Investigating the off-target effects of *Sclerotinia sclerotiorum* double-stranded RNAs with other important fungal pathogens, *Botrytis cinerea* and *Leptosphaeria maculans.*

by

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ABSTRACT

Sclerotinia sclerotiorum, *Botrytis cinerea*, and *Leptosphaeria maculans* are three devastating plant fungal pathogens that affect a wide array of economically important crops. Current control strategies rely on broad spectrum fungicides; however, they are not a viable long-term solution due to the rise of fungicide resistance and their potential to harm the environment. Consequently, there is a direct and urgent need to develop new solutions to protect our crops. RNA interference (RNAi) is an intrinsic cellular mechanism that can reduce messenger RNA transcript levels through the introduction of double-stranded RNA (dsRNA). Due to the sequence specificity of RNAi, there is potential to design dsRNAs that perturb gene expression in a single pathogenic fungus without affecting other species. Using a combination of quantitative-real time polymerase chain reaction and fungal growth assays, I identified a set of novel *S. sclerotiorum* dsRNAs that are effective at reducing target transcript abundance and fungal growth. The potential off-target effects of these *S. sclerotiorum*-specific dsRNAs were subsequently examined in the closely-related *B. cinerea* and the more distally-related *L. maculans*. Data revealed that topically-applied dsRNAs can indeed be designed to be species-specific, but if even a single 21 nucleotide sequence is shared with another fungal species, cross-species transcript knockdown and reduced fungal growth can occur. This research provides useful data to evaluate the specificity of topically-applied dsRNA molecules and compelling evidence for the efficacy of RNAi-based crop protection strategies.

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NON-COMMON ABBREVIATIONS USED

ABC	ATP-binding cassette
Ago	ARGONAUTE
***Bc*-sRNA**	*B. cinerea* small RNA
BGMV	*Bean golden mosaic virus*
bZIP	basic leucine zipper
DCL	DICER-LIKE
dpi	days post inoculation
dsRNA	double-stranded RNA
DvSnf7	*Diabrotica virgifera virgifera snf7*
FPKM	fragments per kilobase of million mapped reads
GM	genetically modified
HIGS	host-induced gene silencing
hpi	hours post inocualtion
hpRNA	hairpin RNA
Loqs-PD	Loquacious-PD
MBC	methyl benzimidazole carbamate
miRNA	micro-like RNA
mRNA	messenger RNA
nt	nucleotide
piRNA	piwi-interacting RNA
PRSV	*Papaya ringspot virus*
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
SsPG	*S. sclerotiorum* polygalacturonase
SSR	sclerotinia stem rot
TF	transcription factor

CHAPTER 1: BACKGROUND AND RELEVANCE

1.1 Introduction

In the next 30 years, the global population is expected to increase by 2 billion persons, from 7.7 billion to 9.7 billion, and could peak at nearly 11 billion around the year 2100 (United Nations, 2019). This population growth, along with intensive urbanization and increases in economic prosperity, is shaping unprecedented global demands for food and it will be necessary to roughly double global crop yields over the next 40 to 50 years (Cai et al., 2018a; Fisher et al., 2018). However, climate change, soil erosion, and water scarcity threaten to make food more difficult and expensive to produce. The shift from small-scale, diverse, and single-cycle agriculture to large-scale, genetically uniform, and intensive monoculture production, which is now associated with most current farming practices, has favoured large-scale epidemics and outbreaks, and have rendered our agroecosystems more vulnerable to pests and pathogens. The rise of global trade has allowed the free flow of pests and pathogens from continent to continent, bringing them into contact with naïve hosts (Fisher et al., 2012). Globally, each year, pests and pathogens reduce 20 to 40% of crop production (Cai et al., 2018a). Furthermore, destruction caused by post-harvest diseases during processing, transportation, and storage account for 20 to 25% of crop losses in the United States and up to 50% in some developing countries (Kader, 2005; Sharma et al., 2009). Current practices to control these pests and pathogens rely on heavy chemical treatments, but they pose a serious threat to human health and the environment. Additionally, excessive use of fertilizers and pesticides are causing soil, water, and air pollution, as well as fauna and flora biodiversity loss (Md Meftaul et al., 2020). Therefore, there is an urgent need to improve our current food systems, invest in sustainable food sources, and develop alternative methods of protecting our crops from pests and pathogens.

1.2 Canola And Fungal Pathogens Found in Western Canada

In Canada, one of the most important crops is canola (*Brassica napus L.*), a variety of rapeseed known for its oil-rich seed. Canola was developed in the mid 1970s at the Universities of Manitoba and Saskatchewan by Baldur Stephenson and Keith Downey (Lin et al., 2013). Using conventional breeding techniques, Stephenson and Downey were able to select plants with low antinutritional compounds, such as erucic acid, glucosinolates, and seed chlorophyll, while still retaining favourable agronomic characteristics that would allow the crop to be widely adapted to the Canadian prairies (Barthet, 2013; Canola Council of Canada, 2017). Now, canola is grown extensively in Australia, Canada, China, Europe, India, and the United States. Currently, canola is Canada's most valuable oilseed and contributes to a significant portion of the national economy. Overall, canola generates $26.7 billion of economic activity each year, and provides over 250,000 jobs nationwide (LMC International, 2016). In 2017, Canadian canola production exceeded 21 million tonnes and accounted for nearly 70% of the world's canola imports (Canola Council of Canada, 2017). In addition to being economically important, canola oil has the lowest saturated fat content of all common vegetable oils and is considered to be a heart-healthy cooking oil (Lin et al., 2013). The consumption of canola oil has been reported to show positive effects on insulin sensitivity, fasting blood sugar, and blood pressure (Kruse et al., 2015). When compared to other edible oils, canola oil significantly improved several cardiometabolic risk factors, including significantly reducing total cholesterol, low-density lipoprotein cholesterol, and apolipo-protein B levels (Amiri et al., 2020).

Besides being a popular source of cooking oil, canola has emerged as a new source for plant-based proteins. As global food demands have increased, population requirements for animal proteins have also increased (Delgado, 2003). It is projected that the demand for animal-derived

protein will double by 2050 (Chmielewska et al., 2020). However, meat production has many negative environmental impacts. It has been reported that 12% of worldwide greenhouse gas emissions are derived from livestock production and 30% of human-induced terrestrial biodiversity loss can be linked to animal production (Henchion et al., 2017). Plant-based proteins can help meet the nutritional requirements of our growing population, while also reducing the negative impacts of livestock production. Canola is a promising source for plant-based protein due to its nutritional value, well-balanced amino acid profile, and functional properties (Chmielewska et al., 2020). The leftover canola meal from pressing oil has traditionally been used for animal and aquaculture feed, but has been shown to meet the human demand for essential amino acids and contains nutritionally complete proteins (Wanasundara et al., 2016). As a result, canola is an ideal candidate as a new source of plant-based protein. Canola protein also has many valuable non-food uses, such as biodiesel, bio-degradable plastics, films, packaging material, and adhesives (Bandara et al., 2018). Evidently, canola as a crop is invaluable to Canada and any threats to canola production will have serious economic impacts.

1.2.1 *Sclerotinia sclerotiorum*

Like many crops, canola is indeed threatened by a variety of pests and pathogens. Among the most serious of pathogens to affect canola is *Sclerotinia sclerotiorum* (Lib.) de Bary. *S. sclerotiorum* is a necrotrophic plant fungal pathogen that is known for its wide host range and environmental persistence. This pathogen affects over 500 host plants, including a wide array of economically important crops such as chickpea, soybean, sunflower, and most *Brassica* crops (Bolton et al., 2006; Pankaj et al., 2016). *S. sclerotiorum* can induce different disease symptoms depending on the host plant. In general, ascospores are produced by germinating sclerotia found in the soil or soil surface. The windborne ascospores adhere to senescing petals and develop into

watery lesions that quickly drop onto the leaves' surfaces. The lesions develop into necrotic zones and pathogen-induced host cell death occurs. Eventually, the lesions enter the vasculature, and the infection becomes systemic with white fluffy mycelium, or white mould, growing on the surface of infected tissues. At the end of the growing season, new sclerotia will form and the lifecycle will repeat (Purdy, 1979).

In canola, Sclerotinia stem rot (SSR) not only reduces the yield and quality of the seed, but also the overall oil content (Pankaj et al., 2016). SSR occurs in all major Canadian canola growing regions and is generally considered one of the most economically damaging canola pathogens (Derbyshire & Denton-Giles, 2016). The severity of SSR is highly variable, but under epidemic conditions, yield losses in canola growing regions affected by *S. sclerotiorum* have been reported to exceed 50% (Seifbarghi, 2019). SSR has become a more serious problem in recent years due to increased canola production and management practices that produce dense foliage canopies that are optimal for fungal growth. In 2016, SSR was widespread across Western Canada and over 90% of surveyed canola fields showed signs of fungal infection (Canola Council of Canada, n.d.-b). The disease management of *S. sclerotiorum* has been difficult and inconsistent due to its wide host range and long-term survival of sclerotia, the primary overwintering structure of *S. sclerotiorum*, in soil. In addition, selective breeding for completely resistant genotypes has been very challenging and fully resistant cultivars have yet to be produced (Kamal et al., 2016; Pankaj et al., 2016). Partial, quantitative resistance of SSR has been observed in field trials of commercial soybean varieties and through quantitative trait locus mapping (Bastien et al., 2014; Conley et al., 2018; Zhao et al., 2015). However, reliance on this type of quantitative resistance can result in limited control. Current *S. sclerotiorum* control methods rely primarily on cultural control practices and chemical fungicide application (Murray & Brennan, 2012). Cultural control practices include the

use of organic soil amendments, soil sterilization, zero tillage and crop rotation, tillage and irrigation, wider row spacing, and smaller plant populations (Kamal et al., 2016). The majority of these agronomic practices aim to reduce the level of *S. sclerotiorum* inoculum in the soil, or create a local environment that is not conducive to SSR growth and development (Pankaj et al., 2016). The use of foliar fungicides is common and several different fungicide classes are effective against SSR, including anilinopyrimidines, benzimidazoles, dicarboxamides, demethylation inhibitors, quinone outside inhibitors, and succinate dehydrogenase inhibitors (Derbyshire & Denton-Giles, 2016). However, the efficacy of foliar fungicides depends on several factors including time of application, spraying coverage, weather conditions, disease cycle, crop phenology, and protection duration (Mueller et al., 2002). Overall, fungicide application is quite costly and if the grower fails to apply the fungicide at the most appropriate time, it can lead to significant economic loss. Exposure to chemical fungicides also has many negative environmental and health impacts, including embryotoxicity, germ cell apoptosis, teratogenesis, infertility, and development toxicity in different mammalian species (Singh et al., 2016). The development of fungicide resistance also remains a threat and many reports of fungicide resistance in *S. sclerotiorum* have been documented (Gossen et al., 2001; Wang et al., 2014; Zhou et al., 2014). For example, repeated singular applications of dicarboximide and benzimidazole fungicides have caused the emergence of resistant strains (Duan et al., 2014; Ma et al., 2009). For all these aforementioned reasons, there is a need to develop new technologies to protect canola from this devastating fungal pathogen.

1.2.2 *Botrytis cinerea*

Botrytis cinerea Pers. Fr. is another necrotrophic plant fungal pathogen and the causal agent of grey mould. Similar to *S. sclerotiorum*, *B. cinerea* has a wide host range and can infect over 200 crop species, including many agriculturally important crops such as cucumbers, grapes,

strawberries, and tomatoes (Williamson et al., 2007). This pathogen affects many of the plant's tissues, causing cutting and crown rot, stem cankers, blight on leaf and petal tissues, and damping off (Ayoub et al., 2018). *B. cinerea* is difficult to control due to its wide host range and variety of modes of attack (Williamson et al., 2007). Similar to *S. sclerotiorum*, this pathogen has the ability to overwinter and can form sclerotia on plant debris and other necrotic tissues (Jacometti et al., 2010). Grey mould epidemics are particularly common in greenhouses and stems girdled by disease result in significant yield loss (Shtienberg & Elad, 1997). In addition to causing disease during the growing season, *B. cinerea* contributes especially to post-harvest problems. Its fungal spores can reside on the surface of fruits and latent infection can occur during storage, marketing, and even after customer purchase of produce (Simone et al., 2020). *B. cinerea* annually causes $10 to $100 billion in losses worldwide and is ranked second in the "world's top 10 fungal pathogens in molecular plant pathology" in terms of economic and scientific relevance (Dean et al., 2012; Weiberg et al., 2013). In absence of crop varieties resistant to *B. cinerea*, growers still rely on multiple fungicide applications to control this fungal pathogen. Different classes of fungicides have been used to manage grey mould, including benzimidazoles, dicarboximides, anilinopyrimidines, hydroxyanilides, quinone outside inhibitors, and succinate dehydrogenase inhibitors (Hu et al., 2016). However, due to improper fungicide application and the diversity in morphology and genetic variation of *B. cinerea*, fungicide resistance has emerged. It has been reported that *B. cinerea* is resistant against 15 different classes of fungicides, including pyraclostrobin, boscalid, dicarboximide, aniline-pyrimidine, phenylpyrrole, and hydroxyaniline (Fernández-Ortuño et al., 2012; Fisher et al., 2018). For example, benzimidazole fungicides, which act by binding fungal microtubules and inhibiting hyphal growth, were highly effective against grey mould in the late 1960s but strains resistant to these fungicides started to emerge within a few

years of their application (Shim et al., 2014). Therefore, the use of chemical fungicides as the only control method is unlikely to be successful. Alternatives to fungicides include biological control, essential oils, plant hormones and extracts, abiotic stimulants, and microbial induction (Jacometti et al., 2010). *B. cinerea* is also managed using a variety of cultural techniques that aim to reduce canopy humidity and inoculum sources. Physical methods to control *B. cinerea* in post-harvest crops have also been used, including modification of several physical parameters such as temperature, gas pressure, UV irradiation, and sonication (Simone et al., 2020). For example, table grapes are usually stored in refrigerated chambers with strictly controlled temperature and humidity levels. However, *B. cinerea* survives at low temperatures, and any variation in temperature can promote water condensation and result in fungal growth and sporulation. Overall, current *B. cinerea* management strategies are lacking and there needs to be a more effective way to control this fungal pathogen during pre- and post-harvest conditions.

1.2.3 *Leptosphaeria maculans*

Leptosphaeria maculans (Desm.) Ces. & de Not. is the causal agent of blackleg, or phoma stem canker. Unlike *S. sclerotiorum* and *B. cinerea*, *L. maculans* is a hemi-biotrophic fungal pathogen with a complex life cycle. This fungal pathogen infects a wide variety of *Brassica* crops, including cabbage and canola. Blackleg has been reported as a major constraint to canola production in Africa, Australia, Europe and North America (Fitt et al., 2005). *L. maculans* is a virulent species, infecting canola from the seedling stage and progressively damaging the crop by girdling stems and restricting moisture and nutrient uptake, eventually leading to significant yield loss (Canola Council of Canada, n.d.-a). In addition, *L. maculans* overwinters as saprophytes on infected canola stubble, which allows the fungus to reproduce sexually and produce fruiting bodies, called pseudothecia and pycnidia, in the following spring (Aubertot et al., 2004). Blackleg

can overwinter for over two years on infected canola stubble and continue to produce pseudothecia. The severity of a blackleg epidemic is dependent on several factors, such as variation in the canola cultivars grown, climate and agriculture practices, geographic region, and pathogen population structure (Hwang et al., 2016). In Europe, blackleg is considered as a monocyclic disease, whereas secondary cycles of infection can occur in Australian and Canadian fields (West et al., 2001). In western Canada, yield losses up to 50% have been reported in individual fields and in Australia, blackleg can cause yield losses of up to 90% (Canola Council of Canada, n.d.-a; Marcroft & Bluett, 2020). Blackleg disease severity can be controlled by manipulating farming and cultural practices, such as early sowing, residue management, longer crop rotations, and adequate crop spacing (Hwang et al., 2016). Seed treatment and foliar application of fungicides are often used to reduce blackleg disease, but these methods do not provide full protection to crops (Kutcher et al., 2013). In Canada, blackleg has often been managed by the deployment of resistant canola cultivars (Kutcher et al., 2010). More specifically, qualitative resistance can be mediated by interactions between a race-specific R protein within the plant and corresponding avirulent proteins produced by the fungal pathogen (Fu et al., 2020). However, reliance on any one strategy alone, such as genetic resistance, is not a viable long-term strategy. Resistant varieties exert a selection pressure on the fungus and *L. maculans* has a high potential to overcome gene resistance due to its annual cycle of sexual recombination, production of widely dispersed wind-borne ascospores, and large number of conidia spread through rain splash (Fitt et al., 2005). In 2012, this loss of resistance was especially evident when many canola crops that were considered moderately resistant cultivars were nevertheless highly infected with blackleg, resulting in severe yield losses (Hwang et al., 2016). Even when integrated management practices are being employed, intensive canola cropping cycles are eroding *L. maculans* genetic resistance (Khangura et al., 2019). Thus, new management

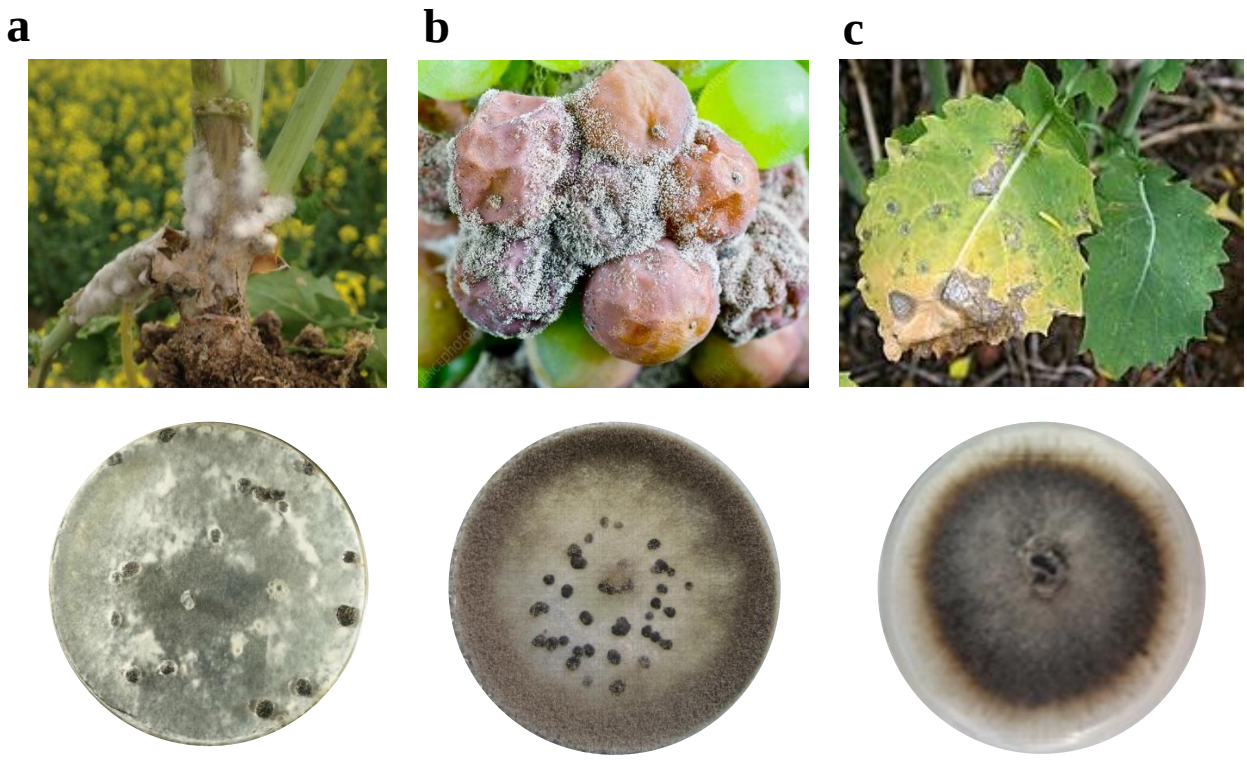


Figure 1.1. Comparison of *Sclerotinia sclerotiorum, Botrytis cinerea,* and *Leptosphaeria maculans*. (**a**) *S. sclerotiorum* grown on canola and a potato dextrose agar (PDA) plate. (**b**) *B. cinerea* grown on grapes and a PDA plate. (**c**) *L. maculans* grown on canola and a PDA plate.

practices are needed to protect canola from *L. maculans*.

1.3 General Mechanisms of RNAi

Currently, plant pathogenic fungi and fungi-like oomycetes account for roughly 20% of worldwide perennial crop yield losses and an additional 10% of loss occurs due to post-harvest fungal disease (Fisher et al., 2018). Therefore, there is a demand to develop an alternative and more sustainable method of fungal pathogen control. Recently, scientists have demonstrated that eukaryotic pests and pathogens can be inhibited by small non-coding RNAs (sRNAs) targeting their essential and/or pathogenicity genes. This has raised the possibility that our crops can be protected by a new generation of eco-friendly RNA-based fungicides, which are highly specific and can be easily adapted to control multiple pathogens simultaneously.

RNA interference (RNAi) is a conserved eukaryotic gene regulatory mechanism that uses sRNAs to mediate post-transcriptional gene silencing (PTGS). RNAi was initially thought to be a host defense mechanism against invading viruses and transposons, however studies have now demonstrated that RNAi pathways use sRNAs to regulate diverse cellular processes, such as development, RNA stability and processing, host defense, chromosome segregation, transcription, and translation (Lax et al., 2020). Among the identified sRNAs there are three major classes: small interfering RNA (siRNA), microRNA (miRNA), and piwi-interacting RNA (piRNA). SiRNAs are involved in exogenous and endogenous pathways for viral and transposon defense, respectively. MiRNAs are involved in a pathway that regulates gene expression at the transcription level and piRNAs function in epigenetic control of genomic elements.

RNAi was first described in *Caenorhabditis elegans* (Fire et al., 1998) and has now been well documented in most eukaryotic organisms, including protozoans, fungi, algae, plants, invertebrates, and vertebrates (Wytinck et al., 2020a). Before the discovery of RNAi, the phenomenon of RNA silencing was first observed in plants and described as co-suppression or PTGS (Napoli et al., 1990). In parallel to the discoveries in plants, transgene-induced silencing was also reported in the fungus *Neurospora crassa* and was first described as quelling (Romano & Macino, 1992). Now, the term "RNAi" is the most widely used, and its application to reduce gene expression has emerged as a powerful tool to study gene function in many organisms. The core components of RNAi have been identified in all major branches of eukaryotes (Cerutti & Casas-Mollano, 2006). Dicer, or a Dicer-like (DCL) protein, is the first major protein involved in the RNAi pathway. It is an endonuclease that belongs to the RNase III family, an evolutionarily conserved protein group that shows specificity for double-stranded RNAs (dsRNAs) and is responsible for processing long dsRNAs into siRNAs that are typically 21 to 25 nucleotides (nt)

long (Bernstein et al., 2001; Zamore et al., 2000). While Dicer is evolutionarily conserved, it has diversified structurally. Animals such as nematodes and humans possess a single Dicer gene that can produce both siRNAs and miRNAs (Zhang et al., 2002). Conversely, arthropods and fungi possess two Dicer paralogs and *Arabidopsis thaliana* has four distinct DCL proteins (Bologna & Voinnet, 2014; Schauer et al., 2002). Once Dicer has processed the dsRNA into siRNA duplexes, each siRNA duplex is loaded onto the RNA-induced silencing complex (RISC), where the duplex is unwound (Joga et al., 2016). The exact molecular composition of the RISC has yet to be defined, however, the minimal RISC sufficient for RNA recognition and cleavage requires an Argonaute (Ago) protein bound to a sRNA (Rivas et al., 2005). Ago is the main component of the RISC and it discards the passenger (sense) strand and the guide (antisense) strand remains associated with the RISC (Hammond et al., 2000). Plant Ago proteins are functionally diverse and are involved in a variety of different gene silencing processes (Carbonell, 2017). Plant Ago proteins have also been shown to be involved in antiviral or bacterial defense mechanisms and responses to herbivore attack (Pradhan et al., 2017; Sibisi & Venter, 2020). The guide strand of the siRNA allows Watson-Crick base-pairing of the complex to complementary target messenger RNA (mRNA), which is subsequently cleaved by Ago and then degraded by cytosolic exonucleases (Rosa et al., 2018). Some species also possess RNA-dependent RNA polymerases (RdRPs), which catalyze the replication of dsRNA from aberrant single stranded-RNA transcripts. They play an important role in RNAi in eukaryotes, however they are not as widely distributed as Dicer and Argonaute (Cerutti & Casas-Mollano, 2006). RdRPs have only been discovered in *S. pombe*, *C. elegans*, plants, and fungi (Ketting, 2011).

More recent discoveries have shown that sRNAs can move across the cellular boundaries between a host and interacting pests and pathogens and induce gene silencing, described as cross-

kingdom RNAi (Weiberg et al., 2017). Weiberg *et al.* (2013) showed that *B. cinerea* siRNAs (*Bc*-sRNAs) can suppress *A. thaliana* and tomato genes involved in immunity. Some *Bc*-sRNAs can target multiple host immunity genes to enhance *B. cinerea* pathogenicity. For example, *Bc*-siR37 suppresses host immunity by targeting at least 15 *A. thaliana* genes, which includes WRKY transcription factors, cell wall modifying enzymes, and receptor-like kinases (Wang et al., 2017b). The *Bc*-sRNAs utilize the host RNAi machinery by binding to Arabidopsis ARGONAUTE1 (AGO1) to silence host immunity genes (Weiberg et al., 2013). Consistent with this finding, *B. cinerea* causes much less disease symptoms on the Arabidopsis *ago1-27* mutant compared to wildtype plants as sRNA effectors could no longer function without the host AGO1 (Weiberg et al., 2013). Furthermore, the *B. cinerea dcl1 dcl2* double mutant strain that fails to produce sRNA effectors is compromised in pathogenicity on various plant species, including vegetables, fruits, and flowers, when compared to wildtype *B. cinerea* (Wang et al., 2016). In comparison, *B. cinerea dcl1* or *dcl2* single mutant exhibited significant fungal growth defects when grown on plates and plants but can still produce sRNA effectors and maintain aggressive virulence, supporting the premise that sRNA effectors are essential for *B. cinerea* pathogenicity (Wang et al., 2016). *Puccinia striiformis*, a destructive wheat pathogen, has also been shown to deliver sRNAs into host cells to suppress wheat *Pathogenesis-related 2* genes in the cellular defense pathway. Wang et al. (2017a) showed that silencing of the *P. striiformis* microRNA-like RNA1 precursor led to enhanced wheat resistance to the virulent pathogen (Wang et al., 2017a).

Conversely, animals and plants can deliver host sRNAs into pathogens to suppress their virulence. Cai *et al.* (2018) showed that host Arabidopsis cells secrete exosome-like extracellular vesicles to deliver sRNAs into *B. cinerea.* Using transgenic plants expressing green fluorescent protein, they showed that sRNA-containing vesicles accumulated at the infection sites and are

taken up by the fungal cells (Cai et al., 2018b). *Verticillium dahlia* collected from infected cotton plants contained 28 miRNAs from cotton, implying that host-derived sRNAs were transmitted into the pathogen during infection (Zhang et al., 2016). Two of those cotton miRNAs, miR166 and miR159, target the fungal genes *Ca^{2+}-dependent cysteine protease calpain* (*VdClp-1*) and *Isotrichodermin C-15 hydroxylase* (*VdHiC-15*), respectively. Consistent with host-mediated silencing, *VdClp-1* and *VdHiC-15* transcripts were reduced in the *V. dahlia* hyphae recovered from *V. dahlia* infected cotton. Moreover, fungal mutants *vdclp-1* and *vdhic-15* were reduced in virulence, confirming that these genes contribute to fungal pathogenicity (Zhang et al., 2016).

Externally-applied and endogenously-supplied dsRNAs can induce RNAi in various organisms, which has been termed as "environmental RNAi". More specifically, environmental RNAi refers to sequence-specific gene silencing in response to environmental encountered dsRNA (Whangbo & Hunter, 2008). Environmental RNAi was first observed in *C. elegans* when the injection of dsRNA into the body cavity led to target gene silencing throughout the injected animal and its progeny, which provided evidence that environmental dsRNA can enter into an organism's cells (Fire et al., 1998). Subsequently, alternative dsRNA delivery methods in *C. elegans* demonstrated that RNAi could be triggered by soaking the animals in a dsRNA solution or by feeding on bacteria expressing dsRNAs (Lisa Timmons & Andrew Fire, 1998; Tabara et al., 1998). Mutant screenings in *C. elegans* have identified several *SID* genes that are responsible for both environmental RNAi and systemic RNAi (Feinberg & Hunter, 2003; McEwan et al., 2012; Winston et al., 2002, 2007). However, most of these genes are exclusively present in invertebrates and homologous genes have not been found in plants or fungi (Wang et al., 2015a). *SID-1* and *SID-2* are two important genes that mediate *C. elegans* dsRNA uptake. *SID-1* is a dsRNA specific membrane channel that facilitates endocytosis-independent dsRNA uptake (Feinberg & Hunter,

2003; Winston et al., 2002) and *SID-2* is a single pass transmembrane protein that mediates endocytosis-dependent dsRNA uptake (McEwan et al., 2012). In several insect species, orthologous proteins to *C. elegans SID-1*, called *SID-like* (*SIL-A*, *SIL-B*, and *SIL-C*), have been identified, however their involvement in environmental RNAi have yet to be determined (Wytinck et al., 2020a) Clathrin-mediated endocytosis has been shown to be involved in the uptake of exogenous dsRNA in several insects (Li et al., 2015; Xiao et al., 2015), as well as some fungal species, including *S. sclerotiorum* (Wytinck et al., 2020b). Although the mechanism for fungal dsRNA uptake is still not well understood, the phenomenon is well documented.

1.4 Host-Induced Gene Silencing vs. Spray-Induced Gene Silencing

Host-induced gene silencing (HIGS) is an alternative method of crop protection, involving the generation of transgenic plants that produce sRNAs to silence target genes of plant pests or pathogens. HIGS-based technologies are currently being developed to protect crop plants from nematodes (Huang et al., 2006), insects (Baum et al., 2007; Mao et al., 2007), fungi (Koch et al., 2016; McLoughlin et al., 2018; Wang et al., 2016), and oomycetes (Vega-Arreguín et al., 2014). For HIGS, a dsRNA or a hairpin structured dsRNA expression construct targeting a specific pathogen gene is transformed into the host plant. The transgenic plant produces dsRNAs and siRNAs, which find their entry into the plant pathogen during host-pathogen interactions. The *in vivo* interference phenomenon was first demonstrated in the pathogenic fungus *Fusarium verticillioides*. Tinoco *et al.* (2010) inoculated mycelial cells into transgenic tobacco engineered to express siRNAs from a dsRNA corresponding to a particular fungal transgene and showed the fungal transgene's transcript levels were reduced (Tinoco et al., 2010). Since then, the HIGS approach has shown to be effective at protecting host plants against a variety of fungal pathogens, including *Aspergillus flavus, B. cinerea, Bremia lactucae, Colletotrichum gloeosporioides,*

Festuca arundinacea Schreb, *Magnaporthe oryzae*, *Nicotiana tabacum 'Xanthi'*, *S. sclerotiorum*, *V. dahliae*, and *Blumeria*, *Fusarium*, *Phytophthora*, and *Rhizoctonia* species (Rajam & Chauhan, 2021). The HIGS approach has been shown to protect a variety of host plants and important crops, including *A. thaliana*, *Brachypodium distachyon*, *Capsicum annuum L.* (peppers), *Gossypium hirsutum* (cotton), *Hordeum vulgare* (barley), *Lactuca sativa L.* (lettuce), *Musa spp.* (bananas and plantains), *Nicotiana benthamiana*, *Oryza sativa* (rice), *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Triticum aestivum* (wheat), and *Zea mays L.* (corn) (Rajam & Chauhan, 2021).

However, HIGS has many limitations. One of the major constraints of HIGS is its requirement for stable genetic transformation of the host plant, which is dependent on the transformability and genetic stability of the crop species (Weiberg et al., 2013). Most crop species do not have an established transformation protocol, and its development in new crop plants can take years to optimize and have good efficiency rates (Mitter et al., 2017b). Furthermore, expression of a transgene is not always stable, and they are often suppressed or silenced at the transcriptional level after a few generations. Even when stable transformation is feasible, considerable time is required to develop and use an economically valuable cultivar in the field, including a long regulatory process to obtain government approval. The regulatory framework for the assessment of genetically modified (GM) crops varies from country to country, and many countries in Europe have a highly restrictive regulatory environment. Currently, the insect-resistant Bt maize MON810 is the singular GM plant authorized for cultivation in the EU (Lucht, 2015). In addition, the general public still has many concerns regarding GM crops. Consequently, it is highly desirable to develop a new means of disease control without the generation of GM crops or extensive use of chemical inputs.

The use of environmental RNAi and spray-induced gene silencing (SIGS) is a non-

transformative strategy for plant protection. The dsRNA targeting the pathogen is sprayed directly onto the plant surface and the fungal pathogen can directly take up the dsRNA, and induce its RNAi machinery (Sang & Kim, 2020). SIGS has been used to control fungal pathogens found in several crop plants. Wang *et al.* (2016) generated *in vitro* dsRNAs and sRNAs targeting *B. cinerea* Dicer-like protein 1 (*Bc-DCL1*) and Dicer-like protein 2 (*Bc-DCL2*) and showed that the dsRNA and sRNA spray treatment significantly controlled grey mould disease on fruits (tomato, strawberry, and grape), vegetables (onion and lettuce), and flower (rose) (Wang et al., 2016). McLoughlin *et al.* (2018) showed that multiple topically-applied dsRNAs targeting *S. sclerotiorum* genes can reduce *S. sclerotiorum* and *B. cinerea* fungal growth in detached canola and *Arabidopsis* leaves (McLoughlin et al., 2018). Koch *et al*. (2016) showed that a 791 bp dsRNA targeting three ergosterol biosynthesis genes (CYP51A, CYP51B, and CYP51C) conferred resistance to Fusarium head blight on detached barley leaves (Koch et al., 2016).

SIGS is an appealing alternative for pest control as it avoids any modification of the plant genome, provides a high flexibility for multi-target strategies, and can be exploited for any pest or pathogen that is sensitive to RNAi-approaches. More importantly, pathogens cannot easily generate target-resistance mutations to escape RNAi. If mutations do occur, a different target gene could be selected to induce mortality or reduce pathogenicity. In addition, topical application of RNAs does not leave toxic residues in the field or environment and due to their chemical properties, RNAs have a shorter half-life than most chemical pesticides (Mitter et al., 2017b). Additional technologies have been developed to improve the strength and duration of SIGS. The use of nanoparticle technology, such as the incorporation of dsRNAs into BioClay, a layered double hydroxide clay nanosheet, can protect dsRNAs from RNases and sunlight. These incorporated compounds were able to effectively control viral diseases in plants and its duration

was extended to more than 20 days (Mitter et al., 2017a). Therefore, SIGS provides a safe and powerful plant protection for not only pre-harvest crops but also on post-harvest products.

1.5 Safety of RNAi and Potential of Off-Target Effects

The sequence-specific mechanism of RNAi allows dsRNAs to be designed with high specificity to avoid affecting other organisms in the environment. The increasing availability of genomic and transcriptomic sequence data has helped with designing highly specific dsRNAs. *In silica* analyses can identify non-target organisms that share some level of sequence complementarity with the derived siRNAs from the target gene of the target pest or pathogen. High levels of sequence complementarity between the derived siRNAs and non-target organism transcripts would suggest the potential for off-target effects. An off-target effect refers to any gene being silencing that is not the intended target, either in the organism producing the dsRNA or in an organism exposed to the dsRNA that is not the intended target organism (Roberts et al., 2015). A common approach to identify off-target effects has been to use the NCBI BLAST search tool to compare target sequences against genome sequences available in the public domain. Many regulatory agencies, such as The European Food Safety Authority, have developed bioinformatics-based strategies for risk assessment and the identification of off-target effects (Papadopoulou et al., 2020). In addition, research groups have also developed their own off-target software, such as OfftargetFinder, a web application developed by Good *et al.* (2016). However, there are major limitations to bioinformatic analyses, such as availability of sequence information, differences between organisms in terms of RNAi machinery and base pair mismatches, and the uncertainties surrounding the mechanism of sRNA-mRNA matches and interactions (Roberts et al., 2015). Additional risk assessments such as food and feed safety assessment and environmental risk assessment, must be conducted to evaluate the safety of RNAi-based technologies and ensure no

off-target effects (Papadopoulou et al., 2020).

For transcript knockdown and off-target effects to occur from a dsRNA-expressing plant or environmental RNAi, non-target organisms must be susceptible to the dsRNA expressed by the plant and ingest it in sufficient concentrations (Christiaens et al., 2018). Exposure to dsRNA can occur when non-target organisms feed on living plant material, or consume plant-fed herbivores, or are exposed through plant root exudates into soil or aquatic environments (Romeis et al., 2019). Once the dsRNA is ingested by the non-target organism, it must resist degradation in the gut, and be absorbed in sufficient quantities to activate the non-target organism's endogenous RNAi machinery. Fortunately, the safety of ingesting nucleic acids and sRNA for mammals is well understood from several perspectives. Humans and mammals routinely consume significant amounts of dietary RNA (siRNAs, miRNAs, and longer dsRNAs) from plant and animal derived ingredients (Petrick et al., 2013). Dietary RNAs are known to be rapidly degraded soon after ingestion due to the extreme pH conditions and nucleases present in the gastrointestinal tract (Petrick et al., 2016). Furthermore, there are several biological barriers that exist at the cellular and intercellular level that prevent systemic absorption of sRNAs (Juliano et al., 2009; O'Neill et al., 2011; Papadopoulou et al., 2020). The efficacy of biological barriers against ingested dietary RNAs has been demonstrated by mammal *in vivo* feeding studies with plant and endogenous miRNAs (Dickinson et al., 2013; Snow et al., 2013; Witwer et al., 2013). Further confirmation of the effectiveness of these barriers is provided by researchers and the noted difficulties in achieving successful oral delivery of nucleic acid drugs for intestinal tract diseases (Knipe et al., 2016). Therefore, the amount of dietary RNAs absorbed after food/feed ingestion can be considered negligible in humans and other mammals. However, conditions in the gastrointestinal tract of arthropods can differ considerably from that of humans and mammals, and hence, there are

numerous research groups developing ingestible dsRNAs that can kill insects but not harm other species (reviewed in Bachman et al., 2016). Subsequently, various international regulatory bodies have developed risk assessment considerations for RNAi-based genetically modified plants, such as the Center for Environmental Risk Assessment, the European Food Safety Authority, the Food Standards Australia New Zealand, and the US Environmental Protection Agency (US EPA) (Casacuberta et al., 2015).

Several RNAi-mediated virus-resistant and insect-resistant plant varieties have been deregulated or approved by international regulatory agencies for commercial cultivation and consumption (Baranski et al., 2019). More specifically, the Brazilian National Technical Commission on Biosafety has approved a transgenic bean crop resistant to *Bean golden mosaic virus* (BGMV) (Lima Aragão, 2014). BGMV is transmitted by the whitefly *Bemisia tabaci* and affects the common bean (*Phaseolus vulgaris* L.). Bonfim *et al.* (2007) designed an intron-spliced hairpin RNA (hpRNA) vector that targets *AC1*, which encodes the complex multifunctional protein Rep, that acts as a rolling-circle replication initiation factor (Bonfim et al., 2007). The US Department of Agriculture has deregulated three GM papaya (*Carica papya* L.) cultivars resistant to the *Papaya ringspot virus* (PRSV) and one has subsequently been registered by the US EPA (Baranski et al., 2019). In addition, China's Ministry of Agriculture has commercialized a GM papaya cultivar that produces a dsRNA that targets PRSV isolates found in South China (Wu et al., 2018). PRSV is an aphid-transmitted potyvirus and causes visible rings on fruits and smaller deformed leaves. Consequently, tree growth is restricted and yield is reduced (Basso et al., 2016). All American GM cultivars target the PRSV coat protein using RNAi, whereas the Chinese cultivar targets the PRSV replicase *NIb* gene (Davis et al., 2003; Lius et al., 1997; Tennant et al., 2005; Wu et al., 2018).

The first commercial RNAi product has been developed and approved by the US EPA (Environmental Protection Agency, 2017). Monsanto (acquired by Bayer CropScience in 2018) developed a transgenic maize (*Zea mays* L.) crop that expresses a 240 bp dsRNA fragment targeting the *snf7* gene in the Western corn rootworm (*Diabrotica virgifera virgifera*) (Petrick et al., 2016). This product has been marketed under the trade name SmartStax PRO® and is expected to be ready for a full commercial launch in the United States in 2022 (*Bayer's Third Generation Corn Rootworm Trait Gains Final Approval; SmartStax® PRO Technology to Launch in US in 2022*, 2021). SmartStax PRO® is considered to be a major milestone in the use of RNAi technology in pest management (Head et al., 2017). Several ecological risk assessments have been performed regarding *D. v. virgifera snf7* (DvSnf7) dsRNA and the mode of action has been shown to have a narrow spectrum of activity, controlling only a small subset of beetles within the Galerucinae subfamily (Bachman et al., 2013). In addition, the potential toxicity of DvSnf7 dsRNA was evaluated with a variety of non-target organisms, including invertebrate predators, parasitoids, pollinators, arthropods, soil biota, and aquatic and terrestrial vertebrate species (Bachman et al., 2016). Bachman *et al.* (2016) evaluated all ecologically relevant endpoints such as survival, growth, development, and reproduction. Velez *et al.* (2016) examined the impact on honeybees (*Apis mellifera* L.) of maize pollen expressing a dsRNA targeting *V-ATPase A* transcripts of either the target Western corn rootworm or the same transcript in the bee itself (Vélez et al., 2016). There were no observable impacts on survival of larval or adult insects by either dsRNA, indicating honeybees are not readily susceptible to environmental dsRNA, even with complete sequence homology. Petrick *et al.* (2016) investigated the impact of DvSnf7 RNA on mice and determined that the No Observed Adverse Effect Level of DvSnf7 RNA was 100 mg/kg, which was the highest dose administered (Petrick et al., 2016). Overall, the dsRNA used for insect control does not

produce adverse health effects in mammals, even when given as oral doses millions and billions of times higher than anticipated human and mammal exposure, and poses negligible risk.

However, to date, very few studies have examined the potential off-target effects of RNAi with closely and distally related fungal pathogen species. Recently, the Belmonte and Whyard labs have focused on investigating fungal pathogens of canola (Becker et al., 2017, 2019; McLoughlin et al., 2018; Wytinck et al., 2020b). *S. sclerotiorum* and *L. maculans* are two economically important pathogenic fungi that affect canola and are found in Canadian canola growing fields. *B. cinerea* is another economically important fungal pathogen. It does not affect canola but is closely related to *S. sclerotiorum*; the two species share approximately 83% amino acid identity and belong in the Helotiales order (Figure 1.2) (Amselem et al., 2011). A comparative analysis of the *S. sclerotiorum* and *B. cinerea* genomes shows a high level of co-linearity and identity between the two necrotrophs. They also share a similar group of genes associated with necrotrophic processes, such as plant cell wall degradation, which involves pectinase and oxalic acid production (Amselem et al., 2011). *L. maculans* is more distally related to *S. sclerotiorum* and all three fungal species belong in the Ascomycota division (Figure 1.2). Due to the more distant relationships across these fungal pathogens, essential genes may be conserved and shared amongst the three species. As a result, conserved genes would not be ideal RNAi candidates and these targets would likely have an impact on *S. sclerotiorum*, *B. cinerea*, and *L. maculans.* Whereas, less conserved genes may be more species-specific and we may be able to use those gene targets to control one fungus and have little to no impact on a closely or distally related fungal pathogen.

1.6 Objectives of Research

With the development of more RNAi-based technologies, there is an increasing demand from

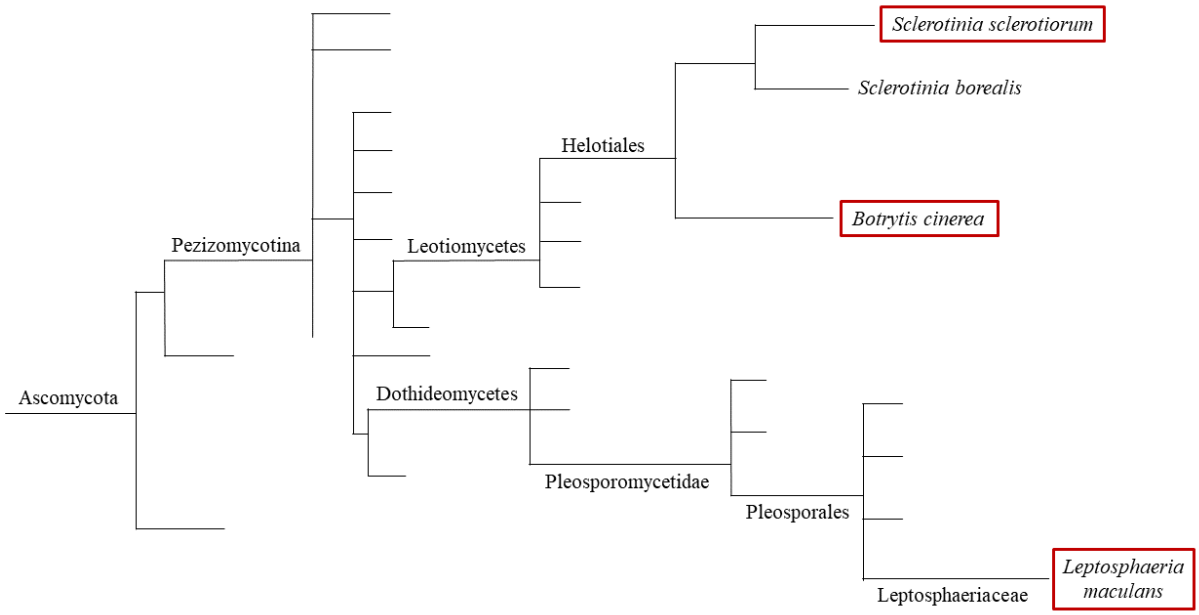


Figure 1.2. Phylogenetic relationships of *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Leptosphaeria maculans*. *S. sclerotiorum* and *B. cinerea* belong in the Helotiales order and all three fungal pathogens belong in the Ascomycota division.

our regulatory agencies to determine whether topically applied dsRNAs will produce off-target effects and have impacts on non-target organisms. However, there is a large gap in the literature and very little is known about the conditions that would allow off-target effects to occur. Studies have shown that the initiation of silencing occurs when the dsRNA is recognized by internal machinery and converts the molecule into 21 or 22 nucleotide RNA fragments (Elbashir et al., 2001). Therefore, I will examine if gene targets with shared 21 nt fragments between different fungal species will result in transcript degradation. More specifically, I aim to test the off-target effects of *S. sclerotiorum* RNAi molecules against a panel of other fungal species that are found in Western Canada. I will also test and develop new dsRNAs against other economically important pathogenic fungi like *B. cinerea* and *L. maculans*. The goal is to develop and test a new generation of dsRNA-based, species-specific foliar fungicides that can be designed to target fungal pathogens

that affect crop species, reduce our reliance on broad-spectrum chemical fungicides, and provide canola growers with safer alternatives to our existing conventional chemistries. The development of topically applied RNAi technologies will reduce excessive chemical inputs being released into the environment and promote agro-ecological health.

1.6.1 *S. Sclerotiorum* Target Identification and Development of New Topical RNAi Technologies

Question: *What are some novel S. sclerotiorum gene targets?*

I hypothesize that *S. sclerotiorum* genes related to pathogenicity or genes that are required for essential function will be effective RNAi targets. More specifically, dsRNAs targeting these genes will be effective at reducing target transcript levels, as well as reducing fungal growth when topically applied on to *B. napus*. For *S. sclerotiorum*, genes related to pathogenicity include cell-wall degrading enzymes and toxin production. For instance, SS1G_01703 is involved in the aflatoxin biosynthesis pathway and SS1G_10167 is an endo-polygalacturonase, which degrades pectin. Genes required for essential cellular function in *S. sclerotiorum* includes SS1G_00005, which encodes a 60S ribosomal subunit, SS1G_03490 is involved in DNA biosynthesis, SS1G_04652 encodes beta tubulin (β-tubulin), and SS1G_06617 is involved in translation initiation. All *S. sclerotiorum* genes targets were identified from the Belmonte lab *S. sclerotiorum* gene database. Using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), I will demonstrate that synthesized dsRNA molecules can reduce *in vitro S. sclerotiorum* transcript levels. Using detached *B. napus* leaf assays, I will also demonstrate the functional utility of these dsRNA molecules to reduce lesion size and uncover trends for target identification for RNAi.

1.6.2 Homologous Gene Targets

Question: Can homologous genes in closely related fungal species be targeted for dsRNA-based control technologies?

Due to the close genetic relatedness of *S. sclerotiorum* and *B. cinerea*, I hypothesize that it will be possible to develop effective *B. cinerea* control using homologous dsRNAs that were previously found effective at controlling *S. sclerotiorum*. Furthermore, there is some genetic relatedness between *S. sclerotiorum* and *L. maculans*. I hypothesize that conserved genes in *S. sclerotiorum* and *L. maculans* will be effective RNAi targets. I will test if efficacious *S. sclerotiorum* gene targets will also be effective targets in *B. cinerea* and *L. maculans*, by examining whether species-specific dsRNAs can reduce their respective transcript levels in the different fungal species.

1.6.3 Potential Off-Target Effects of RNAi

Question: How many shared 21-mers is too many?

Although extensive research has been conducted on the efficacy of RNAi as a molecular fungicide, there has been limited research on the potential off-target effects of RNAi and the safety of topically applied dsRNAs in the agriculture field. This information is especially important for regulatory agencies, as they are responsible for determining the safety of new technologies. This study will help determine the number of shared 21-mers required between gene homologs to produce off-target effects in closely and distally related fungal species. More specifically, I will be investigating three economically important fungi that are currently present in Canadian crop fields: *S. sclerotiorum*, *B. cinerea*, and *L. maculans*. I hypothesize that gene targets that have a high number of shared 21-mers will produce off-target effects, whereas gene targets that have little to zero shared 21-mers will not produce off-target effects.

CHAPTER 2: MATERIALS AND METHODS

2.1 Selection of RNAi Gene Targets

Using NCBI Nucleotide BLAST (National Center for Biotechnology Information Nucleotide Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi), *S. sclerotiorum* genes with varying sequence identity to their *B. cinerea* and *L. maculans* homologs were selected to observe the potential off-target effects of RNAi. All selected *S. sclerotiorum* genes have high fragments per kilobase of million mapped reads (FPKM) values (between 45 – 19992) both *in vitro* and when grown on *B. napus* cv. Westar plants (Table 3.2) (McLoughlin et al., 2018). All possible 21 nt siRNAs (21-mers) for each *S. sclerotiorum* gene and its *B. cinerea* and *L. maculans* homolog were generated *in silico* using NCBI Nucleotide (https://www.ncbi.nlm.nih.gov/nuccore) and Microsoft Excel (Microsoft Corporation, Redmond, WA, US) using the LEFT and RIGHT functions to shift through the mRNA transcripts. The 21-mers for each *S. sclerotiorum* gene and its *B. cinerea* and *L. maculans* homolog were compared using Venn-diagram software (Venny, https://bioinfogp.cnb.csic.es/tools/venny/) and mapped out visually with Geneious Prime (Version 5. 6. 3.; Biomatters, New Zealand). The *S. sclerotiorum* gene targets were subsequently categorized into 4 distinct groups: shared 21-mer overlaps across all three fungal species (*S. sclerotiorum, B. cinerea,* and *L. maculans*), shared 21-mer overlaps between *S. sclerotiorum* and *B. cinerea* and *S. sclerotiorum* and *L. maculans*, shared 21-mer overlaps between *S. sclerotiorum* and *B. cinerea* only, and no shared 21-mer overlaps. Using Geneious, the dsRNA regions were designed to fall under one of the four categories. The number of shared 21-mers across the three fungal species were limited to the designed dsRNA region, and not the whole gene. Putative functions and accessions were determined and confirmed with NCBI (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/).

2.2 *Brassica napus* Growth Conditions

All experiments with *B. napus* were conducted using the Sclerotinia susceptible Westar cultivar grown in a controlled chamber set for a 16-hour light (150-200 µE/m^2/s) and 8-hour dark photoperiod at 22 °C. Seeds were sown onto plastic trays (8 cm × 12 cm × 6 cm) and grown in Sunshine® Mix #1 (SunGro Horticulture, Agawam, MA, US).

2.3 Fungal Growth Conditions

S. sclerotiorum ascospores were collected and generated at the Morden Research and Development Centre (Agriculture and Agri-Food Canada, Morden, MB, Canada). *B. cinerea* plates were generously donated from the Saskatoon Research and Development Centre (Agriculture and Agri-Food Canada, Saskatoon, SK, Canada) and *L. maculans* spores were provided by scientists at the University of Melbourne (Melbourne, Vic, Australia). *S. sclerotiorum*, *B. cinerea*, and *L. maculans* cultures were maintained on full strength potato dextrose agar (PDA) media (Difco Laboratories Inc., Detroit, MI, USA) at 23 °C in the dark.

2.4 *In Vitro* Production of dsRNAs

S. sclerotiorum, *B. cinerea*, and *L. maculans* gene sequences were retrieved from NCBI and the primers used to PCR amplify the gene fragments were designed using Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/). Primer quality was assessed using the IDT OligoAnalyzer Tool (https://www.idtdna.com/pages/tools/oligoanalyzer). The primer sets were designed to avoid regions of homology to *Apis mellifera*, *Mus musculus*, and *Homo sapiens* using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) RefSeq accessions. Additionally, a BLASTN query was performed to ensure each dsRNA molecule was complementary to a single transcript within the fungus. A complete list of primers used are found in Tables 2.1 and 2.2. The primers were designed to include the restriction enzyme sites *Xho1*

Table 2.1. Primer sequences for cloning *S. sclerotiorum*-specific dsRNAs. Product size does not include restriction enzyme sites or GTATA overhang.

Target	Primer sequence (5' – 3')	Product size
SS1G_00005 F	GTATACTCGAGGCCCAAGTTGGTCTTCGTTAT	380
SS1G_00005 R	GTATATCTAGACTTCCTCTGGCCATTCAAGC	
SS1G_01411 F	GTATACTCGAGGAGCGTCCTCACAGAACACA	221
SS1G_01411 R	GTATATCTAGACGACACTTCCCTTCTTAGCG	
SS1G_01703 F	GTATACTCGAGACGGAATCCCAGTTCGT	336
SS1G_01703R	GTATATCTAGAGATCGTTTGCTACGGTGAGAG	
SS1G_03490 F	GTATACTCGAGGGCTGTCAAATGGATTCAA	303
SS1G_03490 R	GTATATCTAGACAAGGAAGAGCCTCGGTTAAG	
SS1G_04652 F	GTATACTCGAGTTCCAGAGTTGACCAA	399
SS1G_04652 R	GTATATCTAGACCAAATCGTTCATGTTGGACT	
SS1G_06487 F	GTATACTCGAGCGTAGCATATCCGACCGAGTC	211
SS1G_06487 R	GTATATCTAGACAGCAGCTCAATTCTCCGTCT	
SS1G_06487 F (1 21mer)	GTATAGGTACCCAAGCGTGCTAGACAAGCTTT	219
SS1G_06487 R (1 21mer)	GTATACTCGAGTGACATTCTTATATGCCTCTGTCT	
SS1G_06617 F	GTATACTCGAGATTATGGACCGGGATGT	383
SS1G_06617 R	GTATATCTAGACCTCGGCAATCTTGAATGTTA	
SS1G_10167 F	GTATACTCGAGTAACTCCCTCGGACACA	356
SS1G_10167 R	GTATATCTAGAATCTTGCTCGATGACAACACC	

Table 2.2. Primer sequences for cloning *B. cinerea*-specific (BCIN) and *L. maculans*-specific (LEMA) dsRNAs. The targets genes are homologs of *S. sclerotiorum* gene targets.

Target	Primer sequence (5' – 3')	Product size
BCIN_02g03480 F (SS1G_01703)	GTATAGGTACCTCCAACATAGTCGGAACCTTG	334
BCIN_02g03480 R (SS1G_01703)	GTATACTCGAGCACGTCCTCTTCTCGTAGGTG	
BCIN_11g03740 F (SS1G_06617)	GTATAGGTACCATCTTTTGTCCGGTGGAGAAT	329
BCIN_11g03740 R (SS1G_06617)	GTATACTCGAGGAATCGCAAGTTCTCAGTTGG	
LEMA_P069770.1 F (SS1G_01703)	GTATAGGTACCTCCAACATAGTCGGAACCTTG	331
LEMA_P069770.1 R (SS1G_01703)	GTATACTCGAGCACGTCCTCTTCTCGTAGGTG	
LEMA_P053820.1 F (SS1G_06617)	GTATAGGTACCTAGCATGAAGCCTCAGACG	343
LEMA_P053820.1 R (SS1G_06617)	GTATACTCGAGATAAAATCGTGCTCCACAACG	

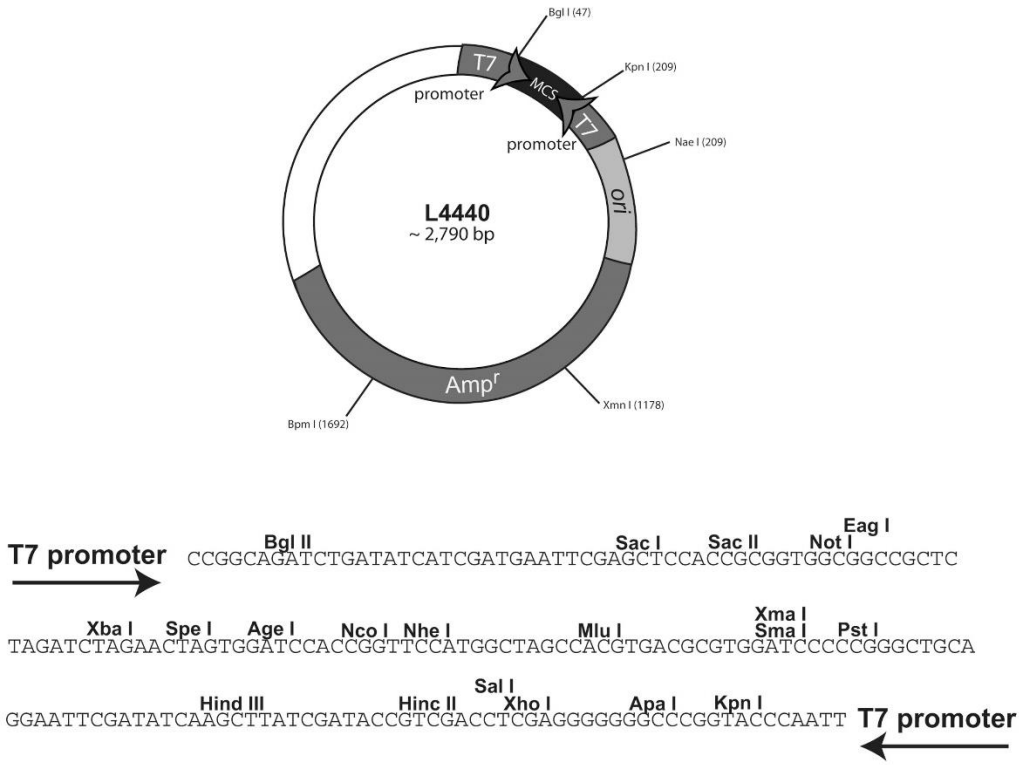


Figure 2.1. Vector map of pL4440. The vector includes T7 promoter sites and a multiple cloning site.

(CTCGAG) or *Kpn1* (GGTACC) and *XbaI* (TCTAGA). The dsRNA target gene fragments ranged from 211 to 399 base-pairs (bp) in length (Tables 2.1 and 2.2). Target gene sequences were amplified using a T100 PCR Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) and EconoTaq® PLUS 2X Master Mix (Biosearch Technologies, Hoddesdon, UK, EU) under the following conditions: 95 °C for 3 min; followed by 35 cycles of: 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 15 s; and a final extension of 72 °C for 3 min. The PCR products were resolved on a 1.5% agarose gel supplemented with ethidium bromide and visualized using the ChemiDoc™ Imaging System (Bio-Rad Laboratories, Hercules, Ca, USA). The PCR products were gel excised and purified using the E. Z. N. A.® Plasmid Mini Kit (Omega Bio-tek, Norcross, GA, USA) and then digested using FastDigest *XhoI* or *KpnI*, and *XbaI* (Thermo Fisher Scientific, Waltham, MA,

US) according to the manufacturer's protocol. The products were ligated into the similarly digested pL4440 vector (a gift from Andrew Fire, Addgene Plasmid #1654) (Figure 2.1) using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The prepared plasmids were transformed into Subcloning Efficiency™ DH5α Chemically Competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA, US) using the heat-shock method as described by the manufacturer. The cells were plated onto lysogeny 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) and incubated at 37 °C overnight. A colony PCR screen, using primer specific and plasmid specific primers was performed using the conditions stated above but with the initial step of 95 °C for 10 min. The samples were resolved on a 1.5% agarose gel supplemented with ethidium bromide to confirm transformation. Once confirmed to contain the expected sized insert, bacterial colonies were grown in a shaking incubator (230 rpm, 37 °C overnight) in 4 mL of LB broth substituted with 50 µg/mL ampicillin. The plasmid DNA was isolated from the bacterial cells and purified using the E. Z. N. A.® Plasmid Mini Kit. The sequence inserts were confirmed using Sanger sequencing (The Centre for Applied Genomics Sick Kids, Toronto, ON, Canada). DsRNA molecules were synthesized using the MEGAScript™ RNAi kit (Invitrogen, Carlsbad, CA, US) according to the manufacturer's protocol, although the volumes were doubled, except for the wash steps, to achieve roughly 1000 ng/µL dsRNA concentrations. Agarose gel electrophoresis, spectrophotometric analysis (NanoDrop™ One; Thermo Fisher Scientific, Waltham, MA, US), and the Aligent 2100 BioAnalyzer with a DNA 1000 chip were used to determine the size, concentration, and purity of the dsRNA molecules, respectively.

2.5 Fungal *In Vitro* Assays

For *S. sclerotiorum in vitro* assays, a 1 mm plug was taken from the leading edge of a freshly

cultured 3-day old *S. sclerotiorum* colony grown on PDA plates and placed in 3 mL of quarter strength PDB (Difco Laboratories Inc., Detroit, MI, USA) containing ampicillin (50 µg/mL; MP Biomedicals Inc., Santa Ana, CA, USA) and dsRNA applied at a dose of 500 ng/mL in a 15 mL polypropylene tube (Thermo Fisher Scientific, Waltham, MA, US) and placed on a rocking platform shaker (Benchmark Scientific Inc., Sayreville, NJ, USA) covered with aluminum foil and grown at 17 °C for 3 days. For *B. cinerea* and *L. maculans in vitro* assays, three 1 mm plugs were taken from the leading edge of a freshly cultured *B. cinerea* or *L. maculans* colony grown on PDA plates and placed in 3 mL of full-strength PDB containing ampicillin and dsRNA applied at a dose of 500 ng/mL in a 15 mL polypropylene tube and placed on a rocking platform shaker covered at aluminum foil and grown at 23 °C for 3 days. For collection, all fungal samples were spun down using a Sorvall™ ST 16R Centrifuge (Thermo Fisher Scientific, Waltham, MA, US) at 12,000 xg for 2 min at room temperature and rinsed with water twice to remove excess PDB broth, then frozen and stored at -80 °C.

2.6 Tissue Collection, RNA Extraction, and cDNA Synthesis

Frozen fungal tissue was ground using 1.4 mm ceramic beads (Fisher Scientific, Pittsburgh, PA, USA), 1.5 mL microcentrifuge tubes with screw caps (Fisher Scientific, Pittsburgh, PA, USA) and a bead mill homogenizer (Fisher Scientific, Pittsburgh, PA, USA) with the following settings: speed = 6:00, time = 4:00, cycles = 1, and dwell = 0:00. RNA was isolated using RLT lysis buffer supplemented with 2% β-mercaptoethanol, QIAshredder columns (Qiagen, Venlo, Netherlands), and a RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions. Extracted RNA was eluted in 30 to 50 µL of RNase-free water and its quality and quantity were assessed using a spectrophotometer (NanoDrop™ One, Thermo Fisher Scientific, Waltham, MA, US). The RNA was then treated with Turbo DNA *free*™ (Thermo Fisher Scientific,

Waltham, MA, US) to remove any contaminating genomic DNA. Removal of genomic DNA was verified by PCR amplification using EconoTaq® PLUS 2X Master Mix (Lucigen, Middleton, WI, US) with β-Tubulin primers and subsequent agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized with qScript™ cDNA SuperMix (Quantabio, Beverly, MA, US) according to the manufacturer's protocol.

2.7 Relative Transcript Abundance Following dsRNA Application *In Vitro*

Relative transcript abundance was quantified using qRT-PCR, the Bio-Rad CFX96 Connect Real Time System (Bio-Rad Laboratories, Hercules, CA, USA), and SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol in a total reaction volume of 15 µL. No-reverse transcriptase (no-RT) controls were used to confirm absence of genomic DNA contamination. All relevant primers and their primer efficiencies are listed in Tables 2.3, 2.4, and 2.5. Conditions used were as followed: 95 °C for 3 min, and 39 cycles of 95 °C for 5 s, 56 °C for 5 s, and 72 °C for 5 s. Melt curve analysis, involving 0.5 °C increments over a range of 60 °C to 95 °C, for each primer set was performed to confirm absence of splice variants, primer dimers, and non-specific amplification. Fungal load was assessed relative to fungal tissue treated with the negative control of water. *S. sclerotiorum* and *B. cinerea* transcript levels were measured in reference to fungus-specific β-Tubulin or Sac7 housekeeping genes using the $2^{-\Delta\Delta CT}$ method. *L. maculans* transcript levels were measured in reference to the fungus-specific Actin housekeeping gene using the $2^{-\Delta\Delta CT}$ method. A student's t-test was performed for each gene examined to determine significant fold changes between the negative control and the dsRNA treatment ($p < 0.05$). All reactions were repeated in triplicate (technical replicates) and a minimum of 6 biological replicates were analyzed for each gene target with dsRNA.

Table 2.3. *S. sclerotiorum* primer sequences and their primer efficiencies used for qRT-PCR.

Target	Primer sequence (5' – 3')	Primer efficiency
β-Tubulin F	TTGAAGCTCAGCCACCCATC	87.55
β-Tubulin R	GACCAGGGAAACGGAGACAG	
SS1G_00005 F	TCTCATCCCTGAGACGCTTT	103.35
SS1G_00005 R	GACCTTCTCGGTCTCAGCAG	
SS1G_01411 F	TTCCATCCGAATTTGAGGTC	90.62
SS1G_01411 R	AAGAGGGGGAGTTGATGGTT	
SS1G_01703 F	GTAAGCGGCAGGGATGGAAG	98.7
SS1G_01703R	CCATAGCGGCTCGATCTAGAATC	
SS1G_03490 F	CTCCAAGAGAACCCAAATCG	102.16
SS1G_03490 R	CTTCTGCGGTCCAGAAAGAG	
SS1G_04652 F	CCGTTTCCCTGGTCAACTTA	95.29
SS1G_04652 R	TCAAAGGAGCAAATCCAACC	
SS1G_06487 F	TGGACTTGGGAAACTCCTGT	97.94
SS1G_06487 R	TGCCTCTGTCTGTCGAATTG	
SS1G_06617 F	GGTTGGTTCCAACGGTATTG	107.41
SS1G_06617 R	TTGCCAGTCTGGTGGGTTAT	
SS1G_10167 F	TCTTGCAGCAGTCGAGAAGA	98.17
SS1G_10167 R	AGGAAGCCTTGGACTTGATG	

Table 2.4 *B. cinerea* primer sequences and their primer efficiencies used for qRT-PCR.

DsRNA target	Gene target	Primer sequence (5' – 3')	Primer efficiency
β-Tubulin	β-Tubulin F	CACTCTCTCGGTGGTGGAAC	98.89
	β-Tubulin R	CGACGGAGAAGGTAGCCATC	
SS1G_00005	BCIN_03g07630 F	AAGAAGCGCAAGAGTCAAGC	95.80
	BCIN_03g07630 R	TCAGCACGCTTGAAGATGAC	
SS1G_01411	BCIN_02g04140 F	GGCGAGAATCCTTTGCAGTA	104.99
	BCIN_02g04140 R	ATTGTTGGATGCCATTGGTT	
SS1G_01703	BCIN_02g03480 F	GGACCCGAGAGTTTTTGTG	104.19
	BCIN_02g03480 R	TTTCCAACACCTGACCATC	
SS1G_03490	BCIN_08g01360 F	TGCTGATCGTCTCCTCC	98.85
	BCIN_08g01360 R	TTTCCAGCCAAGGAGATG	
SS1G_04652	BCIN_01g08040 F	AGGTACCATGGATGCTGTCC	97.82
	BCIN_01g08040 R	AGCCCAGTTGTTACCAGCAC	
SS1G_06487	BCIN_11g02580 F	GTTGGTAAGGGAGCTGCTTG	92.18
	BCIN_11g02580 R	ACATTTGCTGTGGCATTGAC	
SS1G_06487 **(**1 shared 21-mer)	BCIN_11g02580 F	CCTCATTCCAAATGGAACCTT	92.97
	BCIN_11g02580 R	GAGCTTGAGGGTTTCGGTATC	
SS1G_06617	BCIN_11g03740 F	CCCGACTGGCAAGATGTT	97.68
	BCIN_11g03740 R	CAACATATTGGGGCTTCACA	
SS1G_10167	BCIN_14g00850 F	AATCGTCTCTGCTGCTCC	110.60
	BCIN_14g00850 R	CACCAGATCCCGAGAAGG	

Table 2.5. *L. maculans* primer sequences and their primer efficiencies used for qRT-PCR.

DsRNA Target	Gene Target	Primer sequence (5' – 3')	Primer efficiency
Actin	LEMA_P099940.1 F	GTATGGGCCAGAAGGACTCA	104.53
	LEMA_P099940.1 R	TCAAGATACCACGCTTCGACT	
SS1G_00005	LEMA_P016310.1 F	CCTGAAGAAGAAGCGTGAGG	99.42
	LEMA_P016310.1 R	CTGGGCCTTGAGGTACTCCT	
SS1G_01411	LEMA_P096720.1 F	GGAAGGTCTGGACTTTTTGC	101.62
	LEMA_P096720.1 R	GATGATGGTGGGCAGGAAT	
SS1G_01703	LEMA_P069770.1 F	ACTACGGTCCCTCGTCCAAT	103.44
	LEMA_P069770.1 R	TTTTGCTGCATGTAGGCTTG	
SS1G_03490	LEMA_P107610.1 F	TCTCAAGATGACCGACTCACC	107.34
	LEMA_P107610.1 R	AAATCTGGTATGCCGACAATG	
SS1G_04652	LEMA_P053950.1 F	CTGGCCATTCAAGCTGTCT	103.30
	LEMA_P053950.1 R	TGGTTGATGAACTCCTCACG	
SS1G_06487	LEMA_P054200.1 F	GCAGCATGGACTTGGGATAC	93.64
	LEMA_P054200.1 R	CACTGGCCGTGTAGCATAGT	
SS1G_06617	LEMA_P053820.1 F	GGGTGAGAACGGTACTGGAA	91.86
	LEMA_P053820.1 R	CATCTTGGGAACCTTGGTGT	
SS1G_10167	LEMA_P016820.1 F	GGTATCTGCTATGCCCACGA	105.46
	LEMA_P016820.1 R	ATGACAATGGAACTGCACGA	

2.8 Foliar Application of dsRNAs to the Leaf Surface

For *S. sclerotiorum* leaf assays, senescing petals of *B. napus* were placed in a sterile empty petri plate and inoculated with 10 µL of *S. sclerotiorum* spores (1 x 10^6 spores mL^{-1}) resuspended in PDB and Silwet- 77 (Lehle Seeds, Round Rock, TX, US), sealed with parafilm, and incubated for 1 day to allow ascospore germination. A 12 µL solution of 500 ng dsRNA or water and 0.03% Silwet-77 was applied (approximately 1 cm^2) and spread on the leaf surface using a plastic pipette tip (n = 12). The dsRNA solution was allowed to dry completely (approximately 30 min) before a petal covered with visible fungal hyphae was placed on the same area as the dsRNA treatment. *S. sclerotiorum* lesion size was recorded 2 days after dsRNA treatment (Girad et al. 2017).

For *B. cinerea* leaf assays, a 12 µL solution containing 500 ng dsRNA or water and 0.03% Silwet-77 was applied (approximately 1 cm^2) and spread on the leaf surface using a plastic pipette tip (n = 12). The dsRNA solution was allowed to dry completely (approximately 30 min) before 12 µL of *B. cinerea* spores (1 x 10^6 spores mL^{-1}) suspended in PDB broth was placed on the same area as the dsRNA treatment and allowed to incubate for 4 days.

For *L. maculans* leaf assays, *B. napus* leaves were wounded with a 1 mm pipette tip and then a 12 µL solution containing 500 ng dsRNA or water and 0.03% Silwet-77 was applied (approximately 1 cm^2) on the leaf surface using a plastic pipette tip (n = 12). The dsRNA solution was allowed to dry completely (approximately 30 min) before 12 µL of *L. maculans* spores was placed onto the lesion and allowed to incubate for 10 days. For all leaf assays, fungal lesion growth was measured using ImageJ software (https://imagej.nih.gov) and compared statistically using a student's t-test ($p < 0.05$).

CHAPTER 3: RESULTS

3.1 Selection of RNAi Gene Targets

Eight *S. sclerotiorum* genes with varying shared 21-mer overlaps with *B. cinerea* and *L. maculans* were selected to examine the potential off-target effects of dsRNAs (Table 3.1). Selection criteria of gene targets involved essential genes or genes involved in common biological processes (i.e., mitochondria, reactive oxygen species response, protein modification, pathogenicity factors, DNA replication, etc.) and avoided genes that are lowly expressed during infection (FPKM < 25, Table 3.2). SS1G_00005 (60S ribosomal subunit) and SS1G_04652 (β-tubulin) have shared 21-mer overlaps across all three fungal species. SS1G_06617 (translational initiation & termination) and SS1G_03490 (DNA biosynthesis) have shared 21-mer overlaps between *S. sclerotiorum* and *B. cinerea* and *S. sclerotiorum* and *L. maculans*. SS1G_01411 (basic leucine zipper (bZIP) transcription factor (TF)) and SS1G_10167 (endo-polygalacturonase) have shared 21-mer overlaps between *S. sclerotiorum* and *B. cinerea*. SS1G_01703 (aflatoxin biosynthesis) and SS1G_06487 (TIM44) have no shared 21-mers with *B. cinerea* or *L. maculans*. SS1G_01703 and SS1G_06487 gene targets were previously identified to be effective gene targets for *S. sclerotiorum* transcript knockdown and reducing *S. sclerotiorum* lesion size (McLoughlin et al., 2018). Therefore, they were included to further investigate the potential off-target effects of RNAi-derived molecules.

Predicting the number of different siRNAs generated from a single long dsRNA may at first glance seem straightforward, as it has been argued that Dicer positions itself on the dsRNA termini, and cuts every 21 nt downstream, thereby generating a limited number ([length of dsRNA]/21) of end-to-end siRNAs for each dsRNA (Zhang et al., 2002). However, recent RNA sequencing of siRNAs derived from the dicing of long dsRNAs revealed that many more different siRNAs can

Table 3.1. The 8 *Sclerotinia sclerotiorum* target genes used to test the off-target effects of RNAi. These gene targets will be used to make dsRNA and tested with *Botrytis cinerea* and *Leptosphaeria maculans* and their predicted or known biological processes.

Gene ID	Biological Process	Source
SS1G_00005	60S ribosomal subunit	NCBI[1]
SS1G_04562	β-tubulin	NCBI[1]
SS1G_06617	Translational initiation & termination	InterPro[2]
SS1G_03490	DNA biosynthesis	InterPro[2]
SS1G_10167	Endo-polygalacturonase	NCBI[1]
SS1G_01411	bZIP transcription factor	UniProt[3]
SS1G_01703	Aflatoxin biosynthesis	TIP[4]
SS1G_06487	TIM44	NCBI[1]

(**1**) NCBI *Sclerotinia sclerotiorum* 1980 UF-70 (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=665079); (**2**) InterPro (https://www.ebi.ac.uk/interpro/); (**3**) UniProtKB (https://www.uniprot.org/uniprot/); (**4**) TIP = Target identification pipeline (McLoughlin et al., 2018)

Table 3.2. The 8 *Sclerotinia sclerotiorum* target genes and their FPKM values. FPKM is an indicator of expression level, these FPKM values are for *S. sclerotiorum* genes when grown *in vitro* or on *B. napus*. Low FPKM values range from 0 – 5, medium FPKM values range from 6 – 25, and high FPKM values range from 25 and above.

Gene ID	FPKM *in vitro*	FPKM Westar
SS1G_00005	6057	4338
SS1G_04652	1161	1271
SS1G_06617	218	331
SS1G_03490	560	403
SS1G_01411	187	359
SS1G_10167	13739	19582
SS1G_06487	85	80
SS1G_01703	44	301

be produced than would be predicted by the simple end-to-end cleaving by Dicer. Instead, some siRNAs show small overlaps with their neighbouring siRNA, which implies that Dicer may not always position itself at one end of the dsRNA (Power et al., 2020), or alternatively, the long dsRNA templates can be variable in their exact lengths, thereby resulting in a frameshifted series of siRNAs. Due to this uncertainty, we provide two different numbers to count shared 21-mers, "x ; y". The first number, "x", assumes a single nucleotide frameshift count of 21-mers within the shared nt regions and the second number, "y", assumes an end-to-end counting of 21-mers within the shared nt regions (Figure 3.1, Table 3.3).

3.2 *S. sclerotiorum In Vitro* Assay Development

Initial *S. sclerotiorum in vitro* assays were grown at 23 °C for 3 days with full strength PDB and three fungal plugs, but this resulted in rapid and extensive fungal growth, and measurements of gene transcript levels were highly variable. Different combinations of growth temperatures and the amount of starting fungal tissue were tested and the most consistent growth and transcript level measurements were obtained using quarter strength PDB and one fungal plug grown at 17 °C for 3 days.

3.3 QRT-PCR Assessment of RNAi in *S. sclerotiorum*

To assess the efficacy of using dsRNAs for RNAi-mediated transcript knockdown, *S. sclerotiorum* was grown in liquid cultures containing dsRNA molecules targeting one of the following eight *S. sclerotiorum* genes: SS1G_00005 (60S ribosomal subunit), SS1G_04652 (β-tubulin), SS1G_10167 (endo-polygalacturonase), SS1G_01411 (bZIP TF), SS1G_06617 (translational initiation & termination), SS1G_03490 (DNA biosynthesis), SS1G_06487 (TIM44), and SS1G_01703 (aflatoxin biosynthesis). QRT-PCR was used to determine the level of transcript change for each dsRNA treatment (Figure 3.2). Six out of the eight dsRNA treatments,

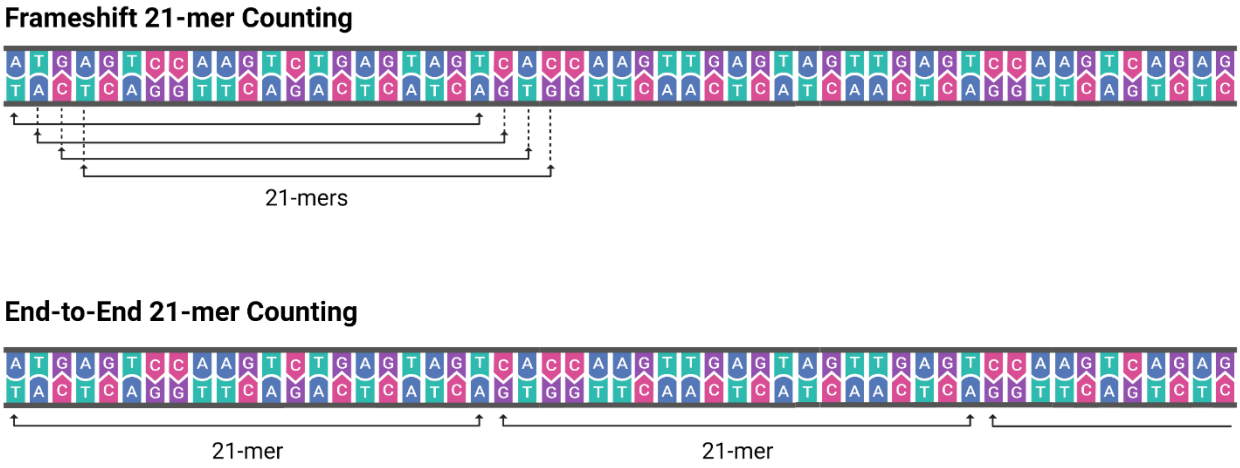


Figure 3.1. Comparison of frameshift and end-to-end 21-mer counting. When counting shared 21-mers between the dsRNA and its target gene, we provide two different numbers, “x;y”. The frameshift 21-mer counting corresponds to the “x” counting method and assumes a frame-shifted series of siRNAs. The end-to-end 21-mer counting corresponds to “y” and assumes end-to-end cleaving of Dicer. Created with BioRender.com.

Table 3.3. The 8 *Sclerotinia sclerotiorum* target genes and number of shared 21-mers with *Botrytis cinerea* and *Leptosphaeria maculans*. The numbers in the last three columns indicate the number of shared 21-mers between the *S. sclerotiorum*-specific dsRNA and the indicated fungal species' homologous gene target. Within each column the left number assumes a single nucleotide frameshift count of 21-mers within the shared nt regions and the second number assumes an end-to-end counting of 21-mers within the shared nt regions.

Gene ID	*Ss* x *Bc* x *Lm*	*Ss* x *Bc*	*Ss* x *Lm*
21-mer overlap across all 3 fungal species			
SS1G_00005	20 ; 3	161 ; 11	22 ; 3
SS1G_04652	12 ; 2	156 ; 11	33 ; 2
21-mer overlap between *Ss* x *Bc* and *Ss* x *Lm*			
SS1G_06617	0	36 ; 6	3 ; 1
SS1G_03490	0	30 ; 4	9 ; 1
21-mer overlap between *Ss* and *Bc*			
SS1G_10167	0	64 ; 5	0
SS1G_01411	0	44 ; 5	0
No 21-mer overlap			
SS1G_01703	0	0	0
SS1G_06487	0	0	0

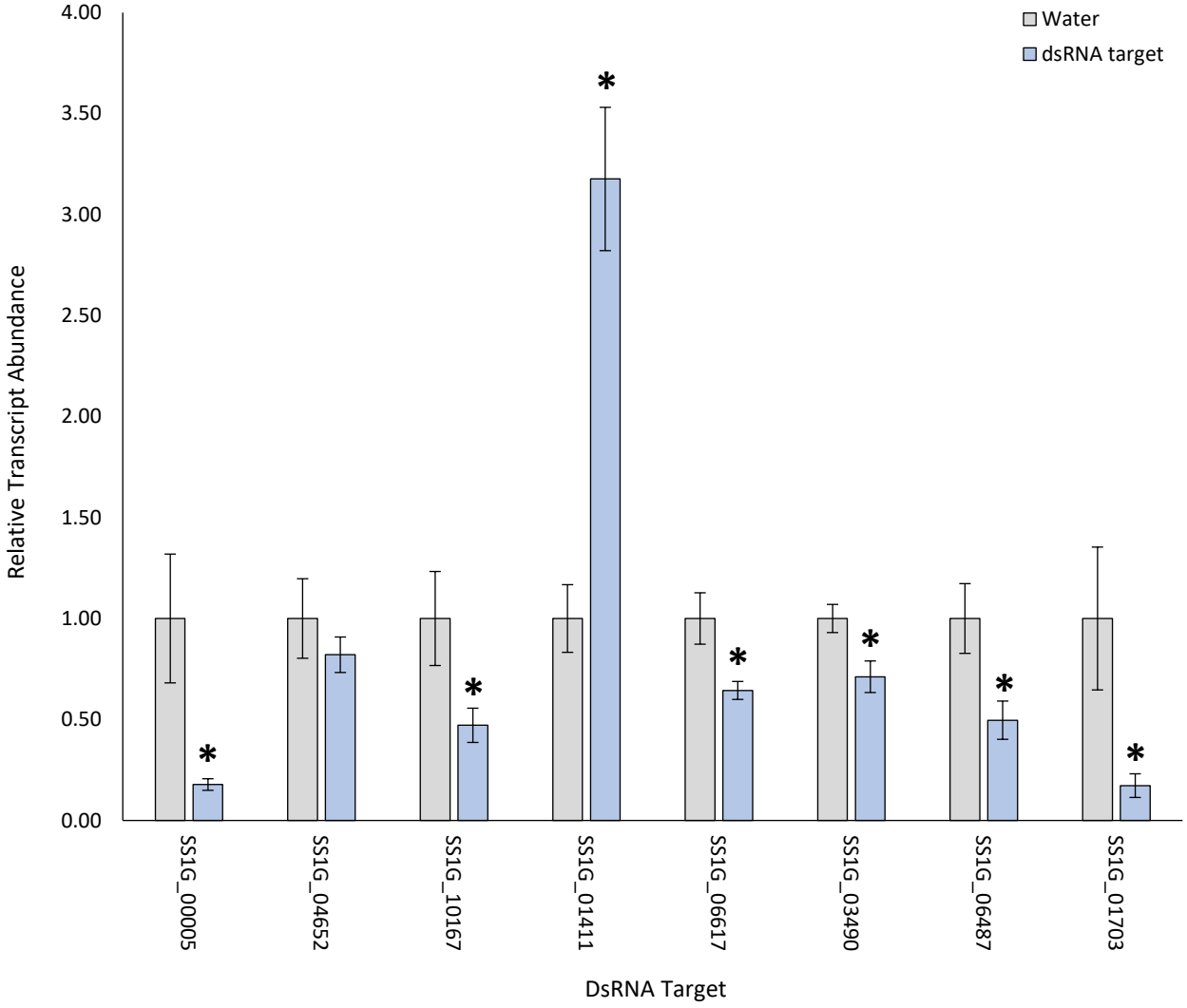


Figure 3.2. Effects of *Sclerotinia sclerotiorum*-specific dsRNA on *S. sclerotiorum* grown in liquid cultures. For the select targets: SS1G_00005 (60S ribosomal subunit), SS1G_04652 (β-tubulin), SS1G_10167 (endo-polygalacturonase), SS1G_01411 (basic leucine zipper (bZIP) transcription factor), SS1G_06617 (translational initiation & termination), SS1G_03490 (DNA biosynthesis), SS1G_06487 (TIM44), and SS1G_01703 (aflatoxin biosynthesis). The transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. Each targeted gene's transcript levels were normalized relative to the corresponding water (non-target control) treatment using Ssβ-tubulin (SS1G_04652) as the reference gene for all treatments, except SS1G_04652, where SsSac7 (SS1G_12350) was used. The values represent the means and standard errors for 6 biological replicates. Seven out of the eight dsRNA treatments resulted in significant transcript changes (student's t-test, $p < 0.05$), denoted by the asterisk (*).

SS1G_00005, SS1G_10167, SS1G_06617, SS1G_03490, SS1G_06487, and SS1G_01703, resulted in significant reduction of transcript abundance when compared to the water control and their efficacy in transcript knockdown ranged from 29 to 83% (student's t-test, $p < 0.05$). SS1G_00005 (60S ribosomal subunit) and SS1G_01703 (aflatoxin biosynthesis) dsRNA treatments were the most effective at reducing transcript levels, with 82 and 83% knockdown, respectively. SS1G_06487 (TIM44) and SS1G_10167 (endo-polygalacturonase) had roughly half of their transcript levels reduced, with 50 and 53%, respectively. SS1G_03490 (DNA biosynthesis) and SS1G_06617 (translational initiation & termination) had the least amount of transcript knockdown, with 29 and 36% knockdown, respectively. Interestingly, the dsRNA targeting a *SsbZIP* TF (SS1G_01411) resulted in significant overexpression of transcript levels (student's t-test, $p < 0.05$), specifically a 213% increase. Conversely, the dsRNA targeting SS1G_04652 (β-tubulin) caused no significant transcript reduction.

3.4 Foliar Application of dsRNAs and *S. sclerotiorum* on *B. napus*

Once it was confirmed that most of the *S. sclerotiorum* dsRNAs examined could reduce transcript abundance in *S. sclerotiorum*, I tested the level of protection these dsRNAs could provide *B. napus* plants using a petal infection assay that facilitated aggressive *S. sclerotiorum* infection (Girard et al., 2017). Fungal lesion size was assessed 2 days post infection (dpi) and compared to a water control treatment. All eight genes showed a significant reduction in lesion size, ranging from 45 to 62% (student's t-test, $p < 0.05$, Figure 3.3). Although two of the dsRNA treatments, SS1G_04652 and SS1G_01411, failed to show transcript knockdown in *in vitro* assays, they significantly reduced *S. sclerotiorum* fungal lesion growth when applied to *B. napus* leaves.

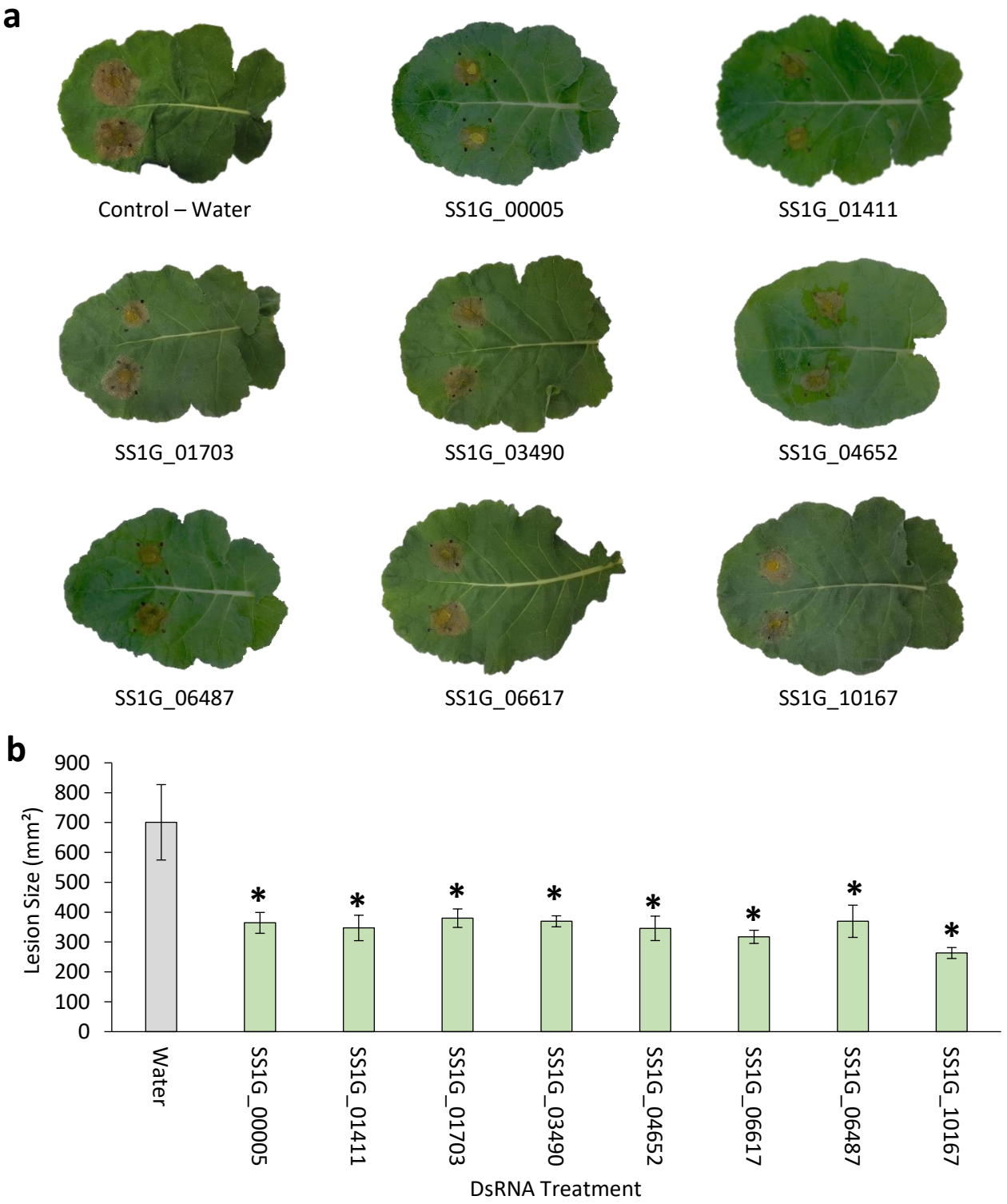


Figure 3.3. *Sclerotinia sclerotiorum*-specific dsRNA targeting *S. sclerotiorum* genes on *Brassica napus* Sclerotinia susceptible Westar cultivar. (**a**) *S. sclerotiorum* infection lesions on *B. napus* following a treatment of 500 ng dsRNA targeting *S. sclerotiorum* genes at 2 dpi. (**b**) Average lesion size (n = 12) relative to water control with standard error bars. Significant lesion reduction (student's t-test; $p < 0.05$) compared to the control treatment is noted by the asterisk (*).

3.5 QRT-PCR Assessment of Off-Target Effects of RNAi with *B. cinerea*

B. cinerea is a necrotrophic fungal pathogen that is closely related to *S. sclerotiorum*. Therefore, it is an ideal candidate to examine if topically applied dsRNAs can produce off-target effects in a closely related species. More specifically, I assessed whether *B. cinerea* genes could be supressed using *S. sclerotiorum*-specific dsRNA. To assess the potential off-target effects of dsRNAs on RNAi-mediated transcript knockdown, *B. cinerea* was grown in liquid cultures containing dsRNA molecules targeting one of the following six *S. sclerotiorum* genes that have shared 21-mer overlaps between the *S. sclerotiorum* and *B. cinerea*: SS1G_00005 (161;11 shared 21-mers), SS1G_04652 (156;11 shared 21-mers), SS1G_10167 (64;5 shared 21-mers), SS1G_01411 (44;5 shared 21-mers), SS1G_06617 (36;6 shared 21-mers), and SS1G_03490 (30;4 shared 21-mers). QRT-PCR was used to determine the level of transcript change for each dsRNA treatment (Figure 3.4). Four out of the six dsRNA treatments, SS1G_00005, SS1G_04652, SS1G_06617, and SS1G_03490, resulted in significant reduction of transcript abundance when compared to the water control and their efficacy in transcript knockdown ranged from 48 to 85% (student's t-test, $p < 0.05$). The SS1G_03490 (DNA biosynthesis) dsRNA treatment was the most effective at reducing *B. cinerea* transcript levels, with 85% knockdown. SS1G_06617 (translational initiation & termination), SS1G_04652 (β-tubulin), and SS1G_00005 (60S ribosomal subunit) dsRNA treatments resulted in roughly half *B. cinerea* transcript levels reduced, with 48, 51 and 58%, respectively. Interestingly, the dsRNA targeting a SS1G_10167, an endo-polygalacturonase, resulted in significant overexpression of transcript levels (student's t-test, $p < 0.05$), specifically a 485% increase. SS1G_06487 (TIM44) and SS1G_01703 (aflatoxin biosynthesis) are two dsRNAs that do not have shared 21-mer overlaps between *S. sclerotiorum* and *B. cinerea*. Both dsRNA treatments did not result in significant transcript changes

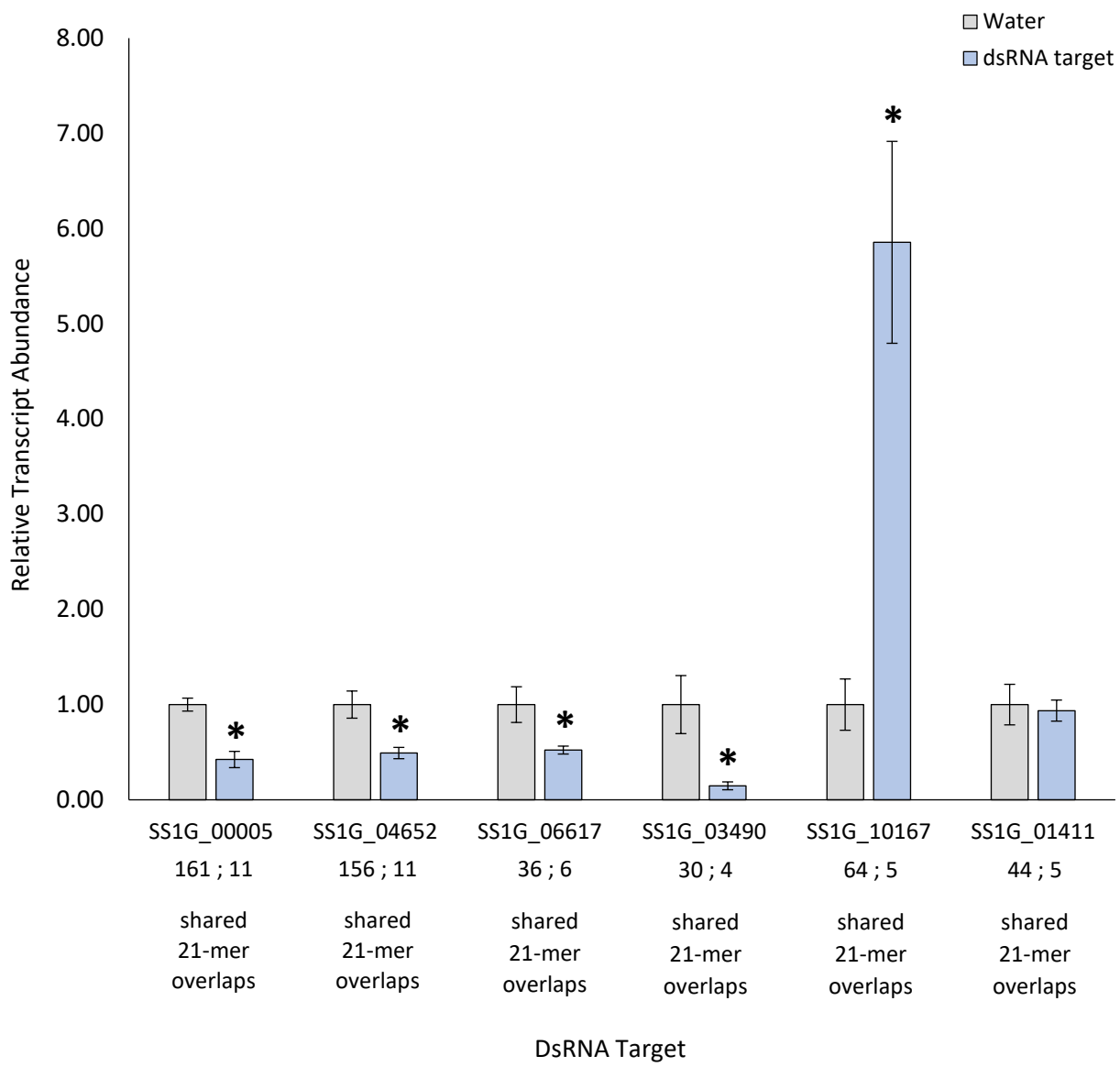


Figure 3.4. Effects of *Sclerotinia sclerotiorum*-specific dsRNA on *Botrytis cinerea* gene targets that have shared 21-mer overlaps. For the select targets: SS1G_00005 (60S ribosomal unit), SS1G_04652 (β-tubulin), SS1G_06617 (translational initiation & termination), SS1G_03490 (DNA biosynthesis), SS1G_10167 (endo-polygalacturonase), and SS1G_01411 (bZIP transcription factor). All six dsRNAs have shared 21-mer overlaps with *B. cinerea*. The transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. Each targeted gene's transcript levels were normalized relative to the corresponding water (non-target control) treatment, using BctubA (BC1G_00122) as the reference gene for all treatments, except SS1G_04652, where BcSac7 (BC1G_01690) was used. The values represent the means and standard errors of 6 biological replicates. Five out of the six dsRNA treatments resulted in significant transcript changes (student's t-test, $p < 0.05$), noted by the asterisk (*).

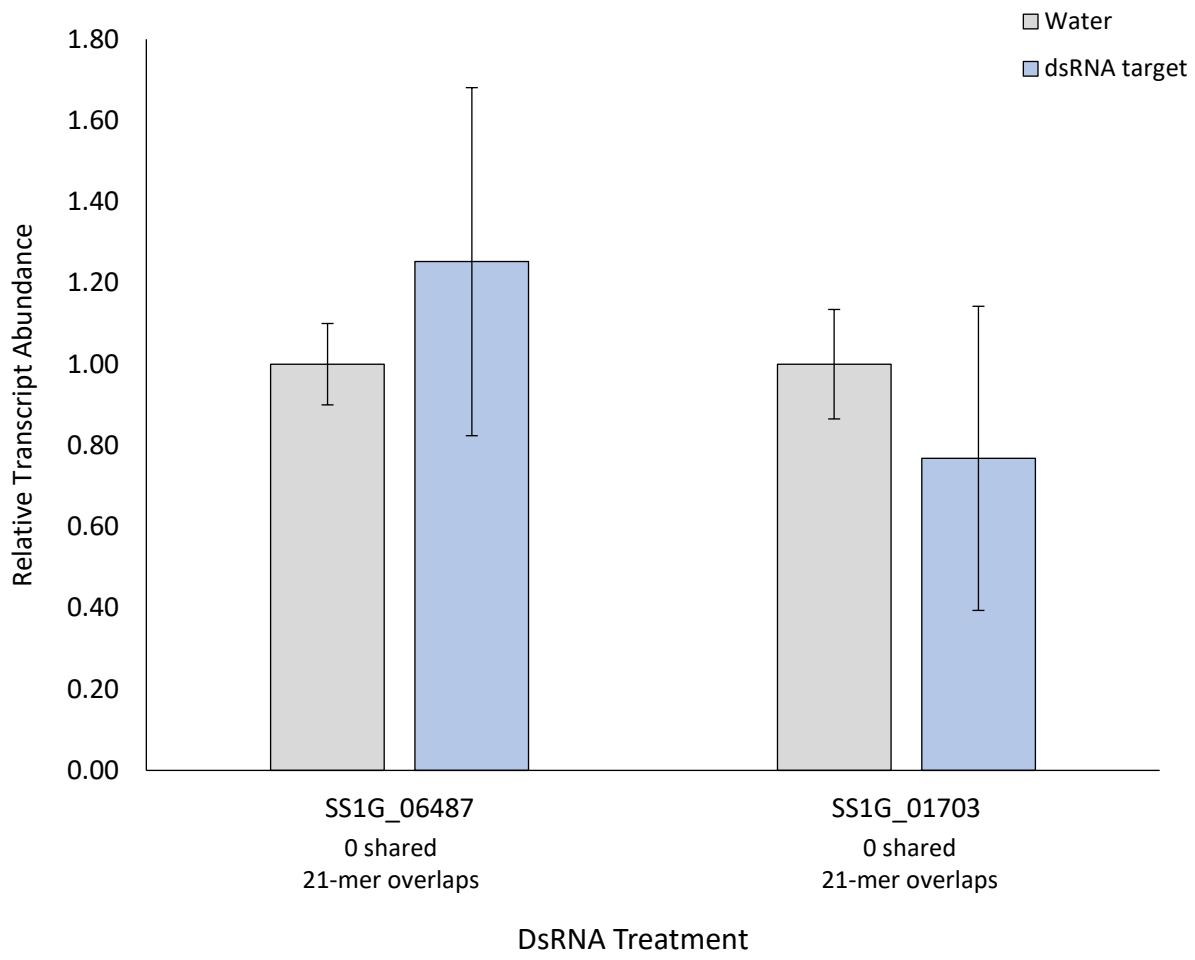


Figure 3.5. Effects of *Sclerotinia sclerotiorum*-specific dsRNA on *Botrytis cinerea* gene targets that do not have shared 21-mer overlaps. For the select targets: SS1G_06487 (TIM44) and SS1G_01703 (aflatoxin biosynthesis). SS1G_06487 and SS1G_01703 dsRNAs do not have shared 21-mer overlaps with *B. cinerea*. The transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA Each targeted gene's transcript levels were normalized relative to the corresponding water (non-target control) treatment, using BctubA (BC1G_00122) as the reference gene for all treatments. The values represent the means and standard errors of 6 biological replicates. The two dsRNA treatments did not result in significant transcript changes (student's t-test, $p > 0.05$).

(student's t-test, $p > 0.05$, Figure 3.45).

3.6 Foliar Application of dsRNAs and *B. cinerea* on *B. napus*

Once it was confirmed that the *S. sclerotiorum* dsRNAs could reduce transcript abundance in *B. cinerea*, I tested the level of protection these dsRNAs could provide for *B. napus* plants using a leaf assay that facilitated *B. cinerea* infection. Fungal lesion size was assessed 4 dpi and compared to a water control treatment. Three different *S. sclerotiorum* dsRNAs with varying shared 21-mer overlaps with their *B. cinerea* homolog were tested, SS1G_00005 (60S ribosomal subunit), SS1G_10167 (endo-polygalacturonase), and SS1G_01703 (aflatoxin biosynthesis) (Figure 3.6). The SS1G_00005 dsRNA has high shared 21-mers (161;11) with *B. cinerea* and significantly reduced fungal lesion size by 57% (student's t-test, $p < 0.05$). The SS1G_10167 dsRNA has medium shared 21-mers (36;6) with *B. cinerea* and significantly reduced fungal lesion size by 53% (student's t-test, $p < 0.05$). The SS1G_01703 dsRNA has zero shared 21-mers with *B. cinerea* and the treatment did not result in changes to fungal lesion size when compared to the water control (student's t-test, $p > 0.05$).

3.7 QRT-PCR Assessment of Off-Target Effects of RNAi with *L. maculans*

L. maculans is a phytopathogenic fungus and is more distantly related to *S. sclerotiorum* compared to *B. cinerea*. To assess the potential off-target effects of using *S. sclerotiorum* dsRNAs on *L. maculans*, the fungus was grown in liquid cultures containing dsRNA molecules targeting one of the following four *S. sclerotiorum* genes that have shared 21-mer overlaps between the *S. sclerotiorum* and *L. maculans*: SS1G_00005 (22;3 shared 21-mers), SS1G_04652 (33;2 shared 21-mers), SS1G_03490 (9;1 shared 21-mers), and SS1G_06617 (3;1 shared 21-mers). QRT-PCR was used to determine the level of transcript knockdown for each dsRNA treatment (Figure 3.7). Four out of the six dsRNA treatments, SS1G_00005, SS1G_04652, SS1G_06617, and SS1G_03490

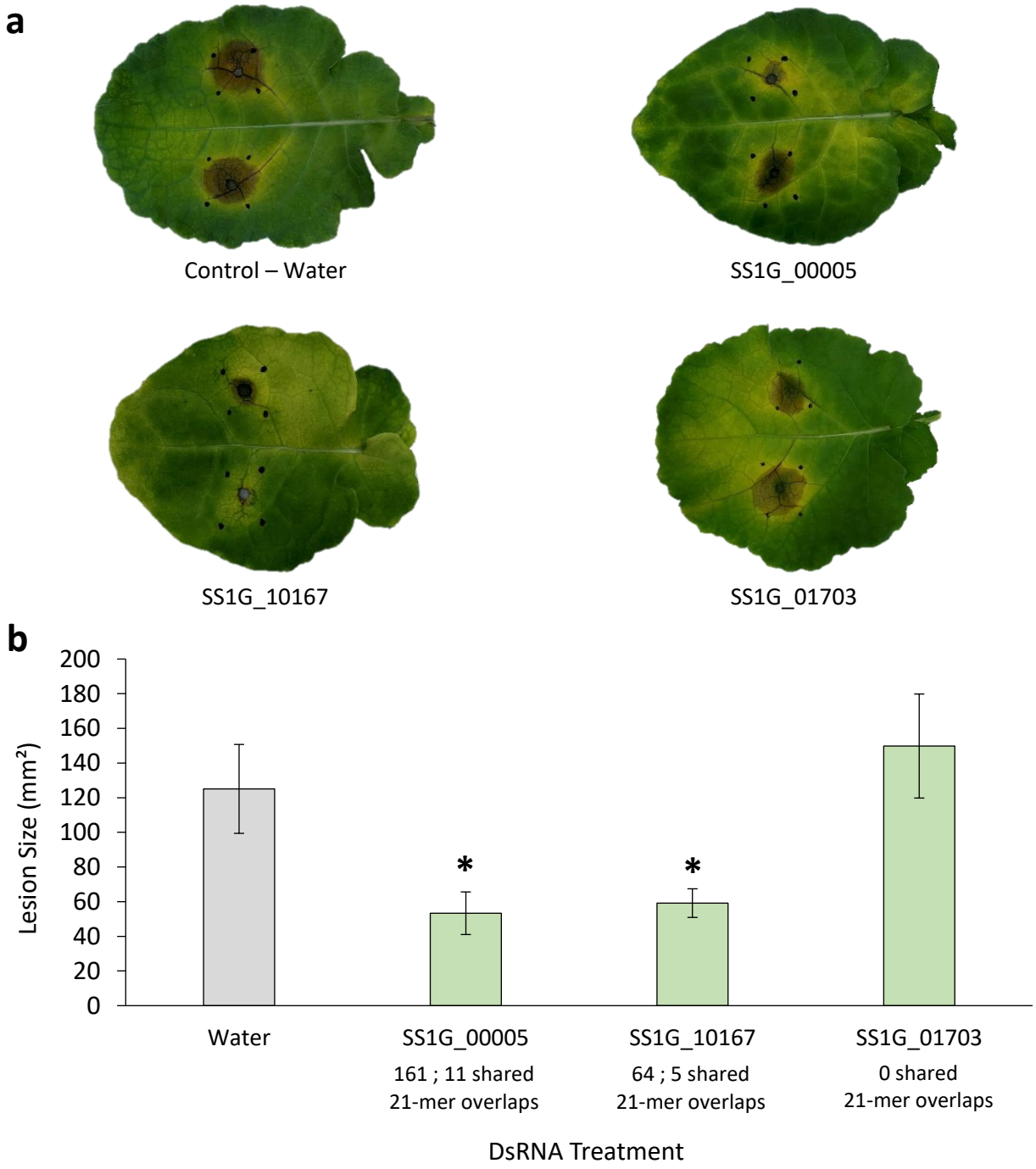


Figure 3.6. *Sclerotinia sclerotiorum*-specific dsRNA targeting *Botrytis cinerea* genes on *Brassica napus* Sclerotinia-susceptible Westar cultivar. (**a**) *B. cinerea* infection lesions on *B. napus* following a treatment of 500 ng dsRNA targeting *S. sclerotiorum* genes at 4 dpi. (**b**) Average lesion size (n = 12) relative to water control with standard error bars. SS1G_00005 has high shared 21-mer overlaps (161;11), SS1G_10167 has medium shared 21-mer overlaps (64;5) and SS1G_0703 has no shared 21-mers between *S. sclerotiorum* and *B. cinerea.* Significant lesion reduction (student's t-test; $p < 0.05$) compared to the control treatment is noted by the asterisk (*).

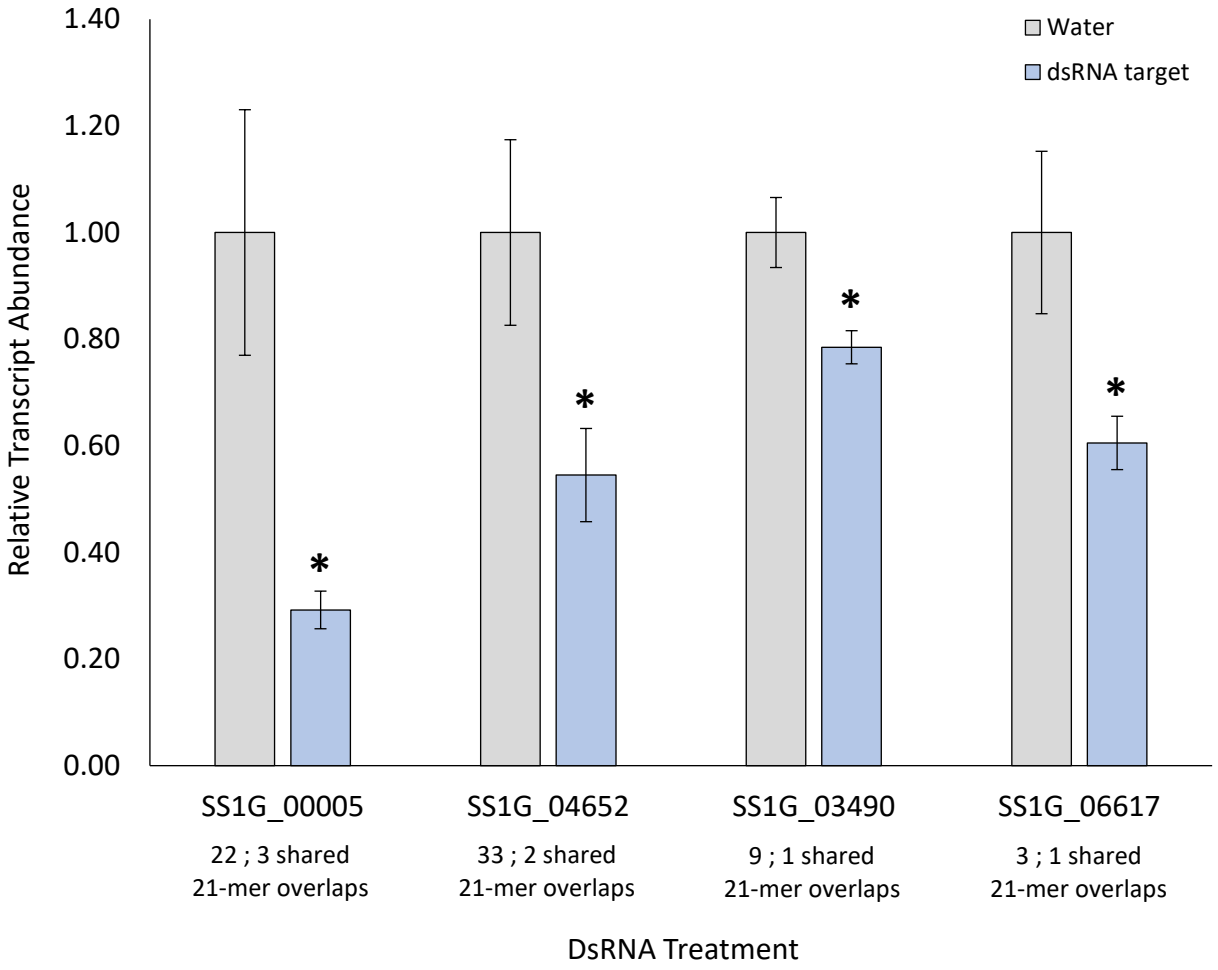


Figure 3.7. Effects of *Sclerotinia sclerotiorum*-specific dsRNA on *Leptosphaeria maculans* gene targets that have shared 21-mer overlaps. For the select targets: SS1G_00005 (60S ribosomal unit), SS1G_04652 (β-tubulin), SS1G_03490 (DNA biosynthesis), and SS1G_06617 (translational initiation & termination). All four dsRNAs have shared 21-mer overlaps with *L. maculans*. The transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. Each targeted gene's transcript levels were normalized relative to the corresponding water (non-target control) treatment, using LmActin (LEMA_P099940.1) as the reference gene for all treatments. The values represent the means and standard errors for 6 biological replicates. All four dsRNA treatments resulted in significant transcript changes (student's t-test, $p < 0.05$), denoted by the asterisk (*).

resulted in significant reduction of transcript abundance when compared to the water control and their efficacy in transcript knockdown ranged from 22 to 71% (student's t-test, $p < 0.05$). The SS1G_00005 (60S ribosomal subunit) dsRNA treatment was the most effective at reducing transcript levels, with 71% knockdown. SS1G_03490 (DNA biosynthesis), SS1G_06617 (translational initiation & termination), and SS1G_04652 (β-tubulin) had less than half of their transcript levels reduced, with 22, 39, and 45%, respectively. SS1G_10167 (endo-polygalacturonase) and SS1G_01703 (aflatoxin biosynthesis) are two dsRNAs that do not have shared 21-mer overlaps between *S. sclerotiorum* and *L. maculans.* Both dsRNA treatments did not result in significant transcript changes (student's t-test, $p > 0.05$, Figure 3.8).

3.8 Foliar Application of dsRNAs and *L. maculans* on *B. napus*

Once it was confirmed that the *S. sclerotiorum* dsRNAs could reduce transcript abundance in *L. maculans*, I tested the level of protection these dsRNAs could provide for *B. napus* plants using a leaf assay that facilitated *L. maculans* infection. Fungal lesion size was assessed 10 dpi and compared to a water control treatment. Three different *S. sclerotiorum* dsRNAs with varying shared 21-mer overlaps with their *L. maculans* homolog were tested, SS1G_00005 (60S ribosomal subunit), SS1G_10167 (endo-polygalacturonase), and SS1G_01703 (aflatoxin biosynthesis) (Figure 3.9). The SS1G_00005 dsRNA has high shared 21-mers (22/3) with *L. maculans* and significantly reduced fungal lesion size by 43% (student's t-test, $p < 0.05$). The SS1G_10167 and SS1G_01703 dsRNAs have zero shared 21-mers with *L. maculans* and the treatment did not result in changes to fungal lesion size when compared to the water control (student's t-test, $p > 0.05$). Based on visual inspection, the *B. napus* leaf assays with *L. maculans* showed more chlorosis when compared to the *S. sclerotiorum* and *B. cinerea* leaf assays, presumably due to the loss of nutrients to the detached leaves sustained over the longer assay time.

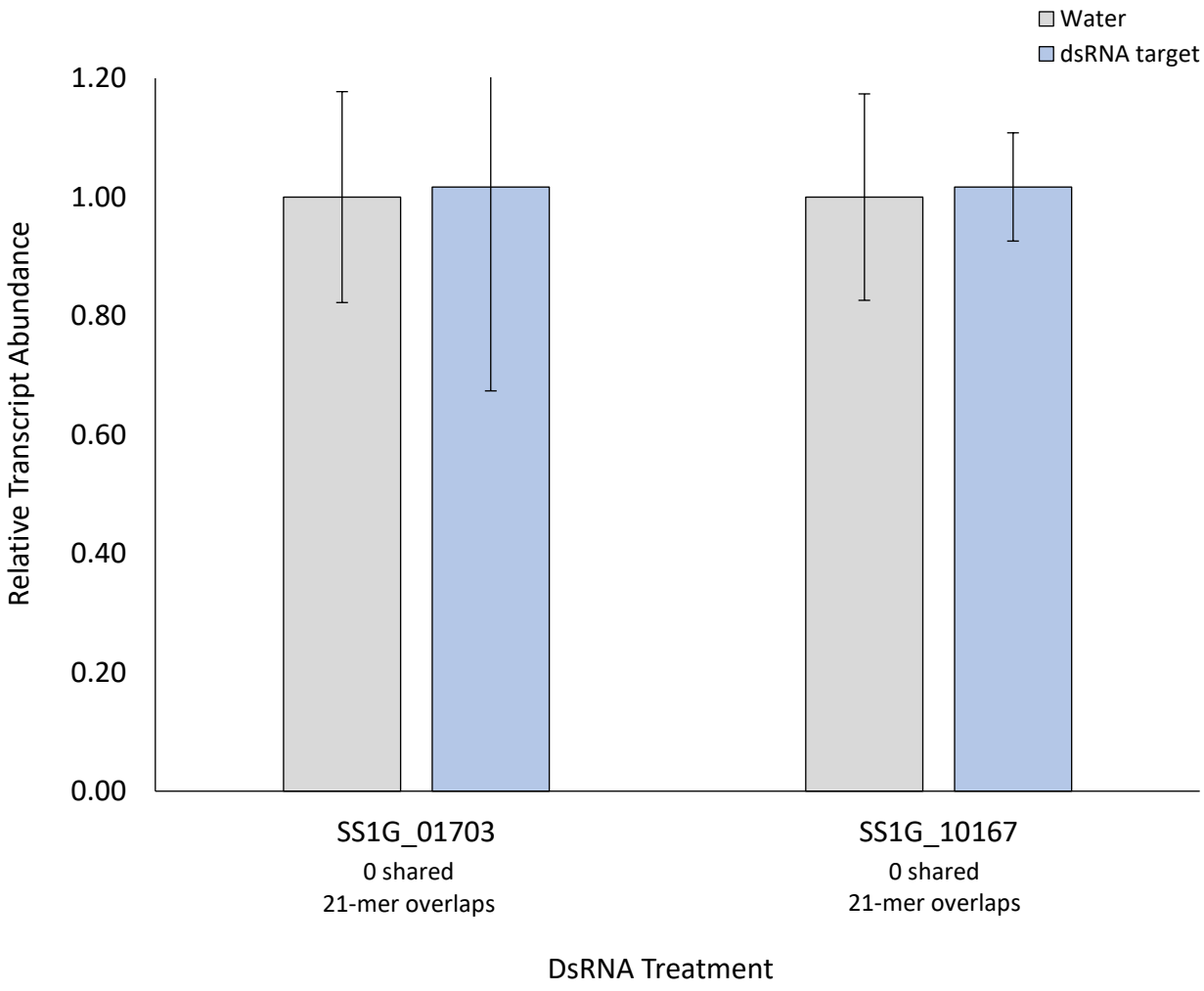


Figure 3.8. Effects of *Sclerotinia sclerotiorum*-specific dsRNA on *Leptosphaeria maculans* gene targets that do not have shared 21-mer overlaps. For the select targets: SS1G_01703 (aflatoxin biosynthesis) and SS1G_10167 (endo-polygalacturonase). SS1G_01703 and SS1G_10167 dsRNAs do not have any shared 21-mer overlaps with *L. maculans*. The transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. Each targeted gene's transcript levels were normalized relative to the corresponding water (non-target control) treatment, using LmActin (LEMA_P099940.1) as the reference gene for all treatments. The values represent the means and standard errors for 6 biological replicates. The two dsRNA treatments did not result in significant transcript changes (student's t-test, $p > 0.05$).

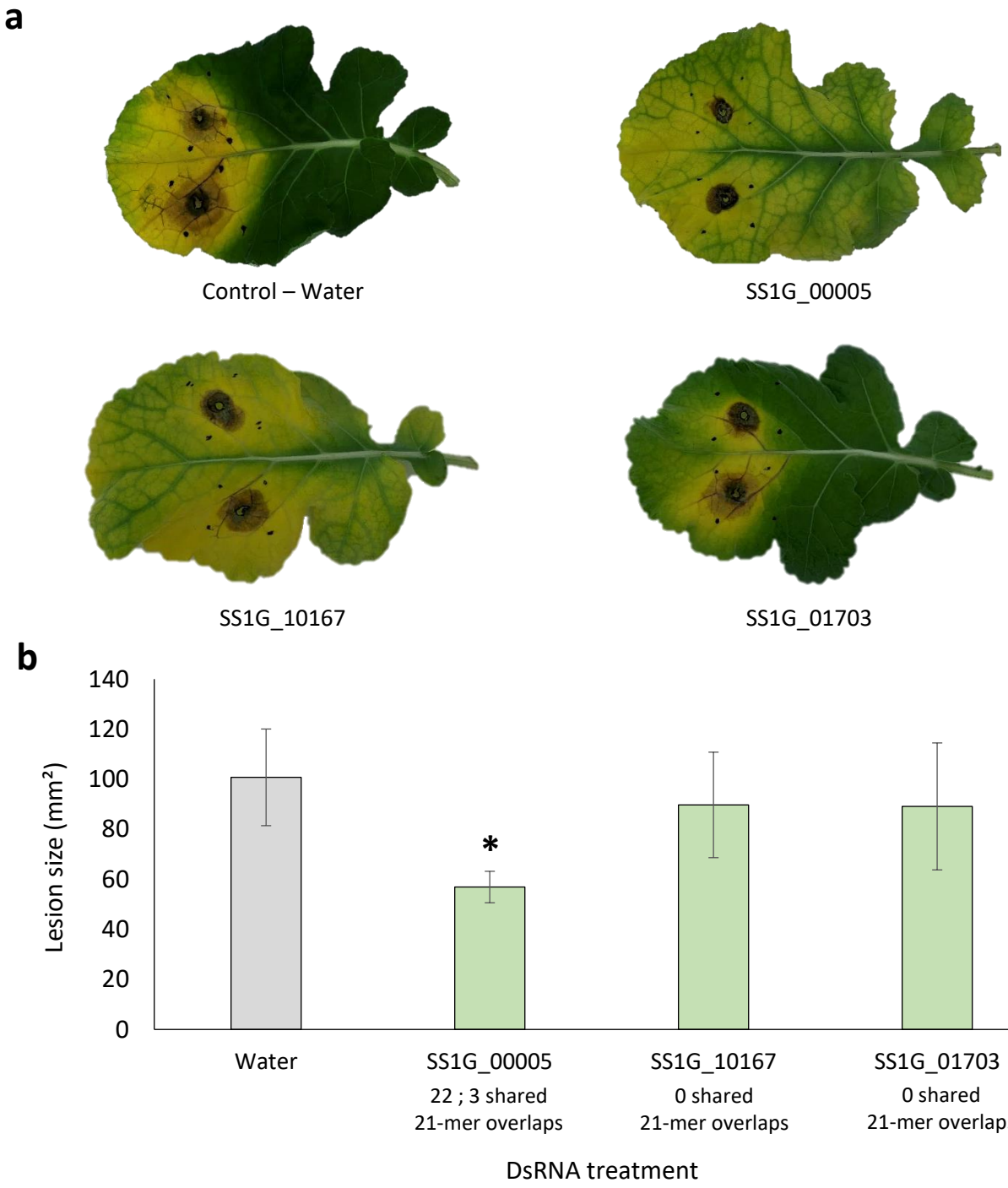


Figure 3.9. *Sclerotinia sclerotiorum*-specific dsRNA targeting *Leptosphaeria maculans* genes on *Brassica napus* Sclerotinia susceptible Westar cultivar. (**a**) *L. maculans* infection lesions on *B. napus* cv. Westar following a treatment of 500 ng dsRNA targeting *S. sclerotiorum* genes at 10 dpi. (**b**) Average lesion size (n = 12) relative to water control with standard error bars. SS1G_00005 has high shared 21-mer overlaps (22;3), and SS1G_10167 and SS1G_0703 has no shared 21-mers between *S. sclerotiorum* and *L. maculans.* Significant lesion reduction (student's t-test; $p < 0.05$) compared to control treatment is noted by the asterisk (*).

3.9 Relationship Between Shared 21-Mers and Off-Target Effects

Overall, there was no significant correlation between the number of shared 21-mers and the amount of off-target effects a dsRNA would produce in the non-target organism. For *B. cinerea*, there was negligible negative correlation when using the frameshift counting of shared 21-mers (Pearson's correlation coefficient, $r(4) = -0.13$, $p < 0.05$) and a low negative correlation when using the end-to-end counting method (Pearson's correlation coefficient, $r(4) = -0.30$, $p < 0.05$). For *L. maculans* there was a moderate positive correlation when using the frameshift counting of shared 21-mers (Pearson's correlation coefficient, $r(2) = 0.49$, $p < 0.05$) and a high positive correlation for the end-to-end method (Pearson's correlation coefficient, $r(2) = 0.92$, $p < 0.05$). Similar to how the level of transcript knockdown varied for each *S. sclerotiorum* dsRNA treatment in *S. sclerotiorum*, it is quite likely that the degree of knockdown response in *B. cinerea* and *L. maculans* is more related to each dsRNA's potency, which may be dependent on sequence and structural properties, than the number of shared 21-mers between two species.

3.10 *B. cinerea* and *L. maculans* Specific dsRNAs

Having confirmed that *S. sclerotiorum*-specific dsRNAs could reduce transcript abundance in *S. sclerotiorum*, as well as in *B. cinerea* and *L. maculans* gene targets with shared 21-mer overlaps, it was of interest to determine whether *B. cinerea*-specific and *L. maculans*-specific dsRNAs could reduce transcript abundance in their respective fungal species. To assess the efficacy of *B. cinerea*-specific dsRNAs, two dsRNAs targeting the *B. cinerea* homolog of SS1G_01703 (aflatoxin biosynthesis) and SS1G_06617 (translational initiation & termination) were designed, which target BCIN_02g03480 and BCIN_11g03740, respectively (Table 2.2). QRT-PCR was used to determine the level of transcript change for each dsRNA treatment (Figure 3.10). Both dsRNA

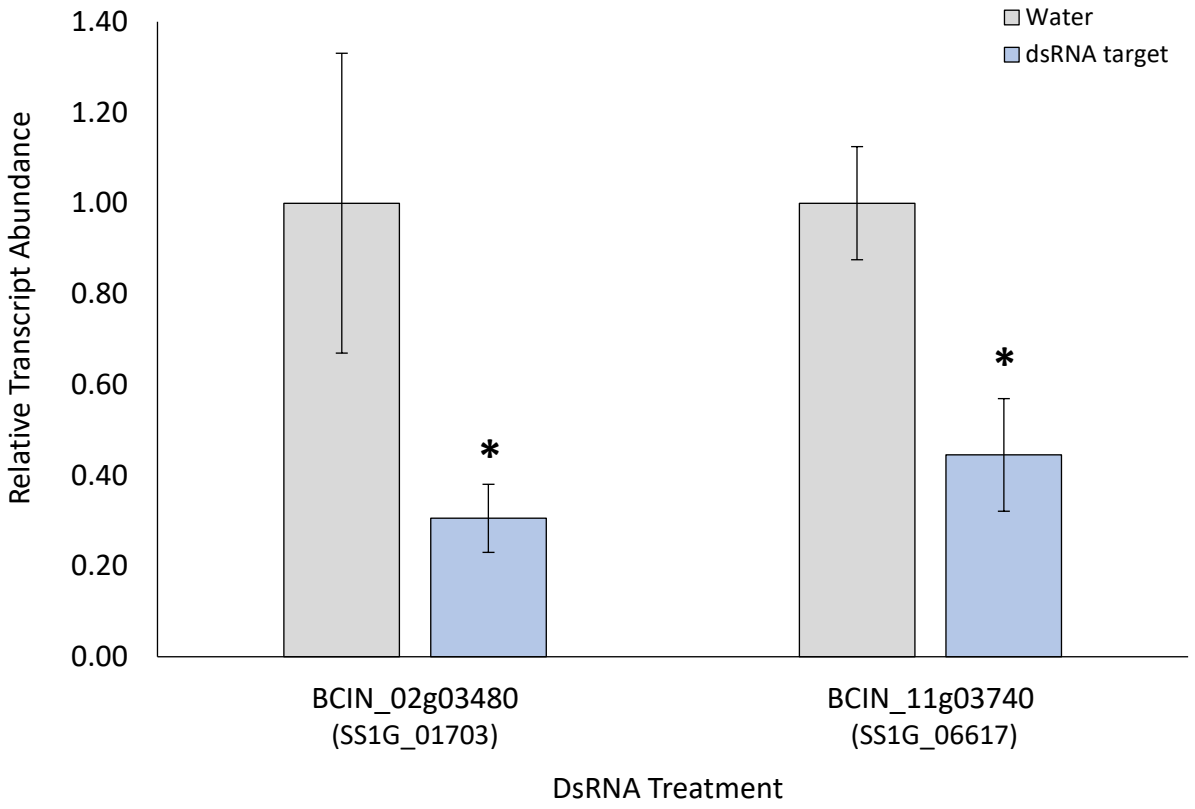


Figure 3.10. Effects of *Botrytis cinerea*-specific dsRNA on *B. cinerea* liquid cultures. For the select targets: BCIN_02g03480 (aflatoxin biosynthesis) and BCIN_11g03740 (translation initiation factor RLI1). BCIN_02g03480 is the *B. cinerea* homolog to *S. sclerotiorum* SS1G_01703 and BCIN_11g03740 is the *B. cinerea* homolog to *S. sclerotiorum* SS1G_06617. The transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. Each targeted gene's transcript levels were normalized relative to the corresponding water (non-target control) treatment, using BctubA (BC1G_00122) as the reference gene for all treatments. The values represent the means and standard errors of 6 biological replicates. Both dsRNA treatments resulted in significant transcript changes (student's t-test, $p < 0.05$), denoted by the asterisk (*).

treatments resulted in significant reduction of transcript abundance when compared to the water control (student's t-test, $p < 0.05$). Their efficacy in transcript knockdown ranged from 56 to 69%.

To assess the efficacy of *L. maculans*-specific dsRNAs, two dsRNAs targeting the *L. maculans* homolog of SS1G_01703 (aflatoxin biosynthesis) and SS1G_06617 translational initiation &termination) were designed, which target LEMA_P069770.1 and LEMA_P053820.1, respectively (Table 2.2). QRT-PCR was used to determine the level of transcript change for each dsRNA treatment (Figure 3.11). LEMA_P069770.1 (SS1G_01703) dsRNA treatment resulted in significant reduction of transcript abundance when compared to the water control, with an 83% reduction (student's t-test, $p < 0.05$). In contrast, the dsRNA targeting LEMA_P053820.1 (SS1G_06617) resulted in no transcript reduction.

3.11 qRT-PCR Assessment of Shared 21-Mer Overlaps

To examine whether a single shared 21-mer fragment between a *S. sclerotiorum*-specific dsRNA and its homologous gene target in *B. cinerea* is sufficient to induce off-target effects, *B. cinerea* was grown in liquid cultures containing dsRNA molecules with zero or one shared 21-mer overlap. QRT-PCR was used to determine the level of transcript change for each dsRNA treatment (Figure 3.12). The SS1G_06487 (TIM44) dsRNA with no shared 21-mer overlaps did not result in significant transcript changes, whereas the SS1G_06487 (TIM44) dsRNA with one shared 21-mer overlap resulted in significant reduction of transcript abundance when compared to the water control, with a 59% in transcript reduction (student's t-test, $p < 0.05$, figure 3.12).

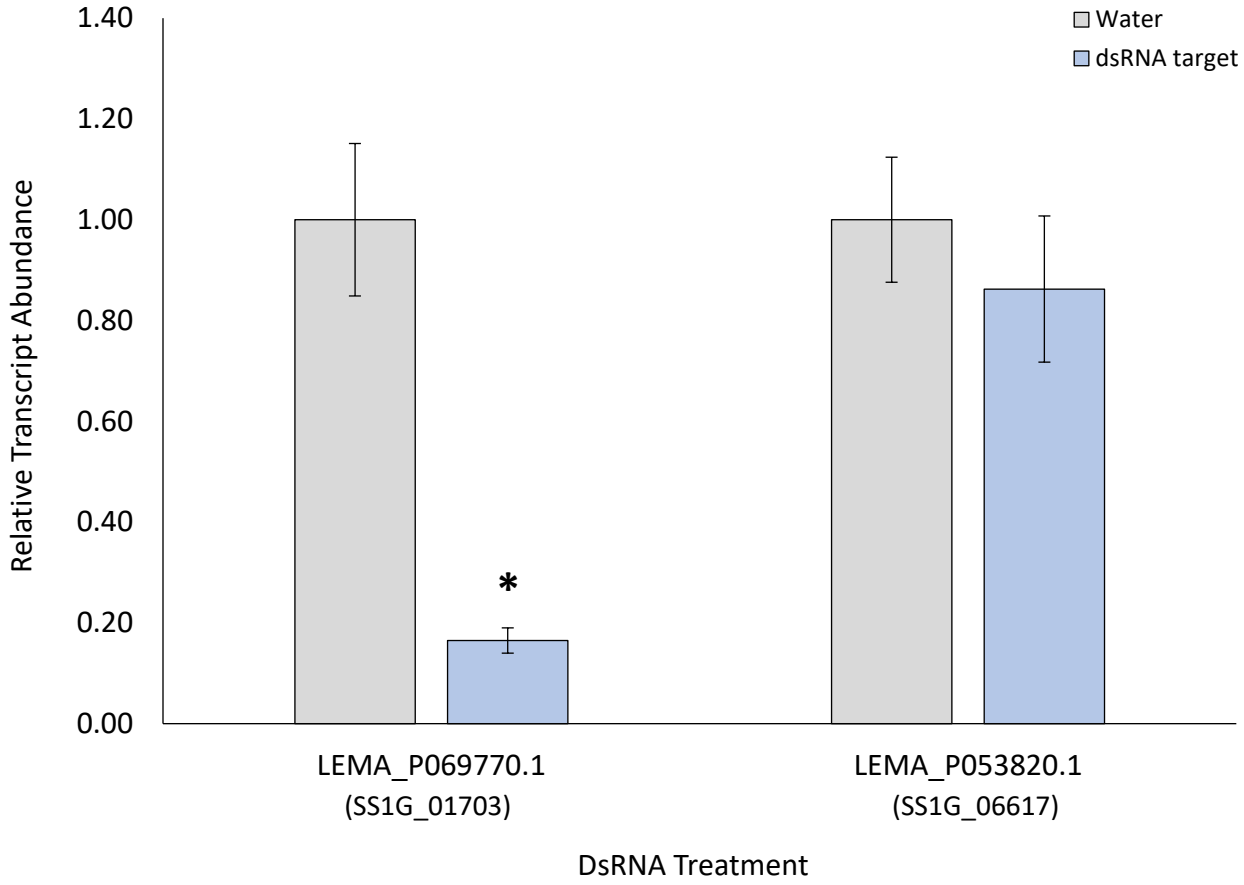


Figure 3.11. Effects of *Leptosphaeria maculans*-specific dsRNA on *L. maculans* liquid cultures. For the select targets: LEMA_P069770.1 (aflatoxin biosynthesis) and LEMA_P053820.1 (translation initiation factor RLI1). LEMA_P069770.1 is the *L. maculans* homolog to *S. sclerotiorum* SS1G_01703 and LEMA_P053820.1 is the *L. maculans* homolog to *S. sclerotiorum* SS1G_06617. The transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. Each targeted gene's transcript levels were normalized relative to the water (non-target control) treatment using LmActin (LEMA_P099940.1) as the reference gene for all treatments. The values represent the means and standard errors for 6 biological replicates. The LEMA_P069770.1 dsRNA treatment resulted in significant transcript changes (student's t-test, $p < 0.05$), denoted by the asterisk (*).

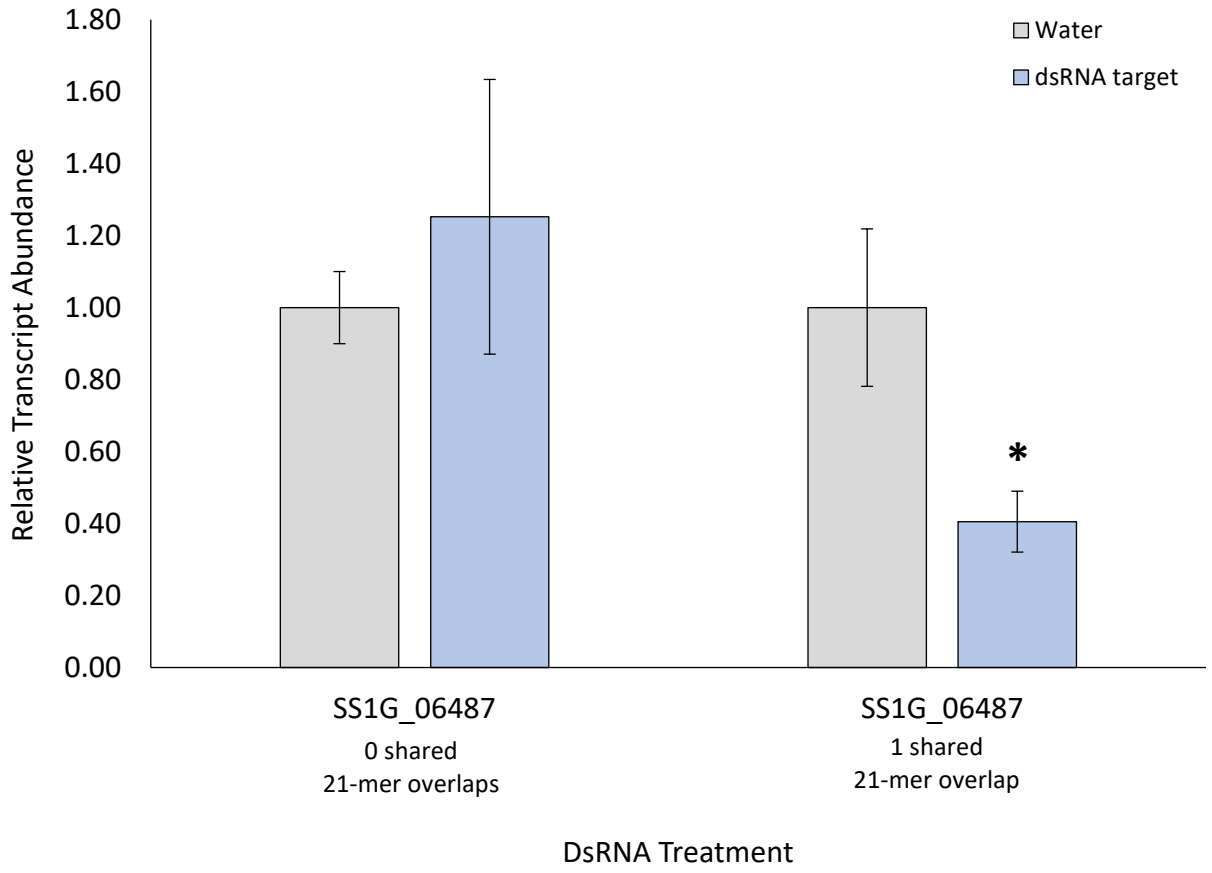


Figure 3.12. Comparison of *Sclerotinia sclerotiorum*-specific dsRNA with zero or one shared 21-mer with *Botrytis cinerea*. For the select targets: SS1G_06487 (TIM44) with no shared 21-mer overlaps and SS1G_06487 (TIM44) with one shared 21-mer overlap. The transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. Each targeted gene's transcript levels were normalized relative to the corresponding water (non-target control) treatment using BctubA (BC1G_00122) as the reference gene. The values represent the means and standard errors for 6 biological replicates. The SS1G_06487 dsRNA treatment with one shared 21-mer resulted in significant transcript changes (student's t-test, $p < 0.05$), denoted by the asterisk (*).

CHAPTER 4: DISCUSSION

4.1 Novel RNAi Targets in *S. sclerotiorum*

RNAi technology has the potential to provide crop producers with a flexible and environmentally friendly solution to combat a wide array of devastating pests and pathogens. HIGS and SIGS are two sustainable next-generation crop protection strategies that are capable of targeting pests and pathogens that affect global food production. The HIGS approach has been used extensively to control fungal and oomycete diseases, however the SIGS approach is still in early development. Previous work in the Belmonte and Whyard labs have identified four *S. sclerotiorum* RNAi targets effective at reducing *S. sclerotiorum* fungal growth on *B. napus*, as well as fungal transcript abundance (McLoughlin et al., 2018). Here, I tested six novel *S. sclerotiorum* RNAi targets and two previously investigated targets, and their ability to reduce *S. sclerotiorum* fungal growth and transcript abundance. DsRNAs targeting SS1G_01703 and SS1G_06487 were previously investigated by McLoughlin *et al.* (2018), and in that study, they both showed a 50% transcript knockdown for both dsRNAs 72 hours post inoculation (hpi) *in vitro* (McLoughlin et al., 2018). In this current study, SS1G_06487 showed the same degree of transcript knockdown, but SS1G_01703 showed a stronger (83%) knockdown. However, a direct quantitative comparison between these two studies cannot be made, as the *in vitro* assay parameters were altered (i.e., the amount and concentration of growth medium used and stationary vs. rotating cultures during growth), the dsRNA sequences differ, and a different reference gene was used to compare transcript levels within *S. sclerotiorum*. Despite these technical differences, a general trend in the results was observed, in that both of these dsRNAs caused statistically significant reductions of the targeted transcript levels.

Overall, I was able to detect knockdown of transcript levels within the first 3 days of dsRNA exposure, similar to other studies conducted in the Belmonte and Whyard labs (McLoughlin et al., 2018; Wytinck et al., 2020b). When the *S. sclerotiorum*-specific dsRNAs were incubated with *S. sclerotiorum in vitro*, significant transcript changes were observed for seven out of the eight dsRNA treatments (SS1G_00005, SS1G_01411, SS1G_01703, SS1G_03490, SS1G_06487, SS1G_06617, and SS1G_10167), providing clear evidence of dsRNA uptake and a RNAi response within *S. sclerotiorum*. The dsRNA treatment SS1G_04652 did not result in significant transcript changes. Using live cell imaging, a recent study demonstrated that the dsRNA uptake mechanism in *S. sclerotiorum* occurs through clathrin-mediated endocytosis (Wytinck et al., 2020b). Clathrin-mediated endocytosis has been shown to be involved in dsRNA uptake in various insect species, such as the Colorado potato beetle (*Leptinotarsa decemlineata*), red flour beetle (*Tribolium castaneum*), oriental fruit fly (*Bactrocera dorsalis*), and Western corn rootworm (*D. virgifera*) (Cappelle et al., 2016; Li et al., 2015; Pinheiro et al., 2018; Xiao et al., 2015). The level of transcript knockdown varied for each dsRNA treatment and each individual gene responded differently. This phenomenon has been previously observed in studies investigating fungal pathogens and insects (Asokan et al., 2014; Gu et al., 2019; McLoughlin et al., 2018; Song et al., 2018). Gu *et al.* (2019) showed that RNAi molecules derived from different regions of the *F. asiaticum* β_2-tubulin gene had different effects on mycelial growth, asexual reproduction, and virulence (Gu et al., 2019). Furthermore, transcript knockdown of *β_2-tubulin* varied between the different RNAi segments. Differences in knockdown response may be due to target site accessibility, GC content, differences in mRNA secondary structures, mRNA turnover rates, site of dsRNA accumulation, dsRNA delivery method, and other properties associated with dsRNA structure (Chan et al., 2009; Larsson et al., 2010). The presence or absence of RdRPs may also play a role, as they are involved in

amplifying silencing signals (Asokan et al., 2014). One main challenge of developing effective RNAi pest management strategies will be identifying suitable target genes that are conducive to transcript knockdown and reduction of fungal growth. Screening gene targets and *in vivo* and *in vitro* testing of dsRNAs will continue to be essential for the identification of efficient SIGS targets, until more robust rules for identifying optimal targets can be developed.

SS1G_00005 (60S ribosomal subunit) and SS1G_01703 (aflatoxin biosynthesis) dsRNA treatments were the most effective at reducing transcript levels, with 82 and 83% knockdown, respectively. SS1G_00005 encodes the ribosomal protein L7. Ribosomal proteins are abundant RNA-binding proteins essential for protein synthesis and are also likely involved in extra-ribosomal auxiliary functions (Takada & Kurisaki, 2015). Previous research on ribosomal transcripts in insects have shown that ribosomal proteins may be effective targets for RNAi-mediated pest control. For example, knockdown of ribosomal transcripts in the Western predatory mite (*Metaseiulus occidentalis*) resulted in significant loss of female egg production (Wu & Hoy, 2014) and dsRNA treatment targeting a ribosomal protein in ticks (*Rhipicephalus haemaphysaloides*) significantly affected blood feeding, molting, and reproduction (Zhang et al., 2018). In addition, Jing *et al.* (2020) showed that feeding the 28-spotted ladybeetle (*Henosepilachna vigintioctopunctata*) dsRNA targeting two ribosomal protein-encoding genes significantly decreased their survival (Jing et al., 2020). Therefore, SS1G_00005 and ribosomal genes in general may serve as good candidate genes for RNAi-based biological control and should be investigated in more detail.

SS1G_01703 has been previously investigated in the Belmonte and Whyard labs and based on Kegg (https://www.kegg.jp/pathway/ssl00254) and NCBI, SS1G_01703 is a versiconal hemiacetal acetate esterase involved in an aflatoxin biosynthesis pathway (McLoughlin et al., 2018).

However, it is uncertain whether SS1G_01703 indeed encodes an aflatoxin biosynthesis protein, as to date, no aflatoxins have been reported in *S. sclerotiorum*. Aflatoxins are a family of highly toxic polyketide-derived secondary metabolites and are primarily produced by *Aspergillus* spp., including *Aspergillus parasiticus, A. nomius*, and *A. flavus*, all of which parasitize various crop plants (Chang et al., 2001). Several studies have shown that HIGS is an effective approach to lowering aflatoxin levels within *Aspergillus* spp., without affecting host plant growth, first in peanuts (*Arachis hypogaea*) and then in maize (Arias et al., 2015; Thakare et al., 2017). However, despite lowering aflatoxin levels, both crops were still infected with the pathogen, and it seems that aflatoxins are not a pathogenicity factor in *Aspergillus* species. Other studies have shown that an agricultural fungus with an altered toxin profile can still be pathogenic, such as the maize pathogen *Cochliobolus carbonum* (Idnurm & Howlett, 2001). In contrast, the Belmonte and Whyard labs have shown that transgenic *A. thaliana* plants expressing SS1G_01703 hpRNAs significantly decreased foliar disease severity (Belmonte, unpublished data). Here, I show that topical application of SS1G_01703 dsRNA significantly reduces transcript abundance *in vitro*, as well as reduces *S. sclerotiorum* fungal infection on *B. napus* leaves. SS1G_01703 has two conserved domains and they are associated with lipid transport and metabolism (acetyl esterase/lipase), and alpha/beta hydrolase folds, but these domains do not precisely define the encoded protein's function. The function of SS1G_01703 thus remains unclear. SS1G_01703 gene's product could, for example, be involved in an essential cellular process for *S. sclerotiorum* growth or development. Alternatively, SS1G_01703 may indeed encode a novel aflatoxin that, unlike those of *Aspergillus* spp., facilitates the fungus's pathogenicity on some host plants. Or, the gene enables the production some other secondary metabolites that are not related to fungal pathogenicity, but nevertheless permit growth in plant host.

Many studies have shown a co-regulation of secondary metabolite production (i.e., aflatoxin biosynthesis) and sclerotial development in *Aspergillus* spp. (Calvo, 2008; Chang et al., 2001). In addition, it has been shown that oxidative stress is involved in both sclerotial development and aflatoxin biosynthesis. It has been hypothesized that fungi sequester a number of secondary metabolites in sclerotia as a chemical defense system against fungivorous predators (Calvo & Cary, 2015; Grintzalis et al., 2014). It would be of interest to examine this relationship in *S. sclerotiorum* and investigate whether there is a positive relationship between aflatoxin production and sclerotia formation. Future studies could inoculate transgenic *B. napus* plants expressing SS1G_01703 dsRNA with *S. sclerotiorum* and compare the number of formed sclerotia to wildtype inoculated *B. napus* plants. If targeting SS1G_01703 could result in reduced fungal growth as well as reduced sclerotia production, it would an ideal candidate for RNAi-based biological control.

Interestingly, the dsRNA targeting SS1G_01411, a SsbZIP TF, resulted in more than a two-fold increase in this gene's transcript levels. Based on RNA-sequencing data, there is an up-regulation of SS1G_01411 gene expression during *S. sclerotiorum* infection of *B. napus* (McLoughlin et al., 2018; Seifbarghi et al., 2017). The bZIP protein family is one of the largest and most diverse TF families and proteins with bZIP domains are present in all eukaryotes (Sornaraj et al., 2016). Recent studies have shown that members of the bZIP TF family play an important regulatory role in filamentous fungi. More specifically, fungal bZIP TFs are known for their ability to mediate stress responses triggered by developmental or environmental cues, and adaption to oxidative stress is thought to be an important virulence-associated cellular process (Guo et al., 2010; Wee et al., 2017; Yin et al., 2013). In *Aspergillus* spp., bZIP proteins such as AtfA, AtfB, and AP-1, play key roles in regulation of oxidative stress and secondary metabolism (Roze et al., 2013). Wee *et al.* (2017) demonstrated that AtfB is a master regulator of at least four

cellular processes, including secondary metabolism, stress response, conidiospore development, and intracellular transport machinery (Wee et al., 2017). In *Pestalotiopsis fici*, the bZIP TF PfZipA, regulates secondary metabolism and oxidative stress (Wang et al., 2015b). And, in *Fusarium pseudograminearum*, the bZIP TF FpAda1 is essential for fungal growth and conidiation (Chen et al., 2020). Thus, it is quite possible that SS1G_01411 is involved in *S. sclerotiorum* oxidative stress and secondary metabolism pathways. TFs often form homo- or hetero-dimers with other proteins and interacting partners may also contribute to transcript regulation. For instance, signaling pathways that participate in the activation of Nrf2, an evolutionarily conserved bZIP protein that binds to antioxidant response elements, has been implicated in various pathological conditions such as inflammation, cardiovascular dysfunction, neurodegenerative disorders, premature aging, and cancer (Wee et al., 2017). Given the importance of bZIP TFs and their role in the regulation of gene expression patterns, and SS1G_01411 is heavily involved in fungal pathogenicity, this gene's expression may be regulated in a complex manner, and hence, it could overcome the impacts of the siRNAs targeting its transcripts. Transcript knockdown of SS1G_01411 may have initially occurred during the early hours of exposure to the dsRNA (well before I sampled the fungus to measure transcript knockdown), and the fungus was subsequently able to rebound from the dsRNA treatment through internal cellular responses. Although an overexpression of SS1G_01411 transcript levels was observed *in vitro* at 3 dpi, the SS1G_01411 dsRNA treatment still had an adverse effect on *S. sclerotiorum* growth when applied onto detached *B. napus* leaves. To better understand the role of SS1G_01411 dsRNA and its ability to affect fungal transcript abundance *in vivo*, future studies could investigate gene transcript levels when the dsRNA is applied onto *B. napus* leaves. Future work should also investigate SS1G_01411 transcript levels at different timepoints after dsRNA application, such as 12, 24, and 48 hpi, to

determine if knockdown is occurring earlier than 3 dpi. In some of my preliminary studies, I observed that the sampling time was quite important when pinpointing knockdown, especially when dealing with *S. sclerotiorum*, a fungal pathogen that has a rapid growth rate.

Curiously, transcript knockdown of SS1G_04652 (β-tubulin) was not detectable 3 dpi *in vitro*. β-tubulins are a major component of microtubules and are essential for various cellular activities, including intracellular transport, ciliar and flagellar motility, and cell division (Zhao et al., 2014). One possible explanation for this phenomenon is that transcript knockdown may have occurred at an earlier timepoint, but due to its importance in cellular functions, the fungus was able to recover from the dsRNA treatment through intrinsic regulatory networks. While the dsRNA dose had no impact *in vitro*, SS1G_04652 dsRNA had an adverse effect on *S. sclerotiorum* growth when applied onto detached *B. napus* leaves. Overall, SS1G_04652 may be an effective RNAi target and β-tubulin has been used as the molecular target for methyl benzimidazole carbamate (MBC) fungicides (Hollomon et al., 1998). MBCs exert antifungal activities by targeting the β-tubulin subunit of microtubules, which results in a failure of microtubule formation and cell division, leading to cell death (Davidse & Flach, 1977). Gu *et al.* (2019) demonstrated that a dsRNA treatment targeting a *F. asiaticum* β_2-tubulin gene increased fungal sensitivity to MBCs (Gu et al., 2019). In addition to SS1G_01411, future studies should investigate SS1G_04652 transcript levels after dsRNA application on *B. napus* leaves to provide a better understanding of how this dsRNA affects gene transcript levels *in vivo*. Additional studies should also investigate SS1G_04652 transcript abundance levels at different timepoints subsequent to dsRNA treatment to confirm if knockdown is occurring at an earlier timepoint.

When *S. sclerotiorum*-specific dsRNAs were topically applied to *B. napus*, significant lesion reduction was observed for all eight dsRNA treatments. While all the tested dsRNAs significantly

reduced disease symptoms in *B. napus*, some targets showed greater reductions in disease severity. SS1G_10167 (*SsPG1*), targeting an endo-polygalacturonase, was the most effective at reducing fungal lesion size, with a reduction of 62%. *SsPG1* is a well documented *S. sclerotiorum* gene and belongs to an important group of plant cell wall degrading enzymes. Specifically, *S. sclerotiorum* polygalacturonases (SsPGs) are the main group of hydrolytic enzymes involved with fungal virulence and are considered as one of the important pathogenic factors of *S. sclerotiorum* (Z. Wang et al., 2021). *S. sclerotiorum* is a necrotrophic fungus and necrotrophs secrete a variety of toxic molecules and hydrolytic enzymes to dissolve plant tissue ahead of pathogen invasion and use the disrupted plant tissue as a source of nutrients for its growth (Seifbarghi, 2019). During *S. sclerotiorum* infection, *SsPG1* activity precedes that of *SsPG3*, *SsPG5*, and *SsPG6*, suggesting that *SsPG1* may be important for establishing infection (Li et al., 2004). Therefore, knockdown of *SsPG1* may reduce *S. sclerotiorum's* ability to degrade plant cell wall and limit the level of fungal infection.

4.2 Homologous Gene Targets in *B. cinerea* and *L. maculans*

Previous studies have shown that effective RNAi gene targets can be adapted as homologous gene targets in closely related fungal species. Wang *et al.* (2016) showed that transgenic *A. thaliana* plants expressing hpRNAs could simultaneously target *DCL* genes in *B. cinerea* and *V. dahliae* (Wang et al., 2016). McLoughlin *et al.* (2018) demonstrated that *S. sclerotiorum* RNAi targets could also be used as homologous targets in *B. cinerea* and were effective at reducing fungal lesion size (McLoughlin et al., 2018). In this study, I investigated whether this principle could be applied to both closely and distally related fungal species. Overall, the sequence complementarity of some of the *S. sclerotiorum*-specific dsRNAs to transcripts in *B. cinerea* and *L. maculans* could potentially be used for cross-species control. The *B. cinerea*-specific dsRNAs

targeting BCIN_02g03480 (SS1G_01703) and BCIN_11g03740 (SS1G_06617) were both successful at reducing transcript abundance *in vitro*, as well as the *L. maculans*-specific dsRNA targeting LEMA_P069770.1 (SS1G_01703). However, this study did not investigate whether these dsRNAs could reduce fungal lesion size when applied to *B. napus* leaves. Therefore, conclusions cannot be made about their efficacy in reducing fungal growth. Future studies should test these dsRNAs on *B. napus* leaves and this would provide further insight into their ability to control fungal growth.

SS1G_06617, BCIN_11g03740 (SS1G_06617), and, LEMA_P053820.1 (SS1G_06617) are ATP-binding cassette (ABC) transporter proteins and belong to the largest transporter gene family (Davidson et al., 2008). ABC proteins are membrane transporters that use ATP hydrolysis to transport molecules across biological membranes (Kovalchuk et al., 2015). In fungi, ABC transporters are implicated in numerous physiological functions, including pathogenesis, fungicide baseline sensitivity, and multidrug resistance (De Waard et al., 2006; Stergiopoulos et al., 2002). For instance, the *F. graminearum* ABC transporter gene *FgABC1* is responsible for the secretion of fungal metabolites to suppress plant defense (Zhang et al., 2013). One well documented mechanism of fungal multi-drug resistance involves constitutive overexpression of ABC transporters to increase efflux activity (De Waard et al., 2006). The *B. cinerea* ABC transporter atrB (BcatrB) has been well studied and Schoonbeek *et al.* (2001) showed that BcatrB is involved in protection against resveratrol, a grapevine phytoalexin, and the fungicide fenpiclonil (Schoonbeek et al., 2001). Additionally, Leroux and Walker (2012) showed that overproducing BcatrB in multidrug resistant *B. cinerea* strains increased *B. cinerea* fungicide resistance to carbendazim, fluazinam, and malonoben (Leroux & Walker, 2013). Ortuño *et al.* (2015) also showed that *BcartB* expression levels were increased in multidrug resistant isolates (Fernández-

Ortuño et al., 2015). It would be of interest to investigate if this set of ABC proteins (SS1G_06617, BCIN_11g03740, and LEMA_P053820.1) also play a role in protection against exogenous toxic compounds. Future studies could investigate whether the application of dsRNA and subsequent knockdown of ABC transporter proteins would render *S. sclerotiorum*, *B. cinerea*, and *L. maculans* more sensitive to certain fungicides. If so, RNAi technology could be used in tandem with traditional fungicide applications and still reduce the amount of fungicide needed thus, reducing possible environmental impacts on the agroecological environment.

4.3 Potential Off-Target Effects of RNAi Molecules

Despite more than a decade of research investigating the use of RNAi as a pest management tool, there are still many unanswered questions about the cross-reactivity of dsRNAs and the possibility of off-target effects. While the mechanism of RNAi is reasonably well understood in most systems, the RISC machinery is relatively complex and there are still many unknown molecular players in this intricate intracellular network. In addition, there are still many unanswered questions about Dicer and the mechanisms by which it cleaves long dsRNAs into siRNAs. For researchers and government regulatory agencies, is of interest to determine how shared homology between species will affect cross-reactivity between closely and distally related species. Understanding these processes more clearly will aid in the regulatory process and mitigate off-target effects of RNAi-based technologies and anticipate negative environmental impacts. Current off-target assessments have relied heavily on bioinformatic tools, and seldomly have ecological risk assessments been conducted to evaluate off-target effects of RNAi between closely and distally fungal related species.

This study shows that shared 21-mer overlaps between closely and distally related fungal species will lead to specific off-target effects. More specifically, *S. sclerotiorum*-specific dsRNA

will reduce transcript abundance in other fungal pathogens if there are shared 21-mer overlaps, regardless of the relatedness of the pathogens. Conversely, *S. sclerotiorum*-specific dsRNAs that do not have shared 21-mer overlaps did not produce off-target effects in either *B. cinerea* or *L. maculans*, showing that RNAi can indeed provide species-specific crop protection. Despite these findings, the possibility of off-target effects at the molecular level still exists for organisms we have yet to study, as well as non-specific off-target effects. SS1G_00005 dsRNA, targeting a *S. sclerotiorum* 60S ribosomal subunit, has shared 21-mer overlaps with its *B. cinerea* and *L. maculans* homologs and was able to reduce fungal growth and transcript abundance for both *B. cinerea* and *L. maculans* (Supplementary Figure 3.1). The dsRNA targeting a *S. sclerotiorum* endo- polygalacturonase, SS1G_10167, has shared 21-mer overlaps with its *B. cinerea* homolog and was able to reduce fungal growth and transcript abundance in *B. cinerea*. However, SS1G_10167 does not have shared 21-mer overlaps with its *L. maculans* homolog and does not affect fungal growth or transcript abundance in *L. maculans* (Supplementary Figure 3.2). SS1G_01703 dsRNA, targeting a gene in the *S. sclerotiorum* aflatoxin biosynthesis pathway, does not have shared 21-mers with its *B. cinerea* and *L. maculans* homolog and did not produce any off-target effects when tested with *B. cinerea* or *L. maculans* (Supplementary Figure 3.3). It is clear that even a single shared 21-mer can result in off-target effects (Figure 3.12). However, we can use these off-target effects to our advantage, and this leaves many opportunities to use SIGS and HIGS as a broad-spectrum plant protection strategy. Alternatively, if crop producers only want to target a single pest species, RNAi can still provide a very narrow spectrum of activity and sequence-specific gene silencing.

Curiously, the application of SS1G_10167 dsRNA on *B. cinerea* resulted in an approximately 6-fold transcript overexpression of the *B. cinerea* homolog gene BCIN_14g00850 (*Bcpg1*). *Bcpg1*

has 64;5 shared 21-mers with the SS1G_10167 dsRNA and is a well documented endo-polygalacturonase gene. Similar to SS1G_10167, *Bcpg1* is highly expressed *in planta* and is required for full virulence of *B. cinerea* (Soulie et al., 2020; Ten Have et al., 1998). Ten Have *et al.* (1998) showed that disruption of *Bcpg1* resulted in significant reduction of secondary fungal infection (Ten Have et al., 1998). Although dsRNA application led to an overexpression of *Bcpg1* transcript abundance, SS1G_10167 was quite effective at reducing *B. cinerea* fungal growth. Ultimately, SS1G_10167 may be a great RNAi target for crop protection against *S. sclerotiorum* and *B. cinerea* due to its involvement in fungal pathogenicity and host plant colonization. This study showed that this SS1G_10167 dsRNA has the ability to provide protection against these fungal pathogens. Further studies should continue to investigate the effectiveness of this gene target and determine if it can be effective against additional fungal pathogens with homologous endo-polygalacturonase genes.

In this study, I showed that RNAi is highly sensitive and that a single shared 21-mer between two fungal species will lead to off-target effects. The shared 21-mer between *S. sclerotiorum* and *B. cinerea* for SS1G_06487 dsRNA was designed at the 5’ end of the dsRNA sequence. As other studies have shown that dsRNA design does affect the level of transcript knockdown and knockdown phenotypes, it would be of interest to explore the effect of the positioning of a single shared 21-mers on their relative efficacies (Gu et al., 2019; Koch et al., 2016). More specifically, future studies could compare knockdown effects of a single 21-mer when placed at the 5’, middle, or 3’ end of a dsRNA molecule. This would help further our understanding of the mechanisms in which Dicer binds and processes long dsRNA into siRNAs. Overall, Dicer mechanisms are better understood in mammals and insects. In *Drosophila*, biochemical studies have proposed that Dcr-2 distinguishes dsRNAs based on their termini. Blunt end dsRNAs promote Dcr-2 cleavage

without dissociation (processive cleavage) whereas dsRNAs with 2-nt 3’ overhangs cause Dcr-2 to dissociate after the first cleavage and requires Dcr-2 to rebind for subsequent cleavage (distributive cleavage) (Cenik et al., 2011; Sinha et al., 2018; Welker et al., 2011). In general, Dcr-2 cleaves dsRNAs with blunt ends more efficiently and this model is consistent with cryo-electron microscopy structures of Dcr-2 (Naganuma et al., 2021). The dsRNA-binding protein Loquacious-PD (Loqs-PD) is a partner protein of *Drosophila* Dcr-2 and enhances the dsRNA cleavage reaction of Dcr-2 (Cenik et al., 2011; Sinha et al., 2018). Using a single-molecule imaging system, Naganuma *et al.* (2021) showed that the terminal structures of long dsRNAs and the presence or absence of Loqs-PD change the probability of Dcr-2 to initiate processive cleavage (Naganuma et al., 2021). Future research could determine if there is a Loqs-PD homolog or similarly-functioning protein in fungi and if processive and distributive cleavage mechanisms are performed by Dicer. In some fungi (*N. crassa*), sRNAs can be generated from Dicer-dependent and Dicer-independent pathways, which could add another source of variation on how many different siRNAs are produced within a fungus, and hence, how effective RNAi-based methods of fungus control could be for some species (Li et al., 2010).

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Bioinformatic tools and *in silico* analyses have allowed researchers and regulatory agencies to predict the potential off-target effects of RNAi technology and identify non-target organisms that share some level of sequence complementarity with the designed dsRNA. In our study, we used these predictive tools to select gene targets to perform *in vitro* and *in planta* assays to investigate the off-target effects of *S. sclerotiorum*-specific dsRNA on the closely and distally related fungal pathogens, *B. cinerea* and *L. maculans*, respectively. The results of this study suggest that dsRNA molecules can be designed to target a single fungal species, as well as multiple fungal pathogens, based on shared 21-mers between the dsRNA and its target mRNA. For example, *S. sclerotiorum*-specific dsRNAs that have shared 21-mers with *S. sclerotiorum, B. cinerea*, and *L. maculans* can provide broad-spectrum protection and reduce fungal growth in all three species. Conversely, *S. sclerotiorum*-specific dsRNAs that do not have shared 21-mers will only provide species-specific protection. In this study, I was also able to identify novel *S. sclerotiorum* gene targets that are effective at reducing *S. sclerotiorum* growth.

The off-target analyses performed as part of this thesis provides the first detailed report of how shared 21-mers between a dsRNA and its target mRNA will result in transcript knockdown and reduction in fungal growth. Although we show that *S. sclerotiorum*-specific dsRNAs can be designed to avoid off-target effects in the *B. cinerea* and *L. maculans* homologous genes, we did not investigate whether the dsRNAs had impacts on any other genes within the genome of the organisms. Future studies could use bioinformatic tools to investigate whether there are additional shared 21-mers outside of the target gene, given that the genomes of these three species have been fully sequenced and are publicly available. If there are more shared 21-mers, qRT-PCR could be used to explore whether transcript levels of these non-targeted genes are affected. Such analyses

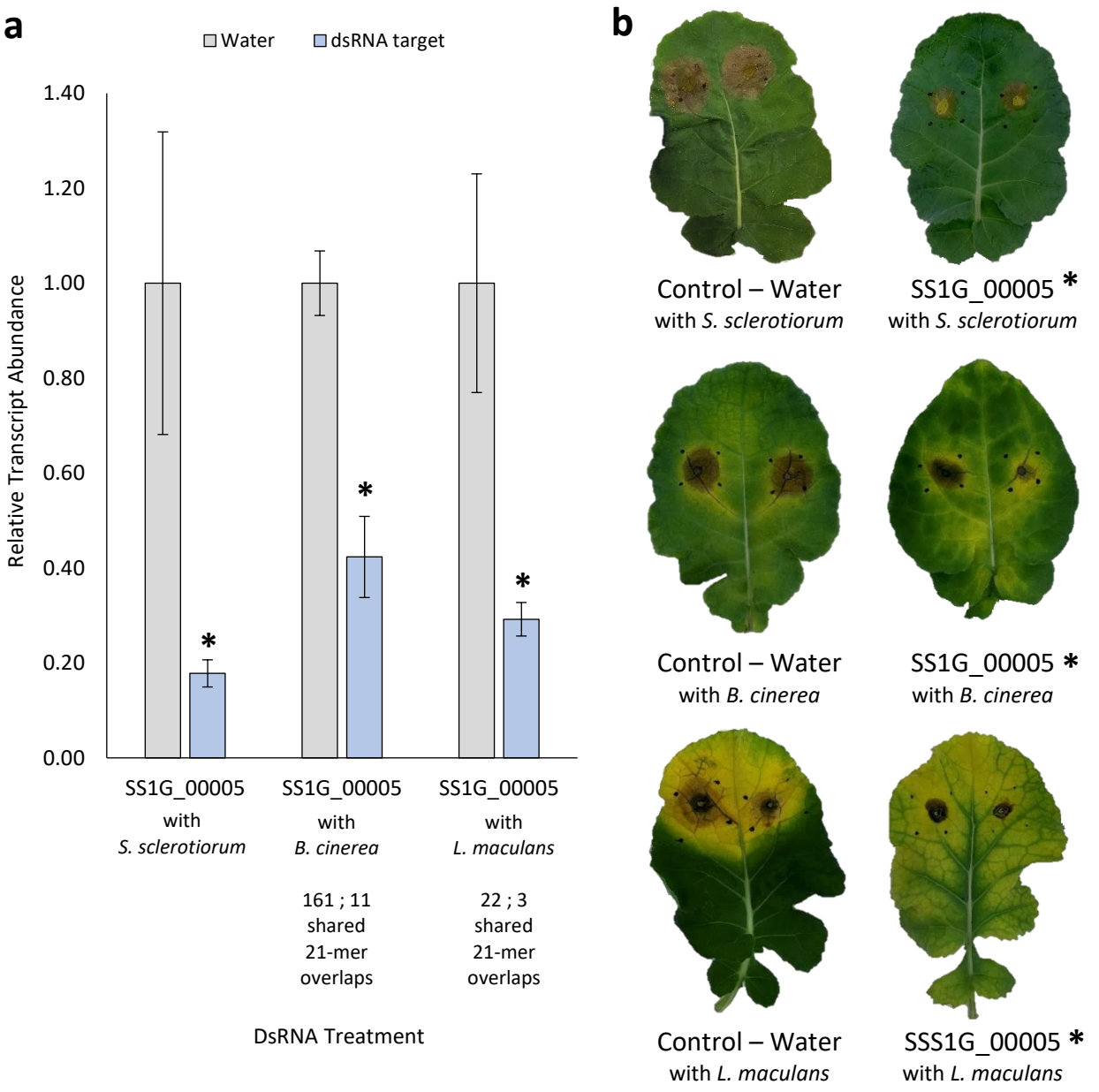
will be of importance for ecological risk assessments and to assure regulatory agencies that SIGS can provide a narrow spectrum of activity. *In silico* analyses, however, are only predictive of possible off-target effects, and until more of these analyses have been performed, *in vivo* testing is necessary to assess off-target effects. For species lacking genomic data, this testing is the only option to evaluate the safety of dsRNA-based fungicides.

There are still many unanswered questions on how Dicer binds and cuts dsRNA into siRNAs. In this study, I considered two different Dicer processing mechanisms. One mechanism assumes simple end-to-end cleavage by Dicer and allows for a clear number of shared 21-mers between the dsRNA and target gene. The other mechanism factors in a frameshifted series of siRNAs and the potential for nucleotide overlap. Depending on the Dicer mechanism, the number of shared 21-mers between the dsRNA and the target mRNA will differ. A factor that I did not evaluate in this study was the possibility of shorter siRNA fragments, as siRNAs can range from 19 to 24 nts in length. To investigate these two unknown questions and the overall molecular mechanism of Dicer in fungi, future studies should sequence the resulting siRNAs of a long dsRNA. The findings from those analyses should provide valuable information on how Dicer processes dsRNA and the molecular composition of cellular siRNAs. Further understanding of how this mechanism occurs in fungal systems will also enable more accurate evaluations of potential off-target effects.

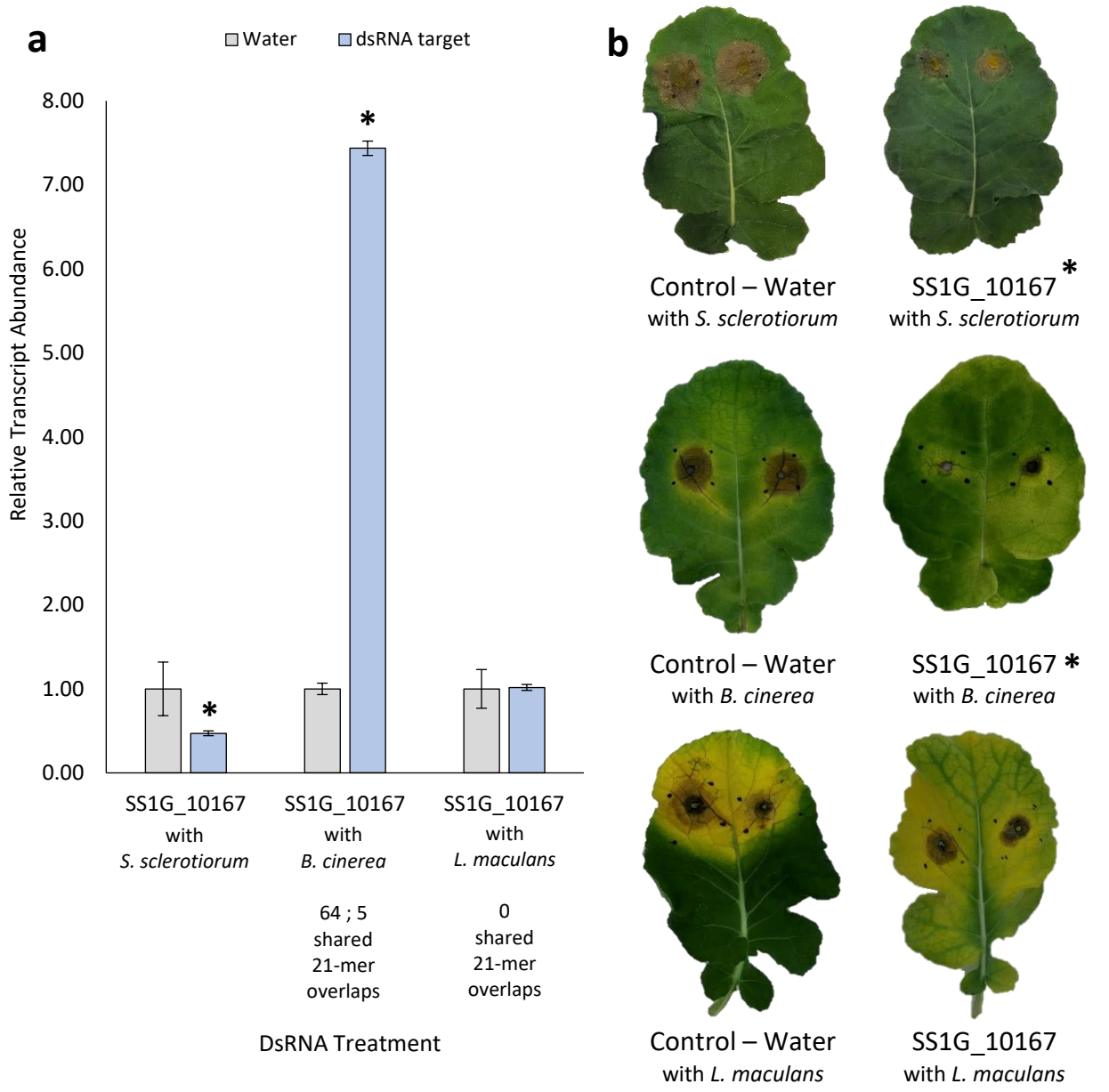
In this study, I explored the possibility of targeting homologous genes of effective *S. sclerotiorum* RNAi targets in *B. cinerea* and *L. maculans*. This can be used as a method of selecting RNAi targets in other fungal pathogens. I was able to show that the *B. cinerea*- and *L. maculans*-specific dsRNAs were successful at reducing transcript abundance in their respective species-specific target genes. However, I was not able to evaluate their ability to reduce fungal growth

when applied to *B. napus* leaves. Future studies should investigate their ability to reduce fungal growth to provide additional evidence that these dsRNAs would provide adequate crop protection. It would also be interesting to investigate these *B. cinerea*- and *L. maculans*-specific dsRNAs and their ability to reduce *S. sclerotiorum* transcript levels. This would provide evidence that the ability for dsRNAs to provide broad-spectrum protection is reciprocal, and not exclusive to *S. sclerotiorum*-specific dsRNA.

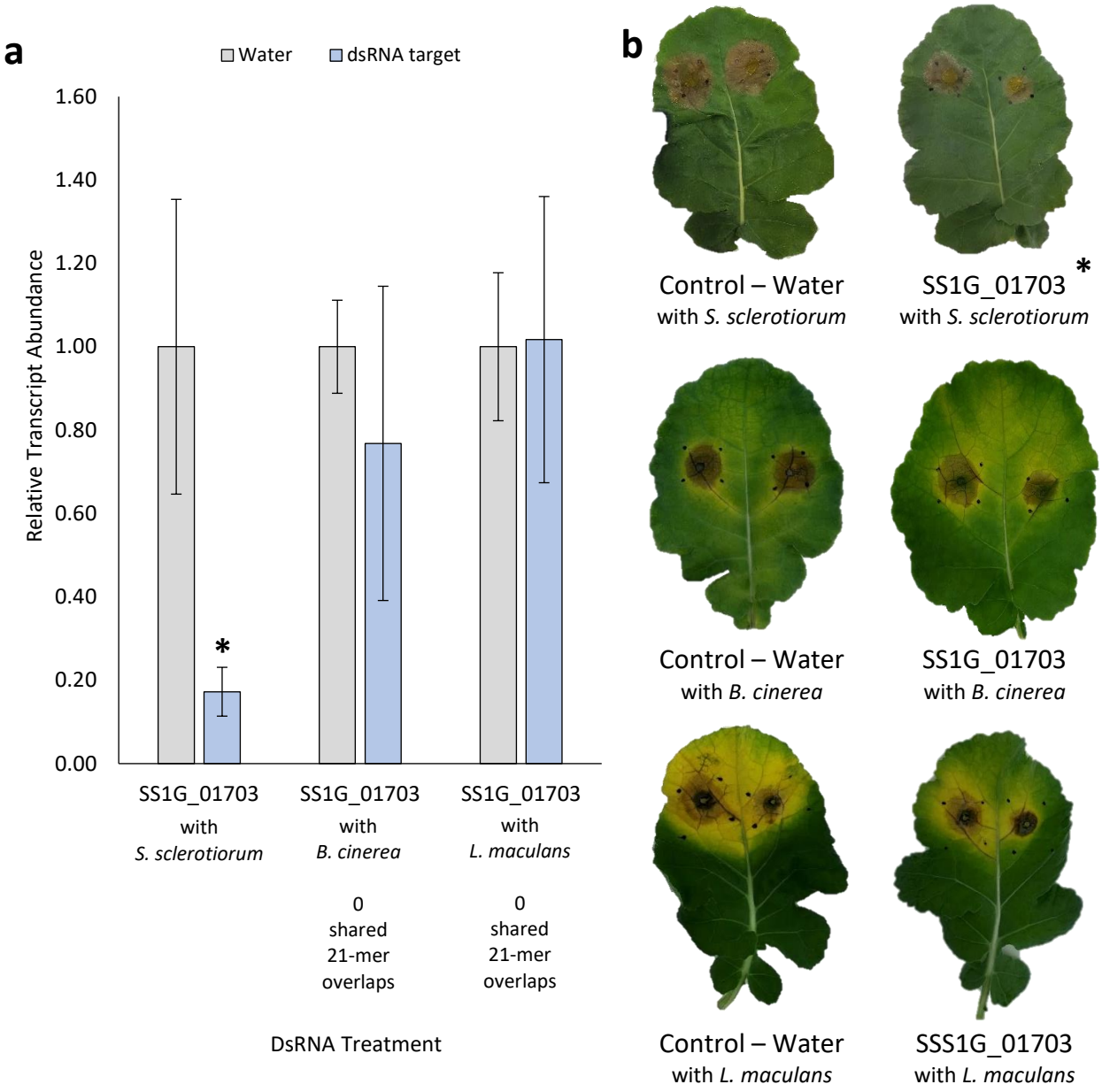
In conclusion, the data generated in this study provide information required to evaluate the potential off-target effects of dsRNA molecules in closely and distally related fungal pathogens. The combination of *in silico* analyses and ecological risk assessments is necessary to fully evaluate the safety of RNAi technology and additional studies will lead to safer and more effective methods of fungal control.



Supplementary Figure 3.1. Comparison of *Sclerotinia sclerotiorum*-specific dsRNA targeting SS1G_00005 (60S ribosomal subunit). Comparison of *S. sclerotiorum*, *Botrytis cinerea* and *Leptosphaeria maculans*. SS1G_00005 has 161;11 shared 21-mer overlaps with *B. cinerea* and 22;3 shared 21-mer overlaps with *L. maculans*. All dsRNA treatments resulted in significant transcript changes (student's t-test, $p < 0.05$) are denoted by an asterisk (*). (**a**) *S. sclerotiorum* dsRNA grown in liquid cultures with *S. sclerotiorum*, *B. cinerea* or *L. maculans*. Transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. (**b**) *S. sclerotiorum* dsRNA applied on *B. napus* Sclerotinia susceptible Westar cultivar with *S. sclerotiorum*, *B. cinerea* or *L. maculans*. Lesion size was measured 3, 4, and 10 dpi, respectively.



Supplementary Figure 3.2. Comparison of *Sclerotinia sclerotiorum*-specific dsRNA targeting SS1G_10167 (endo-polygalacturonase). Comparison of *S. sclerotiorum*, *Botrytis cinerea* and *Leptosphaeria maculans*. SS1G_10167 has 64;5 shared 21-mer overlaps with *B. cinerea* and 0 shared 21-mer overlaps with *L. maculans*. All dsRNA treatments resulted in significant transcript changes (student's t-test, $p < 0.05$) are denoted by an asterisk (*). (**a**) *S. sclerotiorum* dsRNA grown in liquid cultures with *S. sclerotiorum*, *B. cinerea* or *L. maculans*. Transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. (**b**) *S. sclerotiorum* dsRNA applied on *B. napus* Sclerotinia susceptible Westar cultivar with *S. sclerotiorum*, *B. cinerea* or *L. maculans*. Lesion size was measured 3, 4, and 10 dpi, respectively.



Supplementary Figure 3.3. Comparison of *Sclerotinia sclerotiorum* specific dsRNA targeting SS1G_01703 (aflatoxin biosynthesis). Comparison of *S. sclerotiorum*, *Botrytis cinerea* and *Leptosphaeria maculans*. SS1G_01703 has 0 shared 21-mer overlaps with *B. cinerea* and *L. maculans*. All dsRNA treatments resulted in significant transcript changes (student's t-test, $p < 0.05$) are denoted by an asterisk (*). (**a**) *S. sclerotiorum* dsRNA grown in liquid cultures with *S. sclerotiorum*, *B. cinerea* or *L. maculans*. Transcript levels were measured 3 days post treatment of 500 ng/mL dose of dsRNA. (**b**) *S. sclerotiorum* dsRNA applied on *B. napus* Sclerotinia susceptible Westar cultivar with *S. sclerotiorum*, *B. cinerea* or *L. maculans*. Lesion size was measured 3, 4, and 10 dpi, respectively.

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