Symptomatic plant viroid infections in phytopathogenic fungi

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Viroids are pathogenic agents that have a small, circular noncoding RNA genome. They have been found only in plant species; therefore, their infectivity and pathogenicity in other organisms remain largely unexplored. In this study, we investigate whether plant viroids can replicate and induce symptoms in filamentous fungi. Seven plant viroids representing viroid groups that replicate in either the nucleus or chloroplast of plant cells were inoculated to three plant pathogenic fungi, *Cryphonectria parasitica*, *Valsa mali*, and *Fusarium graminearum*. By transfection of fungal spheroplasts with viroid RNA transcripts, each of the three, hop stunt viroid (HSVd), iresine 1 viroid, and avocado sunblotch viroid, can stably replicate in at least one of those fungi. The viroids are horizontally transmitted through hyphal anastomosis and vertically through conidia. HSVd infection severely debilitates the growth of *V. mali* but not that of the other two fungi, while in *F. graminearum* and *C. parasitica*, with deletion of dicer-like genes, the primary components of the RNA-silencing pathway, HSVd accumulation increases. We further demonstrate that HSVd can be bidirectionally transferred between *F. graminearum* and plants during infection. The viroids also efficiently infect fungi and induce disease symptoms when the viroid RNAs are exogenously applied to the fungal mycelia. These findings enhance our understanding of viroid replication, host range, and pathogenicity, and of their potential spread to other organisms in nature.

plant viroid | fungus | transmission | pathogenicity

Viroids are infectious pathogenic agents possessing small, nonencapsidated, circular single-stranded RNAs that, to date, have been found to naturally infect only plants (1, 2). Viroids infect a wide variety of higher plant species, causing devastating diseases in many crops, particularly vegetables, fruits, and ornamental plants (3). In crop plants, viroids are known to spread by vegetative propagation; by mechanical agricultural practices; and, in certain cases, through seeds, pollen, and insect transmission (3, 4). As viroids do not encode any proteins and do not require a helper agent for their multiplication and survival, the biological activities of viroids are entirely dependent on direct interactions of their RNA genome or its derivatives with cellular host components (5–9). Viroid replication or processing of its RNAs in the yeast, *Saccharomyces cerevisiae*, and cyanobacterium, *Nostoc* (*Nostocales*), have been demonstrated (10–12).

Currently, at least 34 viroid species have been identified and are classified into two families, *Avsunviroidae* and *Pospiviroidae* (13–15). The members of *Avsunviroidae* (type species: *Avocado sunblotch viroid*) replicate in the chloroplasts or plastids through symmetric rolling-circle replication using the host nuclear-encoded polymerase. Their RNAs form highly branched secondary structures and have ribozyme activities. Members of *Pospiviroidae* (type species: *Potato spindle tuber viroid*) replicate and accumulate in the nucleus through asymmetric rolling-circle replication using host RNA polymerase II (Pol II) as the replication enzyme. Their RNAs form rod-shaped secondary structures but likely lack ribozyme activities (2, 16). Potato spindle tuber viroid (PSTVd) requires a unique splicing variant of transcription factor IIIA (TFIIIA-7ZF) to replicate by Pol II (17) and optimizes expression of TFIIIA-7ZF through a direct interaction with a TFIIIA splicing regulator (ribosomal protein L5, a negative regulator of viroid replication) (18). The molecular basis of viroid pathogenicity is not fully understood, although some mechanisms have been suggested, including the down-regulation of host gene expression via RNA silencing-mediated degradation guided by viroid-derived small RNAs (19–21).

The majority of plant diseases are caused by fungi and fungus-like organisms (oomycetes). Plant pathogenic fungi mainly belong to *Ascomycetes* and *Basidiomycetes*, and are generally categorized according to whether they absorb sugar or nutrients from the dead host cells (necrotrophic) or living host cells (biotrophic) (22, 23). It has been long known that pathogenic fungi, particularly the biotrophs, secrete effector proteins into host cells to promote fungal infection (24, 25). More recently, it was determined that pathogenic fungi transfer small RNA effectors to suppress host defense-related genes (26, 27) as well as acquire small RNAs transferred from plants during

Significance

Viroids are the only known autonomously replicating pathogenic agents that do not encode proteins. As viroids are known only to naturally infect plants, their infectivity and pathogenicity in other eukaryotes are largely unexplored. Herein, we demonstrate the stable infection of three viroid species in different plant pathogenic filamentous fungi and show that viroid infection can reduce the growth and virulence of fungi. In addition to successful viroid RNA inoculation of fungal spheroplasts, viroid infection of fungus could occur through viroid transmission from the plant and when viroid RNAs are directly applied to fungal mycelia. These findings are relevant to our understanding of viroid replication, transmission, and pathogenicity.

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infection (28, 29), revealing bidirectional horizontal transfers of genetic information between fungi and plant hosts (30).

Like other higher eukaryotes, fungi could host other parasites, including fungi (31), bacteria (32, 33), and viruses (34). Mycoviruses infecting phytopathogenic fungi have been extensively characterized, in part, owing to their prospect for the use as biological control agents of plant fungal diseases (34–36). Indeed, there are a number of examples of the successful or promising use of mycoviruses to control fungal diseases (37–39). Interestingly, a number of studies have recently demonstrated that phytopathogenic fungi could be a suitable host of some plant viruses (40–42), which extends the previously known compatibility of plant viruses and yeast (*S. cerevisiae*) as hosts (43, 44). Conversely, mycoviruses originating from marine fungi (*Penicillium aurantiogriseum* var. *viridicatum*) associated with sea plants were shown to replicate in plant cells (45). Together, these observations give rise to an interesting notion that certain parasitic agents could cross kingdom barriers and invade both plants and fungi.

In this study, we demonstrate the stable replication of three plant viroids in phytopathogenic fungi. Importantly, viroid infection could reduce the growth and virulence of a defined pathogenic fungus. Moreover, we found that viroid infections of fungi could be established through *in planta* transmission during fungal infection or when the viroid RNAs were directly applied to fungal mycelia. We discuss the significance of these findings for extending our knowledge of the host ranges and pathogenesis of viroids.

Results

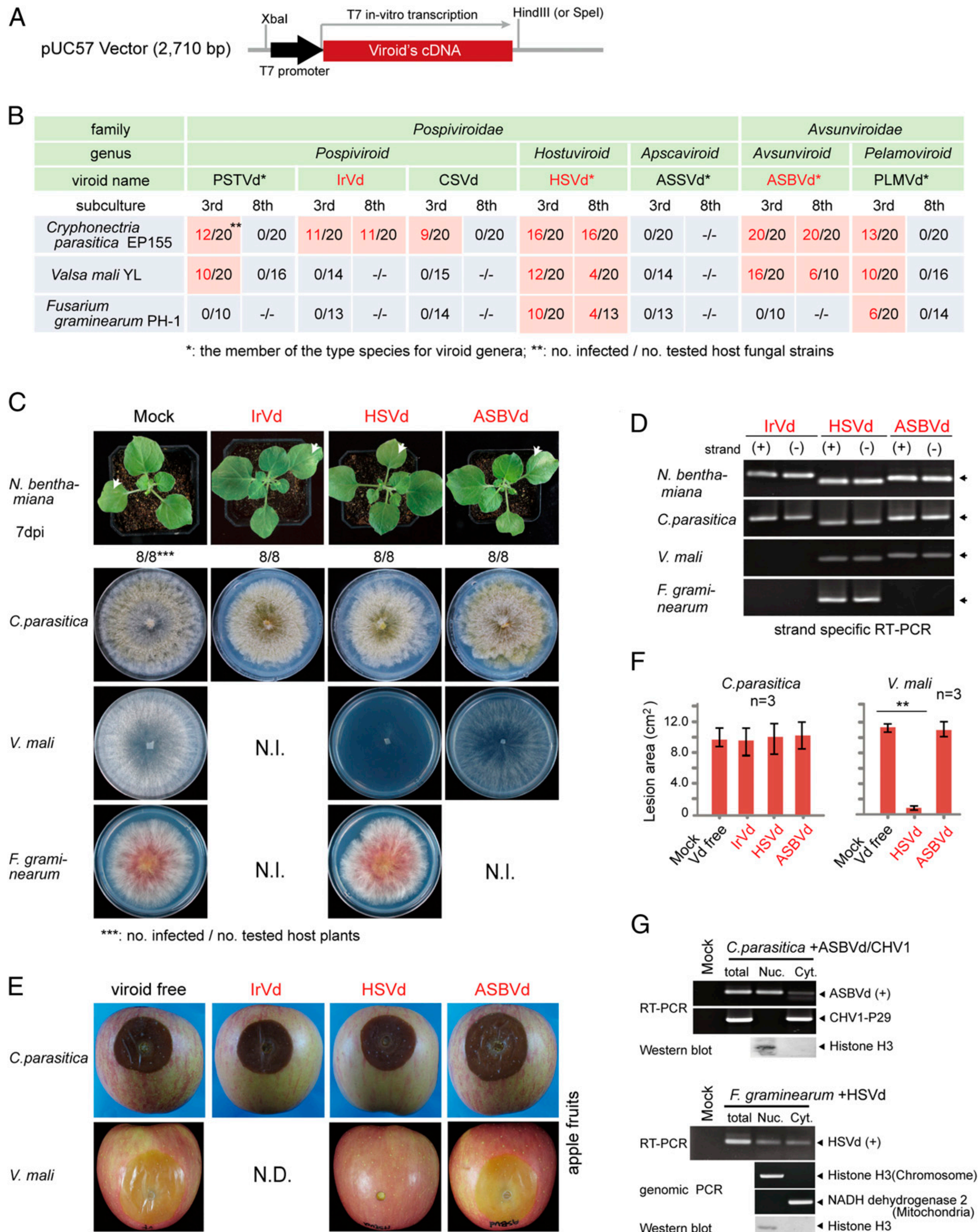
Production of Infectious Viroid cDNA Clones. Using oligonucleotide synthesis and cloning technique, we produced full-length monomeric cDNA clones of seven plant viroids belonging to the *Pospiviroidae* [PSTVd; iresine 1 viroid (IrVd-1), chrysanthemum stunt viroid (CSVd), hop stunt viroid (HSVd), and apple scar skin viroid (ASSVd)] and *Avsunviroidae* [avocado sunblotch viroid (ASBVd) and peach latent mosaic viroid (PLMVd)]. To facilitate in vitro transcription, T7 RNA polymerase promoter sequences and a restriction site (HindIII or SpeI) were incorporated into the 5′ and 3′ termini, respectively, of each cDNA clone (Fig. 1*A* and *SI Appendix*, Fig. S1). First, the infectivity of uncapped plus (+) strand in vitro transcripts of viroid cDNA clones was tested by mechanical inoculation to the leaves of *Nicotiana benthamiana* (Solanaceae) plants. At 7 d postinoculation, viroid RNAs were detected by RT-PCR in the upper systemic leaves of all plants inoculated with the seven viroid transcripts, although no visible symptoms were observed on these plants (Fig. 1 *C* and *D* and *SI Appendix*, Fig. S2 *A* and *B*). When the viroid transcripts were introduced to yeast cells (*S. cerevisiae* strain AH109) by transfection, viroid RNA accumulations were detected by RT-PCR after successive subcultures of the cells (*SI Appendix*, Fig. S2 *C* and *D*), indicating viroid replication in this host similar to what was previously demonstrated for ASBVd (10). These results confirm the infectivity of in vitro RNA transcripts derived from all seven viroid cDNA clones.

Infection of Plant Pathogenic Fungi with Viroids. We investigated the possibility of viroid infection in three phytopathogenic ascomycete fungi, *Cryphonectria parasitica*, *Valsa mali*, and *Fusarium graminearum*, which are the causative agents of chestnut blight, apple tree canker, and wheat/barley head blight and maize ear rot diseases, respectively (46–48). Spheroplasts prepared from these three fungi were transfected with in vitro RNA transcripts derived from each of the seven viroid cDNA clones and then regenerated and followed by subcultures (*SI Appendix*, Fig. S3*A*). Several fungal transfectants (10–20 isolates) derived from each transfection (total of 21 viroid–fungal host combinations) were analyzed for the presence of viroid RNAs using RT-PCR and sequencing of the amplification products. During the first round of screening (third fungal subcultures), the majority of viroid–fungus combinations (12 of 21 transfections with six viroids) yielded fungal transfectants that were carrying viroids (Fig. 1*B*). After the third fungal subculture, *C. parasitica* was infected by six of seven viroids tested, *V. mali* by four of seven, and *F. graminearum* by two of seven (Fig. 1*B*). However, after the eighth subculture, only three viroids were stably maintained in these filamentous fungi: HSVd accumulated in all three fungi, while ASBVd accumulated in *C. parasitica* and *V. mali*, and IrVd-1 could be detected only in *C. parasitica* (Fig. 1 *B* and *D* and *SI Appendix*, Fig. S3*C*). RNA blotting analysis was able to detect HSVd and ASBVd RNA accumulation in *N. benthamiana* plants, but not in fungi (*SI Appendix*, Fig. S4*A*). The negative (−) strand viroid RNAs were also detected by RT-PCR (Fig. 1*D* and *SI Appendix*, Fig. S3*C*; confirmation of strand specificity of RT-PCR is shown in *SI Appendix*, Fig. S3*B*). Likewise, circular (and/or linear oligomeric) (+) genomic RNAs were detected by inverse RT-PCR (*SI Appendix*, Fig. S5). Moreover, viroids were efficiently transmitted through hyphal anastomosis and conidia (*SI Appendix*, Fig. S6), which are similar to horizontal and vertical transmissions of mycoviruses (49). Thus, taken together with the stable accumulation of these viroid RNAs, these observations strongly indicate the replication of these viroids in these fungal hosts.

C. parasitica infected with IrVd-1, HSVd, or ASBVd exhibited slightly reduced growth on potato dextrose agar (PDA) medium compared with viroid-free fungus (Fig. 1*C* and *SI Appendix*, Fig. S3*D*), but viroid infections had no effect on *C. parasitica* virulence in an apple-fruit inoculation assay (Fig. 1 *E* and *F*). Likewise, viroid infections had no apparent effect on growth and virulence of *V. mali* (ASBVd) and *F. graminearum* (HSVd), respectively (Figs. 1 *C*, *E*, and *F* and 2*B* and *SI Appendix*, Fig. S3*D*). In contrast, HSVd infection severely debilitated *V. mali* growth and virulence (Fig. 1 *C*, *E*, and *F* and *SI Appendix*, Fig. S3*D*), indicating that HSVd confers pathogenicity to *V. mali*.

As a member of the *Avsunviroidae*, ASBVd replicates in the chloroplasts of plant cells (5). As fungal cells do not have chloroplasts or plastids (nonphotosynthetic organisms), it is thus not predictable where ASBVd replication takes place within the fungal cell. To investigate this aspect, spheroplasts prepared from *C. parasitica* coinfected with ASBVd and Cryphonectria parasitica hypovirus 1 [CHV1, a well-studied single-stranded RNA (ssRNA) mycovirus belonging to the genus *Hypovirus*] were subjected to a cell fractionation procedure to separate nuclear and cytoplasmic contents. RT-PCR detection showed that ASBVd RNAs were mainly present in the nuclear-enriched fraction, whereas the coinfecting CHV1 RNAs were exclusively detected in the cytoplasmic fraction (Fig. 1*G*), which is expected from cytoplasmically replicating ssRNA viruses (50). In a fractionation experiment using *F. graminearum*, HSVd, a nuclear-replicating viroid, was shown to be present in both nuclear and cytoplasmic fractions (Fig. 1*G*). These findings suggest that both types of plant viroids replicate in the nuclear compartment of fungal cells and might utilize fungal nuclear polymerase for their replication (10).

RNA Silencing Is Implicated in Defense Against Viroids in Fungi. RNA silencing is the primary defense mechanism against viruses in plants, fungi, and insects (51–53). This mechanism has been shown to operate against viroids in plants (19), and it could conceivably also operate in fungi. To elucidate this aspect, HSVd was introduced to *F. graminearum* knockout mutants defective in the gene encoding for Dicer-like (DCL) protein, essential for RNA silencing through generation of small-interfering RNAs (54). Many filamentous fungi encode two *dcl* genes, *dcl1* and *dcl2*



family	*Pospiviroidae*										*Avsunviroidae*			
genus	*Pospiviroid*						*Hostuviroid*		*Apscaviroid*		*Avsunviroid*		*Pelamoviroid*	
viroid name	PSTVd*		IrVd		CSVd		HSVd*		ASSVd*		ASBVd*		PLMVd*	
subculture	3rd	8th	3rd	8th	3rd	8th	3rd	8th	3rd	8th	3rd	8th	3rd	8th
Cryphonectria parasitica EP155	12/20**	0/20	11/20	11/20	9/20	0/20	16/20	16/20	0/20	-/-	20/20	20/20	13/20	0/20
Valsa mali YL	10/20	0/16	0/14	-/-	0/15	-/-	12/20	4/20	0/14	-/-	16/20	6/10	10/20	0/16
Fusarium graminearum PH-1	0/10	-/-	0/13	-/-	0/14	-/-	10/20	4/13	0/13	-/-	0/10	-/-	6/20	0/14

*: the member of the type species for viroid genera; **: no. infected / no. tested host fungal strains

Fig. 1. Viroid infection and pathogenicity in filamentous fungi. (*A*) Sequence construct for generating plus (+) strand in vitro transcripts of viroid cDNA clones. (*B*) Detection of viroid in fungal isolates regenerated from fungal spheroplasts that have been transfected using in vitro transcripts of viroid cDNA clones. Viroid RNA accumulations were detected using RT-PCR at third and eighth fungal subcultures. (*C*) Phenotypic growth of plants and fungi infected with viroids. Fungi were grown on PDA medium (10-cm plate) for 3 d and photographed. N.I., not infected. (*D*) RT-PCR analysis of viroid RNA accumulation in the plants and fungi described in *C*. (*E*) Fungal virulence assay on apple. Apple fruits were inoculated with mycelial plugs, and fungal lesions were photographed 5 d after inoculation. N.D., no data. (*F*) Fungal lesion area measured on inoculated apple fruits described in *E*. $^{**}P < 0.01$ (Student's *t* test). Vd, viroid. (*G*) Accumulation of viroids in nuclear (Nuc.)- and cytoplasmic (Cyt.)-enriched fractions. Spheroplasts of infected fungi were subjected to cell fractionation using differential centrifugation. Viroid (ASBVd or HSVd) and virus (CHV1) RNAs were detected by RT-PCR, while chromosomal and mitochondrial DNAs (cytosolic DNAs) were detected by genomic PCR. The presence of histone H3 protein in the nuclear fraction was confirmed by Western blotting analysis.

(55). HSVd was inoculated to *F. graminearum* Δ*dcl1*, Δ*dcl2*, and Δ*dcl1/2* double mutants via transfection of spheroplasts with viroid RNAs. Relative to HSVd-infected wild-type strain, infected Δ*dcl1* strain exhibited only slightly reduced growth on PDA medium, while infected Δ*dcl2* and, Δ*dcl1/2* strains showed a strong reduction in growth (Fig. 2*A*); furthermore, *dcl* knockout did not noticeably alter the growth of viroid-free *F. graminearum* (*SI Appendix*, Fig. S7*A*). A fungal inoculation assay on maize seedlings demonstrated that HSVd-infected Δ*dcl2* and Δ*dcl1/2* strains, but not Δ*dcl1* strain, showed reduced virulence (Fig. 2*B*). Accordingly, HSVd RNA accumulation was more than 20-fold higher in Δ*dcl2* and Δ*dcl1/2* than in Δ*dcl1* and wild-type strains (Fig. 2*C*), and was readily detectable in the Δ*dcl1/2* mutant by RNA blotting (*SI Appendix*, Fig. S4*B*), indicating that DCL2 is the primary DCL component for defense against viroids in *F. graminearum*. In addition, inactivation of *dcl1* or *dcl2* enhanced the efficiency of HSVd transmission through conidia (*SI Appendix*, Fig. S6*B*).

To further investigate whether DCL2 protein also plays a defense role against viroids in other fungi, ASBVd was inoculated to the *C. parasitica* Δ*dcl2* mutant (56). ASBVd-infected wild-type and Δ*dcl2* mutant strains of *C. parasitica* showed similar levels of growth and virulence (Fig. 2 *D* and *E* and *SI Appendix*, Fig. S7*B*); nevertheless, ASBVd RNAs accumulated around fivefold higher in Δ*dcl2* mutant versus wild-type strain (Fig. 2*F*), and ASBVd RNAs in Δ*dcl2* mutant were detectable by RNA blotting (*SI Appendix*, Fig. S4*B*), showing that *C. parasitica* DCL2 also contributes to defense against viroid infection, similar to mycovirus infections (56, 57). CHV1 infection was previously shown to have enhancing effects on a coinfecting double-stranded RNA (dsRNA) mycovirus through the activity of CHV1 p29, an RNA-silencing suppressor (58). Interestingly, coinfection with CHV1 also elevated ASBVd RNA accumulation (Fig. 2*F*). This result further underlines the role of RNA silencing in fungal host defense against viroid infection.

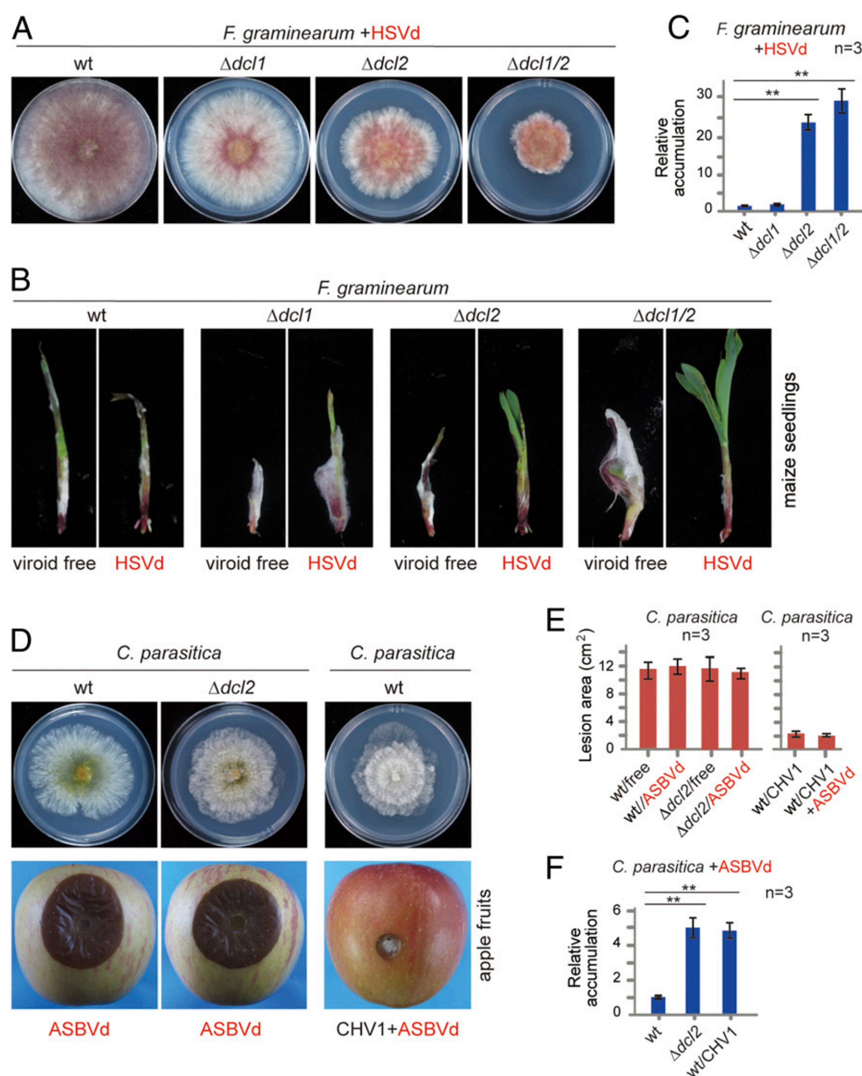


Fig. 2. Viroid accumulation and pathogenicity in *dcl* mutant fungal strains. (*A*) Phenotypic growth of wild-type (wt) and *dcl* mutants of *F. graminearum* infected with HSVd. Fungi were grown on PDA medium (6-cm plate) for 3 d and photographed. (*B*) Disease severities on maize seedlings induced by infection of the fungal strain described in *A*. Seedlings were photographed 7 d after inoculation. (*C*) Quantitative RT-PCR analysis of HSVd accumulation in *F. graminearum dcl* mutants. $^{**}P < 0.01$ (Student's *t* test). (*D*) Phenotypic growth and virulence on apple of wt and *dcl2* mutants of *C. parasitica* infected with ASBVd and wt *C. parasitica* coinfected with ASBVd and CHV1. Fungi were grown on PDA medium for 3 d and photographed. Fungal lesions on apples were photographed 5 d after inoculation. (*E*) Fungal lesion area measured on inoculated apple described in *D*. (*F*) Quantitative RT-PCR analysis of ASBVd accumulation in *C. parasitica* strains described in *D*. $^{**}P < 0.01$ (Student's *t* test).

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Viroids Could Be Bidirectionally Transferred Between Plants and Fungi. The finding that plant viroids can infect phytopathogenic fungi raises the question of whether viroids could be horizontally transferred from the plant to fungus during colonization of plant tissue by the fungal pathogens. In fact, under laboratory conditions, we have previously demonstrated the transfer of a plant RNA virus from the plant to fungus, an event that possibly occurs in natural settings (42). To explore this possibility, we inoculated viroid-free wild-type and Δ*dcl2* mutant strains of *F. graminearum* to HSVd-infected *N. benthamiana* plants. After allowing *F. graminearum* to infect and colonize the leaf tissue, the fungus was then reisolated from plants and subcultured before viroid detection using RT-PCR (Fig. 3*A*). Analysis showed that, respectively, ~44% and 36% of wild-type and Δ*dcl2* fungal isolates derived from HSVd-infected plants contained HSVd (Fig. 3 *B* and *C*), indicating that *F. graminearum* could acquire HSVd from plants during infection.

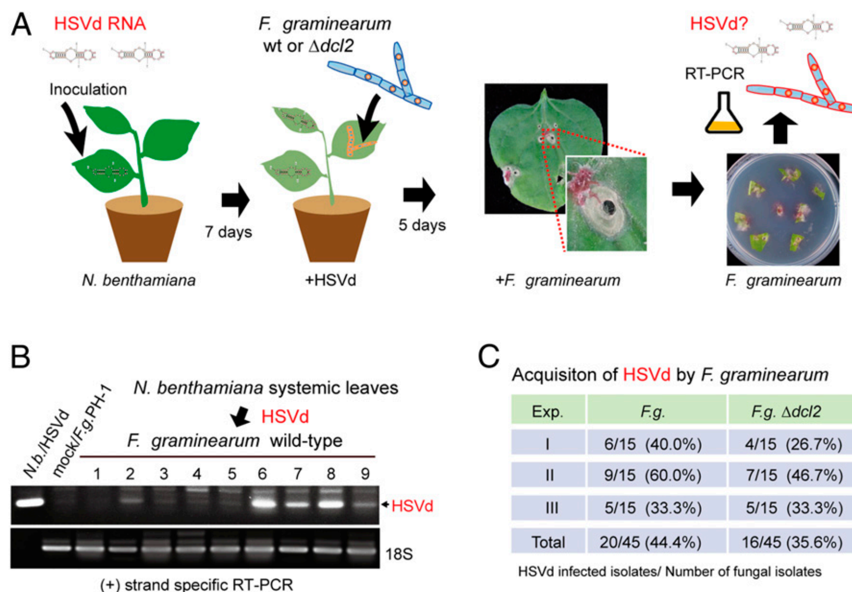
Next, we investigated whether *F. graminearum* could introduce HSVd to the plant by inoculating HSVd-infected fungus to viroid-free *N. benthamiana* plants. Seven days later, viroid RNAs were detected in the uninoculated upper leaves by RT-PCR (*SI Appendix*, Fig. S8*A*). RT-PCR detected HSVd RNAs in the upper systemic leaves of all fungal inoculated *N. benthamiana* plants (*SI Appendix*, Fig. S8*B*). Lack of spread of the fungus to the upper leaves was confirmed by RT-PCR for detection of *F. graminearum* 18S rRNA (*SI Appendix*, Fig. S8*B*), as well as no growth of fungal mycelia from the portions of uninoculated upper leaves placed on medium (*SI Appendix*, Fig. S8*C*). This result demonstrates that viroid-infected *F. graminearum* can efficiently transmit HSVd to a plant.

Viroid Infection Through Direct Inoculation of Fungal Mycelia. Previous studies showed that fungal and plant viruses can infect fungi when virions are externally applied to mycelia (38, 41), but it is not known whether naked viral RNAs are infectious when similarly applied to mycelia. However, there have been reports showing that various fungi can uptake exogenous dsRNAs (59, 60). We therefore investigated whether viroid RNA could be introduced to the fungus through exogenous application. As a first attempt, solutions containing HSVd or ASBVd RNAs (100 μg/μL) were directly applied to the mycelia of *F. graminearum* and *V. mali* grown on PDA medium by spraying with the viroid solutions or water. After allowing the fungus to grow more for 2 d, several mycelial plugs were taken and subcultured for phenotyping and RT-PCR analysis (*SI Appendix*, Fig. S9*A*). Fungal isolates derived from *F. graminearum* and *V. mali* that were sprayed with HSVd and ASBVd RNAs, respectively, were confirmed to be infected with the corresponding viroids, although no clear symptoms were observed (Fig. 4 *A*–*D*). In a parallel experiment, the presence of viroid RNAs was not detected when a similar spray inoculation procedure was carried out using ASSVd RNAs and *V. mali* (*SI Appendix*, Fig. S9 *B* and *C*), which are incompatible viroid and fungal host combinations (Fig. 1*B*), ruling out the possibility that RT-PCR amplified RNA in the inoculum that was applied to the cultures.

Next, we extended the experiment by inoculating HSVd RNAs to *F. graminearum* Δ*dcl2* mutant and *V. mali*, which are the susceptible hosts of HSVd (Figs. 1*C* and 2*A*), so that the effects of viroid infection on the host fungi would be visible following the viroid RNA spray. As expected, *F. graminearum* Δ*dcl2* and *V. mali* exhibited an obvious reduction in growth following HSVd RNA treatments (Fig. 4 *A* and *B*), and HSVd infection was confirmed by RT-PCR (Fig. 4 *C* and *D*). Prompted by these results, we then sprayed HSVd RNAs on *F. graminearum* Δ*dcl2* and *V. mali* colonies that grew on maize seedlings and apples, respectively. Strikingly, compared with water treatment (+H_2O), HSVd RNA treatment (+HSVd) reduced fungal growth and virulence, as indicated by the larger growth of *F. graminearum*-infected maize seedlings (Fig. 4*E*) and the reduced size of *V. mali* lesions on apples (Fig. 4*F*). Taken together, these results demonstrate the direct application of viroid RNAs on fungal mycelia as an effective viroid inoculation method.

Discussion

Viroids are considered as the smallest and simplest infectious pathogen. Despite the small size (~250–400 nucleotides) and noncoding nature of their RNA genome, viroids are capable of self-replication, cell-to-cell and long-distance spread, and inducing disease symptoms in host plants. PSTVd (a pospiviroid) is



Exp.	F.g.	F.g. Δdcl2
I	6/15 (40.0%)	4/15 (26.7%)
II	9/15 (60.0%)	7/15 (46.7%)
III	5/15 (33.3%)	5/15 (33.3%)
Total	20/45 (44.4%)	16/45 (35.6%)

Fig. 3. Acquisition of HSVd by *F. graminearum* from plants. (*A*) Experimental procedure for investigating HSVd acquisition by wild-type (wt) and *dcl2* mutants of *F. graminearum*. (*B*) RT-PCR detection of HSVd accumulation in *F. graminearum* (*F.g.*) strains isolated from inoculated leaves of *N. benthamiana* (*N.b.*) plants. (*C*) Efficiency of HSVd acquisition by wt and *dcl2* mutants of *F.g.* from *N. benthamiana* plants. Exp., experiment.

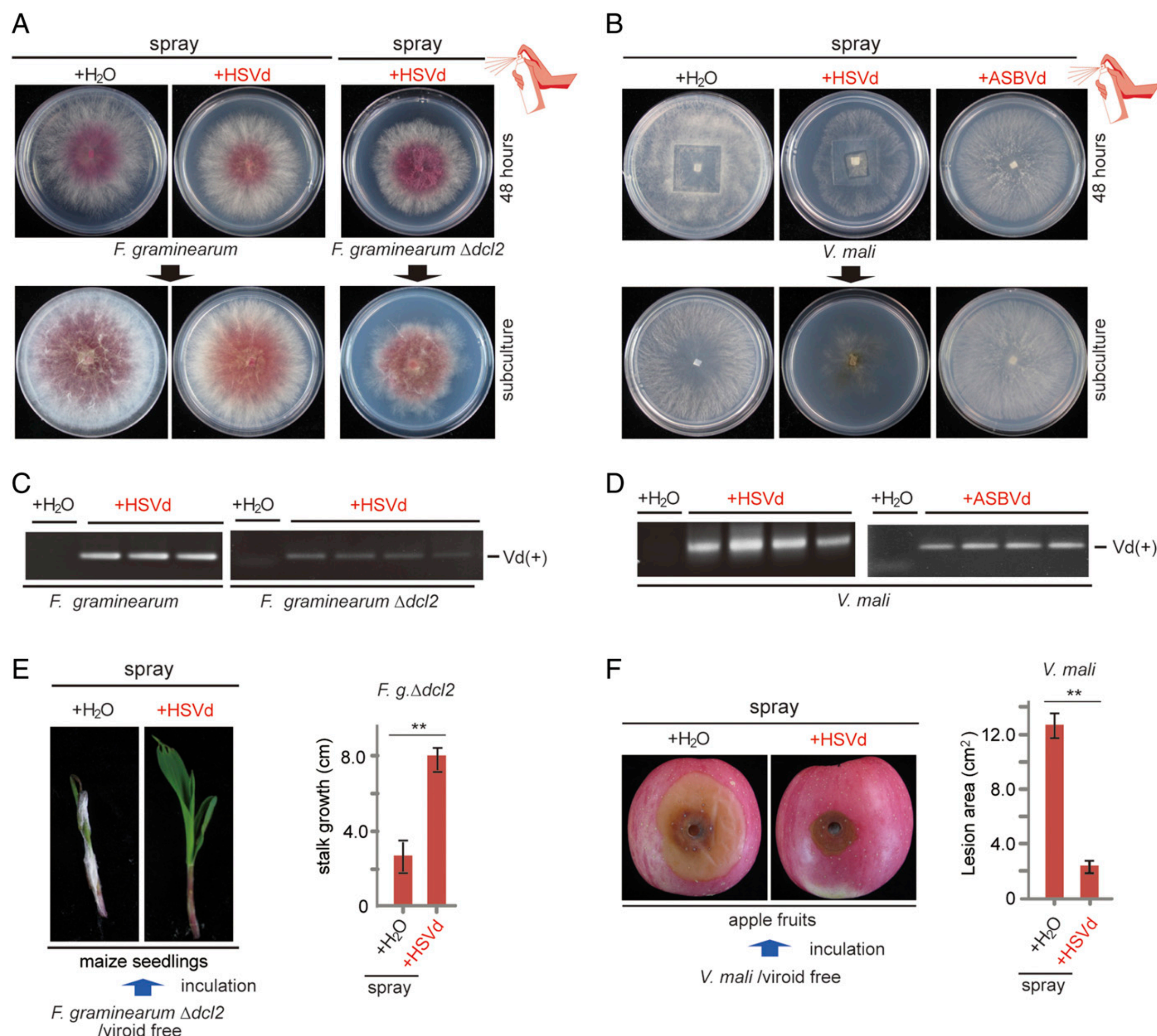


Fig. 4. Viroid infection through exogenous inoculation. (*A* and *B*) Phenotype of fungal strains after direct application of viroid RNAs on mycelia (as illustrated in *SI Appendix*, Fig. S9). (*C* and *D*) RT-PCR detection of viroid (Vd) accumulations in the fungal strains described in *A* and *B*. (*E* and *F*) Disease severities of fungal strains grown on maize seedlings (*E*) and apple fruits (*F*) following direct application of viroid RNAs are illustrated. $^{**}P < 0.01$ (Student's *t* test).

the most intensively studied viroid, and its RNA secondary structure is well understood. Mutational analyses have identified the loops in PSTVd's structure that are critical for its replication, along with its cell-to-cell and systemic spread (61, 62), demonstrating the direct role of the RNA genome in regulating biological activities of viroids. Although viroids generally infect a limited range of natural plant hosts, experimentally, they have been shown to replicate in a wide range of plant species (63), yeast (10), and cyanobacteria (12). In this study, we have shown they can also infect filamentous fungi. These results indicate the ability of some viroids to infect a wide variety of organisms across biological kingdoms. Thus, viroids probably coopt highly conserved polymerases and other factors to replicate.

In this study, seven viroids were inoculated to three filamentous phytopathogenic fungi belonging to different families. Unlike the full compatibility of all viroids with a unicellular fungus (yeast) (*SI Appendix*, Fig. S2), viroid infection in filamentous fungi was apparently more host-specific and also seems to be affected by host immune responses (Fig. 1). We found that not all viroids tested were infectious in the same fungus, suggesting incompatibility with the host factors required for replication in some viroid–fungus combinations (5–9). In addition, some viroids initially accumulated in the specific fungal hosts, but they were then eliminated after successive fungal cultures, likely by the activities of a fungal defense mechanism(s) against invading pathogens. For example, PLMVd (a pelamoviroid), was detected in all three fungi in the third subculture but not in the eighth subculture, suggesting that PLMVd is sensitive to the fungal immune responses. Only one viroid, HSVd (a hostuviroid), was able to stably replicate in all three fungi, while ASBVd (an avsunviroid) replicated in *C. parasitica* and *V. mali*, which both belong to the order *Diaporthales*, but not in *F. graminearum* (order *Hypocreales*), suggesting that HSVd has a rather wide fungal host range, while the host range of ASBVd is more limited. We showed

that both nuclear (HSVd)- and chloroplast (ASBVd)-replicating plant viroids replicate in filamentous fungi, and were both detected in fungal nuclear fractions (Fig. 1), suggesting the adaptability of both types of plant viroids to the nuclear environment of fungal cells.

Among the five fully compatible viroid–fungus combinations, only HSVd infection in *V. mali* caused severe disease symptoms, as seen by the drastic reduction of fungal growth (Fig. 1). This viroid-induced phenotypic symptom is quite similar to the commonly observed reduced growth and "hypovirulence" associated with mycovirus infection in filamentous fungi (64). In fact, the symptom diseases induced by viroid infection in plants quite faithfully resemble those caused by plant viruses, such as plant stunting, leaf variegation, yellowing, necrosis, and sometimes plant death (3). Viral pathogenicity proteins are commonly responsible for development of viral symptoms through disturbances of various biological processes, such as gene expression, metabolism, and immune responses (65), while the mechanism underlying viroid pathogenesis is not well understood. However, it is generally known that viroid infection is associated with the alteration of expression of host plant genes (66–68) and accumulation of viroid-derived small RNAs (69, 70). Some studies suggest the role of viroid-derived small RNAs in modulating viroid symptoms (20, 21, 71, 72). Further work is needed to investigate whether the RNA-silencing pathway or other mechanisms are involved in HSVd pathogenicity in *V. mali*. Given the practicality of filamentous fungi as an experimental model that has a short life cycle and allowance of productive genetic manipulations, a viroid–fungus pathosystem would provide an advantageous platform to elucidate the molecular mechanism underlying pathogenic effects of viroid infection on the nonplant host.

Inactivation of genetic components of the RNA-silencing pathway usually lead to enhanced host susceptibility manifested as an increase of virus accumulation and symptoms (56, 73, 74). Plants encode multiple DCL proteins that are implicated in distinct RNA-silencing pathways (75–77). For example, experimental model plants, such as *Arabidopsis thaliana* and the *Nicotiana* species, encode four DCL proteins (75, 78). Studies of PSTVd using transgenic *N. benthamiana* plants with down-regulated *dcl* genes revealed a complex relationship between DCL proteins and viroid infections. DCL1 and DCL4, which play essential roles in the microRNA pathway and antiviral defense against RNA viruses, respectively, are required for optimum PSTVd accumulation, whereas the combined activity of DCL2 and DCL3 inhibits PSTVd accumulation (79–81). Hence, different from plant RNA viruses, viroids may utilize particular DCL proteins to support their infection; meanwhile, they may be suppressed by other DCL components. Our inoculation experiment using *dcl* mutant strains of *F. graminearum* and *V. mali* indicated that DCL2, but not DCL1, is likely the primary DCL component in fungal defense against viroids (Fig. 2). Similarly, DCL2 is also critical for antiviral defense in *C. parasitica* and *Colletotrichum higginsianum* (a hemibiotrophic ascomycete fungus) (56, 82); in other fungi (*F. graminearum*), DCL1 and DCL2 can functionally complement each other (83). Therefore, it seems that the filamentous fungi exert similar RNA-silencing mechanisms to suppress viral and viroid infections, although it is necessary to further investigate the roles of other core components of the RNA-silencing pathway, such as Argonaute and RNA-dependent RNA polymerase, which have been shown to contribute to defense against viroids in plants (84, 85).

We previously discovered a natural infection of a plant virus, cucumber mosaic virus [CMV; a plant alpha-like (+) ssRNA virus superfamily] in a strain of the phytopathogenic basidiomycete fungus, *Rhizoctonia solani*, obtained from the field (42). Moreover, under laboratory conditions, we demonstrated that *R. solani* can acquire CMV from an infected plant and, vice versa, can transmit CMV to the plant. Thus, based on our current findings on the potential viroid transfer between plants and fungi (Fig. 3 and *SI Appendix*, Fig. S8), it is tempting to speculate the occurrence of viroid infections in plant-associated fungi in the natural environment. Thus, varieties of fungi, including phytopathogenic, mycorrhizal, and endophytic fungi, could possibly serve as the reservoir or biological vector of viroids. Except for certain mycoviruses with viroid-like RNA satellites (86, 87), viroid or self-replicating viroid-like agents have not been found in fungi. Presumably, the common use of dsRNA fractions that exclude low-molecular-weight RNAs for mycovirus identifications or viral metagenomics may have prevented the discovery of viroids from fungi.

The application of mycoviruses for biological control of plant fungal diseases is hampered by the lack of practical methods to introduce the hypovirulence-causing virus to pathogenic fungi in the field (38). Although mycoviruses could be transmitted intracellularly via hyphal anastomosis, different fungal isolates often show vegetative incompatibility that prevents the spread of the virus (88, 89). Therefore, exogenous inoculation through direct application of the virus, such as successfully demonstrated for inoculation of a circular ssDNA mycovirus, Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (family *Genomoviridae*, nonenveloped spherical virion), to the phytopathogenic ascomycete fungus *Sclerotinia sclerotiorum* (38), is a promising method for biocontrol application. Moreover, fungal cells could uptake external dsRNAs that activate the down-regulation of fungal mRNA targets through an RNA-silencing mechanism, and this has been exploited as an alternative fungal control method (59, 90, 91). Our inoculation experiments showed efficient viroid infection of the fungi when the viroid RNAs were directly applied to the mycelia (Fig. 4). The high stability of dsRNAs and highly structured viroid RNAs in unprotective extracellular conditions may partly facilitate efficient entry of those RNA molecules to fungal cells. Importantly, reduced growth and virulence were apparent on fungal colonies grown on plants or fruit following exogenous inoculation of viroid RNAs (Fig. 4). Considering that certain viroids are not infectious or pathogenic to particular crop plants, this implies the potential use of viroids for biocontrol of fungal diseases, but biosafety considerations need to be resolved prior to using viroids for such applications.

Materials and Methods

Fungal Strains, Virus Isolate, and Plant Materials. *C. parasitica* strain EP155 virus-free, infected with CHV1, and Δ*dcl2* mutant strains (56, 92) were generous gifts from Nobuhiro Suzuki, Okayama University. *V. mali* YL strain was described previously (42). *F. graminearum* PH1, Δ*dcl1*, Δ*dcl2*, and Δ*dcl1*/Δ*dcl2* mutants (93) were kindly provided by Jinrong Xu, Northwest A&F University. All fungal strains were grown on PDA medium for 3–6 d at 24–26 °C for morphological observation or on cellophane-covered PDA medium for RNA, DNA, and protein extractions. *Zea mays* (maize) var. Luoyu 818 and *N. benthaniana* plants were grown in a growth room at 22 ± 2 °C with a photoperiod of 16 h/8 h (day/night).

Generation of Viroid cDNA Clones. The full-length monomeric cDNAs of seven plant viroids were generated by oligonucleotide synthesis and performed by Jin Weizhi Co. To construct the infectious cDNA clone, the full-length monomeric cDNA fragments of viroids were PCR-amplified with primers incorporating the restriction site, XbaI, and T7 promoter sequences at 5′ and HindIII/SpeI (Fig. 1*A* and *SI Appendix*, Fig. S1). The PCR products were inserted into the pUC57 Vector (GenScript, Inc.). After linearization of plasmids with restriction enzymes, the monomeric viroid RNAs were synthesized using an in vitro transcription kit (RiboMAXLarge Scale RNA Production Systems-SP6 and T7; Promega) according to the manufacturer's instructions.

Yeast and Fungal Protoplast Isolation and Viroid Transfection. Preparation of competent cells of *S. cerevisiae* AH109 (Clontech) and transfection of in vitro transcripts of viroid RNAs (0.1 μg) using plasmid transformation methods were carried out according to the CLONTECH yeast protocols handbook. The transfected yeast cells were grown on yeast peptone dextrose agar (YPDA; Sigma) and incubated at 28 °C for roughly 72 h. Four independent colonies were randomly selected and cultured in the YPD liquid medium for 72 h.

Protoplasts of *V. mali*, *C. parasitica*, and *F. graminearum* strains were prepared following the method described previously (94). Transfection of fungal protoplasts with in vitro-transcribed RNAs (0.1 μg) was performed as described previously (95).

Viroid and Fungal Inoculations to Plants. For mechanical inoculation of viroids to plants, in vitro transcripts of viroid cDNA clones (0.1 μg/μL) were rubbed onto carborundum-dusted leaves of *N. benthamiana* plants. For inoculation of *F. graminearum* strains to *N. benthamiana* plants, lower leaves were wounded with sterilized toothpicks, and mycelia-containing gel plugs (~0.5 × 1 cm), which were picked up from the edge of a 3-d-old culture colony, were placed on the wounded area. For inoculation of *F. graminearum* to maize plants, maize seeds were germinated on sterilized Petri plates layered with wet filter papers. After the shoots reached a height of 4 cm, the stems were wounded and mycelia-containing gel plugs were placed on the wounds. The inoculated part of the stem was wrapped with parafilm for 24 h. Inoculated plants were grown in pots with soil at 23 ± 2 °C at 70–80% humidity and a photoperiod of 16 h/8 h (day/night).

Exogenous Inoculation of Fungal Mycelia with Viroid RNAs. In vitro transcripts of viroid RNAs were diluted in RNase-free water (100 μg/μL), and solution was then transferred to a small spray bottle (~1 mL). The viroid RNA solution (~200 μL) was directly sprayed onto fungal colonies grown on PDA plates (1-d-old), apples (2 d), or maize seedlings (2 d) until the solution visibly covered the mycelial area. For subculturing the fungal colonies, mycelial plugs outside the sprayed region were transferred to new PDA plates.

Viroid Acquisition by *F. graminearum*. To investigate HSVd acquisition by *F. graminearum*, lower leaves of *N. benthamiana* plants were first mechanically rub-inoculated with HSVd (12–16 plants were inoculated in each experiment), and viroid infection was then confirmed by RT-PCR at 7 d after inoculation. Virus-free *F. graminearum* was then inoculated onto the noninoculated upper leaves. Seven days after fungal inoculation, *F. graminearum* was retrieved from the inoculated plants with a similar procedure as described previously (42).

RNA Extraction, RT-PCR, and RNA Blot Analysis. Extraction of ssRNA and total RNA from fungal mycelia followed the procedure described previously (96). Total RNAs were extracted from leaves of *N. benthamiana* plants using TRIzol (Invitrogen). For RT-PCR detection, first-strand cDNAs were synthesized using ReverTra Ace reverse transcriptase (Toyobo) and amplified by using 2× mixture DNA polymerase (Kangwei). For quantitative RT-PCR, the 18S RNA of *C. parasitica* and *F. graminearum* was employed as an internal control. Quantitative RT-PCR was performed with the GoTaq Green Master Mix kit (Promega) on a CFX96TM Real-Time PCR Detection System apparatus (Bio-Rad). Three biological replicate samples were analyzed. For Northern blot analysis, total RNAs were separated on 7.5% polyacrylamide gel electrophoresis (PAGE) or denaturing agarose gel (2%). Digoxigenin (DIG)-labeled DNA probes specific for HSVd (nucleotides 2–301) or ASBVd (nucleotides 6–233) were used for detection. The probes were prepared using the PCR DIG Probe Synthesis Kit (Roche). Gel electrophoresis and blotting were carried out as described previously (97). Hybridization conditions and detection of mRNAs were as described in the DIG application manual supplied by Roche. All of the primers used in this study are listed in *SI Appendix*, Table S1.

Fractionation of Cytosolic and Nuclear Components and Western Blot Analysis. Fractionations of cytosolic and nuclear components were conducted according to the subcellular fractionation protocol provided by Abcam (https://www.abcam.com/ps/pdf/protocols/subcellular_fractionation.pdf). Briefly, fungal spheroplasts were suspended in a fractionation buffer [250 mM sucrose, 20 mM Hepes (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM dithiothreitol, and protease inhibitor mixture (Roche)] and passed through a 25-gauge needle 10 times. Nuclear pellet (nuclear-enriched fraction, P1) was obtained by centrifugation at 720 × *g* for 5 min. The supernatant was centrifuged at 10,000 × *g* for 15 min, and the resulting pellet was used as the cytoplasmic fraction.

Preparation of protein samples, sodium dodecyl sulfate/PAGE, electroblotting, and immunodetection for Western blot analysis were carried out as described previously (96). Histone H3 was detected using primary polyclonal anti-Histone H3 antibody (1:2,000; Abcam) and secondary polyclonal horseradish peroxidase-conjugated mouse anti-rabbit IgG (1:10,000; Abcam). Protein bands were visualized with a western ECL substrate kit (Bio-Rad).

Fungal Pathogenicity Assays. The virulence assay for *C. parasitica* and *V. mali* on apples was described previously (95). The pathogenicity assay for *F. graminearum* on maize shoots followed the method described previously (98).

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