Optimization of *in vitro* RNA interference assays to reduce variability in target gene knockdown in the phytopathogen *Sclerotinia sclerotiorum*

by

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ABSTRACT

Sclerotinia sclerotiorum is a broad host range fungal phytopathogen that is the causal agent of Sclerotinia stem rot in many crop plants, causing devastating yield losses worldwide. Sclerotinia is partially controlled using broad-spectrum chemical fungicides, but new, more species-specific fungicides, such as double-stranded RNAs, are being considered as safer alternatives. This study evaluated the effectiveness of long double-stranded RNAs (dsRNAs) and paperclip RNAs (pcRNAs) to induce gene silencing in two different fungal inoculants of *S. sclerotiorum*. The three different genes that were targeted in this study, SS1G_01703, SS1G_00005, SS1G_14116, are all involved in different aspects of *S. sclerotiorum*'s infection process i.e. pathogenicity, encoding 60S ribosomal unit and mycelial growth respectively. Initial dsRNA experiments using small fungal plugs collected from solid media generally resulted in highly variable levels of transcript knockdown, potentially arising from unequal hyphal amounts in treatment tubes. This led to the development of fungal slurries as the inoculum to provide a more uniform distribution of hyphae across all the samples. The slurry method resulted in significant transcript knockdowns using all three long dsRNAs at different doses, although some variability persisted. The variability using the slurry method was linked to potential differences in hyphal developmental stages, age of hyphae and differences in expression levels of each target gene. In contrast, structurally different and shorter in length pcRNAs failed to induce consistent transcript knockdown in both plug and slurry assays, despite earlier reports of their efficacy in reducing lesion sizes on canola leaves. This inefficacy may stem from pcRNAs' reliance on a single siRNA for gene targeting, potentially insufficient to overcome the structural complexity of target mRNAs. These findings highlighted the potential of long dsRNAs for gene knockdown in *S. sclerotiorum* using optimized slurry-based assays, while suggesting that pcRNAs require further modifications. Future work can explore alternative siRNA designs, increased pcRNA doses, and identifying accessible mRNA regions to improve knockdown efficiency. Additionally, optimizing the methods to assess hyphal developmental stages and minimizing variability during sample preparation are crucial for reproducibility and accuracy in RNAi-based studies.

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NON-COMMON ABBREVIATIONS USED

Ago	ARGONAUTE
DCL	DICER-LIKE
dpi	days post inoculation
dsRNA	double-stranded RNA
FPKM	fragments per kilobase of million mapped reads
HIGS	host-induced gene silencing
hpRNA	hairpin RNA
miRNA	micro-like RNA
mRNA	messenger RNA
nt	nucleotide
PTGS	post-translational gene silencing
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA inteference
SIGS	spray-induced gene silencing
siRNA	small interfering RNA
sRNA	small non-coding RNA
SSR	sclerotinia stem rot
TF	transcription factor

CHAPTER 1: BACKGROUND AND RELEVANCE

1.1 Significance of Canola in Canadian agriculture:

Canola plays a vital role in Canadian agriculture, contributing to the economy, fostering innovation, and supporting sustainable practices. Developed from the traditional rapeseed, Canada's canola industry utilizes advanced breeding techniques and agronomic innovations to cater to both domestic needs and international markets. Canada is a recognized global leader in canola production and exportation, with the Prairie Provinces of Manitoba, Saskatchewan, and Alberta serving as key contributors to this sector. This leadership significantly boosts Canada's GDP while fostering rural employment and driving advancements in agricultural technology. Canola plays a major role in strengthening the Canadian economy by boosting exports, increasing farm revenues, and supporting related industries. The adoption of biotechnological advancements, including herbicide-resistant canola strains, between 1969 and 2012, markedly improved productivity and economic returns (Brewin & Malla Stavroula, 2012). Research by Smyth et al. (2011) revealed that the adoption of herbicide-tolerant canola has provided substantial agronomic advantages while minimizing environmental impacts, thereby promoting sustainable agricultural practices. Due to their favourable climate, soil conditions, and advanced farming practices, the Canadian Prairies are renowned for their ideal conditions for cultivating canola, establishing it as the second most widely grown crop in the region after wheat (Morrison et al., 2016). The integration of canola into crop rotation strategies contributes to enhanced soil health by disrupting pest and disease cycles, improving nutrient dynamics, and promoting biodiversity within the soil ecosystem (Shrestha et al., 2014). Canola cultivation has been associated with a reduction in carbon footprints, as modern agronomic methods and efficient resource use minimize greenhouse gas emissions during production. Hence, the combination of ecological and economic benefits makes canola an integral crop for both sustainable farming and economic resilience in Canadian agriculture.

1.2 Overview of Sclerotinia Infestation in Canola in Canada:

The necrotrophic fungus *Sclerotinia sclerotiorum* is the causal agent of stem rot disease in canola and can survive in the cropping ecosystem by overwintering in soil without depending on any host in the form of a resting spore called sclerotia (Ordóñez-Valencia et al., 2015). In a favorable

environment, i.e., prolonged humidity and temperature (12 to 24°C), sclerotia germinate into mushroom shaped apothecia which produce thousands of air-borne spores that land on senescent canola flower petals (Kabbage et al., 2015). The infected petals fall on leaves, spreading the Sclerotinia infection throughout the host tissues of stems, leaves, and pods of canola plants. As the infection advances, the fungi develop sclerotia in the vasculature of the host plant and the plant stem becomes bleached, weak, and wilted, which leads to premature death (Kabbage et al., 2015; Liang & Rollins, 2018). Petal infestation is a pivotal stage in the disease cycle, serving as a standard indicator for predicting Sclerotinia outbreaks (Turkington et al., 1991). These outbreaks, while dependent on spore abundance, are also highly dependent on optimal moisture and temperature conditions during the flowering period (McDonald & Boland, 2004).

1.3 Pre-existing measures to control Sclerotinia:

Many practices have been adopted to control Sclerotinia stem rot including canopy management (McDonald et al., 2013), crop rotation (Peltier et al., 2012), chemical fungicides (O'Sullivan et al., 2021), and resistant plant breeding against *S. sclerotiorum* (Derbyshire & Denton-Giles, 2016). Because of the prolonged survival capacity of sclerotia (seven years), agronomic practices are not effective enough to control Sclerotinia disease (Dolatabadian et al., 2022). Additionally, plant resistance to Sclerotinia is governed by the interaction of multiple minor quantitative trait loci (QTLs) that are involved in detoxification, ROS production and oxidative protection that are part of defence responses. While each minor gene has a limited effect, their combined action contributes to plant's overall defense hence, making it difficult to breed completely resistant canola cultivars. Besides, conventional breeding can potentially introduce undesirable traits in resistant plants among the offspring (Wang et al., 2019).

Chemical fungicides such as anilinopyrimidines, methyl benzimidazole carbamates, dicarboxamides, demethylation inhibitors (DMIs), strobilurins, and succinate dehydrogenase inhibitors (SDHIs) have widely been applied to plants before flowering to prevent Sclerotinia infection. The active ingredient of the fungicides inhibits Sclerotinia infection in plants in various ways, including disruption of microtubule formation, inhibition of membrane sterol biosynthesis of functional cell walls, and inhibition of mitochondrial respiration, to name only a few (Derbyshire & Denton-Giles, 2016; Peltier et al., 2012). Fungicides are applied several times to crops to ensure the prevention or delay of disease incidence. Thus, selective pressure has been

observed in Sclerotinia and other non- target pathogens that evolve into resistant strains due to extensive use of chemical fungicides (Jo et al., 2008; Liu et al., 2021). In addition, the residues of fungicides are washed away into freshwater bodies and can become toxic to algae, fish, and invertebrates in aquatic ecosystems (Zubrod et al., 2019). Thus, for integrated disease management for Sclerotinia, RNA interference (RNAi), a natural type of eukaryotic innate immunity, can be another possible approach to reduce the fungal infestation (De Schutter et al., 2022; Degnan et al., 2023; Majumdar et al., 2017; McLoughlin et al., 2018).

1.4 Mechanism of RNA interference:

RNA interference (RNAi) is an innate defense mechanism in eukaryotes initiated by the introduction of double-stranded RNA (dsRNA) that causes cells to silence genes complementary to the dsRNA's sequence (Baulcombe, 2004). The concept of RNAi was illuminated in 1990, with the silencing of the purple pigment-encoding genes in petunia, resulting in white or patchy petunia flowers (Napoli et al., 1990). Later, the role of dsRNA as the cause of the gene silencing was described for the first time in a nematode, *Caenorhabditis elegans* (Fire et al., 1998), and in 2006, the lead investigators, Andrew Fire and Craig Mello, were awarded the Nobel Prize for Physiology or Medicine for their pioneering work.

The RNAi mechanism is categorized into three branches depending on the non-protein coding RNAs i.e small interfering RNA (siRNA) mediated pathway, micro-RNA (miRNA) mediated pathway, and piwi- interacting RNA (piRNA) mediated pathway that transcriptionally or post-transcriptionally regulates the gene expression (Matranga C & Zamore PD, 2007). MiRNAs are primarily associated with regulating gene expression, while siRNAs are involved in defense against viruses, transposable elements, and other aberrant RNA molecules (Shabalina & Koonin, 2008). However, they differ based on their origin and the degree of their complementarity to the target sequence. While miRNAs emerge from endogenous non-coding RNA, siRNAs are derived from viral dsRNA or the host's own RNA-dependent RNA polymerase. RNAi machinery, found in virtually all eukaryotes, consists of three key proteins - Argonaute proteins, Dicer-like proteins that typically consist of RNaseIII and helicase domains, and RNA-dependent RNA polymerases (RdRP) (Gaffar et al., 2019). The mechanism is initiated by the cleavage of dsRNA producing small interfering RNA (siRNA) duplexes by an RNAase III enzyme named 'Dicer' (Figure 1.1). The resultant siRNA duplexes are 21–23 nucleotides long and loaded into an Argonaute (AGO)

protein, which is a part of the RNA- induced silencing complex (RISC). Subsequently, the siRNA duplexes get unwound in the RISC complex, AGO then releases the passenger strand and activates the guide strand while connected with the RISC complex. The guide strand of siRNA recognizes the target mRNA by complementary base pairing, which subsequently allows AGO to cleave the mRNA, hence preventing translation. In some organisms, including plants, a secondary cycle of RNAi is started by recruiting RdRP at the 3' end of cleaved mRNA that serves as a template. This template is used in the biogenesis of secondary siRNAs, thereby amplifying the RNAi signal (Nien et al., 2024; Svoboda, 2020).

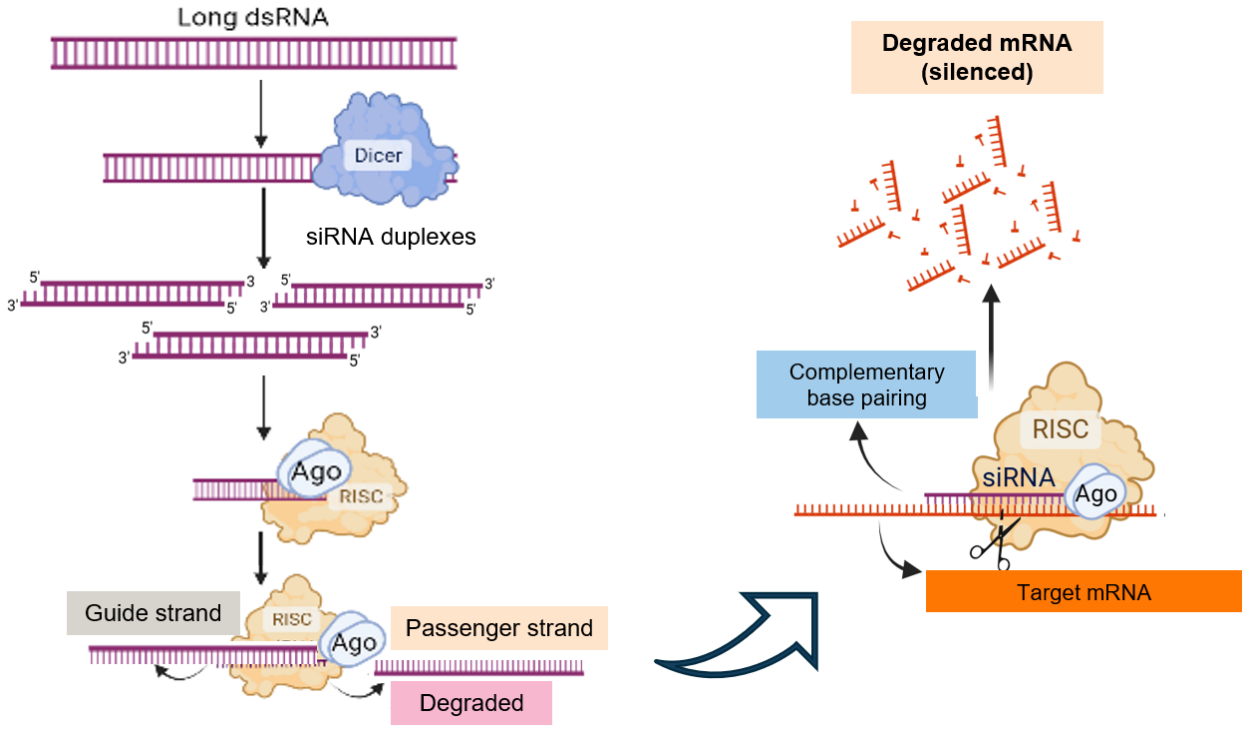

Figure 1.1: Schematic illustration of RNAi mechanism. Double stranded RNA (dsRNA) binds with Dicer protein and is cleaved into small interfering RNA (siRNA). The siRNAs bind with Argonaute (Ago) protein and are loaded into RNA-Induced Silencing Complex (RISC). Double strands of siRNAs are separated, producing guide RNA and passenger RNA. While the passenger RNA gets degraded, guide RNA serves as a search probe for the base pairing of guide RNA to complementary target messenger RNA (mRNA) for degradation of the target sequence. Illustration was created with BioRender.com.

1.5 Cross kingdom RNAi in plants and fungi

In many species, siRNAs are recognized as regulatory molecules that can leave their host cell and move between cells, organisms, and species (Sarkies & Miska, 2014). In plants, siRNAs can easily spread short distances from their source to 10-15 adjacent cells by plasmodesmata (Wang & Dean, 2020). Furthermore, by producing secondary siRNAs by RdRP, the abundance of siRNAs can transport long distances within the plant, for example, from leaves to anthers and flowers (Zhang et al., 2014), from grafted materials to the host plant (Molnar et al., 2010), and from shoot to root through the phloem (Wassenegger & Krczal, 2006). In plants, the siRNA mediated resistance from viral disease can persist for prolonged periods due to the transport of dsRNA and generation of primary and secondary siRNAs derived from diced viral RNA (Baulcombe, 2004; Dalmay et al., 2000; Johansen & Carrington, 2001).

Similar to plants, a functional RNAi pathway exists in fungi that can respond to stress (Calo et al., 2014; Mochama et al., 2018a; Yin et al., 2020a), mediate the interactions with other organisms, limit transposon movement (Burke et al., 2019; Navarro-Mendoza et al., 2019; Yamanaka et al., 2013), confer viral defence (Hammond et al., 2008), regulate gene expression (Lau et al., 2018; Son et al., 2017), pathogenesis (Dunker et al., 2020; Gaffar et al., 2019; Jo et al., 2018; Mochama et al., 2018; Weiberg et al., 2013; Yin et al., 2020), and drug tolerance (Lax et al., 2020). DsRNA or siRNA, may move throughout the mycelial network of fungi by septal pores, microtubules, and endosomes that are involved in cargo transfer in cells of filamentous fungi (Bloemendal & Kück, 2013; Wang & Dean, 2020). Evidence of dsRNA-mediated gene silencing in fungi was initially demonstrated in the fungus *Neurospora crassa* (Romano & Macino, 1992). Fluorescein-labelled dsRNA or small RNA was detected in germinating spores of *Botrytis cinerea,* providing evidence of direct uptake of dsRNA in fungal cells (Wang et al., 2016). Subsequently, it was demonstrated that the dsRNA uptake mechanism was done by clathrin-mediated endocytosis in *Sclerotinia sclerotiorum* (Wytinck et al., 2020). Clathrin-mediated endocytosis is a cellular mechanism that enables the uptake of metabolites, hormones, and proteins by forming invaginations of the plasma membrane, thereby creating vesicles that import extracellular contents into the cell (Calvar et al., 2024). Furthermore, 24 hours of dsRNA treatment to *Fusarium graminearum* conidia led to silencing of targeted ergosterol biosynthesis genes proving the direct uptake of dsRNA by fungal cells (Koch et al., 2016). Uptake and silencing of essential fungal genes by dsRNA were also

demonstrated in *Magnaporthe oryzae, Colletotrichum truncatum,* and *Phytophthora capsici* (Cheng et al., 2022; Gu et al., 2019).

When fungal pathogens breach the initial physical barrier of plant cells, plants can detect pathogen-associated molecular patterns (PAMPs) (Mierziak & Wojtasik, 2024). In response to PAMPs, plants initiate PAMP-triggered immunity (PTI) to produce antifungal compounds or small RNAs (sRNAs). To counteract the plant's defensive response, the fungal pathogen produces toxins, effector proteins, and enzymes trafficked to plant cells. This plant-fungi interaction leads the plant to activate resistance (R) genes that recognize fungal effector proteins to initiate plant natural immunity. During the infection process, both the plant and fungal pathogen export sRNAs in response to pathogen virulence responses and host resistance responses, respectively (Cai et al., 2018). Fungal sRNAs are one of the pathogen effectors that ensure successful infection by inhibiting host immunity. Weiberg et al., (2013) showed that *Botrytis cinerea* hijacked RNAi machinery of Arabidopsis and tomato plants by transferring fungal sRNAs to bind with plant AGO, which resulted in the silencing of plant defence-related genes. On the other hand, host plant cells have demonstrated the capacity to transport small RNA molecules (sRNAs) into insects, oomycetes, fungi, and nematodes to induce gene silencing in the pest or pathogen (Nien et al., 2024). For example, when cotton plants were infected by *Verticillium dahliae*, the host showed increased miRNAs (miR166 and miR159), which were transported to the fungus to knock down two essential virulence-related genes encoding $Ca2^{+}$-dependent cysteine protease, and an isotrichodermin C-15 hydroxylase (Zhang et al., 2016). Similarly, sRNAs has also been documented to undergo cross-kingdom transport from Arabidopsis and rice to the fungus *Magnaporthe oryzae*, silencing the pathogenic genes (Jin et al., 2024).

1.6 Host induced gene silencing vs spray induced gene silencing:

Cross-kingdom RNAi prompted the development of an eco-friendly approach to manage plant disease that can be specific to the intended pathogen and capable of targeting multiple pathogens. One of the strategies to introduce RNAi into plants is called host-induced gene silencing (HIGS), where genetically engineered plants can express dsRNA targeting essential genes in pathogens upon infection to plants. (Niu et al., 2021; Wang et al., 2016). The dsRNA-expressing transgenes are introduced to plants via *Agrobacterium tumefaciens* transfection, and the dsRNAs then accumulate and can be processed within plant cells into siRNAs. The intact dsRNA and resulting

siRNAs are then absorbed by infecting fungal cells, to trigger RNAi within the phytopathogen, to silence genes critical for the fungi's infection process or its survival. One of the earliest examples of HIGS to protect a plant from fungal disease was conducted in transgenic barley plants expressing antifungal RNAi constructs against powdery mildew fungal disease. T1 plants exhibited successful mitigation of disease symptoms in comparison to non-transgenic control plants, confirming the potential effectiveness of HIGS. (Nowara et al., 2010). HIGS has also been employed in plant varieties of wheat, maize, barley, rice, and banana, to develop resistance against fungal diseases such as *Blumeria graminis*, *Fusarium*, *Puccinia*, *Magnaporthe oryzae* (Jin et al., 2024). A single transgenic rice variety was developed to target six pathogenic genes (*CRZ1, PMC1, MAGB, LHS1, CYP51A, CYP51B*) of *M. oryzae* to control rice blast disease (M. Wang & Dean, 2022). The transgenic plants successfully inhibited fungal growth and reduced the transcripts of fungal genes compared to negative control plants. Transgenic *Nicotiana benthamiana* and *Arabidopsis thaliana* were developed that expressed RNAi constructs targeting the *SsGAP1* gene responsible for sclerotia formation, appressoria production, and virulence in *S. sclerotiorum.* Transgenic leaves expressing the RNAi constructs targeting *SsGAP1* exhibited reduced lesions caused by *S. sclerotiorum* (Xu et al., 2024). Persistent silencing of *SsGAP1* was noted, as mycelia rescued from the infected leaves exhibited notable reduction in sclerotia formation when subsequently grown *in vitro*, and qRT-PCR analyses confirmed that the *SsGAP1* transcripts were reduced in the rescued mycelia.

To develop successful HIGS, RNAi constructs can be designed to target either pathogenesis-related genes or housekeeping genes of the fungal pathogen. A study showed that silencing the expression of the host-selective toxin-producing gene *ACTTS2* in *Alternaria alternata* unequivocally leads to the complete loss of pathogenicity by the organism (Ajiro et al., 2010). As a result, transformed *A. alternata* that lost the capacity to produce any ACT- toxins showed less than 50% infection to tangerine leaves. The findings confirm *ACTTS2* as a promising target to develop HIGS protection against citrus. High throughput silencing of calcium signalling genes in *Magnaporthe oryzae* affirmed their involvement in fungal hyphal growth, sporulation, and pathogenicity, and RNAi mediated knockdown of many of these genes attenuated fungal infestation in plants (Nguyen et al., 2008).

Although it is a promising disease management approach, HIGS is still subject to approval by regulatory bodies and must overcome public concerns. The approval of the application of genetically modified organisms to produce transgenic plants requires biosafety assessment before commercialization. A papaya variety expressing dsRNA conferring protection against Papaya ring spot virus (PRSV), and a plum variety 'HoneySweet' expressing hairpin RNA suppressing Plum pox potyvirus (PPV) have both undergone evaluation and been approved to cultivate in USA, Canada and Brazil. However, the varieties have not yet received approval to release in the European Union (Levy et al., 2000; Vadlamudi et al., 2020). In addition, methods to develop transgenic plants may differ from species to species, making HIGS a laborious effort. The efficiency of HIGS in plants, like other types of RNAi, broadly depends on the selection of genes targeted by RNAi constructs, but also on the design of the transgene construct with an appropriate promoter, length of target sequence and the site of integration of the construct within the genome of the target plants. Moreover, several generations of plants are required to obtain stable dsRNA-expressing plants, thus making it a lengthy process (Cai et al., 2018; Choudry et al., 2024).

As an alternative to HIGS, spray-induced gene silencing (SIGS) is likewise effective in controlling plant disease, as fungi can also take up environmental RNA (Qiao et al., 2021). In SIGS applications, lab-produced dsRNA, generated using either *in vitro* or microbial expression systems, is applied to host plants' surfaces and can be taken up by the pathogens when they attempt to infect the hosts. Infection of the plants can be reduced if the dsRNAs/siRNAs trigger the degradation of fungal target mRNAs involved in pathogenicity or growth of the pathogen as (Choudry et al., 2024). SIGS can confer protection to not only the tissues sprayed with the dsRNA, but also to distal, untreated portions of the plant (Morozov et al., 2019). Unlike HIGS, SIGS eliminates the technical challenges of genetically transforming the host plants, which is a distinct advantage, as there are many crop plants for which transformation techniques have not been established. The topical administration of dsRNA solutions offers a faster optimization for each plant species and may be more readily accepted by the public and regulators in countries that prohibit genetic modification technologies. Compared to many chemical fungicides, which can persist and readily spread beyond the agricultural ecosystem (Sánchez-Bayo et al., 2011), RNA-based pesticides may pose fewer environmental risks, as dsRNAs can quickly degrade in soil and water. While dsRNA was observed to be stable for 168 hours under sterile laboratory conditions (Wang & Jin, 2017), dsRNAs in soils degraded with several hours due to the presence of dsRNA

degrading enzymes from soil microbes (Bachman et al., 2016). The rapid biodegradability of dsRNA under natural conditions necessitates further in-depth research to ensure that the dsRNAs can last at least long enough to provide a degree of long- term and persistent plant protection (Fletcher et al., 2020; Zhang et al., 2020).

SIGS-mediated control of a fungal phyopathogen was first demonstrated in *Fusarium graminearum* infectecd barley, by targeting the *cytochrome p450* gene to limit the fungal growth in the leaves (Koch et al., 2016). Later, the SIGS approach was successfully demonstrated once again in *Fusarium graminearum*- barley (Höfle et al., 2020), in *S. sclerotiorum*- canola (McLoughlin et al., 2018), in *Botrytis cinerea*- tomato (Niño-Sánchez et al., 2022; Qiao et al., 2021), in *Rhizoctonia solani*- rice (Qiao et al., 2021), and *B. cinerea*- strawberry (Islam et al., 2021) patho-systems. SIGS also reduced *B. cinerea* disease formation in a wide range of hosts i.e. vegetables, fruits and flowers (Qiao et al., 2021).

The efficacy of SIGS can depend upon various factors, including the selection of the target genes of the dsRNA (Gebremichael et al., 2021). *In vitro* culture of *F. culmorum* incubated with dsRNA targeting the cytochrome P-450 gene CYP51A revealed that 90% silencing of *FcCYP51A* expression did not inhibit growth of the pathogen, presumably because *FcCYP51A* is not critical for development and survival of Fusarium in the *in vitro* cultures (Koch et al., 2018). In contrast, 40% silencing of a related gene, *FcCYP51B,* resulted in abnormal hyphal morphology and 30% reduced fungal growth, suggesting that the selection of the target gene is critical for effective control of the phytopathogen. Another factor affecting the success of SIGS in fungi is RNA uptake efficiency. High uptake efficiency was seen in *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Aspergillus niger*, and *Verticillium dahlia*. On the contrary, SIGS was ineffective at controlling diseases caused by *Colletotrichum gloeosporioides* and *Ustilago maydis*, as these two fungi showed no detectable uptake of fluorescein-labelled dsRNA (Qiao et al., 2021). The primary uptake pathway for dsRNA identified in *S. sclerotiorum* is clathrin-mediated endocytosis (CME), which appears to be a conserved dsRNA uptake mechanism in many eukaryotes examined to date (Wytinck et al., 2020). However, this observation does not imply that CME is the only dsRNA uptake pathway other mechanisms may be involved in different species, each with variable efficiencies. It was noted by Wytinck et al. (2020) that dsRNA entry was typically localized at the tips of the growing Sclerotinia hyphae, which suggests that uptake

of dsRNA could be affected by the growth phase of the fungus. Even in fungi that can take up dsRNA, targeting the same gene can have differential effects in different species. For example, the silencing of the *β2-tubulin* gene in *F. asiaticum* infecting wheat caused 90% transcript reduction, but in *C. truncatum* infecting soybean caused only 54% transcript reduction (Gu et al., 2019). The reasons for this difference in the two fungi could be many-fold, ranging from differences in the dsRNA uptake pathways, differences in the RNAi machinery, differences in the regulatory mechanisms of the target gene, and differences in the number of related or redundant genes that may be able to compensate for the loss of this gene's product. Based on all these observations, it is clear the multiple factors can contribute to the success of SIGS and each patho-system may need to be uniquely optimized for effective plant disease management.

1.7 Different structures of double stranded RNA:

In many RNAi applications in non-vertebrate species, synthetic long dsRNAs ranging in length from 200-600 nucleotides are used (Figure 1.2). Once internalized by the eukaryotic cells, Dicer cleaves the dsRNA into ~22-26 nucleotides to produce short interfering RNAs (siRNAs) (Vermeulen et al., 2005). In vertebrates, delivery of long dsRNAs will induce an interferon-mediated cellular response, leading to apoptosis of the exposed cells. To circumvent that problem, only siRNAs are used for RNAi in vertebrates, as these short double-stranded RNAs resemble naturally occurring miRNAs and thus do not induce the apoptotic mechanisms (Berkhout, 2018; Schuster et al., 2019). A modified version of siRNAs, short hairpin RNAs (shRNAs), have also been used in vertebrates and non-vertebrates. These shRNAs are so-named because their structure typically consists of a stem of 19-29 base paired complementary sequence separated by a loop of 7-9 nucleotides that give a hairpin- like shape. ShRNAs are recognized by Dicer by the two nucleotides overhang at the 3' end. The precision of Dicer cleavage is ensured when the Dicer can identify single- stranded RNA either in the form of a loop region or an internal bulge at a fixed distance (two nucleotides) from the cleavage site (Vermeulen et al., 2005). ShRNAs are more durable than siRNAs because the loop prevents the exonuclease interaction with dsRNA at the 5' or 3' ends, hence reducing dsRNA nuclease degradation (Solodushko & Fouty, 2023). Various studies in insects have demonstrated that dsRNAs are prone to attack by nucleases present in the gut, saliva and hemolymph, which results in degraded dsRNA and reduced the efficiency of the silencing (Arimatsu et al., 2007; Christiaens & Smagghe, 2014; Singh et al., 2017). In most insect applications, dsRNAs are delivered to the insects through their food, but the uptake of dsRNA into

the host gut cells may can be severely compromised if dsRNA molecules are degraded by nuclease enzymes before the dsRNA can reach its target mRNA within the cells (Chen et al., 2021). Injecting naked dsRNA directly into the body of the mosquito *Aedes aegypti* (Cirimotich et al., 2009), and the desert locust *Schistocerca gregaria* (Wynant et al., 2014) triggered strong RNAi-mediated gene knockdown, whereas orally delivered dsRNA was largely ineffective in these insects due to presence of the nuclease-rich saliva. In the fungus *S. sclerotiorum*, silencing of a nuclease encoded gene improved the RNAi silencing of target genes and reduced leaf necrosis, which indicated that nucleases can also hinder RNAi efficacy in organisms other than insects (Walker, 2022).

Considering this, a novel structure of dsRNA named a paperclip RNA (pcRNA) was developed that is characterized by complementary sequences of 25 nucleotides separated by two loops each having 9 nucleotides. Owing to the presence of the closed ends, pcRNA is also speculated to be more durable than conventional linear dsRNA or shRNA (Abbasi et al., 2020).

An interesting difference was observed between long dsRNA and pcRNA with respect to their cellular uptake mechanisms. A primary pathway for long dsRNA uptake in a range of species is clathrin-mediated endocytosis (CME), observed in Drosophila cells (Saleh et al., 2006), corn rootworms (Pinheiro et al., 2018), potato beetles (Cappelle et al., 2016), and the fungus *S. sclerotiorum* (Wytinck et al., 2020). Similarly, reduced uptake of long dsRNA (>200 nucleotides) was observed in mosquito (*Aedes aegypti*) cultured cells and larvae by chemically inhibiting the CME pathway. The paperclip RNA (pcRNA), however, was still able to induce target transcript knockdown where the CME pathway was blocked, indicating that the pcRNA entered cells through a clathrin- independent pathway (Abbasi et al., 2020).

A comparative study was carried out to evaluate the effectiveness of long dsRNA and pcRNA through foliar application on Arabidopsis leaves in reducing the leaf lesions induced by *S. sclerotiorum* (Walker, 2022). While pcRNA demonstrated efficacy in reducing lesion areas, its performance exhibited variability in comparison to long dsRNA, depending on different genes.

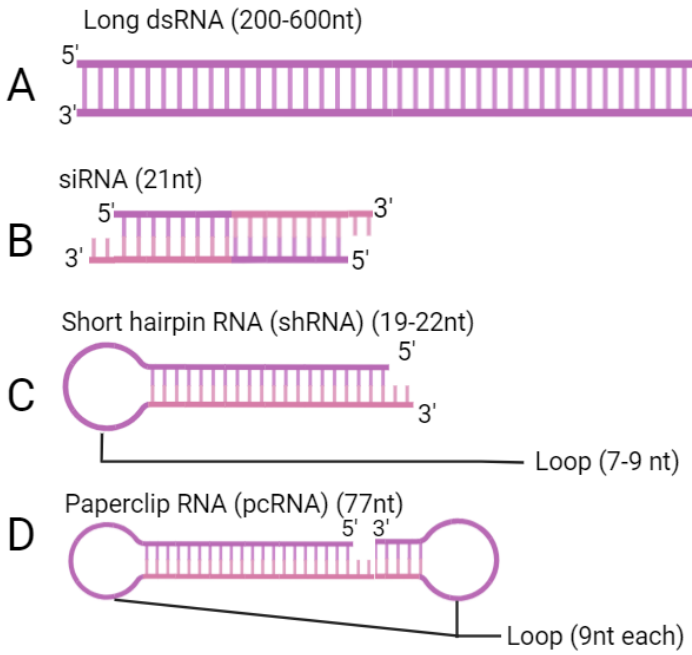

Figure 1.2: Structure of A) long double stranded RNA, B) small interfering RNA (siRNA), C) short hairpin RNA (shRNA) and D) paperclip RNA (pcRNA). A) Long dsRNA, 200-600 nucleotides long with open ends at both sides, B) siRNA, 21 nucleotide long having 2 nucleotide 3' overhangs, C) Short hairpin RNA (shRNA), 19-22 nucleotide long with one loop having 7-9 nucleotides and 2 nucleotide overhands on 3' end, D) Paperclip RNA (pcRNA), 23 nucleotide long having 2 closed ends and lacks covalent bond between 5' end and 3'end. Illustration was created with BioRender.com.

1.8 Safety of RNAi and Potential of Off-Target Effects:

Advanced genomic and transcriptomic sequence data have facilitated the development of highly specific dsRNAs, enabling the screening of potential off-target organisms sharing some similarity in the sequence of dsRNA that can be screened beforehand. This precautionary measure effectively reduces the risk of targeting organisms not intended to be affected by RNAi silencing. RNAi's target sequence specificity offers one layer of potential safety of this new class of pesticides, and the regular intake of RNA in most organisms' diets, including a variety of endogenous small double stranded RNAs suggests that RNAi-based crop protection methods may be safe for human and non-target organisms (Fletcher et al., 2020). There has been a well-documented history of humans and animals consuming exogenous and endogenous RNA that has 100% sequence complementarity with no adverse biological effects. Corn produces small RNAs that has

complementary sequences to 450–2300 unique protein coding RNA transcripts present in rats, mice, and humans (Petrick et al., 2016). Small RNAs found in food degrade once ingested by mammals by nucleases in saliva and blood plasma, the acidic environment of the stomach, and digestive enzymes in the small intestine. Besides, the lipid bilayer membranes of cells in the gastrointestinal tract and other tissues form barriers to ensure homeostasis after ingesting exogenous dsRNA instead of posing any health risk to the vertebrates (Petrick et al., 2013). Recently, RNAi-mediated resistance has been developed and successfully commercialized in cassava against ipomovirus (Yadav et al., 2011), in corn against corn rootworm (Bachman et al., 2016), in tobacco against plum pox virus, in potatoes against black spot and late blight, and in papaya against papaya ringspot virus (Halder et al., 2022). The European Food Safety Authority (EFSA) stated RNAi molecules exhibited limited bioavailability and quick turnover and no harmful effect after oral consumption in mammals (Ballarín-González et al., 2013).

To ensure effective plant disease control and to minimize environmental impact, developing dsRNA solutions necessitates comprehensive risk assessments. Given that both target and non-target organisms will be exposed to dsRNA solutions in the crop field, the gene sequence of dsRNA molecules must be specifically complementary to the target organism, thereby leaving beneficial organisms unharmed within the ecosystem. Furthermore, the application rate and timing of the dsRNA solution application for disease control must be optimized for each plant species. Additionally, the stability of sprayed dsRNA in the field is a critical factor, since UV rays, loss by rain, or nucleases present in soil and water can limit the effectiveness of dsRNA before it enters its intended target organism. Therefore, it is crucial to consider the sustained efficacy of the dsRNA solution in protecting plants against diseases. Another factor that can limit the efficacy of dsRNA pesticides is the development of resistance in pest populations. Like any other pesticide used in agriculture, repeated use of dsRNA will likely result in the development of resistance against dsRNA. To illustrate this point, lab-bred Western corn rootworms developed >10,000-fold resistance to dsRNA by increasing dosage exposures over as few as 11 generations. The resistance was attributed to an alteration of the insects' uptake machinery of dsRNA (Khajuria et al., 2018). Understanding the mechanism of resistance is the first step in anticipating resistance in the field, and in designing approaches to overcome this impending problem. The different route of cellular entry of the pcRNAs may be one approach to counter resistance mediated by changes in the CME

pathway, and it will be worth exploring if the lab-bred resistant insects are sensitive to this or other structurally altered dsRNAs.

Before releasing dsRNA as a biopesticide, it is crucial to assess for i) sensitivity, where the target organism will demonstrate susceptibility to RNAi; ii) relevance, wherein the target organism must be representative of a significant species or genus that can be found in field exposure; and iii) availability and reliability, requiring the target organisms to obtain sufficient quantity at the appropriate life stage, with readily accessible validated test protocols. (De Schutter et al., 2021).

Sclerotinia infection in canola contributes to decreased crop yields that negatively affects the national economy of Canada. The management of *S. sclerotiorum* disease in canola has emerged as a prominent area of interest, particularly considering environmentally sustainable practices, such as RNAi. Given the high target specificity and applicability to diverse pathogens, dsRNA bio-fungicides may perform more successfully than some of our current crop protection strategies. Understanding the underlying mechanism of different structured dsRNA could allow us to achieve improved target-dsRNA durability, providing better insights to develop effective RNAi bio-fungicides. The current study aims to elucidate intriguing aspects of the performance of dsRNA molecules, which eventually will be valuable knowledge to control *S. sclerotiorum* diseases. In particular, the ability of different structured dsRNA to reach its target mRNA and induce gene silencing is explored. To develop sustainable and safe dsRNA fungicides, exploring potentials of different structured dsRNA can be conducive to the increased RNAi efficiency. These studies will not only provide new options for worldwide canola protection against Sclerotinia but also will formulate a blueprint for advanced crop protection methods for other plant fungal diseases.

1.9 Objective:

Objective 1. Examine the effects of dsRNA dose on transcript knockdown in Sclerotinia

While previous studies conducted here at the University of Manitoba have provided evidence that exogenously delivered dsRNA can caused targeted gene knockdown and reduced growth of the fungus *Sclerotinia sclerotiorum* (Li, 2021; McLoughlin et al., 2018; Wytinck et al., 2020), further research is needed before the technology can be applied in a field crop setting. Several studies have observed that different doses of dsRNA may be required to effectively knock down different genes in any one species (Abbasi et al., 2020; Hong et al., 2005; Petrick et al., 2016; Shi et al., 2016; K. Zhang et al., 2020), but this issue has not been adequately addressed in Sclerotinia. In this study,

I set out to assess what doses of dsRNA were required to induce a significant knockdown of a small set of genes, by trying to optimize a liquid-based fungal growth assay that could be adapted for higher throughput screening of different dsRNAs. Using some assay methods previously developed in the Belmonte lab, I observed some highly variable responses of the fungus to different dsRNAs, and hence, part of my research became focused on modifying the assay to reduce the variability initially observed in my preliminary assays.

Research Question 1A: Using a previously established assay method, what dose of long dsRNA and pcRNA can cause significant transcript knockdown of the candidate genes?

Hypothesis: Each of the long dsRNA or pcRNA will exhibit significant knockdown in a specific dose and the dose will differ for different genes.

McLoughlin et al. (2018) used small agar plugs containing Sclerotinia hyphae to inoculate a liquid-medium suitable for Sclerotinia growth to evaluate the effects of dsRNA on transcript knockdown. Replicating this assay method, I examined several different dsRNAs' abilities to knockdown their target transcripts. Due to unexpected variation in the data, the assay was modified (see Research Question 1B).

Research Question 1B: Can we reduce the variability of the observed knockdown by using a modified liquid media-based assay?

Hypothesis: Homogenizing a large piece of agar containing Sclerotinia hyphae, to create a slurry of homogenously distributed fungal fragments, will result in less variable knockdown values.

The method used by McLoughlin et al. (2018) could potentially deliver very different amounts of fungus to each assay tube. By creating a large volume of homogenized agar, I expected that more consistent amounts hyphae would be added to each sample, which could reduce the variation previously observed using the previous method.

Objective 2. Compare the efficacy of long vs pcRNA to knock down different gene transcripts

Prior to the start of this study, pcRNAs had only been demonstrated to knock down transcripts in cells of the mosquito *Aedes aegypti* (Abbasi et al., 2020). Here, the ability of short pcRNAs to knock down transcripts in Sclerotinia will be assessed, and compared to knockdowns achieved using conventional, longer linear dsRNAs.

Research Question 2A: What dose of long dsRNA and pcRNA can cause significant transcript knockdown of the candidate genes using the revised liquid media method?

Hypothesis: Each of the long dsRNA or pcRNA will exhibit significant knockdown in a specific dose and the dose will differ for different genes.

Research Question 2B: Can we reduce the variability of the knockdown datapoints of each of the treatment using the revised liquid media method?

Hypothesis: By homogenizing the agar in which Sclerotinia was grown, a more consistent amount of hyphae will be provided to each assay tube and thus produce more consistent transcript knockdown values in each treatment.

Research Question 2C: Does the pcRNA knockdown the transcripts better than the long dsRNA?

Hypothesis: PcRNA will reduce the target transcripts more effectively than long dsRNA.

CHAPTER 2: MATERIALS AND METHODS

2.1 Selection of RNAi targets:

Targets for RNAi were selected from upregulated genes of *S. sclerotiorum* while grown *in planta Brassica napus* (Westar) and *in vitro* solid media Potato Dextrose Agar (PDA) (McLoughlin et al., 2018). A study done by McLoughlin et al., (2018), selected RNAi target genes based on their involvement in different fungal biological processes i.e. ROS response, pathogenicity factors, cell wall modification, protein modification, transcription, splicing, and translation. In the current study, gene targets were selected based on Fragments Per Kilobase per Million mapped (FPKM) of sequence reads of mRNA values ranging between 33 and 7000 avoiding genes with multiple homologs, functional redundancy, and high levels of expression while Sclerotinia infecting leaves to achieve successful RNA interference silencing of the target gene. In the current study, three target genes fulfilling those criteria, SS1G_01703, SS1G_00005 and SS1G_14116 from a subset of genes from McLoughlin et al. (2018) were selected for the further experiments, based on their ability to both suppress fungal growth in *in vivo* studies and their ability knock down the target transcripts by more than 50% compared to negative control in *in vitro* experiments in previous studies (Li, 2021; Walker, 2022; Wytinck et al., 2020) (Table 2.1).

Table 2.1: Selected list of *S. sclerotiorum* target genes for RNA interference, their function and FPKM values in vitro and in planta

Target gene	Function	FPKM value of the gene *in vitro* (PDA)	FPKM value of the gene *in planta* (*Brassica napus*)
SS1G_01703	Aminoacyl-tRNA ligase activity, *ABHYDROLASE-3*	44	301
SS1G_00005	Encode 60S ribosomal subunit	6057	4338
SS1G_14116	Mycelial growth	33	89

2.2 Maintenance of fungal cultures:

S. sclerotiorum ascospores were collected and generated at the Morden Research and Development Centre (Agriculture and Agri-Food Canada, Morden, MB, Canada). The ascospores (1×10^5 mL^{-1}) were allowed to germinate in full strength Potato Dextrose Agar (PDA) (Difco Laboratories Inc., Detroit, MI, USA) in 100 mm Petri dishes. Using a P1000 pipette tip, a 1 mm diameter agar plug was collected from the growing edge of the mycelia in the abovementioned PDA plates media was taken. The agar plug was then released from the P1000 pipette tip by aspiration into 25ml of solidified quarter strength PDA media (9.75g PDA, 11.25g BD Bacto dehydrated agar dissolved in 1L distilled water) to re-culture *S. sclerotiorum* in a liquid culture. Fungal mycelia were allowed to grow in the quarter strength PDA media at 23°C, in the dark, condition for three days. Three days post inoculation, another single plug was collected from the growing edge of fungal mycelia to maintain the next subculture and allowed the mycelia to grow for the next three days on quarter strength PDA solid media at 23°C, in the dark condition.

2.3 Design and in vitro synthesis of long dsRNA:

S. sclerotiorum sequences were retrieved from NCBI and primer pairs were designed to PCR amplify the exons of the target genes that will be used as a template for dsRNA (250bp- 400bp). The quality of the primer pairs i.e. melting temperature, GC content, hairpin formation, and dimer formation was assessed using the IDT OligoAnalyzer Tool (https://www.idtdna.com/pages/tools/oligoanalyzer). To avoid regions of complete 20-mer homology to *Brassica napus*, *Apis mellifera*, *Mus musculus*, and *Homo sapiens*, the dsRNA-targeted regions were compared to these organisms' transcriptomes using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) RefSeq accessions. Furthermore, a BLASTN query was done to ensure that the dsRNA target region is complementary to a single transcript of the target gene in *S. sclerotiorum*. After selecting the primers, the T7 promoter sequence (TAATACGACTCACTATAGGG) was added to the 5' end of both primers so that the target PCR template could be recognized by the T7 RNA polymerase during the dsRNA synthesis reaction. The length of IVT primers, melting temperature (Tm) and GC content for the primers are 44- mer, 52-60°C and 50-60% respectively. Primers for cloning and synthesizing long dsRNA are listed in Table 2.2.

Table 2.2: Primer sequences for cloning and synthesizing *S. sclerotiorum*-specific dsRNAs.

Sequences in upper cases are the transcription site of T7 polymerase. Six random nucleotides were added upstream of the T7 polymerase sequence to enable the enzyme to interact with the template more effectively and thus enhance the efficiency of transcription initiation. Sequences in lower cases are the primers to amplify the target regions that will be used as a dsRNA template.

Target	Accession number	Sequence (5'-3')	Product size
SS1G_01703_F	XM_001597459.1	ACCTCATAATACGACTCACTATAGGGttctgccggaaaccctcttc	259bp
SS1G_01703_R		ACCTCATAATACGACTCACTATAGGGaccgccgattgtgaagactt	
SS1G_00005_F	XM_001597869.1	ACCTCATAATACGACTCACTATAGGgcccaagttggtcttcgtta	380bp
SS1G_00005_R		ACCTCATAATACGACTCACTATAGGgcttgaatggccagaggaag	
SS1G_14116_F	XM_001584969.1	GGATCCTAATACGACTCACTATAGGgttaacgcccgttggaagta	285bp
SS1G_14116_R		GGATCCTAATACGACTCACTATAGGGcacgtaaaagcccaggtgat	

Total RNA was extracted using a Qiagen RNeasy Plant Mini Kit (Cat# 74904) from three days old *S. sclerotiorum* grown in a liquid broth of Potato Dextrose Broth (PDB). RNA was treated with DNase to remove any genomic contamination immediately followed by the synthesis of complementary DNA (cDNA) using a Maxima First Strand cDNA Synthesis Kit provided with the inclusion of DNase enzyme (K1672). Later, the cDNA was PCR amplified (Q5 High Fidelity Kit; NEB #E0555S/L) with the IVT primers under the following conditions: 95 °C for 3 min; followed by 35 cycles of: 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s; and a final extension of 72 °C for 3 min. PCR products were purified and resolved by electrophoresis using 1% agarose supplemented with Syber Safe DNA gel stain (Thermo S33102) and visualized using a ChemiDoc™ Imaging System (Bio-Rad Laboratories, Hercules, Ca, USA). The PCR products

were gel excised and purified using the E. Z. N. A. Gel Extraction Kit (Omega Bio-Tek) according to the manufacturer's instructions.

The PCR products were inserted into the pJET1.2/blunt Cloning Vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) according to the manufacturer's specifications. To confirm that the plasmids contained the correct inserts, the plasmids were first linearized by digesting the plasmids using *Nco*I, and sequenced using the T7 primer at the Centre for Applied Genomics in Toronto, Ontario (http://www.tcag.ca/facilities/dnaSequencingSynthesis.html).

The plasmids were transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells (Thermofisher #C404010) as described by the manufacturer. The cells were plated onto Luria broth (LB) agar plates (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, and 1.5% bacto-agar) and selected using ampicillin (50 µg/mL; MP Biomedicals Inc., Santa Ana, CA, USA) to incubate at 37 °C overnight.

A colony PCR screen was conducted using primer specific and plasmid specific primers (Forward Sequencing Primer: 5'-CGACTCACTATAGGGAGAGCGGC-3' and Reverse Sequencing Primer 5'-AAGAACATCGATTTTCCATGGCAG-3') using the conditions stated above but with the initial step of 95 °C for 10 min. To confirm the transformation, the samples were resolved on a 1.5% agarose gel stained with SYBR Safe DNA gel stain. Bacterial colonies that were confirmed to contain the expected insert, were grown in a shaking incubator (230 rpm, 37°C overnight) in 5mL of LB broth supplemented with 50 µg/mL ampicillin. Plasmid DNA was extracted and purified from the liquid cultures (4mL) following the instructions of the Monarch Plasmid Miniprep Kit. The purified plasmid was PCR amplified using the T7-linked primers (Table 2) by Q5 High Fidelity Kit to obtain the desired template to produce dsRNA molecules. The sequence of PCR products was confirmed by Sanger sequencing (http://www.tcag.ca/facilities/dnaSequencingSynthesis.html). To prepare the glycerol stock of each of the dsRNA template, 500µl from the bacterial liquid culture was mixed with 500µl of 50% glycerol and subsequently stored in -80°C.

To synthesize dsRNA, the MEGAscript RNAi kit (Invitrogen, Carlsbad, CA, US) was used to produce long dsRNA 00005, long dsRNA 01703, and long dsRNA 14116 from their respective PCR product amplified (Figure 2.1) by their respective IVT primers (Table 2.2). NanoDrop One UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to quantify the

long dsRNA. The size and quality of long dsRNAs were visualized using agarose gel electrophoresis and the Agilent 4150 TapeStation System.

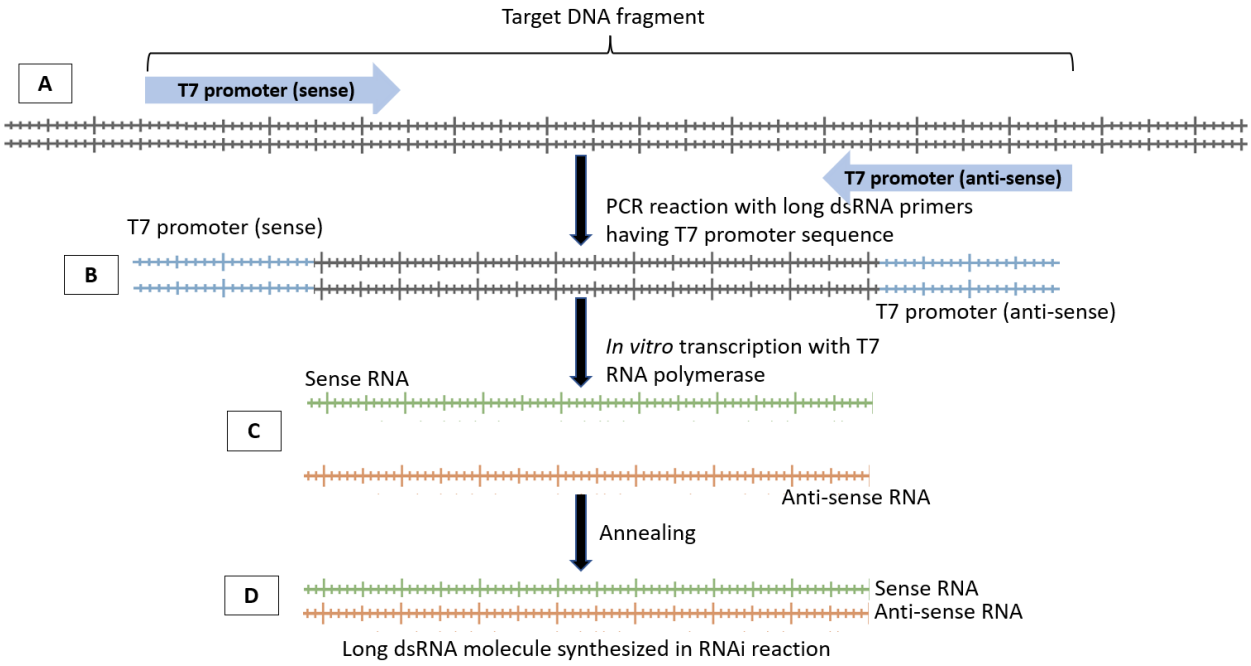

Figure 2.1: Synthesis of long dsRNA from pJET plasmid. A) The primers having T7 promoter sequence amplify the target sequence from pJET plasmid. B) PCR product now having T7 promoter sequence will be used as template for synthesis of long dsRNA molecules. C) *In vitro* transcription with T7 RNA polymerase to synthesis sense and anti-sense RNA. D) Annealing of complementary RNA strands to form the long dsRNA

2.4 Design and synthesis of paper clip RNA (pcRNA):

The target region (25bp) for pcRNA was selected within the target region of long dsRNA for each gene using IDT's siRNA design algorithm using default parameters. A single DNA oligo (101bp) to synthesize pcRNA (77bp), consists of two complementary sequences (each 25bp) selected from their respective long dsRNA target region and linked by two noncomplementary short loops of adenosine each containing 9 nucleotides. In addition to two loops, additional nucleotides were added to enable the RNA to fold into the pcRNA (Figure 2.2). The 5' region of each of the oligonucleotides contains a T7 promoter sequence to allow the transcription reaction. Both sense

and antisense strands were used as a template to synthesize each pcRNA for each gene. The sequences of DNA oligos to synthesize pcRNA are listed in Table 2.3.

Table 2.3: DNA oligo sequences used as template for pcRNA synthesis

Target	Sequence (5'-3')	Product size
SS1G_01703_sense	5'CATGTTTTTTTTTCATGTTGGATGATAGTCTTAAGGCTTTTGAATTTTTTTTTTTCAAAAGCCTTAAGACTATCATCCTATAGTGAGTCGTATTAGGATCC 3'	77bp
SS1G_01703_antisense	5'GGATCCTAATACGACTCACTATAGGATGATAGTCTTAAGGCTTTTGAAAAAAAAAAATTCAAAAGCCTTAAGACTATCATCCAACATGAAAAAAAAACATG 3'	
SS1G_00005_ sense	5'GGATCCTAATACGACTCACTATAGGTATTGTCTGTATGGAAGATTTGAAAAAAAAAATCAAATCTTCCATACAGACAATACCGTCATGAAAAAAAAACATG 3'	77bp
SS1G_00005_ antisense	5'CATGTTTTTTTTTCATGACGGTATTGTCTGTATGGAAGATTTGATTTTTTTTTTCAAATCTTCCATACAGACAATACCTATAGTGAGTCGTATTAGGATCC 3'	
SS1G_14116_ sense	5'CATGTTTTTTTTTCATGAGGTGGCAACGCAAAAGCTGTTGTCAATTTTTTTTTTTGACAACAGCTTTTGCGTTGCCACTATAGTGAGTCGTATTAGGATCC 3'	77bp
SS1G_14116_ antisense	5'GGATCCTAATACGACTCACTATAGTGGCAACGCAAAAGCTGTTGTCAAAAAAAAAAATTGACAACAGCTTTTGCGTTGCCACCTCATGAAAAAAAAACATG 3'	

Both the sense and antisense strands of DNA oligos for a pcRNA were heated at 94°C for 2 minutes to remove any secondary structure. To anneal the strands, the DNA templates were allowed to cool down slowly to 22°C by reducing the temperature -0.2°C in every 10 seconds. Later, the annealed template was quantified in a spectrophotometer and one microgram of the template was used to synthesis pcRNA with MEGAscript RNAi kit (Invitrogen, Carlsbad, CA, US). The column purification was replaced with an ethanol-sodium acetate precipitation according to Abbasi et al.,

(2020) to purify pcRNA molecules. pcRNA molecules were quantified with a Qubit 4 Fluorometer by Qubit microRNA Assay Kit (Thermo Fisher# Q32880). The quality of pcRNA molecules were assessed using 2% agarose gel electrophoresis and the Agilent 4150 TapeStation System.

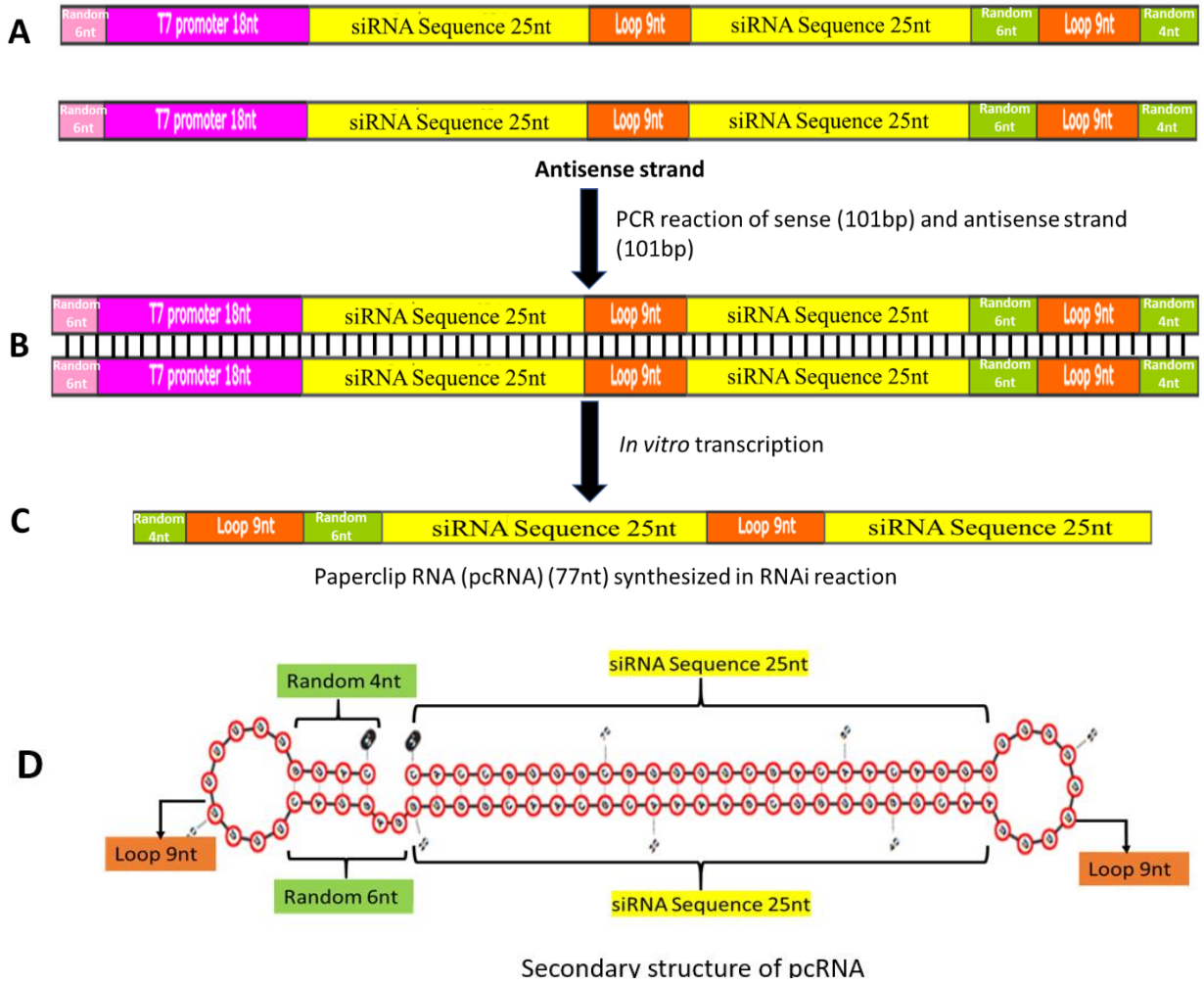

Figure 2.2: Structure of paperclip RNA (pcRNA) having 25 nucleotides target sequence enclosed by two loops rendering a paperclip shape. A) Sense and antisense of DNA oligo (101bp) each having T7 promoter sequence were annealed. B) The annealed template was used in RNAi reaction to produce pcRNA. C) Synthesis of pcRNA (77nt) by *in vitro* transcription reaction. D) Secondary structure of pcRNA.

2.5 In vitro assay with fungal plug:

For *in vitro* assays with the fungus, a 1 mm fungal plug was collected from the leading edge of freshly grown (three days old) *S. sclerotiorum* using a p1000 pipette tip and transferred into 3mL

of quarter-strength Potato Dextrose Broth (PDB) (Difco Laboratories Inc., Detroit, MI, USA) (Figure 2.3). The PDB was supplemented with tetracycline (100 µg/mL) into each 15ml tube. The PDB liquid media was later mixed with either long dsRNA or pcRNA at varying doses. All the replicates were placed on a rocking platform shaker and incubated in dark conditions at 17°C for 40 hours according to Li (2021).

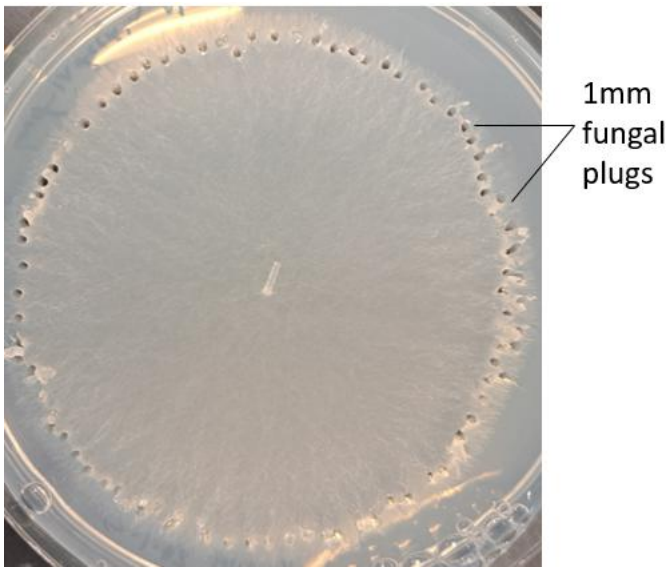

Figure 2.3: Collection of 1mm fungal plugs from leading edge of three days old *S. sclerotiorum* cultured on quarter strength of PDA media. In each replicate, a fungal plug was incubated with either long dsRNA or pcRNA mixed with quarter strength PDB liquid media.

2.6 In vitro assay with fungal slurry:

Agar rings of diameter 1cm (for long dsRNA) or 0.5cm (for pcRNA) from the leading edge of three days' old *S. sclerotiorum* (explained in Result section) cultured on quarter strength of PDA media supplemented with tetracycline (100 µg/mL) were collected (Figure 2.4). The agar was divided into pieces weighing one gram, which were collected into 2mL Eppendorf tubes containing stainless steel beads (0.2g of 3.2mm and 0.2g of the 0.9mm – 2.0mm beads) and one mL of quarter strength of PDB. The pieces were ground in a Bullet blender Homogenizer to homogenize the mixture for 2 minutes with speed 10. The prepared slurries from multiple 2mL tubes were pooled together in a 5mL tube and vortexed to ensure the homogeneous distribution of the mycelia. For *in vitro* assays, 20µL of the slurry was pipetted into each replicate of 15 mL Falcon tube containing

3mL of quarter strength PDB mixed with different doses of either long dsRNA or pcRNA i.e. 250ng/mL, 500ng/mL, 750ng/mL, 1000ng/mL. All of the replicates were placed on a rocking platform shaker (Benchmark Scientific Inc., Sayreville, NJ, USA) and incubated in dark conditions at 17°C for 72 hours.

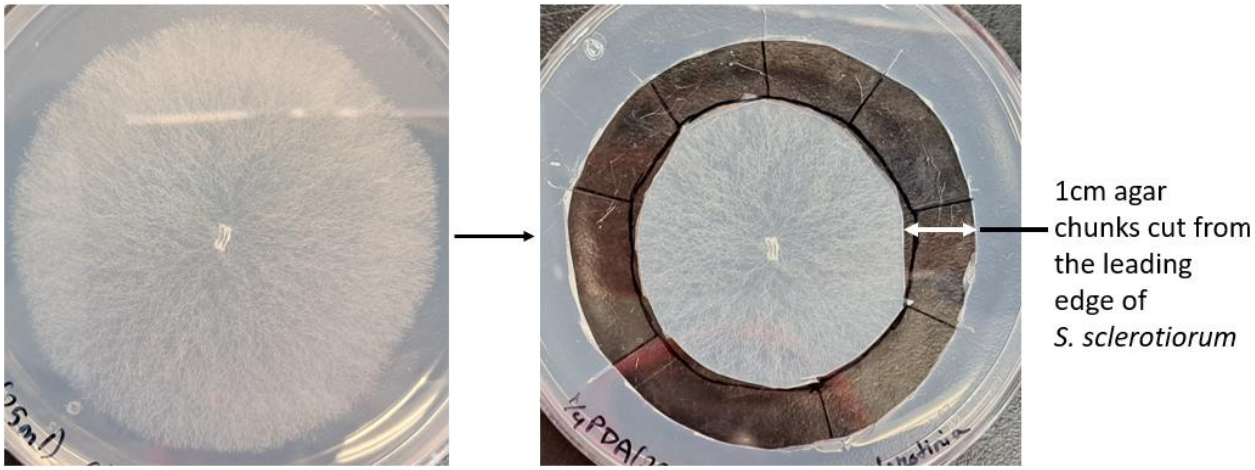

Figure 2.4: Collection of 1cm fungal agar chunk from leading edge of three days old *S. sclerotiorum* cultured on quarter strength of PDA media. In each replicate, 20µL of fungal slurry was incubated with either long dsRNA or pcRNA mixed with quarter strength PDB liquid media.

2.7 Tissue collection, RNA extraction, cDNA preparation and RT-qPCR:

After 72 hours of incubation, tubes containing fungal mycelia from the *in vitro* assays were centrifuged at 4000 rpm at 4°C for 15 minutes. Following this, the liquid growth media PBD was carefully removed from each tube. To eliminate any remaining PDB, the fungal mycelia underwent two separate washes, with 3mL of autoclaved water added to each tube. During the rinsing process, a brief vortex was performed to ensure thorough water permeation into the fungal mycelia, followed by centrifugation at 4000 rpm for 5 minutes. The water was then discarded, and the fungal tissue was collected from each tube with the help of a p1000 pipette tip into 1.5mL microcentrifuge tubes with screwcap (Fisher Scientific, Pittsburgh, PA, USA) containing 0.6g -0.8g of 1.4mm ceramic beads (Fisher Scientific, Pittsburgh, PA, USA). The fungal tissue was immediately flash frozen into liquid nitrogen and then homogenized in a Bead Mill 24 Homogenizer (Fisher brand) for 30 seconds at a speed of 6. Subsequently, the tissue was immediately processed for RNA extraction by using the TRIzol extraction method (Cat #15596026). The extracted RNA was eluted

in 30µL of molecular-grade water. The quality of RNA was assessed by spectrophotometer and 1.5% gel. The RNA samples were DNase-treated and complementary DNA (cDNA) was prepared from the DNase-treated RNA samples following the protocol of the Maxima First Strand kit (K1672).

2.8 Relative Transcript Abundance measured by Quantitative reverse transcription polymerase chain reaction (qRT-PCR):

Relative transcript abundance was measured using the cDNA by qRT-PCR. The reaction was done with SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol in a total reaction volume of 10 µL. The Bio-Rad CFX96 Connect Real Time System (Bio-Rad Laboratories, Hercules, CA, USA), was used with the conditions as followed: 95 ℃ for 3 min, and 30 cycles of 95 ℃ for 5 s, 56 ℃ for 5 s, and 72 ℃ for 5 s. No-reverse transcriptase (no-RT) controls were used to confirm absence of genomic DNA contamination. Melt curve analysis, involving 0.5 ℃ increments over a range of 60 ℃ to 95 ℃, for each primer set was performed to confirm absence of splice variants, primer dimers, and non-specific amplification. *S. sclerotiorum* transcript levels were measured in reference to fungus-specific Sac7 housekeeping genes using the $2^{-\Delta\Delta CT}$ method. The primers to amplify the target region in qRT-PCR are listed in Table 2.4. For each *in vitro* assay, a one-way ANOVA test followed by Turkey's test was performed for each gene to determine significant fold changes between the negative control and the dsRNA/pcRNA treatment ($p < 0.05$). All treatments had 4-6 biological replicates and each biological replicate was repeated in triplicate (technical replicates) for each gene target.

Table 2.4: Primer sequences used in qRT- PCR

Target	Sequence (5'-3')	Product size
SS1G_01703_F	TCTTAAAGGATCTTTCCCACACG	84bp
SS1G_01703_R	TCTTAAAGGATCTTTCCCACACG	
SS1G_00005_F	CCACAACCACCCCAACC	84bp
SS1G_00005_R	CCTCTCTCTCCTTGGCTTGAC	
SS1G_14116_F	GTCACCTGTCTACCTGGTTGTTTT	107bp
SS1G_14116_R	ACGCACTCGCTGTACTCATC	
SS1G_12350 (Sac7) _F	CGATACTGTGCCTGTGACCA	91bp
SS1G_12350 (Sac7) _R	CCTCTCCTCAAGCGCCATAG	

CHAPTER 3: RESULTS

3.1 Long dsRNA treatments of Sclerotinia grown from agar plugs:

RNAi-mediated silencing efficacies of long dsRNAs were first evaluated using three long dsRNAs targeting SS1G_01703, SS1G_00005, and SS1G_14116 (see Table 2.1). These dsRNAs were incubated with Sclerotinia that was derived from small (1mm diameter) agar plugs collected from PDA plates. Each long dsRNA was administered at three different concentrations (500ng/ml, 1000ng/ml, and 1500ng/ml) in PDB broth. The qRT-PCR analyses indicated that only one dose (1000ng/ml) of the long dsRNA 01703 caused a significant (56% ± 3.5% SE) reduction of transcripts. The other two doses (500 and 1500 ng/ml) failed to produce a statistically significant knockdown, although the 1500 ng/ml dose caused a modest, yet not statistically significant decrease in transcripts (Figure 3.1a). It is important to note that the negative controls showed large variances, making it difficult to detect significant changes in transcript levels using this type of assay. In the case of long dsRNA 00005 and 14116, none of the doses of dsRNA caused statistically significant knockdowns of the targeted transcripts (Figure 3.1b and 3.1c). In these assays, the extent of knockdown was highly variable in both treatments and negative controls, and it was clear that the assay would need to be modified to reduce sources of variation. For long dsRNA 14116, the potential effects of other doses remain unexplored, as no additional doses were assessed.

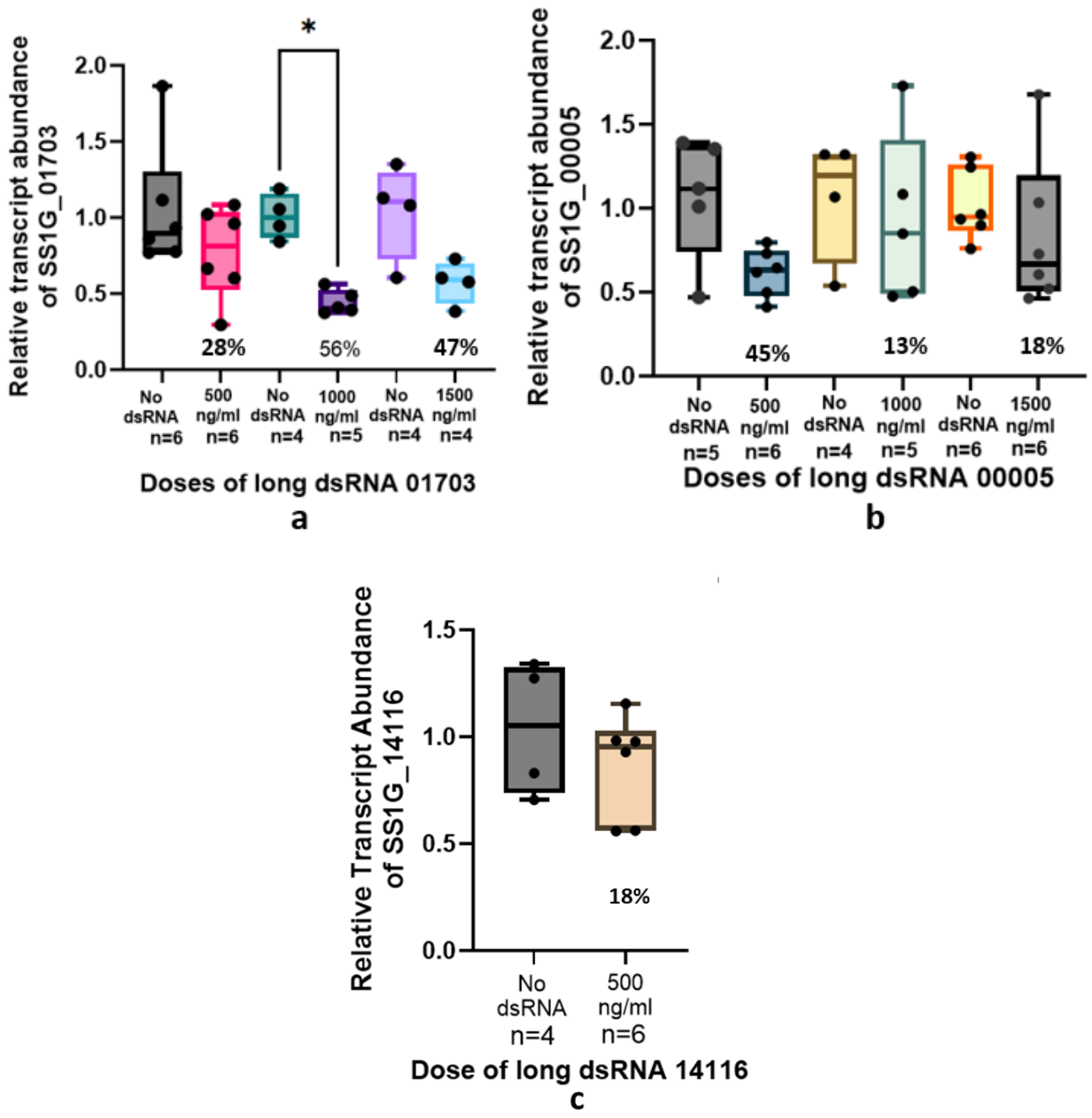

Figure 3.1: Relative transcript abundance of a) SS1G_01703, b) SS1G_00005, and c) SS1G_14116 tested in vitro assay of *S. sclerotiorum* plugs with their respective long dsRNA molecules. A fungal plug was incubated with 500ng/ml, 1000ng/ml, 1500ng/ml long dsRNA for 40 hours. The percent transcript knockdowns, relative to a no dsRNA treatment (negative control), listed below each whisker plot, were calculated using the ΔΔCT method and n denotes to the biological replicates. Each targeted gene's transcript levels were normalized relative to the corresponding negative control (no dsRNA), using Sac7 (SS1G_12350) as the reference gene for all treatments. The asterisk denotes a significant difference between the dsRNA treatment and

the negative control treatment; all other dsRNA treatments caused no significant knockdown (one- way ANOVA test followed by Tukey's post-hoc test, *: $p < 0.05$).

3.2 pcRNA treatments of Sclerotinia grown from agar plugs:

As was observed with the long dsRNA treatments, liquid cultures of small agar plug of Sclerotinia also failed to show significant transcript knockdowns in most of the pcRNA treatments. In samples treated with a range of concentrations of pcRNA 01703, no knockdown was observed (Figure 3.2a). Using pcRNA 00005, only one dose (500ng/ml) caused a significant (34% ± 5.7% SE) knockdown of the targeted transcripts, while the other doses failed to induce a statistically significant change (Figure 3.2b). It is worth noting that in these preliminary experiments, the doses used for the pcRNAs were different from those used in the long dsRNA treatments, so it is not possible to make any direct comparisons about the relative efficacy of the pcRNA relative to its corresponding dsRNA. However, given that these pcRNA assays, like those of the long dsRNA treatments, produced highly variable effects, it was concluded that a change in the methodology should be attempted to eliminate or at least reduce some sources of variation in the assay.

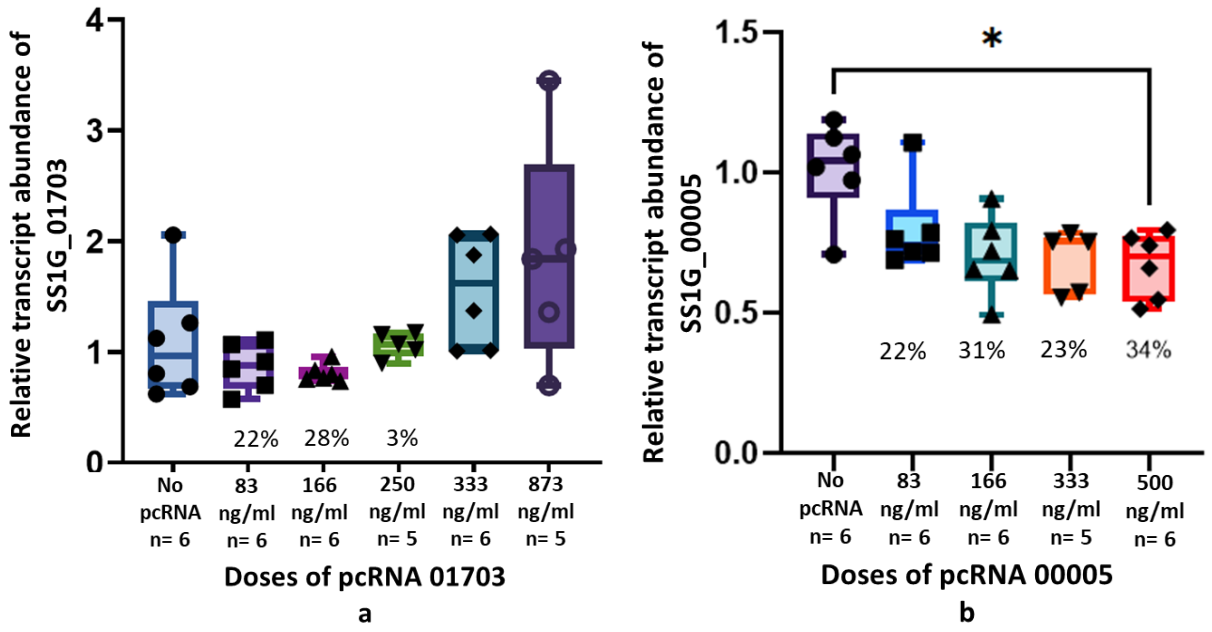

Figure 3.2: Relative transcript abundance of a) SS1G_01703 and b) SS1G_00005 tested in vitro assays of *S. sclerotiorum* plugs with different doses of pcRNA molecules. One fungal plug from *S. sclerotiorum* cultured in Potato Dextrose Agar plate (PDA) was incubated with different doses of pcRNA for 40 hours. The percent transcript knockdowns, relative to a no dsRNA treatment (negative control), listed below each whisker plot, were calculated using the ΔΔCT method and n denotes to the biological replicates. Each targeted gene's transcript levels were normalized relative to the corresponding negative control (no dsRNA), using Sac7 (SS1G_12350) as the reference gene for all treatments. The asterisk denotes a significant difference between the dsRNA treatment and the negative control treatment; all other dsRNA treatments caused no significant knockdown (one- way ANOVA test followed by post-hoc Tukey's test, *: $p < 0.05$).

3.3 Long dsRNA treatments using a fungal slurry inoculum:

To address the issue of high variability and non- significant reduction of target transcripts in the previous assays, a new assay method was developed. The method involves homogenizing larger pieces of the PDA containing Sclerotinia mycelia diluted with buffer to create a slurry of fragmented hyphae. Each treatment received an equal volume of fungal slurry to ensure a similar mass of hyphae. The purpose of using a fungal slurry as the inoculum instead of the fungal plug

was to eliminate the possibility of inoculating each replicate with different numbers/masses of hyphae.

Different volumes (10ul, 20ul, 30ul) of slurry were allowed to grow for two and three days in liquid media (¼ strength PDB), in the same manner as the *in vitro* assay with the agar plugs. Among the three volumes of slurry evaluated, the 20 µL fungal slurry incubated for a duration of three days yielded enough fungal biomass to facilitate the subsequent experiments. Hence, the *in vitro* assays with long dsRNA and pcRNA targeting SS1G_01703, SS1G_00005 and SS1G_14116 were incubated with 20ul fungal slurry for three days at 17°C.

The initial assays using the fungal slurry method were conducted with long dsRNA 01703. Three trials were conducted, using three different agar plates of Sclerotinia, and as the starting source of fungus was another possible source of biological variation, the data from these three trials were analyzed separately. While some large variances in transcript levels were still observed in the negative control samples using this modified assay, statistically significant knockdown of SS1G_01703 transcripts, relative to the negative control, were observed in Trial 1 (70% ± 5.5% SE knockdown; Figure 3.3a) using 750 ng/ml dsRNA, and in Trial 3 (65% ± 4.8% SE knockdown; Figure 3.3c) using 1000 ng/ml dsRNA. The second trial, while showing a trend towards knockdown using all three doses of dsRNA, was not significantly different from the transcript levels of the negative controls. In the third trial, when the transcript levels for the higher dose treatments were compared to the 250 ng/ml treatments, significant reductions in SS1G_01703 transcripts were also noted at the 500ng/ml and 1000 ng/ml dosages (Figure 3.3c). There was no significant difference between the negative controls of the three trials with long dsRNA 01703. To evaluate the differences in transcript knockdown results between the *in vitro* plug assay and the *in vitro* slurry assay, data from all three trials in the slurry approach were combined (Figure 3.3 d) to provide a comprehensive overview of variability and the knockdown of the transcript SS1G_01703. In the combined graph significant knockdown was observed only at the dose of 750ng/ml, with an average 65 % ± 4.6% SE.

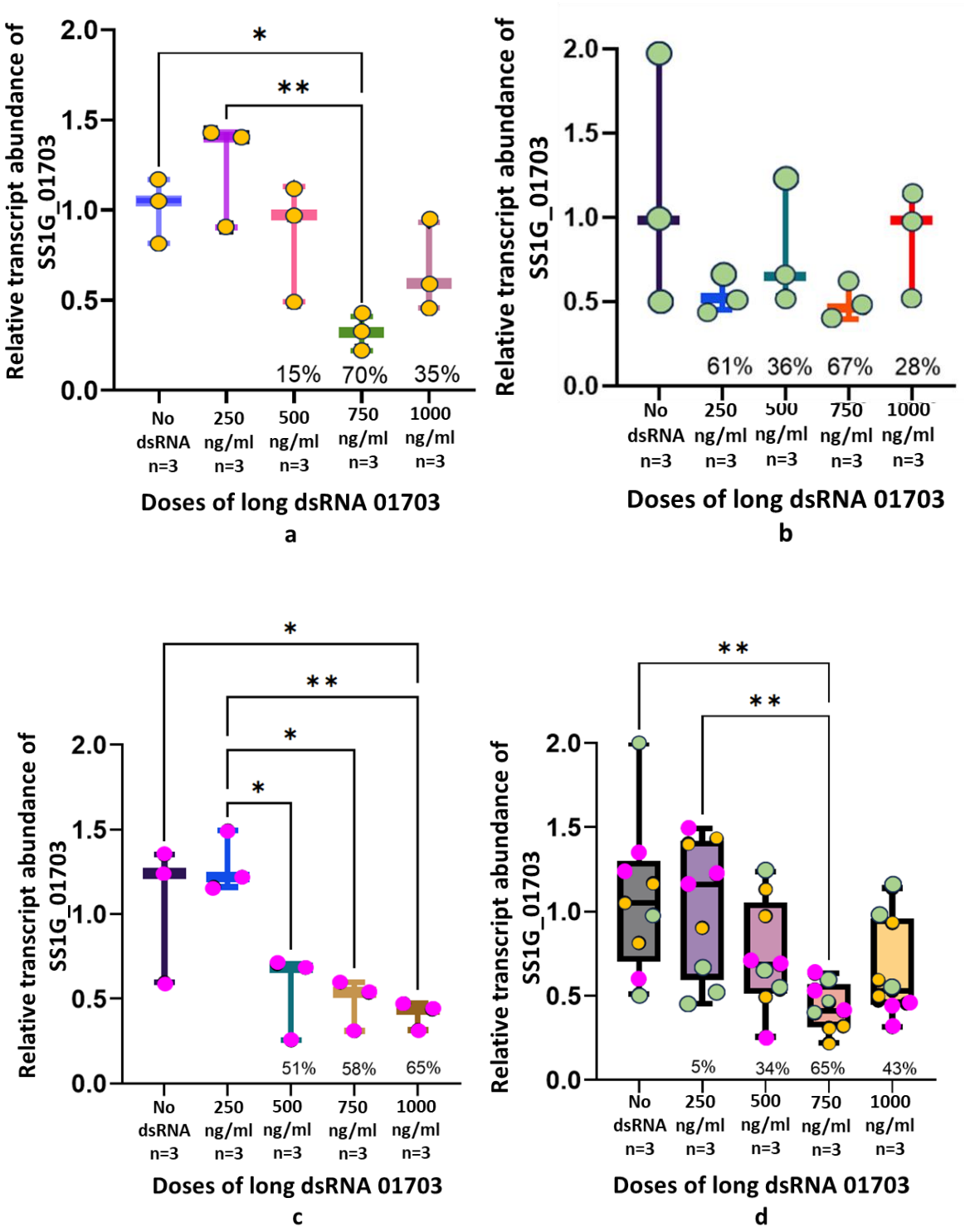

Figure 3.3: Relative transcript abundance of SS1G_01703 tested three different in vitro assays of *S. sclerotiorum* slurry a) Trial-1, b) Trial-2, c) Trial-3 and d) Combination of all trials with long dsRNA 01703. Fungal slurry of 20ul was incubated with 250ng/ml,

500ng/ml,750ng/ml, 1000ng/ml long dsRNA for 72 hours. The percent transcript knockdowns, relative to a no dsRNA treatment (negative control), listed below each whisker plot, were calculated using the ΔΔCT method and n denotes to the biological replicates. Each targeted gene's transcript levels were normalized relative to the corresponding negative control (no dsRNA), using Sac7 (SS1G_12350) as the reference gene for all treatments. The asterisk denotes a significant difference between the dsRNA treatment and the negative control treatment; all other dsRNA treatments caused no significant knockdown (one- way ANOVA test followed by post-hoc Tukey's test), *: $p < 0.05$, **: $p \leq 0.01$).

Similarly, tests with long dsRNA 00005 exhibited significant knockdown using three doses, i.e. 500ng/ml reduced transcripts by 33% ±7% SE, 750ng/ml by 35% ± 9.2% SE and 1000ng/ml by 30% ±5.5% SE (Figure 3.4a). While the 250ng/ml dose was not enough for long dsRNA 00005 and 01703 to knock down their transcripts, for long dsRNA 14116, that dose exhibited 33%± 5% SE knockdown of SS1G_14116 transcripts. The 1000ng/ml dose long dsRNA 14116 also showed a significant knockdown 35%± 10.9% SE (Figure 3.4b). Notably, at a dose of 750ng/ml, both long dsRNA 01703 and long dsRNA 14116 displayed less variability, however, high variability was still observed with some treatments.

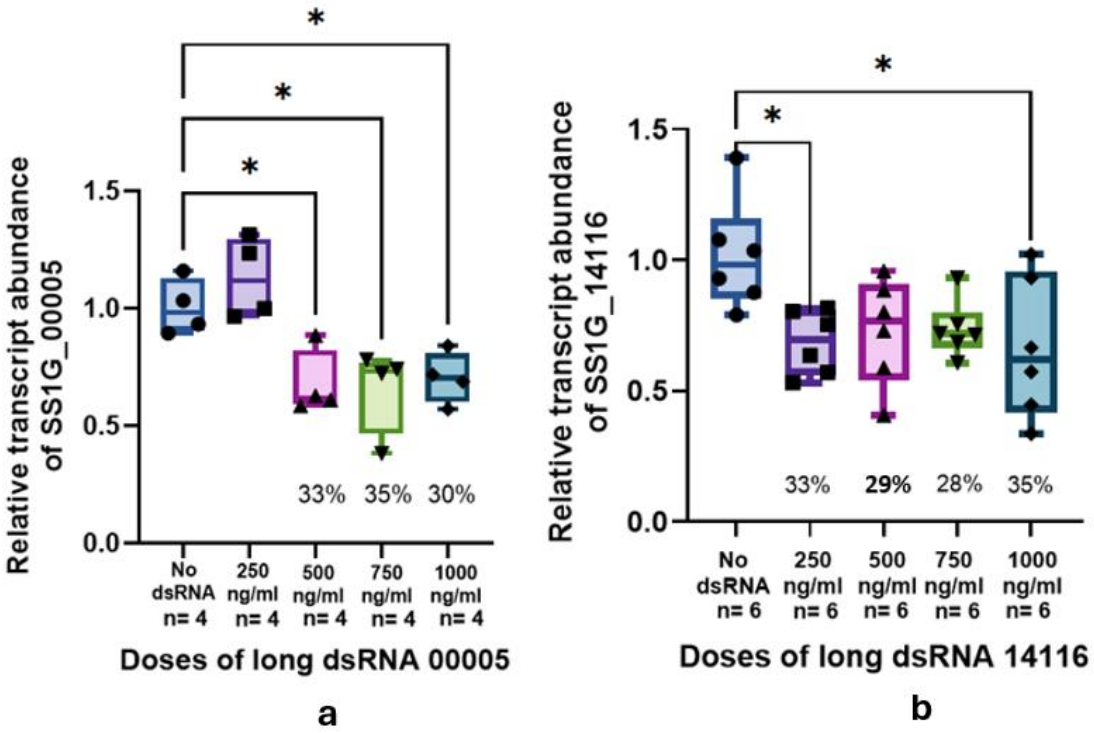

Figure 3.4: Relative transcript abundance of a) SS1G_00005 and b) SS1G_14116 tested *in vitro* assay of *S. sclerotiorum* slurry with their respective long dsRNA molecules. Fungal slurry of 20ul was incubated with 250ng/ml, 500ng/ml,750ng/ml, 1000ng/ml long dsRNA for 72 hours The percent transcript knockdowns, relative to a no dsRNA treatment (negative control), listed below each whisker plot, were calculated using the ΔΔCT method and n denotes to the biological replicates. Each targeted gene's transcript levels were normalized relative to the corresponding negative control (no dsRNA), using Sac7 (SS1G_12350) as the reference gene for all treatments. The asterisk denotes a significant difference between the dsRNA treatment and the negative control treatment; all other dsRNA treatments caused no significant knockdown (one- way ANOVA test, *: $p < 0.05$).

3.4 In vitro assay of pcRNA with fungal slurry:

To reduce the possible variability of different developmental stages of hyphae observed in the assays of long dsRNA with the fungal- slurry method, the size of the fungal agar rings was reduced to 0.5cm from 1cm for the pcRNA assays. Slurries prepared in this slightly modified method showed less variability in transcript levels, but unexpectedly, the pcRNAs targeting all three genes in these assays were not able to knock down their target transcripts significantly (Figure 3.5). At

the dose of 1000ng/ml, pcRNA 00005 induced increased accumulation of SS1G_00005 transcripts, relative to the other doses tested, but not to the negative control samples (Figure 3.5b).

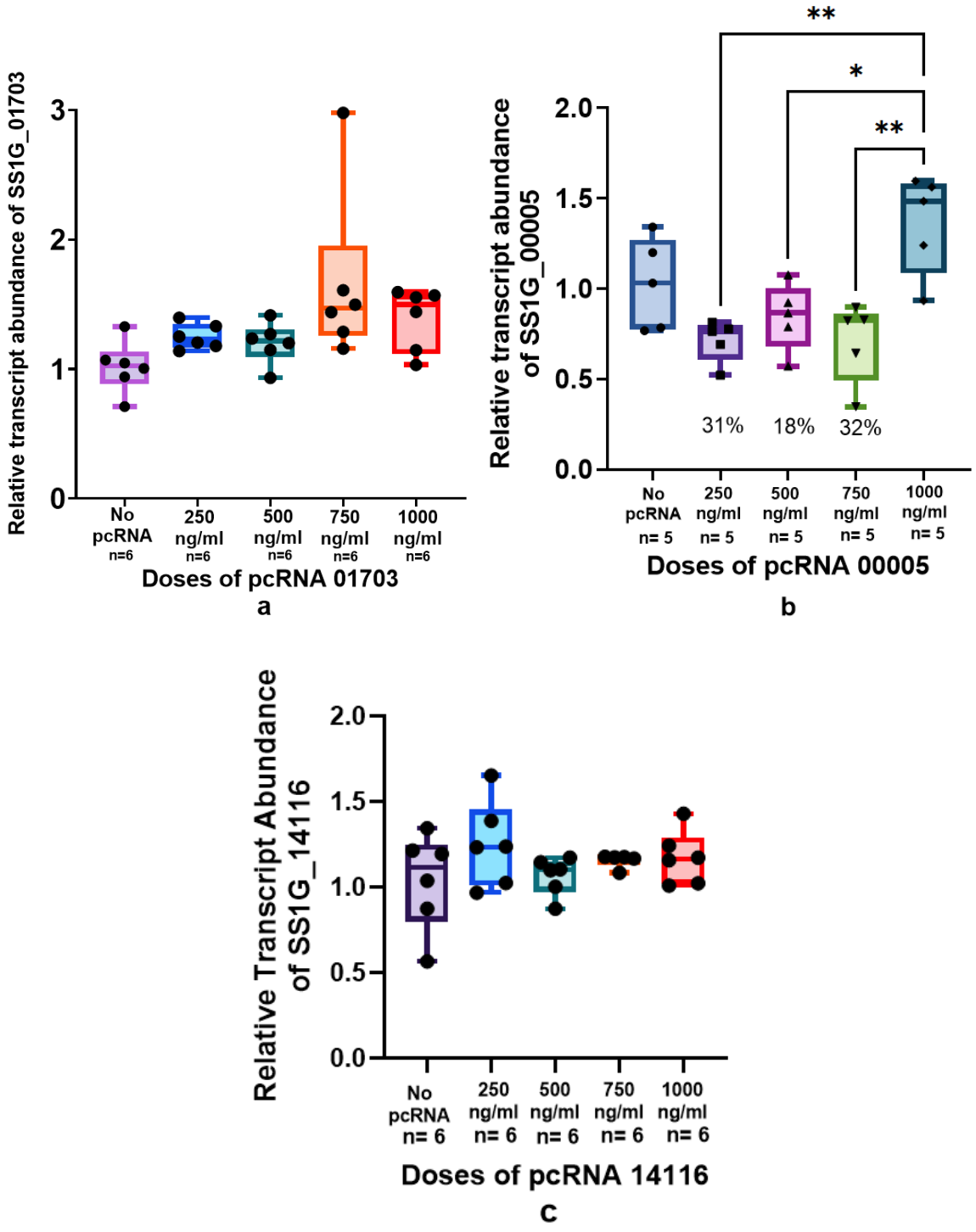

Figure 3.5: Relative transcript abundance of a) SS1G_01703, b) SS1G_00005 and c) SS1G_14116 tested in vitro assay of *S. sclerotiorum* slurry with their respective pcRNA

molecule. 20ul of fungal was incubated with 250ng/ml, 500ng/ml,750ng/ml, 1000ng/ml pcRNA for 72 hours. The percent transcript knockdowns, relative to a no dsRNA treatment (negative control), listed below each whisker plot, were calculated using the ΔΔCT method and n denotes to the biological replicates. Each targeted gene's transcript levels were normalized relative to the corresponding negative control (no dsRNA), using Sac7 (SS1G_12350) as the reference gene for all treatments. The asterisk denotes a significant difference between the dsRNA treatment and the negative control treatment; all other dsRNA treatments caused no significant knockdown (one- way ANOVA test followed by post-hoc Tukey's test, *: $p < 0.05$, **: $p \leq 0.01$).

CHAPTER 4: DISCUSSION

RNA interference (RNAi) technology presents a promising and environmentally sustainable solution for agricultural producers to effectively manage a diverse range of damaging pests and pathogens. The application strategy of RNAi known as spray-induced gene silencing (SIGS) represents advanced, sustainable approaches to crop protection, targeting the various pests and pathogens that impact global food production. SIGS strategies have successfully been demonstrated by various research groups under controlled conditions to mitigate fungal pathogen infections, including *Fusarium graminearum, Botrytis cinerea, Sclerotinia sclerotiorum, Rhizoctonia solani, Aspergillus niger* and *Verticillium dahlia* (Choudry et al., 2024; Höfle et al., 2020b; Islam et al., 2021; Qiao et al., 2021) as well as the oomycetes *Hyaloperonospora arabidopsidis, Phytophthora capsica, Phytophthora infestens* (Cheng et al., 2022; Dunker et al., 2020; Kalyandurg et al., 2021). While many of these studies highlighted the potential for exogenous dsRNA to cause gene knockdown and slow the growth of these phytopathogens, further optimizations will be required before SIGS-mediated control methods can be implemented in field settings. In this current study, I set out to explore this issue of optimization by examining whether RNAi-mediated knockdown of Sclerotinia's transcripts could be controlled in a dose-dependent manner, and whether alternatively structured dsRNAs, namely pcRNAs, offered any improvement in the efficacy of RNAi in this fungus. While I found that some doses of dsRNAs could cause significant transcript knockdown, the responses were not always improved with increasing doses of dsRNA, and there was considerable variability in the different genes' responses to dsRNA treatments. Identifying the sources of variation in RNAi responses in Sclerotinia thus became the focus of this study.

Previously, the Belmonte lab identified a list of RNAi target genes in *S. sclerotiorum* using RNA sequencing (McLoughlin et al., 2018). The study conducted RNA-seq to identify fungal genes associated with pathogenicity, infection, or proliferative growth in different plant tissues. Sclerotinia-induced leaf lesions were reduced up to 85%, compared to negative control treatments, when 20 out of 64 candidate genes were targeted using SIGS (Walker et al., 2022). It was obvious from this modest screening effort that not all genes are effective targets for RNAi-mediated control of the fungus. Despite all the genes targeted in that study being genes considered essential for

fungal growth and development; other factors are obviously important in determining which are susceptible to RNAi-mediated transcript knockdown. Among those targets, SS1G_01703, SS1G_00005, and SS1G_14116 were selected for the current study based largely on their moderate transcript knockdown efficiencies recorded in the McLoughlin et al. (2018) study. Having a moderate level of RNAi would permit changes (increases or decreases) in RNAi efficiency to be readily detected, as I tested the efficacies of different dsRNA dosages and differently structured dsRNA molecules. I tested the knockdown efficiency of dsRNA and pcRNA in two different fungal inocula assays, using either fungal plugs or fungal slurries, targeting SS1G_01703, SS1G_00005 and SS1G_14116 genes in *Sclerotinia sclerotiorum*. The efficiency of the molecules was assessed by comparing the mRNA transcript levels, compared to a negative control, measured in quantitative reverse transcription polymerase chain reaction (qRT-PCR). Using these two assay methods, it was observed that the fungal plug method yielded largely inconsistent results, whereas the slurry method demonstrated effective knockdown of all three target genes, but the dsRNA dose required to knock down each gene differed across the genes, and that there was not always a direct correlation of the dose administered and the extent of transcript knockdown.

4.1 Effect of gene expression and FPKM values on RNAi:

The effectiveness of a dsRNA to induce a measurable level of transcript knockdown is undoubtedly dependent on many factors, one of which is the relative transcriptional level of the target genes. In a study by the Belmonte lab group, Walker et al. (2022) proposed that genes with moderate FPKM values, which are normalized measures of transcript levels derived from RNA sequencing data analyses, might serve as the best RNAi target genes. The rationale for this prediction was that very highly expressed genes (with FPKM values over 10,000) (Fagerberg et al., 2014) may require excessive amounts of dsRNA to cause a noticeable knockdown, and that very lowly expressed genes' transcripts (with FPKM values less than 1) may be too scarce for the RISC machinery to find them. Previous studies have indeed demonstrated that genes with very high levels of transcripts or very low levels of transcripts are challenging to knock down by RNAi. Travella et al., (2006) argued that knockdown of lowly expressed genes in wheat was difficult to measure, simply due to the challenges of distinguishing the expression of homologous genes from the target gene expression. Chen et al. (2021) also described difficulties in knocking down lowly expressed genes in the flour beetle, *Tribolium castaneum*, while more abundantly expressed genes

were comparatively easier. In contrast, various reports have suggested that for very highly expressed genes, it can be challenging to provide sufficient dsRNA to eliminate all transcripts, for example, some researchers have simply increased the dosage of dsRNA to knock down highly expressed genes (Guo et al., 2023; Parrish et al., 2000; Shi et al., 2016). But there are a growing number of examples describing more widespread impacts on gene expression when more dsRNA is used, which has been attributed to perturbations to the miRNA levels within the cells, as the RISC machinery becomes overwhelmed by an abundance of dsRNAs and their associated siRNAs (Arora et al., 2021; Grimm, 2011; Parrish et al., 2000). Mcloughlin et al. (2018), somewhat surprisingly, found 39 of the RNAi-responsive genes in Sclerotinia in *in vivo* leaf lesion assays were highly expressed (≥ 25 FPKM), 15 genes were moderately expressed (5 > 25 FPKM), and 10 genes were lowly expressed (1 ≥ 5 FPKM). Although this current study focused on *in vitro*, rather than *in vivo* assays, it was decided that the best genes to focus upon to optimize a SIGS approach to fungus control would be more dsRNA-responsive genes that were highly expressed, with FPKM values greater than 25.

For the genes under investigation in this current study, the observed FPKM values for SS1G_14116, SS1G_01703, SS1G_00005, under *in planta* growth conditions, were 89, 301, and 4338, respectively (Table 4.1). These values, strictly speaking, would be considered slightly high to moderately high. However, their FPKM values under in vitro growth conditions were relatively low, which should be the primary criterion for selecting RNAi target genes for the current the *in vitro* assays instead of FPKM values observed *in planta* conditions. Upon reviewing the knockdown of the three genes using the fungal slurry method, the FPKM values alone did not help predict which gene would be most effectively knocked down using different doses of dsRNA (Table 4.1). All three genes were responsive to the administered dsRNA, but the one gene showing the greatest knockdown was 01703, with the one dose of 750 ng/ml causing a 65% knockdown. The other two, 14116 and 00005, with highly different FPKM values to each other, showed nearly identical maximal knockdown values, although the doses required to induce those knockdowns were not the same. Interestingly, SS1G_00005, with an FPKM more than 150 times greater than that of the other two, showed modest and consistent transcript knockdowns, using three doses of dsRNA. An FPKM value is thus the not sole factor to be considered to select target genes for enhanced RNAi-mediated knockdown. Reliance on this value to select a target gene should be approached with caution, as FPKM values can be affected by not just endogenous biological

factors, but by experimental procedure factors. The duration between sample collection and RNA extraction can notably impact FPKM values. RNA degradation during this timeframe may lead to significant variations in the relative abundances of transcripts (Romero et al., 2014). Depending on the gene length, FPKM normalization can be affected by amplified noise or reduction of signal.

Table 4.1: Summary of maximum knockdown of gene achieved after dsRNA treatment and their respective FPKM values

Gene	FPKM value (*in vitro* condition)	FPKM value of the gene *in planta* (*Brassica napus*)	Maximum knockdown (%)	Concentration of dsRNA (ng/ml) inducing significant knockdown (* denotes maximum knockdown)
SS1G_1703	44	301	65	750*
SS1G_00005	6057	4338	35	500, 750*, 1000
SS1G_14116	33	89	35	250, 1000*

Measuring a gene's transcript level may prove helpful in selecting an RNAi sensitive gene, but a gene's expression rate may vary over time. Relying on a static measure of transcript levels at a single timepoint, the FPKM values from the McLoughlin et al. (2018) study lack information about the dynamics of gene expression changes (Arora et al., 2020). If the targeted genes expression levels can be quickly modulated to respond to perturbations such as an RNAi-mediated knockdown, then those genes may prove to be refractory to increasing or sustained doses of dsRNA. Cedden et al. (2024), for example, observed that different genes can alter their responses to dsRNA over time. In their study, they delivered equal doses of dsRNA targeting the Sec23 and the VatpG genes in the cabbage stem flea beetle (*Psylliodes chrysocephala*), and while both genes showed similar silencing effects in the beetles at three days post-treatment, the Sec23 gene continued to show sustained knockdown for several more days, while the VatpG gene's transcript levels rebounded (Cedden et al., 2024). Clearly, different genes respond differently to RNAi-mediated knockdown, which highlights the importance of understanding each target gene's capacity to regulate its transcript levels, particularly following perturbations, such as RNAi-mediated post-transcriptional gene silencing.

Hong et al. (2005), in contrast, observed that excessive amounts of dsRNA can result in reduced RNAi efficacy for not just one gene, but for many. They studied RNAi effects in mice liver tissue

and found that while siRNA-induced gene silencing is typically dose-dependent, higher doses of esiHBVP unexpectedly reduced the silencing effect. They showed that the cells responded to the excess of siRNAs by activating dsRNA degrading enzymes like ERI-1 and ADAR, which likely reduced the amount of siRNA (Hong et al., 2005). This observation suggests that excessive siRNA doses may trigger unwanted mechanisms that limit RNA interference. This observation may partially explain why some of the higher doses of dsRNA used in my study failed to induce effective transcript knockdown. It would also suggest there is likely an optimal dose of dsRNA for each target gene as too little dsRNA may fail to significantly reduce the transcripts, whereas too much dsRNA may trigger non-specific mechanisms that eliminate any excess dsRNAs/siRNAs. Therefore, in addition to considering FPKM values, assessing gene expression at multiple time points during the growth and development of the fungus may help predict the necessary dsRNA dosage and the timing of dosage to maximize transcript knockdown and to achieve effective and long-lasting RNAi-based treatments. Until such data are available, I cannot readily predict the optimal dsRNA treatments for future treatments, but it is worth considering what is known about each of the target genes, to help determine why I observed considerable variation in gene expression in negative control treatments, and different responses to the dsRNA doses.

4.2 Effect of function of gene on RNAi:

The SS1G_01703 gene, also known as ABHYDROLASE-3, plays a vital role in *S. sclerotiorum* ability to infect and colonize host plants (Wytinck et al., 2022). This gene is believed to function as a pathogenicity factor and is predicted to be involved in aflatoxin biosynthesis, based on sequence similarities to aflatoxin synthesis genes in other fungi (Wytinck et al., 2022). Previous research in the Belmonte lab demonstrated that suppressing the expression of the *ABHYDROLASE-3* gene significantly impairs the *S. sclerotiorum* infection process and reduces its ability to colonize plant tissues (Walker et al., 2023; Wytinck et al., 2022). McLoughlin et al. (2018) observed that the transcript abundance of this gene, based on FPKM values, was almost 7 times higher in the fungus grown on the plant compared to fungus grown on agar plates, which also supports the premise that *ABHYDROLASE-3* helps promote the infection process within a plant, and may not be as essential on a nutrient-rich agar medium. It also suggests that the gene is inducible, depending on environmental conditions. The precise factors that induce this gene during infection are unknown but may include nutrient availability or the presence of plant-derived factors

that may trigger infection processes. Understanding the inducing signals for this gene may help understand how the transcript levels can vary and may help explain why large variances in transcript levels were observed in my analyses of this gene, with and without exposure to the dsRNA.

The gene SS1G_00005, encoding components of the 60S ribosomal subunit, is generally considered a constitutively expressed gene, but ribosomal proteins and rRNAs are typically expressed at high levels in actively growing cells to support protein synthesis (Dörner et al., 2022; Gregory et al., 2019). Some studies have shown that the expression of certain ribosomal proteins can be cell cycle-regulated, with higher expression during phases of active growth and division (Tuch et al., 2008; Zhang et al., 2024). In multicellular organisms, there can be some tissue-specific variations in the expression of ribosomal proteins, though they are generally expressed constitutively in all cell types (Dörner et al., 2022). In my assays, variable levels of expression of this gene might also be encountered, depending on whether the fungus harvested from the agar plate was in a rapid state of growth or was in a more quiescent state due to hyphal crowding. The earlier stages of fungal growth during incubation with dsRNA may have exhibited more pronounced transcript knockdowns. While allowing 72 hours provided enough time for fungal cells to grow sufficiently to harvest RNA, it probably also allowed the fungus to recover from the transcript knockdowns, and hence, some treatments showed no significant transcript reduction. The fungus from the plate was subsequently immersed in a nutrient-rich liquid medium, but as it might take some time for it to adapt to new environmental conditions, the dsRNA may have different impacts on the fungus, and hence, variable responses in transcript knockdown.

The gene SS1G_14116 is associated with chitin synthase in fungi, playing a crucial role in the production of chitin, an essential component that contributes to the rigidity, structure, and hyphal extension of the fungal cell wall (Ding et al., 2019). Genes involved in chitin synthase are involved in bud formation, stress responses, and directly involved in virulence (Lenardon et al., 2010). The regulation of mycelial growth is governed by specific transcription factors that respond to various environmental and developmental cues (Liu et al., 2022). For example, the availability of nutrients can significantly affect the expression of genes responsible for controlling hyphal extension and branching. Furthermore, the expression levels of the genes involved in mycelial growth exhibit variation across different developmental stages. For example, carbohydrate metabolic genes

showed higher expression in the vegetative growth stage of hyphae, while glycosyltransferase genes were upregulated during the sexual stage i.e. primordium and fruiting body stages of edible burgundy mushroom *Stropharia rugosoannulata* (Hao et al., 2022). The chitin synthase gene in Sclerotinia can be considered an inducible gene, responding to cues stimulating growth, and in my assays, it could also be expressed at variable levels in the fungus that was harvested from the agar plates, depending on whether the fungus was actively growing or was quiescent. Hence, this gene could also display variable responses to dsRNA in my assays, which could account for some of the variation observed in different treatments.

Earlier research in the Belmonte lab demonstrated substantial gene knockdown in *Sclerotinia sclerotiorum* (60-80%) using long dsRNAs 01703, 00005, and 14116, and using the fungal plug inoculum method. However, the current study did not replicate these high levels of knockdown, suggesting potential differences in experimental conditions or methodology. The reason behind not achieving similar significant knockdown in the present study with plugs could be due to different cultures or strains of the fungus used that led to varying results. It has been more than three years since the initial assays were conducted, and although the same *Sclerotinia* strain was used, consecutive culturing over time may have led to the accumulation of mutations. However, these changes have not yet been significant enough to classify it as a distinct strain. Among fungi of the same species, various strains can show different mRNA transcript abundances of the same gene (McGrath et al., 2024). Strains of the same species can produce different metabolites under the same conditions, which may be reflected in varying mRNA transcript levels for relevant biosynthetic genes (Hatmaker et al., 2022).

4.3 Effect of fungal growth on RNAi:

Another possible difference in the different studies' results is the source of the primary inoculum used in the liquid cultures. I observed large variances of gene expression, even in the negative control treatments, using either the fungal plug or fungal slurry methods. These differences may reflect variability in both the stage of development of the fungus (i.e. was it still growing rapidly, or had it slowed or even stopped as it became crowded?), and in the density of hyphae within the agar. The previous studies were conducted using a range of fungal inoculants, with some derived from ascospores, some from hyphae grown on the petals of canola flowers, some from canola or Arabidopsis leaves, and some from solid growth agar-based media. These different nutrient

sources could activate different metabolic pathways, leading to changes in gene expression profiles and potentially responses to the dsRNA. The type of nutrient source can influence the production of secondary metabolites, affecting the expression of biosynthetic genes (Jia et al., 2024). Some of the variation can also be due to differences in the timing of harvest of the fungus, as the development of the fungus over time may affect expression of the targeted genes. Faster or slower growth rates due to varying environmental parameters like temperature, pH, or nutrient sources can lead to substantial changes in the expression levels of target genes (Zopf et al., 2013).

The *in vitro* assays using fungal plugs faced challenges in optimizing the hyphal content within each plug, since each plug contained only a small amount of hyphae, and small variances could equate to substantial percentage changes in the mass of viable hyphae. This made it very difficult to ensure consistent hyphal quantities were used across different dsRNA treatments. Plugs containing a higher hyphal density might exhibit accelerated fungal growth, potentially leading to variations in the dsRNA concentration per hyphal unit. This inconsistency could have resulted in varying degrees of gene silencing among samples, ultimately contributing to the lack of significant transcript knockdown observed across the dsRNA treatment tubes. To resolve the issue, measuring the optical density of the slurry prior loading it in the assay can be one possible solution.

The intracellular localization of the target RNAs can also affect the extent of RNAi. Ferguson et al. (2020) demonstrated that when cells retained apolipoprotein E (ApoE) transcripts within the nucleus, they were resistant to RNAi, whereas in cells where the same gene's transcripts were largely cytoplasmic, substantial RNAi-mediated knockdown of the transcripts occurred. The cultured Sclerotinia used in my study likely had multiple cell types, and it would be interesting to determine if the different cells showed differential retention of the target genes transcripts within their nuclei, thereby affecting RNAi efficiency.

To minimize the variation in fungal inoculum quantity observed with plug-based methods, the fungal slurry method was developed for the *in vitro* assays. This method was developed by homogenizing old and young fungal mycelia ensuring a mixture of hyphae of various sizes and types. The underlying hypothesis was that providing an equal proportion of the hyphal mixture to each biological replicate will achieve significant knockdown while minimizing variability. Although the fungal slurry method yielded more significant knockdowns compared to plug-based assays, some degree of variability persisted. Fungal cells typically absorb nutrients at the growing

tip of their hyphae, and the hyphal tip is thus the primary site of growth (Steinberg, 2007). Moreover, younger hyphae are characterized by relatively simpler cell wall structures, which may enhance their permeability to dsRNA. Conversely, older hyphae often develop thicker and more complex cell walls due to increased lipids and fatty acids, which could limit the efficiency of molecule uptake. Study on *Sclerotium bataticola* provided evidence of developing increased lipids and fatty acid in cell membrane that contributes to the integrity of cell wall with increasing age (Steinberg, 2007). At the hyphal tip, the cell wall is more flexible as chitin at the tip is in a non-crystalline state, allowing for greater flexibility and expansion. Wytinck et al., (2020) showed that fluorescently labeled dsRNA was taken up by younger, more actively growing hyphae. The fluorescence accumulated at the hyphal tip, indicating this area could be a primary entry point or site of dsRNA localization and activity. In the causal agent of Dutch elm disease, *Ophiostoma novo-ulmi*, a significant shift in gene expression occurs during its transition from yeast-to-hyphae development, with 22% of the genome differentially expressed. This change is most notable in the early hyphal stages, with genes related to the cell cycle and cell wall remodeling being upregulated (Nigg & Bernier, 2016). In my fungal slurry assays, there were very likely hyphae of many different developmental stages. Since hyphae of varying ages can exhibit differences in cell wall structure and permeability, this variation could contribute to varying efficiency in the uptake of dsRNA, resulting in differential knockdowns in different samples.

4.4 Performance of paperclip RNA (pcRNA):

As was observed with the long dsRNA treatments, there was a high variability of responses in the samples treated with pcRNAs using the fungal plug method to assay knockdown. *In vitro* assays with pcRNA 01703 and pcRNA 14116 failed to show significant knockdown using a range of dsRNA concentrations with this assay. The only treatment that showed a significant decrease in transcript levels was the one treated with pcRNA 00005 at a dose of 500ng/ml, which showed a 34% knockdown. Previously, pcRNA-mediated knockdown achieved a 75% reduction in its target transcript, which was similar to that achieved using the same mass as a linear dsRNA that was 10 times longer in cultured mosquito cells (Abbasi et al., 2020). In the fungus *S. sclerotiorum*, pcRNAs targeting 01703 were previously demonstrated to be effective in reducing the fungal lesion size on canola leaves, which suggests that pcRNAs may be effective in *in vitro* assays (Walker, 2022). Using the fungal slurry method, the *in vitro* assays with pcRNAs exhibited less

variation of transcripts in some treatments but still failed to achieve significant knockdown. Given that the pcRNAs used in the current study were short, and capable of producing only a single siRNA, it is quite possible that one siRNA was insufficient to induce significant knockdown. Since the pcRNAs did not effectively knock down any transcripts, further dosages should be tested. However, given that high siRNA concentrations have produced unintended global gene upregulation or downregulation, increasing the doses used should be approached with some caution (Lin et al., 2005).

The pcRNAs used in this study were designed to generate a single siRNA, following Dicer processing (Abbasi et al., 2020), and their structure was based on the premise that short hairpin RNAs (shRNAs) could be more durable than siRNAs when delivered to cells exogenously. A few research groups have used siRNAs to knock down genes in the fungus *Aspergillus nidulans* (Jöchl et al., 2009; Kalleda et al., 2013; Khatri & Rajam, 2007), targeting genes that resulted in significant inhibition of fungal growth, conidiation, and spore germination. Short hairpin RNA (shRNA)-mediated silencing has also been successfully applied in *Fusarium graminearum* (Chen et al., 2015). That study first demonstrated that a transgene expressing a shRNA targeting the FgCNB1 gene could induce strong transcript knockdowns and confirmed that it was mediated by RNAi by showing that deletion of two RNAi components, *FgDCL2* or *FgAGO1,* restored the expression of the hpRNA-targeted genes to wild-type levels. A shRNA was also effective in down-regulating gene expression in the filamentous fungus *Coprinopsis cinerea* (Costa et al., 2008). To investigate the role of semaphorin-1a (sema1a) in the olfactory development of the mosquito *Aedes aegypti*, chitosan/siRNA nanoparticles were used that resulted in knockdown of sema1a, resulting in significant defects in the antennal lobes of the adult insects (Mysore et al., 2013). A comparative study of dsRNA and siRNA silencing efficiency was conducted, to silence the *tyramine receptor 1* (*tyr1*) gene in honeybee brains. Both approaches successfully reduced *tyr1* expression at both the mRNA and protein levels, with siRNAs producing faster knockdown effects than dsRNA. The authors hypothesized that siRNAs bypass the preprocessing step required for dsRNAs, and hence the silencing effect occurs faster than dsRNA (Guo et al., 2018). DsRNA and siRNAs are generally vulnerable to degradation by extracellular and intracellular nucleases, which can rapidly break down the RNA molecules (Kang et al., 2023). Unmodified siRNAs typically have a short half-life in biological fluids, such as serum and tissues, ranging from several minutes to an hour (Gavrilov & Saltzman, 2012). Hence, chemical modifications are often used to enhance their stability and

resistance to nucleases when siRNAs are applied in biomedical applications (Abbasi et al., 2020; Aluri et al., 2024; Chiu & Rana, 2003; Gangula et al., 2024; Kremer et al., 2024). While there have been no studies confirming the involvement of fungal nucleases degrading siRNAs, RNA degradation by nucleases was considered a potential factor affecting RNAi efficiency in Aspergillus and Sclerotinia (Kalleda et al., 2013; Walker, 2022).

Efficient delivery of siRNA to target organisms remains a major hurdle. Environmental factors such as UV radiation, alkaline hydrolysis and nucleases can degrade dsRNA before it reaches the target (Komarova et al., 2023; Möller et al., 2016). ShRNAs were demonstrated more stable and provided more consistent target gene down-regulation than siRNAs in the filamentous fungus *Blakeslea trispora* (Li et al., 2021), the yeast *Cryptococcus neoformans* (Chen et al., 2023), mosquitoes (Abbasi et al., 2020), humans (Rao et al., 2009) and mice (McAnuff et al., 2007). This property makes shRNAs particularly useful for creating stable cell lines or transgenic organisms for long-term RNAi studies. The delivery of shRNA to mosquito larvae by feeding them transgenic yeast successfully induced gene silencing upon ingestion, presenting a promising strategy for controlling diseases like dengue and malaria. (Mysore et al., 2019). Like siRNAs, shRNAs are still vulnerable to many abiotic and biotic factors that can reduce their stability, and transgenic approaches to deliver dsRNA to a target, either directly or through the food it consumes, will provide some additional protection of the shRNAs until they can reach their target cells.

Another factor affecting the success of siRNA- or shRNA-mediated silencing is the relative accessibility of the target sequence within the mRNA to the guide strand of the siRNA within RISC. Shao et al. (2007) showed that by examining the structure of the target mRNA, siRNAs could be selected that had a greater likelihood of improving transcript knockdown efficiency (Shao et al., 2007). Using a population-based approach to model mRNA secondary structures with the Sfold program, they found that the secondary structure of mRNA can greatly influence RNAi efficiency by limiting or improving the accessibility of target sites and the binding strength of complementary nucleic acids (Bohula et al., 2003; Kretschmer-Kazemi Far & Sczakiel, 2003; Krueger et al., 2007; Overhoff et al., 2005; Schubert et al., 2005; Shao et al., 2007; Vickers et al., 2003; Westerhout et al., 2005; Yoshinari et al., 2004). Due to intramolecular folding, it has been estimated that only 5-10% of a typical transcript is accessible for binding with siRNA (Bohula et al., 2003). Hybridization of siRNA with targets of accessible regions showed effective gene

silencing, whereas siRNAs directed at less accessible regions produced limited silencing effects. This phenomenon may explain why the pcRNAs in my experiments were unable to induce knockdown, as the siRNA derived from the pcRNA may have been unable to access the mRNA. The presence of secondary or tertiary structures in the mRNA may have prevented the pcRNA-derived siRNA to bind with the target and thus failed to induce significant knockdown in the current assays. The pcRNAs were designed using Integrated DNA Technology's algorithms, which identify siRNAs with predicted high affinities to their target mRNA. However, these predictions do not consider the complexity of the mRNA target's natural structure, and hence, not all siRNAs may gain effective access to their target to initiate RNAi.

Therefore, when selecting target sequences, it will be important to identify regions with more exposed nucleotides, avoid target sites with high local free energy, and stable hairpin structures, since these factors can highly influence the RNAi efficacy (Schubert et al., 2005; Shao et al., 2007). In future, pcRNA can be designed by using bioinformatic tools to predict mRNA secondary structures and evaluate various siRNA sequences for the target gene to optimize the silencing efficiency.

4.5 Conclusion and future directions:

To achieve successful RNAi-based bio-pesticides, *in vitro* testing serves as a crucial initial step in evaluating the efficacy of dsRNA molecules. The integration of molecular and computational approaches has facilitated the assessment of dsRNA silencing effectiveness. Furthermore, the design of different dsRNA structures offers new perspectives on RNAi mechanisms, gene expression, and the elucidation of intricate biological pathways. The present study examined the performance of two distinct dsRNA structures long dsRNA and pcRNA under *in vitro* conditions. Initial assays in this study using a fungal plug inoculation method yielded inconsistent results, prompting the development of a revised methodological approach. This new method, termed the fungal slurry method, aimed to incubate dsRNA molecules with fungal hyphae of varying ages, resulting in different rates of growth. Newly growing hyphae, for example, appear more receptive to dsRNA uptake (Wytinck et al., 2020). In contrast, older hyphae with highly chitinized cell walls may be less responsive to dsRNA compared to younger hyphae. While the slurry method provided more consistency with some dsRNA treatments, variability in transcript knockdowns persisted for both long dsRNA and pcRNA structures. This ongoing variability drew

attention to the complexity of RNAi-based biopesticide development and the need for further refinement of experimental techniques.

The observed variability and absence of significant knockdowns in all treatments can be attributed to the quantity of hyphae in each biological replicate, the diverse developmental stages of cells within the hyphae, the expression levels of individual genes at the time of mycelial collection, and the accessibility of the target gene. These elements potentially contributed to variability and consequently influenced the knockdown effectiveness in the current study. The lack of a straightforward method to evaluate hyphal age or growth phase on PDA plates prevents the confirmation of this possibility. While FPKM values can serve as a primary criterion for selecting RNAi targets, they are likely only to be useful measures of a gene's activity if they can be obtained multiple times during the fungus' development. To effectively implement RNAi-mediated control of this significant plant pathogen, it is crucial to have a thorough understanding of the target gene's transcriptional patterns. Focus should be given on selecting targets that demonstrate moderate and consistent expression levels. Genes with inducible expression should be excluded to ensure more stable and reliable outcomes. Further research on fungal gene expression will ultimately help us identify the best RNAi targets for RNAi-mediated control of this serious phytopathogen.

In experimental assays, it is crucial to maintain uniformity in the age, developmental stage, and growth phase of the fungal specimens to ensure reliable and reproducible results. In future assays, Sclerotinia used as an inoculant should be selected such that it is actively growing and of similar age and development. Harvesting the fungus from the plate at an earlier time point from the periphery of fungal growth may prove effective in this regard. Additionally, by harvesting dsRNA treated fungal biomasses by 72 hours, the cells may no longer be responding effectively to dsRNA. Hence, harvesting the fungal biomass at an earlier time point than 72 hours could provide clearer insights into variability and significant transcript knockdowns. A precise methodology should be established to ensure the accurate quantification of hyphal biomass incorporated into the assay, thereby enhancing the reliability and reproducibility of experimental outcomes. Conducting the assays using equal amounts of ascospores can be investigated as means to resolve this issue. A wider range of dsRNA and pcRNA doses can be screened to determine the optimal concentration for the silencing of each target gene. It would also be interesting to investigate the factors that hindered the efficiency of pcRNA to knock down the genes in *S. sclerotiorum*. A systematic

analysis evaluating diverse siRNA designs for a specific gene, coupled with an assessment of increased siRNA length, could yield valuable insights. Such a comprehensive study would contribute to the development of optimized pcRNA constructs, potentially leading to more robust and effective gene silencing outcomes in *S. sclerotiorum*.

In conclusion, the slurry method presents as a potentially efficacious technique for reducing the knockdown variability. However, this approach necessitates further rigorous investigation and comprehensive optimization protocols. The positive outcomes observed in *in vitro* assays may potentially be extrapolated to *in vivo* conditions, thereby facilitating a more accurate assessment of the RNA interference (RNAi) efficacy of both long double-stranded RNA (dsRNA) and paperclip RNA (pcRNA). This transfer of RNAi information from *in vitro* to *in vivo* conditions could provide valuable insights into the practical applications and limitations of the RNAi approaches.

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