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*Article*

# Mycoviruses as triggers and targets of RNA silencing in white mold fungus *Sclerotinia sclerotiorum*

**Pauline Mochama [1], Prajakta Jadhav [1], Achal Neupane[1] and Shin-Yi Lee Marzano [1,2,*]**

[1] Department of Biology and Microbiology, South Dakota State University, Brookings, SD USA
[2] Department of Horticulture, Agronomy, and Plant Sciences, South Dakota State University, Brookings, SD USA
* Correspondence: shinyi.marzano@sdstate.edu; Tel.: +01-605-688-5469

**Abstract:** This study aimed to demonstrate the existence of antiviral RNA silencing mechanisms in *Sclerotinia sclerotiorum* by probing wild-type and RNA-silencing-deficient strains of the fungus with an RNA virus and a circular DNA virus. Key silencing-related genes, specifically dicers, were disrupted in order to dissect the RNA silencing pathway and provide useful information on fungal control. Dicers *Dcl-1*, *Dcl-2*, and both *Dcl-1*/*Dcl-2*- genes were displaced by selective marker(s). Disruption mutants were then compared for changes in phenotype, virulence, susceptibility to viral infection, and small RNA accumulation compared to the wild-type strain. Disruption of *Dcl-1* or *Dcl-2* resulted in no changes in phenotype compared to wild-type *S. sclerotiorum*; however, the double dicer mutant strain exhibited slower growth. To examine the effect of viral infection on strains containing null-mutations of *Dcl-1*, *Dcl-2* or both genes, mutants were transfected with full-length RNA transcripts of a hypovirus SsHV2L and copies of a single-stranded DNA mycovirus-SsHADV-1 as a synthetic virus. Results indicate that the *ΔDcl-1*/*Dcl-2* double mutant which was slow growing without virus infection exhibited much more severe debilitation following virus infection. Altered colony morphology including: reduced pigmentation, significantly slower growth, and delayed sclerotial formation. Additionally, there is an absence of virus-derived small RNAs in the virus-infected *ΔDcl-1*/*Dcl-2* mutant compared to the virus-infected wild-type strain which displays a high percentage of virus-derived small RNA. The findings of these studies suggest that if both dicers are silenced, invasive nucleic acids which include mycoviruses ubiquitous in nature- can greatly debilitate the virulence of fungal plant pathogens.

**Keywords:** RNA silencing; gemycircularvirus; mycovirus; antiviral; dicer

## 1. Introduction

RNA-directed gene silencing down-regulates gene expression at the transcriptional and post-transcriptional level. RNA silencing or RNA interference is a mechanism involving the recognition of dsRNA by an RNase III domain containing Dicer enzyme which processes the dsRNA into small RNA (sRNA) duplexes of 18-30 nt in length. These sRNA duplexes are separated into two strands with one of the strands being loaded onto Argonaute proteins to target complementary nucleic acids in a sequence-specific manner.

There are two main biological functions of RNA silencing: the first is endogenous gene regulation in development, stress response, and suppression of transposons and repetitive elements to maintain genome integrity. The second role is to confer defense against invasive nucleic acids including viruses [1-3]. Endogenous gene regulation through RNA silencing has been confirmed in plants and animals but is still debatable for fungi because RNA-silencing gene disruption mutants often do not suffer lethal effects as in plants or animals. However, it is when these mutants are challenged with viruses that the antiviral role of RNA silencing genes becomes evident [4, 5]. Therefore, the most noticeable role of RNA silencing in fungi has been identified as an adaptive

defense function [5, 6]. Although the canonical RNA silencing pathway is deeply conserved, the presence of RNA silencing genes is less uniform in Kingdom Fungi. For instance, *Saccharomyces cerevisiae* has lost all the RNA silencing genes required to internalize a dsRNA mycovirus, L-A: a killer virus that produces a toxin which kills uninfected neighbor cells and leaves the infected cells immune to the toxin (reviewed in [7]). Within the same genera, one fungal species may be predicted to encode RNA silencing genes but another species may not (eg. *Ustillago hordei* vs. *U. maydis*) [8]. It could be circumstantial that endogenous gene regulation in fungi does not involve RNA silencing mechanisms, but this could partially be due to the existence of unidentified domains producing miRNAs that carry out this function.

The cellular components of RNA silencing have been elucidated in the model fungus *Neurospora crassa*. Two dicer orthologs were identified as *Dcl1* and *Dcl2* and shown to play a redundant role in transgene silencing [6]. However, efforts to demonstrate a role for RNA silencing in antiviral defense are lacking due to the absence of a mycovirus experimental system for this fungus. Although it has been determined that *Dcl2* is responsible for antiviral RNA silencing in the ascomycete, *Cryphonectria parasitica* [5], and *Dcl1* has been found to play the antiviral defense role in another ascomycete *Colletotrichum higginsianum* [4], there are currently no reports of evolutionarily conserved dicer homolog specific targets in fungi. Furthermore, no canonical PAZ (Piwi-Argonaute-Zwille) domain has been found in these fungal dicers which is atypical for Class III enzymes that are considered to be RNA silencing initiators in model organisms such as *Drosophila* (reviewed in [9]). Clearly more studies are needed to dissect the roles of RNA silencing in fungi.

*Sclerotinia sclerotiorum* is phylogenetically related to *N. crassa* and *C. parasitica* under phylum *Ascomycota* but in a different class, and its genome has been sequenced and annotated [10]. DNA transformation of *S. sclerotiorum* is straightforward. Moreover, *S. sclerotiorum* has been shown to support the replication of members of more than ten virus families including uniquely, a single stranded (ss)DNA virus, Sclerotinia sclerotiorum hypovirulence-associated DNA virus (SsHADV-1). This virus belongs to a new family, *Genomoviridae*, and has been associated with several infections caused by unknown agents (reviewed in [11]). Previously, a reverse genetics system was developed for a member of the *Hypoviridae* virus family, Sclerotinia sclerotiorum hypovirus 2 – lactuca (SsHV2-L) [12]. This diversity in mycoviruses that infect *S. sclerotiorum* allows for an examination of the effect of RNA silencing on viruses with a range of replication strategies in the same host. Antiviral RNA silencing protects an organism against virus infection, however, an outstanding question remains whether the core features against RNA and DNA viruses differ in fungi. In addition, a recent study demonstrated that by simultaneously silencing *Dcl-1* and *Dcl-2* genes in *Botrytis cinerea*, a close relative of *S. sclerotiorum*, the virulence of *B. cinerea* is greatly hampered due to the reduction in small RNA mediated cross-kingdom RNAi [13]. The two fungal dicer genes are redundant in generating pathogen small RNA effectors that hijack plant immunity [14] . As *Sclerotinia sclerotiorum* is closely related to *B. cinerea* [10], it is intriguing whether corresponding dicer gene(s) have the same effects on *S. sclerotiorum* virulence, small RNA processing, and antiviral defense. We now report the use of the *S. sclerotiorum* experimental system to investigate the role of antiviral RNA silencing in fungi.

## 2. Materials and Methods

**Fungal strains and culture conditions.** Cultures of *Sclerotinia sclerotiorum* wild-type strain DK3 and *Dicer* mutant strains were grown on potato dextrose agar (Sigma) at 20-22°C. The *ΔDcl-1* and *ΔDcl-2* mutant strains were maintained on PDA supplemented with 100µg/mL hygromycin B (Alfa Aesar) and the *ΔDcl-1/Dcl-2* strain was maintained on PDA supplemented with 100µg/ml hygromycin and 250µg/ml Geneticin (G418) [15].

**Construction of *Dcl-1*, *Dcl-2* and *Dcl-1/Dcl-2* null alleles.** *Sclerotinia sclerotiorum Dicer* genes (Ss1G_13747 and Ss1G_10369, respectively) were predicted based on homology to those identified in *Neurospora crassa* [8]. Deletion of Dicer genes was accomplished using the split marker recombination method which requires two DNA constructs for each gene deletion. To generate the *ΔDcl-1* disruption mutant, an 814bp long upstream region of the gene was amplified using primers F1-DCL1 and F2-DCL2 and a 663bp long downstream region of the gene was amplified using primers F3-DCL1

and F4-DCL1. F2 and F3 primers include 26-32bp of complementary sequence to the *Aspergillus nidulans* trpC promoter and terminator respectively. Plasmid pCSN43 containing the *hygb* gene flanked by the *Aspergillus nidulans* trpC promoter and terminator [16], obtained from Fungal Genetics Stock Center (Manhattan, KS, USA), was used to amplify the marker gene and promoter and terminator sequences. Primers PtrpC-F and HY-R were used to amplify a 1.2kb region of the marker gene including the promoter and primers YG-F and TrpC-R were used to amplify a 1.3kb region of the gene including the terminator. Both amplicons represent roughly two thirds of the marker gene and contain 400bp of overlapping sequence. The F1-F2 amplicon was then fused to the PrtpC-HY amplicon and the F3-F4 amplicon was fused to the YG-TrpC amplicon using the overlap extension PCR protocol described by Fitch et al. [17]. In the final round of PCR, nested primers were used to give the final gene deletion constructs representing 600bp of upstream homologous sequence fused to two- thirds of the *hygb* gene in the first construct and 600bp of downstream sequence fused to two-thirds of the *hygb* gene in the second construct. Disruption of the *Dcl-2* gene was accomplished with constructs generated as described above using a separate set of primers (Supplemental Table 1). Final *Dcl-2* gene deletion constructs included 830bp of sequence homologous to the upstream region of the gene and 1kb of downstream homologous sequence.

The *ΔDcl-1/Dcl-2* mutant was generated by knocking out the *Dcl-1* gene in a *ΔDcl-2* mutant without using the split marker method. *ΔDcl-2* protoplasts were transformed with a single gene-deletion DNA cassette generated using overlap extension PCR. (Primers listed in Supplemental Table 1). The DNA construct contained 600bp of sequence homologous to the upstream region of the *ΔDcl-1* gene and 600bp of downstream homologous sequence fused to the G418 resistance gene under the control of the *Aspergillus nidulans* trpC promoter. Recombination occurred at the homologous arms flanking the resistance gene and the *Dcl-1* gene was subsequently replaced by the G418 gene. G418 is an aminoglycosidic antibiotic similar to hygromycin but with no cross-resistance. The G418 resistance gene was amplified from pSCB-TrpC-G418 [15].

**Fungal transformation.** Gene deletion cassettes were transformed into wild-type *S. Sclerotiorum* protoplasts using polyethylene glycol (PEG)-mediated transformation. Protoplasts were prepared as described by Chen et al [18] with a digestion time of 3h at RT using the lysing enzyme from *Trichoderma harzianum* (Sigma). PEG-mediated transformation of gene deletion constructs into fungal protoplasts was performed following the protocol described by Rollins et al. [19] with some modifications [20]. Briefly, following PEG transformation 3mL of liquid regeneration media (RM) was added to protoplasts and the suspension incubated at 28°C with shaking (100rpm, 2-4hrs). Molten RM (45°C) was then added to a final volume of 20mL and the mixture poured into a petri dish. Plates were grown at 28°C for 12h and then overlaid with 5mL molten RM containing hygromycin for single *Dicer* gene mutants and hygromycin and G418 for the double *Dicer* mutant. Final antibiotic concentrations used for fungal selection were 100µg/mL for hygromycin and 250 µg/mL for G418. Colonies were transferred to potato dextrose agar (PDA) plates supplemented with the appropriate antibiotic and transferred at least three times.

To confirm gene deletions, DNA was extracted from transformants and PCR was conducted using primer pairs- F1 and F4, F1 and HY-R, and YG-F and F4 to amplify the target regions (Supplemental Table 1). PCR amplicons were compared in size to the wild-type gene amplicon. Amplicons of the correct size (indicating successful gene deletion) were sequenced to confirm integration of the marker gene into the correct region. Repeated hyphal tipping and nested PCR were performed to ensure monokaryotic gene deletions in each gene disruption experiment.

**Phenotypic characterization of gene deletion mutants.** Growth assays were conducted on at least 3 replicates each of wtDK3, *ΔDcl-1*, *ΔDcl-2* and *ΔDcl-1/Dcl-2* cultures. 5-mm PDA discs were taken from the edges of actively growing 2-day old mutant and wild-type cultures and inoculated onto fresh PDA plates. Hyphal diameter was measured 24h, 48h and 72h post inoculation.

**Virulence assay of gene deletion mutants.** Pathogenicity assays were conducted by placing a single 5-mm PDA disc from the edge of an actively growing, 2-day-old culture on the center of a freshly harvested canola leaf or a detached center leaflet (4 to 5 cm long) from the first trifoliate leaf of a soybean or sunflower seedling. At least 3 replicates of the leaves were incubated at 20±1ºC in a

growth chamber with a 12h light-12h dark photoperiod. Lesion size was calculated 24h, 48h, and 72h post inoculation by averaging two perpendicular lesion diameters.

**Transfection of mutants with in vitro transcripts of SsHV2**. *In vitro* transcripts of SsHV2-L were synthesized and transfected into wtDK3 and *Dicer* mutant protoplasts following a published procedure [12]. After >6 transfers, viral infection was confirmed by extraction of total RNA using RNeasy Mini Kit (Qiagen) followed by reverse transcription using Maxima H Minus Reverse Transcriptase (Thermofisher) and PCR to amplify a 1.1kb region corresponding to the viral genome. PCR amplicons were sequenced to confirm identity with the SsHV2-L genome.

**Construction of an infectious clone of SsHADV-1 and transfection of mutants with SsHADV-1.** The 2,166 nt genome of SsHADV-1 was chemically synthesized by GeneArt (ThermoFisher Scientific) in three segments with the ends flanked by overlapping unique restriction enzyme cutting sites, based on GenBank accession NC_013116.1. The 1-mer genome of SsHADV-1 was reconstructed by ligating three fragments containing restrictions sites *Spe*I, *Apa*I, *Eag*I internal to the viral genome. Using primers 33F and 3R, the viral genome was amplified and cloned into pJET1.2 as a 1 copy (1-mer) clone. A second copy of the genome was amplified by primers SV2F and 3R'-NotI (Supplemental Table 1 with procedure follows). Both the 1-mer clone and the second copy of the genome were digested with *Spe*I and *Not*I and ligated to form a tandem 1.9-mer clone which was then used for transfection (Fig. S2(A)). Detailed procedure is described in Supplemental Data. There was no long concatemers formed because directional cloning with noncomplementary sticky ends was performed. Fungal protoplasts (wtDK3) were transfected by PEG-mediated transformation. Infectivity was confirmed by inverse PCR to amplify a 2,166 bp fragment (Fig. S2(B)), which suggests a recombined DNA template was formed. Fungal DNA was extracted and served as the template for rolling circle amplification (RCA) (Illustra Templiphi, GE Health, CA) using random primers and a single cut restriction enzyme digestion, resulted in a 2,166 bp fragment, indicating no concatemer exists after transfection. The RCA product was subjected to Sanger sequencing to confirm the infectivity. Additionally, after >6 serial transfers to fresh PDA plates, the presence of the replicating virus in fungal hyphae was confirmed by PCR amplification using SsHADV-1-specific primers and sequencing. Mutant cultures were infected with SsHADV-1 by extracellular transmission or virus particles from infected wtDK3 growth medium into fungal hyphae. Specifically, plugs were taken from the agar surrounding an SsHADV-1 infected culture of wtDK3 and placed adjacent to plugs taken from the edges of actively growing mutant cultures on fresh PDA plates with corresponding selective antibiotics.

**Preparation of small RNA libraries and sequencing analysis.** To understand the roles played by dicer genes in small RNA processing, we sequenced libraries prepared from small RNA extracted from wtDK3 and *ΔDcl-1/Dcl-2* strains infected with SsHADV-1 and SsHV2L as well as virus-free strains. Three replicates of each virus-infected strains were sequenced and two replicates of each virus-free strains. Small RNAs were extracted from 4-day old mycelia using mirVana miRNA Isolation kit (ThermoFisher Scientific) following the manufacturer's protocol. Libraries were prepared using the NEBNext small RNA Library Kit (NEB). The libraries were pooled and sequenced in one lane for 50-nt single-end reads on an Illumina HiSeq4000 at Keck Center, University of Illinois. The sequences have been deposited in NCBI (the accession will be available during review). Demultiplexed reads were removed of the 3' adaptors by Trimmomatic [21]. Loci producing sRNAs were identified by ShortStack [22].

## 3. Results

### *3.1. Generation of disruption mutants for DCL genes.*

Dicer-like genes in *S. sclerotiorum* were disrupted using the homologous recombination method for gene displacement (Fig.1A) to generate *ΔDcl-1*, *ΔDcl-2* and *ΔDcl-1/Dcl-2* mutants directly from wild-type strain DK3 without using a *Δ*Ku80 strain. Dicer genes were confirmed to be disrupted by extracting DNA from multiple transformants and performing PCR amplification using F1 and F4 primers for initial screening. When the target locus was amplified, wild-type and mutant PCR

amplicons differed in size confirming gene deletion (Fig. 1B). PCR screening and Sanger sequencing of PCR amplicons confirmed integration of the gene-replacement cassettes into the target region and ruled out ectopic integration of the *hygb* gene. Finally, nested PCR was used to rule out heterokaryotic mutation in which both the original dicer genes and disrupted genes occur in different nuclei within fungal hyphae (Supplemental Table 1). This step was necessary because each transformed protoplast can contain multiple nuclei. Once a monokaryotic mutation was confirmed, further characterization of colony morphology and pathogenicity was carried out.

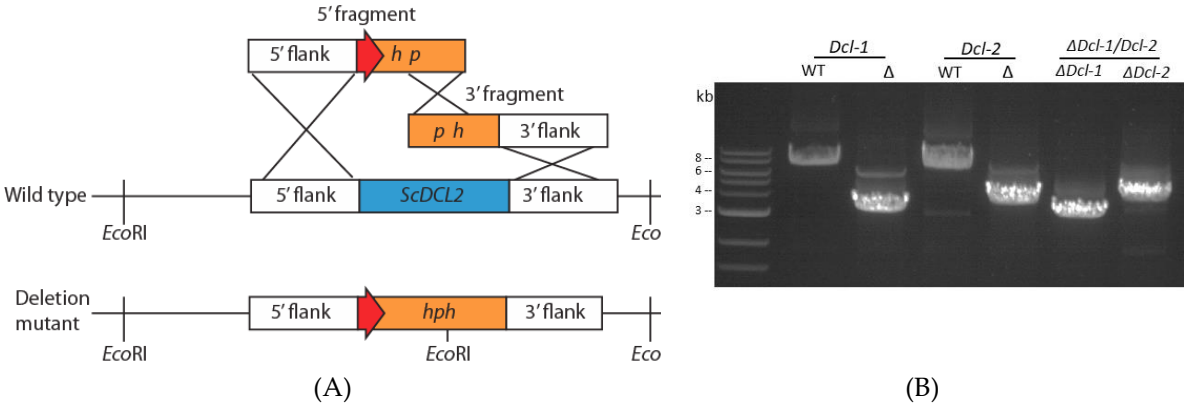


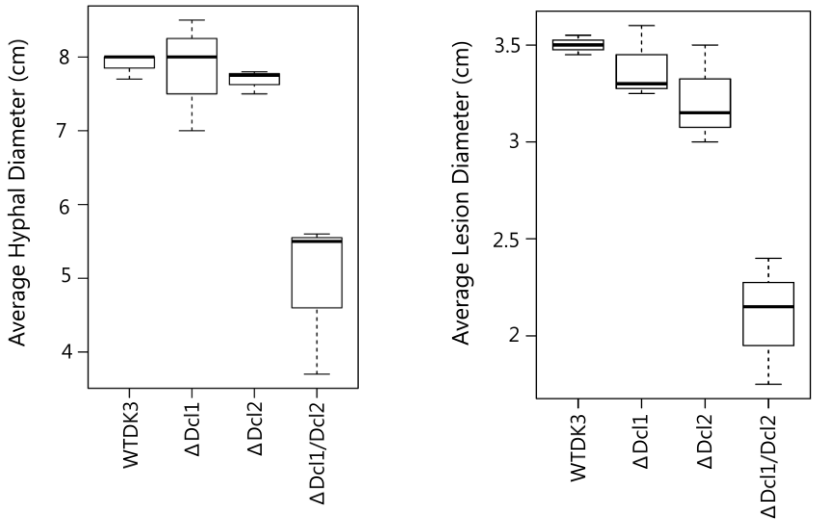

**Fig.1** (A) Generation of deletion mutants for dicer genes in *S. sclerotiorum* using the split-marker gene replacement method and (B) Electrophoresis gel image of PCR amplification to confirm Dicer gene disruption using F1-F4 primer pairs. As shown, amplicons of wild-type *Dcl-1* and *Dcl-2* genes (7.7kb and 7kb respectively) and deletion alleles (3.3 and 3.9kb) differ in size. Lanes 5 and 6 show deletion alleles (3.1 and 3.9kb) in the double Dicer mutant.

### *3.2. Effect of Dicer-like gene disruption on S. sclerotiorum phenotype.*

We compared growth rate and colony morphology in dicer mutants and the wild-type strain, DK3 (denoted as wtDK3) on PDA. Single mutants of *ΔDcl-1*, *ΔDcl-2*, or wtDK3 exhibited similar growth rates, whereas the double *ΔDcl-1/Dcl-2* disruption mutant exhibited significantly slower growth at 72h as indicated by measurements of hyphal diameter ($p<0.05$) (Fig. 2A). No significant difference in phenotype was observed in *ΔDcl-1* or *ΔDcl-2* compared to wtDK3, whereas *ΔDcl-1/Dcl-2* mutant showed more hyphal branching and feathery colony morphology.

### *3.3. Effects of Dicer-like gene disruptions on S. sclerotiorum pathogenicity.*

To test the pathogenicity of *S. sclerotiorum* dicer mutants, plugs taken from actively growing cultures were used to inoculate detached leaves. Lesion size data collected 24 h, 48 h, and 72 h post inoculation showed that there was no difference in the sizes of lesions produced on canola leaves by the single mutants *ΔDcl-1* or *ΔDcl-2* compared to wtDK3. However, significantly smaller lesions were produced by the *ΔDcl-1/Dcl-2* double mutant compared to those produced by wtDK3 ($p<0.05$) (Fig. 2B).




(A) (B)

**Fig. 2** (A) Average mycelial growth of *S. sclerotiorum* wild type and dicer gene disruption mutants grown on PDA for 4 days. (B) Lesion diameter measurements 72 hpi comparing wtDK3, Δ*Dcl-1*, Δ*Dcl-2* and Δ*Dcl-1*/*Dcl-2* virus-free cultures inoculated on canola leaves.

*3.4. Transfection of Dicer gene deletion mutants with SsHV2-L or SsHADV-1 viruses consistently result in severe debilitation in the ΔDcl-1/Dcl-2 mutant.*

To examine the effect of viral infection on strains containing disruption of *Dcl-1*, *Dcl-2* or both genes, mutants were transfected by hyphal fusion with SsHV2-L or SsHADV-1 infected mycelia. SsHADV-1 was generated by a synthetic dimer genome approach. As shown in Fig. 3A, the *ΔDcl-1* or *ΔDcl-2* mutants infected with either mycoviruses, SsHV2-L or SsHADV-1, showed no significant difference in growth and morphology compared to virus-infected wtDK3. In sharp contrast, the *ΔDcl-1/Dcl-2* mutant showed severe debilitation following virus infection as evidenced by significantly slower growth and hypovirulence on three different crop species (Fig. 3B-3D).

*3.5 Infectious clone of SsHADV-1 causes severe debilitation and significantly reduced virulence in wtDK3 at lower temperatures*

The transformed and serial transferred fungal DNA was extracted to determine the infectivity of the infectious clone. Rolling circle amplification with XbaI digestion resulted in a 2.2 kb band. Also, Inverse PCR amplification of the viral genome and sequencing confirmed that the synthetic virus is identical to the Chinese strain also found in soil DNA extracts from two locations of South Dakota, USA (data not shown). Initial viral infection at room temperature (~24 $^{0}$C) resulted in asymptomatic infection in wtDK3, however incubation at lower temperatures (20 $^{0}$C) in a growth chamber resulted in severe debilitation including slow growth and reduced virulence.

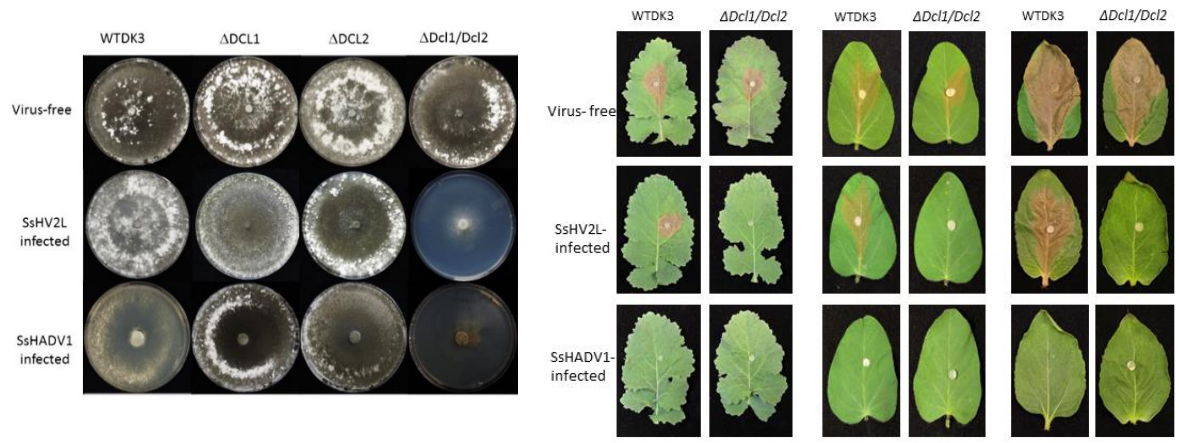



**Fig. 3** (A) Colony morphology of virus-free and virus- infected gene deletion mutants. (Top row) Virus-free wtDK3, *ΔDcl-1*, *ΔDcl-2* and *ΔDcl1/Dcl2*. (Middle row) wild-type and mutant strains infected with hypovirus SsHV2-L. (Bottom row) wild-type and mutant strains infected with SsHADV-1. Cultures were grown for 7days on PDA at room temperature. (B) Virulence assays on detached canola leaves, (C) detached soybean leaves and (D) detached sunflower leaves. Plugs were taken from the edge of actively growing wtDK3, *ΔDcl-1* (not shown), *ΔDcl-2* (not shown) and *ΔDcl-1/Dcl-2* cultures and inoculated onto detached leaves stored at 20±1ºC. Lesion size was measured 36h post-inoculation.

### *3.6. Double dicer disruption mutant had reduced 21-24 nt sRNA accumulation.*

To examine whether sRNA accumulation is affected by disrupting both dicers, sRNA sequences were profiled by size distribution and 5′ terminal nucleotide in the virus-free *ΔDcl1/Dcl2* mutant and wild-type strain. Although the 5′ terminal nucleotide remained uracil-biased, the size distribution of small RNAs was drastically changed in the double dicer mutant compared to the wild-type strain (Fig 4A and 4B). Specifically, there was a reduction in the 21-24 nt sRNA fraction in the double mutant compared to the wild-type strain. Notably, similar to *B. cinerea*, sRNA production in *S. sclerotiorum* is not completely eliminated even after both dicers are disrupted.

### *3.7. SsHADV-1 and SsHV2-L are both processed by dicer(s)*

Sequence analysis of the small RNAs produced by either SsHADV-1 or SsHV2-L infected wtDK3 and double dicer mutants showed major differences. In each barcoded library, 5 to 10 million of reads passed the QC. In average, 14.4% of the total small RNA reads from the SsHV2L- infected wild-type strain were derived from SsHV2L, whereas 2.26% of the total small RNA reads in the SsHADV-1 infected wild-type strain were derived from SsHADV-1. 22-nt sRNAs were the most abundant for both virus-infected wild-type strains (Fig. 4C and D) with a preference (>90%) for uracil at the 5′ position. 77.89% of SsHV2-L derived sRNA aligned to the negative strand, and 22.01% to the positive strand (Fig. 4E). SsHADV-1 derived sRNA reads were aligned non-uniformly from both strands (Fig. 4F) with strand biases for the negative strand in the first 350 nt of the coat protein encoding gene, and for the positive strand between 1000 nt – 2200 nt for the replicase protein encoding gene, but overall 51.6% of the reads aligned to the published positive strand sequence and 48.3% to the negative strand.

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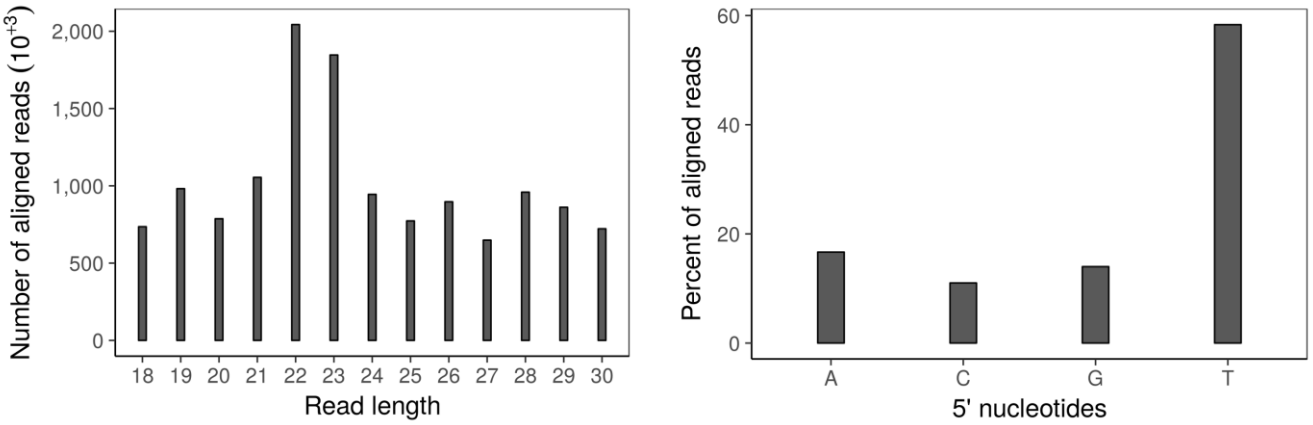
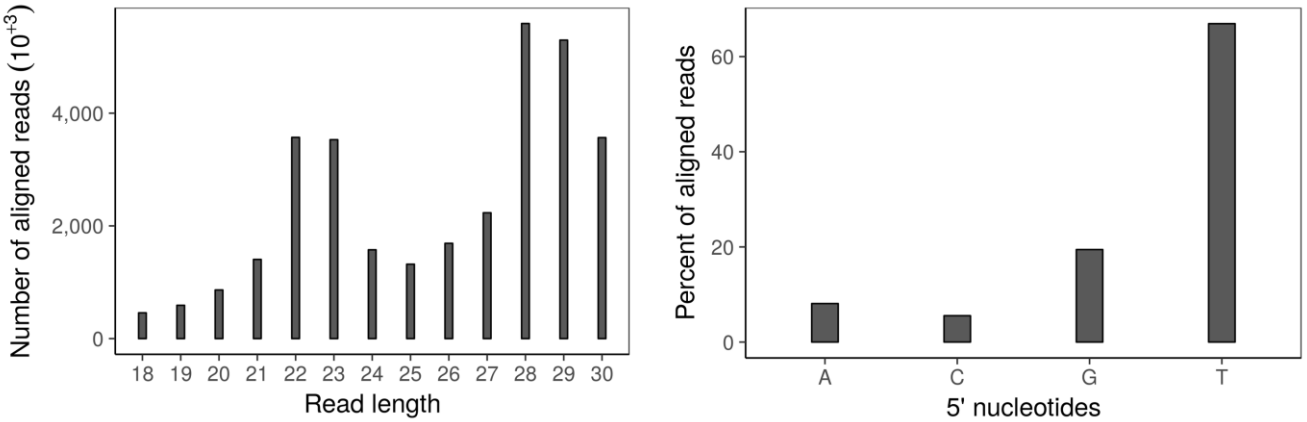
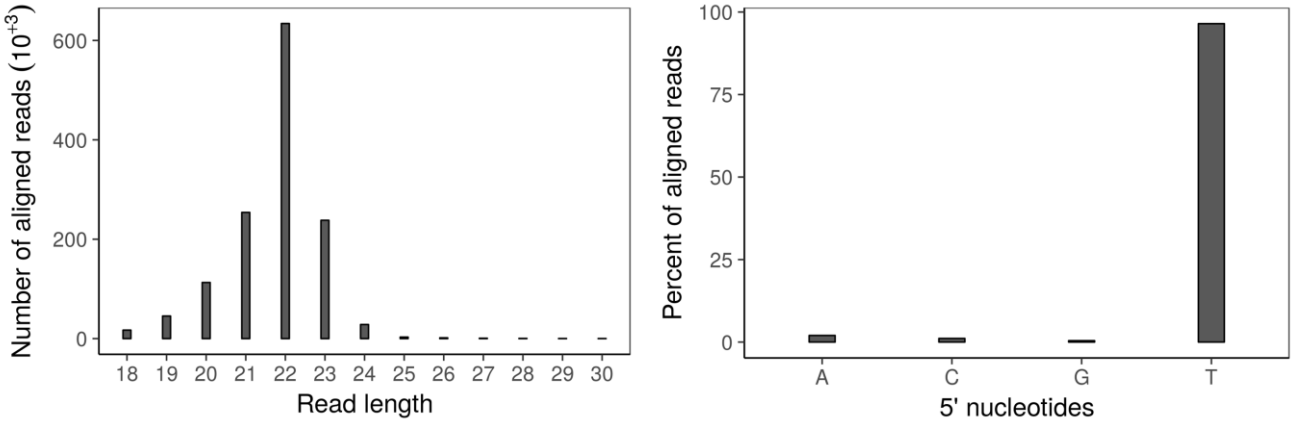



(A)




(B)




(C)

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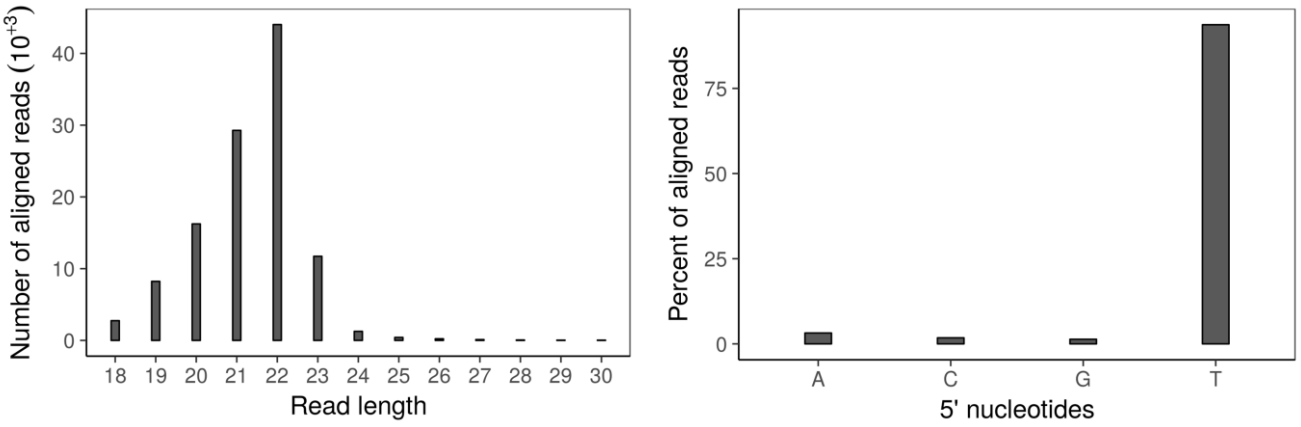
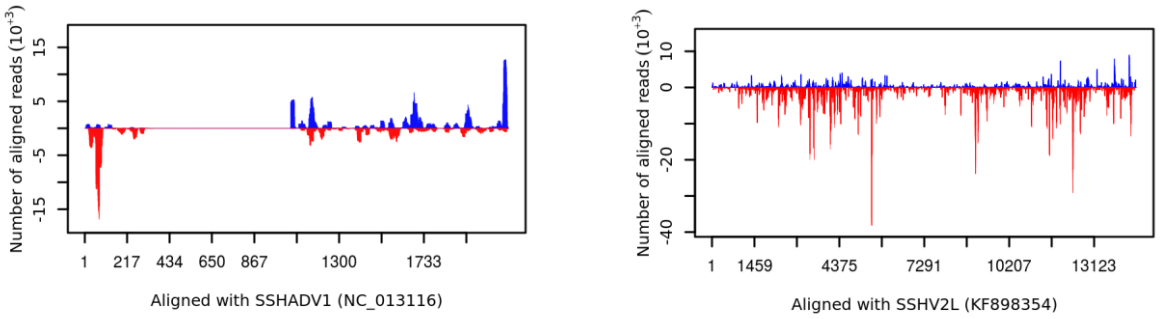



(D)




(E)

**Fig. 4** Small RNA (A) Size distribution (left) and frequency of 5′ terminal nucleotides (right) of small RNAs in wtDK3. (B) Size distribution (left) and frequency of 5′ terminal nucleotides (right) of small RNAs in *Δdcl1/dcl2* disruption mutant. (C) Size distribution (left) and frequency of 5′ terminal nucleotides (right) of small RNAs aligned to SsHV2-L genome. (D) Size distribution (left) and frequency of 5′ terminal nucleotides (right) of small RNAs aligned to SsHDV-1 genome. (E) Distribution of small RNA reads that aligned to the SsHV2-L genome in plus and minus strands (left). Distribution of small RNA reads that aligned to the SsHADV-1 genome in plus and minus strands (right). Bars above zero indicate alignment to the positive strand, and bars below zero indicate alignment to the negative strand.

## 4. Discussion

We previously compared the three hypovirus strains of SsHV2 and detected inter- and intra-specific recombination near the 5′ end of the genome where a putative virus silencing suppressor is predicted to be located, suggesting the existence of RNA silencing in the host fungus [12]. Here, we have determined that a double dicer disruption mutant, but not single dicer disruption mutants, has slower hyphal growth than the wild type. This can be partially explained by the significant shift in sRNA accumulation observed in the double mutant since small RNAs generated by dicers may play important roles in gene regulation and other biological functions (reviewed in [23]). A similar change in phenotype was also observed in another member of *Sclerotiniaceae*, *Botrytis cinerea*, [13] that resulted in slower growth and reduced pathogenicity when both dicer genes are disrupted. Further, we demonstrate that the double dicer disruption mutant is highly debilitated upon virus infection. Redundancy in dicer antiviral function has not been reported in fungal species; only a redundancy

in dicer function in transgene-induced gene silencing has been found in *Neurospora crassa* so far [6]. Dicer redundancy in antiviral RNA silencing of *S. sclerotiorum* could be validated by small RNA sequence analysis in virus-infected single dicer knockout mutants to demonstrate that the small RNA accumulations are identical to the wild-type strain due to the presence of an intact dicer gene in each mutant. This further investigation is outside the scope of this study, however. Once validated, then dicer redundancy would appear to have evolved in a specific lineage of ascomycetes as a conserved anti-invasive nucleic acid mechanism because it is not the case for *Cryphonectria parasitica* [5] nor *Colletotrichum higginsianum* [4].

In this study, we demonstrate that a ss(+)RNA virus and notably, a ssDNA virus are not only the triggers but also the targets of RNA silencing based on virus-derived small RNA production in the wild-type strain that is not observed in the double dicer disruption mutant. This provides solid evidence that *S. sclerotiorum* can degrade both a ss(+)RNA virus and a ssDNA virus by either Dcl-1 or Dcl-2 enzymes. Further studies will involve introducing mutations in either DNA or RNA viral genomes and constructing disruption mutants of RNA-Dependent-RNA-Polymerase (RDR) proteins. The latter will confirm the involvement of host RDR proteins in the production of dsRNA using RNA transcripts from both DNA and RNA viruses. These RDR-produced dsRNA act as templates to trigger the RNA silencing pathway which effectively targets the invasive nucleic acid. A hypothesis that awaits to be tested is whether the production of dsRNAs from viral RNA transcripts of the ssDNA virus presumably by fungal RDR proteins, sequesters the viral transcripts and hinders translation of viral proteins.

Only one of the three small RNA libraries from SsHADV-1 infected wtDK3 cultures had a well accumulated small RNA profile. Acute/initial SsHADV-1-infection is marked by severe debilitation, with sectoring growth and an absence of virus-derived small RNA production. This is followed by a strong host immune reaction resulting in the silencing of viral nucleic acids and the remission of acute symptoms. Virus-derived small RNAs become detectable in this latter stage. The two samples with no virus-derived small RNAs detected were possibly obtained from debilitated, sectoring hyphae that had not progressed to symptom remission. Plant geminivirus, Pepper gold mosaic virus, is also associated with a recovery phenotype in plant hosts accompanied by decreased viral DNA and RNA titers which correspond with the presence of virus-derived small RNAs [24]. Hotspots were observed in virus-derived small RNAs from this virus with a 700 bp gap similar to the small RNAs profiled for tomato yellow leaf yellow curl china virus [25]. Opposite strand biases were also observed between the two clusters, possibly due to the fact that the direction of transcription for the two genes is opposite.

A somewhat surprising finding is that upon the deletion of both dicer genes (without virus infection), sRNAs are still being produced. A similar observation was made in *Botrytis cinerea* following deletion of both its dicer genes [13]. By conserved domain search, we found a putative RNaseL (Ire1p) gene (GenBank Ss1G_04823), also an RNA endonuclease III that is similar to a yeast protein, that might be responsible for the remaining small RNA processing. This protein could be involved in fungal RNA splicing [26].

Mycoviruses belonging to the families *Hypoviridae* and *Genomoviridae* are widespread, especially the later having been reported to associate with eukaryotes including vertebrates and invertebrates, in addition to fungi. *Genomoviridae* is considered part of an emerging group of infectious agents {Halary, 2016 #149}. The host of the sole representative of the Family *Genomoviridae* so far, i.e. SsHADV-1, is *S. sclerotiorum*. The results presented here suggest the therapeutic potential of dicer proteins, and the importance of other RNA silencing genes that interact with viral nucleic acids to produce dsRNA triggers of RNA silencing. An understanding of how and when the RNA silencing pathway is triggered to target this group of DNA viruses will provide insights into the arms race of virus-host co-evolution. The importance stems from that this group of circular ssDNA viruses have polyphyletic origin [27] and can replicate in distant hosts [28].

**Supplementary Materials:** The following are available online, Figure S1: title, Table S1: Primers used in this study. Table S2: Primers and expected sizes of nested PCR reactions performed to confirm monokaryotic deletion of dicer genes with the corresponding gel image in Figure S1. Figure S1: Electrophoresis gel image to confirm monokaryotic deletion of dicer genes by nested PCR. Lanes as indicated in table S1. Lane 1~12 from right to left on the upper half of the agarose gel image. Lane 13~18 from right to left on the lower half of the agarose gel image. Ladders are shown on the farthest right and left lanes with corresponding size labelled. Figure S2: Inverse PCR product amplified using SsHADV-1-specific primers to confirm the infectivity of the infectious clone from a >6 times transferred culture. A 2.2 kb band was amplified and confirmed by Sanger sequencing. The product shows the dimer clone has recombined circular template of SsHADV-1 genome and demonstrates the infectivity of the infectious clone assembled from synthetic DNA. Left Lane: 1kb ladder. Right Lane: 2.2 kb amplicon.

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**Author Contributions:** PKM and SLM conceived and designed the experiments; PKM and PKJ performed the experiments; AN analyzed the small RNA data; PKM and SLM wrote the paper.

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