The development of RNA interference-based biotechnologies for the sustainable management of *Sclerotinia sclerotiorum*

by

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ABSTRACT

Necrotrophic pathogens, such as *Sclerotinia sclerotiorum* and *Botrytis cinerea*, threaten global agricultural production. Traditional agrochemicals may negatively impact the environment and chemical resistance is rising. Therefore, novel strategies are needed to overcome these obstacles. RNA interference (RNAi), a cellular defence pathway for targeted mRNA degradation using double stranded RNA (dsRNA), could be a next-generation fungicide. RNA sequencing of the host-pathogen interface identified hundreds of genes for RNAi-based control development. I first demonstrated the dsRNA molecules elicited gene silencing *in vitro*. Next, I formulated foliar applications to protect both *Brassica napus* and *Arabidopsis thaliana* from *S. sclerotiorum* and *B. cinerea* using designer molecules targeting homologous transcripts. Finally, I engineered host induced gene silencing (HIGS) to suppress fungal colonization on *A. thaliana* and explored host defense and death responses during HIGS. Together, these findings provide compelling evidence for an RNAi-based biotechnological management strategy and will be a valuable resource for future research endeavours.

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NON-COMMON ABBREVIATIONS USED

AGO	ARGONAUTE
DCL	DICER-LIKE
dsRNA	double stranded RNA
FPKM	fragments per kilobase of million mapped reads
GO	Gene ontology
HIGS	Host induced gene silencing
hpRNA	hairpin RNA
JA	Jasmonic acid
milRNA	micro-like RNA
miRNA	micro RNA
mRNA	messenger RNA
nt	nucleotides
OA	Oxalic acid
QIP	QUELLING DEFICIENT-2 INTERACTING PROTEIN
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RdRP	RNA dependent RNA Polymerase
RISC	RNA induced silencing complex
RNAi	RNAi interference
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
SA	Salicylic acid
siRNA	small interfering RNA
sRNA	small RNA

CONTRIBUTION OF AUTHORS

Chapter 1

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Preface, Chapter 3, and Chapter 4

I performed all the experiments stated in Chapter 3 and prepared thesis. Mark F Belmonte and Steve Whyard contributed to the final document preparation.

CHAPTER 1 : AN INTRODUCTION TO DEVELOPING NEW RNA INTERFERENCE TECHNOLOGIES TO CONTROL FUNGAL PATHOGENS

Austein G Mcloughlin, Phil L Walker, Nick Wytinck, Daniel Sullivan, Steve Whyard, and Mark Belmonte

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1.1 ABSTRACT

Current agricultural output is challenged by increasing fungal pressure that can lead to considerable losses in crop yield and post-harvest storage. Traditional chemical fungicides used to treat pathogenic fungi can be ineffective and harmful to the environment if not managed properly. With fungicide resistance increasing, new environmentally-friendly and sustainable technologies are required to protect some of the world's most important crops. RNA interference (RNAi) is an intrinsic cellular mechanism, mediated by double-stranded RNA (dsRNA), which can suppress protein expression through targeted destruction of mRNAs, and with recent advances in dsRNA delivery or expression in plants, this mechanism has the potential to revolutionize crop management strategies. Examples of RNAi-based control to manage pathogenic fungal species are steadily increasing, as the technology offers new options for the development of new fungicides with improved species-specificity and/or potency against fungi for which existing fungicides have been ineffective. RNAi technology can be adapted to provide either robust and multi-crop plant protection using topical sprays or can provide more durable resistance through transgene expression of dsRNAs within susceptible plant tissues. Using RNA

sequencing to identify fungal gene targets, RNAi-based control technology will continue to develop as a potent and environmentally-conscious alternative to traditional agrochemicals for crop protection

1.2 INTRODUCTION

Access to safe, healthy, and sustainable food sources is one of the defining challenges of our time. Given the rapid increase of the human population, it is estimated that we will require an additional production of 200 billion calories, equating to a 100-110% increase in crop demand to meet nutritional needs by the year 2050 (Bebber and Gurr, 2015; Tilman *et al.*, 2011; United Nations, 2017). Currently, 40% of ice-free land is used to grow crops; however, global climatic changes challenge our current production systems by decreasing yield potentials and shrinking arable land resources (Myers *et al.*, 2017; Pugh *et al.*, 2016). Moreover, biotic agents, such as necrotrophic and biotrophic fungi, further complicate agronomic production, causing global losses of up to 30-40% in crop yield in field and post-harvest (Bebber and Gurr, 2015; Myers *et al.*, 2017). With rapid globalization and migration across the globe, fungal pathogens are predicted to spread rapidly to virgin lands, presenting new challenges for crop production globally (Bebber *et al.*, 2014).

Traditional chemical treatments used to combat fungal disease epidemics and control necrotrophic pathogens, like *Sclerotinia sclerotiorum* (Lib.) de Bary, achieve mixed success in field (Huzar-Novakowiski *et al.*, 2017). For example, chemical applications at sub-lethal levels were shown to promote fungicide resistance (Amaradasa and Everhart, 2016). Common fungicides like boscalid, iprodine, thiophanate methyl, azoxystrobin, and pyracostrobin, when diluted to sub-lethal levels resulted in increased mutation rates of up to 60-fold due to cellular

stress in *S. sclerotiorum* (Amaradasa and Everhart, 2016). Additional treatments of fungicides resulted in reduced fungal sensitivity due to genetic mutations suggesting that common agricultural techniques could promote fungicide resistance (Amaradasa and Everhart, 2016). Development of resistance may lead to a positive feedback loop, where chemical use selects for further resistance, attributing to the rise of fungicide resistant *S. sclerotiorum*, *Botrytis cinerea* Pers., and *Magnaporthe oryzae* Couch (Castroagudín *et al.*, 2015; Penaud and Walker, 2015; Rupp *et al.*, 2017). For these reasons, there is a clear need to develop new methods to control disease outbreaks using sustainable technologies.

Despite the suggestion that precise chemical applications could reduce environmental impacts, fungicide use has deleterious effects on the surrounding agro-ecological landscape due to its general biocidal nature, as well as the dispersal and persistence within the environment (Le Cointe *et al.*, 2016; Sabatier *et al.*, 2014; Smalling *et al.*, 2013). The use of fungicidal compounds was noted to alter the structure and function of aquatic communities, culminating in severe physiological pathologies, such as increased mortality, reduced reproductive rates, and decreased enzyme activity, for zooplankton, gastropods, amphibians, and earthworms (McMahon *et al.*, 2012; Rico *et al.*, 2016; Zubrod *et al.*, 2011). Furthermore, fungicidal compounds have been implicated in reduced bee health and abnormal behaviors, such as reduced nest recognition, decreased colony initiation, and uncoupled mitochondrial respiration, all of which may contribute to the decline of bee populations (Elston *et al.*, 2013; Simon-Delso *et al.*, 2014; Syromyatnikov *et al.*, 2017). The development of any new species-specific fungicides should provide both environmentally-safer control strategies, but also provide effective control of the fungus to ensure improved crop yields.

Recently, a new generation of species-specific control methods, taking advantage of a cellular defence mechanism called RNA interference (RNAi), demonstrated successful control of insects, nematodes, viruses, and parasitic plants (Alakonya *et al.*, 2012; Papolu *et al.*, 2013; Schmitt-Engel *et al.*, 2015; Whyard *et al.*, 2009). The first commercially approved, transgenic plants carrying RNAi constructs against corn rootworm (*Diabrotica virgifera virgifera* LeConte) and bean golden mosaic virus were approved for cultivation in the US and Brazil, respectively (Tollefson, 2011; United States Environmental Protection Agency, 2017). Despite the successes of RNAi approaches, few studies have applied this revolutionary technique for the management of fungal phytopathogens, likely due to the lack of reasonable target identification tools and poor fungal genomic annotation. Here, we describe how RNAi technology exploits intrinsic cellular pathways in eukaryotes for the development of novel fungal control strategies. A summary of aspects relating to safety of this new technology will be explored, as well as how integrating modern genomics techniques could help guide the development of next-generation RNAi-based control of fungal pathogens.

1.3 THE MECHANISM OF RNA INTERFERENCE

RNAi is a conserved pathway in eukaryotes that protects cells from viruses and controls transposon activity. The mechanism utilizes short interfering RNAs (siRNAs) to guide the targeted degradation of transcripts using sequence homology (Torres-Martínez and Ruiz-Vázquez, 2017). The description of RNAi in *Caenorhabditis elegans* (Maupas) Dougherty by Andrew Fire and Craig Mello earned them the Nobel Prize for Medicine in 2006 (Fire *et al.*, 1998; Nobel Media AB, 2017). However, similar phenomena had been noted in other organisms. Romano and Macino (1992) (Romano and Macino, 1992) earlier had described a

post-transcriptional silencing phenomenon, which they termed quelling, in the ascomycete *Neurospora crassa* Shear & B.O. Dodge. The core proteins in the quelling pathway are the same proteins implicated in the RNAi pathway: ARGONAUTE (AGO), QUELLING DEPENDENT-2 (QDE-2), DICER-like (DCL), and RNA-dependent RNA Polymerase (RdRP) (Torres-Martínez and Ruiz-Vázquez, 2017).

Since RNAi is an intrinsic cellular defence process against invading double stranded RNA (dsRNA) viruses, introducing *in vitro* synthesized dsRNAs or producing the molecules *in planta* exploits this cellular reaction as a crop management strategy naturally (Wang and Jin, 2017) (Fig. 1.1a). Transport of long dsRNA and shorter, small interfering RNA (siRNA) into *B. cinerea* spores was observed using fluorescein-labeled nucleotides (nt) (Wang *et al.*, 2016a); however, the mechanism of transport remains undefined in fungi. In both humans and *C. elegans*, dsRNA diffuses passively through a dsRNA-specific channel, SID1 (Duxbury *et al.*, 2005; Whangbo *et al.*, 2017). Homologues of the SID1 channel do not exist in fungi, and thus, dsRNA transport must occur via an alternative mechanism(Wang *et al.*, 2016a). In various insects, exogenous dsRNA is transported using receptor-mediated clathrin endocytosis (reviewed in Huvenne and Smagghe, 2010) (Huvenne and Smagghe, 2010). An "*RNAi of RNAi*" approach provided evidence for endocytic involvement in dsRNA transport. Initially, cells were treated with dsRNA targeting a specific component implicated in clathrin-mediated endocytosis, before a following treatment of GFP-dsRNA was applied. Using a GFP-reporter system, components involved in uptake of the molecules could be elucidated by observing a diminished fluorescent signal. Thus, the components of receptor mediated endocytosis were inferred to be clathrin heavy chain, clathrin adaptor protein 50, vacuolar H^+ ATPase, and ADP ribosylation factor ARF72A (Saleh *et al.*, 2006). Similarly, application of chemical inhibitors confirmed endocytosis as a

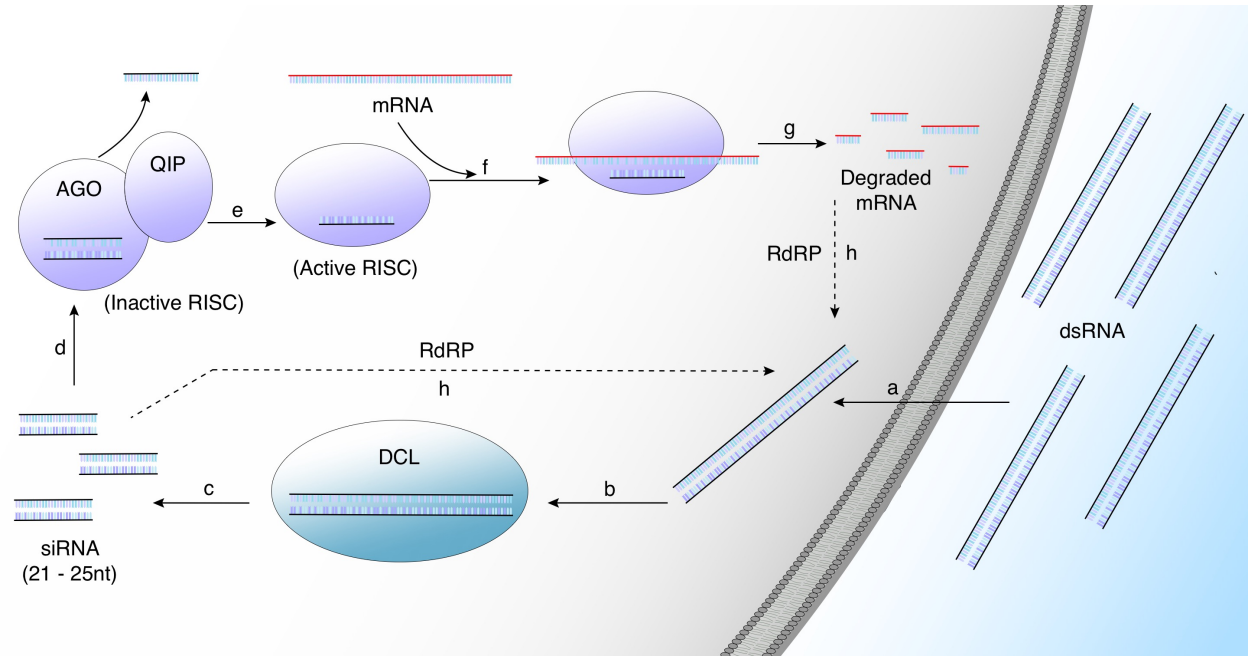


Figure 1.1. Overview of the mechanism of RNAi within fungal hyphae. Upon encountering double stranded RNA (dsRNA), the molecules are transported into the cytoplasm through an undefined mechanism (**a**). Once in the cytoplasm, the molecules are recognized by DICER-LIKE (DCL) (**b**), which cleaves the molecules into small interfering RNA (siRNA) 21-25 nucleotides (nt) in length (**c**). The siRNA molecules then complex with ARGONAUTE (AGO) (**d**), which nicks the siRNA and recruits QUELLING DEFICIENT-2 INTERACTING PROTEIN (QIP) to degrade the passenger strand (**e**). With the removal of the passenger strand, RISC becomes activated to seek messenger RNA (mRNA) transcripts with complementary sequences to the internal siRNA (**f**) for degradation (**g**). The degraded mRNA and the siRNA can function as primers in secondary dsRNA synthesis using RNA-dependent RNA Polymerase (RdRP) (**h**) to further amplify gene silencing.

secondary pathway of dsRNA uptake in *C. elegans* (Xiao *et al.*, 2015). Without SID1, clathrin-mediated endocytic pathways may play an integral function in the transport of dsRNA in fungi.

Once in the cytoplasm, the presence of a dsRNA molecule is recognized by the dsRNA binding domain of DCL1 or DCL2 (Lee *et al.*, 2010; Li *et al.*, 2010) (Fig. 1.1b). Upon recognition of dsRNA, DCL recruits the SAGA complex with histone acetyltransferase activity to increase transcription from the *dcl* and *ago* promoters, and mobilize RNAi machinery within the fungus (Andika *et al.*, 2017). To cleave dsRNA molecules, the 5' end of the dsRNA anchors in the PAZ (Piwi-Argonaute-Zwille/Pinhead) domain within DCL, allowing two consecutive RNase III domains to cleave the ribose-phosphate backbone, resulting in siRNAs of 21-25 nt in length with a 5' monophosphate and a 3' 2 nt overhang (Kandasamy and Fukunaga, 2016) (Figure 1.1c). Once the double stranded siRNA is generated, AGO complexes with the siRNA to recruit QIP (QDE-2-INTERACTING PROTEIN) and form the RNA-induced silencing complex (RISC) (Dang *et al.*, 2011) (Fig. 1.1d). Once bound, AGO nicks the siRNA duplex; QIP recognizes and degrades the nicked passenger strand with exonuclease activity (Cheng *et al.*, 2016; Maiti *et al.*, 2007) (Fig. 1.1e). The RISC complex then becomes activated to seek transcripts with complementary sequences to the remaining siRNA strand, termed the guide strand (Fig. 1.1f). When a messenger RNA (mRNA) base pairs to the guide strand in RISC, exonuclease activity is activated to degrade complementary RNA, resulting in a reduction of mRNA accumulation within the fungal hyphae (Dang *et al.*, 2011) (Fig. 1.1g).

Some of the resulting siRNAs produced during long dsRNA processing and the degraded target mRNA could act as a primer to initiate RdRP activity and temporarily sustain silencing (Fig. 1.1h). siRNAs anneal to complementary mRNA transcripts in the absence of DCL to act as a primer for second strand synthesis through recruited RdRPs. The secondary dsRNAs produced

by RdRPs invoke further RISC complex formation to amplify RNAi-mediated silencing (Ghildiyal and Zamore, 2009; Villalobos-Escobedo *et al.*, 2016). The existence of RdRPs vary greatly amongst fungi, with species like *Fusarium graminearum* (Schwienitz) Petch having five, while others, like *N. crassa,* have two (Chen *et al.*, 2015; Zong *et al.*, 2009). The presence of RdRP may be required for potent silencing to occur, as is the case for *Mucor circinelloides*. However, for most fungi, RdRPs are likely nonessential, but can serve a role in RNAi signal amplification as well as being involved in miRNA (microRNA) and transposon silencing (Calo *et al.*, 2012; Dang *et al.*, 2011).

1.4 THE SAFETY OF RNA INTERFERENCE TECHNOLOGY

The sequence-specific mechanism of RNAi provides unparalleled evidence that RNAi-based technologies offer safer and more environmentally-conscious alternatives to more traditional agrochemicals. DsRNA can be designed to avoid sequences of other organisms within the environment. Thorough ecological testing of the recently approved transgenic corn expressing RNAi constructs targeting corn rootworm best demonstrates the specificity of RNAi technology (United States Environmental Protection Agency, 2017). For example, both implementations of RNAi technology, transgenic plant material and topical *in vitro* synthesized dsRNA molecules, were used to assess cross-reactivity amongst a variety of invertebrates (e.g. *Apis mellifera* L., *Eisenia Andrei* Bouché, *Coleomegilla maculate* De Geer), vertebrates (e.g. *Gallus domesticus* L., *Ictalurus punctatus* Rafinesque), and soil microorganisms. No observable change in physiology, nutrient assimilation, or reproduction in the tested organisms was observed, regardless of the dsRNA source (Bachman *et al.*, 2016). The expression of interfering RNA in the pollen did not adversely affect honeybees (*A. mellifera*) during the experiment, and previously, diet-derived miRNA from pollen demonstrated negligible transfer to and

dissemination within honeybees (Bachman *et al.*, 2016; Masood *et al.*, 2016). Furthermore, honeybee feeding assays of long dsRNA not specific for any honeybee gene also failed to demonstrate an effect on the bees (Tan *et al.*, 2016). The findings from these studies suggest that honeybees exposed to exogenously delivered dsRNAs, whether derived from either transgenic plants or foliar applications, may not pose serious threats to these important pollinators. Further studies will likely be required to satisfy government regulators that any dsRNA applied to a crop does not affect honeybees or any other species within the surrounding environment.

One asset of RNAi-based control is the stability of the dsRNA molecule within the phyllosphere (Miguel and Scott, 2015). Synthetic molecules can be readily made in the laboratory and are more thermodynamically stable than single stranded RNA (Nicholson, 2014; Wang and Jin, 2017). Due to the double stranded structure of the molecules, dsRNAs are also more resilient to nuclease degradation than mRNA (Aryani and Denecke, 2015; Hoerter *et al.*, 2011). As a topical application, dsRNA molecules were bioactive against Colorado potato beetle (*Leptinotarsa decemlineata* Say) on the surface of potato (*Solanum tuberosum* L.) leaves for over 28 days under greenhouse conditions (Miguel and Scott, 2015). Using natural chemistries, such as clay nanosheets (BioClay)(Mitter *et al.*, 2017), dsRNA efficacy was improved against cucumber mosaic virus and pepper mild mottle virus under adverse environmental conditions. The clay nanosheets shielded the dsRNA molecules from environmental RNase III degradation and improved adhesion to the leaf surface (Ladewig *et al.*, 2009; Mitter *et al.*, 2017). Thus, dsRNA induced gene silencing could be a suitable solution as foliar application against phyllophillic pathogens.

Another advantage of RNAi management is the lack of persistence within the pedosphere (Dubelman *et al.*, 2014). Regardless of dsRNA molecule size or the composition of the

agricultural soil, the majority of dsRNA rapidly degrades within 24 hours (Dubelman *et al.*, 2014; Fischer *et al.*, 2016). Similarly, dsRNAs were demonstrated to dilute and degrade greatly within 96 hours upon entering water systems (Albright *et al.*, 2017). Likely, bacterial RNA metabolic processes and RNase III enzymes cause the dsRNA degradation in the soil and aquatic environments (Cho, 2017; Urich *et al.*, 2008). Therefore, dsRNA sourced from plant material or foliar sprays will not likely disseminate far from the point of application through soil leaching. Coupled with its sequence specificity, RNAi-based fungicides are unlikely to have environmentally adverse effects compared to traditional chemical methods of fungal control and therefore, they represent an attractive alternative to the broad-spectrum fungicides currently in use.

For consumer food production, RNAi technology would not differ from the current consumption of small RNAs (sRNAs) that naturally populate grains, fruits, vegetables, and meats. Large pools of sRNAs, which include siRNAs, miRNAs, piRNA, and endogenous dsRNA, are produced from a variety of sources, such as viruses, transposons, or the host cell itself (Frizzi *et al.*, 2014; Ivashuta *et al.*, 2009). *In silico* analyses predict siRNAs with 100% complementarity to human mRNA transcripts, yet no pathological effects have been associated with daily consumption of sRNA (Jensen *et al.*, 2013). Humans inherently have evolved many barriers that would prevent or limit the transport of sRNA, such as nucleases in the saliva and gastrointestinal track, acid in the stomach, and the unfavourable transport of large, polar molecules across a hydrophobic membrane (Juliano *et al.*, 2009; O'Neill *et al.*, 2011). The lack of dietary siRNA efficacy was supported in mouse model research. Mice were dosed daily with either siRNA or long dsRNA targeting an essential vacuolar ATPase over a 28-day period and no adverse cellular pathologies were noted (Petrick *et al.*, 2015). The lack of bioavailability could

be attributed to instability passing through the gastrointestinal track or rapid metabolism in the bloodstream (Christensen *et al.*, 2013; Dickinson *et al.*, 2013). Therefore, any dsRNA or siRNA molecules present should not elicit adverse effects from dietary sources rich in nucleic acid.

Furthermore, many food products undergo many processing techniques before consumer consumption (Chemat *et al.*, 2017; Misra *et al.*, 2017). The majority of processing techniques (baking, microwaving, solvent extraction, thermal treatment, fermentation, acidification, alkalization, and bleaching) result in effective nucleic acid destabilization and degradation prior to the final food product (Gryson, 2010; Vijayakumar *et al.*, 2009). For example, edible oils undergo multiple steps involving heat, pressure, and solvent treatments, which exclude polar molecules, such as nucleic acid, and/or result in molecule fragmentation (Belur *et al.*, 2017; Mba *et al.*, 2015). Similarly, heating and purification in sugar production eliminates DNA by a factor of 10^{14} (Klein *et al.*, 1998). Based on the chemical and physical similarities, dsRNA would likely have a similar fate to DNA during food processing (Forbes and Peppas, 2012; Lipfert *et al.*, 2014). Thus, any nucleic acid introduced from RNAi technology would not survive food processing. Taken together, RNAi technology, due to both the chemistry of the RNA molecules and the sequence-specificity of the molecule, could be considered a safe, green technology, which can be expressed as a novel trait (transgene) or through topical formulations.

1.5 DEVELOPMENT OF NOVEL TRAITS THOUGH HOST-INDUCED GENE SILENCING

Host-induced gene silencing (HIGS) is an emerging biotechnology in which plants are engineered to produce sRNAs capable of silencing target genes of a target organism. HIGS utilizes the RNAi pathway by equipping the host plant with hairpin RNAs (hpRNAs) containing

sequence homology to target genes. Upon transcription, these hpRNA molecules mimic dsRNA and initiate the inherent cellular RNAi pathway. Export of hpRNA from the plant nucleus to the cytoplasm is hypothesized to be facilitated by the binding of an exportin protein HASTY to guide successful nucleocytoplasmic transport (Bollman, 2003; Tuan Le and Wang, 2011). Once in the cytoplasm, hpRNA initiates the host RNAi pathway leading to the generation of approximately 21 nt siRNA molecules, as in the case for fungi. For siRNAs to function in target gene silencing, they must undergo successful transfer from host plant cell to the pest or pathogen. While evidence suggests target gene silencing in pathogenic fungal species may operate through host derived siRNAs (Panwar *et al.*, 2013), the mechanism of siRNA transfer from host to pathogen remains unclear. Studies in animal systems show that secreted miRNA may be associated with host-derived exosomes and lead to successful transfer between organisms (Valadi *et al.*, 2007). Exosomal uptake by the receiving cell is hypothesized to utilize exosome-mediated endocytosis, where the vesicular membrane of the sRNA containing exosome fuses with the receiving plasma membrane leading to the release of sRNA into the pathogen's cytoplasm (Valadi *et al.*, 2007). Another hypothesis for potential sRNA transfer involves transmembrane transporter-mediated uptake without utilization of host derived vesicles. Previously, membrane-free sRNAs were found within the extracellular space and associated with high density lipoproteins. These lipoproteins may facilitate successful transfer of extracellular sRNA to recipient cells (Vickers *et al.*, 2011). While there is still much to discover about the mechanisms of HIGS, this technology represents a promising strategy to protect plants against difficult to control fungal pathogens.

The safety and efficacy of HIGS technology led to the approval of two RNAi crops for commercial production in the United States and Brazil. The Brazilian National Technical

Commission approved RNAi pinto beans (*Phaseolus vulgaris*) for commercial production in 2011 (Tollefson, 2011). Pinto beans were engineered to disrupt early viral replication of the Bean Golden Mosaic Virus by targeting the viral gene AC1 (Bonfim *et al.*, 2007). Similarly, the Environmental Protection Agency approved maize plants expressing dsRNA targeting DvSnf4, a component of the ESCRT-III complex involved in endosomal sorting and lysosomal degradation in corn rootworms, *D. virgifera virgifera*, for production in the US (Bolognesi *et al.*, 2012; United States Environmental Protection Agency, 2017). DvSnf4 dsRNA was potent at low doses, leading to accumulation of ubiquinated proteins in the midgut cells of larvae. Since autophagy was impaired, the cells malfunctioned, the gut's digestive processes ceased, and the insects failed to grow and eventually died (Baum *et al.*, 2007; Ramaseshadri *et al.*, 2013). Given the flexibility and utility of the technology, when applied to fungal pathogens affecting crops, RNAi-HIGS technology has the potential for regulatory approval and full scale agronomic production.

1.6 *IN PLANTA* EXPRESSION OF HPRNA OVERCOMES FUNGAL PRESSURE

The creation of stably transformed plants expressing dsRNA molecules could impart full plant protection. HIGS has been implemented to reduce fungal pressure and toxin biosynthesis for a variety of phytopathogenic fungi (Koch *et al.*, 2013; Thakare *et al.*, 2017; Zhang *et al.*, 2016). Engineering novel traits like fungal resistance using genetic techniques is an efficient strategy to build resistance in crop plants. While traditional germplasm screening and breeding strategies have achieved limited success in providing resistance against *S. sclerotiorum* (Disi *et al.*, 2014), the use of HIGS in *Nicotinia tobacum* L. expressing hpRNA targeting fungal chitin synthase (*Sschs*) proved effective in controlling *S. sclerotiorum*. Expression of hpRNA reduced the level of *Sschs* mRNA within the fungus, indicating *S. sclerotiorum* could take up dsRNA

from the host through yet to be determined cellular mechanisms (Andrade *et al.*, 2016). Since phytopathogens are likely capable of taking up environmental dsRNA from host cells, HIGS could provide a functional strategy to reduce fungal pressure on the plant.

The root-pathogen interface could also be protected from many fungal pathogens that initiate root infections from the soil, such as *Verticillium sp.*, *Fusarium sp.,* and *Rhizoctonia solani* J.G. Kühn (De Coninck *et al.*, 2015; Tedersoo *et al.*, 2014). While topical formulations of dsRNA would likely degrade rapidly within the soil (Dubelman *et al.*, 2014), GM plants could deliver siRNA to the pathogen at the root-pathogen interface, as exemplified by transgenic RNAi cotton (*Gossypium sp.*). Roots of cotton expressing hpRNA targeting *Verticillium dahlia*e Kleb hygrophobins1 gene were able to resist severe root infection sufficiently compared to the wilted non-transformed control (Zhang *et al.*, 2016). Additionally, plants engineered with RNAi constructs expressing hpRNA could convey resistance throughout the plant lifecycle. Transgenic banana plants (*Musa spp.*) expressing hpRNA directed at either *F. oxysporum* f. sp. *cubense* genes *velvet* or *Fusarium transcription factor 1*, resisted infection for 8 months post-inoculation (Ghag *et al.*, 2014). Thus, engineered plants expressing novel RNAi traits conferring resistance against economically important plant pathogens represents an additional level of durability that would benefit growers interested in sustainable crop protection technologies.

1.7 FOLIAR APPLICATIONS OF DSRNA REDUCED FUNGAL DISEASE SYMPTOMS

Foliar dsRNA applications offer shorter term protection from fungal infections, relative to transgene mediated resistance, but nevertheless, they could be particularly useful to protect agri-food products during post-harvest storage and in protecting plants without defined or

efficient transformation protocols for HIGS (Wang and Jin, 2017). Despite recent advances to control insect pests and viral pathogens, few studies have implemented *in vitro* synthesized molecules for fungal control. In a pioneering study by Mumbanza *et al.* (2013), 14 different genes involved in processes such as transcription, RNA modification, DNA replication, and intracellular transport were tested *in vitro*, using nutrient plates rather than plants, for spore germination inhibition in *F. oxysporum* f. sp. *cubense* and *Mycosphaerella fijiensis* Morelet. Treatment of fungal spores with dsRNA molecules inhibited germination of the two banana pathogens by up to 95.9% and 65.8% respectively. *In vitro* testing provided compelling evidence for the potential for fungal suppression, despite not having tested these molecules on their plant hosts.

Recently, topical application of dsRNA to the surface of treated barley (*Hordeum vulgare* L.) leaves reduced *F. graminearum* growth. By targeting three fungal cytochrome P450 lanosterol C-14α demethylases, required for fungal ergosterol synthesis and a common fungicide target, reductions over 50% of both target transcript and fungal DNA accumulation were achieved (Koch *et al.*, 2016). Similarly, using long dsRNA targeting Dcl1 and Dcl2 simultaneously in *B. cinerea,* Wang *et al.* (2016)(Wang *et al.*, 2016a) demonstrated remarkable levels of protection in *Arabidopsis thaliana* (L.) Heynh, tomato, grape, strawberry, onion, and rose petals from infection, with average lesion sizes, transcript levels, and fungal DNA all being reduced by over 80%. The observed protection in a wide-range of host tissues suggests broad utility of the technology in the protection of food and ornamental species. The protection of barley leaves and tomato leaves demonstrates the utility for field management of crops, while reduced fungal presence on various fruits and vegetables could be useful after harvest, protecting food in transport, storage, or on store shelves (Koch *et al.*, 2016; Wang *et al.*, 2016a). Taken

together, these studies provide solid evidence of the flexibility of RNAi-based molecular sprays that confer protection along the food production pipeline.

1.8 RNA SEQUENCING AS AN INFORMATIVE GUIDE FOR RNA INTERFERENCE

Despite the advances in the development of RNAi-based phytopathogenic control, the selection of target genes still represents a significant challenge in the design and effectiveness of fungal suppression. Previously, RNAi targets were selected using gene deletions or chemical inhibition. For example, Koch *et al.* (2016) chose a common fungicidal target, while Wang *et al.* (2016) developed foliar applications based on previous genetic deletions of *B. cinerea* strains (Weiberg *et al.*, 2013). Fungal transformation protocols are lengthy and at times inefficient, thereby limiting the effective size of target gene identification screens (Meyer, 2008). However, dual RNA-sequencing of both fungal pathogen and host plant now provides unprecedented opportunities to identify novel RNAi targets based on the transcriptome atlas of the pathogen lifecycle, infection state, tissue type, host defense response, or treatment conditions (Westermann and Barquist, 2017). Genes implicated in plant resistance response provide useful information for plant breeding and manipulation, while specific fungal responses essential for pathogenesis could eventually be used as targets for RNAi-based fungal control (Girard *et al.*, 2016). For example, transcriptomic sequencing across major developmental stages of *S. sclerotiorum* uncovered genes important for pathogenicity and development. In particular, two small cysteine-rich effector proteins, SsCVMH and SsSSVP1, as well as a microbial opsin homologue were upregulated during infection (Lyu *et al.*, 2015). *S. sclerotiorum* transformed with RNAi silencing vectors targeting these effector proteins significantly inhibited the ability of the fungus to colonize *Brassica napus* L.. (Lyu *et al.*, 2015; Lyu *et al.*, 2016a; Lyu *et al.*, 2016b). Clearly,

RNA-seq identification of transcripts essential for pathogenesis could be very useful in the development of future RNAi target genes for fungal control.

The lack of development of RNAi-based fungal strategies is likely due to poor genomic annotation of many fungi and a lack of accessible bio-computational platforms. User-friendly programs, such as FungiFun2 (elbe.hki-jena.de/fungifun/fungifun.php; Priebe *et al.*, 2015) and SeqEnrich (belmontelab.com; Becker *et al.*, 2017), have overcome many of the problems associated with both a lack of functional annotation and complicated bioinformatics analyses. Once gene ontology (GO) is resolved using FungiFun2, researchers could use SeqEnrich, to identify significantly upregulated GO terms and genes. Melding both programs together would provide a solid basis for the execution of RNAi-based technologies. Upregulated transcripts and processes could then be selected for the development of either foliar implementations or HIGS technology. RNA-seq provides greater depth and efficiency for the rapid identification of hundreds of critical, upregulated transcripts and processes within infecting pathogens. Moreover, RNA-seq offers a marked improvement over previous searches reliant on labor-intensive fungal transformation studies. The use of RNA sequencing technology has the potential to revolutionize and expedite the next-generation of species-specific fungal management strategies.

1.9 OUTLOOK

RNAi technology provides a flexible and environmentally-conscious solution to combat an array of devastating pathogens. Both HIGS and foliar dsRNAs represent potent and sustainable next-generation strategies capable of controlling pathogens that affect global food production. Although further research exploring the interaction of dsRNA within food should be explored, RNAi strategies have proven to be safe through its species-specificity and molecule

degradation during food processing. Fundamental aspects of small molecule transport have yet to uncover the underlying molecular mechanism of RNAi-based control of fungal species. Additionally, the identification of new targets for pathogen control may provide additional clues into conserved pathogenesis genes or common targets for broad levels of efficacious fungal control. Furthermore, RNA sequencing could provide an effective and efficient method for recognizing targets for RNAi, accelerating the further establishment of novel, species-specific fungicides. The field of RNAi technology is developing for fungi, RNAi is a sustainable solution to dealing with fungal resistance and food security challenges.

1.10 RESEARCH OBJECTIVES

Canada's canola (*B.napus*) fields are greatly threatened by the necrotrophic pathogen, *S. sclerotiorum*. Traditional strategies to control fungi have relied on chemical fungicides, which cause deleterious effects on the agro-ecological landscape and promote fungicide resistance. Given the economic importance of the crop and the need for green management strategies, there is an opportunity to develop modern RNAi tools to ameliorate fungal pressure. RNAi-based controls have been implemented previously to control the necrotrophic phytopathogens *F. graminearum* and *B. cinerea*. Most previous *S. sclerotiorum* research focused on fungal transformations, which do not represent a pragmatic approach for target identification or control. Therefore, the objective of my research was to develop a higher throughput approach to RNAi-based control against *S. sclerotiorum*. Fusing the modern technologies, RNAi and RNA-seq, I will elucidate the criteria that define potent RNAi control and provide a demonstration of the utility of both foliar dsRNA molecules and the protection imparted by *in planta* production of hpRNA using host-induced gene silencing (HIGS).

1.11 BIOLOGICAL QUESTIONS AND HYPOTHESES

1.11.1 Target Identification and development of topical RNAi-based technology for fungal control

Question: Can foliar applications of in vitro synthesized dsRNA control fungal pathogens effectively? Are there patterns between well- and poor- performing targets? Can homologous targets in related species offer protection?

Hypothesis:

I hypothesize that RNAi targets can be identified from an RNA-seq dataset, which compared *S. sclerotiorum* infection on susceptible and tolerant *B. napus* cultivars. Additional targets could also be determined from the Database of Essential Genes. Using qRT-PCR, I will demonstrate capability of synthesized molecules to reduce transcript levels *in vitro*. Using *B. napus* and *Arabidopsis thaliana* infection screens, I will demonstrate the functional utility of foliar dsRNA molecules to reduce lesion size, uncovering trends for target identification for RNAi. Finally, I expect to show board-level control when targeting homologous targets using dsRNA in the close-related fungus, *B. cinerea*.

Relevance:

RNAi technology is more specific and environmentally-conscious than traditional agrochemicals. However, there is a lack of RNAi-based control for fungal phytopathogens, despite the characterization of functional RNAi pathways. The major issue of target identification represents a significant gap for RNAi fungal control. RNA-seq could provide the pragmatic link between transcripts implicated in pathogenesis and

target identification for the development of RNAi management. A similar method informed by transcriptomics could be deployed in a variety of other economically important species.

1.11.2 Host-induced gene silencing (HIGS) of *S. sclerotiorum*

Question: Can plants engineered with silencing constructs convey potent protection to resist fungal infection pressure?

Hypothesis:

I hypothesize that engineering plants with RNAi silencing vectors will repress fungal proliferation through the transcription and subsequent processing of hpRNA. The siRNA molecules produced *in planta* will be sufficient to diminish lesion size, fungal DNA presence, and transcript abundance in the leaf tissue.

Relevance:

Current germplasm screening and breeding approaches to develop host resistance have achieved limited success. Using HIGS, resistance could be engineered against *S. sclerotiorum* in a more targeted and efficient manner. The *in-planta* application of RNAi could protect plants throughout the duration of the lifecycle. Together, my body of work will provide a platform for additional applications of informed RNAi biotechnologies to control fungal pathogens.

1.12 REFERENCES

Alakonya, A., Kumar, R., Koenig, D., et al. (2012) Interspecific RNA Interference of SHOOT MERISTEMLESS-Like Disrupts *Cuscuta pentagona* Plant Parasitism. *Plant Cell* **24**, 3153–3166.

Albright, V.C., Wong, C.R., Hellmich, R.L. and Coats, J.R. (2017) Dissipation of double-stranded RNA in aquatic microcosms. *Environ. Toxicol. Chem.* **36**, 1249–1253.

Amaradasa, B.S. and Everhart, S.E. (2016) Effects of Sublethal Fungicides on Mutation Rates and Genomic Variation in Fungal Plant Pathogen, *Sclerotinia sclerotiorum*. *PLoS One* **11**, e0168079.

Andika, I.B., Jamal, A., Kondo, H. and Suzuki, N. (2017) SAGA complex mediates the transcriptional up-regulation of antiviral RNA silencing. *Proc. Natl. Acad. Sci.* **114**, E3499–E3506.

Andrade, C.M., Tinoco, M.L.P., Rieth, A.F., Maia, F.C.O. and Aragão, F.J.L. (2016) Host-induced gene silencing in the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*. *Plant Pathol.* **65**, 626–632.

Aryani, A. and Denecke, B. (2015) In vitro application of ribonucleases: comparison of the effects on mRNA and miRNA stability. *BMC Res. Notes* **8**, 164.

Bachman, P.M., Huizinga, K.M., Jensen, P.D., Mueller, G., Tan, J., Uffman, J.P. and Levine, S.L. (2016) Ecological risk assessment for DvSnf7 RNA: A plant-incorporated protectant with targeted activity against western corn rootworm. *Regul. Toxicol. Pharmacol.* **81**, 77–88.

Baum, J. a, Bogaert, T., Clinton, W., et al. (2007) Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* **25**, 1322–6.

Bebber, D.P. and Gurr, S.J. (2015) Crop-destroying fungal and oomycete pathogens challenge food security. *Fungal Genet. Biol.* **74**, 62–64.

Bebber, D.P., Holmes, T. and Gurr, S.J. (2014) The global spread of crop pests and pathogens. *Glob. Ecol. Biogeogr.* **23**, 1398–1407.

Becker, M.G., Walker, P.L., Pulgar-Vidal, N.C. and Belmonte, M.F. (2017) SeqEnrich: A tool to predict transcription factor networks from co-expressed *Arabidopsis* and *Brassica napus* gene sets. *PLoS One* **12**, 1–13.

Belur, P.D., Iyyasami, R., Sampath, C. and Chandrasekhar, V. (2017) Refining Technologies for Edible Oils. In Edible Oils Estraction, Processing, and Applications., pp. 99–128. Boca Raton, FL, USA: Taylor & Francis Group.

Bollman, K.M. (2003) HASTY, the Arabidopsis ortholog of exportin 5/MSN5, regulates phase change and morphogenesis. *Development*.

Bolognesi, R., Ramaseshadri, P., Anderson, J., et al. (2012) Characterizing the Mechanism of Action of Double- Stranded RNA Activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). **7**.

Bonfim, K., Faria, J.C., Nogueira, E.O.P.L., Mendes, É.A., Aragão, F.J.L., Recursos, E., Norte, P.W. and Brasília, U. De (2007) RNAi-Mediated Resistance to Bean golden mosaic virus in Genetically Engineered Common Bean (*Phaseolus vulgaris*). **20**, 717–726.

Calo, S., Nicolás, F.E., Vila, A., Torres-Martínez, S. and Ruiz-Vázquez, R.M. (2012) Two distinct RNA-dependent RNA polymerases are required for initiation and amplification of RNA silencing in the basal fungus *Mucor circinelloides*. *Mol. Microbiol.* **83**, 379–394.

Castroagudín, V.L., Ceresini, P.C., Oliveira, S.C. de, Reges, J.T.A., Maciel, J.L.N., Bonato, A.L. V, Dorigan, A.F. and McDonald, B.A. (2015) Resistance to QoI Fungicides Is Widespread in Brazilian Populations of the Wheat Blast Pathogen *Magnaporthe oryzae*. *Phytopathology* **105**, 284–94.

Chemat, F., Rombaut, N., Meullemiestre, A., Turk, M., Perino, S., Fabiano-Tixier, A.S. and Abert-Vian, M. (2017) Review of Green Food Processing techniques. Preservation,

transformation, and extraction. *Innov. Food Sci. Emerg. Technol.* **41**, 357–377.

Chen, Y., Gao, Q., Huang, M., Liu, Y., Liu, Z., Liu, X. and Ma, Z. (2015) Characterization of RNA silencing components in the plant pathogenic fungus *Fusarium graminearum*. *Sci. Rep.* **5**, 12500.

Cheng, L., Ling, J., Liang, L., Luo, Z., Zhang, J. and Xie, B. (2016) Qip gene in *Fusarium oxysporum* is required for normal hyphae morphology and virulence. **1203**.

Cho, K.H. (2017) The structure and function of the gram-positive bacterial RNA degradosome. *Front. Microbiol.* **8**, 1–10.

Christensen, J., Litherland, K., Faller, T., Kerkhof, E. Van De, Natt, F., Hunziker, J., Krauser, J. and Swart, P. (2013) Metabolism Studies of Unformulated Internally [3 H] - Labeled Short Interfering RNAs in Mice s.

Cointe, R. Le, Simon, T.E., Delarue, P., Hervé, M., Leclerc, M. and Poggi, S. (2016) Reducing the use of pesticides with site-specific application: The chemical control of *Rhizoctonia solani* as a case of study for the management of soil-borne diseases. *PLoS One* **11**, 1–18.

Coninck, B. De, Timmermans, P., Vos, C., Cammue, B.P.A. and Kazan, K. (2015) What lies beneath: Belowground defense strategies in plants. *Trends Plant Sci.* **20**, 91–101.

Dang, Y., Yang, Q., Xue, Z. and Liu, Y. (2011) RNA Interference in Fungi: Pathways, Functions, and Applications. *Eukaryot. Cell* **10**, 1148–1155.

Dickinson, B., Zhang, Y., Petrick, J., Heck, G. and Ivashuta, SergeyMarshall, W.S. (2013) Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. *Nat. Biotechnol.* **31**, 965–967.

Disi, J.O., Mei, J., Wei, D., Ding, Y. and Qian, W. (2014) Inheritance of leaf and stem

resistance to *Sclerotinia sclerotiorum* in a cross between *Brassica incana* and *Brassica oleracea* var. alboglabra. *J. Agric. Sci.* **152**, 146–152.

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., Levine, S. and Carson, D. (2014) Environmental fate of double-stranded RNA in agricultural soils. *PLoS One* **9**, 1–7.

Duxbury, M.S., Ashley, S.W. and Whang, E.E. (2005) RNA interference: A mammalian SID-1 homologue enhances siRNA uptake and gene silencing efficacy in human cells. *Biochem. Biophys. Res. Commun.* **331**, 459–463.

Elston, C., Thompson, H.M. and Walters, K.F.A. (2013) Sub-lethal effects of thiamethoxam, a neonicotinoid pesticide, and propiconazole, a DMI fungicide, on colony initiation in bumblebee (*Bombus terrestris*) micro-colonies. *Apidologie* **44**, 563–574.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.

Fischer, J.R., Zapata, F., Dubelman, S., Mueller, G.M., Jensen, P.D. and Levine, S.L. (2016) Characterizing a novel and sensitive method to measure dsRNA in soil. *Chemosphere* **161**, 319–324.

Forbes, D.C. and Peppas, N.A. (2012) Oral delivery of small RNA and DNA. *J. Control. Release* **162**, 438–445.

Frizzi, A., Zhang, Y., Kao, J., Hagen, C. and Huang, S. (2014) Small RNA profiles from virus-infected fresh market vegetables. *J. Agric. Food Chem.* **62**, 12067–12074.

Ghag, S.B., Shekhawat, U.K.S. and Ganapathi, T.R. (2014) Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance

against Fusarium wilt in banana. *Plant Biotechnol. J.* **12**, 541–53.

Ghildiyal, M. and Zamore, P.D. (2009) Small silencing RNAs: an expanding universe. *Nat. Rev. Genet.* **10**, 94–108.

Girard, I.J., McLoughlin, A.G., Kievit, T.R. De, Fernando, D.W.G. and Belmonte, M.F. (2016) Integrating large-scale data and RNA technology to protect crops from fungal pathogens. *Front. Plant Sci.* **7**.

Gryson, N. (2010) Effect of food processing on plant DNA degradation and PCR-based GMO analysis : a review. , 2003–2022.

Hoerter, J.A.H., Krishnan, V., Lionberger, T.A. and Walter, N.G. (2011) SiRNA-like double-stranded RNAs are specifically protected against degradation in human cell extract. *PLoS One* **6**, 1–9.

Huvenne, H. and Smagghe, G. (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *J. Insect Physiol.* **56**, 227–235.

Huzar-Novakowiski, J., Paul, P.A. and Dorrance, A.E. (2017) Host resistance and chemical control for management of Sclerotinia stem rot of soybean in Ohio. *Phytopathology* **107**, 937–949.

Ivashuta, S.I., Petrick, J.S., Heisel, S.E., Zhang, Y., Guo, L., Reynolds, T.L., Rice, J.F., Allen, E. and Roberts, J.K. (2009) Endogenous small RNAs in grain: Semi-quantification and sequence homology to human and animal genes. *Food Chem. Toxicol.* **47**, 353–360.

Jensen, P.D., Zhang, Y., Wiggins, B.E., Petrick, J.S., Zhu, J., Kerstetter, R. a, Heck, G.R. and Ivashuta, S.I. (2013) Computational sequence analysis of predicted long dsRNA transcriptomes of major crops reveals sequence complementarity with human genes. *GM Crops Food* **4**, 90–7.

Juliano, R., Bauman, J., Kang, H. and Ming, X. (2009) Biological Barriers to Therapy with Antisense and siRNA. *Mol. Pharm.* **6**, 686–695.

Kandasamy, S.K. and Fukunaga, R. (2016) Phosphate-binding pocket in Dicer-2 PAZ domain for high-fidelity siRNA production. *Proc. Natl. Acad. Sci.* **113**, 14031–14036.

Klein, J., Altenbuchner, J. and Mattes, R. (1998) Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets. **60**, 145–153.

Koch, A., Biedenkopf, D., Furch, A., et al. (2016) An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery. *PLoS Pathog.* **12**, 1–22.

Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J. and Kogel, K. (2013) Host-induced gene silencing of cytochrome P450 lanosterol C14 α -demethylase – encoding genes confers strong resistance to *Fusarium species*. **110**, 19324–19329.

Ladewig, K., Xu, Z.P. and Lu, G.Q. (Max) (2009) Layered double hydroxide nanoparticles in gene and drug delivery. *Expert Opin. Drug Deliv.* **6**, 907–922.

Lee, H.C., Li, L., Gu, W., et al. (2010) Diverse Pathways Generate MicroRNA-like RNAs and Dicer-Independent Small Interfering RNAs in Fungi. *Mol. Cell* **38**, 803–814.

Li, L., Chang, S.S. and Liu, Y. (2010) RNA interference pathways in filamentous fungi. *Cell. Mol. Life Sci.* **67**, 3849–3863.

Lipfert, J., Skinner, G.M., Keegstra, J.M., et al. (2014) Double-stranded RNA under force and torque: Similarities to and striking differences from double-stranded DNA. *Proc. Natl. Acad. Sci.* **111**, 15408–15413.

Lyu, X., Shen, C., Fu, Y., Xie, J., Jiang, D., Li, G. and Cheng, J. (2016a) A Small Secreted Virulence-Related Protein Is Essential for the Necrotrophic Interactions of *Sclerotinia*

sclerotiorum with Its Host Plants Ma, W., ed. *PLOS Pathog.* **12**, e1005435.

Lyu, X., Shen, C., Fu, Y., Xie, J., Jiang, D., Li, G. and Cheng, J. (2015) Comparative genomic and transcriptional analyses of the carbohydrate-active enzymes and secretomes of phytopathogenic fungi reveal their significant roles during infection and development. *Sci. Rep.* **5**, 15565.

Lyu, X., Shen, C., Fu, Y., Xie, J., Jiang, D., Li, G. and Cheng, J. (2016b) The microbial opsin homolog sop1 is involved in Sclerotinia sclerotiorum development and environmental stress response. *Front. Microbiol.* **6**, 1–14.

Maiti, M., Lee, H.-C. and Liu, Y. (2007) QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. *Genes Dev.* **21**, 590–600.

Masood, M., Everett, C.P., Chan, S.Y. and Snow, J.W. (2016) Negligible uptake and transfer of diet-derived pollen microRNAs in adult honey bees. *RNA Biol.* **13**, 109–118.

Mba, O.I., Dumont, M.-J. and Ngadi, M. (2015) Palm oil: Processing, characterization and utilization in the food industry – A review. *Food Biosci.* **10**, 26–41.

McMahon, T.A., Halstead, N.T., Johnson, S., Raffel, T.R., Romansic, J.M., Crumrine, P.W. and Rohr, J.R. (2012) Fungicide-induced declines of freshwater biodiversity modify ecosystem functions and services. *Ecol. Lett.* **15**, 714–722.

Meyer, V. (2008) Genetic engineering of filamentous fungi - Progress, obstacles and future trends. *Biotechnol. Adv.* **26**, 177–185.

Miguel, K.S. and Scott, J.G. (2015) The next generation of insecticides : dsRNA is stable as a foliar-applied insecticide. *Pest Manag. Sci.*

Misra, N.N., Koubaa, M., Roohinejad, S., Juliano, P., Alpas, H., Inácio, R.S., Saraiva, J.A.

and Barba, F.J. (2017) Landmarks in the historical development of twenty first century food processing technologies. *Food Res. Int.* **97**, 318–339.

Mitter, N., Worrall, E.A., Robinson, K.E., et al. (2017) Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* **3**, 16207.

Mumbanza, F.M., Kiggundu, A., Tusiime, G., Tushemereirwe, W.K., Niblett, C. and Bailey, A. (2013) In vitro antifungal activity of synthetic dsRNA molecules against two pathogens of banana, *Fusarium oxysporum f. sp. cubense* and *Mycosphaerella fijiensis*. *Pest Manag. Sci.* **69**, 1155–1162.

Myers, S.S., Smith, M.R., Guth, S., Golden, C.D., Vaitla, B., Mueller, N.D., Dangour, A.D. and Huybers, P. (2017) Climate Change and Global Food Systems: Potential Impacts on Food Security and Undernutrition. *Annu. Rev. Public Health* **38**, 259–277.

Nicholson, A.W. (2014) Ribonuclease III mechanisms of double-stranded RNA cleavage. *Wiley Interdiscip. Rev. RNA* **5**, 31–48.

Nobel Media AB (2017) The Nobel Prize in Physiology or Medicine 2006.

O'Neill, M.J., Bourre, L., Melgar, S. and Driscoll, C.M.O. (2011) Intestinal delivery of non-viral gene therapeutics : physiological barriers and preclinical models. *Drug Discov. Today* **16**, 203–218.

Panwar, V., McCallum, B. and Bakkeren, G. (2013) Host-induced gene silencing of wheat leaf rust fungus *Puccinia triticina* pathogenicity genes mediated by the Barley stripe mosaic virus. *Plant Mol. Biol.* **81**, 595–608.

Papolu, P.K., Gantasala, N.P., Kamaraju, D., Banakar, P., Sreevathsa, R. and Rao, U. (2013) Utility of host delivered RNAi of two FMRF amide like peptides, flp-14 and flp-18, for the management of root knot nematode, *Meloidogyne incognita*. *PLoS One* **8**, 1–16.

Penaud, A. and Walker, A.-S. (2015) Oilseed Rape Pathogens in France. In Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. (Ishii, H. and Hollomon, D.W., eds), pp. 389–399. Tokyo: Springer Japan.

Petrick, J.S., Moore, W.M., Heydens, W.F., Koch, M.S., Sherman, J.H. and Lemke, S.L. (2015) A 28-day oral toxicity evaluation of small interfering RNAs and a long double-stranded RNA targeting vacuolar ATPase in mice. *Regul. Toxicol. Pharmacol.* **71**, 8–23.

Priebe, S., Kreisel, C., Horn, F. and Guthke, R. (2015) Databases and ontologies FungiFun2 : a comprehensive online resource for systematic analysis of gene lists from fungal species. **31**, 445–446.

Pugh, T.A.M., Müller, C., Elliott, J., Deryng, D., Folberth, C., Olin, S., Schmid, E. and Arneth, A. (2016) Climate analogues suggest limited potential for intensification of production on current croplands under climate change. *Nat. Commun.* **7**, 12608.

Ramaseshadri, P., Segers, G., Flannagan, R., Wiggins, E., Clinton, W., Ilagan, O., McNulty, B., Clark, T. and Bolognesi, R. (2013) Physiological and Cellular Responses Caused by RNAi- Mediated Suppression of Snf7 Orthologue in Western Corn Rootworm (*Diabrotica virgifera virgifera*) Larvae. *PLoS One* **8**, 1–10.

Rico, A., Sabater, C. and Castillo, M.-A. (2016) Lethal and sub-lethal effects of five pesticides used in rice farming on the earthworm *Eisenia fetida*. *Ecotoxicol. Environ. Saf.* **127**, 222–229.

Romano, N. and Macino, G. (1992) Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol. Microbiol.* **6**, 3343–3353.

Rupp, S., Weber, R.W.S., Rieger, D., Detzel, P. and Hahn, M. (2017) Spread of botrytis

cinerea strains with multiple fungicide resistance in german horticulture. *Front. Microbiol.* **7**, 1–12.

Sabatier, P., Poulenard, J., Fanget, B., et al. (2014) Long-term relationships among pesticide applications, mobility, and soil erosion in a vineyard watershed. *Proc. Natl. Acad. Sci.* **111**, 15647–15652.

Saleh, M.-C., Rij, R.P. van, Hekele, A., Gillis, A., Foley, E., O'Farrell, P.H. and Andino, R. (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nat. Cell Biol.* **8**, 793–802.

Schmitt-Engel, C., Schultheis, D., Schwirz, J., et al. (2015) The iBeetle large-scale RNAi screen reveals gene functions for insect development and physiology. *Nat. Commun.* **6**, 7822.

Simon-Delso, N., Martin, G.S., Bruneau, E., Minsart, L.A., Mouret, C. and Hautier, L. (2014) Honeybee colony disorder in crop areas: The role of pesticides and viruses. *PLoS One* **9**, 1–16.

Smalling, K.L., Reilly, T.J., Sandstrom, M.W. and Kuivila, K.M. (2013) Occurrence and persistence of fungicides in bed sediments and suspended solids from three targeted use areas in the United States. *Sci. Total Environ.* **447**, 179–185.

Syromyatnikov, M.Y., Kokina, A. V., Lopatin, A. V., Starkov, A.A. and Popov, V.N. (2017) Evaluation of the toxicity of fungicides to flight muscle mitochondria of bumblebee (*Bombus terrestris* L.). *Pestic. Biochem. Physiol.* **135**, 41–46.

Tan, J., Levine, S.L., Bachman, P.M., et al. (2016) No impact of DvSnf7 RNA on honey bee (*Apis mellifera* L.) adults and larvae in dietary feeding tests. *Environ. Toxicol. Chem.* **35**, 287–294.

Tedersoo, L., Bahram, M., Põlme, S., et al. (2014) Global diversity and geography of soil fungi. *Science (80-.).* **346**, 1052–1053.

Thakare, D., Zhang, J., Wing, R.A., Cotty, P.J. and Schmidt, M.A. (2017) Aflatoxin-free transgenic maize using host-induced gene silencing. , 1–9.

Tilman, D., Balzer, C., Hill, J. and Befort, B.L. (2011) Global food demand and the sustainable intensi fi cation of agriculture. **108**.

Tollefson, J. (2011) Brazil cooks up transgenic bean. *Nature* **478**, 168–168.

Torres-Martínez, S. and Ruiz-Vázquez, R.M. (2017) The RNAi Universe in Fungi : A Varied Landscape of Small RNAs and Biological Functions. *Annu. Rev. Microbiol.* **71**, 371–391.

Tuan Le, N. and Wang, M.-B. (2011) The Role of RNA Silencing in Plant Stress Responses. *Omi. Plant Abiotic Stress Toler.*, 151–162.

United Nations (2017) *World Population Prospects: The 2017 Revision, Key Findings and Advance Tables*,.

United States Environmental Protection Agency (2017) EPA Registers Innovative Tool to Control Corn Rootworm.

Urich, T., Lanzén, A., Qi, J., Huson, D.H., Schleper, C. and Schuster, S.C. (2008) Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PLoS One* **3**.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J. and Lötvall, J.O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **9**.

Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D. and Remaley, A.T. (2011) MicroRNAs are Transported in Plasma and Delivered to Recipient Cells by High-Density

Lipoproteins. *Nat. Cell Biol.* **13**, 423–433.

Vijayakumar, K.R., Martin, A., Gowda, L.R. and Prakash, V. (2009) Detection of genetically modified soya and maize : Impact of heat processing. *Food Chem.* **117**, 514–521.

Villalobos-Escobedo, J.M., Carreras-Villaseñor, N. and Herrera-Estrella, A. (2016) The interaction of fungi with the environment orchestrated by RNAi. *Mycologia* **108**, 15-246-.

Wang, M. and Jin, H. (2017) Spray-Induced Gene Silencing: a Powerful Innovative Strategy for Crop Protection. *Trends Microbiol.* **25**, 4–6.

Wang, M., Weiberg, A., Lin, F.-M., Thomma, B.P.H.J., Huang, H.-D. and Jin, H. (2016a) Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* **2**, 16151.

Weiberg, A., Wang, M., Lin, F.-M., et al. (2013) Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* **342**, 118–23.

Westermann, A.J. and Barquist, L. (2017) Resolving host – pathogen interactions by dual. , 1–19.

Whangbo, J.S., Weisman, A.S., Lu, J., Chae, J. and Hunter, C.P. (2017) SID-1 domains important for dsRNA import in *C. elegans*. *bioRxiv* **2**.

Whyard, S., Singh, A.D. and Wong, S. (2009) Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* **39**, 824–832.

Xiao, D., Gao, X., Xu, J., Liang, X., Li, Q., Yao, J. and Zhu, K.Y. (2015) Clathrin-dependent endocytosis plays a predominant role in cellular uptake of double-stranded RNA in the red flour beetle. *Insect Biochem. Mol. Biol.* **60**, 68–77.

Zhang, T., Jin, Y., Zhao, J.-H., Gao, F., Zhou, B.-J., Fang, Y.Y. and Guo, H.-S. (2016) Host-

Induced Gene Silencing of the Target Gene in Fungal Cells Confers Effective Resistance to the Cotton Wilt Disease Pathogen *Verticillium dahliae*. *Mol. Plant*, 1–4.

Zong, J., Yao, X., Yin, J., Zhang, D. and Ma, H. (2009) Evolution of the RNA-dependent RNA polymerase (RdRP) genes: Duplications and possible losses before and after the divergence of major eukaryotic groups. *Gene* **447**, 29–39.

Zubrod, J.P., Bundschuh, M., Feckler, A., Englert, D. and Schulz, R. (2011) Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. *Environ. Toxicol. Chem.* **30**, 2718–2724.

PREFACE TO EXPERIMENTAL CHAPTERS: AN INTRODUCTION TO *SCLEROTINIA SCLEROTIORUM*

Brassica napus L.*,* commonly referred to as canola or rapeseed, contributes $26.7 billion and 250,000 jobs to the Canadian economy annually (Canola Council of Canada, 2017). The high oil content of the seed and the relatively low degree of saturation of its fatty acids make canola a valued crop and its oil a healthy cooking product. Consumption of canola oil has been linked to improved cardiovascular health through the lowering of the glycemic index, low density lipids, and serum cholesterol (Jenkins *et al.*, 2014; Kruse *et al.*, 2015; Lin *et al.*, 2013). Following lipid extraction, a nutritious, high protein animal feed for the dairy and porcine industries can also be produced from the seed (Broderick *et al.*, 2015; Zhou *et al.*, 2013). Additionally, a variety of industrial bio-products have also been manufactured from the seeds, such as bio-lubricant, bio-diesel, and new bio-degradable packaging (Madankar *et al.*, 2013; McVetty and Duncan, 2015; Zhang *et al.*, 2017). Due to its variety of applications, canola has been and will undoubtedly continue to be one of Canada's most economically significant crops.

Despite the growing demand for canola's products, canola fields are threatened by necrotrophic fungal pathogens, such as *Sclerotinia sclerotiorum* (Lib.) de Bary*,* which can also infect an additional 500 plant species around the globe (Kamal *et al.*, 2016). The infection cycle initiates in the early spring under damp conditions. Hyphal congregates, termed sclerotia, germinate within the soil. The lifecycle then progresses through one of two ways: spore or infective mycelial production. Root infections, which are common attack strategies for crops such as sunflowers and carrot, are uncommon in canola (Derbyshire and Denton-Giles, 2016; Foster *et al.*, 2008; Peluffo *et al.*, 2010). Once germinated, the fungal hyphae form spore-bearing structures called apothecia, which release millions of ascospores. The ascospores are carried in

wind currents up to 10 km away from release, and land on senescing tissues of the canola plant (Kamal *et al.*, 2016; Sharma *et al.*, 2015). The ascospores then germinate, using the freely available nutrients to launch an infection against the host plant. The fungus forms an infection peg to penetrate the leaf cuticle, entering into the host plant (Davidson *et al.*, 2016). Due to the necrotrophic nature of the fungus, the subsequent infection is characterized by pathogen-induced host cell death to procure nutrients, appearing as necrotic tissue on the leaf. The lesion spreads, as the fungus enters into the vasculature, and the infection becomes systemic (Kabbage *et al.*, 2015; Silva *et al.*, 2009). Once in the stem, new sclerotia form and the host plant becomes susceptible to lodging and shattering, which deposit sclerotia into the soil, where they will overwinter and infect crops in the subsequent years (Bolton *et al.*, 2006).

Since *S. sclerotiorum* does not exhibit a gene-for-gene response during interactions with the host, a plant cannot simply develop resistance by altering a single target protein; often, many gene products would need to mutate, such that the small contributions of many genes cumulatively confer a more effective host defence. Pathogens that require this so-called quantitative resistance are inherently more difficult to develop control (Corwin and Kliebenstein, 2017). Secreted fungal proteins protect the pathogen against host defences (e.g. peptidase inhibitors, superoxide dismutases), induce apoptosis (e.g. necrosis-inducing peptides), and digesting host cell walls (e.g. pectinesterases) during infection (Guyon *et al.*, 2014; Heard *et al.*, 2015; Veluchamy *et al.*, 2012). Additionally, oxalic acid (OA), a key contributor to fungal pathogenicity, has three main roles in modulating the host environment: acidification, chelation of ions, and host cell death. The secretion of OA acidifies host tissues to pH 4, providing an optimal pH for the activity of secreted fungal enzymes and depletes the pectin of plant cell walls of calcium ions simultaneously. Therefore, the host cells essentially unglue, facilitating access of

digestive enzymes for nutrient acquisition and allowing hyphae to grow intercellularly (Uloth *et al.*, 2015b; Williams *et al.*, 2011). Lastly, OA modulates the host environment by initially dampening host respiratory burst response to allow for fungal establishment, before initiating an apoptotic programmed cell death during the colonization process to procure nutrients (Kabbage *et al.*, 2013; Kabbage *et al.*, 2015). Using this multitude of pathogenicity factors, *S. sclerotiorum* is a successful and aggressive phytopathogen.

The plethora of factors utilized by *S. sclerotiorum* has thus far defeated our attempts to breed robust host resistance. Tolerant cultivars that demonstrated reduced infection symptoms have been bred, but true resistance has yet to be attained (Disi *et al.*, 2014; Garg *et al.*, 2010). Commercial cultivars labelled as resistant show increased cell wall deposition and intense lignification to restrain fungal infection within host tissues (Garg *et al.*, 2010; Uloth *et al.*, 2015a). While some of these cultivars show improved resistance against the pathogen, yield and oil quality were negatively affected (Bradley *et al.*, 2006; Derbyshire and Denton-Giles, 2016). In general, the discovery of tolerant cultivars involves lengthy germplasm screening protocols (Taylor *et al.*, 2015). Resistance could be more rapidly developed within more agronomically favoured lines using genetic engineering techniques.

Other traditional management strategies of *S. sclerotiorum* involve crop rotations and chemical field treatments. However, crop rotations may fail due to the presence of sclerotia in the soil or the wind dispersion of ascospores from adjacent fields. Due to the large host range of *S. sclerotiorum*, many weed species can also propagate the sclerotial load within the soil, despite the use of non-host crops (Derbyshire and Denton-Giles, 2016; Kamal *et al.*, 2016). The sclerotia can persist for 7 – 10 years, surviving periods of anoxia and temperatures above 107°C (Hind-Lanoiselet *et al.*, 2005; Wu *et al.*, 2008). Additionally, common agrochemicals have achieved

variable success suppressing *S. sclerotiorum* disease incidence (Bradley *et al.*, 2006). Due to the expense of applying chemical treatments, diluted doses used by some producers have lead to the rise of fungicide resistant strains (Amaradasa and Everhart, 2016; Penaud and Walker, 2015). Furthermore, due to the biocidal nature of fungicidal compounds and persistence in the environment, chemical applications are not a conscious choice for fungal management (Maltby *et al.*, 2009; Smalling *et al.*, 2013). Taken together, there is a need to develop new methodologies and novel strategies to tip the scales against this devastating pathogen.

REFERENCES

Amaradasa, B.S. and Everhart, S.E. (2016) Effects of Sublethal Fungicides on Mutation Rates and Genomic Variation in Fungal Plant Pathogen, *Sclerotinia sclerotiorum*. *PLoS One* **11**, e0168079.

Bolton, M.D., Thomma, B.P.H.J. and Nelson, B.D. (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: Biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol.* **7**, 1–16.

Bradley, C.A., Lamey, H.A., Endres, G.J., Henson, R.A., Hanson, B.K., McKay, K.R., Halvorson, M., LeGare, D.G. and Porter, P.M. (2006) Efficacy of Fungicides for Control of Sclerotinia Stem Rot of Canola. *Plant Dis.* **90**, 1129–1134.

Broderick, G.A., Faciola, A.P. and Armentano, L.E. (2015) Replacing dietary soybean meal with canola meal improves production and efficiency of lactating dairy cows1. *J. Dairy Sci.* **98**, 5672–5687.

Canola Council of Canada (2017) Economic Impact of the Canola Industry.

Corwin, J.A. and Kliebenstein, D.J. (2017) Quantitative Resistance : More Than Just Perception of a Pathogen.

Davidson, A.L., Blahut-Beatty, L., Itaya, A., Zhang, Y., Zheng, S. and Simmonds, D. (2016) Histopathology of *Sclerotinia sclerotiorum* infection and oxalic acid function in susceptible and resistant soybean. *Plant Pathol.* **65**, 878–887.

Derbyshire, M.C. and Denton-Giles, M. (2016) The control of Sclerotinia stem rot on oilseed rape (*Brassica napus*): Current practices and future opportunities. *Plant Pathol.* **65**, 859–877.

Derbyshire, M.C. and Denton-Giles, M. (2016) The control of sclerotinia stem rot on oilseed rape (*Brassica napus*): current practices and future opportunities. *Plant Pathol.* **65**, 859–877.

Disi, J.O., Mei, J., Wei, D., Ding, Y. and Qian, W. (2014) Inheritance of leaf and stem resistance to *Sclerotinia sclerotiorum* in a cross between Brassica incana and Brassica oleracea var. alboglabra. *J. Agric. Sci.* **152**, 146–152.

Foster, A.J., McDonald, M.R. and Boland, G.J. (2008) Disease progression of Sclerotinia rot of carrot, caused by Sclerotinia sclerotiorum, from shoot to root before and after harvest. *Can. J. Plant Pathol.* **30**, 206–213.

Garg, H., Li, H., Sivasithamparam, K., Kuo, J. and Barbetti, M.J. (2010) The infection processes of *Sclerotinia sclerotiorum* in cotyledon tissue of a resistant and a susceptible genotype of Brassica napus. *Annu. Bot.*, 897–908.

Guyon, K., Balagué, C., Roby, D. and Raffaele, S. (2014) Secretome analysis reveals effector candidates associated with broad host range necrotrophy in the fungal plant pathogen *Sclerotinia sclerotiorum*. *BMC Genomics* **15**, 336.

Heard, S., Brown, N.A. and Hammond-kosack, K. (2015) An Interspecies Comparative Analysis of the Predicted Secretomes of the Necrotrophic Plant Pathogens *Sclerotinia*

sclerotiorum and *Botrytis cinerea*. *PLoS One*, 1–27.

Hind-Lanoiselet, T.L., Lanoiselet, V.M., Lewington, F.K., Ash, G.J. and Murray, G.M. (2005) Survival of *Sclerotinia* sclerotia under fire. *Australas. Plant Pathol.* **34**, 311.

Jenkins, D.J.A., Kendall, C.W.C., Vuksan, V., et al. (2014) Effect of lowering the glycemic load with canola oil on glycemic control and cardiovascular risk factors: A randomized controlled trial. *Diabetes Care* **37**, 1806–1814.

Kabbage, M., Williams, B. and Dickman, M.B. (2013) Cell Death Control: The Interplay of Apoptosis and Autophagy in the Pathogenicity of *Sclerotinia sclerotiorum*. *PLoS Pathog.* **9**.

Kabbage, M., Yarden, O. and Dickman, M.B. (2015) Pathogenic attributes of *Sclerotinia sclerotiorum*: Switching from a biotrophic to necrotrophic lifestyle. *Plant Sci.* **233**, 53–60.

Kamal, M.M., Savocchia, S., Lindbeck, K.D. and Ash, G.J. (2016) Biology and biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary in oilseed Brassicas. *Australas. Plant Pathol.* **45**, 1–14.

Kruse, M., Loeffelholz, C. von, Hoffmann, D., et al. (2015) Dietary rapeseed/canola-oil supplementation reduces serum lipids and liver enzymes and alters postprandial inflammatory responses in adipose tissue compared to olive-oil supplementation in obese men. *Mol. Nutr. Food Res.* **59**, 507–519.

Lin, L., Allemekinders, H., Dansby, A., Campbell, L., Durance-Tod, S., Berger, A. and Jones, P.J. (2013) Evidence of health benefits of canola oil. *Nutr. Rev.* **71**, 370–385.

Madankar, C.S., Dalai, A.K. and Naik, S.N. (2013) Green synthesis of biolubricant base stock from canola oil. *Ind. Crops Prod.* **44**, 139–144.

Maltby, L., Brock, T.C.M. and Brink, P.J. Van Den (2009) Fungicide risk assessment for aquatic ecosystems: Importance of interspecific variation, toxic mode of action, and

exposure regime. *Environ. Sci. Technol.* **43**, 7556–7563.

McVetty, P.B.E. and Duncan, R.W. (2015) Canola, Rapeseed, and Mustard: For Biofuels and Bioproducts. In Industrial Crops. Handbook of Plant Breeding. (Cruz, V.M.V. and Dierig, D.A., eds), pp. 133–156. New York, NY, US: Springer, New York, NY.

Peluffo, L., Lia, V., Troglia, C., et al. (2010) Metabolic profiles of sunflower genotypes with contrasting response to *Sclerotinia sclerotiorum* infection. *Phytochemistry* **71**, 70–80.

Penaud, A. and Walker, A.-S. (2015) Oilseed Rape Pathogens in France. In Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. (Ishii, H. and Hollomon, D.W., eds), pp. 389–399. Tokyo: Springer Japan.

Sharma, P., Meena, P.D., Verma, P.R., Saharan, G.S., Mehta, N., Singh, D. and Kumar, A. (2015) *Sclerotinia sclerotiorum* (Lib .) de Bary causing Sclerotinia rot in oilseed Brassicas : A review. *J. Oilseed Brassica* **6**, 1–44.

Silva, A.P. De, Bolton, M.D. and Nelson, B.D. (2009) Transformation of Sclerotinia sclerotiorum with the green fluorescent protein gene and fluorescence of hyphae in four. , 487–496.

Smalling, K.L., Reilly, T.J., Sandstrom, M.W. and Kuivila, K.M. (2013) Occurrence and persistence of fungicides in bed sediments and suspended solids from three targeted use areas in the United States. *Sci. Total Environ.* **447**, 179–185.

Taylor, A., Coventry, E., Jones, J.E. and Clarkson, J.P. (2015) Resistance to a highly aggressive isolate of *Sclerotinia sclerotiorum* in a *Brassica napus* diversity set. , 932–940.

Uloth, M.B., Clode, P.L., You, M.P. and Barbetti, M.J. (2015a) Attack modes and defence reactions in pathosystems involving *Sclerotinia sclerotiorum* , *Brassica carinata* , *B. juncea* and *B. napus*. *Ann. Bot.*, mcv150.

Uloth, M.B., Clode, P.L., You, M.P. and Barbetti, M.J. (2015b) Calcium Oxalate Crystals: An Integral Component of the *Sclerotinia sclerotiorum*/*Brassica carinata* Pathosystem. *PLoS One* **10**, e0122362.

Veluchamy, S., Williams, B., Kim, K. and Dickman, M.B. (2012) The CuZn superoxide dismutase from *Sclerotinia sclerotiorum* is involved with oxidative stress tolerance, virulence, and oxalate production. *Physiol. Mol. Plant Pathol.* **78**, 14–23.

Williams, B., Kabbage, M., Kim, H.J., Britt, R. and Dickman, M.B. (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLoS Pathog.* **7**.

Wu, B.M., Subbarao, K. V, Liu, Y., Bary, L. De and Jagger, S. (2008) Comparative Survival of Sclerotia of *Sclerotinia mino*r and *S . sclerotiorum*. **98**, 659–665.

Zhang, Y., Liu, Q. and Rempel, C. (2017) Processing and characteristics of canola protein-based biodegradable packaging: A review. *Crit. Rev. Food Sci. Nutr.* **0**, 1–11.

Zhou, X., Oryschak, M.A., Zijlstra, R.T. and Beltranena, E. (2013) Effects of feeding high- and low-fibre fractions of air-classified, solvent-extracted canola meal on diet nutrient digestibility and growth performance of weaned pigs. *Anim. Feed Sci. Technol.* **179**, 112–120.

CHAPTER 2 : IDENTIFICATION AND APPLICATION OF RNA INTERFERENCE-BASED FOLIAR CONTROL OF *SCLEROTINIA SCLEROTIORUM* AND *BOTRYTIS CINEREA* IN PLANTS

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2.1 ABSTRACT

Sclerotinia sclerotiorum, the causal agent of white stem rot, is responsible for significant losses in crop yield around the globe. While our understanding of *S. sclerotiorum* pathogenicity is becoming clearer, genetic control of the pathogen has been elusive and effective control of pathogen colonization using traditional broad-spectrum agro-chemical protocols are less effective than desired. In the current study, we developed species-specific RNA interference-based biocontrol treatments capable of reducing fungal infection. Development of a target identification pipeline using global RNA sequencing data for selection and application of double stranded RNA (dsRNA) molecules identified single gene targets of the fungus. Using this approach, we demonstrate the utility of this technology through foliar applications of dsRNAs to the leaf surface that significantly reduce fungal infection and *S. sclerotiorum* disease symptoms. Select target gene homologs were also tested in the closely related species, *Botrytis cinerea*, reducing lesion size and providing compelling evidence of the adaptability and flexibility of this technology in protecting plants against devastating fungal pathogens.

2.2 INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal pathogen and the causal agent of white stem rot in canola (*Brassica napus* L.). This ascomycete infects over 500 different plant species and causes major economic losses around the globe (Bolton *et al.*, 2006; Kamal *et al.*, 2016). Current methods to control *S. sclerotiorum* infection through the application of broad-spectrum fungicides remains ineffective due to poor penetration and may have deleterious or unwanted effects on the surrounding agro-ecological landscape, if not properly managed. Other management practices, such as crop rotation, have proven ineffective due to the formation of overwintering sclerotia structures that can remain in the soil for several years (Bradley *et al.*, 2006; Derbyshire and Denton-Giles, 2016; Schwartz and Singh, 2013). In addition, few plant cultivars are considered completely genetically resistant to *S. sclerotiorum* and further complicate effective disease management (Sharma *et al.*, 2015). For these reasons, new, environmentally-safe, species-specific fungicides capable of suppressing *S. sclerotiorum* are eagerly being sought.

Species-specific molecular insecticides, using RNA interference (RNAi) approaches, have been shown to control insect pests in the laboratory (Killiny *et al.*, 2014; Price and Gatehouse, 2008; San Miguel and Scott, 2016; Whyard *et al.*, 2009), and recently, the first transgenic plants with RNAi-based genetic constructs have been approved for field use (United States Environmental Protection Agency, 2017). RNAi-based control technologies are dependent on double stranded RNA (dsRNA) molecules, which are designed with complementary sequences to a given mRNA within the target organism. Once the dsRNA molecules enter the cell they complex with DICER-LIKE and the molecule is fragmented into small interfering RNAs (siRNAs) of 21-24 nucleotides in length. These siRNAs then associate with

ARGONAUTE within the RNA-induced silencing complex (RISC), which acts as an endonuclease, cleaving the target mRNA molecules that share complementarity with the siRNA sequences (Billmyre *et al.*, 2013; Majumdar *et al.*, 2017; Nicolás and Garre, 2016). While the number of studies that describe the application of RNAi to control insect pests is increasing steadily, there are considerably fewer studies that describe the potential of RNAi to control fungal plant pathogens, despite the characterization of fungal RNAi machinery in different species (Campo *et al.*, 2016; Chen *et al.*, 2015; Koch *et al.*, 2016; Wang *et al.*, 2016).

Recently, *S. sclerotiorum* engineered to express an RNAi construct targeting *SsITL* (integrin-like immune suppressor), *SsMADS* (MADS transcription factor), *SsSl2* (a cell wall protein), and *SsBi1* (Bax-inhibitor protein) showed compromised pathogenicity and altered cellular development (Qu *et al.*, 2014; Yu *et al.*, 2012; Yu *et al.*, 2015; Zhu *et al.*, 2013). Another study demonstrated limited fungus-induced lesion formation in tobacco plants (*Nicotiana tabacum*) expressing hairpin RNA (hpRNA) molecules targeting *S. sclerotiorum Sschs* (chitin synthase) (Andrade *et al.*, 2016). Despite these advances, the ability to control *S. sclerotiorum* is still limited and no study has shown effective control of this fungus on the leaf surface using topical applications of dsRNA molecules.

A pioneering study used *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen and *Mycosphaerella fijiensis* Morelet to demonstrate the potency of synthesized dsRNA molecules under *in vitro* conditions to target essential genes of the fungi (Mumbanza *et al.*, 2013). dsRNAs have also been used to control *Botrytis cinerea* Pers. and *Fusarium graminearum* Schwabe infections *in planta*. For example, dsRNAs that targeted Dicer-like 1 and 2 transcripts in *B. cinerea,* reduced disease symptoms in a range of plant tissues (Wang *et al.*, 2016). Similarly, dsRNA targeting three *F. graminearum* cytochrome P450 lanosterol C-14-α

demethylases, a common fungicide target, protected barley (*Hordeum vulgare* L.) against fungal colonization (Koch *et al.*, 2016). These studies established the feasibility of topical applications of dsRNA to control pathogenic fungi. However, in both studies, the researchers demonstrated that targeting more than one protein-encoding mRNA was more effective than targeting a single mRNA, although in each case, they targeted genes encoding proteins involved in shared biological processes. To date, no study has demonstrated effective fungal foliar suppression targeting only a single transcript or has identified multiple unique targets for RNAi-based control.

In this study, RNA sequencing (RNA-seq) was used to identify genes associated with fungal pathogenicity in the *B. napus – S. sclerotiorum* pathosystem to identify novel targets for fungal control using RNAi. Bioinformatics analysis identified global changes of gene expression of *S. sclerotiorum* on both susceptible (*B. napus cv. Westar*) and moderately tolerant (*B. napus cv. ZhongYou821*) cultivars of canola. Global changes in gene expression revealed biological processes associated with cell growth, cellular homeostasis, and infection. DsRNA molecules were designed to target genes associated with reactive oxygen species (ROS) responses, transcription, and host colonization, in addition to those identified as essential in *Aspergillus fumatigus* (Hu *et al.*, 2007). Target transcripts were successfully knocked down *in vitro* and topical applications of dsRNA reduced lesion progression on *B. napus* leaves. Knockdown of many of these target mRNAs also proved effective in suppressing *S. sclerotiorum* growth on leaves of *Arabidopsis thaliana.* The versatility of the RNAi targets was demonstrated by similarly reducing the growth of another phytopathogenic fungus, *B. cinerea.* Taken together, the results clearly demonstrate the utility of RNA-seq technology to guide selection of multiple

target genes for RNAi and to extend the utility of large scale datasets to protect agronomically-important plants against phytopathogenic fungi.

2.3 METHODS

2.3.1 *Brassica napus* growth conditions for RNA sequencing

Seeds of susceptible cv. Westar and tolerant cv. Zhongyou821 (ZY821) *B. napus* were grown in Sunshine Mix No.1 at 22˚C, 50-70% humidity under long day conditions (16 hours light, 8 hours dark 150-200 $\mu E/m^2/s$). ZY821 plants were subjected to a 1-month vernalization treatment after planting (8 hours light, 16 hours dark, 8-10˚C, 40% humidity and 100 $\mu E/m^2/s$), before being transferred back to long day conditions (Girard *et al.*, 2017).

2.3.2 Leaf inoculation and *S. sclerotiorum* tissue collection for RNA sequencing

Sclerotinia. sclerotiorum ascospores were collected at the Morden Research and Development Centre, Agriculture and Agri-Food Canada, Morden, MB, Canada and stored at 4˚C in desiccant in the dark. *S. sclerotiorum* ascospores (8×10^4 mL^{-1}) were suspended in a 0.02% Tween80 (Sigma-Aldrich, St. Louis, MO, USA) solution. 25µL of the ascospore solution was transferred onto senescing *B. napus* petals in a petri plate and sealed with Parafilm. Ascsospore-inoculated petals were stored at room temperature (21˚C) for 72 hours and allowed to germinate prior to being inoculated on the leaf surface.

Brassica napus petals were then transferred to Westar and ZY821 leaves between 1-3 PM at the 30% bloom stage and covered with a clear plastic bag to maintain high relative humidity. After 24 hours, at least ten lesions (1 cm^2) were collected from the site of inoculation for each of three biological replicates to identify early infection stage pathogenicity factors. To identify genes associated with *S. sclerotiorum* grown *in-vitro*, sclerotia were cut into halves and placed

open side down on PDA media (Kamesh Krishnamoorthy *et al.*, 2016). Mycelium tissue was collected from the leading hyphal edge after 3 days and flash frozen in liquid nitrogen prior to RNA isolation.

2.3.3 RNA extraction and sequencing

RNA was isolated using Invitrogen Plant RNA Purification Reagent and treated with Ambion Turbo DNA-free DNase according to the manufacturer's protocol (Thermo Fisher, Waltham, MA, USA). Quantity and purity were assessed spectrophotometrically and the quality of RNA samples verified using electropherogram profiles and RNA Integrity Numbers (RIN) with an Agilent 2100 Bioanalyzer and RNA Nano Chip (Aligent, Santa Clara, CA, USA). RNA-sequencing cDNA libraries were prepared from 5µg of total RNA according to the methods of Kumar et al. (2012) with some modifications. Isolation of mRNA from was performed using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, US) according to manufacturer's instructions with the following modifications: all reaction volumes were 7.5µL of Oligo $d(T)_{25}$ beads per sample. The remaining preparation steps were performed according to the HTR protocol starting with the first strand cDNA synthesis. NEXTflex™ ChIP-Seq Barcodes (Bio Scientific, Austin, TX, USA) were used as adaptors for the adapter ligations and NEXTflex™ PCR Primer Mix for the library enrichment PCR step. Library quality was assessed using a High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer and size selected using E-Gel® SizeSelect™ 2% agarose gel (Life Technologies, Carlsbad, CA, USA) to target fragments from 250-500 bp in length. 100 bp single-end RNA sequencing was carried out using the Illumina HiSeq2000 platform (Génome Québec Innovation Centre, McGill University, Montreal, Canada).

2.3.4 Bioinformatics Pipeline

FastQ files were trimmed using Trimmomatic 0.33 (Bolger *et al.*, 2014): adapter sequences, initial 12 bases of raw reads, low quality reads with a quality score under 20 over a sliding window of 4 bases, and reads with an average quality score under 30 removed during the trimming process. Remaining reads shorter than 50 nucleotides were also removed. The splice junction mapping software TopHat (v2.1.0, http://ccb.jhu.edu/software/tophat/index.shtml; Trapnell *et al.*, 2012) was then used to align trimmed reads to the *S. sclerotiorum* genome (Amselem *et al.*, 2011). Gene expression was quantified using Cuffquant (v2.2.1, https://github.com/cole-trapnell- lab/cufflinks) and expression values normalized to FPKM (fragments per kilobase per million mapped reads) using Cuffnorm (v2.2.1, https://github.com/cole-trapnell-lab/cufflinks). Differential expression analysis was performed using Cuffdiff (v2.2.1, https://github.com/cole-trapnell-lab/cufflinks) and resulting significantly differentially expressed genes used as input for GO term enrichment using SeqEnrich (https://github.com/nagreme/SeqEnrich; Becker *et al.*, 2017). GO terms were collected from UniProt KB (http://www.uniprot.org/; Bateman *et al.*, 2015) and kindly made available by Nicolas Lapalu (L'Institut national de la recherché argonomique, Versailles, France; Amselem *et al.*, 2011). Clustering analyses were performed using the pvclust package in R studio (https://cran.r-project.org/web/packages/pvclust/index.html) for hierarchical clustering and the DESeq package in R studio for principle component analysis clustering, in both analyses the Cuffnorm outputted FPKM transcript expression values with a value > 1 were used as input values for clustering (http://www-huber.embl.de/users/anders/DESeq/; Anders and Huber, 2010). GO term heat map visualization was carried out using the gplots package in R studio (https://cran.r-project.org/web/packages/gplots/index.html), terms were considered enriched at

$P < 0.001$ with a blue-red color scale representing levels of statistical enrichment. Venn diagram visualization was performed using Venny 2.1(http://bioinfogp.cnb.csic.es/tools/venny/; Oliveros, 2015).

2.3.5 Selection of gene targets and Target Identification Pipeline (TIP)

Targets for RNAi were identified from a list of differentially upregulated genes shared between *S. sclerotiorum* grown on Westar and ZY821 compared to *in vitro* control. Essential genes from close relatives were also identified using the Database of Essential Genes (www.essentialgene.org; Luo *et al.*, 2014) and known regulators of infection (Liang *et al.*, 2014).

Genes were then selected based on enrichment of biological processes (GO terms) associated with cell wall modification, mitochondria, ROS response, protein modification, pathogenicity factors, transcription, splicing, protein modification, and translation while those associated with growth, transport, transcription factors, electron carriers, signal transduction, pigment synthesis, and carbohydrate metabolism were avoided due to the complex nature, functional redundancy, and non-compromising roles the biological process at play. Putative functions and accessions were determined and confirmed using NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) and KEGG (Kyoto Encyclopedia of Genea and Genomes; http://www.genome.jp/kegg/) (Kanehisa *et al.*, 2017). Only targets of at least 200 nucleotides were selected to avoid natural sequence variations that could impair RNAi silencing (Joga *et al.*, 2016).

The list of target genes was reduced further based on FPKM values of 1-500 and log2-fold change thresholds of -0.5 and 4. Highly regulated targets, targets with functional redundancy and genes with multiple homologues were also avoided. Genes encoded within

organelles, such as mitochondria were also avoided since mitochondria cannot process or import the dsRNA from the cytoplasm. The Target Identification Pipeline is summarized in Table S2.

2.3.6 RNA extraction, cDNA synthesis and *in vitro* production of dsRNAs

Actively growing fungal hyphae grown *in vitro* were ground in liquid nitrogen and RNA extracted using Invitrogen Plant RNA Reagent (Invitrogen, Carlsbad, CA, US) and treated with Turbo DNase (Ambion, Carlsbad, CA, US). cDNA was synthesized with the Maxima First Strand reverse transcriptase (Thermo Scientific, Waltham, MA, US) using 500 ng of RNA in a 10 μL reaction.

Sclerotinia. sclerotiorum and *B. cinera* gene sequences (Genbank; http://www.ncbi.nlm.nih.gov/) and primers were designed using Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) to PCR amplify gene fragments ranging between 180 and 480 bp in length and quality assessed using Primer3 (http://bioinfo.ut.ee/primer3/) (Untergasser *et al.*, 2012). Primer sets were designed to limit regions of homology (>20 bases) to other Eukaryotes by searching BLASTN (http://blast.ncbi.nlm.nih.gov/Blast) RefSeq accessions to discover sequence homologies in putative dsRNA. A BLASTN query using *Sclerotinia sclerotiorum UF-80* RefSeq entries was performed to ensure each dsRNA molecule reacted with a single transcript within the fungus (Mocellin and Provenzano, 2004). A complete list of primers used in the paper are found in Additional file 3.

Target gene sequences were amplified using Phusion Taq (Thermo Scientific, Waltham, MA, US) under the following conditions: 98°C for 30 s; 35 cycles of: 98°C for 10s, 57°C for 20s, and 72°C for 20s; and a final extension of 72 °C for 7 min. Amplicons were gel purified (New England Biolabs, Ipswich, MA, US) and digested using FastDigest KpnI and XbaI or XhoI (Thermo Scientific, Waltham, MA, US) according to the manufacturer's protocols. The products

were ligated into the similary digested pL4440 vector (kindly donated by Andrew Fire, Stanford University) using T4 ligase (Invritogen, Carlsbad, CA, US) according to the manufacturer's protocol. Prepared plasmids were used to transformed *E. coli* MachI cells (Thermo Scientific, Waltham, MA, US) and sequence inserts were confirmed using Sanger sequencing (The Centre for Applied Genomics. Toronto, ON, Canada).

Primers (F: CAACCTGGCTTATCGAA; R: TAAAACGACGGCCAGTGA) designed to amplify T7 promoters flanking each insert were used in a Phusion PCR: 98°C for 3 min, 35 cycles of: 98°C for 15s, 57°C for 15s, and 72°C for 40s; and a final extension of 72 °C for 10 min. The PCR reaction was purified using a PCR cleanup kit (New England Biolabs, Ipswich, MA, US) and dsRNA synthesized using the MEGAScript^{TM} RNAi kit (Invitrogen, Carlsbad, CA, US) according to manufacturer's instructions.

2.3.7 Quantification of relative transcript abundance following dsRNA application *in vitro*

A 1 mm plug was taken from the leading edge of freshly cultured 3-day old fungal colony and placed in stationary 7 mL of potato dextrose broth (PDB) containing ampicillin (50 μg/mL) in a 60mm x 15mm petri dish for 48 hours. DsRNAs were applied at a dose of 500 ng mL^{-1} and tissue collected at 0, 24, 48, 72, and 96 hours post-inoculation (hpi). To examine the effect of RNA concentration on target gene knockdown, 100 ng ml^{-1}, 200 ng ml^{-1} , 500 ng ml^{-1} , and 1000 ng ml^{-1} of dsRNA were added to a 3 mL liquid medium, shaking at 200 rpm, and tissue collected 3 dpi.

MRNA transcripts for the target genes were determined using qPCR on the Bio-Rad CFX96 Connect Real-Time system using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, US) in 10 μL reactions according to the manufacturer's protocol under the

following conditions: 95°C for 30s, and 45 cycles of: 95°C for 2s and 60°C for 5s. Melt curves with a range of 65 – 95°C with 0.5°C increments were used to assess nonspecific amplification, primer dimers, and aberrant amplifications. Primers and corresponding efficiencies are given in Additional file 3. Relative accumulation was calculated using the ΔΔCq method, relative to Sac7 (SS1G_12350) and *GFP*-dsRNA control with the corresponding dose (Livak and Schmittgen, 2001; Llanos *et al.*, 2015).

2.3.8 Foliar applications of dsRNAs to the leaf surface

Senescing petals of *B. napus cv. Westar* were inoculated with 20 ng μL^{-1} dsRNA, 0.015% Silwet L-77 (Lehle Seeds, Round Rock, TX, US), and 10 µL of *S. sclerotiorum* spores in water (5×10^5 spores ml^{-1}) and allowed to incubate for 3 days (Girard *et al.*, 2017). A 25 µL solution of 200 ng dsRNA and 0.03% Silwet L-77 was applied to the leaf surface of approximately 4 week old plants (30-50% flowering; n =10). Then, the senescing petal was applied to the same spot and allowed to incubate under high humidity for 2 days.

For the Arabidopsis assays, 25 day old leaves were treated with 10 µL of 200 ng of dsRNA and 0.02% Silwet L-77 and allowed to dry. A 10 µL *S. sclerotiorum* spore solution (5.5 mM glucose, 62.5 mM KH_2PO_4 (Sigma Life Science, St. Louis, MO, US); 1×10^6 spores mL^{-1} was spotted on the surfaces of the dsRNA coated leaves (n=30) and allowed to incubate under high humidity for 4 days (Garg *et al.*, 2010).

For *B. cinera* assays, a 12 µL solution containing 500 ng of dsRNA and 0.03% Silwet L-77 was applied to the leaf surface of approximately 4 week old (30-50% flowering) *B. napus*. Following a complete drying period, 10 µL of buffered *B. cinera* spores (5.5 mM glucose, 62.5 mM KH_2PO_4; 1×10^5 spores mL^{-1}) were placed on the same spot (n=40) and allowed to incubate for 4 days (Garg *et al.*, 2010). In all cases, lesion size was quantified using ImageJ software

(imagej.nih.gov). Water and *GFP*-dsRNA were both used as controls during the foliar assays and neither were significantly different from each other (Figure S2; one way ANOVA; p = 0.6).

2.4 RESULTS

2.4.1 Gene expression of *S. sclerotiorum* grown *in vitro* and on susceptible and tolerant hosts of *B. napus*

Next generation RNA sequencing was used to identify similarities and differences in gene expression between *in vitro* plate-grown cultures and *in planta*-grown *S. sclerotiorum* (Supplementary Table 2.1). Hierarchical clustering of FPKM values revealed *S. sclerotiorum* grown *in vitro* was transcriptomically distinct from cultures grown for 24 hours on *B. napus* leaves (Fig. 2.1). Specifically, *S. sclerotiorum* grown on either tolerant (cv. ZY821) or susceptible (cv. Westar) plants clustered together with a bootstrapping score of 100, indicating, that the expression of many *S. sclerotiorum* genes was dependent on the nature of the nutrition source, be it the nutrient-accessible *in vitro* culture or the more complex host canola cultivars.

2.4.2 Differential expression and GO term enrichment analysis in target gene identification

The hierarchical clustering of *S. sclerotiorum* gene expression in the three transcriptomes likely reflects differences in how the fungus responds to and infects different plant cultivars. To identify genes that may be responsible for the plant-infection process, a comparison of global gene expression of *S. sclerotiorum* grown on susceptible and tolerant leaves of *B. napus* to those grown *in vitro* was performed. The analyses identified 1,858 genes that were significantly up and 1,637 that were significantly down-regulated in both Westar and

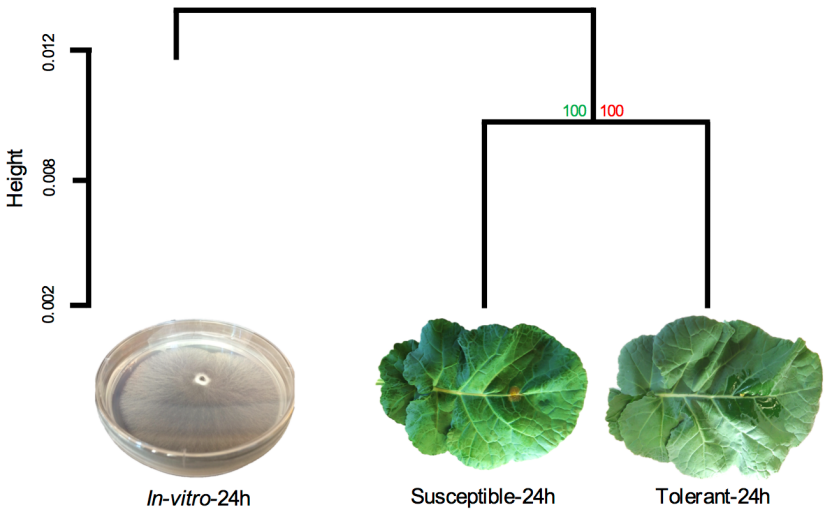


Figure 2.1 Hierarchical clustering of gene expression of the growth and penetration of *Sclerotinia. sclerotiorum in-vitro* (PDA) and in-planta (*Brassica napus* cv. Westar (Susceptible) and cv. ZhongYou821 (Tolerant)). Hierarchical clustering analysis of global gene expression was based on FPKM transcript abundances and a minimum detection value of 1 FPKM. Approximately unbiased (au) values found in green and bootstrapping p-values (bp) in red. Height represents correlation value between sub-branches.

ZY821, relative to the *in vitro*-growth fungus (Fig. 2.2). Differential expression was also observed between the susceptible Westar and partially resistant ZY821 cultivars of *B. napus,* with 487 and 448 genes being up and down-regulated, respectively, in the Westar cultivar, and 277 and 402 genes being up and down-regulated, respectively, in the ZY821 cultivar.

A gene ontology term enrichment analysis was performed on the significantly up and down-regulated genes to identify biological processes associated with infection. Processes that showed conserved enrichment in both *in-planta* and *in-vitro* included *oxidation-reduction processes, mycelium development,* and *cell division* (Fig. 2.2b). Significant enrichment of several processes involved in host-pathogen interaction within *in planta*-grown *S. sclerotiorum,* relative to *in vitro*-grown fungus were also observed, including; *carbohydrate metabolic process* (e.g. mannosidase, amylase, trehalose synthase, beta-glucosidase), *hydrolase activity* (e.g. triacylglycerol lipase, glycosyl hydrolase) and *transmembrane transport* (e.g. Major Facilitator Superfamily transporters, aminoacyl permeases). In contrast, when looking at processes that were down-regulated within *in-planta S. sclerotiorum,* processes related to protein synthesis and energy production including; *structural constituent of ribosome, translation* (e.g. initiation factors, elongation factors, RNA binding) *and cytochrome-c oxidase activity* were identified. Differences in enriched biological processes were also observed between S. *sclerotiorum* grown on susceptible and tolerant *B. napus*. For example, genes associated with *carbohydrate metabolism*, *hydrolase activity*, and *transcription factor activity* were differentially expressed in susceptible and partially resistant cultivars of *B. napus.* Complete lists of significantly differentially expressed genes and their respective FPKM values, as well as significantly enriched GO terms and their respective p-values can be found in Additional file 1.

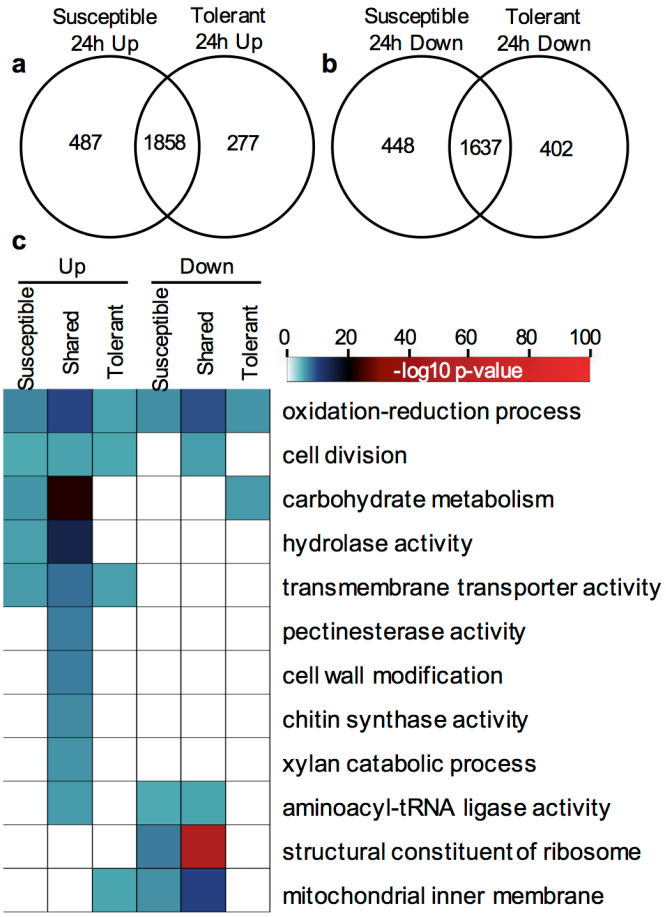


Figure 2.2 Identification of *Sclerotinia sclerotiorum* genes and biological processes significantly up and down regulated during infection (*in-planta*). (a) Venn diagram showing up-regulated genes in *S. sclerotiorum* 24hpi on susceptible (*Westar*) and tolerant (*ZY821*) genotypes of *Brassica napus*. (c) Venn diagram showing down-regulated genes in *S. sclerotiorum* 24hpi on susceptible (*Westar*) and tolerant (*ZY821*) genotypes of *B. napus.* (c) Heatmap of enriched GO terms associated with significantly up and down regulated genes *in-planta* grown *S. sclerotiorum* during infection. GO terms are considered statistically significant if the hypergeometric p-value < 0.05.

2.4.3 QRT-PCR assessment of RNAi in *S. sclerotiorum*

To assess the duration of dsRNA target gene knockdown, *S. sclerotiorum* was grown in liquid cultures containing dsRNA molecules targeting one of the following three genes: SS1G_01703, amino acyl tRNA ligase; SS1G_05899, thioredoxin reductase; and SS1G_06487, TIM44 (Fig. 2.3).

While a significant reduction in dsRNA target gene activity was observed 24 hpi ($p<0.05$), by 48 hpi, all three genes' transcripts were reduced by 60-70% ($p<0.01$), and remained at that level of suppression up to 96 hpi.

To examine dsRNA dose effects on target gene knockdown, liquid cultures of *S. sclerotiorum* were exposed to a range of doses (100-1000 ng/mL) of dsRNAs targeting the previously mentioned three genes and one additional gene; SS1G_11912, necrosis/ethylene-inducing peptide 2. The dsRNA targeting SS1G_05899 showed a 79 - 85% ($p<0.01$) reduction in transcript abundance across all doses tested. Similarly, SS1G_06487 showed a 45 - 60% ($p<0.05$) reduction in transcript accumulation. The dose-response varied amongst the genes, where both SS1G_01703 and SS1G_11912, which showed stronger expression during infection conditions compared to the *in vitro* cultures, required a dose of at least 200 ng/mL to achieve a maximum reduction of 61% and 97% ($p<0.05$) respectively (Fig. 2.4). It is worth noting that SS1G_05899 in the RNA-seq analyses had a 0.05 to 0.14 -fold change decrease in expression during infection on the susceptible and tolerant cultivars of *B. napus,* respectively, and was reduced 80% at the lowest dose tested, 100 ng/mL. In contrast, SS1G_11912, with fold changes of 3.9 to 4.5 during the plant infections, needed a dose of at least 200 ng/mL to suppress expression to 91%. From this limited analysis, it appears that genes with higher induction during infection may require more dsRNA to adequately knockdown expression levels.

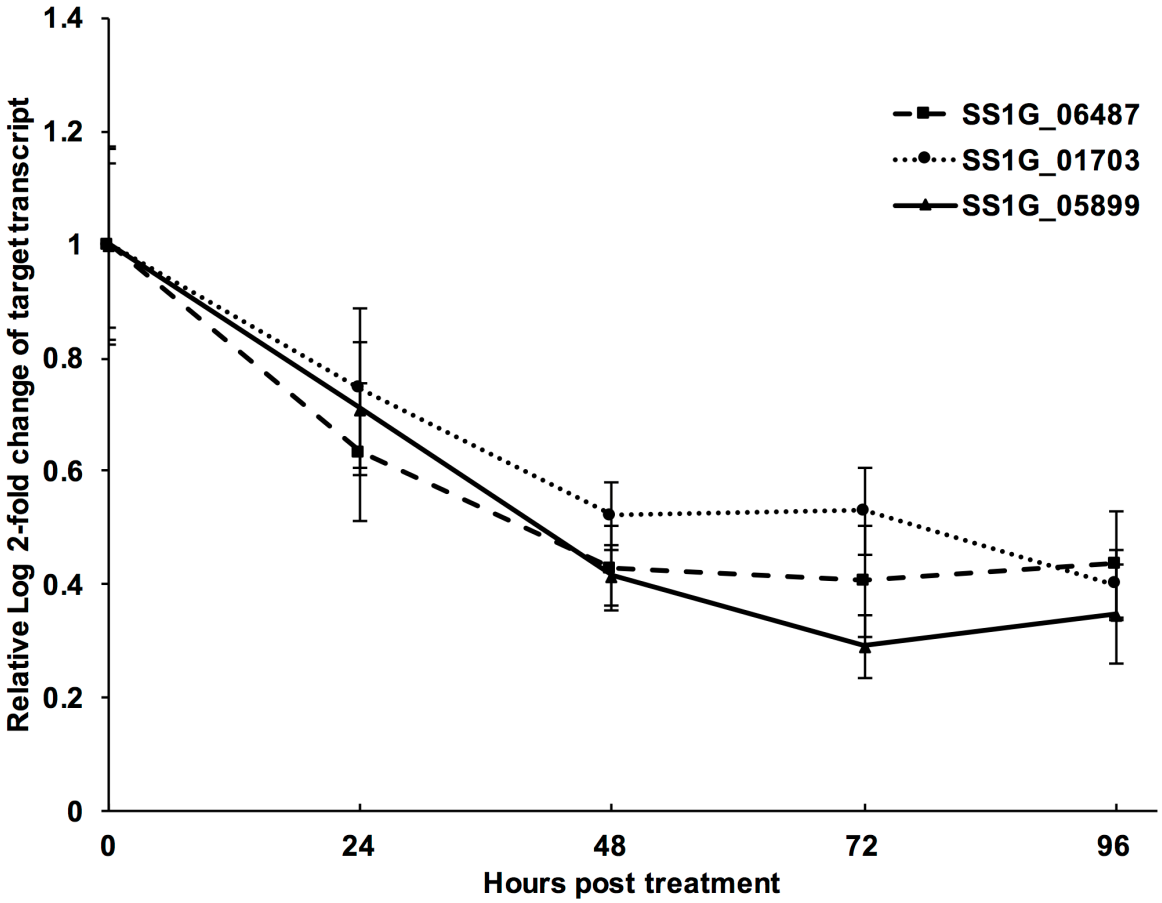


Figure 2.3 Timing of RNAi silencing in *Sclerotinia sclerotiorum* for the select targets SS1G_01703, SS1G_05899, and SS1G_06487. Transcript levels were measured at time points 0, 24, 48, 72, and 96 hours post treatment of 500 ng/mL of dsRNA in liquid culture. Data are relative to time point 0 hrs and GFP-dsRNA sample. SsSac7 (SS1G_12350) was used as reference gene. Data represents 3 biological replicates. Error bars represent standard error and reductions were significant over time (Student's t-test; $p < 0.05$).

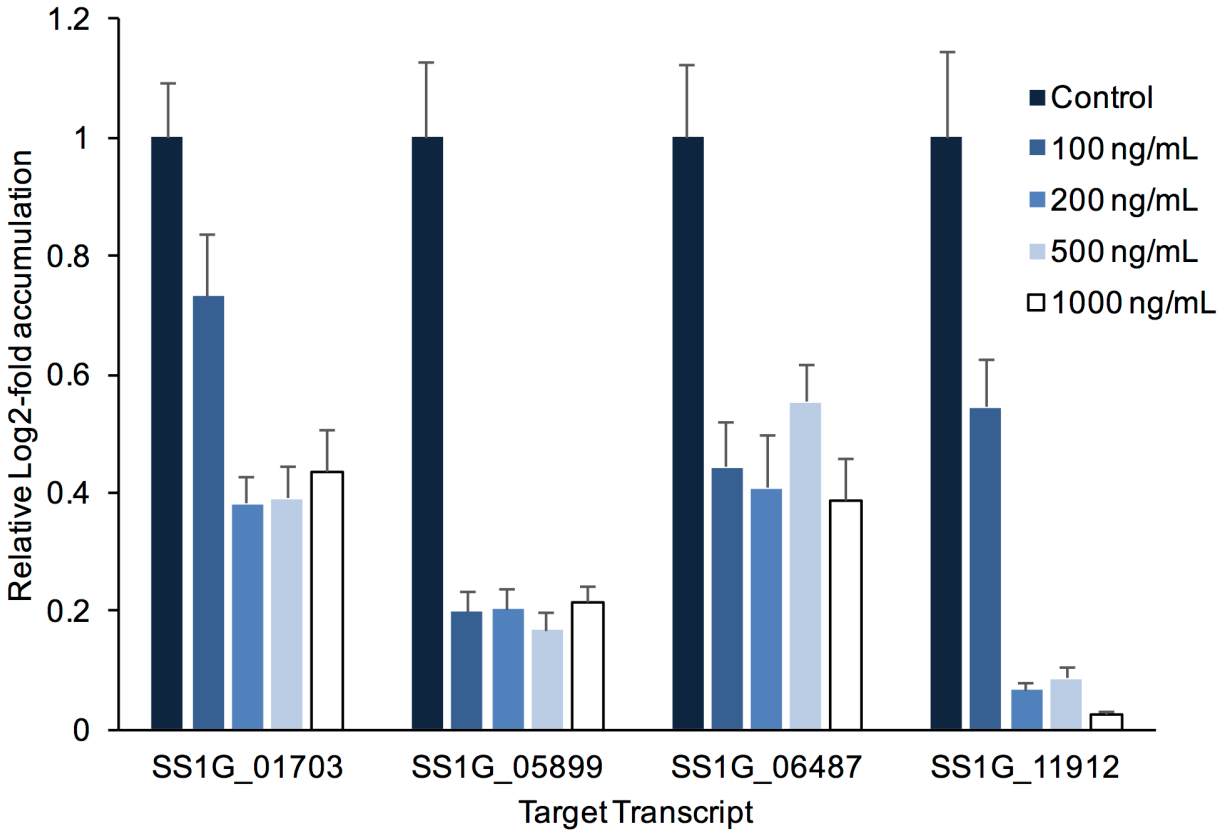


Figure 2.4 Effect of dsRNA dose on *Sclerotinia sclerotiorum* grown in liquid cultures for the select targets SS1G_01703, SS1G_05899, SS1G_06487, and SS1G_11912. Transcript levels were measured 3 days post treatment of 100, 200, 500, or 1000 ng/mL dose of dsRNA. Data relative to corresponding GFP-dsRNA (non-targeting control) dose and SsSac7 (SS1G_12350) reference gene. Data represents 3 biological replicates. Error bars represent standard error and all treatments were significant (Student's t-test, $p < 0.05$).

2.4.4 Foliar applications of dsRNA reduce *S. sclerotiorum* infection in *B. napus*

Having confirmed that dsRNAs could reduce transcript abundance in *S. sclerotiorum* for at least 96 hpi using relatively low concentrations, the level of protection imparted to *B. napus* plants using a petal infection assay that facilitated aggressive *S. sclerotiorum* infection was then assessed (Girard *et al.*, 2017). A total of 61 *S. sclerotiorum* genes targets were selected from i) the Database of Essential Genes (DEG; Luo *et al.*, 2014), ii) literature searches (Liang *et al.*, 2014), and iii) the Target Identification Pipeline (TIP; Supplementary Table 2.2). TIP genes were nominated based on a range of selection criteria, including: common significant expression within the RNA-seq dataset; biological function; moderate expression levels (between 10 and 500 FPKM); fold changes between -0.5 and 4 (infection relative to *in vitro*); and biological processes summarized in Supplementary Table 2.2. The dsRNAs were applied to leaves, and *S. sclerotiorum*-infected petals were then placed over the dsRNA-treated surfaces. Fungal lesion size was scored 2 dpi, and of the 61 dsRNAs tested, 24 showed a significant reduction in lesion size, ranging from 24 to 85 % (one way ANOVA, $p < 0.05$; Fig. 2.5b; Table 2.1; Additional file 2). Of these 24 dsRNA molecule treatments, 19 conformed to the criteria outlined in TIP.

Some of the dsRNA molecules nominated using the TIP selection criteria included genes involved in such biological processes as toxin biosynthesis (SS1G_01703), ROS response (SS1G_02495), and cell cycle regulation (SS1G_09897). Targeting these genes' transcripts with dsRNA resulted in significant reductions in lesion size by 85%, 71%, and 45% respectively (one way ANOVA, $p<0.001$ for all 3 dsRNA treatments; Fig. 2.5b; Table 2.1). Similarly, *A. fumatigus* essential gene homologues associated with ribosomal biogenesis (SS1G_07873) and mitochondrial protein import (SS1G_06487) also significantly reduced fungal lesion formation by 64% ($p<0.001$) and 85% ($p<0.001$), respectively (Fig. 2.5b; Table 2.1). Leaf surfaces treated

Table 2.1 Selected list of S*clerotinia. sclerotiorum* target genes for RNA interference testing in liquid culture, and infection assays on *Brassica napus* and *A. thaliana*.

Source	Gene	FPKM *in vitro*	FPKM Westar 24 hpi	FPKM ZY821 24 hpi	Process
Literature [1]	SS1G_08218	7913.2	5211.0	6743.8	Oxaloacetate acetylhydrolase
KEGG [2]	SS1G_00543	782.7	411.3	350.2	SCF complex subunitSkp1
Essential Genes [3]	SS1G_05899	268.7	259.1	244.4	Thioredoxin reductase
Essential genes [3]	SS1G_06487	87.3	82.0	114.3	TIM44
Essential genes [3]	SS1G_07873	47.1	41.5	47.8	pre-40S ribosomal particle
Low expression values	SS1G_00509	1.8	6.6	3.2	Hydrolase activity, carbohydrate metabolic process
Low expression values	SS1G_06055	0.0	0.8	2.0	Prenyltransferase activity
Low expression values	SS1G_13720	0.0	1.4	0.5	MFS sugar transporter
Low expression values	SS1G_13982	0.1	0.2	2.0	Triacylglycerol lipase
Moderate expression values	SS1G_09261	14.20	22.56	22.80	Bub1-Bub3 complex localization to kinetochore
High accumulation values	SS1G_10167	14126.6	19992.7	24179.3	Endo-polygalacturonase
Positive expression fold change [4]	SS1G_02791	2.0	19.1	23.8	Transcription factor activity
Positive expression fold change [4]	SS1G_04551	1.9	12.6	16.4	Pectinesterase
Positive expression fold change [4]	SS1G_14298	17.9	31.6	23.0	bHLH transcription factor
TIP [5]	SS1G_01703	45.1	307.0	164.7	Aminoacyl-tRNA ligase activity / alfatoxin biosynthesis
TIP [5]	SS1G_02495	61.2	336.1	353.7	Peroxidase activity
TIP [5]	SS1G_03208	11.5	51.4	48.3	Pre-mRNA splicing factor 8
TIP [5]	SS1G_03991	16.7	39.5	39.4	Srb8 component of mediator complex
TIP [5]	SS1G_04966	30.6	64.3	59.5	Histone modification
TIP [5]	SS1G_06830	13.7	101.8	71.3	Aminoacyl permease
TIP [5]	SS1G_09680	50.8	61.2	70.3	60S ribosome biogenesis
TIP [5]	SS1G_09897	28.53	61.36	59.24	Cdc25
TIP [5]	SS1G_11912	24.5	551.2	359.3	Necrosis/ethylene inducing peptide 2
TIP [5]	SS1G_12021	149.3	397.4	363.8	1,3 glucan synthase
TIP [5]	SS1G_12640	53.1	114.2	130.1	Protein disulphide oxidoreductase process
TIP [5]	SS1G_12992	13.5	57.5	39.0	Transglutaminase protein modification in ER
TIP [5]	SS1G_13702	127.1	98.2	101.9	TIM23
TIP [5]	SS1G_13746	15.1	33.7	32.5	Peroxisome

(**1**) Liang et al. (2014); (**2**) Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/ ;Kanehisa *et al.*, 2017); (**3**) Database of Essential Genes (http://www.essentialgene.org/ ;Luo *et al.*, 2014); (**4**) Positive fold change in response to in planta growth (**5**) TIP = Target identification pipeline (Table S2)

with either water + Silwet L-77 or a non-fungal dsRNA + Silwet L-77 *(GFP*-dsRNA) equally resulted in rapid *S. sclerotiorum* infection that developed large necrotic lesions by 2 dpi (Fig. 2.5a; Supplementary Figure 2.1). In contrast, when the leaf surface was treated with an application of *S. sclerotiorum*–specific dsRNAs, dramatic reductions in lesion size and morphology was observed for these aforementioned dsRNAs (Fig. 2.5a).

Interestingly, 6 of the dsRNAs tested significantly increased ($p<0.05$) the lesion size compared to the control treatment (Fig. 2.5b). Two of these dsRNAs targeted *S. sclerotiorum* transcription factor genes (SS1G_14298; SS1G_02791), and lesion size increased 64 and 88%, respectively (one way ANOVA, $p<0.05$). The dsRNA targeting *SsSkp1* (SS1G_00543) caused an increase in lesion size by 133% (one way ANOVA, $P<0.01$). As SKP1 plays a role in both chromosome separation and ubiquitin-mediated protein degradation (Kanehisa *et al.*, 2017), RNAi-mediated knockdown was expected to adversely affect fungal growth, but on closer inspection of the RNA-seq data, *SsSkp1* transcripts were not observed to accumulate to a significant extent during infection on *B. napus* (Table 2.1), which may explain a lack of fungal suppression with this particular dsRNA. In contrast, SS1G_14298 and SS1G_02791, which had increased expression during infection conditions, were possibly nonessential to the infection process or facilitated pathogenicity and thus failed to reduce fungal pressure *in planta*.

Furthermore, 31 genes targeted by some dsRNA molecules caused no significant impact on fungal lesion sizes on the leaves; these dsRNAs targeted genes involved in a range of cellular processes, including carbohydrate catabolism (SS1G_00509; endo-1,4-beta-xylanase), hydrolysis (SS1G_13982; triacylglycerol lipase), and kinetochore functions (SS1G_09261). Even a previously characterized genetic deletion target(Liang *et al.*, 2014), SS1G_08218 (*Ssoah*; oxaloacetate acetylhydrolase) had no significant impact on lesion size

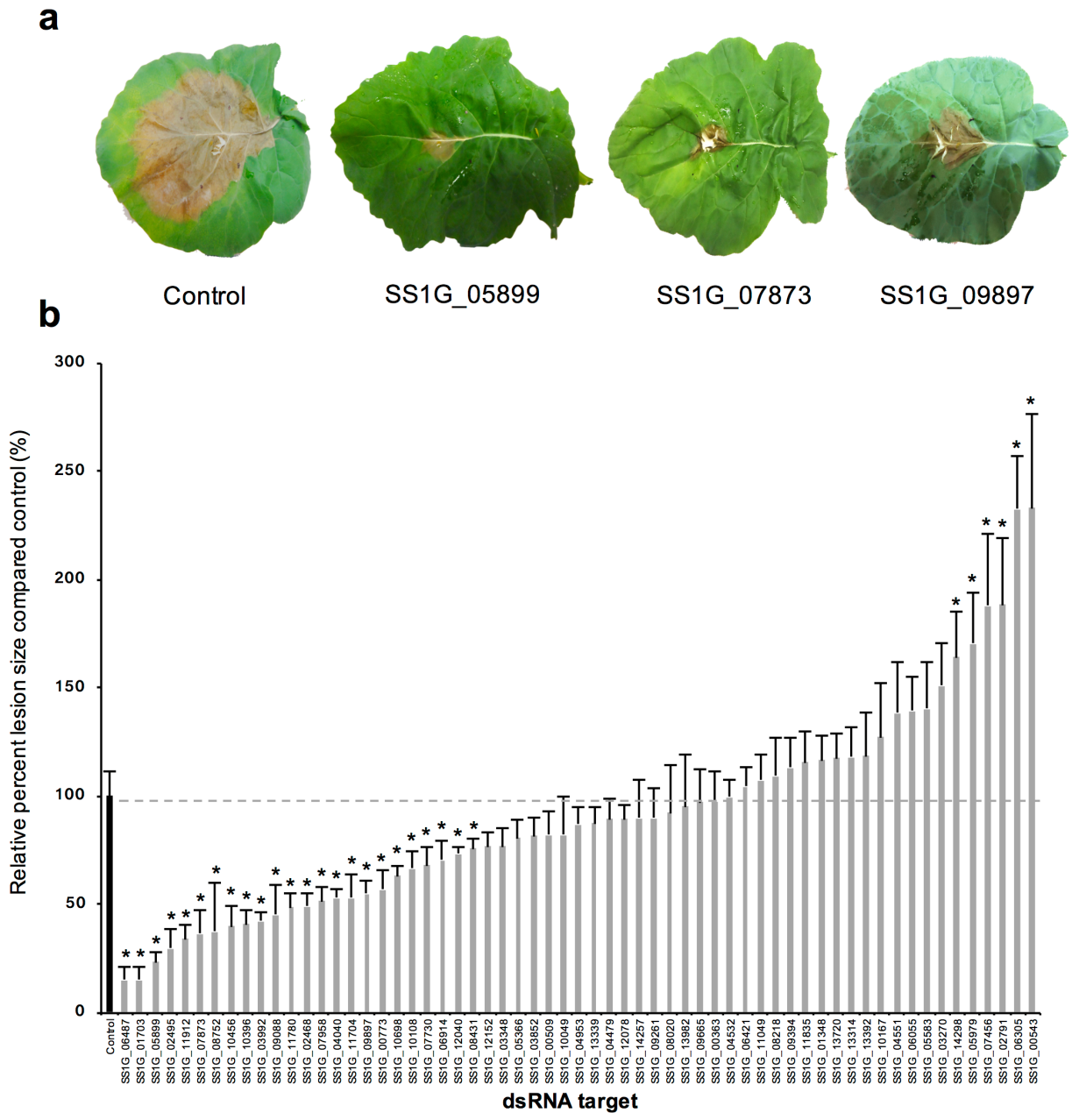


Figure 2.5 DsRNA targeting *Sclerotinia sclerotiorum* suppresses lesion size on *Brassica napus* susceptible cultivar Westar. (**a**) *Sclerotinia sclerotiorum* infection lesions on *B. napus cv. Westar* following a treatment of 200 ng dsRNA targeting *S. sclerotiorum* genes at 2 dpi. (**b**) Average lesion size (n = 10 leaves) relative to control (black bar) with standard error bars. Significant difference (One-way ANOVA; $p < 0.05$) from control represented by asterix (*).

(one way ANOVA, $p = 0.7$) under the conditions tested. While these targets did not show significant modulations in lesion size, they helped define criteria for TIP.

2.4.5 Topical dsRNAs mitigate *S. sclerotiorum* infection on *A. thaliana*

Given the extensive host range of *S. sclerotiorum*, it was of interest to determine whether this fungus could be similarly suppressed using dsRNAs in another plant species. Using the model plant *A. thaliana*, 10 new *S. sclerotiorum* genes, nominated using TIP selection criteria (Table 2.1; Supplementary Table 2.2), were assessed in parallel with 6 of the best performing targets in *B. napus* (SS1G_01703, SS1G_02495, SS1G_05899, SS1G_06487, SS1G_07873, and SS1G_11912; Fig. 5b). Significant reductions in lesion size between 34 – 66 % were observed (one-way ANOVA, $p < 0.05$; Fig. 2.6b) by targeting genes associated with processes such as mRNA splicing (SS1G_03208), ribosome biogenesis (SS1G_09680), protein disulphide oxidoreductase (SS1G_12640) and a peroxisomal protein (SS1G_13746). DsRNA treatments reduced the fungal progression on the spore-inoculated leaves, suggesting that dsRNA used to protect one plant species can also be applied to protect another with near-equivalent success.

The most effective dsRNA treatments on *B. napus* leaves were also effective at reducing infection on *A. thaliana* significantly, such as SS1G_11912, which reduced lesion sizes by 64-66% in both plant species. However, there were noticeable differences in efficacy of the other five dsRNAs. Specifically, the dsRNAs targeting SS1G_02495 and SS1G_07873, which reduced lesions 71 and 64%, respectively in *B. napus*, only reduced lesions by 34 and 46% in *A. thaliana,* respectively. The disparities in lesion size between *B. napus* and *A. thaliana* may be attributed to differences in infection rates, defence responses, or leaf structures.

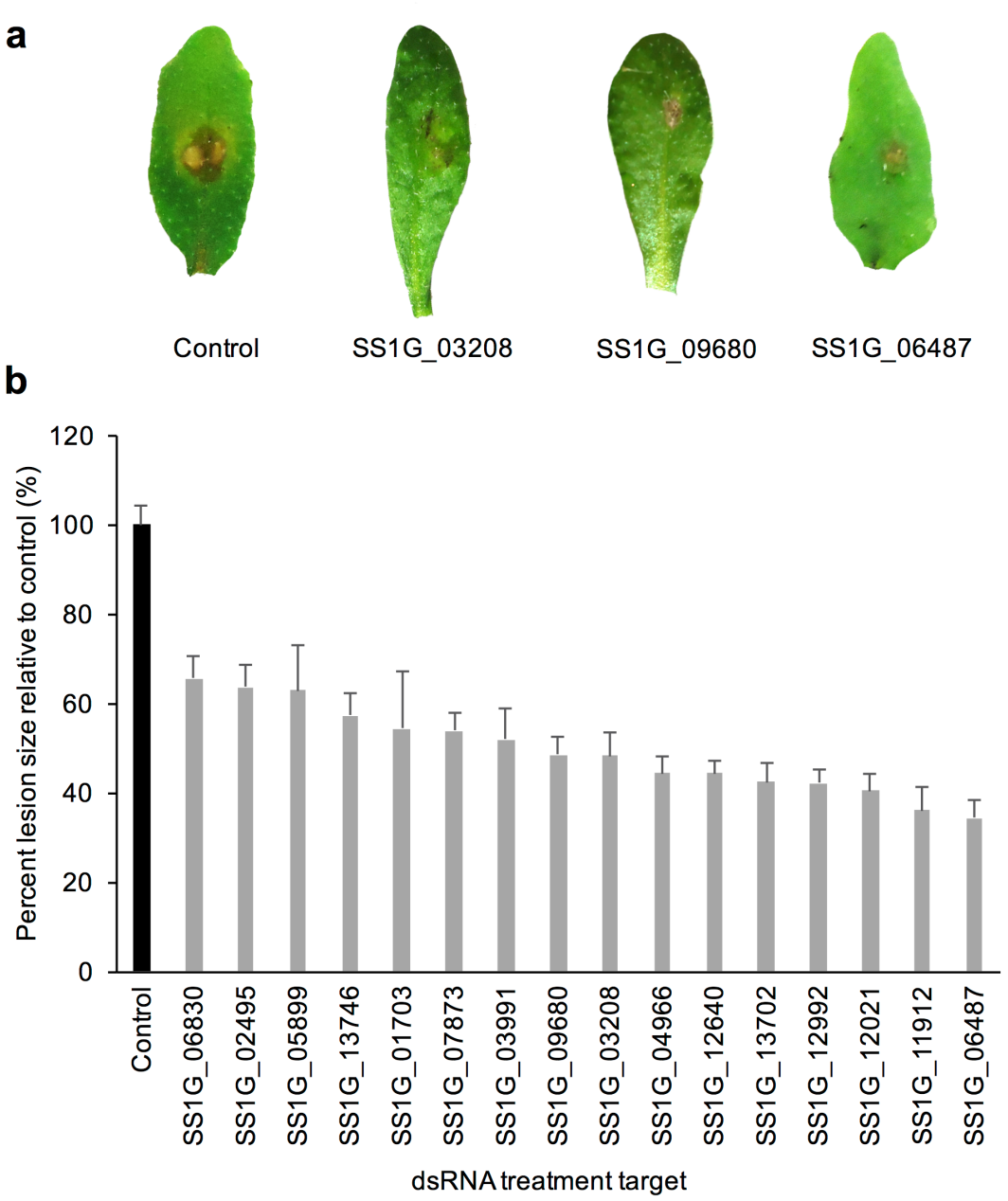


Figure 2.6 DsRNA treatment on *Arabidopsis thaliana* leaves reduces *Sclerotinia sclerotiorum* lesion area. (**a**) *Sclerotinia sclerotiorum* spore inoculation on *A. thaliana* leaves after a foliar application 200 ng of specific dsRNA spread over the entire leaf surface at 4 dpi. (**b**) Average lesion relative to control (darker bar) with standard error bars of 3 bio-replicates of 10 leaves (n =30). All targeting dsRNA treatment were significantly different from control (One-way ANOVA, $p < 0.05$).

2.4.6 DsRNAs targeting homologues in *Botrytis cinerea* attenuate fungal infection

Botrytis cinerea is a phytopathogenic fungus closely related to *S. sclerotiotum,* and hence, it was interest to determine whether some of the *S. sclerotiotum* target genes identified during earlier screens could be similarly used as RNAi targets to suppress *B. cinerea* infections on *B. napus.* *Botrytis cinerea*-specific dsRNA molecules were designed to target five of the most effective targets identified in *S. sclerotiotum* (Additional file 2). Leaves treated with dsRNA targeting BC1G_04775 (SS1G_06487 homologue) and BC1G_01592 (SS1G_05899 homologue) formed smaller brown necrotic regions than the control leaves (Fig. 2.7a). Treatments of dsRNA targeting BC1G_04955 (SS1G_02495 homologue), BC1G_04775, BC1G_01592, BC1G_07805 (SS1G_07873 homologue), and BC1G_10306 (SS1G_11912 homologue), on average, reduced lesion sizes by 66% (Fig. 2.7b; one-way ANOVA, $p<0.05$ for all treatments). Interestingly, the dsRNA targeting the SS1G_11912 homologue, BC1G_010306, showed only moderate reductions in lesion area, suggesting that the gene product may be more important for *S. sclerotiorum* infection rather than *B. cinerea.* A complete summary of foliar results can be found in Additional file 2.

2.5 DISCUSSION

Transcriptome interrogation of the *B. napus*-*S. sclerotiorum* interface uncovered selective targets of fungal growth and pathogenicity that helped guide the development of an RNAi-based control target identification pipeline (TIP; Supplementary Table 2.2). The demonstrated dsRNA-mediated knockdown of *S. sclerotiorum* transcripts provided evidence of the utility of RNAi to selectively and specifically target individual genes in this agriculturally important fungal pathogen. Topical applications of dsRNAs targeting *S. sclerotiorum* transcripts significantly

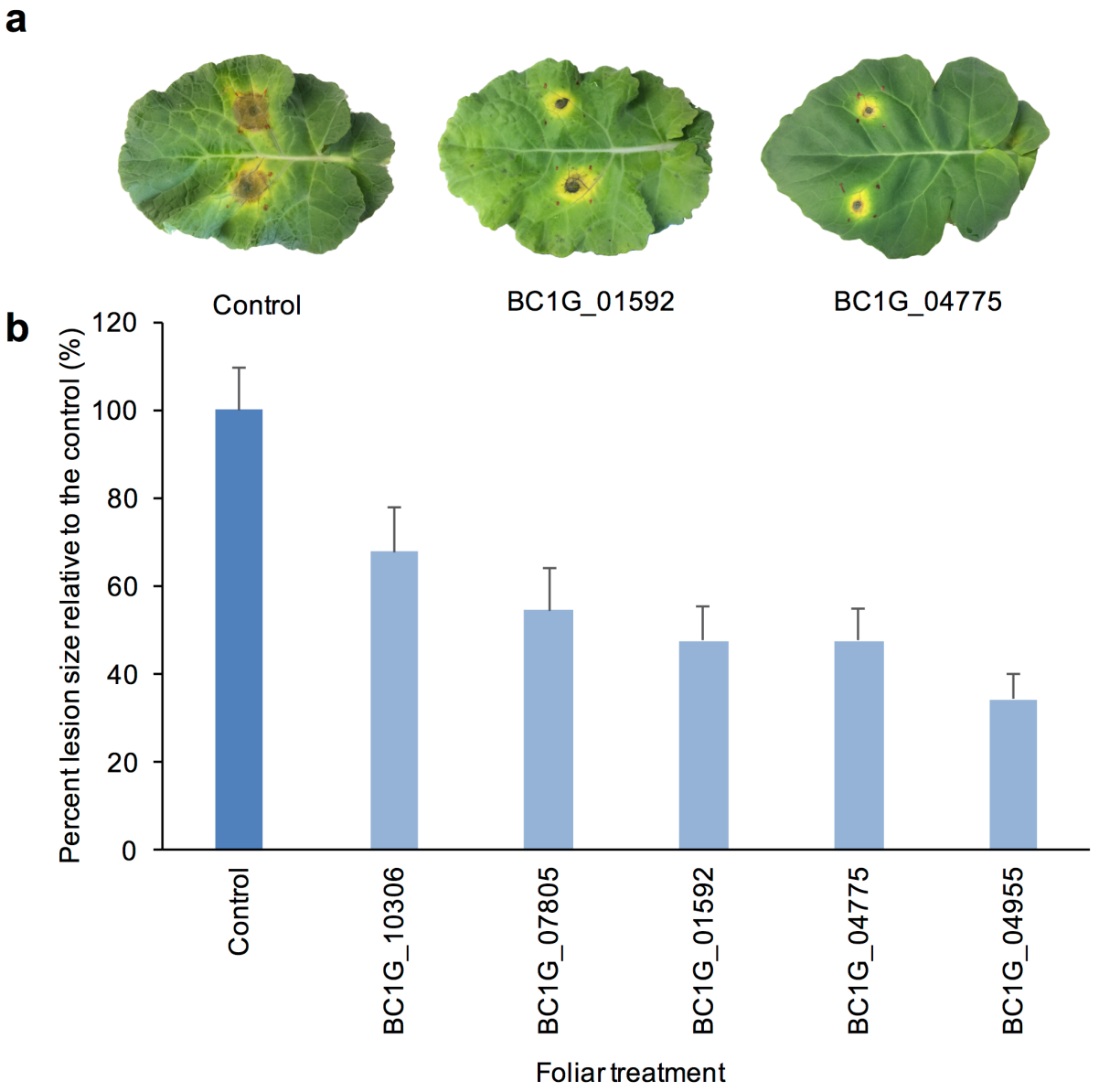


Figure 2.7 *Botrytis cinerea* homologues targeted with foliar dsRNA controls fungal infection. (a) *Botrytis cinerea* spore inoculation of *Brassica napus cv.* Westar leaves after an application of 500 ng of dsRNA targeting *B. cinerea* genes was spread over a 4 cm^2 area at 4 dpi. (b) Average lesion size compared to control (darker bar)(n = 40 leaves) with standard error bars. All targeting dsRNA treatment were significantly different from *GFP*-dsRNA control (One-way ANOVA, $p < 0.05$).

reduced the size of fungal lesions in both *B. napus* and the model plant system *A. thaliana.* Extension of this technology to homologous transcripts of *B. cinerea* revealed unprecedented

cross-species control of fungal pathogenesis using topical applications of dsRNAs targeting a single gene.

When dsRNAs were incubated with *S. sclerotiorum in vitro*, transcript level reductions were observed for all genes tested, providing clear evidence of fungal dsRNA uptake and an RNAi response. Although the dsRNA uptake mechanism has yet to be established in fungi, the results are consistent with other eukaryotic organisms, where knockdown of transcript levels occurred within 48 hours (Gong *et al.*, 2014; Neumann *et al.*, 2006; Štefanic *et al.*, 2010; Zimmermann *et al.*, 2006). In addition, the dose-response for individual transcripts varied, with each individual gene responding differently, and very high doses failing to elicit further transcript reductions, which has been observed in previous insect and flatworm research (Asokan *et al.*, 2014; Killiny *et al.*, 2014; Meyering-Vos and Müller, 2007; Štefanic *et al.*, 2010). Differences in knockdown response may be attributed to mRNA secondary structure, mRNA turnover rate, GC content, or other properties associated with dsRNA structure (Chan *et al.*, 2009; Larsson *et al.*, 2010; Shao *et al.*, 2007; Vermeulen *et al.*, 2005). Without modifying the delivery or chemistry of the dsRNA molecules, notable reductions in infection symptoms were achieved. With recent developments utilizing modified nucleotides and novel delivery methods (Joga *et al.*, 2016; Love *et al.*, 2010; Semple *et al.*, 2010), future chemistries could increase silencing potency of RNAi-based control strategies.

Interrogation of global changes in gene expression at the host-pathogen interface in both susceptible and tolerant hosts of *S. sclerotiorum* identified novel targets for fungal control using

RNAi-based biotechnologies. Using transgenic techniques, endogenously-produced dsRNAs within fungi targeting single genes can suppress fungal growth (Andrade *et al.*, 2016; Ghag *et al.*, 2014). In contrast, previous efforts to develop topically-applied dsRNA-based fungicides have been effectively limited in application and gene specificity by requiring multiple target gene knockdowns to reduce pathogen progression (Koch *et al.*, 2016; Wang *et al.*, 2016). No previous research has provided a clear and rational link between transcript profiling and effective dsRNA molecule design. The identification of single RNAi targets allows for broader agriculture applications, through the flexible design of dsRNA molecules targeting selective fungal genes using the target identification pipeline, and the specificity to control a single gene from a single target organism.

Global RNA profiling and GO term enrichment highlighted specific genes and biological processes associated with cell division (e.g. SS1G_09897), cell wall modification (e.g. SS1G_12021), oxidoreductase (e.g. SS1G_05899), and peroxidase activity (e.g. SS1G_02495) as novel targets for fungal control and selection criteria for TIP. By suppressing gene expression of processes such as cell wall modification, required for fungal growth and maintenance of cellular integrity (Werner *et al.*, 2007), and fungal ROS response proteins, required to protect the advancing fungal hyphae from plant respiratory ROS defense (Heller and Tudzynski, 2011), gene silencing impeded fungus establishment on the leaf surface. Previous genetic deletions of thioredoxin reductase and peroxidases in *Magnaporthe oryzae* Couch striked parallels to the dsRNA treatment of SS1G_05899 (thioredoxin reductase) and SS1G_02495 (peroxidase) (Fernandez and Wilson, 2014; Mir *et al.*, 2015), which also restricted fungal advance. Furthermore, by identifying homologues of essential genes from the model organism *A. fumatigus* (Luo *et al.*, 2014), despite the overall processes being downregulated during infection,

flexibility was added to TIP-guided selection for dsRNA control. By exploring fungal transcriptional reprogramming, a more complete understanding of the genes and cellular processes associated with pathogenicity was gained and provided a guide to the flexible and adaptable design of dsRNA molecules targeting key processes using TIP.

RNAi of a single gene product may not always significantly limit fungal infection, as evidenced by efforts to knockdown 32 genes that failed to prevent fungal lesion expansion. During infection, *S. sclerotiorum* secretes a diverse arsenal of pathogenicity factors including hydrolases and nutrient acquisition enzymes to secure nutrient sources during host infection, while maintaining a constant store of carbohydrates (Heard *et al.*, 2015; Jobic *et al.*, 2007; Lyu *et al.*, 2015). Consequently, when targeting processes such as carbohydrate catabolism (e.g. SS1G_00509), cell wall degrading enzymes (e.g. SS1G_04551) and hydrolase activity (e.g. SS1G_13982), despite being upregulated in the infected tissues, no significant impacts on lesion sizes were observed, highlighting the fact that not all cellular processes can provide good RNAi control targets. Similarly, a high level of gene regulation may also reduce the effect of RNAi-based control. For example, the pathogenicity factor *Ssoah* (SS1G_08218; oxaloacetate acetylhydrolase), although essential for pathogenicity, is also known to be heavily regulated by *SsPac1* (Rollins, 2003). Thus, the RNAi-induced reduction in *Ssoah* gene activity may be rescued by the intrinsic regulatory network responsible for *S. sclerotiorum* pathogenesis. Additionally, other factors such as protein half-life, which could outlast an RNAi silencing event, and negative pathogenicity regulation, already observed in *S. sclerotiorum*, could fail to elicit an observable physiological response despite successful transcript knockdown (Pan *et al.*, 2015; Rinkevich and Scott, 2013). Thus, by screening a great variety of genes sharing single biological processes, poor targets can be excluded from the RNAi target selection.

Transcript accumulation levels could have affected the efficiency of RNAi silencing in *S. sclerotiorum* and thus was an important consideration for the construction of TIP. For the genes SS1G_13720, SS1G_06055, and SS1G_13982, which accumulated at low levels under all conditions, no significant changes in lesion formation were observed. Previously, low transcript accumulation levels resulted in poor siRNA-mediated knockdown in human cells, suggesting that a threshold level of target mRNA must be present for the activation of the RISC complexes (Hong *et al.*, 2014; Hu *et al.*, 2004). In contrast, transcripts of the genes *Ssoah* and SS1G_10167 accumulated at high levels under all conditions, also failed to respond to the administered dsRNA in the infection assays. In other organisms, genes with high transcription rates have similarly been difficult to knock down using RNAi, presumably because the dose of dsRNA was insufficient to eliminate enough of the target transcripts (Dornseifer *et al.*, 2015; Larsson *et al.*, 2010). At the dsRNA dose tested, the majority of significant reductions in lesion size were associated with dsRNAs that targeted moderately expressing genes (10-500 FPKM); genes that showed large deviations in expression levels (up or down-regulated) in the infected plants, relative to the *in vitro* –grown fungus, were not significantly affected by the dsRNA treatments. Effective RNAi can be achieved when targeting genes that cannot reliably increase transcription in response to a silencing event. Transcriptomic analyses are useful to uncover which genes' transcripts are induced to increase under infection conditions, as observed by drastic transcript fold-change levels. A higher accumulation of mRNA would result from stronger promoter induction and activity under infection conditions (Coradetti *et al.*, 2012; Kawahara *et al.*, 2012). Therefore, a gene with the capability to elicit a strong transcriptional response may have the capacity to buffer RNAi-induced perturbations upon exposure to dsRNA. Using RNA-seq,

highly induced genes can be avoided, and genes with moderate upregulation during infection can be preferentially selected.

Synthesizing the results into TIP, 80% of the dsRNA targets, which reduced lesion sizes in *B. napus* significantly, adhere to the TIP protocol. Furthermore, all of the ten additional dsRNAs designed utilizing the TIP criteria, mitigated disease on an alternate plant, *A. thaliana*, validating TIP as a novel fungal RNAi identification guide for control of the devastating phytopathogen *S. sclerotiorum.* Additionally, six dsRNAs previously assayed on *B. napus* also protected *A. thaliana.* The protection imparted by the dsRNA molecules on both plant species suggests a common fungal infection strategy is being deployed by *S. sclerotiorum* and similar target knockdown may be responsible for improved plant protection. While there was a level of protection shared between the two host species, the differences in the extent of protection may be attributed to variations in leaf architecture, such as larger cell size, cell wall and thicker cuticle of *B. napus*, which together, can structurally limit fungal growth (Miedes *et al.*, 2014; Serrano *et al.*, 2014; Uloth *et al.*, 2015). Furthermore, in the closely related fungus, *B. cinerea*, the same topical dsRNA protected a variety of horticultural species (Wang *et al.*, 2016). Taken together, the array of dsRNAs that produced strong transcript reductions could be sufficient to confer protection for other major agronomic crops. Identifying a variety genes provides multiple tactics for successful phytoprotection regardless of potential differences in host architecture.

A recent report by Wang *et al.* (2016) showed that dsRNAs targeting transcripts encoding both Dicer-like protein 1 and 2, two proteins responsible for small RNA processing in eukaryotic cells, were able to suppress *B. cinerea* and *Verticillium dahliae* Kleb infection of transgenic *A. thaliana* containing a construct to produce dsRNA molecules targeting both species (Wang *et al.*, 2016). Here, the results demonstrated that a single dsRNA, when applied to the leaf surface, was

able to suppress the expansion of leaf lesions of both *S. sclerotiorum* and *B. cinerea*. While most of the tested dsRNAs of *S. sclerotiorum* and *B. cinerea* could significantly reduce disease symptoms in *B. napus,* some targets showed greater reductions in disease severity. Despite both necrotrophic pathogens likely sharing a common cluster of genes to control pathogenicity in a broad range of host plant species, *B. cinerea* likely operates through alternative pathogenic pathways to *S. sclerotiorum* to control infection in different host plants (Amselem *et al.*, 2011; Billon-Grand *et al.*, 2012). Thus, additional target discovery of any host-pathogen system is warranted to identify the best targets for RNAi-based control of fungal pathogenesis.

RNA-seq technology offers comprehensive insights into the genes and processes involved in fungal pathogenesis for the design of specific RNAi biotechnology. Identifying numerous alternative target transcripts allows for greater design flexibility, such that topical applications of dsRNA can be extended to control other fungal pathogens and protect other agronomically important plant species. The target identification pipeline provides a new, adaptable platform for the design of RNAi control and marks a substantial evolution of next-generation fungal phytopathogenic control. Further examinations of pathosystems will identify additional efficient RNAi targets across multiple fungi to improve broad fungal control.

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2.7 REFERENCES

Amselem, J., Cuomo, C.A., Kan, J.A.L. van, et al. (2011) Genomic Analysis of the Necrotrophic Fungal Pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS Genet.* **7**, e1002230.

Anders, S. and Huber, W. (2010) Differential expression analysis for sequence count data. *Genome Biol.* **11**, 1–12.

Andrade, C.M., Tinoco, M.L.P., Rieth, A.F., Maia, F.C.O. and Aragão, F.J.L. (2016) Host-induced gene silencing in the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*. *Plant Pathol.* **65**, 626–632.

Asokan, R., Sharath Chandra, G., Manamohan, M., Krishna Kumar, N.K. and Sita, T. (2014) Response of various target genes to diet-delivered dsRNA mediated RNA interference in the cotton bollworm, *Helicoverpa armigera*. *J. Pest Sci. (2004)*. **87**, 163–172.

Bateman, A., Martin, M.J., O'Donovan, C., et al. (2015) UniProt: A hub for protein information. *Nucleic Acids Res.*

Becker, M.G., Walker, P.L., Pulgar-Vidal, N.C. and Belmonte, M.F. (2017) SeqEnrich: A tool to predict transcription factor networks from co-expressed *Arabidopsis* and *Brassica napus* gene sets. *PLoS One* **12**, 1–13.

Billmyre, R.B., Calo, S., Feretzaki, M., Wang, X. and Heitman, J. (2013) RNAi function, diversity, and loss in the fungal kingdom. *Chromosom. Res.* **21**, 561–572.

Billon-Grand, G., Ve, Rascle, C., Droux, M., Rollins, J.A. and Poussereau, N. (2012) pH modulation differs during sunflower cotyledon colonization by the two closely related necrotrophic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*. *Mol. Plant Pathol.* **13**, 568–578.

Bolger, A.M., Lohse, M. and Usadel, B. (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120.

Bolton, M.D., Thomma, B.P.H.J. and Nelson, B.D. (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: Biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol.* **7**, 1–16.

Bradley, C. a., Lamey, H. a., Endres, G.J., Henson, R. a., Hanson, B.K., McKay, K.R., Halvorson, M., LeGare, D.G. and Porter, P.M. (2006) Efficacy of Fungicides for Control of Sclerotinia Stem Rot of Canola. *Plant Dis.* **90**, 1129–1134.

Campo, S., Gilbert, K.B. and Carrington, J.C. (2016) Small RNA-Based Antiviral Defense in the Phytopathogenic Fungus *Colletotrichum higginsianum*. *PLoS Pathog.* **12**, 1–36.

Chan, C.Y., Carmack, C.S., Long, D.D., Maliyekkel, A., Shao, Y., Roninson, I.B. and Ding, Y. (2009) A structural interpretation of the effect of GC-content on efficiency of RNA interference. *BMC Bioinformatics* **10 Suppl 1**, S33.

Chen, Y., Gao, Q., Huang, M., Liu, Y., Liu, Z., Liu, X. and Ma, Z. (2015) Characterization of RNA silencing components in the plant pathogenic fungus *Fusarium graminearum*. *Sci. Rep.* **5**, 12500.

Coradetti, S.T., Craig, J.P., Xiong, Y., Shock, T., Tian, C. and Glass, N.L. (2012) Conserved and essential transcription factors for cellulase gene expression in ascomycete fungi. *Proc. Natl. Acad. Sci.* **109**, 7397–7402.

Derbyshire, M.C. and Denton-Giles, M. (2016) The control of Sclerotinia stem rot on oilseed

rape (*Brassica napus*): Current practices and future opportunities. *Plant Pathol.* **65**, 859–877.

Dornseifer, S., Willkomm, S., Far, R.K.K., et al. (2015) RNAi revised - Target mRNA-dependent enhancement of gene silencing. *Nucleic Acids Res.* **43**, 10623–10632.

Fernandez, J. and Wilson, R.A. (2014) Characterizing roles for the glutathione reductase, thioredoxin reductase and thioredoxin peroxidase-encoding genes of *Magnaporthe oryzae* during rice blast disease. *PLoS One* **9**.

Garg, H., Li, H., Sivasithamparam, K., Kuo, J. and Barbetti, M.J. (2010) The infection processes of *Sclerotinia sclerotiorum* in cotyledon tissue of a resistant and a susceptible genotype of *Brassica napus*. *Annu. Bot.*, 897–908.

Ghag, S.B., Shekhawat, U.K.S. and Ganapathi, T.R. (2014) Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against Fusarium wilt in banana. *Plant Biotechnol. J.* **12**, 541–53.

Girard, I.J., Tong, C., Mao, X., Becker, M.G., Huang, J., Kievit, T. De, Dilantha Fernando, W.G., Liu, S. and Belmonte, M.F. (2017) RNA sequencing of *Brassica napus* reveals cellular redox control of Sclerotinia infection. *J. Exp. Bot.*

Gong, Y.H., Yu, X.R., Shang, Q.L., Shi, X.Y. and Gao, X.W. (2014) Oral Delivery Mediated RNA Interference of a Carboxylesterase Gene Results in Reduced Resistance to Organophosphorus Insecticides in the Cotton Aphid, *Aphis gossypii* Glover. *PLoS One* **9**, 23–25.

Heard, S., Brown, N.A. and Hammond-kosack, K. (2015) An Interspecies Comparative Analysis of the Predicted Secretomes of the Necrotrophic Plant Pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS One*, 1–27.

Heller, J. and Tudzynski, P. (2011) Reactive Oxygen Species in Phytopathogenic Fungi: Signaling, Development, and Disease. *Annu. Rev. Phytopathol.* **49**, 369–390.

Hong, S.W., Jiang, Y., Kim, S., Li, C.J. and Lee, D. (2014) Target gene abundance contributes to the efficiency of siRNA-mediated gene silencing. *Nucleic Acid Ther.* **24**, 192–8.

Hu, W., Sillaots, S., Lemieux, S., et al. (2007) Essential Gene Identification and Drug Target Prioritization in *Aspergillus fumigatus*. **3**.

Hu, X., Hipolito, S., Lynn, R., Abraham, V., Ramos, S. and Wong-Staal, F. (2004) Relative gene-silencing efficiencies of small interfering RNAs targeting sense and antisense transcripts from the same genetic locus. *Nucleic Acids Res.* **32**, 4609–4617.

Hulsen, T., Vlieg, J. de and Alkema, W. (2008) BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC Genomics* **9**, 1–6.

Jobic, C., Boisson, A.M., Gout, E., Rascle, C., Fèvre, M., Cotton, P. and Bligny, R. (2007) Metabolic processes and carbon nutrient exchanges between host and pathogen sustain the disease development during sunflower infection by *Sclerotinia sclerotiorum*. *Planta* **226**, 251–265.

Joga, M.R., Zotti, M.J., Smagghe, G. and Christiaens, O. (2016) RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: What we know so far. *Front. Physiol.* **7**, 1–14.

Kamal, M.M., Savocchia, S., Lindbeck, K.D. and Ash, G.J. (2016) Biology and biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary in oilseed Brassicas. *Australas. Plant Pathol.* **45**, 1–14.

Kamesh Krishnamoorthy, K., Sankaralingam, A. and Nakkeeran, S. (2016) Standardization

of Culture Media and pH for the Rapid Growth of *Sclerotinia sclerotiorum* causing Head Rot Disease of Cabbage Standardization of Culture Media and pH for the Rapid Growth of Sclerotinia sclerotiorum causing Head Rot Disease of Cabbage. *Adv. Life Sci.* **5(22)**, 10659–10661.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. and Morishima, K. (2017) KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* **45**, D353–D361.

Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T. and Minami, E. (2012) Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. *PLoS One* **7**, e49423.

Killiny, N., Hajeri, S., Tiwari, S., Gowda, S. and Stelinski, L.L. (2014) Double-stranded RNA uptake through topical application, mediates silencing of five CYP4 genes and suppresses insecticide resistance in *Diaphorina citri*. *PLoS One* **9**, 1–8.

Koch, A., Biedenkopf, D., Furch, A., et al. (2016) An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery. *PLoS Pathog.* **12**, 1–22.

Kumar, R., Ichihashi, Y., Kimura, S., Chitwood, D.H., Headland, L.R., Peng, J., Maloof, J.N. and Sinha, N.R. (2012) A High-Throughput Method for Illumina RNA-Seq Library Preparation. *Front. Plant Sci.* **3**, 1–10.

Larsson, E., Sander, C. and Marks, D. (2010) mRNA turnover rate limits siRNA and microRNA efficacy. *Mol. Syst. Biol.* **6**, 433.

Liang, X., Liberti, D., Li, M., Kim, Y.-T., Hutchens, A., Wilson, R. and Rollins, J. a (2014) Oxaloacetate acetylhydrolase gene mutants of Sclerotinia sclerotiorum do not accumulate

oxalic acid, but do produce limited lesions on host plants. *Mol. Plant Pathol.* **16**, 1–13.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and. *Methods* **25**, 402–408.

Llanos, A., François, J. and Parrou, J. (2015) Tracking the best reference genes for RT-qPCR data normalization in filamentous fungi. *BMC Genomics* **16**, 71.

Love, K.T., Mahon, K.P., Christopher, G., et al. (2010) Lipid-like materials for low-dose, in vivo gene silencing. *Proc. Natl. Acad. Sci.* **107**, 9915–9915.

Luo, H., Lin, Y., Gao, F., Zhang, C.T. and Zhang, R. (2014) DEG 10, an update of the database of essential genes that includes both protein-coding genes and noncoding genomic elements. *Nucleic Acids Res.* **42**, 574–580.

Lyu, X., Shen, C., Fu, Y., Xie, J., Jiang, D., Li, G. and Cheng, J. (2015) Comparative genomic and transcriptional analyses of the carbohydrate-active enzymes and secretomes of phytopathogenic fungi reveal their significant roles during infection and development. *Sci. Rep.* **5**, 15565.

Majumdar, R., Rajasekaran, K. and Cary, J.W. (2017) RNA Interference (RNAi) as a Potential Tool for Control of Mycotoxin Contamination in Crop Plants: Concepts and Considerations. *Front. Plant Sci.* **8**, 200.

Meyering-Vos, M. and Müller, A. (2007) RNA interference suggests sulfakinins as satiety effectors in the cricket *Gryllus bimaculatus*. *J. Insect Physiol.* **53**, 840–848.

Miedes, E., Vanholme, R., Boerjan, W. and Molina, A. (2014) The role of the secondary cell wall in plant resistance to pathogens. *Front. Plant Sci.* **5**, 358.

Mir, A.A., Park, S.-Y., Sadat, M.A., Kim, S., Choi, J., Jeon, J. and Lee, Y.-H. (2015) Systematic characterization of the peroxidase gene family provides new insights into fungal

pathogenicity in *Magnaporthe oryzae*. *Sci. Rep.* **5**, 11831.

Mocellin, S. and Provenzano, M. (2004) RNA interference: learning gene knock-down from cell physiology. *J. Transl. Med.* **2**, 39.

Mumbanza, F.M., Kiggundu, A., Tusiime, G., Tushemereirwe, W.K., Niblett, C. and Bailey, A. (2013) In vitro antifungal activity of synthetic dsRNA molecules against two pathogens of banana, *Fusarium oxysporum f. sp. cubense* and *Mycosphaerella fijiensis*. *Pest Manag. Sci.* **69**, 1155–1162.

Neumann, B., Held, M., Liebel, U. and Erfle, H. (2006) High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat. Methods* **3**, 385–390.

Nicolás, F.E. and Garre, V. (2016) RNA Interference in Fungi : Retention and Loss. , 1–15.

Oliveros, J.C. (2015) Venny. An interactive tool for comparing lists with Venn's diagrams.

Pan, Y., Xu, Y., Li, X., Yao, C. and Gao, Z. (2015) SsPemG1 encodes an elicitor-homologous protein and regulates pathogenicity in *Sclerotinia sclerotiorum*. *Physiol. Mol. Plant Pathol.* **92**, 70–78.

Price, D.R.G. and Gatehouse, J.A. (2008) RNAi-mediated crop protection against insects. *Trends Biotechnol.* **26**, 393–400.

Qu, X., Yu, B., Liu, J., et al. (2014) MADS-box transcription factor SsMADS is involved in regulating growth and virulence in *Sclerotinia sclerotiorum*. *Int. J. Mol. Sci.* **15**, 8049–8062.

Rinkevich, F.D. and Scott, J.G. (2013) Limitations of RNAi of α6 nicotinic acetylcholine receptor subunits for assessing the in vivo sensitivity to spinosad. *Insect Sci.* **20**, 101–108.

Rollins, J. A (2003) The *Sclerotinia sclerotiorum* pac1 gene is required for sclerotial development and virulence. *Mol. Plant. Microbe. Interact.* **16**, 785–795.

San Miguel, K. and Scott, J.G. (2016) The next generation of insecticides: DsRNA is stable as

a foliar-applied insecticide. *Pest Manag. Sci.* **72**, 801–809.

Schwartz, H.F. and Singh, S.P. (2013) Breeding common bean for resistance to white mold: A review. *Crop Sci.* **53**, 1832–1844.

Semple, S.C., Akinc, A., Chen, J., et al. (2010) Rational design of cationic lipids for siRNA delivery. *Nat Biotechnol* **28**, 172–176.

Serrano, M., Coluccia, F., Torres, M., L'Haridon, F. and Métraux, J.-P. (2014) The cuticle and plant defense to pathogens. *Front. Plant Sci.* **5**, 274.

Shao, Y., Chan, C.Y., Maliyekkel, A., Lawrence, C.E., Roninson, I.B. and Ding, Y. (2007) Effect of target secondary structure on RNAi efficiency. *RNA* **13**, 1631–1640.

Sharma, P., Meena, P.D., Verma, P.R., Saharan, G.S., Mehta, N., Singh, D. and Kumar, A. (2015) *Sclerotinia sclerotiorum* (Lib .) de Bary causing Sclerotinia rot in oilseed Brassicas : A review. *J. Oilseed Brassica* **6**, 1–44.

Štefanic, S., Dvořák, J., Horn, M., et al. (2010) RNA interference in *Schistosoma mansoni schistosomula*: Selectivity, sensitivity and operation for larger-scale screening. *PLoS Negl. Trop. Dis.* **4**.

Trapnell, C., Roberts, A., Goff, L., et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–78.

Uloth, M.B., Clode, P.L., You, M.P. and Barbetti, M.J. (2015) Attack modes and defence reactions in pathosystems involving *Sclerotinia sclerotiorum* , *Brassica carinata* , *B. juncea* and *B. napus*. *Ann. Bot.*, mcv150.

United States Environmental Protection Agency (2017) EPA Registers Innovative Tool to Control Corn Rootworm.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. and

Rozen, S.G. (2012) Primer3-new capabilities and interfaces. *Nucleic Acids Res.* **40**, 1–12.

Vermeulen, A., Behlen, L., Reynolds, A., Wolfson, A., Marshall, W.S., Karpilow, J. and Khvorova, A. (2005) The contributions of dsRNA structure to Dicer specificity and efficiency. *RNA* **11**, 674–82.

Wang, M., Weiberg, A., Lin, F.-M., Thomma, B.P.H.J., Huang, H.-D. and Jin, H. (2016) Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* **2**, 16151.

Werner, S., Sugui, J. A, Steinberg, G. and Deising, H.B. (2007) A chitin synthase with a myosin-like motor domain is essential for hyphal growth, appressorium differentiation, and pathogenicity of the maize anthracnose fungus *Colletotrichum graminicola*. *Mol. Plant. Microbe. Interact.* **20**, 1555–1567.

Whyard, S., Singh, A.D. and Wong, S. (2009) Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* **39**, 824–832.

Yu, Y., Jiang, D., Xie, J., Cheng, J., Li, G., Yi, X. and Fu, Y. (2012) Ss-Sl2 , a Novel Cell Wall Protein with PAN Modules , Is Essential for Sclerotial Development and Cellular Integrity of *Sclerotinia sclerotiorum*. *PLoS One* **7**.

Yu, Y., Xiao, J., Yang, Y., Bi, C., Qing, L. and Tan, W. (2015) Physiological and Molecular Plant Pathology Ss-Bi1 encodes a putative BAX inhibitor-1 protein that is required for full virulence of *Sclerotinia sclerotiorum*. *Physiol. Mol. Plant Pathol.* **90**, 115–122.

Zhu, W., Wei, W., Fu, Y., Cheng, J., Xie, J., Li, G. and Yi, X. (2013) A Secretory Protein of Necrotrophic Fungus *Sclerotinia sclerotiorum* That Suppresses Host Resistance. **8**.

Zimmermann, T.S., Lee, A.C.H., Akinc, A., et al. (2006) RNAi-mediated gene silencing in non-human primates. **441**, 111–114.

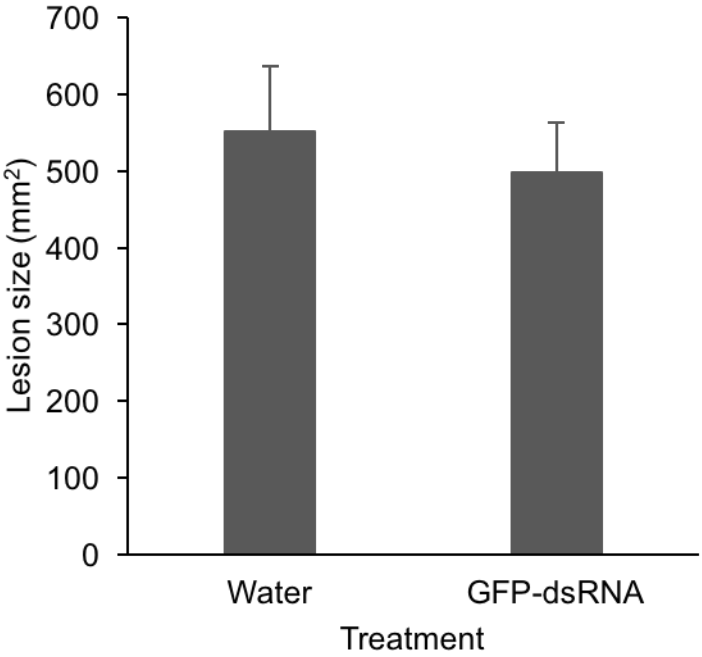
Supplementary Table 2.1 . Mapping summary for all biological replicates of in-vitro and in-planta samples. Raw RNA-sequencing reads trimmed using Trimmomatic 0.33 and aligned to the *Sclerotinia sclerotiorum* genome using TopHat v2.1.0.

	in-vitro (1)	*in-vitro* (2)	*in-vitro* (3)	ZY821 24hpi (1)	ZY821 24hpi (2)	ZY821 24hpi (3)	Westar 24hpi (1)	Westar 24hpi (2)	Westar 24hpi (3)
Trimmed reads	6537111	6339049	11826235	31473436	44428372	20453088	40287633	37020385	52025758
Mapped reads	5388394	5216455	10273216	6282525	6724081	3061161	7810054	6478810	8125640
% Mapped	82.4	82.3	86.9	20	15.1	15	19.4	17.5	15.6

Supplementary Table 2.2 Target identification pipeline guidelines for selecting targets to control phytopathogenic fungi using RNAi

Criteria	Choose	Avoid
RNA-seq dataset	Upregulated genes common amongst infection conditions	Down regulated genes in response to infection
Essential Genes	Genes of closely related species. Genes can be found in the Database of Essential Genes [1]	Essential genes from distant or unrelated relatives
Biological processes	Mitochondria, ROS response, protein modification, pathogenicity factors, transcription, splicing, protein modification, translation, cell wall modification	General growth, transcription factors, transport, electron carriers, signal transduction, pigment synthesis, carbohydrate metabolism
Expression levels	FPKM values between 1 and 500	Lowly (FPKM <1) and Highly (FPKM > 500) expressed during infection
Log2-fold change (control vs. infection)	Fold changes between -0.5 and 4	Fold changes below -0.5 and above 4
Transcript length	Above 200 nucleotides	Below 200 nucleotides
Gene location	Nuclear-encoded	Organelle-encoded
Redundancy	Single function or without homologues	Genes with multiple homologues and functionally similar roles
Regulation		Genes that are heavily regulated

(1) www.essentialgene.org (Luo *et al*., 2014)



Supplementary Figure 2.1 *Sclerotinia sclerotiorum* infection on *Brassica napus* leaves after treatment of water or GFP-dsRNA. No significant difference was observed at 2 dpi with a sample size of n =10 ($p = 0.6$, one-way ANOVA).

CHAPTER 3 HOST-INDUCED GENE SILENCING AS AN RNA INTERFERENCE BIOTECHNOLOGY FOR *SCLEROTINIA SCLEROTIORUM* MANAGEMENT

3.1 ABSTRACT

Sclerotinia sclerotiorum infects over 500 plant species and causes agronomic losses globally. Previous breeding strategies in most crops have been laboriously lengthy and have not yet uncovered completely effective host resistance against this pathogen. Recently, plants were engineered to produce hairpin RNA molecules, which invoke an intrinsic cellular defence mechanism, RNA interference, within the assailing pest or pathogen and results in targeted mRNA destruction. With plant transformation techniques, host-induced gene silencing (HIGS) could provide a more efficient solution for developing host resistance against *S. sclerotiorum*. Previously, I demonstrated the capacity of long dsRNA targeting SS1G_01703 (aflatoxin biosynthesis) and SS1G_05899 (thioredoxin reductase) to impart protection against *S. sclerotiorum* infection on the surface of *Arabidopsis thaliana* leaves. To test HIGS as an implementation for host resistance, I created two transgenic *A. thaliana* lines, $SS1G01703_{RNAi}$ and *A. thaliana* $SS1G05899_{RNAi}$, using *Agrobacterium tumefaciens*. Infection on both *A. thaliana* $SS1G01703_{RNAi}$ and *A. thaliana* $SS1G05899_{RNAi}$ lines demonstrated reductions in lesion size, fungal DNA presence, and fungal transcript levels. Furthermore, I assessed host cell death and defence markers to search for differences between transformed and wild-type *A. thaliana.* Overall, HIGS could be a new solution for developing host resistance against *S. sclerotiorum.*

3.2 INTRODUCTION

Canola (*Brassica napus* L.), is threatened by the devastating necrotrophic fungus, *Sclerotinia sclerotiorum* (Lib.) de Bary (Canola Council of Canada, 2017; Kamal *et al.*, 2016). Traditional breeding strategies involved lengthy germplasm screens that have proven to be largely ineffective in developing host resistance (Garg *et al.*, 2010; Taylor *et al.*, 2015). Arguably, more tolerant cultivars with increased oxidative responses, intense lignification, and greater cell wall deposition have been bred to physically restrain fungal colonization at the cellular level (Garg *et al.*, 2013; Uloth *et al.*, 2015). However, without a truly resistant cultivar, producers rely on chemical fungicides, which are neither environmentally-benign nor economical when managed ineffectively. (Derbyshire and Denton-Giles, 2016; Huzar-Novakowiski *et al.*, 2017; Smalling *et al.*, 2013). Due to the toxicological mode of action, some fungicides are classified as general biocides (Maltby *et al.*, 2009). These compounds have been observed to cause mitochondrial respiratory uncoupling in bumblebees (*Bombus terrestris* L.), induced necrosis of epithelial cells in earthworms (*Lumbricus terrestris* L.), and increased mortality in Blanchard's cricket frogs (*Acris blanchardi*) (Cusaac *et al.*, 2016; Kılıç, 2011; Syromyatnikov *et al.*, 2017). Furthermore, due to the high costs of traditional chemical treatments, growers may apply diluted fungicidal products to their crops, that have been implicated in the rise of chemical resistance (Amaradasa and Everhart, 2016; Penaud and Walker, 2015). Thus, new strategies for developing resistance are required.

New technologies that take advantage of an innate cellular defense pathway, RNA interference (RNAi), may offer a novel solution to traditional agrochemicals and have been applied successfully to control agronomic pests (Baum *et al.*, 2007; Bonfim *et al.*, 2007; Papolu *et al.*, 2013). Recently, two crops, maize (*Zea mays* L.) and pinto beans (*Phaseolus vulgaris* L.),

used Host-induced gene silencing (HIGS) to control corn rootworm (*Diabrotica virgifera virgifera* LeConte) and Bean Golden Mosaic Virus and were approved for commercial production in the United States and Brazil, respectively (Tollefson, 2011; United States Environmental Protection Agency, 2017). This technology functions to protect plants against pests and pathogens through the host plant's RNAi pathway. Plant cells are transformed with RNAi constructs to produce hairpin RNA (hpRNA) with sequence homology to a specific pathogen transcript. These hpRNA molecules act as molecular mimics of the RNAi-inducing double stranded RNA (dsRNA) and invoke RNAi (Nunes and Dean, 2012). After transcription and export of the hpRNA into the host cytoplasm, DICER proteins process the molecules into small interfering RNA (siRNA), 21 – 25 nucleotides in length. These siRNAs are then delivered to the pathogen cell and are incorporated into ARGONAUTE, forming the RNA-induced silencing complex (RISC) (Chapter 1, Fig. 1.1d; Nunes and Dean, 2012; Panwar *et al.*, 2017). Using the internal siRNA as a guide, RISC targets messenger RNA (mRNA) with a complementary sequence for degradation within the attacking pathogen, resulting in gene silencing (Chapter 1, Fig. 1.1e-g; Nunes and Dean, 2012; Panwar *et al.*, 2013). Therefore, the sequence specificity of the mechanism allows RNAi technologies to be species-specific, unlike most traditional chemical approaches.

HIGS implementations have been successful in controlling various plant pathogenic fungi (Koch *et al.*, 2013; Tiwari *et al.*, 2017; Zhang *et al.*, 2016). The main advantage of HIGS is the continual production, and henceforth protection, across the lifecycle of the host plant. For example, banana plants (*Musa spp.*) expressing hpRNA targeting either *Fusarium transcription factor 1* or *velvet* resisted *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen infection 8 months post-exposure (Ghag *et al.*, 2014). A similar employment of *in planta* hpRNA

expression targeting *S. sclerotiorum* chitin synthase, critical for fungal cell wall synthesis, confirmed the use of HIGS for *S. sclerotiorum* host resistance. Transgenic *Nicotiana tabacum* L. reduced lesion size on the leaf surface and diminished *S. sclerotiorum* transcript levels compared to the wild-type infection (Andrade *et al.*, 2016). While advances in the development of HIGS transgenic plants have been successful, there is still no knowledge of the use and functionality of this defence process in the control of *S. sclerotiorum* using the model plant, *Arabidopsis thaliana*. The use of this model organism could provide insight into how defence interactions with necrotrophic fungi may occur amongst a variety of important crop species, especially after the successful formulation of topical dsRNAs to mitigate infection on the leaf surface (Chapter 2, Fig. 2.6).

Previously, topical sprays of dsRNA targeting two *Botrytis cinerea* Dicer-like proteins and three *Fusarium graminearum* cytochrome P450 lanosterol C-14α demethylases showed improved levels of protection against fungal infection (Koch *et al.*, 2016; Wang *et al.*, 2016). However, HIGS technology was concurrently or previously implemented to demonstrate equal or greater levels of host protection against *B. cinerea* or *F. graminearum* infection (Koch *et al.*, 2013; Wang *et al.*, 2016). Since topical applications of RNAi technologies to control *S. sclerotiorum* proved effective (Chapter 2), I was now interested in developing novel traits in *A. thaliana* that would protect plants against the same fungus. In this chapter, I transformed *A. thaliana* with a RNAi-expression vector targeting either SS1G_01703, encoding a putative versiconal hemiacetal acetate esterase involved in the aflatoxin biosynthesis (Kanehisa *et al.*, 2017), or SS1G_05899, a thioredoxin reductase (Luo *et al.*, 2014). Both transgenic lines restricted lesion progression compared to the wild-type control. Furthermore, using quantitative PCR (qPCR), I demonstrated reduced fungal colonization and reduction in target transcript

accumulation providing unparalleled evidence that HIGS is responsible for improved plant performance following infection with *S. sclerotiorum*. Finally, I began exploring potential differences in host plant cell death and defense between the HIGS and wild-type *A. thaliana.* In summary, HIGS engineers resistance against *S. sclerotiorum* and provides a valuable alternative for RNAi-based control to manage this devastating fungal disease.

3.3 METHODS

3.3.1 Growth Conditions for *Arabidopsis thaliana*

A. thaliana seeds were surface sterilized and plated on Murashige and Skoog (MS) basal media (4.4g/L Murashige and Skoog with vitamins (Caisson Labs, Smithfield, UT, USA) + 2% sucrose (Sigma Aldrich, St. Louis, Missouri, USA)) supplemented with 150 μg/mL of kanamycin (Sigma Aldrich, St. Louis, Missouri, USA). Seeds were then vernalized for 3 days at 4°C to synchronize germination rates and placed under constant light. Seedlings were then transferred to Sunshine® soil mix #1 (Sungro Horticulture, Agawam, Massachusetts, US) and stored at 21°C with an alternating light cycle of 16 hours light, 8 hours dark, until the plants reached maturity.

3.3.2 Transformation and Identification of *A. thaliana* transformants

Preparation of Agrobacterium tumefaciens

Primers for cloning SS1G_01703 and SS1G_05899 can be found in Table 3.1. Cloning into *Agrobacterium tumefaciens* followed the Gateway cloning protocol (Invitrogen, Carlsbad, CA, US). Target gene sequences were amplified using Phusion Taq (Thermo Scientific, Waltham, MA, US) under the following conditions: 98°C for 30 s; 35 cycles of: 98°C for 10s, 57°C for 30s, and 72°C for 30s; and a final extension of 72 °C for 5 min. Amplicons were gel purified (New

England Biolabs, Ipswich, MA, US) and digested using FastDigest KpnI and XhoI (Thermo Scientific, Waltham, MA, US) according to the manufacturer's protocols. The products were ligated into the similarly digested pENTR4 vector (Invitrogen, Carlsbad, CA, US) using T4 ligase (Invritogen, Carlsbad, CA, US) according to the manufacturer's protocol. Prepared plasmids were used to transform *E. coli* MachI cells (Thermo Scientific, Waltham, MA, US), selected using 50 mg/L chlorophenicol (MP Biomedicals Inc., Santa Ana, CA, USA) + LB solid media (Difco Laboratories, Inc, Detroit, MI, USA), and sequence inserts were confirmed using Sanger sequencing (The Centre for Applied Genomics, Toronto, ON, Canada). Once sequence was confirmed, Gateway LR clonase II reactions (Invritogen, Carlsbad, CA, US) with an input ratio of 4:1 (pENTR4:pHellsgate8) were used to shuttle inserts into pHellsgate (Invitrogen, Carlsbad, CA, US) and prepared plasmids were used to transformed *E. coli* MachI cells (Thermo Scientific, Waltham, MA, US). Colonies were selected using 50 mg/L spectinomycin (MP Biomedicals Inc., Santa Ana, CA, USA). To confirm transformation, colony PCR screens were conducted using GoTaq® Green Master Mix (Promega, Madison WI, USA) with the following conditions: 94°C for 2 min.; 35 cycles of: 94°C for 30 s, 55°C for 30 s., 72°C for 35 s.; final extension of 72°C for 5 min. Plasmids were isolated from positive colonies using Monarch Miniprep kit (New England Biolabs, Ipswich, MA, US) and then FastDigest XbaI or XhoI separately (Thermo Scientific, Waltham, MA, US) according to the manufacturer's protocols to confirm double recombination. Plasmids were transformed into *Agrobacterium tumefaciens* GV3201 (Thermo Scientific, Waltham, MA, US) and selected using 50 mg/L spectinomycin and 50 mg/L gentamycin to maintain the pMP90 plasmid (MP Biomedicals Inc., Santa Ana, CA, US). A colony PCR was performed as stated above with the initial 94°C step for 10 min to confirm transformation.

Transformation of Arabidopsis thaliana

Agrobacterium tumefaciens cultures were grown in 100 mL of yeast extract peptone broth (Difco Laboratories, Inc, Detroit, MI, US) until an OD_{600} of 1.5 to 2 was achieved. Cells were collected using centrifugation at 4000*g* for 10 min at 21°C. Cells were re-suspended in 100 mL of MS medium with 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX, US). Inflorescences of *A. thaliana* (Col-0) were dipped at the flowering stage into bacterial resuspension for 10 s and kept under high humidity for 2 days. Dipping was repeated 3 times before plants were dried and seeds collected.

Tissue Collection/DNA Extraction and Genotyping

DNA was extracted from leaf samples of 14 day old *A. thaliana* using a GenoGrinder 2000 (Spex CertiPrep, Metuchen, New Jersey, USA) and DNA extraction buffer [0.25M NaCl, 1M 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl pH 7.5, 25mM EDTA pH 8, 0.5% SDS] as described by Edwards *et al.* (1991). DNA was precipitated with isopropanol, washed with 75% ethanol, and suspended in water. PCR was performed using fungal gene specific primers (Table 3.1) using GoTaq® Green Master Mix (Promega Corporation, Madison, WI, US) to according to the manufacturer's instructions. Thermocycler conditions were as follows: 94°C for 2 min.; 35 cycles of: 94°C for 30 s., 55°C for 30 s., 72°C for 35 s.; final extension of 72°C for 5 min. Product sizes were assessed using gel electrophoresis.

3.3.3 Arabidopsis Infection Assays, fungal load quantification, and target gene knockdown

The surface of 20 day old leaves of *A. thaliana* were point inoculated with a10 µL droplet of 1 x 10^6 *S. sclerotiorum* spores/mL suspended in sterilized media (24 g/L potato dextrose broth + 10 g/L peptone; Garg *et al.*, 2013). Lesion size was scored 3 days post inoculation (dpi) using

Table 3.1 Primer sequences for cloning *Sclerotinia sclerotiorum* target genes and confirmation of genetic insertion within *Arabidopsis thaliana* lines

Target Gene	Accession	Sequences
SS1G_01703	XM_001597459.1	**L**: TTCTGCCGGAAACCCTCTTC **R**: ACCGCCGATTGTGAAGACTT
SS1G_05899	XM_001592927.1	**L**: GCTCACACTGCTGCCGTATA **R**: CTCCGCAGCACTATCTCCAC

Table 3.2 Quantitative PCR primer sequences and efficiencies for *Sclerotinia sclerotiorum* and *Arabidopsis thaliana* target transcripts and *S. sclerotiorum* 18S rDNA

Organism	Target	Sequence	Efficiency
S. sclerotiorum	18S rDNA	**L:** TGGTGGAGTGATTTGTCTGCT **R:** CCTCTAAGAAGCCAGCGACC	107.4
S. sclerotiorum	SS1G_01703	**L:** CTTCCATCCCTGCCGCTTAC **R:** CCATAGCGGCTCGATCTAGAATC	98.7
S. sclerotiorum	SS1G_05899	**L:** ACACGGTTGGAGGCGAAATT **R:** TCCTCTCCAGTAACGACGTTC	96.2
S. sclerotiorum	SS1G_12350	**L:** CGATACTGTGCCTGTGACCA **R:** CCTCTCCTCAAGCGCCATAG	101.7
A. thaliana	*EEF-1BB1*	**L:** CTGGAGGTTTTGAGGCTGGTAT **R:** CCAAGGGTGAAAGCAAGAAGA	99.3
A. thaliana	*ATG8B*	**L:** TGGCCAATTTGTGTACGTTGT **R:** AATGCCGCAGTTGGTGGTAA	92.1
A. thaliana	*BI1*	**L:** TGGTGGCTCTGCATCTATCT **R:** ATCTCTTGTGTGTCCACCACC	98.9
A. thaliana	*SEN1*	**L:** ATCCGGTTTAGGACAAGCGG **R:** GTCCAAGCGACGTATCCTCC	99.0
A. thaliana	*PR1*	**L:** CTCGGAGCTACGCAGAACAA **R:** CGCTACCCCAGGCTAAGTTT	92.2
A. thaliana	*PDF1.2*	**L:** TTCTCTTTGCTGCTTTCGACG **R:** TTACTGTTTCCGCAAACCCCT	93.2

ImageJ software (imagej.nih.gov) and normalized to *A. thaliana* wild-type control. Infected tissue was collected and pooled with 3 leaves per biological replicate and stored at -80°C.

Collected tissue was ground in liquid nitrogen and DNA extraction was performed using a modified fungal DNA extraction buffer [0.5M KCl, 1M 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl pH 7.5, 125 mM EDTA pH 8] (Chi *et al.*, 2009). Fungal load was quantified using Quantitative PCR (qPCR) and the Bio-Rad CFX96 Connect Real Time System. 10 µL reactions were performed using PerfeCTa SYBR® Green SuperMix (Quanta Bio, Beverly, MA, US) and 1 ng of DNA in each reaction and the relevant primers, listed in Table 3.2. Conditions used were as follows: 95°C for 30 s, and 40 cycles of: 95°C for 5 s, and 60°C for 30 s. Fungal load was assessed relative to wildtype infected tissue (Becker *et al.*, 2017; Livak and Schmittgen, 2001).

Transcript knockdown was measured 3 dpi using quantitative real time PCR (qRT-PCR). RNA from each infected leaf sample (3 leaves per biological replicate) was isolated using the Invitrogen Plant RNA Isolation and Ambion Turbo DNase treatment protocol (Thermo Fisher, Waltham, MA, USA). Complementary DNA strand synthesis was performed using qScript cDNA synthesis kit (QuantaBio, Beverly, MA, US) following the manufacturer's protocol, using a 10 µL reaction volume and 1000 ng of RNA. Thermocycler conditions for cDNA synthesis were as follows: 25°C for 10 min., 50°C for 15 min., 85°C for 5 min. Using the Bio-Rad CFX96 Connect Real Time System, 10 µL qRT-PCR reactions were performed using PerfeCTa SYBR® Green SuperMix and the primers listed in Table 3.2. Conditions used were as follows: 95°C for 30 s; 40 cycles of: 95°C for 5s and 60°C for 30s. Transcript levels were measured in reference to the SsSac7 (SS1G_12350) housekeeping gene using the ΔΔCq method (Livak and Schmittgen, 2001; Llanos *et al.*, 2015).

Plant cell death and defense markers of infected samples used for measuring fungal transcript were also assessed using qRT-PCR. Non-infected leaf tissue for each line was also collected. RNA extraction and cDNA preparation was performed as described above. 10µL qRT-PCR reactions were run on the Bio-Rad CFX96 Connect Real Time System using Perfecta SYBR Green® SuperMix under the aforementioned conditions and primers in Table 3.2. Transcript levels were referenced to the *A. thaliana ELONGATION FACTOR 1B BETA* (*EEF-1BB1*) housekeeping gene and compared infected to non-infected using the ΔΔCq method (Kabbage *et al.*, 2013; Komaki *et al.*, 2010; Livak and Schmittgen, 2001).

3.4 RESULTS

3.4.1 Host-induced gene silencing technology lessens lesion progression

To assess the extent of host resistance, *A. thaliana* leaves were inoculated with an aggressive *S. sclerotiorum* spore inoculation technique (Garg *et al.*, 2013). *A. thaliana* *SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$ decreased lesion size 76 and 64 %, respectively, compared to the wild-type control (one-way ANOVA, $p < 0.01$; Fig. 3.1b). The reduction of infection was greater by 26% and 27% on *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana* *SS1G05899*$_{RNAi}$ compared to dsRNA foliar applications of SS1G_01703 and SS1G_05899, respectively (*student's t-test*, $p<0.05$; Chapter 2, Fig. 2.6). The data suggest HIGS may be a more successful application of RNAi technology to control fungal pathogens.

Phenotypic differences between the pathogenic progression on the wild-type and the RNAi lines were also observed (Fig. 3.1a). The control, non-hpRNA producing plants had advanced lesions, covering the majority of the leaf surface. Evidence of systemic fungal

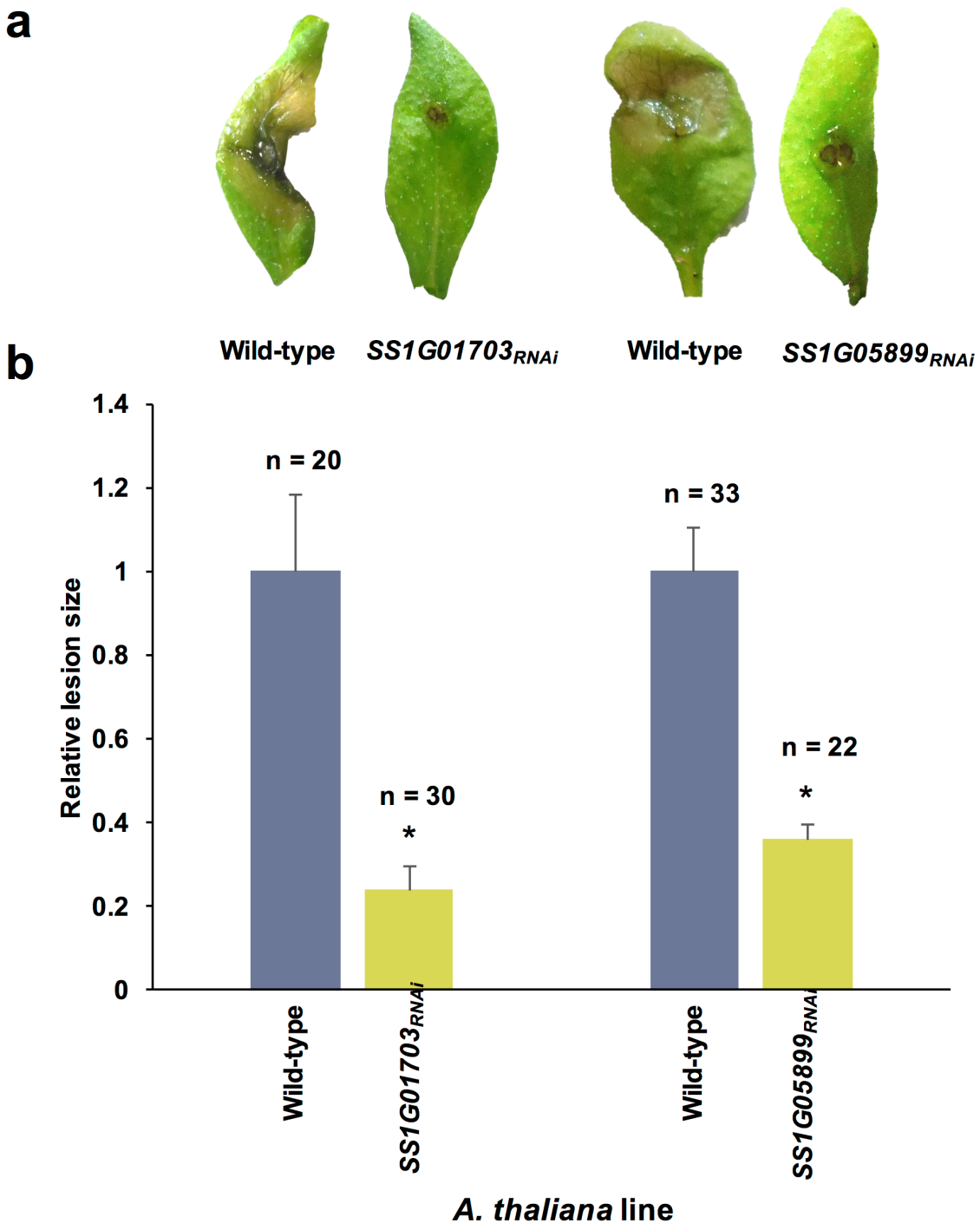


Figure 3.1 *Sclerotinia sclerotiorum* infection was limited on hpRNA-producing *Arabidopsis thaliana*. (**a**) Infected leaf tissue 3 dpi after spore inoculation. (**b**) Average lesion size of *A. thaliana* plants 3 dpi relative to wildtype control plants with standard error bars. Significant reductions of lesion size were observed and denoted with "*" (one-way ANOVA, $p<0.05$).

infection was also apparent as the lesions appeared to progress into the vasculature of the petiole. In contrast, the infected *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$ leaves had smaller, localized lesions on the surface of the leaf. The lesion appeared drier compared to the damp lesions on the wild-type control

3.4.2 Infection under host–induced gene silencing decreases the presence of fungal DNA

The relative amount of *S. sclerotiorum* 18S rDNA extracted from infected leaf tissue between the wild-type and the transgenic lines was assessed using qPCR to confirm the magnitude of fungal colonization. Fungal load of infected *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$ was reduced by 96 and 49 %, respectively, compared to the wild-type tissue (*student's t-test* $p < 0.05$; Fig 3.2). The difference in lesion size and fungal load are in concordance with the phenotypic observations of the infected leaves and suggest the infection was impeded in *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$. The greater reduction of fungal load in the *A. thaliana SS1G01703*$_{RNAi}$ suggests that the infection may have been superficial with limited colonization within the interior of the leaf. In contrast, the *A. thaliana SS1G05899*$_{RNAi}$ leaves developed more prominent lesions. The reduction in fungal DNA confirmed the infection was less developed in comparison to the wild-type plants and further strengthens the evidence that hpRNA expression was sufficient to suppress fungal invasion into the leaf's tissues.

3.4.3 Specific fungal transcripts were reduced during infection on *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$

Next, transcript levels of SS1G_01703 and SS1G_05899 were measured during infection on *A. thaliana SS1G01703*$_{RNAi}$, *A. thaliana SS1G05899*$_{RNAi}$, and wild-type lines to assess hpRNA production indirectly. *S. sclerotiorum*-treated *A. thaliana SS1G01703*$_{RNAi}$ and

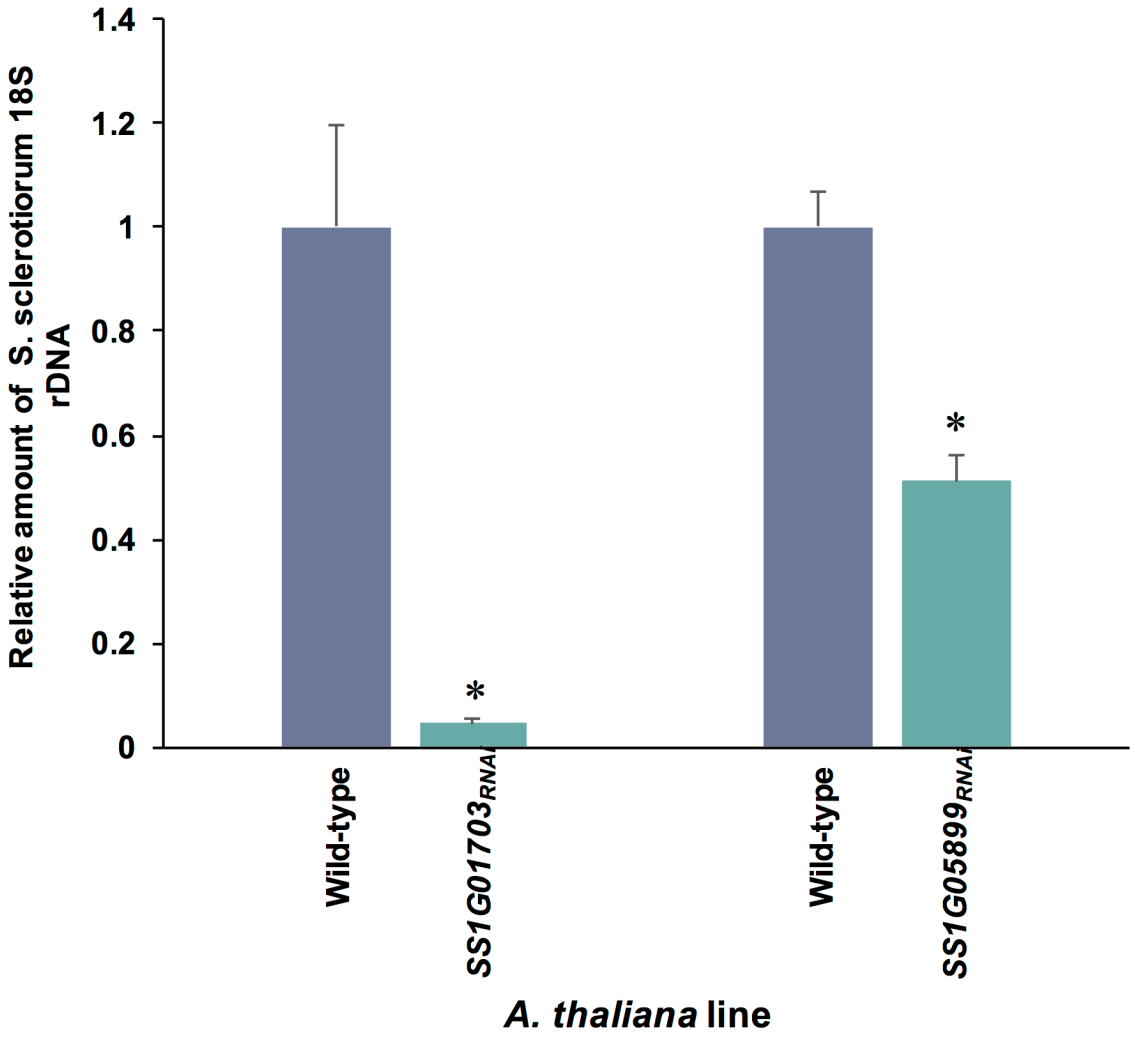


Figure 3.2 Relative presence of *Sclerotinia sclerotiorum* 18S rDNA was reduced on *Arabidopsis thaliana* $SS1G01703_{RNAi}$ *and A. thaliana* $SS1G05899_{RNAi}$ compared to wild-type *A. thaliana.* DNA was extracted from spore inoculated tissue 3 days post inoculation. DNA levels were assessed using quantitative PCR. Data represent 3 biological replicates relative to wild-type tissue collection and error bars represent standard error. Significance is denoted with "*" (*student's t-test*, $p<0.05$).

A. thaliana SS1G05899$_{RNAi}$ demonstrated transcript reductions of 83% and 75% for SS1G_01703, and SS1G_05899, respectively, compared to the non-transformed lines (*student's t-test* $p < 0.01$; Fig.3.3). Thus, *A. thaliana SS1G01703$_{RNAi}$* and *A. thaliana SS1G05899$_{RNAi}$* were likely expressing hpRNA targeting SS1G_01703 and SS1G_05899 respectively, which delivered dsRNA to the fungus infecting the host tissue, and resulted in RNAi-mediated mRNA knockdown within the fungal hyphae.

3.4.4 Defence markers are elevated and cell death markers are repressed differentially during infection of hpRNA producing and non-hpRNA producing *A. thaliana*

qRT-PCR was used to compare mRNA levels of the infected and non-infected states of the following cell death and defence markers: *AUTOPHAGY 8B* (*ATG8B;* autophagy), *BAX-INHIBITOR 1* (*BI1*; negative regulator of BAX-induced cell death), *PLANT DEFENSIN 1.2* (*PDF1.2*; jasmonic acid (JA) mediated defence), *PATHOGENESIS-RELATED GENE 1* (*PR1*; salicylic acid (SA) response) and *SENESCENCE 1* (*SEN1*; senescence). Compared to the non-infected state, infected *A. thaliana SS1G01703$_{RNAi}$*, *A. thaliana SS1G05899$_{RNAi}$*, and wild-type *A. thaliana* demonstrated significant differences in all markers, except for *ATG8B* and *BI1* in wild-type leaves (*student's* t-test, $p < 0.05$; Fig. 3.4). Both *A. thaliana SS1G01703$_{RNAi}$* and *A. thaliana SS1G05899$_{RNAi}$* demonstrated significant differences in the death and defence markers compared to the infection on the wild-type *A. thaliana* (Fig 3.4). The *A. thaliana SS1G01703$_{RNAi}$* line showed decreases in *ATG8B* (2-fold; student's *t test* $p < 0.01$), *BI1* (1.5-fold; $p < 0.01$), *PDF1.2* (1.7-fold; $p < 0.01$), *PR1* (4.2-fold; $p < 0.01$), and *SEN1* (0.7-fold; $p < 0.05$) compared to the wildtype (Fig. 3.4). Similarly, *A. thaliana SS1G05899$_{RNAi}$* line demonstrated decreases of the markers *ATG8B* (2-fold; $p < 0.01$), *BI1* (1.6-fold; $p < 0.01$), *PDF1.2* (1.7-fold; $p < 0.01$), and

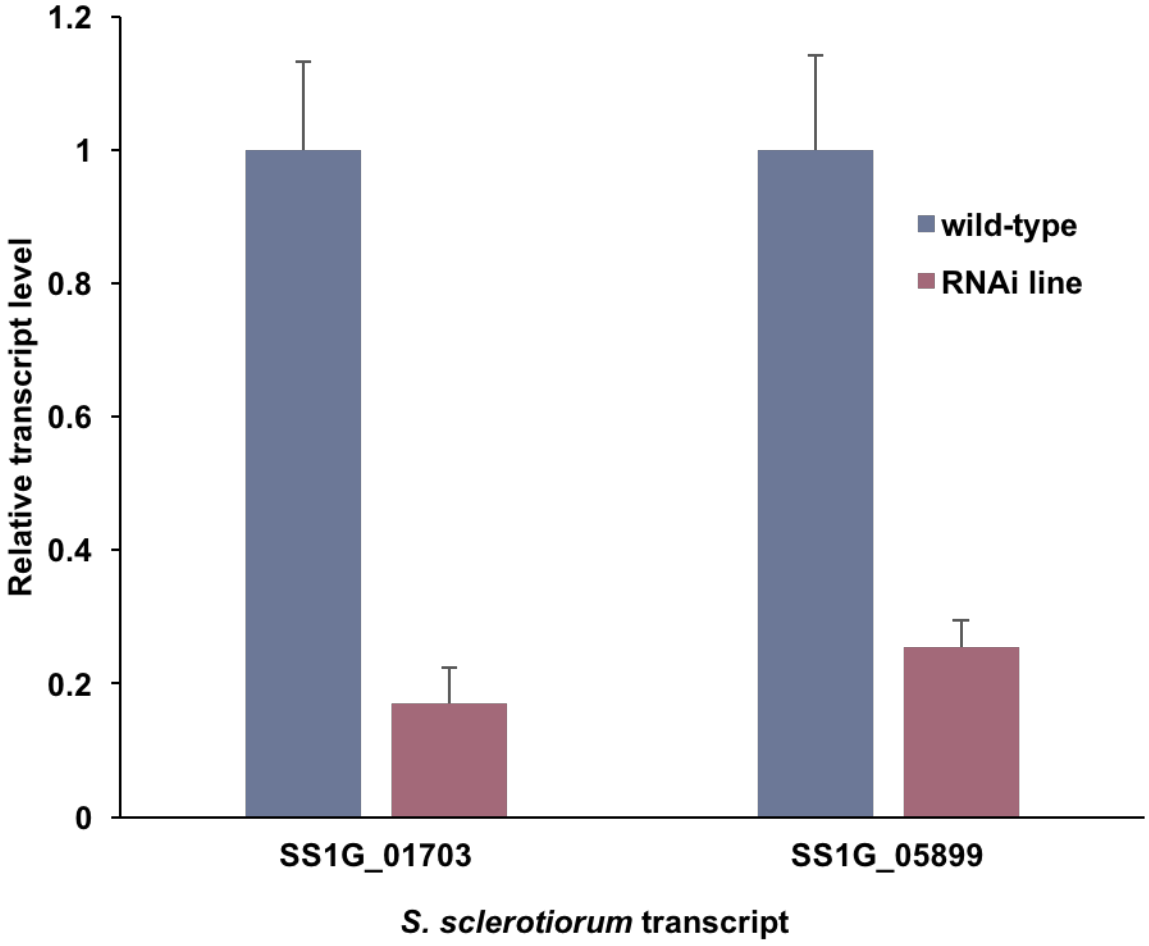


Figure 3.3 Relative transcript levels of SS1G_01703 and SS1G_05899 during infection on *A. thaliana* $SS1G01703_{RNAi}$ and *A. thaliana* $SS1G05899_{RNAi}$ compared to wild-type *A. thaliana*. RNA was extracted from infected tissue 3 dpi after spore inoculation. Data represent 3 biological replicates relative to wild-type tissue collection and normalized *with S. sclerotiorum* reference gene SsSac7 (SS1G_12350). Error bars represent standard error and significant reductions are denoted with "*" (student's t-test, $p<0.05$).

SEN1 (2.6-fold; $p < 0.01$). However, *PR1* was increased by 0.6-fold ($p < 0.05$) compared to the infected wildtype (Fig. 3.4b).

In general, *A. thaliana SS1G01703*$_{RNAi}$, *A. thaliana SS1G05899*$_{RNAi}$, and wild-type *A. thaliana* all showed a strong increase in the JA marker *PDF1.2*, which is characteristic of necrotrophic infections, as well as elevated *PR1* and *SEN1* levels during fungal colonization (Nováková *et al.*, 2014). However, *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$ responded similarly by decreasing mRNA levels of *ATG8B* and *BI1*, unlike the wild-type plants. Between the transgenic lines, the *A. thaliana SS1G01703*$_{RNAi}$ had decreased *PR1* levels, while the *A. thaliana SS1G05899*$_{RNAi}$ demonstrated a weaker *SEN1* induction.

3.5 DISCUSSION

The capability of HIGS to suppress *S. sclerotiorum* disease pressure on *A. thaliana* through the production of hpRNA was demonstrated. Both *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$ lines decreased foliar disease severity effectively. The levels of fungal transcript and DNA were reduced during infection on the RNAi plants, further suggesting *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$ actively attenuated disease symptoms. Using established markers for cell death and defence, plant response to fungal infection was explored. Although the defence and death patterns between the RNAi line and the wild-type plants appeared rather similar, alternative processes are potentially cooperating with HIGS to repress fungal disease.

SS1G_01703 encodes a putative versiconal hemiacetal acetate esterase, an enzyme involved in the fungal aflatoxin biosynthesis pathway, and may be involved in the production of secondary metabolites in *S. sclerotiorum* (Azevedo *et al.*, 2016; Kanehisa *et al.*, 2017). To

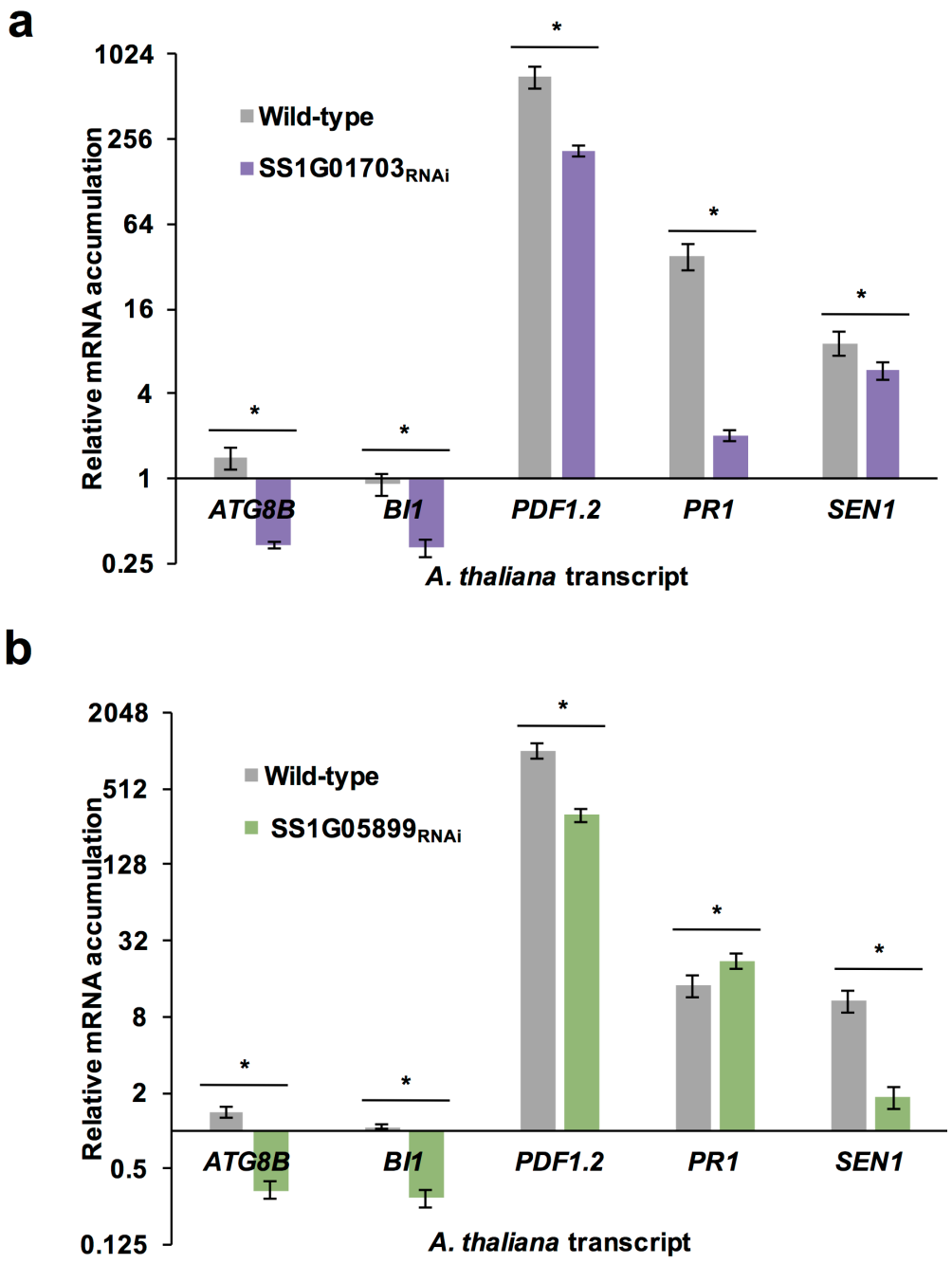


Figure 3.4 Relative *Arabidopsis thaliana* transcript levels of cell death (*ATG8B, BI1, SEN1*) and defense markers (*PDF1.2, PR1*) during *Sclerotinia sclerotiorum* colonization as assessed by quantitative real-time PRC. (**a**) Infection of *A. thaliana SS1G01703$_{RNAi}$* line (purple) and wild-type line (grey). (**b**) Infection of *A. thaliana SS1G05899$_{RNAi}$* (green) and wild-type line (grey). RNA was extracted from infected tissue 3 dpi after spore inoculation. Data represent 3 biological replicates with mRNA levels compared to uninfected control with all mRNA levels normalized to *A. thaliana EEF-1BB1* reference transcript. Error bars represent standard error and significant pairwise comparisons are denoted with "*" (student's t-test, $p<0.05$)

mitigate the effect of these putative toxins, I developed a RNAi vector targeting SS1G_01703 through genetic transformation of *A. thaliana.* Previously, HIGS was used to prevent mycotoxin accumulation in maize and peanut (*Arachis hypogaea*) (Arias *et al.*, 2015; Masanga *et al.*, 2015; Thakare *et al.*, 2017). When maize and peanut were challenged with *Aspergillus flavus*, the pathogen was unable to produce aflatoxins that would contaminate the kernels of the host plant. Despite the elimination of toxigenic capacity, the fungus did not show reductions in pathogenesis when targeting this pathway (Arias *et al.*, 2015; Masanga *et al.*, 2015; Thakare *et al.*, 2017). In contrast, the *A. thaliana SS1G01703*$_{RNAi}$ demonstrated efficacious suppression of fungal disease on the leaf surface. Likely, the production of the *S. sclerotiorum* contributes to the pathogenic biology and potentially the necrotrophic nature of this fungus. Recently, Roze *et al.* (2015) suggested that blocking aflatoxin biosynthesis may limit the production of reactive oxygen species (ROS) and decrease tolerance to oxidative damage. Since host defence creates an oxidative environment, such as the respiratory burst, I predicted knocking down levels of SS1G_01703 mRNA, could contribute to the pathogen's sensitivity to host counterattack (Heller and Tudzynski, 2011; Roze *et al.*, 2015). The dramatic reduction in fungal load further supports the diminished fungal proliferation in the host tissue. Taken together, directed targeting of SS1G_01703 through the RNAi pathway improves plant performance following *S. sclerotiorum* infection. While there is a growing body of evidence that targeting this gene in *S. sclerotiorum* is beneficial to the plant, the specific cellular mechanism remains unclear. However, I speculate that the mode of action is likely through the suppression of host poisoning and increased sensitivity to oxidative damage.

I was also successful in reducing fungal pressure in *A. thaliana* line by targeting a fungal thioredoxin reductase, a gene essential for *Aspergillus fumatigus* (Hu *et al.*, 2007). Thioredoxin

reductase is involved in sulphur tolerance, ROS scavenging, and the reduction of disulphide bonds (Breitenbach *et al.*, 2015; Montibus *et al.*, 2015; Sato *et al.*, 2011). Previous genetic deletions of thioredoxin reductase in *Aspergillus nidulans* resulted in abnormal development (Thön *et al.*, 2007). Similarly, in the rice pathogen *Magnaporthe oryzae*, the thioredoxin reductase system was determined to be essential for appressorium development, intercellular growth, and oxidative tolerance using genetic deletions. The modified strain was unable to severely infect rice leaves (Fernandez and Wilson, 2014). During fungal colonization, the host plant will invoke respiratory bursts and produce ROS that will create an oxidative environment and damage the molecular structure of cellular components (Heller and Tudzynski, 2011; Torres *et al.*, 2006). Therefore, the reduction in thioredoxin reductase by an RNAi-induced silencing event could prevent ROS scavenging and innate cellular responses, leading to an accumulation of oxidized proteins and nucleic acids within advancing hyphae. The host-induced damage may have led to the observed reduction in fungal DNA and reduced lesions, suggesting limited fungal replication within the host tissue. *A. thaliana SS1G05899*$_{RNAi}$ is the first demonstration of HIGS to control fungal infection on a plant species targeting the fungal thioredoxin reductase system.

Transgenic *A. thaliana* expressing hpRNA directed at SS1G_01703 or SS1G_05899 demonstrated greater control of *S. sclerotiorum* compared to a topical treatment of 200 ng of dsRNA to the leaf surface (Chapter 2). Previously, Koch *et al.* (2013) showed dramatic reductions in *F. graminearum* infection using HIGS barley lines (*Hordeum vulgare* L.), which proved to be more effective than the later study attempting to suppress fungal pressure using topical dsRNA of the same target (Koch *et al.*, 2016). Furthermore, the size of the molecules, whether long dsRNA or sRNA, did not have an effect on the closely related fungus *B. cinerea* (Wang *et al.*, 2016). Based on the data, there are two potential mechanisms that could explain

the higher potency of HIGS plants. First, the host plant cells process the transcribed dsRNA into siRNA, which would be delivered to the assailing pathogen (Koch *et al.*, 2013; Panwar *et al.*, 2017). Since the RNAi mechanism is a multi-step process, siRNA could be more efficient as it skips processing by DCL (Knip *et al.*, 2014; Chapter 1, Fig. 1.1). Second, application of dsRNA to the surface of the leaf represents a finite amount of dsRNA compared to the RNAi silencing vectors that constantly produce hpRNA driven by the 35S Cauliflower Mosaic Virus promoter (Khalid *et al.*, 2017). Thus, the plant would provide a constant supply of processed dsRNAs to the advancing fungus, sustaining optimal silencing conditions of the target gene despite changes in fungal cell metabolism or replication. Therefore to complement the diminution of pathogen transcript levels with the production of hpRNA, northern blots should be done to detect both the presence and processing of the hpRNA *in planta* (Koch *et al.*, 2013; Panwar *et al.*, 2017). Gel visualization would provide supporting evidence linking HIGS technology with the attenuation of fungal pathogenesis. Taken together, engineering of HIGS in planta could provide a more efficacious RNAi management strategy to mitigate fungal pressure and could be applied to globally important cropping systems where transformation protocols are available.

Differences in disease severity observed between the two RNAi lines suggest the plant is responding to the fungus and fungal attack through different cellular mechanisms. Previous research using HIGS technology against fungal pathogens failed to explore the host response during infection (Ghag *et al.*, 2014; Koch *et al.*, 2013; Wang *et al.*, 2016). To better understand these differences, we quantified changes in gene activity associated with selected cell death and defense markers. JA-induced senescence could explain the induction of both *SEN1* and *PDF1.2* and provides a possible explanation into the improved plant protection observed in the transgenic lines compared to wild-type plants (Balbi and Devoto, 2008). Furthermore, Kabbage *et al.*

(2013) explored the interaction of a non-pathogenic strain of *S. sclerotiorum A2* during infection on wild-type *A. thaliana.* During host colonization, *S. sclerotiorum A2* induced greater production of *PDF1.2* and *ATG8B* in host *A. thaliana*. Likely, the JA defense response promoted cellular autophagy instead of an apoptotic-like programmed cell death, leading to fungal non-pathogenesis (Kabbage *et al.*, 2013). Despite the evidence, the qRT-PCR results do not support the hypothesis that autophagy was operating as the primary mode of defence against fungal infection, as the autophagy marker was downregulated during infection. Thus, other mechanisms may be responsible for improved plant performance.

Downregulation of *BI1*, a BAX antagonist, suggests the innate defense response was likely independent of BAX-induced programmed cell death, usually invoked by H_2O_2, temperature stress, SA, and biotic factors (Chen *et al.*, 2013; Watanabe and Lam, 2009). Recently, the expression of *BI1* and autophagy was linked through interactions via *AUTOPHAGY 6* (*ATG6*) (Xu *et al.*, 2017). Thus, the reduction of both *BI1* and *ATG8B* may operate via an ATG6-mediated interaction between autophagy and cell death. Other effectors have also been implicated in necrotrophic resistance and cell death, including ethylene signalling, cell wall modification, secondary compound synthesis, or nitric oxide (Broekgaarden *et al.*, 2015; Khare *et al.*, 2017; Miedes *et al.*, 2014; Perchepied *et al.*, 2010). Based on these results and the differential accumulation of defense markers like *PR1*, there exists a possibility that the underlying cellular mechanisms responsible for plant health in *A. thaliana* $SS1G01703_{RNAi}$ and *A. thaliana* $SS1G05899_{RNAi}$ are different, despite leading to the same beneficial outcome. Moreover, the RNAi construct integrates preferentially into euchromatic regions in the host genome such as promoters and coding regions (Gelvin and Kim, 2007; Lim *et al.*, 2007). The interruption of genetic expression and regulation could have also contributed to

the differences within *A. thaliana SS1G01703*$_{RNAi}$ and *A. thaliana SS1G05899*$_{RNAi}$. Therefore, global profiling of RNA populations through RNA-seq analysis should provide the necessary information to dissect how plants expressing dsRNAs that target the fungus respond to fungal attack.

When transformation protocols are available, HIGS biotechnology provides a more potent implementation of an RNAi-based strategy that imparts species-specific resistance from necrotrophic pathogens. The production of hpRNA in planta disables specific cellular processes within the invading pathogen and leads to minimal colonization within host tissue. Although unclear, the underlying molecular processes in the transformed plants contributed to positive host outcomes, allowing for survival instead of death. Taken together, HIGS could be a sentinel of the next-generation of fungal management strategies utilizing RNAi that improve upon traditional protocols for developing host resistance.

3.6 REFERENCES

Amaradasa, B.S. and Everhart, S.E. (2016) Effects of Sublethal Fungicides on Mutation Rates and Genomic Variation in Fungal Plant Pathogen, *Sclerotinia sclerotiorum*. *PLoS One* **11**, e0168079.

Andrade, C.M., Tinoco, M.L.P., Rieth, A.F., Maia, F.C.O. and Aragão, F.J.L. (2016) Host-induced gene silencing in the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*. *Plant Pathol.* **65**, 626–632.

Arias, R.S., Dang, P.M. and Sobolev, V.S. (2015) RNAi-mediated Control of Aflatoxins in Peanut: Method to Analyze Mycotoxin Production and Transgene Expression in the Peanut/Aspergillus Pathosystem. *J. Vis. Exp.* **2015**, 1–11.

Azevedo, L., Chagas-Paula, D.A., Kim, H., Roque, A.C.M., Dias, K.S.T., Machado, J.C., Soares, M.G. and Mertens-Talcott, S.U. (2016) White mold (*Sclerotinia sclerotiorum*), friend or foe: Cytotoxic and mutagenic activities in vitro and in vivo. *Food Res. Int.* **80**, 27–35.

Balbi, V. and Devoto, A. (2008) Jasmonate signalling network in *Arabidopsis thaliana* : crucial regulatory nodes and new physiological scenarios. , 301–318.

Baum, J. a, Bogaert, T., Clinton, W., et al. (2007) Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* **25**, 1322–6.

Becker, M.G., Zhang, X., Walker, P.L., et al. (2017) Transcriptome analysis of the *Brassica napus–Leptosphaeria maculans* pathosystem identifies receptor, signaling and structural genes underlying plant resistance. *Plant J.* **90**, 573–586.

Bonfim, K., Faria, J.C., Nogueira, E.O.P.L., Mendes, É.A., Aragão, F.J.L., Recursos, E., Norte, P.W. and Brasília, U. De (2007) RNAi-Mediated Resistance to Bean golden mosaic virus in Genetically Engineered Common Bean (*Phaseolus vulgaris*). **20**, 717–726.

Breitenbach, M., Weber, M., Rinnerthaler, M., Karl, T. and Breitenbach-Koller, L. (2015) Oxidative stress in fungi: Its function in signal transduction, interaction with plant hosts, and lignocellulose degradation. *Biomolecules* **5**, 318–342.

Broekgaarden, C., Caarls, L., Vos, I.A., Pieterse, C.M.J. and Wees, S.C.M. Van (2015) Ethylene: traffic controller on hormonal crossroads to defense. *Plant Physiol.* **169**, pp.01020.2015.

Canola Council of Canada (2017) Economic Impact of the Canola Industry.

Chen, X.H., Yu, H., Deng, H.J., Chen, J.X., Mi, H.B. and Mao, L.C. (2013) Cucumber BAX inhibitor-1 , a conserved cell death suppressor and a negative programmed cell death

regulator under cold stress. **57**, 684–690.

Chi, M.H., Park, S.Y. and Lee, Y.H. (2009) A quick and safe method for fungal DNA extraction. *Plant Pathol. J.* **25**, 108–111.

Cusaac, J.P.W., Morrison, S.A., Belden, J.B., Smith, L.M. and McMurry, S.T. (2016) Acute toxicity of Headline® fungicide to Blanchard's cricket frogs (*Acris blanchardi*). *Ecotoxicology* **25**, 447–455.

Derbyshire, M.C. and Denton-Giles, M. (2016) The control of Sclerotinia stem rot on oilseed rape (*Brassica napus*): Current practices and future opportunities. *Plant Pathol.* **65**, 859–877.

Edwards, K., Johnstone, C. and Thompson, C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* **19**, 1349.

Fernandez, J. and Wilson, R.A. (2014) Characterizing roles for the glutathione reductase, thioredoxin reductase and thioredoxin peroxidase-encoding genes of *Magnaporthe oryza*e during rice blast disease. *PLoS One* **9**.

Garg, H., Li, H., Sivasithamparam, K. and Barbetti, M.J. (2013) Differentially Expressed Proteins and Associated Histological and Disease Progression Changes in Cotyledon Tissue of a Resistant and Susceptible Genotype of *Brassica napus* Infected with *Sclerotinia sclerotiorum*. *PLoS One* **8**.

Garg, H., Li, H., Sivasithamparam, K., Kuo, J. and Barbetti, M.J. (2010) The infection processes of *Sclerotinia sclerotiorum* in cotyledon tissue of a resistant and a susceptible genotype of Brassica napus. *Annu. Bot.*, 897–908.

Gelvin, S.B. and Kim, S.I. (2007) Effect of chromatin upon Agrobacterium T-DNA integration and transgene expression. *Biochim. Biophys. Acta - Gene Struct. Expr.* **1769**, 410–421.

Ghag, S.B., Shekhawat, U.K.S. and Ganapathi, T.R. (2014) Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against Fusarium wilt in banana. *Plant Biotechnol. J.* **12**, 541–53.

Heller, J. and Tudzynski, P. (2011) Reactive Oxygen Species in Phytopathogenic Fungi: Signaling, Development, and Disease. *Annu. Rev. Phytopathol.* **49**, 369–390.

Hu, W., Sillaots, S., Lemieux, S., et al. (2007) Essential Gene Identification and Drug Target Prioritization in *Aspergillus fumigatus*. **3,** e24.

Huzar-Novakowiski, J., Paul, P.A. and Dorrance, A.E. (2017) Host resistance and chemical control for management of Sclerotinia stem rot of soybean in Ohio. *Phytopathology* **107**, 937–949.

Kabbage, M., Williams, B. and Dickman, M.B. (2013) Cell Death Control: The Interplay of Apoptosis and Autophagy in the Pathogenicity of *Sclerotinia sclerotiorum*. *PLoS Pathog.* **9**.

Kamal, M.M., Savocchia, S., Lindbeck, K.D. and Ash, G.J. (2016) Biology and biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary in oilseed Brassicas. *Australas. Plant Pathol.* **45**, 1–14.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. and Morishima, K. (2017) KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* **45**, D353–D361.

Khalid, A., Zhang, Q., Yasir, M. and Li, F. (2017) Small RNA based genetic engineering for plant viral resistance: Application in crop protection. *Front. Microbiol.* **8**, 1–11.

Khare, D., Choi, H., Huh, S.U., Bassin, B., Kim, J., Martinoia, E., Sohn, K.H., Paek, K.-H. and Lee, Y. (2017) *Arabidopsis* ABCG34 contributes to defense against necrotrophic pathogens by mediating the secretion of camalexin. *Proc. Natl. Acad. Sci.* **114**, E5712–

E5720.

Kılıç, G.A. (2011) Histopathological and biochemical alterations of the earthworm (*Lumbricus terrestris*) as biomarker of soil pollution along Porsuk River Basin (Turkey). *Chemosphere* **83**, 1175–1180.

Knip, M., Constantin, M.E. and Thordal-Christensen, H. (2014) Trans-kingdom Cross-Talk: Small RNAs on the Move. *PLoS Genet.* **10**.

Koch, A., Biedenkopf, D., Furch, A., et al. (2016) An RNAi-Based Control of Fusarium graminearum Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery. *PLoS Pathog.* **12**, 1–22.

Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J. and Kogel, K. (2013) Host-induced gene silencing of cytochrome P450 lanosterol C14 α -demethylase – encoding genes confers strong resistance to Fusarium species. **110**, 19324–19329.

Komaki, S., Abe, T., Coutuer, S., Inzé, D., Russinova, E. and Hashimoto, T. (2010) Nuclear-localized subtype of end-binding 1 protein regulates spindle organization in *Arabidopsis*.

Lim, P.O., Kim, H.J. and Gil Nam, H. (2007) Leaf Senescence. *Annu. Rev. Plant Biol.* **58**, 115–136.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and. *Methods* **25**, 402–408.

Llanos, A., François, J. and Parrou, J. (2015) Tracking the best reference genes for RT-qPCR data normalization in filamentous fungi. *BMC Genomics* **16**, 71.

Luo, H., Lin, Y., Gao, F., Zhang, C.T. and Zhang, R. (2014) DEG 10, an update of the database of essential genes that includes both protein-coding genes and noncoding genomic elements. *Nucleic Acids Res.* **42**, 574–580.

Maltby, L., Brock, T.C.M. and Brink, P.J. Van Den (2009) Fungicide risk assessment for aquatic ecosystems: Importance of interspecific variation, toxic mode of action, and exposure regime. *Environ. Sci. Technol.* **43**, 7556–7563.

Masanga, J.O., Matheka, J.M., Omer, R.A., Ommeh, S.C., Monda, E.O. and Alakonya, A.E. (2015) Downregulation of transcription factor aflR in *Aspergillus flavus* confers reduction to aflatoxin accumulation in transgenic maize with alteration of host plant architecture. *Plant Cell Rep.* **34**, 1379–1387.

Miedes, E., Vanholme, R., Boerjan, W. and Molina, A. (2014) The role of the secondary cell wall in plant resistance to pathogens. *Front. Plant Sci.* **5**, 358.

Montibus, M., Pinson-Gadais, L., Richard-Forget, F., Barreau, C. and Ponts, N. (2015) Coupling of transcriptional response to oxidative stress and secondary metabolism regulation in filamentous fungi. *Crit. Rev. Microbiol.* **41**, 295–308.

Nováková, M., Šašek, V., Dobrev, P.I., Valentová, O. and Burketová, L. (2014) Plant hormones in defense response of *Brassica napus* to *Sclerotinia sclerotiorum* - Reassessing the role of salicylic acid in the interaction with a necrotroph. *Plant Physiol. Biochem.* **80**, 308–317.

Nunes, C.C. and Dean, R.A. (2012) Host-induced gene silencing: A tool for understanding fungal host interaction and for developing novel disease control strategies. *Mol. Plant Pathol.* **13**, 519–529.

Panwar, V., Jordan, M., McCallum, B. and Bakkeren, G. (2017) Host-induced silencing of essential genes in *Puccinia triticina* through transgenic expression of RNAi sequences reduces severity of leaf rust infection in wheat. *Plant Biotechnol. J.*

Panwar, V., McCallum, B. and Bakkeren, G. (2013) Host-induced gene silencing of wheat

leaf rust fungus *Puccinia triticina* pathogenicity genes mediated by the Barley stripe mosaic virus. *Plant Mol. Biol.* **81**, 595–608.

Papolu, P.K., Gantasala, N.P., Kamaraju, D., Banakar, P., Sreevathsa, R. and Rao, U. (2013) Utility of host delivered RNAi of two FMRF amide like peptides, flp-14 and flp-18, for the management of root knot nematode, *Meloidogyne incognita*. *PLoS One* **8**, 1–16.

Penaud, A. and Walker, A.-S. (2015) Oilseed Rape Pathogens in France. In Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. (Ishii, H. and Hollomon, D.W., eds), pp. 389–399. Tokyo: Springer Japan.

Perchepied, L., Balagué, C., Riou, C., Claudel-Renard, C., Rivière, N., Grezes-Besset, B. and Roby, D. (2010) Nitric oxide participates in the complex interplay of defense-related signaling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *Mol. Plant. Microbe. Interact.* **23**, 846–60.

Roze, L. V., Laivenieks, M., Hong, S.Y., Wee, J., Wong, S.S., Vanos, B., Awad, D., Ehrlich, K.C. and Linz, J.E. (2015) Aflatoxin biosynthesis is a novel source of reactive oxygen species—a potential redox signal to initiate resistance to oxidative stress? *Toxins (Basel).* **7**, 1411–1430.

Sato, I., Shimatani, K., Fujita, K., Abe, T., Shimizu, M., Fujii, T., Hoshino, T. and Takaya, N. (2011) Glutathione reductase/glutathione is responsible for cytotoxic elemental sulfur tolerance via polysulfide shuttle in fungi. *J. Biol. Chem.* **286**, 20283–20291.

Smalling, K.L., Reilly, T.J., Sandstrom, M.W. and Kuivila, K.M. (2013) Occurrence and persistence of fungicides in bed sediments and suspended solids from three targeted use areas in the United States. *Sci. Total Environ.* **447**, 179–185.

Syromyatnikov, M.Y., Kokina, A. V., Lopatin, A. V., Starkov, A.A. and Popov, V.N. (2017)

Evaluation of the toxicity of fungicides to flight muscle mitochondria of bumblebee (*Bombus terrestris* L.). *Pestic. Biochem. Physiol.* **135**, 41–46.

Taylor, A., Coventry, E., Jones, J.E. and Clarkson, J.P. (2015) Resistance to a highly aggressive isolate of *Sclerotinia sclerotiorum* in a *Brassica napus* diversity set. , 932–940.

Thakare, D., Zhang, J., Wing, R.A., Cotty, P.J. and Schmidt, M.A. (2017) Aflatoxin-free transgenic maize using host-induced gene silencing. , 1–9.

Thön, M., Al-Abdallah, Q., Hortschansky, P. and Brakhage, A.A. (2007) The thioredoxin system of the filamentous fungus *Aspergillus nidulans*: Impact on development and oxidative stress response. *J. Biol. Chem.* **282**, 27259–27269.

Tiwari, I.M., Jesuraj, A., Kamboj, R., Devanna, B.N., Botella, J.R. and Sharma, T.R. (2017) Host Delivered RNAi, an efficient approach to increase rice resistance to sheath blight pathogen (*Rhizoctonia solani*). *Sci. Rep.* **7**, 7521.

Tollefson, J. (2011) Brazil cooks up transgenic bean. *Nature* **478**, 168–168.

Torres, M.A., Jones, J.D.G. and Dangl, J.L. (2006) Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* **141**, 373–378.

Uloth, M.B., Clode, P.L., You, M.P. and Barbetti, M.J. (2015) Attack modes and defence reactions in pathosystems involving *Sclerotinia sclerotiorum* , *Brassica carinata* , *B. juncea* and *B. napus*. *Ann. Bot.*, mcv150.

United States Environmental Protection Agency (2017) EPA Registers Innovative Tool to Control Corn Rootworm.

Wang, M., Weiberg, A., Lin, F.-M., Thomma, B.P.H.J., Huang, H.-D. and Jin, H. (2016) Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* **2**, 16151.

Watanabe, N. and Lam, E. (2009) Bax inhibitor-1, a conserved cell death suppressor, is a key molecular switch downstream from a variety of biotic and abiotic stress signals in plants. *Int. J. Mol. Sci.* **10**, 3149–3167.

Xu, G., Wang, S., Han, S., Xie, K., Wang, Y., Li, J. and Liu, Y. (2017) Plant Bax Inhibitor-1 interacts with ATG6 to regulate autophagy and programmed cell death. *Autophagy* **13**, 1161–1175.

Zhang, T., Jin, Y., Zhao, J.-H., Gao, F., Zhou, B.-J., Fang, Y.Y. and Guo, H.-S. (2016) Host-Induced Gene Silencing of the Target Gene in Fungal Cells Confers Effective Resistance to the Cotton Wilt Disease Pathogen *Verticillium dahliae*. *Mol. Plant*, 1–4.

CHAPTER 4 : CONCLUSIONS AND FUTURE DIRECTIONS

Throughout my thesis, I have gathered evidence for the utility of RNAi-based management of fungal phytopathogens. I developed two successful implementations of RNAi biotechnology to control *S. sclerotiorum* and *B. cinerea*. RNA sequencing allowed for the largest and most comprehensive identification of RNAi targets for phytopathogenic management. Synthesizing the data from the functional screens performed in Chapter 2 allowed for the creation of a novel target identification pipeline (TIP), providing the first guide for choosing RNAi-based control for fungi. With further usage, the research community will be able to adjust and fine-tune the criteria for effective selection of RNAi targets within fungi. For example, I extended my techniques to the related fungus, *B. cinerea*, to demonstrate homologous targets could offer efficacious protection in Chapter 2. Additional targeting of homologous genes in other fungi could illuminate how different fungal species initiate and sustain pathogenesis. A deeper understanding of fungal infection would be indispensable for future plant engineering.

The experiments performed in Chapter 3 represent the beginning of further explorations into HIGS as a durable strategy for phytopathogenic control. I would like to further evaluate other lines transformed with RNAi vectors that I created based on molecule performance during my foliar screens. However, some key biological questions remain. For example, I would like to use whole genome sequencing to examine which genes were interrupted by the *Agrobacterium tumefaciens*-mediated transformation, and thereby determine how target site integration differences may have affected the RNAi efficacy in the different lineages. Additionally, to validate the lesion phenotype and differential mRNA levels within the host during infection, I would also like to perform RNA-seq to understand what defence and cell death responses could be implicated in the phenotypic differences observed between the RNAi lines. This could be of

interest for plant breeders looking to develop resistance by modulating these pathways. Furthermore, exploring uninfected tissues could uncover how the production of hpRNA and the insertion event effect the basal state of the plant tissue. Pairing RNA-seq with sRNA-seq could also reveal a more detailed view of RNA processing within cells and develop a greater functional knowledge of HIGS biotechnology.

I explored interaction of the dsRNA or hpRNA-producing plants targeting *S. sclerotiorum* or *B. cinerea* (Chapters 2 and 3). My thesis focused on the 'on-target' effects of the dsRNA molecules. The level of homology for a dsRNA to elicit an RNAi response and the severity of the RNAi response has not been teased apart in relation to potential 'off-target' effects. Therefore, it will be interesting to investigate the effect of *S. sclerotiorum* targeting dsRNA on infection and growth on a variety of other fungal species. QRT-PCR could be performed to explore whether transcript levels change significantly from the controls, which would suggest an effect within another organism. Such an exploration is crucial for ecological and molecular design if RNAi technology will advance further towards commercialization.

Together, these additional experiments would add important details to complete a compelling investigation of the potency of RNAi-based approaches for phytopathogenic management. While there are still many unanswered questions concerning the broader impacts of exogenous dsRNA on plants, my thesis nevertheless provides functional evidence that a new generation of sustainable technologies to overcome today's pressing challenges using RNAi biotechnologies is entirely possible.