by white arrow). Lane M, DNA marker; lanes 1–3, DNA from strain DT-8, virus-inoculated Sf9 cells, and noninoculated Sf9 cells. (*B*) RT-PCR detection of viral *Rep* and *CP* gene transcripts in virus-inoculated Sf9 cells. Viral *Rep* and *CP* transcripts were amplified in virus-inoculated Sf9 cells. The *Actin* gene for strain DT-8 (shown with white arrow) and *COI* gene for Sf9 cells were RT-PCR–amplified as positive controls. Lane M, DNA marker; lanes 1–3, RNA from strain DT-8, virus-inoculated Sf9 cells, and noninoculated Sf9 cells. (*C*) Northern blot analysis for expression of the viral *Rep* and *CP* gene. DIG-labeled DNA probes of 853 bp and 901 bp corresponding to viral DNA for the *Rep* and *CP* gene, respectively, were used. Lanes 1–3, RNA from strain DT-8, virus-inoculated Sf9 cells, and noninoculated Sf9 cells. (*D*) Immunofluorescence detection of SsHADV-1 in Sf9 cells. Cells were inoculated with SsHADV-1 particles, and observations were carried out at 72 hpi, as described in Fig. 2. (Scale bar, 10 μm.) (*E*) Virus infection rates of Sf9 cells. Sf9 cells were inoculated with 100 μL (30 ng/μL) virus particle suspension and incubated for different time points to detect virus infection rates; Sf9 cells were inoculated with 100 μL serially diluted virus particle suspension (original concentration was 300 ng/μL) and incubated for 72 hpi. NI, noninoculated cells. The lowercase letters on top of each column indicate differences that are significant ($P < 0.05$).

Transmission of SsHADV-1 to a Virus-Free *S. sclerotiorum* Isolate by *L. ingenua*. Larvae reared on a virus-infected colony were carefully picked up with sterilized forceps or brush and gently washed in sterilized ddH_2O to remove possible hyphal debris attached to the larvae body. To test the possibility of transmission, the water-bathed larvae were further reared on a colony of strain DT-8VF for 1 d, and then hyphal agar plugs were taken from the larvae-feeding galleries (Fig. 1*A*) and placed on fresh potato dextrose agar (PDA) plates. These new subcultures were subjected to virus detection assays. The viral genomic DNA of SsHADV-1 extracted from the mycelial mass of newly infected subcultures was detected by PCR amplification (Fig. S5*A*). We tested 39 subcultures, eight of which were found to be infected with SsHADV-1, and virus infection of three subcultures—LT3, LT4, and LT9—was further confirmed by Southern blot analysis (Fig. 4*C*). Larvae reared on a colony of strain DT-8VF were injected with virus particles and starved for 1 d and then were used to repeat this experiment, and the results showed that 6 subcultures out of 28 tested were infected with SsHADV-1 (Fig. S5*B*). These results suggest that viruliferous larvae could transmit SsHADV-1 when allowed to feed on a virus-free strain.

The acquisition feeding time required for virus transmission by larvae was examined. The results showed that a minimum feeding time of 0.5 h is required, at which time point we checked a total of 12 subcultures and only one subculture was found to be SsHADV-1–infected. The frequency of transmission was enhanced slowly with increasing acquisition feeding time from 1 h to 24 h, eventually following 48 h of acquisition feeding; all examined subcultures were infected with SsHADV-1 (Fig. S2 *B* and *C*).

We suspected that *L. ingenua* adults could transmit virus, as adults also carried SsHADV-1. To test this assumption, adults that emerged from pupae developed on colonies of strain DT-8 were allowed to oviposit on colonies of strain DT-8VF, and the hatched larvae were allowed to feed on the same DT-8VF colonies. Subcultures derived from larvae-feeding galleries were sampled for virus detection, and the results showed that seven subcultures out of 33 tested were infected with SsHADV-1 (Fig. S5*C*). Alternatively, in experiments using pupae that developed on strain DT-8VF and injected with virus, particles showed that 4 subcultures out of 24 tested were infected with SsHADV-1 (Fig. S5*D*). Thus, viruliferous adults could transmit SsHADV-1.

The fact that SsHADV-1 was successfully detected in eggs of *L. ingenua* and that adults of *L. ingenua* do not feed on fungal colonies suggested that adults transmit virus via eggs. The eggs laid by viruliferous adults were collected and placed on colonies of strain DT-8VF, and 3 d later, the emerged larvae crawled into the colonies and fed on them. Colonies derived from larvae-feeding galleries were sampled for virus detection, and the results showed that 4 out of 27 subcultures were infected with SsHADV-1. These results suggest that virus transmission could occur through viruliferous eggs of *L. ingenua* (Fig. S5*E*).

Traits of Virus-Infected Strains Following Insect-Mediated Transmission. Colony morphology, growth rate, sclerotial production, and virulence on rapeseed of newly virus-infected isolates were examined. The results showed that colonies that developed on PDA plates were rich in aerial hyphae (Fig. 4*A*) and produced few or no sclerotia at a late stage of colony growth (Fig. 4*E*). The growth rate of these newly infected isolates was significantly reduced compared with that of strain DT-8VF, but they grew faster than strain DT-8 (Fig. 4*D*). Although these virus-infected isolates still maintained a certain level of virulence, their virulence was

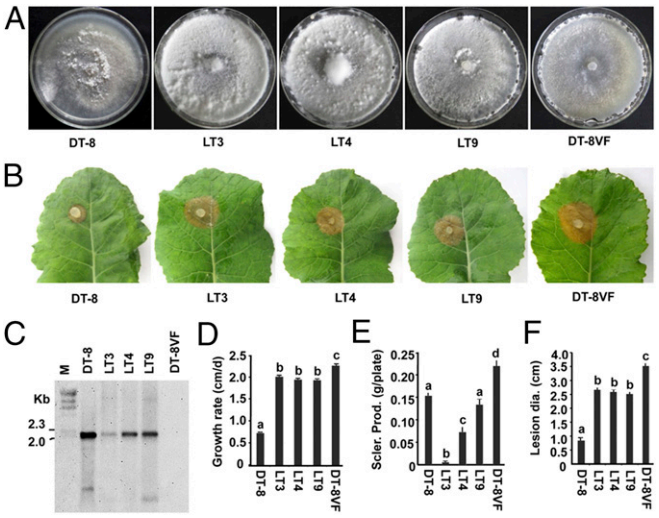

Fig. 4. Transmission of SsHADV-1 to virus-free fungal strain via larvae of *L. ingenua*. (*A*) Colony morphology of virus-infected isolates (colonies were developed on a PDA plate at 20 °C for 7 d). (*B*) Virulence analysis of virus-infected isolates on detached rapeseed leaves; lesion diameters were measured at 48 hpi. (*C*) Confirmation of virus transmission of PCR-positive subcultures (LT3, LT4, and LT9) with Southern blot analysis. See Fig. 1 for probe preparation. Viral DNA was amplified with rolling circle amplification (RCA). DNA samples of strain DT-8 and DT-8VF were used as controls. (*D*–*F*) Sclerotial production and growth rate of newly infected subcultures growing on a PDA plate at 20 °C. Sclerotia were counted on the 15th day of cultivation. The lowercase letters on top of each column indicate differences that are significant ($P < 0.05$).

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significantly reduced compared with strain DT-8VF (Fig. 4 *B* and *F*). These traits were also observed on virus-infected isolates transmitted via adults and eggs.

Virus Transmission from a Fungal Pathogen Residing on Plants via *L. ingenua*. Virus-free larvae were placed on lesions induced by strain DT-8 on rapeseed plants at one side of a plastic box, and plants on the other side were inoculated with strain 1980, a virus-free strain that is labeled with a hygromycin resistance gene. Because strain 1980 is vegetatively incompatible with strain DT-8, virus transmission via hyphal anastomosis could be avoided (Fig. S6*A*). Two weeks later, plants inoculated with strain 1980 were killed, and 48 subcultures of strain 1980 were recovered from diseased plant residues by growing on hygromycin-amended PDA and all subcultures were subjected to virus detection assays. The results showed that viral genomic DNA was detected from six subcultures by PCR amplification (Fig. S5*F*), and virus infection of subcultures AT1, AT7, and AT10 was confirmed by Southern blot analysis (Fig. S6*B*). Compared with SsHADV-1–free strain 1980, these virus-infected subcultures often exhibit abnormal colony morphology, such as abundance of aerial hyphae and significant reduction in growth rate, sclerotia production, and virulence (Fig. S6 *C*–*F*). These traits are similar to those of virus-infected subcultures via larval transmission.

Adults Tend to Lay Eggs on Virus-Infected Colonies. Two glass bottles, one with a colony of strain DT-8VF and another with a colony of strain DT-8, and two small glass beakers with 10 pupae of *L. ingenua* or a piece of black cloth were placed in a large glass beaker and incubated at 23 °C. When adults emerged from pupae, they chose the colony of strain DT-8 for laying eggs. About 10 d later, a large number of larvae appeared on the colony of strain DT-8 and consumed all hyphae, whereas larvae rarely appeared on the colony of strain DT-8VF. However, when activated carbon was added in the large beaker, both virus-free and SsHADV-1–infected colonies were consumed by larvae (Fig. 5*A*). The tendency of insects to prefer virus-infected colonies was further confirmed with a "Y"-shaped tube test with volatiles collected from strains DT-8 and DT-8VF. The results showed that 27 out of 35 female adults chose volatiles released by strain DT-8 (Fig. 5*D*). We collected the volatiles produced by either strain DT-8 or DT-8VF, subjected them to GC–MS analysis, and found that the pattern of volatiles released by strain DT-8 was similar to that by strain DT-8VF (Fig. S7), but the quantity of 1-Octen-3-ol and 3-Octanone produced by strain DT-8 was significantly lower than that by strain DT-8VF (Fig. 5 *B* and *C*). Furthermore, Y-shaped tube tests showed that high concentrations of 1-Octen-3-ol and 3-Octanone were likely to repel *L. ingenua* adults (Fig. 5*D*).

***L. ingenua* Individuals Exist in Rapeseed Fields and Carry SsHADV-1.** Larvae and pupae were frequently found on diseased residuals of *S. sclerotiorum*-infected rapeseed, with preference to the inner parts of diseased stems of rapeseed (Fig. S8*A*). Adults could be found on leaves and petals of rapeseed. Adults of *L. ingenua* were collected either from a strain DT-8–sprayed rapeseed field or from a nonsprayed rapeseed field and then subjected to virus detection. In the strain DT-8–sprayed field, 43 adults were captured, and viral DNA was successfully detected from 35 adults (Fig. S8*B*); 49 adults were captured from the nonsprayed field, which is 19.2 km away from the strain DT-8–sprayed field, and 11 of these 49 adults were found to carry the virus (Fig. S8*C*). The PCR-amplified products from adults in both rapeseed fields were further sequenced and confirmed to originate from SsHADV-1 sequences.

Discussion

In this study, we discovered that the fungal DNA virus SsHADV-1 could infect larvae of *L. ingenua* when reared on colonies of a virus-infected strain and that the virus could replicate in larvae, pupae, adults, and eggs of *L. ingenua*. Furthermore, *L. ingenua*

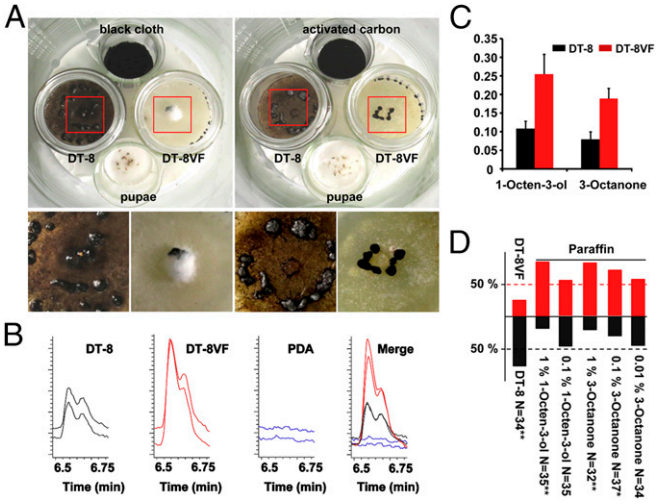

Fig. 5. Preference of *L. ingenua* on virus-infected strain and volatile analysis. (*A*) Adults tended to lay eggs on the virus-infected strain DT-8 colony, and hyphae of strain DT-8 were consumed by emerging larvae. The large glass beaker containing pupae (p), black cloth (bc), and colonies of strains DT-8 and DT-8VF were maintained at 23 °C for 10 d (*Left*). (*Right*) The black cloth was replaced by activated carbon (ac), and others were the same as in *Left*. The *Bottom* photographs were magnified from parts of the red boxes. (*B*) The peak and retention time for 1-Octen-3-ol (6.588 min) and 3-Octanone (6.700 min). The whole profile patterns of volatile chemicals of strains DT-8 and DT-8VF detected by GC–MS were presented in Fig. S7. (*C*) Quantitative analysis of 1-Octen-3-ol and 3-Octanone volatilized by strain DT-8 and DT-8VF. The number on the *y* axis was the relative peak area of 1-Octen-3-ol and 3-Octanone compared with nonyl acetate, which was defined as 1. (*D*) Preference analysis of *L. ingenua* using a Y-shaped tube test. 1-Octen-3-ol and 3-Octanone resolved in paraffin and volatiles collected from strains DT-8 and DT-8VF were used for the test group and paraffin was used as the control. The number on the *y* axis represents the ratio of insects choosing the tested chemicals or volatiles. Numbers choosing chemical or volatiles were given "X^2" test, $*P < 0.05$ and $**P < 0.01$.

could transmit SsHADV-1 transovarially. We propose that *L. ingenua* not only serves as a transmission vector but also is a natural host of SsHADV-1. Additionally, we found that SsHADV-1 could replicate in *S. frugiperda* cells, suggesting *S. frugiperda* is a potential host. Previously, the viral DNA genome of SsHADV-1 was detected in nature in damselflies (26), suggesting that damselflies are likely to be a host of SsHADV-1. Thus, SsHADV-1 is most likely to have a broad insect host range and wide distribution in nature, as *L. ingenua*, *S. frugiperda*, and damselflies are distantly related phylogenetically and they belong to different orders in Insecta.

Our findings imply that gemycircularviruses have transkingdom hosts and could be transmitted via insects. The gemycircularviruses in animal feces might have originated from foods (such as plants and mushrooms). For example, the ancient caribou feces-associated virus (aCFV), which was discovered in 700-y-old caribou feces, could infect *Nicotiana benthamiana* (23). Gemycircularviruses, which were detected in samples of human blood, serum, cerebrospinal fluids, and brain (28, 29), as well as horse liver and spleen (31), most likely replicate and are retained in human and animal cells, suggesting that humans and animals are possible hosts of these viruses. The finding that gemycircularviruses were detected in mosquitoes suggests that mosquitoes and other parasitic insects such as fleas as well as parasitic mites and ticks may serve as vectors for transmission of human and animal gemycircularviruses. Our finding may provide a clue to understanding the ecology and evolution of these newly emerging small circular ssDNA viruses and provide an avenue to study multiple ecological interactions among fungi, viruses, and insects.

Insects as virus transmission vectors are very common in nature, as insects transmit many plant and animal viruses. Most plant viruses are transmitted by insects with piercing sucking mouthparts. The

virus could enter into the body of an insect and be released into the plant through a circulatory system during the next feeding cycle (35). Transmission of mycoviruses via *L. ingenua* may not be specific, but the larvae of *L. ingenua* have chewing mouthparts to uptake hyphae. Virus particles may be released from mechanically broken hyphae during the drawing of hyphae into the mouth, or virus particles may also be released from hyphae digested by enzymes in the intestine of larvae, and then the released virus may infect the insect. This hypothesis is supported by the findings that viral genomic DNA could be detected in starving larvae, pupae, adults, and eggs (Fig. 1) and that SsHADV-1 could replicate in Sf9 cells (Fig. 3). Furthermore, SsHADV-1 has strong extracellular infection ability and is stable (32); SsHADV-1–like viruses are likely to be very stable, as their full-length genomic DNA was detected from 700-y-old animal feces (23). However, details of how SsHADV-1 infects insects and replicates in insect cells need to be studied in the future.

Viruses modulate host secondary metabolism pathways to facilitate their transmission by insect vectors. This is very common in plant/virus/vector systems, such as geminiviruses, which could modulate their hosts to attract whiteflies (36); tomato spotted wilt tospovirus, which could affect its transmission vector *Frankliniella fusca* by regulating its host plant (37); barley yellow dwarf virus, which could change its host to attract aphids (38); and cucumber mosaic virus, which could change the host's volatiles to attract pollinators (39). These natural phenomena may be universal, as SsHADV-1–infected fungal strains could release less repellent volatile compounds to attract the adults of *L. ingenua* than virus-free strains do. Furthermore, we found that viruliferous adults tended to produce more eggs, as 96.4 ± 2.5 eggs were laid per viruliferous female compared with 74.8 ± 1.9 per virus-free female. This phenomenon is of interest and suggests that there is possibly a mutualistic interaction between *L. ingenua* and SsHADV-1.

Hypovirulence-associated mycoviruses have the potential to control fungal plant diseases. A classic example is the successful use of hypoviruses to control chestnut blight caused by *C. parasitica* in Europe. However, this approach for controlling plant diseases has some disadvantages to be overcome. Milgroom and Cortesi concluded that the success of using hypoviruses to control chestnut blight at the population level depended on the natural spread of viruses, and they thought that the application of hypovirulence-associated mycoviruses for biological control of other plant-pathogenic fungi was likely to meet the same constraints as those for chestnut blight (40). Our findings and previous studies on potential mycovirus transmission vectors (14–17) suggest that vectors for mycoviruses are likely to exist in nature, and other mycophagous insects may function as mycovirus transmission vectors, as many fungi could release volatiles to attract insects (41). Thus, *L. ingenua* is not likely to be the only transmission vector for mycoviruses. The existence of other transmission vectors for mycoviruses needs to be explored.

In the rapeseed/*S. sclerotiorum* system, our previous research showed that spraying the SsHADV-1–infected strain under field conditions could control rapeseed stem rot disease and improve the yield of rapeseed (32). Although in the present study our experiments suggested that SsHADV-1 could occur naturally in *L. ingenua*, spraying the virus-infected strain in a rapeseed field could enhance the proportion of viruliferous adults, as the virus is transmitted transovarially. However, we also found that the replication of the insect-transmitted virus in fungi was significantly suppressed. With a better understanding of the multiple interactions among *S. sclerotiorum*, SsHADV-1, *L. ingenua*, and plants—especially a better understanding of the biological and ecological properties of viruliferous *L. ingenua*—the possibility to control rapeseed stem rot disease with SsHADV-1 could be markedly enhanced.

Materials and Methods

Fungal Strains and Maintenance. SsHADV-1–infected strain DT-8 of *S. sclerotiorum*, SsHADV-1–free strain DT-8VF (18), the virus-free strain 1980 labeled with a hygromycin-resistance gene, and strain B05.10 of *B. cinerea* (nonhost fungus to SsHADV-1) were used in this study. Details for this and subsequent methods can be found in *SI Materials and Methods*.

Insect Collection, Identification, and Maintenance. Mycophagous insects were originally collected from a colony of *S. sclerotiorum* growing on a PDA plate. Insect identification was made using traditional methods and sequencing analysis of the *COI* gene (42). Insects were reared and maintained either on a colony of strain DT-8VF or on strain DT-8 in glass bottles.

Virus Particle Preparation. Purified SsHADV-1 particles were prepared as previously described (18) and used for injecting insects and inoculating Sf9 cells.

Insect Injection with Virus Particles. Virus-free larvae and pupae developed on colonies of strain DT-8VF were injected with virus particles by a micromanipulator system, NT-88-V3 (Nikon). The injected larvae were starved for 1 d before rearing on either strain DT-8VF or *B. cinerea*, and the injected pupae were placed in a beaker for emergence.

Virus Replication Assay in Sf9 Cells. To determine whether SsHADV-1 can replicate in insect cells, SsHADV-1 particles either diluted or nondiluted (300 ng/μL) were used to inoculate *S. frugiperda* cells (Sf9). The cell-free supernatant of culture liquid of inoculated Sf9 cells was prepared to conduct passage experiments according to a method described by Hadsbjerg et al. (43). The inoculated Sf9 cells were collected to extract DNA and RNA for RT-PCR amplification, Northern blotting, immunofluorescence, and flow cytometry analyses.

DNA and RNA Extraction; RT-, qRT-, and q-PCR; and Southern/Northern Hybridization Blots. Genomic DNA and RNA from insect, fungi, and Sf9 cells were extracted using a cetyltrimethylammonium bromide (CTAB) method and a TRIzol Kit reagent (Invitrogen), respectively. Specific primer pairs designed based on viral genomic DNA sequence, fungal *Actin* gene (44), and insect *COI* gene (42) were used for RT-, qRT-, and q-PCR amplification and for probe preparation. Primer pairs are listed in Table S1. Southern and Northern blot analysis was conducted with a digoxigenin (DIG) High Prime DNA Labeling and CDP-Star Detection Kit.

Insect Feeding, Egg Production, and Virus Detection. Larvae were fed on colonies of either strain DT-8 or strain DT-8VF. Female adults, 3 d postemergence, were dissected under stereomicroscope, and their egg production was counted. To determine whether the insect could acquire SsHADV-1 by feeding on a colony of strain DT-8, larvae reared on such a colony, and subsequently pupae, adults, and eggs, were randomly selected for DNA extraction, PCR detection, and Southern blot analyses.

Immunofluorescence and Flow Cytometry Analyses. Immunofluorescence detection was performed according to previously described methods (45–47) with larvae, pupae, eggs, ovarian ducts, and midguts of female adults of *L. ingenua* and Sf9 cells. A monoclonal antibody, prepared with SsHADV-1 CP conjugated to virus particles, was used. Goat anti-mouse IgG/FITC (CWBIO) conjugated to the first monoclonal antibody was used as a secondary antibody. Immunofluorescence reaction was observed under a confocal microscope, fv1000mp (Olympus), and infection rates of cells were counted by NovoCyte flow cytometer (ACEA Biosciences) according to previously described methods (48).

Virus Transmission Assay via *L. ingenua*. To determine whether larvae and eggs of *L. ingenua* could transmit the acquired virus to SsHADV-1–free strains, viruliferous larvae and eggs or virus-injected larvae were placed on a colony of strain DT-8VF, and hyphal-agar discs were taken from larvae-feeding galleries and transferred to fresh PDA plates. The new colonies were then subjected to virus detection using PCR amplification and Southern blot analyses using DNA samples from RCA amplification (49).

To determine whether pupae and adults could transmit virus, viruliferous pupae or virus-injected pupae were placed in a small glass beaker to physically separate the pupae and fungal colonies. After emerging, the adults oviposited eggs on colonies of strain DT-8VF, and offspring larvae fed on the same colonies. Subcultures derived from feeding galleries were then subjected to virus detection.

To determine whether virus transmission could occur on plants, rapeseed plants were inoculated with either strain DT-8 or strain 1980 and were placed at two opposite sides of a plastic box with a distance about 30 cm between the two sides to ensure plants in each side do not contact each other. Two weeks later, strain 1980 was recovered from the diseased stem and subjected to virus detection.

Insect Preference of Virus-Infected Fungal Strains. Insect preference was determined by direct observation with naked eyes (Fig. 5*A*) and Y-shaped tube

test. The volatiles released by strain DT-8 and strain DT-8VF were collected as previously described (50) and then analyzed with GC–MS (GC/MS-QP 2010 plus) according to previously described methods (51). To test for possible attractants or repellants specifically released by strain DT-8 or DT-8VF, pure compounds were obtained commercially (Sigma), and their ability to attract or repel insects was conducted with a Y-shaped tube test following a method described previously (52).

Biological Properties of Fungal Subcultures. To assess colony morphology, growth rate, sclerotial production, and virulence on detached rapeseed leaves of insect-mediated virus-infected fungal isolates, the methods described by Yu et al. (18) were followed.

Field Investigation. To determine whether *L. ingenua* could survive in a rapeseed field, larvae and pupae were sampled from stem rot lesions on a diseased rapeseed in a field and identified in the laboratory with a stereoscope. Adults collected either from a strain DT-8–sprayed rapeseed field or from a nonsprayed field were subjected to PCR amplification and sequencing analysis.

Statistical Analysis. Experimental data on fungal growth rate, sclerotial production, and virulence assays of the fungal strains and virus-infection rates of Sf9 cells were subjected to analysis of variance (ANOVA) in SAS (SAS Institute, version 8.1), and treatment means were compared using least significant difference (LSD) test at $P = 0.05$. The peak areas of the GC–MS results were evaluated by a t test at $P = 0.05$. The proportion of *L. ingenua* adults' choices to strain DT-8 or DT-8VF and pure compounds in the Y-shaped tube test were performed via a χ^2 test for an expected proportion of 1:1 for all choice experiments at $P = 0.05$ or $P = 0.01$.

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