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Dissecting RNA Silencing Pathways in *Sclerotinia Sclerotiorum*

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DISSECTING RNA SILENCING PATHWAYS IN *SCLEROTINIA SCLEROTIORUM*

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABSTRACT

DISSECTING RNA SILENCING PATHWAYS IN *SCLEROTINIA SCLEROTIORUM*

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RNA silencing, also known as RNA interference, is an essential mechanism in plants, animals and fungi that functions in gene regulation and defense against foreign nucleic acids. In fungi, RNA silencing has been shown to function primarily in defense against invasive nucleic acids. RNA-silencing- deficient fungi show increased susceptibility to virus infection. Plant pathogenic fungi also utilize RNA silencing to silence plant host immunity genes through the delivery of fungal small RNAs into plants. This cross-kingdom RNA silencing facilitates fungal infection of plants. Overall, these findings demonstrate the significant contributions of fungal RNA silencing pathways to fungal virulence and viral defense. This study dissects the RNA silencing pathway in *Sclerotinia sclerotiorum* by disrupting its key silencing genes using the split-marker recombination method in order to probe the contributions of these genes to fungal virulence and viral defense mechanisms. Following gene disruption, mutants were studied for changes in phenotype, pathogenicity, viral susceptibility, and small RNA processing compared to the wild-type strain, DK3. Results indicated that the double dicer mutant (Δ*dcl-1/dcl-2)* displayed slower growth and reduced pathogenicity before viral infection, and that these symptoms were greatly pronounced following viral infection. Among the argonaute mutants, the Δ*ago-2* mutant had significantly slower growth and virulence prior to and following virus infection. Additional studies indicated that the virus-infected wild-type strain accumulated virus-derived small RNAs (vsiRNAs) with

distinct patterns of internal and terminal nucleotide mismatches. These results together indicate that *S. sclerotiorum* has robust RNA silencing mechanisms that function primarily in antiviral defense but also in endogenous gene regulation processes. This finding expands our overall understanding of *S. sclerotiorum* and has important implications for any current or future uses of mycoviruses as biological control agents, an emerging area of interest in fungal control research.

CHAPTER ONE: LITERATURE REVIEW

Sclerotinia sclerotiorum*: background, problem, pathogenesis and current remedies*

Sclerotinia sclerotiorum is an ascomycetous, necrotrophic fungal plant pathogen that can infect 450 plant species in 75 families including important commercial crops such as oilseed rape, sunflower, soybean, and lettuce (1). *S. sclerotiorum* predominantly infects dicotyledonous plants, however, a handful of monocotyledonous plants such onions and tulips can also be infected by this pathogen (2). *S. sclerotiorum*, also known as white mold, can infect plants at various stages of development including seedlings, flowering plants, and fruits during development and post-harvest (3). Infected plants develop dark lesions which develop into necrotic tissues and eventually patches of fluffy white mycelia- a key indicator of *S. sclerotiorum* infection- appear (2). The losses caused by this devastating pathogen have exceeded $200 million in the United States in some years (2) and there have been reports of Sclerotinia disease infecting 4.7 million ha annually in China (4). The success of this plant pathogen can be attributed to a number of factors including: a large and diverse susceptible host population and the production of sclerotia- hardened masses of mycelia that allow the fungus to withstand adverse conditions and continue to reproduce during favorable conditions

The production of sclerotia is a key pathogenicity and survival determinant for *S. sclerotiorum*. These tough, melanized aggregates of mycelia are produced by the fungus during conditions such as limited nutrient availability and are capable of surviving in soil for up to eight years (5). Sclerotia can withstand adverse conditions including low temperature, microbial activity, low moisture and UV irradiation (6). One of the few detriments to sclerotial survival is flooding. Under flooding conditions, sclerotia may

decay completely within 24-45 days (3). Sclerotial formation is pH dependent and under neutral or alkaline pH, sclerotial formation is inhibited (7). Sclerotia produced at the end of the growing season overwinter in soil and then germinate when conditions are favorable during the next growing season to initiate disease. Sclerotia are capable of germinating into vegetative, infective mycelia under certain conditions. This constitutes myceliogenic germination and can initiate infection in roots and stems that are in proximity to the sclerotia (2). However, sclerotia primarily germinate carpogenically to produce apothecia which produce ascospores that are released in the air and that subsequently infect new plants (6). Ascospores are the primary means by which infection is initiated and spread among crops (6). Environmental conditions that regulate sclerotial germination include soil temperature and moisture (3). For this reason, disease is often initiated when the canopy closes because this maintains cooler temperatures and high soil moisture which encourage sclerotial development. Ascospores require a film of water and an exogenous nutrient source to germinate on plants, and flowering crops with senescing parts are ideal sources of moisture and nutrients (3). Furthermore, flowering of crops occurs around the same time the canopy closes which facilitates the rapid spread of infection.

Mycelia can penetrate the cuticle of the host plant using enzymes, mechanical force via appressoria, or by invading via stomata (2). Cell-wall-degrading enzymes and oxalic acid produced by the fungus facilitate colonization. Oxalic acid production is a key virulence factor for *S. sclerotiorum*, and mutants deficient in oxalic acid production are non-pathogenic (8). Oxalic acid decreases the extracellular pH which enhances the

activity of CWDEs, inhibits early plant defenses, induces stomatal opening by influencing guard cell function and weakens plants due to the acidic conditions (2).

The diseases caused by this fungal pathogen have not been adequately controlled by conventional technologies thus far because: 1) there has been little success in generating resistant cultivars due to low or only partial levels of natural resistance and low heritability in host populations, 2) efficient fungicide application into canopies and soil is challenging and the sporadic nature of ascospore-initiated disease outbreaks makes it difficult to correctly time the fungicide application window, and 3) the development of fungicide-resistant isolates is a growing problem (2, 9, 10). Furthermore, sclerotia which are hardy and important for the spread of infection may be small and hard to detect and eliminate from soil.

Ongoing efforts to address the challenges presented by *S. sclerotiorum* have focused on breeding research aimed at engineering resistant hosts and studies aimes at formulating novel synthetic fungicides that can target and disrupt essential cellular processes in the fungus. However, more effective and sustainable strategies are actively being sought including the use of biological control agents such as mycoviruses. Molecular studies into fungal virulence and survival determinants are also ongoing and techniques such as polyethylene glycol-mediated transformation and agrobacterium-mediated transformation have made gene transfer, gene knockout and insertional mutagenesis studies possible in this pathogen. This study utilizes some of these tools to study the RNA silencing pathway in *S. sclerotiorum.*

RNA silencing in fungi

RNA silencing, also known as RNA interference, was discovered in 1998 and has since been established as an important regulator of gene expression through mRNA degradation, translation inhibition, and chromatin remodeling (11). RNA silencing also functions to control invasive nucleic acids such as viruses, transgenes and transposons (12). RNA silencing occurs in all eukaryotes but has not been observed in prokaryotes (11). It involves the processing of double-stranded RNA (dsRNA) or hairpin RNA into short 21-24nt long RNA molecules by RNAse-III endonucleases known as Dicers. These small RNA molecules known as small-interfering RNA (siRNA) or microRNA (miRNA) - depending on their source- complex with Argonaute proteins to form RNA-induced silencing complexes (RISC) that direct RNA degradation or translational repression of complementary RNA sequences (13). In plants, RISCs also direct the DNA methylation of homologous target genes in a pathway known as the RNA directed DNA methylation (RdDM) pathway (14). The source of miRNAs is imperfect short hairpin RNA formed by complementary regions of an endogenous primary miRNA transcript in the nucleus (14). On the other hand, endogenous or exogenous long dsRNAs are the precursors of siRNAs formed by Dicers (14). These dsRNA siRNA precursors may be of viral origin, and in plants and fungi the siRNA-directed RNA degradation pathway appears to function predominantly in antiviral defense (14, 15). RNA-dependent RNA polymerases (RDRPs) are important components of the RNA silencing pathways of plants, nematodes and fungi. dsRNA precursors can be synthesized from endogenous or viral single-stranded RNA (ssRNA) by RDRPs to initiate the production of secondary siRNAs that amplify and propagate the RNA silencing response (14).

For a long time, fungi were said to lack an miRNA pathway for endogenous gene regulation and it was concluded that RNA silencing systems in fungi functioned almost singularly in defense against viruses, transposons and transgenes. However miRNA-like (milRNAs) molecules, exon-derived siRNAs, and other classes of endogenous small RNAs have been found in fungi, and these have been shown to regulate the expression of fungal genes (16). *Sclerotinia sclerotiorum* and other important phytopathogenic fungi have been shown to produce milRNAs and other endogenous small RNAs generated by the components of the RNA silencing machinery such as Dicer and Argonaute proteins (16).

The number of silencing gene paralogs (dicers, argonautes and RDRPs) varies considerably within fungi. While a number of fungi possess multiple RNA silencing components, others lack all or most of the components. The ascomycetes *Saccharomyces cerevisae, Candida lusitaniae* and the basidiomycete, *Ustilago maydis* lack dicer, argonaute and RDRP homologs (11). The model filamentous fungus *Neurospora crassa* possesses two argonaute homologs, two dicer homologs and three RDRPs (12). Similarly, two argonaute homologs, two dicer homologs and three RDRPs have been identified in *Sclerotinia sclerotiorum.* This suggests that RNA silencing pathways have diversified significantly within fungi.

In the RNA silencing response to viral infection, virus- derived small interfering RNAs (vsiRNA) guide the degradation of complementary viral genomic sequence. Thus viruses are both inducers and targets of RNA silencing. It has been suggested that vsiRNAs are generated from dsRNA replicative forms of viruses, internal hairpin-loop structures within single-stranded viral RNA, or from dsRNA produced by host RDRPs

from viral nucleic acids (12). Viruses on the other hand have evolved strategies to counteract RNA silencing defense mechanisms. These include proteins with dual functions in everyday viral processes and the inhibition of RNA silencing (14). Viral suppressors of RNA silencing (VSR) interfere with RNA silencing by binding dsRNA, physically binding to and inhibiting Argonaute proteins, or inhibiting dsRNA processing by Dicers (14). The debilitation seen in fungal or plant hosts following viral infection is likely partly due to the action of VSRs and the results of disrupted RNA silencing machinery including increases in viral titer and changes in endogenous gene regulation. It has also been reported that vsiRNAs with some amount of sequence homology to host mRNAs can result in the silencing of host genes (16). This phenomenon has been termed virus-induced gene silencing (VIGS).

Recent studies have also demonstrated that plant pathogenic fungi can use RNA silencing to silence plant host immunity genes through the delivery of siRNAs that target these genes (17). These siRNAs are generated by Dicer proteins and delivered into plant cells where they bind plant Argonaute proteins and direct the host RNA silencing machinery to suppress host genes that are involved in immunity such as mitogen-activated protein kinases (MAPKs) (17). Furthermore, this cross-kingdom RNA silencing has been shown to be bidirectional, with plant hosts capable of delivering small RNAs into fungal cells to silence fungal genes, a phenomenon known as host-induced gene silencing (HIGS) (18). HIGs has been utilized as a strategy to reduce the rates of *S. sclerotiorum* infection in several lab studies where plants were transformed with interfering intron- containing hairpin RNA constructs for the silencing of fungal genes (19). The direct uptake of plant-produced small RNAs into fungal cells has been

demonstrated, eliminating the need for plant engineering and leading to the development of RNA fungicides (18, 20). These studies demonstrate the complex, trans-kingdom interplay that occurs involving RNA silencing machineries and the small RNA effectors they generate. Studying RNA silencing pathways will allow us to better understand plant-microbe relationships and spur the development of new pathogen control strategies.

Mycoviruses

Our expanding knowledge about RNA silencing pathways in fungi can largely be credited to the discovery and subsequent studies on mycoviruses and their impacts on fungal hosts. Mycoviruses are ubiquitous in nature and almost all fungi are known to serve as the hosts to one or more mycoviruses (12). Mycovirus infections are persistent and generally asymptomatic, however, some viruses cause virulence attenuation or hypovirulence in their hosts (21). Mycoviruses are transmitted horizontally via hyphal anastomosis and vertically via spores (12). They are predominantly dsRNA or positive-strand RNA viruses, however negative-strand RNA viruses and a ssDNA mycovirus have also been discovered (22). Furthermore, ssRNA mycoviruses mostly occur as dsRNA replicative forms in their hosts (12). Mycoviruses belong to a diverse group of virus families and genera including *Mitoviridae, Totiviridae, Partitiviridae, Chrysoviridae, Hypoviridae* and *Endornaviridae* (23). Many mycoviruses remain unclassified, however. Viruses of the family *Hypoviridae* and *Endornaviridae* do not form true particles while *Totiviridae, Partitiviridae, Chrysoviridae* are packaged in spherical particles (24). Hypoviruses have linear RNA genomes with conserved RDRP, helicase and protease motifs (12). Despite their nomenclature, only some hypoviruses- but not all- confer

hypovirulence on their hosts (25). *S. sclerotiorum* is the host to a diverse range of mycoviruses including positive-sense RNA viruses, a negative-sense RNA virus, dsRNA viruses, and a DNA virus (23). Virus infected strains of *S. sclerotiorum* often involve co-infection or mixed infection and several mycoviruses have been shown to induce hypovirulence in *S. sclerotiorum* (25).

An emerging area of interest is the use of mycoviruses as biological control agents due to the demonstrated ability of several of these viruses to induce hypovirulence in their fungal hosts (12, 21, 26). Hypovirulence is the reduced ability of a pathogen to infect, colonize, kill or reproduce in a host (26). It is not known precisely how viruses induce hypovirulence in their hosts but numerous studies have indicated that a significant number of genes are down-regulated following virus infection including RNA silencing genes (27, 28). Mechanisms involved can include the expression of viral suppressors of RNA silencing (VSRs) that suppress fungal RNA-silencing pathways (possibly including the putative microRNA-like (milRNA) pathway in *S. sclerotiorum*), leading to the disruption of endogenous small RNA metabolism. Furthermore, virus-derived small interfering RNAs (vsiRNAs) could direct the silencing of fungal mRNAs through off-target argonaute-mediated cleavage. Finally, virus-encoded RNA-dependent RNA polymerase (vRdRp) could also convert single-stranded mRNAs into double-stranded RNA (dsRNA), which would serve as a substrate for RNA silencing of the corresponding mRNAs (14). Small RNA sequencing analysis can be used to detect differences in the production and accumulation of vsiRNAs and sRNAs produced by mycovirus infections and disruption of RNA silencing genes.

Mycoviruses can be transmitted to other fungal strains through hyphal anastomoses provided both fungal strains are vegetatively compatible (21). This confers hypovirulence on the new strain. Vegetative incompatibility in fungi is controlled by specific gene loci known as *vic* loci and if one or more alleles differ within these loci then incompatibility occurs (26). *S sclerotiorum* has a relatively high number of vegetative compatibility groups (VCG) and in an individual field several clones can be found, although a few clones often represent the majority of the population (26). Mycovirus-mediated biocontrol was demonstrated in Europe when the application and natural spread of hypovirulent strains of mycovirus-infected *Cryphonectria parasitica* helped curb the spread of chestnut blight disease (29). However, efforts to protect American chestnuts by the same mechanisms have not been as successful due to a higher number of VCGs among fungal strains (29). Despite this, the limitations to viral transmission created by vegetative incompatibility between fungal isolates have been overcome by several mycoviruses that have been shown to be successfully transmitted between vegetatively incompatible strains. These include Sclerotinia sclerotiorum hypovirulence-associated DNA virus-1 (SsHADV-1) (22) and recently a single stranded (+) RNA virus named Sclerotinia sclerotiorum deltaflexivirus 2 (SsDFV2) (30).

Conclusion

Fungal plant pathogens severely limit crop productivity in widespread regions of the world. *Sclerotinia sclerotiorum* is a particularly notorious pathogen due to its survival mechanisms that allow it to propagate from one growing season to the next and its large, diverse and highly susceptible host population. New insights into the virulence

determinants and survival and defense mechanisms of this fungus as well as studies on novel, effective and environmentally-friendly control strategies are imperative. As demonstrated by extensive studies conducted on *Cryphonectria parasitica*, the chestnut blight fungus, RNA silencing pathways in fungi are important pathways that can be exploited in the quest to develop fungal control strategies. This is largely due to the intertwined relationship between RNA silencing pathways and mycovirus infection. Mycoviruses that confer hypovirulence on their fungal hosts can be developed into biological control agents.

The aim of this study was to dissect the RNA silencing pathway in *S. sclerotiorum* in order to understand its role in fungal development, fungal virulence and antiviral defense and to delineate the contributions of key components of the pathway, specifically the dicer homologs and argonaute homologs. These key genes were disrupted using the split-marker recombination method and mutants studied for changes in phenotype, pathogenicity, antiviral defense and changes in small RNA profiles. The results of these studies will broaden our understanding of RNA silencing pathways in *S. sclerotiorum* and shed light on how these pathways may be exploited in the development of robust techniques to manage the spread and virulence of this fungal plant pathogen.

CHAPTER TWO:

KNOCKOUT OF *S. SCLEROTIORUM* DICER AND ARGONAUTE GENES

- Results of the dicer gene knockout experiments presented in this chapter are included in the publication, *Mycoviruses as Triggers and Targets of RNA Silencing in* Sclerotinia sclerotiorum (Mochama, P et al., *Viruses*, 2018)
- Construction of the infectious viral clone of SsHADV-1 mentioned in this chapter was conducted by Prajakta Jadhav. Construction of the infectious viral clone- SsHV2-sx247- mentioned in this chapter was conducted by Dr. Jiuhuan Feng. Bioinformatics analyses were conducted by Achal Neupane and Dr. Shin-Yi Marzano.

INTRODUCTION

In the RNA silencing response to viral infection, fungal Dicer and Argonaute proteins play an indispensable role. Dicer proteins are RNase-III enzymes that cleave double-stranded precursor RNA molecules into short, double-stranded RNA fragments. These 18-30nt fragments become incorporated into RNA-induced Silencing Complexes (RISCs) and guide the sequence specific degradation, translational repression, or transcriptional suppression of target messenger RNAs (mRNAs) (31). Typical Dicer proteins have several functional domains including a PAZ domain that binds double-stranded RNA, a DEAD/Helicase domain that facilitates movement of the protein along long dsRNA molecules, and an RNase-III domain that cleaves dsRNA (32). Dicer proteins have been identified in plants, animals, insects, protozoans and fungi. Vertebrates and nematodes possess a single dicer gene while insects have two and most plant genomes carry four dicer genes (32). The number of silencing related genes vary widely in fungi. The well-studied ascomycetes *Neurospora crassa, Colletotrichum higginsianum* and *Cryphonectria parasitica* have all been shown to encode two dicer

homologs (33-35). Dicer genes as well as other components of the RNA silencing pathway are absent in some fungi including *Saccharomyces cerevisiae* (36). Most fungi that lack RNAi genes, have dsRNA killer viruses that make up for the absence of RNAi by conferring immunity to infected cells (37).

In filamentous fungi, RNA silencing functions primarily in viral defense (35). Disruption of key silencing related genes such as dicers results in increased susceptibility to viral infection. This has been demonstrated in *C. parasitica* and *C. higginsianum* where disruption of one of two dicer genes in these fungi resulted in debilitated phenotypes such as slower growth (34, 35). In the model filamentous fungus, *Neurospora crassa*, two dicer homologs have also been identified, however, the antiviral roles of these genes have not been established due to the lack of a mycovirus experimental system for this fungus. *N. crassa* dicer genes have been shown to play a redundant role in transgene silencing, however (33).

The Argonaute protein family constitutes endonucleases characterized by RNA-binding domains known as PAZ domains and slicer domains known as PIWI domains (12). Argonaute proteins form complexes with small dsRNA molecules produced by Dicer proteins to form RNA-induced silencing complexes (RISC) which are involved in post-transcriptional gene silencing or RNA-induced transcriptional silencing complexes (RITS) which are involved in transcriptional gene silencing including chromatin modification in animals, plants and insects (38). When small dsRNA molecules produced by Dicers are incorporated into these effector complexes, one strand of the RNA molecule is removed and the remaining strand guides the complex to complementary

RNA sequences which are subsequently cleaved by the Argonaute RNase H-like activity (39).

Argonaute homologs have been identified in fungi and they vary in function and number. The basal fungus, *Mucor circinelloides,* has three argonaute genes while *C. parasitica* has four argonaute genes and *C. higginsium* has two (34, 39, 40). *QDE-2* is a fungal argonaute homolog in *N. crassa* that is involved in quelling- the silencing of repetitive sequences such as transgenes (41). In *N. crassa*, a separate silencing pathway called meiotic silencing of unpaired DNA (MSUD) has been characterized, and *N. crassa* RNA silencing components not involved in quelling have been shown to be involved in this pathway (41). Similarly, in other fungi, not all components of the RNA silencing machinery are involved in RNA silencing mediated viral defense mechanisms. In *F. graminearum*, only one of two argonaute genes, *FgAgo1,* is important in RNA silencing of viral nucleic acids (42) while in *C. parasitica* only *agl2* is required for antiviral RNA silencing (39), and in *C. higginsium ago1* but not *ago2* is essential for antiviral RNA silencing (24). The primary functions of the other gene homologs have not been fully characterized.

S. sclerotiorum supports the replication of a number of mycoviruses including a ss(+)RNA virus belonging to the *Hypoviridiae* family- Sclerotinia sclerotiorum hypovirus 2 – lactuca (SsHV2-L)- and a ssDNA virus- Sclerotinia sclerotiorum hypovirulence-associated DNA virus (SsHADV-1)- belonging to the newly formulated *Genomoviridae* family. Both SsHV2-L and SsHADV-1 have been shown to induce hypovirulence in *S. sclerotiorum* (22, 25). Mycoviruses typically have double-stranded or single-stranded RNA genomes, making SsHADV-1 unique among mycoviruses. Furthermore, SsHADV-

1 has been shown to be capable of extracellular transmission, a feature that mycoviruses have traditionally not been known to possess (43). This latter feature makes SsHADV-1 a viable candidate for use as a biological control agent.

SsHV2-L is a recombinant strain of Sclerotinia sclerotiorum hypovirus 2 that was identified from an *S. sclerotiorum* isolate on lettuce (25). SsHV2-L contains a deletion of ~1.2kb near its 5'terminus relative to the other SsHV2 strains and an insertion of 524nt with homology to Valsa ceratosperma hypovirus 1 (21). SsHV2 strains have been shown to contain sequences similar to papain-like proteases which are known viral suppressors of RNA silencing (VSR) (44), and similarly the SsHV2-L genome encodes a putative VSR. This study utilizes an engineered cDNA clone, SsHV2-sx247, in which the putative RNA silencing suppressor has been replaced to examine the effects of VSR on viral infection in fungi.

RNA silencing pathways have not been extensively studied in the plant pathogenic fungus, *Sclerotinia sclerotiorum*, despite the insights that could be gained from exploring this critical pathway. While RNA silencing pathways may serve predominantly as antiviral defense pathways in fungi, there is increasing evidence for the endogenous gene regulation contributions made by these pathways through the actions of small RNA effector molecules generated by Dicers (31). This expands the role of fungal RNA silencing pathways to developmental and physiological functions as well. Furthermore, small RNA molecules are capable of being transmitted into plant host cells and silencing host immunity genes, further diversifying the role of fungal RNA silencing pathways (45). Further studies are needed to elucidate the distinct roles of fungal Dicer and Argonaute proteins due to the evolutionary diversity that exists in orthologs of these

genes among fungal species and the differing roles played by gene homologs within a single species.

To begin deciphering the role(s) of RNA silencing pathways in *S. sclerotiorum*, this study examined the functions of *S. sclerotiorum* dicer and argonaute genes by generating single-gene knockout mutants of the two dicer homologs, *dcl-1* and *dcl-2*, a double-dicer gene knockout mutant, and single gene knockout mutants of the two argonaute genes, *ago-2* and *ago-4*. Mutants were studied for changes in phenotype, virulence, and susceptibility to infection with SsHV2-L, SsHADV-1 (dicer gene mutants) and SsHV2-sx247 (argonaute gene mutants) compared to a wild type strain, DK3. Small RNA profiles were also examined in several mutant and wild-type strains.

MATERIAL AND METHODS

Fungal Strains and Culture Conditions.

Cultures of *Sclerotinia sclerotiorum* wild-type strain DK3 and dicer and argonaute mutant strains were grown on potato dextrose agar (Sigma, St. Louis, MO, USA) at 20–22 °C. The Δ*dcl-1*, Δ*dcl-2*, Δ*ago-2* and Δ*ago-4* mutant strains were maintained on PDA supplemented with 100 µg/mL hygromycin B (Alfa Aesar, Haverhill, MA, USA) and the Δ*dcl-1/dcl-2* strain was maintained on PDA supplemented with 100 µg/mL hygromycin and 250 µg/mL Geneticin (G418).

Construction of dicer and argonaute gene knockout mutants.

Sclerotinia sclerotiorum dicer genes (Ss1G_13747 and Ss1G_10369, respectively) and argonaute genes (Ss1G_00334 and Ss1G_11723, respectively) were predicted based on homology to those identified in *Neurospora crassa* (46).

Deletion of 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 26–32 bp of complementary sequence to the *Aspergillus nidulans* trpC promoter and terminator respectively. Plasmid pCSN43 containing the hygromycin B resistance (*hph*) gene flanked by the *Aspergillus nidulans* TrpC promoter and terminator (47), 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 1.2 kb region of the marker gene including the promoter and primers YG-F and TrpC-R were used to amplify a 1.3 kb 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. (48). 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 *hph* gene in the first construct and 600bp of downstream sequence fused to two-thirds of the *hph* gene in the second construct.

Disruption of the *dcl-2*, *ago-2* and *ago-4* genes was accomplished with constructs generated as described above using a separate set of primers (Appendix 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. Final *ago-2* gene deletion constructs included 1kb of sequence homologous to the upstream region of the gene and 812bp of downstream homologous sequence while final *ago-4* deletion constructs included 805bp of sequence homologous to the upstream region of the gene and 1.1kb 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 Appendix 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-resistance gene. G418 is an aminoglycosidic antibiotic similar to hygromycin but with no cross-resistance. The G418 resistance gene was amplified from pSCB-TrpC-G418 (49).

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. (1) with a digestion time of 3h at RT using the lysing

enzyme from *Trichoderma harzianum* (Sigma, St. Louis, MO, USA). PEG-mediated transformation of gene deletion constructs into fungal protoplasts was performed following the protocol described by Rollins et al. (50) with some modifications (51). Briefly, following PEG transformation, 3mL of liquid regeneration media (RM) was added to protoplasts and the suspension incubated at 28 °C with shaking (100 rpm, 2–4 h). Molten RM (45 °C) was then added to a final volume of 20 mL and the mixture poured into a petri dish. Plates were grown at 28 °C for 12 h and then overlaid with 5 mL molten RM containing hygromycin for single dicer and argonaute gene mutants and hygromycin and G418 for the double dicer mutant. Final antibiotic concentrations used for fungal selection were 100 µg/mL for hygromycin and 250 µg/mL for G418. Colonies were transferred to potato dextrose agar (PDA) plates supplemented with the appropriate antibiotic and hyphal-tip transferred at least three times to generate homokaryotic cultures.

Complementation of dcl-1.

For *dcl-1* complementation, the *Δdcl-1/dcl-2* mutant was transformed with a plasmid (pD-NAT1, Fungal Genetics Stock Center, Manhattan, KS, USA) engineered to contain the full length *dcl-1* open reading frame flanked by 2.3kb of upstream genomic sequence and 1kb of downstream genomic sequence. The *dcl-1* gene and flanking regions were amplified from wtDK3 using primers F1-SacI-Dcl1 and F4-Not1-Dcl1 (Appendix 1) and inserted into the *Sac*I-*Not*I site of the vector downstream to the *Aspergillus nidulans* TrpC promoter and *nat1* gene which confers resistance to nourseothricin. Following transformation with the plasmid construct, protoplasts were grown on RM

media supplemented with nourseothricin to a final concentration of 200 µg/mL. Transformants were then transferred to PDA plates supplemented with 200 µg/mL nourseothricin and phenotypic analysis was conducted. Constructed plasmids were all transformed into *Escherichia coli* strain DH5α for propagation and plasmid isolation. Constructs were verified using PCR amplification and sequencing prior to protoplast transformation.

Phenotypic Characterization of Gene Deletion Mutants.

Growth assays were conducted on 3-5 replicates each of virus-free wtDK3, *Δdcl-1*, *Δdcl-2* and *Δdcl-1/dcl-2* cultures as well as wtDK3, *Δago-2* and *Δago-4* cultures. Five-millimeter 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 24 h, 48 h and 72 h post inoculation. At least three trials were conducted to compare mean hyphal growth.

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 (*Brassica napus*), sunflower leaf ***(****Helianthus annuus)*, forage pea leaf (*Pisum sativum)* or a detached center leaflet (4 to 5 cm long) from the first trifoliate leaf of a soybean seedling (*Glycine max)*. At least 3-5 replicates of the leaves inoculated with wild-type or mutant strains were incubated on moistened Whatman filter paper in sterile petri dishes at 20 ± 1 °C in a growth chamber with a 12h light-12h dark photoperiod.

Lesion size was calculated 24 h, 48 h and 72 h post inoculation by averaging two perpendicular lesion diameter measurements. At least three trials were conducted to compare mean lesion diameters.

Transfection of Dicer and Argonaute Mutants with In Vitro Transcripts of SsHV2-L.

In vitro transcripts of SsHV2-L were synthesized and transfected into wtDK3 and dicer mutant protoplasts following a published procedure (25). After >6 transfers, viral infection was confirmed by extraction of total RNA using RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by reverse transcription using Maxima H Minus Reverse Transcriptase (ThermoFisher, Waltham, MA, USA) 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.

Transfection of Dicer Mutants with Infectious Viral Clone of SsHADV-1

Dicer mutant cultures were infected with SsHADV-1 by extracellular transmission of 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. Fungal DNA was then extracted after >6 serial transfers and virus infection confirmed by inverse PCR to amplify the 2166bp SsHADV-1 viral sequence.

Transfection of Argonaute Mutants with SsHV2-sx247

Transfection of Argonaute mutants with SsHV2-sx247 lacking a putative viral suppressor of RNA silencing was conducted by hyphal fusion between virus-free mutant cultures and virus-infected wtDK3 cultures. Specifically, plugs were taken from an actively growing SsHV2- sx247- infected wtDK3 culture and placed adjacent to plugs taken from the edges of actively growing mutant cultures on fresh PDA plates. Following growth and fusion of mycelia from both cultures, plugs were transferred to fresh PDA plates supplemented with hygromycin. After several transfers, virus infection was confirmed by extraction of total RNA using RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by reverse transcription using Maxima H Minus Reverse Transcriptase (ThermoFisher, Waltham, MA, USA) and PCR to amplify a 1.1 kb region corresponding to the viral genome.

Preparation of Small RNA Libraries and Sequencing Analysis.

Small RNAs were extracted from 4-day-old mycelia using mirVana miRNA Isolation kit (ThermoFisher, Waltham, MA, USA) following the manufacturer's protocol. Libraries were prepared using the NEBNext small RNA Library Kit (NEB, Ipswich, MA, USA). 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. We sequenced two replicates each of virus-free wtDK3 and *Δdcl-1/dcl-2* as well as five replicates each of wtDK3 infected with SsHV2-L and three replicates of wtDK3 infected with SsHADV-1. Demultiplexed reads were removed of the 3' adaptors by Trimmomatic (52). Loci

producing sRNAs were identified by ShortStack (53). The obtained sequences have been deposited in NCBI (SRA accession SRP136666).

Data Analysis

Statistical analysis of hyphal diameter and lesion size measurements were conducted using the latest version of R software. Means were compared using a two sample t-test.

RESULTS

Generation of Disruption Mutants for Dicer and Argonaute Genes

Dicer and argonaute genes in *S. sclerotiorum* were disrupted using the homologous recombination method for gene displacement (Figure 1A) to generate *Δdcl-1*, *Δdcl-2*, Δ*dcl-1/dcl-2, Δago-2* and *Δago-4* mutants directly from wild-type strain DK3. Genes were confirmed to be disrupted by extracting DNA from multiple transformants and performing PCR amplification using F1 and F4 primers for initial screening and F1 – HY and F4 – YG primer pairs for subsequent confirmation. When the target locus was amplified, wild-type and mutant PCR amplicons differed in size confirming gene deletion (Figure 2A and 2B). PCR screening and Sanger sequencing of PCR amplicons confirmed integration of the gene-replacement cassettes into the target region. Finally, nested PCR with primers targeting the coding regions of the genes was used to rule out heterokaryotic mutation in which both the original intact genes and disrupted genes occur in different nuclei within fungal hyphae. Once a homokaryotic mutation was confirmed, further characterization of colony morphology and pathogenicity was carried out.

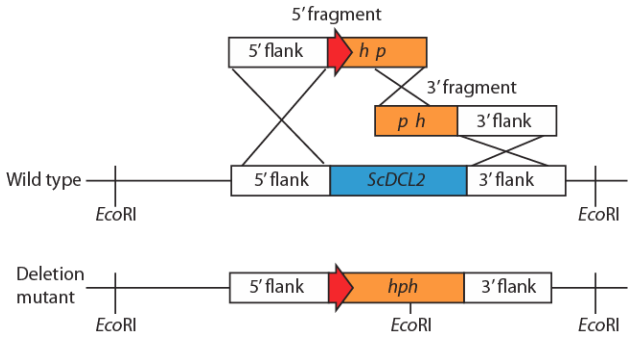


Figure 1. Generation of deletion mutants for dicer and argonaute genes in *S. sclerotiorum* using the split-marker gene replacement method (orange: selective marker, ex. *hph*; blue: gene to be replaced, ex. *dcl-2*; red: TrpC promoter). Figure adapted from Wang et. al, 2012 (14).

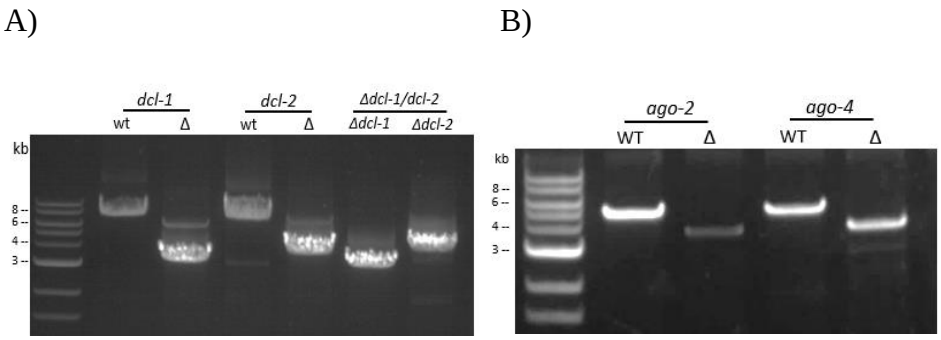


Figure 2. Electrophoresis gel image of PCR amplification to confirm dicer gene disruption using F1–F4 primer pairs. **A)** Amplicons of wild-type *dcl-1* and *dcl-2* genes (7.7 kb and 7 kb, respectively) and deletion alleles (3.3 and 3.9 kb) differ in size. Lanes 5 and 6 show deletion alleles (3.1 and 3.9 kb) in the double dicer mutant. **B)** Amplicons of wild-type *ago-2* and *ago-4* genes (5.1 kb and 5.2 kb, respectively) and deletion alleles (3.9 and 4.1 kb) differ in size.

Effect of Dicer Gene Disruption on S. sclerotiorum *Phenotype*

We compared the growth rate and colony morphology of dicer mutants to the wild-type strain, wtDK3. Single mutants, *Δdcl-1* and *Δdcl-2*, and wtDK3 exhibited similar growth rates, whereas the double-gene *Δdcl-1/dcl-2* disruption mutant exhibited significantly slower growth as indicated by measurements of hyphal diameter ($p < 0.05$) (Figure 3A). 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.

Effects of Dicer 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, 48 and 72 h post inoculation showed that there was no significant 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 ($p < 0.05$) (Figure 3B).

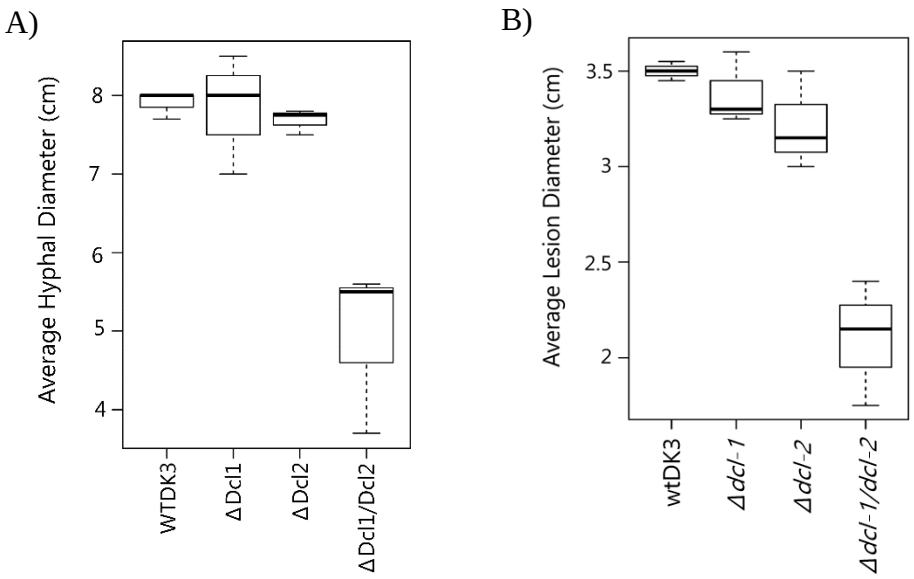


Figure 3. (**A**) Average mycelial growth of wild-type *S. sclerotiorum* and dicer gene mutants grown on PDA for 72h; and (**B**) average lesion diameter measurements 72 hpi comparing wtDK3, Δ*dcl-1*, Δ*dcl-2* and Δ*dcl-1/dcl-2* virus-free cultures inoculated on canola leaves.

Effect of Argonaute Gene Disruption on S. sclerotiorum *Phenotype*

Comparisons of growth rate and colony morphology of argonaute mutants to the wild-type strain, wtDK3 showed that the *Δago-4* mutant had no significant difference in growth rate and phenotype compared to the wild-type strain. However, the *Δago-2* mutant displayed significantly slower growth compared to the wild-type strain ($p < 0.05$) (Figure 4A). In addition, the *Δago-2* mutant produced smaller sclerotia on average (Fig 4C).

Effects of Argonaute Gene Disruptions on S. sclerotiorum *Pathogenicity*

Assays conducted to test the pathogenicity of virus-free *S. sclerotiorum* argonaute mutants showed that the *Δago-4* mutant produced lesions of similar size to the wild-type strain, whereas the *Δago-2* mutant produced significantly smaller lesions than the wild-type strain ($p < 0.05$) (Figure 4B).

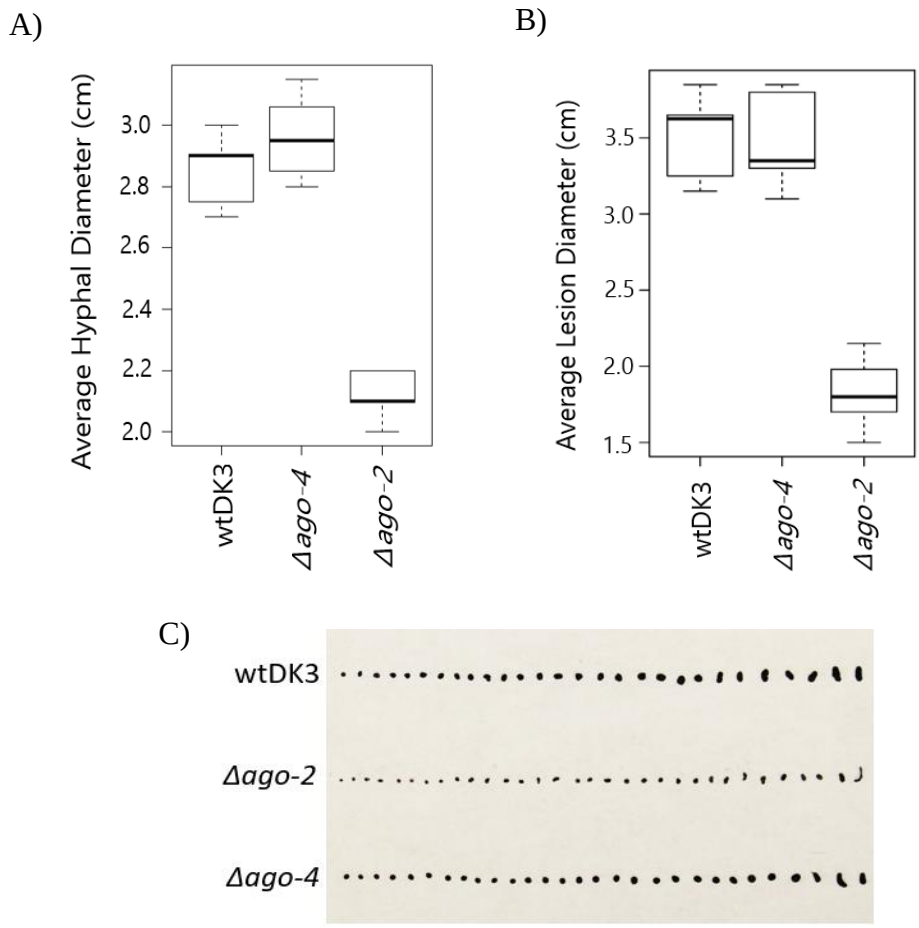


Figure 4. (**A**) Average mycelial growth of wild-type *S. sclerotiorum* and argonaute gene mutants grown on PDA for 24h; (**B**) lesion diameter measurements 48hpi comparing wtDK3, Δ*ago-2*, and Δ*ago-4* virus-free cultures inoculated on canola leaves; **(C)** wtDK3, Δ*ago-2*, and Δ*ago-4* sclerotia obtained from 10day old cultures grown on PDA.

Transfection of Dicer Gene Deletion Mutants with SsHV2-L or SsHADV-1 Viruses Consistently Results in Severe Debilitation in the Δdcl-1/dcl-2 *Mutant*

To examine the effect of viral infection on strains containing deletions of *dcl-1*, *dcl-2* or both genes, mutants were transfected with SsHV2-L or SsHADV-1 via the methods described in the Materials and Methods section. As shown in Figure 5A, the *Δdcl-1* and *Δdcl-2* mutants infected with either mycovirus showed no significant difference in growth or 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 (Figure 5B–D). Complementation of *dcl-1* in the double dicer mutant (named *Comp-dcl-1*) resulted in growth and phenotype similar to the wild-type strain prior to and following virus infection.

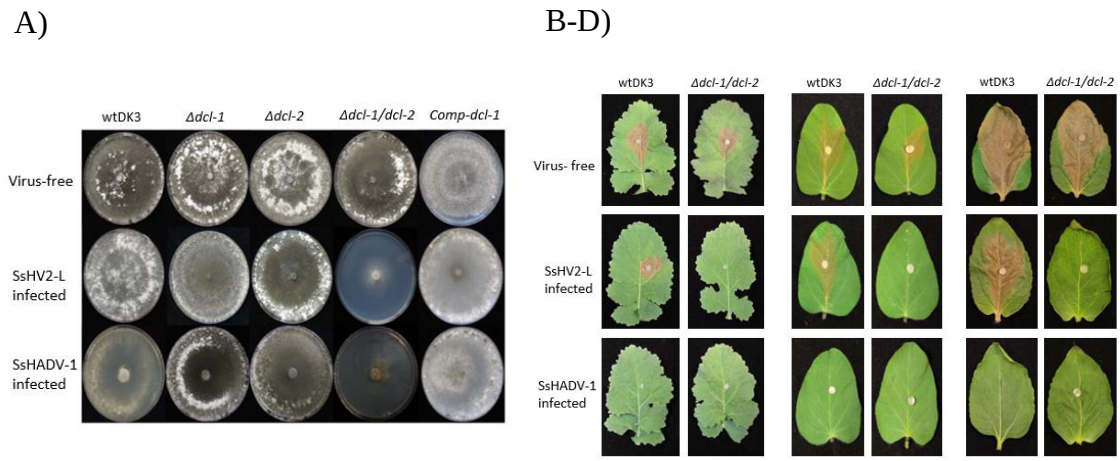


Figure 5. (**A**) Colony morphology of virus-free and virus-infected wild-type, mutant, and complemented strains: (Top row) virus-free wtDK3, Δ*dcl-1*, Δ*dcl-2*, Δ*dcl-1/dcl-2* and *Comp-dcl-1*. (Middle row) strains infected with hypovirus, SsHV2-L; and (Bottom row) strains infected with SsHADV-1. Cultures were grown for seven days on PDA at room temperature. Virulence assays on: (**B**) detached canola leaves; (**C**) detached soybean leaves; and (**D**) detached sunflower leaves. Plugs were taken from the edge of actively growing wtDK3, Δ*dcl-1* (not shown), Δ*dcl-2* (not shown) and Δ*dcl-1/dcl-2* cultures and inoculated onto detached leaves stored at 20 ± 1 °C. Photographs were taken 36 h post-inoculation.

Transfection of Argonaute Gene Deletion Mutants with SsHV2-L Results in Severe Debilitation in the Δago-2 *Mutant*

To examine virus susceptibility in *ago-4* and *ago-2* gene knockout strains, mutants were transfected with SsHV2-L. As shown in Figure 6, the virus-infected *ago-4* mutant showed no significant difference in growth or morphology compared to virus-

infected wtDK3. In sharp contrast, the *Δago-2* mutant showed severe debilitation following virus infection as evidenced by significantly slower growth, delayed sclerotia production and hypovirulence on detached canola leaves and forage pea leaves (Figure 7A and 7B).

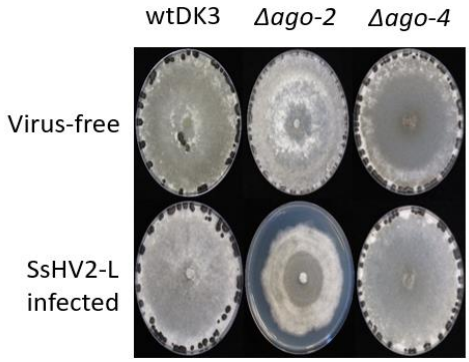


Figure 6 Colony morphology of virus-free and virus-infected wild-type and argonaute mutant strains: (Top row) virus-free wtDK3, Δ*ago-2*, and Δ*ago-4* (Bottom row) strains infected with SsHV2-L. Cultures were grown for nine days on PDA at 22ºC.

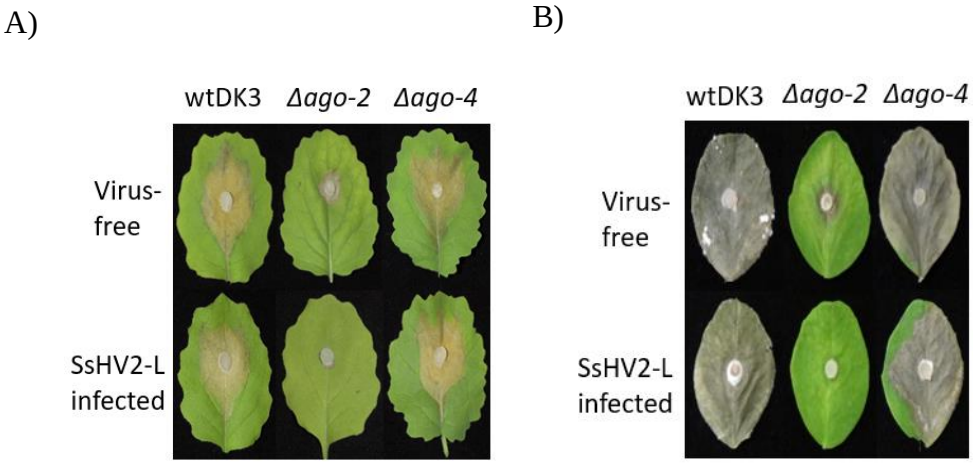


Figure 7 A) Virulence assays on detached canola leaves and **B)** detached forage pea leaves. Plugs were taken from the edge of actively growing wtDK3, Δ*ago-2*, and Δ*ago-4* cultures and inoculated onto detached leaves stored at 20 ± 1 °C. Photographs were taken 48h (canola) and 72h (pea) post-inoculation.

SsHV2-sx247 infection results in less debilitating disease symptoms compared to SsHV2-L infection.

To compare the effect of viral suppressors of RNA silencing on viral disease symptoms, wild-type and argonaute mutant strains were infected with an engineered viral clone lacking a putative RNA silencing suppressor- SsHV2-sx247. Compared to *Δago-2* strains infected with SsHV2-L, SsHV2-sx247 infected *Δago-2* strains were less symptomatic. The SsHV2-sx247-infected *Δago-2* mutants exhibited slower growth on average compared to virus infected wild-type and *Δago-4* strains but showed less debilitation compared to the SsHV2-L- infected *ago-2* mutant (Figure 8).

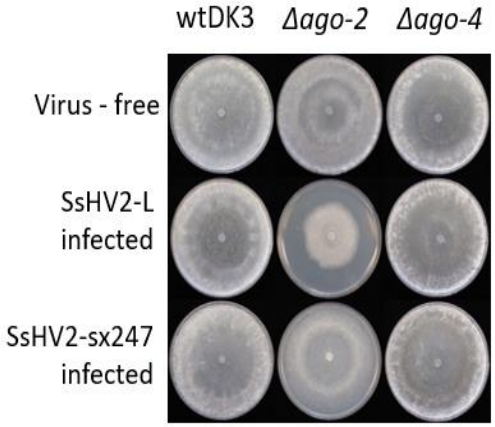


Figure 8. Colony morphology of virus-free and virus-infected wild-type and argonaute mutant strains: (Top row) virus-free wtDK3, Δ*ago-2*, and Δ*ago-4*; (Middle row) strains infected with SsHV2-L. (Bottom row) strains infected with SsHV2-sx247. Cultures were grown for five days on PDA at 22ºC.

Double Dicer Disruption Mutant Has Reduced 21–24nt sRNA Accumulation

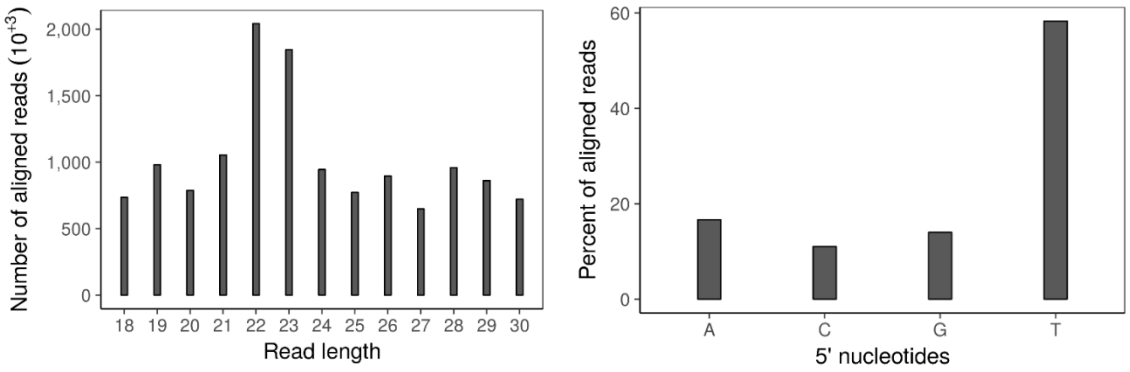
To examine whether sRNA accumulation is affected by disrupting both dicers, sRNA sequences were profiled by size distribution and 5’ terminal nucleotide in the virus-free Δ*dcl-1/dcl-2* mutant and wild-type strain. Although the 5’ terminal nucleotide

remained uracil-biased, the size distribution of small RNAs was drastically changed in the double-dicer mutant compared to the wild-type strain (Figure 9A, B). Specifically, there was a reduction in the 21–24-nt sRNA fraction in the double mutant compared to the wild-type strain. Notably and similar to *B. cinerea*, sRNA production in *S. sclerotiorum* is not completely eliminated after both dicers are deleted.

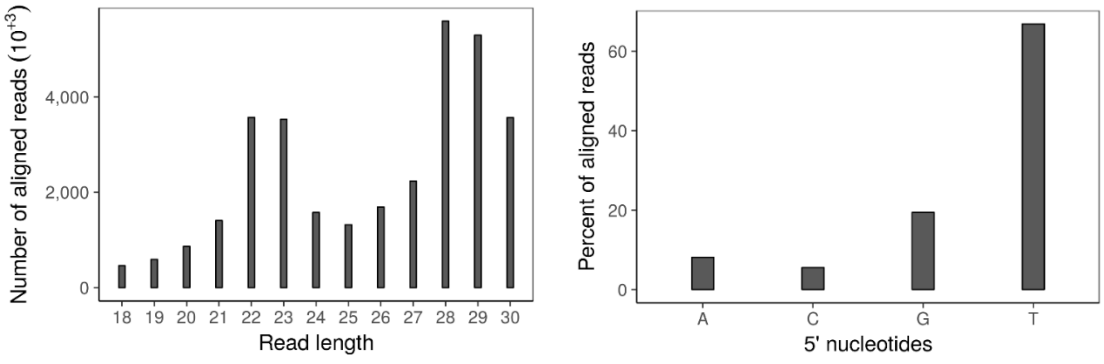
SsHADV-1 and SsHV2-L Are both Processed by Virus-Infected wtDK3

Sequence analysis of the small RNAs produced by either SsHADV-1 or SsHV2-L infected wtDK3 revealed the presence of virus-derived sRNAs (vsiRNAs) within the pool of total small RNAs extracted from these cultures. On average, 14.4% of the total small RNA reads from the SsHV2-L-infected wild-type strain were derived from SsHV2-L, whereas 2.26% of the total small RNA reads from the SsHADV-1 infected wild-type strain were derived from SsHADV-1. For each barcoded library, 5–10 million reads were obtained and passed QC. The 22-nt sRNAs were the most abundant for both virus-infected wild-type strains (Figure 9C, D) with a preference (>90%) for uracil at the 5' position. Overall, 77.89% of SsHV2-L derived sRNA aligned to the negative strand, and 22.01% to the positive strand (Figure 8E). Virus-derived small RNAs from all five replicates of SsHV2-L-infected wtDK3 displayed the same even distribution along the viral genome. SsHADV-1 derived sRNA reads aligned non-uniformly to both strands (Figure 9E) with strand biases for the negative strand in the first 350 bases of the coat protein encoding gene and strand biases for the positive strand between nucleotide bases 1000–2200 of the replicase protein encoding gene; overall, 51.6% of the reads aligned to the published positive strand sequence and 48.3% to the negative strand.

We found that a significant number of virus-derived sRNAs contained 1-nt terminal mismatches. The majority of SsHADV-1 vsiRNAs contained an A or T at the mismatched 3’-terminus and mismatched A nucleotide at the 5’-terminus. SsHV2-L vsiRNAs contained mismatches primarily at the 3’-terminus involving A and T. Mismatches involving G or C were also found but to a much lower extent (Table 1). SsHV2-L vsiRNAs were also found to contain a high number of internal mismatches at specific positions (Figure 10). For example, the 22-nt long sRNAs have an internal peak of mismatches at the 11th nucleotide.



(**A**)



(**B**)

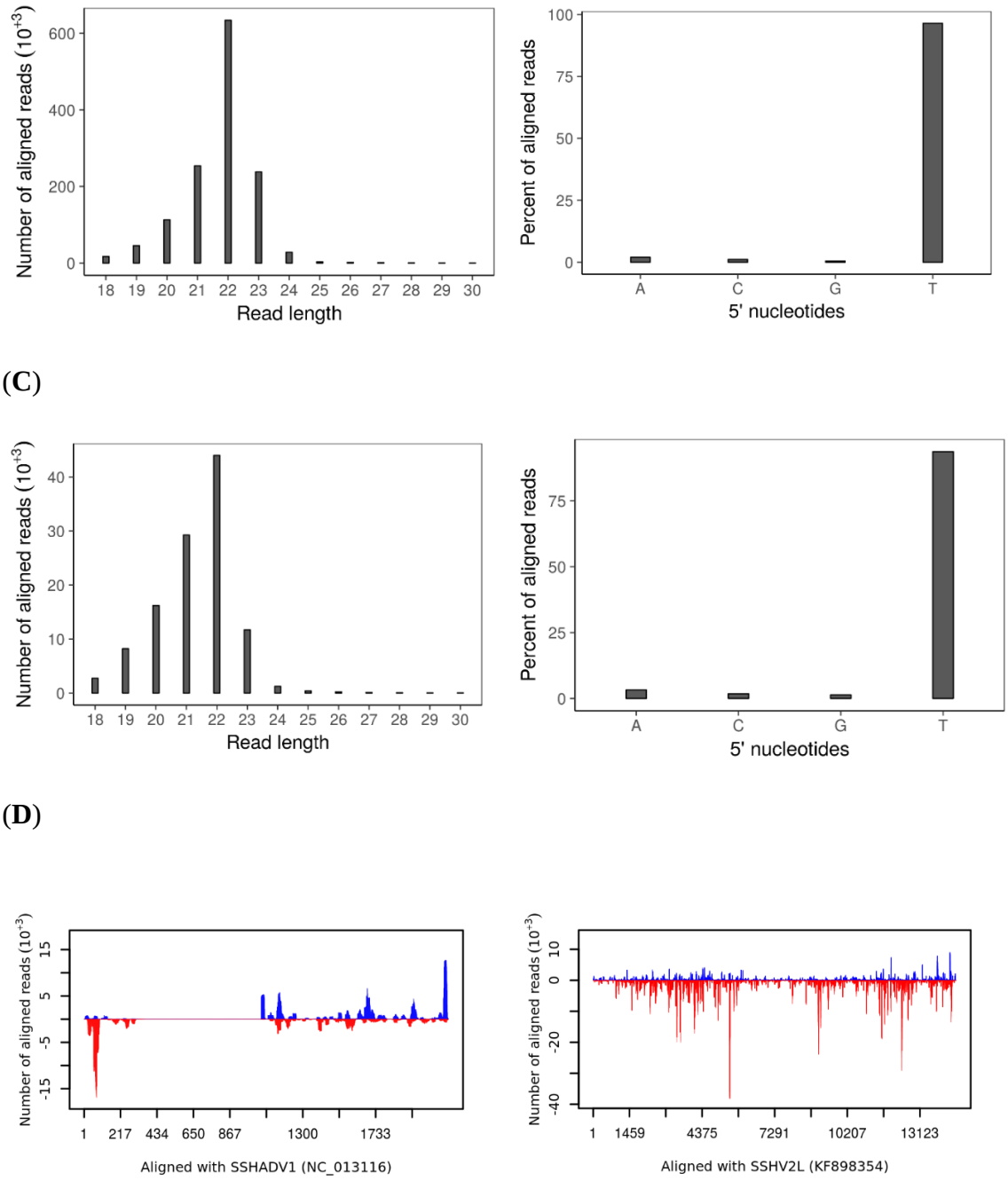


Figure 9. Small RNA: (**A**) Size distribution (left) and frequency of 5' terminal nucleotides (right) of small RNAs in wtDK3; (**B**) size distribution (left) and frequency of 5' terminal nucleotides (right) of small RNAs in Δ*dcl-1*/*dcl-2* disruption mutant; (**C**) size distribution (left) and frequency 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 SsHADV-1 genome; and (**E**) distribution of small RNA reads that aligned to the SsHADV-1 genome plus or minus strands (left) and distribution of small RNA reads that aligned to the SsHV2-L genome plus or minus strands (right). Bars above zero indicate alignment to the positive strand, and bars below zero indicate alignment to the negative strand

Table 1. Percentage of SsHV2-L and SsHADV-1 derived small RNAs containing mismatches relative to viral genomes.

SsHADV-1	**5'-terminal mismatch (%)**				**3'-terminal mismatch (%)**			
vsiRNA Sequence length	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
18	16.9	1.9	1.1	0.8	18.2	5.0	3.0	14.6
19	4.2	1.1	1.0	2.8	21.0	7.3	3.9	19.6
20	10.3	0.8	0.8	1.1	24.5	4.8	3.2	22.4
21	5.0	0.6	0.8	1.6	27.9	3.2	4.7	22.4
22	26.8	0.8	0.8	1.0	20.2	3.0	2.7	12.3
23	46.1	0.6	0.9	0.7	12.5	2.5	1.5	9.1
24	5.9	1.7	2.0	0.6	28.0	3.4	2.0	24.4
SsHV2-L	**5'-terminal mismatch (%)**				**3'-terminal mismatch (%)**			
vsiRNA Sequence length	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
18	1.1	0.4	1.4	0.5	16.6	3.0	6.6	23.1
19	1.2	0.6	1.0	0.3	18.5	3.0	6.1	26.9
20	0.6	0.5	1.1	0.3	21.1	2.6	5.0	26.9
21	0.7	0.5	1.0	0.4	17.1	2.6	5.1	20.6
22	2.3	0.4	1.0	0.4	11.7	3.2	4.5	17.1
23	0.8	0.7	1.7	0.3	14.1	2.5	4.6	19.0
24	0.2	1.3	2.8	0.5	19.6	1.9	5.2	22.0

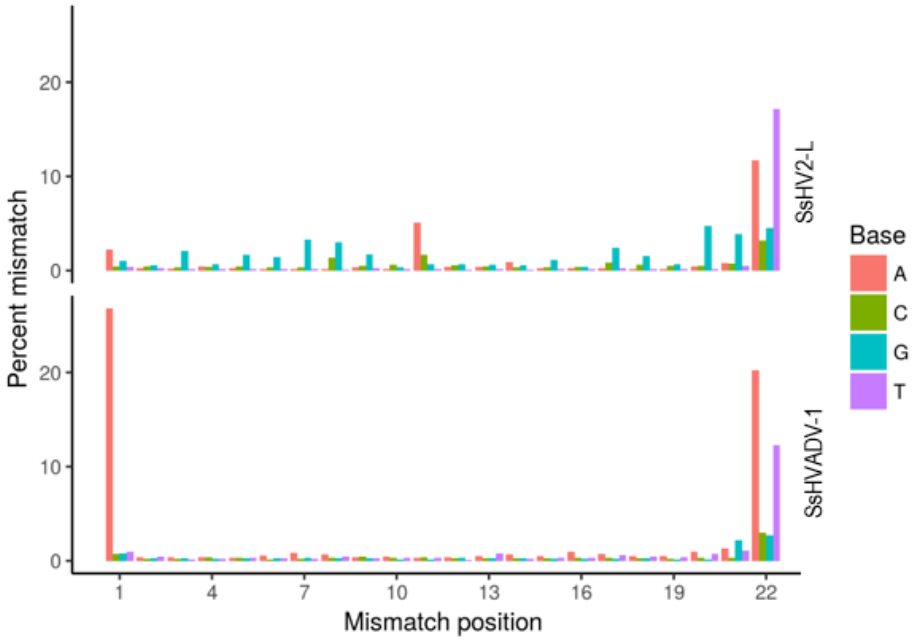


Figure 10: Frequency and distribution of mismatches occurring in 22 nt long SsHADV-1 and SsHV2L-derived sRNAs. A majority of mismatches occur at the 5' and 3' termini; however, a significant number of internal mismatches occur at the 11nt in SsHV2-L- derived vsiRNAs.

DISCUSSION

Studies conducted on a number of fungal species have uncovered robust RNA silencing mechanisms with vital roles in fungal antiviral defense. Similarly, this study demonstrates the existence of RNA silencing mechanisms in the plant pathogenic fungus *Sclerotinia sclerotiorum* and establishes the significant roles played by dicer and argonaute genes in this pathway. Primarily, these findings clearly demonstrate the antiviral function of *S. sclerotiorum* RNA silencing pathways. Wild-type strains of *S. sclerotiorum* displayed fairly normal phenotype and virulence following virus infection, however, RNA-silencing-deficient mutants (specifically the Δ*dcl-1/dcl-2* and Δ*ago-2* mutant) displayed significantly slower growth and decreased virulence upon virus infection.

Besides establishing a role for *S. sclerotiorum* dicer genes in antiviral mechanisms, this study also demonstrated that *S. sclerotiorum* dicers contribute to endogenous gene regulation likely through the action of small RNAs generated by these genes. The important roles played by dicer-generated small RNAs are well documented (31). We found that the deletion of both dicer genes resulted in compromised growth and virulence in the double mutant prior to virus infection suggesting the contributions made by these genes to physiological and developmental processes. Similar changes were observed in another member of *Sclerotiniaceae*, *Botrytis cinerea* (17), where slower growth and reduced pathogenicity were observed when both dicer genes were disrupted. As in *B. cinerea*, the changes observed in the *S. sclerotiorum* double mutant may be attributed to a significant reduction in small RNA effectors produced by the mutant. Indeed, small RNA-seq analysis revealed a reduction in small RNAs 22nt long in the double dicer mutant compared to the wild-type strain. Notably, production of small RNAs is not completely eliminated upon deletion of both dicer genes (again similar to *B. cinerea*), and this indicates that there may be other dicer-independent pathways that contribute to the generation of sRNAs. A class of sRNAs known as dicer-independent-small-interfering RNAs (disiRNAs) which do not require Dicer proteins for generation have been reported (54). By conserved domain search, we found a putative RNaseL gene (GenBank Ss1G_04823), also an RNA-endonuclease-III, which may be responsible for the remaining small RNA processing. RNaseL endonucleases share similarities with yeast Ire1p proteins which are said to be involved in fungal mRNA splicing (55).

The high level of debilitation observed in the double dicer mutant following virus infection was not observed in the virus-infected single dicer mutants. Furthermore,

complementation of a single dicer gene was sufficient to restore viral susceptibility to the wild-type state. These findings suggest that there is redundancy in the antiviral function of *S. sclerotiorum* dicer genes. Redundancy in dicer antiviral function has not been reported in fungal species thus far; however, a redundancy in dicer function in transgene-induced gene silencing has been found in *Neurospora crassa* (33). The antiviral role of *N. crassa* dicers has not been studied due to lack of a mycovirus experimental system for this fungus. Dicer redundancy in antiviral RNA silencing mechanisms in *S. sclerotiorum* could be validated by small RNA sequence analysis of virus-infected single dicer knockout mutants to demonstrate that the small RNA accumulations (particularly vsiRNAs) are identical to the wild-type strain due to the presence of an intact dicer gene (*dcl-1* or *dcl-2*) in each mutant that conducts RNA processing in place of the other. Potential functional redundancy in gene function in *S. sclerotiorum* has been reported before in other gene homologs such as the *ssp1* and *ssp2* genes involved in sclerotial development (6).

The Δ*ago-2* mutant exhibited severe debilitation following virus infection as well. This suggests that the Ago2 protein is primarily responsible for incorporating vsiRNAs into the RISC complex as part of the viral RNA silencing mechanism leading to the silencing of viral RNA. Argonaute proteins have been shown to associate with vsiRNAs in plants to target complementary viral RNAs and in some cases host genes as well (56, 57).

Results also indicated that infection with a virus lacking a putative RNA silencing suppressor resulted in overall less symptomatic infection. This was most obvious in the *Δago-2* mutant which was severely debilitated following infection with SsHV2-L but less

debilitated when infected with SsHV2-sx247. In both these cases *ago-2* expression is absent therefore disease symptoms are explicable following virus infection; however, the greater debilitation caused by the VSR-containing virus, SsHV2-L, may be due to the targeting of other components of fungal silencing defense mechanisms resulting in an increase in disease severity in the Δ*ago-2* mutant infected with SsHV2-L compared to the *Δago-2* mutant infected with SsHV2-sx247 lacking the VSR. Indeed, while Argonaute proteins have been shown to be key targets of viral suppressors of RNA silencing, VSR may also function through other diverse modes of action such as inhibiting dsRNA processing by Dicers (14).

Notably, the *ago-2* mutant displayed slower growth, smaller sclerotia, and reduced virulence before virus infection which suggests that Argonaute proteins also contribute to the regulation of some physiological and developmental processes. miRNA-like molecules with possible gene regulation functions have been found to associate with fungal Argonaute proteins like the QDE-2 protein in *N. crassa* (54). This suggests that argonaute genes may also contribute to endogenous gene regulation guided by this class of small RNA molecules.

Additionally, this study demonstrates that a ss(+)RNA virus (SsHV2-L) and notably, a ssDNA virus (SsHADV-1) are not only the triggers but also the targets of RNA silencing pathways in *S. sclerotiorum* based on the production of virus-derived small RNAs (vsiRNAs) in virus-infected wtDK3. As mentioned, small RNAs are known to influence various cellular functions by altering gene expression at the transcriptional and post-transcriptional level. For this reason, it may be informative to study the impact the accumulation of mycovirus-derived small RNAs may have on *S. sclerotiorum* gene

expression since vsiRNAs can encompass a sizeable proportion of total small RNA accumulation in virus-infected strains. In our study, for example, up to 14% of the total small RNA accumulation in SsHV2-L -infected wtDK3 were vsiRNAs. A small number of studies have shown that vsiRNAs may be able to silence certain plant host genes that share an amount of complementarity to them (58).

It is unlikely that the high percentage of virus derived sRNAs that contained terminal mismatches is due to chance or the introduction of errors during the amplification of small RNAs. This is because an obvious pattern of mismatches involving primarily A or T nucleotides at the 5' and 3' termini is evident. This suggests that non-random modifications of vsiRNAs may have occurred. A similar pattern of terminal mismatches was also discovered in vsiRNA present in virus-infected *C. parasitica* (59). One possibility is that mismatches are generated during the production of secondary siRNAs. This would indicate that a significant portion of SsHV2-L and SsHADV-1 derived siRNAs are associated with secondary silencing. The abundance of 22 nt long vsiRNAs found in our study may further support this hypothesis since in plants 22 nt long miRNAs are associated with secondary siRNA production (60).

Mycoviruses belonging to the families *Hypoviridae* and *Genomoviridae* are widespread. *S. sclerotiorum* is the host of the sole representative of *Genomoviridae*, SsHADV-1, a ssDNA virus. We have demonstrated in our study that SsHADV-1 can be the trigger and target of RNA silencing pathways; however, more studies are needed to help us understand how and when the RNA silencing pathway, which is traditionally triggered by dsRNA molecules, is triggered by DNA viruses. Thus far, one hypothesis that has been put forth for dsDNA viruses is that overlaps in viral transcripts resulting

from overlapping or adjacent genes or secondary structures in viral RNA transcripts may serve as the initiators of the RNA silencing response against these viruses (61). It is unclear how dsRNAs that result in primary siRNA are made in the case of ssDNA viruses but secondary siRNAs are speculated to be made from host-encoded RNA-dependent RNA polymerases and these comprise the majority of siRNAs found in a plant infected with a geminivirus (62).

Overall, the results derived from this study will have broad relevance to efforts to understand the complex interactions between viruses and *S. sclerotiorum* RNA silencing pathways and the contributions made by dicer and argonaute genes to these mechanisms. These findings will pave the way for the development of novel control strategies that exploit RNA silencing mechanisms through HIGS or VIGS techniques.

CHAPTER 3: CONCLUSION

Studies in *Cryphonectria*, *Mucor*, *Aspergillus* and other fungal species indicate that RNA silencing pathways are widely conserved in filamentous fungi but have diversified and evolved among species. The experiments conducted in this study demonstrate that *S. sclerotiorum* has robust RNA silencing mechanisms that function primarily in antiviral defense but that also contribute to endogenous gene function and pathogenicity since virus-free RNA silencing mutants exhibited changes in phenotype such as slower growth and smaller sclerotia. Physiological and developmental changes have also been observed in other fungal RNA silencing mutants; for example, *C. higginsiunum* RNAi mutants showed severe defects in conidiation and conidia morphology (34).

RNA silencing mechanisms and mycoviruses provide viable avenues that can be exploited in the development of biological control agents. Thus far, biological control strategies against *S. sclerotiorum* have utilized parasitic fungi such as *Coniothyrium minitans* which is capable of colonizing and degrading sclerotia (63). A commercial formulation of *C. minitans* known as ConstansWG has been shown to significantly reduce the damage caused by *S. sclerotiorum* (64). Fungi of the genus *Trichoderma* have also been used extensively as biological control agents, however most of these studies were limited to laboratory or green house conditions (3). Still, the efficiency of antagonistic microbes as biological control agents is rarely sufficient or comparable to the efficiency of synthetic fungicides. Furthermore, the activity of antagonistic agents is affected by environmental factors such as temperature, pH, pesticides, and other soil microorganisms. There is clearly a need for more efficient and durable biological control strategies.

Important considerations in the use of mycoviruses as biological control agents are the transmission of mycoviruses between vegetatively incompatible isolates and the means of introducing mycoviruses into fungal host strains. Full-length cDNA based reverse genetics approaches for artificial inoculation are available for only a few fungi (12). Vegetative incompatibility could be overcome by using viral vectors that disrupt genes involved in vegetative incompatibility through virus-induced gene silencing (VIGS). This would facilitate the spread of hypovirulence-inducing mycoviruses. Such vector constructs would also be engineered to disrupt known fungal virulence factors such as oxalic acid production or critical fungal genes such as the chitin synthase gene to devastate fungal infectivity or growth.

Additionally, the continual discovery of mycoviruses with unique features such as SsHADV-1 is certain to speed up progress in this area of research. SsHADV-1 presents a unique opportunity in mycovirus-based biological control due to its capability for extracellular transmission as well as its ability to infect a mycophagous insect, *Lycoriella ingenua*, and use it as a transmission vector (65). Furthermore, SsHADV-1 has been shown to infect isolates from more than one VCG (43).VIGS constructs based on viruses such as SsHADV-1 would help to overcome some of the limits to entry and transmission that VIGS vectors may face.

The ever expanding knowledge of fungal RNA silencing mechanisms and the frequent discovery of novel mycoviruses will bring us closer to a future where mycovirus- based biological control of *S. sclerotiorum* is a widespread and efficient practice.

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APPENDIX

Primer Name	Sequence	Note
YG-F	CGTTGCAAGACCTGCCTGAA	All KO
YG-F-nested	CGATTGCTGATCCCCATGTG	All KO
PtrpC-F	ACGACTCACTATAGGGCGAATTGGGT	All KO
TtrpC-R	TACCTACGATGAATGTGTGTCCTGTAGGCTT	All KO
HY-R	GGATGCCTCCGCTCGAAGTA	All KO
HY-R-nested	GATGTTGGCGACCTCGTATT	All KO
F1-DCL1	AAAAACTAGTCTGGGCCCGT	Dcl1 KO
F1-DCL1-nested	GGCTGGAGCATTTCACATTGG	Dcl1 KO
F2-DCL1	ACCCAATTCGCCCTATAGTGAGTCGTATGAACAGACGATGGCGGAC	Dcl1 KO
F3-DCL1	AAGCCTACAGGACACACATTCATCGTAGGTATTATACCACACCGGGAGAAGC	Dcl1 KO
F4-DCL1	GTGGTGGGGGAATCAGTTGT	Dcl1 KO
F4-DCL1-nested	CAAAACCACCGGAGAATGCG	Dcl1 KO
F2-G418-DCL1	GAAGGGCGAATTCCACAGTGATGAACAGACGATGGCGGAC	Double DCL KO
F3-G418-DCL1	ACTGGCCGTCGTTTTACAACTTATACCACACCGGGAGAAGC	Double DCL KO
F1-DCL2	GGCATGCCCCGTTTGTATTT	Dcl2 KO
F1-DCL2-nested	GGGGCCCCCTTTATTGTTCA	Dcl2 KO
F2-DCL2	ACCCAATTCGCCCTATAGTGAGTCGTTTCCGGGTGCAGTTATCCAT	Dcl2 KO
F3-DCL2	AAGCCTACAGGACACACATTCATCGTAGGTAGTTACTGGATATATATATCA	Dcl2 KO
F4-DCL2	TTCGGCTTGTACTGTCCACC	Dcl2 KO

F1-SacI-Dcl1	TACTCAGAGCTCCATGTCTTCCGAACCACCTT	Dcl1 complementation
F4-Not1-Dcl1	TTACTGCGGCCGCTTGCCCTAAATCTGCAATCC	Dcl1 complementation
F1-AGO2	TGGTGAATTGTGAGTTGAATGGTG	Ago2 KO
F1-AGO2-nested	GTTTGCAACAATCGCAGGTG	Ago2 KO
F2-AGO2	ACCCAATTCGCCCTATAGTGAGTCGTGCTGCTGGATCAAAAGACAT	Ago2 KO
F3-AGO2	AAGCCTACAGGACACACATTCATCGTAGGTACCTGGTCATACCTTCCGCAT	Ago2 KO
F4-AGO2	CAGGTCCAAGTCCTGTCCAC	Ago2 KO
F4-AGO2-nested	TCTCCAACCAGCTACCGATG	Ago2 KO
F1-AGO4	TTTGGTCCAGGCCTTGGTTT	Ago4 KO
F1-AGO4-nested	TTTTCACAACGGGTTTGGGC	Ago4 KO
F2-AGO4	ACCCAATTCGCCCTATAGTGAGTCGTGAGCCATTAGCTTGGATATTCGCA	Ago4 KO
F3-AGO4	AAGCCTACAGGACACACATTCATCGTAGGTAAGTGCCTTCATATCATAATCCTCC	Ago4 KO
F4-AGO4	AAGGTTCGTCGGTTGGTAGT	Ago4 KO
F4-AGO4-nested	CCCTACTTGTCCCACGTGAT	Ago4 KO