The development of RNA interference-based technologies for the control of *Sclerotinia sclerotiorum* in *Brassica napus*

By

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ABSTRACT

Necrotrophic fungal phytopathogens such as *Sclerotinia sclerotiorum* are a detriment to many economically significant crop species like canola, *Brassica napus*, and threaten the ability of the food supply chain to accommodate rising demand. Traditional approaches to control fungal pathogens predominantly involve chemical control through broad-spectrum agents that have been shown to negatively impact the agroecological environment. RNA interference (RNAi), an innate cellular pathway for the targeted silencing of mRNA molecules, may prove to be an effective alternative. I have investigated the utility of this technology both in the application of exogenous double stranded (ds)RNA (spray induced gene silencing – SIGS) to target specific mRNAs of *S. sclerotiorum* as well as through the creation of transgenic *B. napus* to express dsRNA molecules (host induced gene silencing – HIGS). I first investigated the mechanism in which *S. sclerotiorum* uptakes dsRNA. I performed microscopy using fluorescently labeled dsRNA and transgenic *S. sclerotiorum* expressing eGFP as well as molecular experiments using chemical inhibitors of cellular pathways and dsRNAs targeting the expression of specific genes within these pathways. Ultimately, I found clathrin mediated endocytosis to be responsible for dsRNA uptake in *S. sclerotiorum*. Next, I examined whether HIGS would provide effective protection. I created transgenic *B. napus* to target a gene involved in *S. sclerotiorum* pathogenicity and found significantly enhanced tolerance to infection in leaf, stem and whole plant assays. I then characterized the modes of protection by sequencing the mRNA and small RNA transcriptomes as well as performing microscopy at the site of infection. We found that the plant defense response was significantly heightened within the RNAi line, indicating that the produced dsRNA worked in tandem with innate plant defense to provide an effective defense response. Lastly, in order to gain further appreciation for this pathosystem, I studied the small RNA landscape during the *B. napus – S. sclerotiorum* interaction in hopes of using their innate biological mechanisms as a means to identify further gene targets for RNAi. Together, these findings provide compelling evidence and pave the way for further development of RNAi-based pathogen management strategies using both exogenous applications and transgenic approaches.

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TABLE OF CONTENTS

TITLE ..... **i**

ABSTRACT ..... **ii**

ACKNOWLEDGEMENTS ..... **iii**

LIST OF TABLES ..... **ix**

LIST OF SUPPLEMENTARY TABLES ..... **ix**

LIST OF FIGURES ..... **x**

LIST OF SUPPLEMENTARY FIGURES ..... **xi**

NON-COMMON ABBREVIATIONS USED ..... **xii**

CONTRIBUTION OF AUTHORS ..... **xiii**

CHAPTER 1: MODES OF DSRNA UPTAKE IN PLANT PESTS AND PATHOGENS **1**

1.1 ABSTRACT **1**

1.2 INTRODUCTION **2**

1.3 RNA INTERFERENCE CORE COMPONENTS **10**

1.4 MODES OF UPTAKE:

1.4.1 *CAENORHABDITIS ELEGANS: SYSTEMIC RNAI DEFECTIVE (SID) PROTEINS* **11**

1.4.2 INSECT UPTAKE: SID-LIKE PROTEINS **12**

1.4.3 INSECT UPTAKE: ENDOCYTOSIS **13**

1.4.4 FUNGAL UPTAKE **15**

1.5 IMPORTANCE OF UNDERSTANDING DSRNA UPTAKE:

1.5.1 RESISTANCE **17**

1.5.2 FORMULATIONS **17**

1.5.3 NANOPARTICLES **18**

1.5.4 RIBONUCLEOTIDE PROTEIN DSRNA CARRIERS **19**

1.5.6 CATIONIC LIPOSOMES DSRNA CARRIERS……..…….**20**

1.6 CONCLUDING REMARKS……..…**21**

1.7 REFERENCES……...**23**

CHAPTER 2: CLATHRIN MEDIATED ENDOCYTOSIS IS INVOLVED IN THE UPTAKE OF EXOGENOUS DOUBLE-STRANDED RNA IN THE WHITE MOLD PHYTOPATHOGEN *SCLEROTINIA SCLEROTIORUM*…48

2.1 ABSTRACT……..……**48**

2.2 INTRODUCTION……**49**

2.3 MATERIALS AND METHODS:

2.3.1 SCLEROTINIA CULTURE…..……**53**

2.3.2 DESIGN AND *IN VITRO* SYNTHESIS OF DSRNAS……**53**

2.3.3 *IN VITRO* DSRNA FLUORESCENCE LABELLING FOR LIVE CELL IMAGING AND CONFOCAL MICROSCOPY…..……...….**54**

2.3.4 DSRNA UPTAKE INHIBITION USING CHEMICAL INHIBITION AND RNAI OF RNAI EXPERIMENTS ……..……**54**

2.3.5 SCLEROTINIA TRANSFORMATION……..……....……**55**

2.3.6 DATA ANALYSIS…..……...…**56**

2.4 RESULTS:

2.4.1 VISUALIZING REGIONS OF DSRNA UPTAKE……..……**57**

2.4.2 CHEMICAL INHIBITION OF ENDOCYTIC PROCESSES.....**59**

2.4.3 RNAI OF RNAI EXPERIMENT TO ELUCIDATE CANDIDATE UPTAKE GENES……....……**60**

2.4.4 TRANSGENIC SCLEROTINIA ENCODING EGFP TO VALIDATE CME AS THE PRIMARY MODE OF UPTAKE…..…….……**63**

2.5 DISCUSSION……..**65**

2.6 CONCLUSION……...……**70**

2.7 REFERENCES…….……..**71**

CHAPTER 3: HOST INDUCED GENE SILENCING OF THE *SCLEROTINIA SCLEROTIORUM ABHYDROLASE-3* GENE REDUCES DISEASE SEVERITY IN *BRASSICA NAPUS*……..……..82

3.1 ABSTRACT……82

3.2 INTRODUCTION……83

3.3 MATERIALS AND METHODS:

3.3.1 *B. NAPUS* GROWTH CONDITIONS……..……**87**

3.3.2 PREPARATION OF *AGROBACTERIUM TUMEFACIENS*……..…**87**

3.3.3 *B. NAPUS* TRANSFORMATIONS…….……..……**88**

3.3.4 DNA EXTRACTION AND GENOTYPING……..……...……**89**

3.3.5 COPY NUMBER DETERMINATION…….……**89**

3.3.6 RNA ISOLATIONS…….....……..**90**

3.3.7 RT qPCR……...…….……**90**

3.3.8 LINEAR HAIRPIN VARIABLE RT-qPCR……...……...……**90**

3.3.9 LEAF INFECTION ASSAYS……...…**91**

3.3.10 STEM INOCULATIONS…….....…**91**

3.3.11 WHOLE PLANT SPRAY INOCULATIONS…….……**91**

3.3.12 mRNA LIBRARY SYNTHESIS, SEQUENCING AND BIOINFORMATIC ANALYSIS……..**92**

3.3.13 SMALL RNA LIBRARY SYNTHESIS, SEQUENCING AND BIOINFORMATIC ANALYSIS……..**92**

3.3.14 LIGHT MICROSCOPY…….……..**93**

3.3.15 SCANNING ELECTRON MICROSCOPY…….……...…**93**

3.4 RESULTS:

3.4.1 CONFIRMATION OF TRANSGENE EXPRESSION....……...…**94**

3.4.2 HpRNA PRODUCTION IN *B. NAPUS* HOST TARGETING SS1G_01703 SIGNIFICANTLY REDUCES *S. SCLEROTIORUM* INFECTION ……...…**96**

3.4.3 HOST INDUCED GENE SILENCING OF SS1G_01703 CONFERS TOLERANCE TO *S. SCLEROTIORUM* ACROSS THE PLANT LIFECYCLE**……98**

3.4.4 RNA SEQUENCING OF HIGS-BASED PLANT PROTECTION**……..……...……..100**

3.4.5 ACTIVATION OF STRUCTURAL DEFENSE PATHWAYS IN BN1703.2 IN RESPONSE TO *S. SCLEROTIORUM* INFECTION**….....….104**

3.5 DISCUSSION……...……**108**

3.6 REFERENCES……..……**113**

APPENDIX 1: THE INFLUENCE OF HIGS ON SMALL RNA LANDSCAPE WITHIN THE *BRASSICA NAPUS – SCLEROTINIA SCLEROTIORUM* PATHOSYSTEM……..…..**135**

4.1 ABSTRACT……..……**135**

4.2 INTRODUCTION……...……**136**

4.3 MATERIALS AND METHODS:

4.3.1 *B. NAPUS* GROWTH CONDITIONS**……..……139**

4.3.2 PREPARATION OF *AGROBACTERIUM TUMEFACIENS*……..…..**139**

4.3.3 *B. NAPUS* TRANSFORMATIONS**……...……...….139**

4.3.4 DNA EXTRACTION AND GENOTYPING**……..……139**

4.3.5 STEM INOCULATIONS**…...…….…139**

4.3.6 SMALL RNA LIBRARY SYNTHESIS, SEQUENCING**..……...139**

4.3.7 BIOINFORMATIC ANALYSIS**……..……139**

4.4 RESULTS:

4.4.1 SMALL RNA SEQUENCING OF *B. NAPUS* INFECTED WITH *S. SCLEROTIORUM*……..……..**140**

4.4.2 IDENTIFICATION OF SMALL RNA SPECIES INVOLVED IN PLANT DEFENSE AND FUNGAL PATHOGENICITY**……..….....……141**

4.4.3 mRNA TARGETS OF IDENTIFIED SMALL RNA SPECIES**…….....143**
4.5 DISCUSSION……...……147
4.6 REFERENCES……150
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS……..……..157
5.1 OVERVIEW OF IMPACT……157
5.2 IMPLICATIONS AND FUTURE DIRECTIONS…...……158

LIST OF TABLES

Table 4.1. Small RNA species identified in this study and their predicted mRNA targets……**146**

LIST OF SUPPLEMENTARY TABLES

Table S2.1. Gene identifications and functions for each of the gene targets used in this study……..…..**79**

Table S2.2. Cloning primer sequences as well as dsRNA fragment lengths used in this study……..**80**

Table S2.3. qRT PCR primer sequences used in this study……..**81**

Table S3.1. Cloning primer sequences used in this study…….……..**130**

Table S3.2. qRT PCR primer sequences used in this study……**131**

Table S3.3. Gene homologues to SS1G_01703 in related fungal species……..……**132**

Table S3.4. Gene IDs and annotation of differentially expressed genes identified within this study……**132**

LIST OF FIGURES

Figure 1.1. Diagram of dsRNA uptake in three representative species……**16**

Figure 2.1. Real time visualization of dsRNA uptake with fluorescent dsRNA in *S. sclerotiorum*……..……**58**

Figure 2.2. Elucidation of potential uptake mechanism through chemical inhibitors of endocytosis…..…….……...……**60**

Figure 2.3. RNAi of RNAi experiment to identify genes involved in dsRNA uptake……**62**

Figure 2.4. Transgenic *S. sclerotiorum* expressing eGFP as a confirmation of the involvement of clathrin mediated endocytosis in uptake……**64**

Figure 3.1. Confirmation of transgene insertion and characterization……..**95**

Figure 3.2. Constitutive production of dsRNA reduces fungal disease pressure……..**97**

Figure 3.3. Whole plant spray assay further demonstrates enhanced disease protection through HIGS……...……...…**99**

Figure 3.4. Transcriptomic characterization of RNAi-based protection from *S. sclerotiorum*……**103**

Figure 3.5. Activation of structural defenses contributes to enhanced tolerance to S. sclerotiorum……**106**

Figure 4.1. Length distribution of small RNA species identified in this study……...**141**

Figure 4.2. Venn diagrams displaying shared and unique small RNA species between each timepoint……..**142**

LIST OF SUPPLEMENTARY FIGURES

Figure S2.1. Confocal microscopy of above, below and inside *S. sclerotiorum* hyphae during uptake of fluorescent dsRNA……..…**78**

Figure S3.1. Agarose gel images of transgene insert and transgene small RNA fragment amplified through variable linear hairpin variable q RTPCR…….……...…….**124**

Figure S3.2. Distribution of small RNA molecules aligning to the transgene……**125**

Figure S3.3. Lesion size and PR1 transcript accumulation comparison of BN1703.2 to untransformed……..**126**

Figure S3.4. PCA plot of B. napus and S. scleotiorum reads from dual RNA sequencing……**127**

Figure S3.5. Heat map of *S. sclerotiorum* differentially expressed pathogenicity genes in BN1703.2 compared to untransformed……...…….**128**

Figure S3.6. Cross-sections of infected ZY821 and uninfected BN1703.2 and untransformed stained with Sudan IV, aniline blue, or toluidine blue……**129**

Figure S4.1. Venn diagrams displaying unique and shared small RNA species between BN1703 and untransformed at each timepoint for *B. napus*……..**156**

NON-COMMON ABBREVIATIONS USED

AGO	ARGONAUTE
BafA	Bafilomycin A1
bp	base pairs
CME	clathrin mediated endocytosis
CPZ	chlorpromazine
DCL	DICER-LIKE
dsRNA	double stranded RNA
GO	Gene ontology
HIGS	Host induced gene silencing
hpRNA	hairpin RNA
MBCD	Methyl-beta cyclodextrin
miRNA	micro RNA
mRNA	messenger RNA
OA	oxalic acid
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
SIGS	Spray induced gene silencing
siRNA	small interfering RNA
sRNA	small RNA
TIM44	Translocase of inner mitochondrial membrane 44
ThioR	Thioredoxin reductase

CONTRIBUTION OF AUTHORS

Chapter 1

N.W. contributed to the original draft preparation and editing. C.M. contributed to original draft preparation and uptake visualization. V.L. contributed to original draft preparation. M.F.B. and S.W. conceptualized the ideas comprising the manuscript and provided review and editing.

Chapter 2

N.W. performed the chemical inhibitor and RNAi of RNAi experiments, the microscopy, Sclerotinia transformant creation and manuscript preparation. D.S. helped with the chemical inhibitor experiments and with figure design. K.B. helped with RNAi of RNAi and chemical inhibitor experiments. L.C. and P.P. helped to optimize and conduct the live cell imaging with the ImageXpress platform. M.F.B. and S.W. conceived the ideas and prepared the manuscript.

Chapter 3

N.W. performed bioinformatic analyses, primer design, RNA extractions, qRT PCR, plant transformation, *in planta* assays, and manuscript preparation. D.J.Z. performed bioinformatic analyses and microscopy. P.L.W. performed bioinformatic analyses. D.S.S. and K.T.B. performed *in planta* assays. D.K. performed plant transformation. S.K.S and O.W. performed bioinformatic analyses and manuscript preparation. S.W. and M.F.B. conceived the ideas and prepared the manuscript.

Appendix 1

N.W. performed canola transformation, in planta assays, RNA extraction, bioinformatic analysis and manuscript preparation. D.J.Z. performed bioinformatic analysis. S.W. and M.F.B. conceived the ideas and prepared the manuscript.

Preface, Chapter 5

N.W. performed thesis manuscript preparation. M.F.B. and S.W. edited the manuscript.

CHAPTER 1: MODES OF DSRNA UPTAKE IN PLANT PESTS AND PATHOGENS

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1.1 ABSTRACT

Efforts to develop more environmentally friendly alternatives to traditional broad spectrum pesticides in agriculture have recently turned to RNA interference (RNAi) technology. With built-in sequence-specific knockdown of gene targets following delivery of double-stranded RNA (dsRNA), RNAi offers the promise of controlling pests and pathogens without adversely affecting non-target species. Significant advances in the efficacy of this technology have been observed in a wide range of species, including many insect pests and fungal pathogens. Two different dsRNA application methods are being developed. First, host induced gene silencing (HIGS) harnesses dsRNA production through the thoughtful and precise engineering of transgenic plants and second, spray induced gene silencing (SIGS) that uses surface applications of a topically applied dsRNA molecule. Regardless of the dsRNA delivery method, one aspect that is critical to the success of RNAi is the ability of the target organism to internalize the dsRNA and take advantage of the host RNAi cellular machinery. The efficiency of dsRNA uptake mechanisms varies across species, and in some uptake is negligible, rendering them effectively resistant to this new generation of pesticides. If RNAi-based methods of control are to be used widely, it is critically important to understand the mechanisms underpinning dsRNA uptake. Understanding dsRNA uptake mechanisms will also provide insight into the design and formulation of dsRNA for improved delivery and provide clues into the development of potential host resistance to these technologies.

1.2 INTRODUCTION

Crop pests and pathogens cause approximately 300 billion USD of damage to plant-based food supplies each year worldwide (Gautham *et al*. 2020). For five of the major food crops (rice, wheat, maize, soybean and potato), 17 to 30% of annual global yield losses can be directly attributed to these biotic factors (Savary *et al*. 2019). Latest projections suggest that by 2050, we will need to increase food production by more than 50% to feed a population that will be nearing 10 billion people (Searchinger *et al*. 2018). Furthermore, as agriculture has shifted to an intensive, monoculture state to accommodate rising demand, this has favoured the occurrence of widespread epidemics and outbreaks from pests and pathogens (Corredor-Moreno *et al*. 2020). Climate change also creates additional stresses on land suitable for food production. The frequency and intensity of droughts is expected to increase, promoting further desertification, particularly in Africa and Asia (Ameth *et al*. 2017). Rising sea levels also contribute to soil erosion and increased salinity and increased extreme weather events such as floods and cyclones will also reduce arable land mass (Schneider *et al*. 2020). The challenges posed by pest and pathogens, a rapidly growing global population, and unpredictable climactic conditions demands that we find new and innovative solutions to maintain healthy crops without losing yield.

Canada accounts for 15% of the global canola (*Brassica napus*) supply and each year this oilseed occupies over 22 million hectares of Canadian land (Canola Council of Canada, 2017). In 2016 alone, the economic worth of canola in Canada was estimated to be 26.7 billion dollars (Canola Council of Canada, 2016). It's production and value continue to grow, in fact since 2005, canola's impact on the Canadian economy has increased by 19 billion dollars or 250% (Canola Council of Canada, 2016). Canola oil consumption has been linked to improved heart health through the lowering of the serum cholesterol and glycemic index (Jenkins *et al*., 2014, Kruse *et al*., 2015, Lin *et al*., 2013). Following the extraction of the oil, high protein animal feed for beef and pork industries can be produced from the remaining seed material (Broderick *et al*., 2015, Zhou *et al*., 2013). Other industrial bio-products have been manufactured from canola seeds, such as bio-lubricants, bio-diesel, and bio-degradable packaging (Madankar *et al*., 2013, McVetty and Duncan, 2015, Zhang *et al*., 2017). Ultimately, the variety of beneficial uses of the canola seed will ensure its continued prominence within agricultural food production.

Plants coexist with diverse microorganisms, which can associate below ground within root systems in what is referred to as the rhizosphere and above ground in the phyllosphere (Vorholt, 2012, Bulgarelli *et al*. 2013). They are found within the plant as endophytes, attached on the plant surface as epiphytes or around the roots in the nearby soil. Microorganisms can induce beneficial, neutral, or detrimental effects on plant health and development (Newton *et al*. 2010). A perpetual threat to Canadian canola production is a highly aggressive and necrotrophic fungal crop pathogen, *Sclerotinia sclerotiorum*. Sclerotinia stem rot has become an increasingly more serious problem as the production of canola has increased, likely due to more acreage of canola in rotations as well as management practices that maximize yield potentials (Kutcher *et al*. 2011). *S. sclerotiorum* was found to occupy and cause infection in 90% of Canadian canola crops, and loss to growers was estimated to be $600 million (Government of Manitoba, 2014). Yield losses from this pathogen range from 5% to 100% in individual fields (Government of Manitoba, 2014). In addition to its highly aggressive nature, the lifecycle of *S. sclerotiorum* also plays a large role in its ability to wreak havoc on Canadian crops. In particular, its host range of over 500 different plant species, its ability to develop sclerotia resting structures as well as producing a pathogenicity factor, oxalic acid, make control extremely challenging (Kamal *et al*. 2016). Under favourable spring conditions, hard, melanized, sclerotial structures germinate to form spore bearing structures known as apothecia. The ascospores are carried in wind currents up to 10 km away from release, and land on senescing tissues of the canola plant such as the petals (Kamal *et al*. 2016, Sharma *et al*. 2015). The infected petals fall onto the canopy of leaves below, and by using nutrients obtained from the senescing tissue, hyphae will grow and form an infection cushion to attack the host plant (Kabbage *et al*. 2015). Due to the necrotrophic nature of the fungus, the infection is characterized by pathogen-induced necrosis to obtain nutrients, appearing as brown, watery lesions 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). Upon systemic infection, the fungus will deposit new sclerotia into the remaining plant debris. These resting structures are extremely stable, allowing the fungus to overwinter and remain viable for up to ten years in the soil (Bolton *et al*. 2006). Due to this elongated viability, along with the broad host range, *S. sclerotiorum* can persist in the agroecological environment for decades.

The investigation of transcriptomomes within biological systems was revolutionized by the introduction of Next Generation Sequencing (NGS) and RNA sequencing (RNA-Seq) technologies. These technologies have lowered the cost of sequencing and increased the accessibility of sequencing experiments beyond what is possible with traditional dye-terminator methods (Barba *et al*. 2014). Typically, NGS platforms are run on constructed cDNA libraries of sample RNA populations to generate millions to billions of sequenced reads. The study of host pathogen interactions was also dramatically advanced through the influx of this technology. RNA-Seq can be used to measure gene expression within both the host and pathogen simulataneously and this is referred to as dual RNA-Seq. In addition, an emerging technology that is capable of examining small RNA populations of pathogens and hosts, through the selective amplification and sequencing of small RNA libraries is referred to as small RNA-Seq. Small RNAs predominantly perform regulatory roles in modulating gene expression changes during times of cellular flux such as cell differentiation, metabolism, apoptosis and defence (Axtell 2013). Over the last decade, hundreds of RNA-Seq experiments have emerged to gain enhanced insight into host plant defense mechanisms and fungal pathogenicity pathways creating a wealth of information (Westermann *et al*. 2017). In the *S. sclerotiorum – B. napus* pathosystem in particular, several studies have been published detailing the transcriptomic landscape during infection (Joshi *et al*. 2016, Peng *et al*. 2017, Seifbarghi *et al*. 2017, Girard *et al*. 2017, Chittem *et al*. 2020), while small RNA populations within this interaction are also beginning to be examined (Derbyshire *et al*. 2019).

Pathogen perception within the plant is conducted through pathogen associated molecular pattern (PAMP) recognition via pattern recognition receptors (PRRs). Following the activation of PRRs, signals are sent to the nucleus to transcriptionally reprogram the cell (Park and Ronald, 2012, Zipfel, 2014). PAMP detection triggers a signalling cascade through Ca^{2+} channels, nitric oxide signalling, reactive oxide species (ROS) bursts, and mitogen-activated protein kinase (MAPK) cascades (Boller and Felix, 2009, Meng and Zhang, 2013). This signalling leads to stimulation of transcription factors (TFs) that control the activation of bioprocesses responsible for plant defense (Schluttenhofer and Yuan, 2015, Tsuda and Somssich, 2015). Several cellular responses such as the synthesis of phytoalexins, pathogen-related (PR) proteins, and programmed cell death of neighbouring cells and the flux of endogenous hormones are the initial characteristics of

canola defense to *S. sclerotiorum* (Girard *et al*. 2017). Defense to necrotrophic fungi is partially controlled via jasmonic acid (JA)/ethylene (ET) hormone signalling (Denancé *et al*., 2013), however salicyclic acid (SA) has also been shown to be critical to the biotic response of *B. napus* to *S. sclerotiorum (*Novakova *et al*. 2014). These hormones activate defense genes and stimulate production of antimicrobial compounds including glucosinolates and their derivatives (Wu *et al*. 2014). Restriction of pathogen growth via PAMP signalling is referred to as PAMP-triggered immunity (PTI) and is generally synonymous with basal plant defense.

This fungus was long considered as a classic necrotrophic pathogen, where upon the penetration of the host cuticle, pathogenesis immediately follows and acids and hydrolytic enzymes are secreted ahead of the advancing hyphae. However, recent studies suggest that a brief biotrophic phase occurs prior to pathogenesis within the apoplastic space and therefore, it may be more accurate to describe *S. sclerotiorum* as a hemi-biotroph (Kabbage *et al*. 2015). The main pathogenicity factor of *S. sclerotiorum* is oxalic acid (OA). This dicarboxylic acid serves three main purposes. First, *S. sclerotiorum* creates an acidic environment to promote optimal working conditions for secreted cell wall degrading enzymes. Second, by chelating calcium ions and third, causing cell death through the induction of host reactive oxygen species (ROS) production ahead of the advancing hyphae (Liang *et al*. 2015). OA facilitates fungal establishment by reducing the effect of the host respiratory burst response (Kabbage *et al*. 2013). OA acidifies host tissues to a pH of 4, which is the optimal pH for the activity of secreted fungal enzymes and also depletes calcium ions from within the pectin of plant cell walls while also inhibiting the deposition of callose. Therefore, host cells are highly compromised and facilitate access to digestive enzymes for nutrient acquisition and allow hyphae to penetrate intercellularly (Uloth *et al*. 2015b, Williams *et al*. 2011). The production of numerous classes of hydrolytic enzymes such as polygalacturonases, proteases, cellulases, and glucoamylases facilitates host cuticle penetration, tissue degradation and lesion expansion. Several other pathogenicity factors such as secreted integrin like protein (*SSITL*), chorismate mutase (*SSCM1*), necrosis and ethylene inducing peptides (*SSNEP1, SSNEP2*) and cutinase function in the suppression of host defense and the induction of necrosis (Seifbarghi *et al*. 2017). This multitude of pathogenicity factors, facilitates the success and aggressiveness of *S. sclerotiorum* infection.

S. sclerotiorum does not exhibit a gene-for-gene response during interactions with the host unlike other pathogens such as *Leptosphaeria maculans*. Therefore, a plant cannot simply develop resistance by altering a single gene product. Quantitative resistance is required for *S. sclerotiorum*, which means that many proteins would be required to mutate, each providing small contributions that cumulatively confer a more effective host defence (Corwin and Kliebenstein, 2017). Tolerant cultivars that exhibit reductions in disease severity exist, but true complete resistance has yet to be attained (Disi *et al*., 2014, Garg *et al*., 2010). Cultivars with increased cell wall deposition and lignification show improved tolerance against the pathogen, however, yield and oil quality were negatively affected (Bradley *et al*., 2006, Derbyshire and Denton-Giles, 2016). A potential alternative to breeding for *S. sclerotiorum*-resistant plants is the development of genetic engineering strategies by expressing or silencing genes involved in plant–pathogen interactions. The main strategy used so far consists of the expression of genes encoding enzymes capable of degrading oxalic acid or overexpression of defense pathways such as *NPR1* or phenylpropanoids (Dias *et al*. 2006, Cunha *et al*. 2010, Wang *et al*. 2020, Ranjan *et al*. 2019). One drawback in the development of tolerant cultivars is the requirement for lengthy germplasm screening protocols (Taylor *et al*. 2015).

Crop rotations and chemical field treatments are the other traditional management strategies of *S. sclerotiorum* by producers. However, remaining sclerotia in the soil or ascospores dispersed by the wind may cause 3-4 year crop cycle rotations to fail. Sclerotia can persist for up to 10 years, remaining viable through periods of anoxia and temperatures above 107°C (Hind-Lanoiselet *et al*., 2005, Wu *et al*. 2008). The broad host range of *S. sclerotiorum* also includes many common weed species, so when non-host crops are grown, the pathogen can still propagate (Derbyshire and Denton-Giles, 2016, Kamal *et al*. 2016). Additionally, to save costs, diluted doses used by some producers have led to the rise of fungicide resistant strains (Amaradasa and Everhart, 2016, Penaud and Walker, 2015). Fungicides primarily exhibit single site activity, further promoting the development of resistance. Fungicides target processes such as mitochondrial chromosomes, demethylase inhibitors and sterol synthesis (Derbyshire and Denton-Giles, 2016). Resistance to different fungicidal classes were reported in China and France, attributed to heavy single target fungicide use (Wang *et al*. 2014, Sierotzki, 2015). In addition, concerns are growing regarding the broad-spectrum, biocidal nature of fungicides The fungicide vinclozolin, for example, was

recently banned due to concerns of threats to human health (Anway *et al*. 2012), while strobilurin fungicides have been observed to adversely affect non-targeted soil microorganisms and aquatic animals if runoff occurs after spraying (Liu *et al*. 2013). Ultimately, investigation and development of alternative measures and technologies to these chemicals in order to reduce their load and damage upon the agroecological environment is warranted.

The control of phytopathogenic fungi relies heavily on broad spectrum chemistries. Commonly used fungicidal classes include mitosis disruptors (methyl benzimidazole carbamates), cell membrane disruptors (triazoles), and respiration inhibitors (strobilurins) (Lv *et al*. 2017, Vielba-Fernandez *et al*. 2019, Feng *et al*. 2020). Resistance and unintentional off-target effects present a problem to traditional fungal control technologies. In *Botrytis cinerea* for example, rapid resistance to most fungicidal classes has been observed throughout the globe with several incidences where the fungi could no longer be controlled with available fungal control chemistries (Fernandez-Ortuno *et al*. 2014). While these chemical agents can be effective when applied in lethal doses, they are often unintentionally applied repeatedly at lower rates and this has been shown to facilitate the selection of resistant individuals (Guedes and Walse 2017). With increased tolerance to traditional chemistries, additional and more frequent fungicidal applications may be required to control these pathogens and may lead to the selection of fungicide-resistant strains. This development of resistance is seen in a diversity of agricultural pests including many that are resistant to multiple chemistries (Welch *et al*. 2015, Schoville et al 2018, Bolzan *et al*. 2019, Banazeer *et al*. 2020). Although improvements have been made in terms of environmental toxicity compared to early compounds, there are still concerns for current chemicals relating to environmental dispersal and persistence causing lethal non-target effects because of their broad-spectrum nature (Hakeem *et al*. 2016) Evidence suggests populations of beneficial arthropods such as pollinators and aquatic invertebrates have been harmed by the presence of these traditional chemistries leading to several nations placing legislation against the use of the certain classes of chemicals (Henry *et al.* 2015, Wood and Goulson 2017, Bruus *et al.* 2020). Fungicides have also been shown to adversely affect insect species, especially key pollinators such as bees, aquatic species from chemical runoff into waterways, and beneficial soil microorganisms (Zubrod *et al*. 2011, Mcmahon *et al*. 2012, Pech and Heneberg 2015, Syromyatnikov *et al*. 2017, Heneberg *et al*. 2018, Rico *et al*. 2019, Marinho *et al*. 2020).

Ultimately, broad spectrum chemicals targeting fungi are becoming less effective as resistance to traditional chemistries evolves (Shahid *et al*. 2019). Taken together, an environmentally safe alternative that poses less a risk to the agroecological environment may provide a solution to improve crop health moving forward.

One alternative to chemical pesticides with the potential for species-specificity is RNA interference (RNAi). RNAi has been observed in a wide range of eukaryotic organisms and has emerged as a powerful tool to study gene function (Premsrirut *et al.* 2011). RNAi mediates RNA destruction following the introduction of dsRNA molecules, thereby reducing the expression of a target gene. The RNAi pathway serves as an intrinsic cellular defense to viral invasion in plants, nematodes, insects and fungi and researchers have found that synthetic dsRNA can be used to target and knockdown specific genes within an organism (Wang *et al.* 2016, Gammon *et al.* 2017, Schuster *et al.* 2019, Neupane *et al.* 2019). In insects, the order Coleoptera is highly sensitive to RNAi (Arakane *et al.* 2005, Suzuki *et al.* 2008) however the Diptera (Lum *et al.* 2003, Dietzl *et al.* 2007), Hemiptera (Schluns *et al.* 2007, Marco-Antonio *et al.* 2008), and Lepidoptera (Yu *et al.* 2008, Tian *et al.* 2009, Terenius *et al.* 2011) have shown varying levels of sensitivity. In fungi, most species contain the enzymatic machinery necessary for RNAi and gene knockdown is achievable with a few exceptions including *Saccharomyces cerevisiae* and *Ustilago maydis* (Billmyre *et al.* 2013). Researchers have harnessed and applied this technology to control agricultural pests by designing dsRNAs targeting essential genes and thus are able to disrupt cellular functions and kill the organism (Baum *et al.* 2007, Yu *et al.* 2013, Koch *et al.* 2013, Wang *et al.* 2016, McLoughlin *et al.* 2018). The uptake of dsRNA targeting essential genes by insect and fungal pests can lead to growth inhibition, reduced pathogenicity and mortality. Unlike chemical pesticides, which may affect a broad range of species, RNAi is sequence specific and therefore only target organisms are affected. Seminal studies of RNAi-based pest control employed a strategy known as host induced gene silencing (HIGS), where the host plant is engineered to express the dsRNA molecules for insect or fungal protection (Nowara *et al.* 2010, Koch *et al.* 2013, Mamta Reddy *et al.* 2016). For insects, the dsRNA molecules are absorbed through intestinal uptake following feeding, allowing for systemic spread. Currently, the only commercialized example of HIGS technology is in maize and targets the vacuolar sorting protein Snf7 of the western corn rootworm (*Diabrotica virgifera*).

While HIGS offers the promise of long-lasting protection, RNAi can also be used as a topical formulation to avoid difficulties associated with plant transformation and the regulation of genetically modified organisms in different markets. Spray induced gene silencing (SIGS) involves the foliar applications of dsRNA to the plant suraface. A number of studies in both fungi and insects have demonstrated the effectiveness of this technology (Wang *et al.* 2016, Joga *et al.* 2016, McLouglin *et al.* 2018, Zotti *et al.* 2018) Unlike many chemical pesticides, environmental persistence of dsRNAs appears to be limited, which, from an environmental protection perspective, is an attractive feature (Dubelman *et al.* 2014). Studies have shown a half -life of less than 24 hours for dsRNA within the soil, due primarily to bacterial degradation (Dubelman *et al.* 2014), however, they are stable within the phyllosphere, where they can remain biologically active for several weeks (San Miguel and Scott 2016). Unlike transgenic approaches, spray-based control methods are more appropriate for the control of pests or pathogens that affect multiple crops. For example, SIGS does not require the development and approval of genetically engineered technologies for each crop species and does not limit the technology to single gene or application. Due to the large number of coding genes within organisms, this presents the opportunity to design dsRNA to multiple targets. In several studies, multiple gene targets were shown to be effective when dsRNAs were applied as a foliar treatment, thus providing insurance and allowing for alteration of targets between growing seasons (Mumbanza *et al.* 2013, McLoughlin *et al.* 2018, Hu *et al.* 2020).

This review will explore proposed mechanisms of dsRNA uptake in Eukaryotes as a means to control both insect pests and fungal pathogens through RNAi. SIGS provides considerable promise, both in terms of offering a new generation of pesticides that are environmentally more benign than most current pesticides, and in terms of applying RNA technologies in a delivery method that avoids the challenges surrounding the regulation of genetic modification. Understanding the uptake of dsRNAs through SIGS will help accelerate the development of this technology. While recent studies clearly demonstrate the potential of SIGS as tool to control insect pests and fungal pathogens we still require a deep understanding of target species that are sensitive or those refractory to RNAi. We provide additional strategies to improve dsRNA

uptake through the optimization and development of dsRNA formulations and delivery methods that avoids the challenges surrounding the regulation of genetic modification.

1.3 RNA INTERFERENCE CORE COMPONENTS

Since the first description of sequence-specific gene silencing in the nematode *Caenorhabditis elegans* in 1998 (Fire *et al.* 1998), RNAi has been well documented in almost all eukaryotic organisms, including protozoans, invertebrates, vertebrates, plants, fungi, and algae. Before the term RNAi was widely adopted, RNA silencing had been described as post transcriptional gene silencing in plants and quelling in fungi (Napoli *et al.* 1990, Romano and Macino 1992), but each of these different names refer to a common process, with shared intracellular machinery. The core components of RNAi have now been identified in all major branches of eukaryotes (Matveyev *et al.* 2017).

The protein Dicer is the initiator of the RNAi pathway. It belongs to the RNase III family, an evolutionarily conserved protein group with specificity for dsRNAs. It is responsible for processing long dsRNAs into smaller duplex fragments of discrete sizes (Song and Rossi 2017). While Dicer is found in virtually all eukaryotes, it has diversified structurally as well as functionally, producing several types of small RNA (Murphy *et al.* 2008). Dicers are essential for the biogenesis of both small interfering (si)RNA and micro (mi)RNA, however they play differing regulatory roles (Song and Rossi 2017). Organisms such as *C. elegans*, *Saccharomyces pombe*, humans, and nematodes possess one Dicer gene. In contrast, arthropods and fungi possess two Dicer genes and non-polyploid plants such as *Arabidopsis* have four distinct Dicer-like proteins (Mukherjee *et al.* 2013). The Dicer-generated siRNAs are subsequently co-opted by the RNAi-induced silencing complex (RISC), which unwinds the siRNA duplex. The exact molecular composition of RISC has yet to be defined, but must at least contain an Argonaute protein (Svoboda *et al.* 2020). Argonaute cleaves the passenger (sense) strand of the siRNA while the guide (antisense) strand remains connected with the RISC (Hammond *et al.* 2000). The guide strand of the siRNA within RISC binds to base pairs with complementary target mRNAs, which are then cleaved by Argonaute, thereby preventing translation (Chendrimada *et al.* 2005). Some species also possess RNA-dependent RNA polymerases (RdRPs), which catalyze the

replication of dsRNA from aberrant single stranded-RNA transcripts (Marker *et al.* 2010). RDRPs function to amplify the RNA silencing signal, thus promoting systemic spread.

While many of the core RNAi components are conserved throughout taxa, RNAi efficiency varies across species, and many of the variations are due to differences in the efficiency of dsRNA uptake, intracellular distribution, and/or systemic dispersal. For the remainder of this review, the different modes of uptake and dsRNA movements will be examined, highlighting where there are gaps in our understanding, and how these should be addressed if RNAi technologies are to be deployed more widely.

1.4: MODES OF UPTAKE

1.4.1 *CAENORHABDITIS ELEGANS: SYSTEMIC RNAI DEFECTIVE (SID) PROTEINS*

In addition to the discovery of RNAi, the mechanism of dsRNA uptake and cell to cell spread (systemic RNAi) was also first described in *C. elegans*. Soaking nematodes in dsRNA or feeding them bacteria producing dsRNA induces RNAi both within the intestine and in tissues distal from the site of ingestion. This systemic RNAi in nematodes (Tabara *et al.* 1998) is mediated by multiple SID proteins that facilitate transfer of dsRNAs or siRNAs throughout the body. *SID-1*, a dsRNA specific membrane channel, has been studied extensively (Winston *et al.* 2002, Wang *et al.* 2017, Whangbo *et al.* 2017). *sid-1* mutants are insensitive to RNAi and unable to spread the silencing signal to adjacent cells. However, silencing can still be achieved through cell autonomous RNAi using a direct injection delivery or by transgenic expression (Winston *et al.* 2002).

SID-2 is a single pass transmembrane protein located specifically in the luminal membranes of the intestine and is involved in dsRNA uptake within the gut of *C. elegans*. *SID-2* is thought to mediate uptake through interaction and delivery of the dsRNA to *SID-1* or through endocytosis, with *SID-1* thereafter enabling the dsRNA to escape the endosome and enter the cytoplasm (McEwen *et al.* 2012) . *SID-3*, like *SID-1*, is also required for efficient import of dsRNA (76). *SID-3* is ubiquitously expressed in most tissue types, and is important in the uptake of dsRNA in the recipient cell. In addition to being associated with endocytic vesicles, *SID-3* appears to be an

ortholog of *ACK* tyrosine kinase although its phosphorylation targets and its interaction with *SID-1* have yet to be described (Jose *et al.* 2012).

Another SID protein, *SID-5*, has been shown to be required for extracellular spreading of the RNAi signal (Hinas *et al.* 2012). *SID-5* is predicted to have a single transmembrane domain and interacts with late endosomal proteins such as *RAB7* (Gibbings *et al.* 2010). Since Rab7 GTPases are responsible for the regulation of late endosomal trafficking into lysosomes, Hinas *et al.* (2012) proposed that *SID-5* functions to block exosomal fusion with lysosomes and allows for exosomal release from the cell. The complex roles and interactions of the multiple SID proteins in *C. elegans* still need to be fully resolved, but it is clear that endocytic pathways are integral to the uptake and dispersal of dsRNAs in this species.

1.4.2 INSECT UPTAKE: SID-LIKE PROTEINS

Orthologous proteins to *C. elegans SID-1*, called SID-like (*SIL-A, SIL-B* and *SIL-C*), have been identified in several insect species, although their direct involvement in dsRNA uptake has yet to be determined in many cases (Velez and Fishilevich 2018). In cotton and soybean aphids (*Aphis gossypii, Aphis glycines*), for example, the SIL proteins are structurally similar to *C. elegans SID-1*, (Xu *et al.* 2008, Bansal *et al.* 2013), but their role in mediating dsRNA uptake has not been confirmed. In the honey bee (*Apis mellifera*), expression of a SIL gene increased following exposure to dsRNA, suggesting a role in mediating RNAi, but again, the role of the encoded protein in dsRNA uptake was not determined (Aronstein *et al.* 2006). Reduced RNAi efficiencies were observed in the Western corn rootworm *(Diabrotica virgifera)* and Colorado potato beetle (*Letinotarsa decemlineata)* following knockdown of the SIL mRNAs, indicating at least a partial role of SIL proteins in modulating RNAi in these two beetles (Miyata *et al.* 2014, Cappelle *et al.* 2016). In contrast, the flour beetle *Tribolium castaneum* has three orthologs of *SID-1*, but when all three were silenced, there was no effect on RNAi efficiency (Tomoyasu *et al.* 2008). Similarly, the SIL proteins in the desert locust (*Schistocerca gregaria)* or diamondback moth (*Plutella xylostella*) appear to play no role in RNA efficiency (Luo *et al.* 2013, Wang *et al.* 2014). In dipteran insects, no SIL orthologues have been found, and yet RNAi has been demonstrated in many flies and mosquitoes (Goto *et al.* 2003, Petruk *et al.* 2006, Boisson *et al.* 2006), indicating that SIL proteins are likewise not required for dsRNA uptake in these insects.

Based on the range of insects studied thus far, the role of SIL proteins in dsRNA uptake in these organisms is clearly variable, with SIL facilitating RNAi in some species, but not in others.

Reverse BLAST searches between insect SIL proteins and the *C. elegans* proteome suggest that SIL proteins more closely resemble *TAG-I30/CHUP-1* proteins than *SID-1* (Tomoyasu *et al.* 2008). *CHUP-1* genes have no apparent role in uptake or systemic RNAi within the nematode, and when *C. elegans CHUP-1* genes were transfected into Drosophila S2 cells, no dsRNA uptake was observed (Whangbo *et al.* 2017). *CHUP-1* has a role in the cellular uptake of cholesterol (Valdes *et al.* 2012), and there is some speculation that a depletion of cholesterol levels may perturb or disrupt clathrin-mediated endocytosis and vesicle transport, possibly affecting the amount of dsRNA that enters the cell (Kim *et al.* 2017). Moreover, the efficacy of dsRNA uptake may be influenced by the fatty acid composition of the cellular membrane. The ratio of the poly-unsaturated fatty acids, linoleic acid and arachidonic acid in the membrane was shown to be important, and the injection of arachidonic acid improved the RNAi response in *Bactrocera dorsalis* (Dong *et al.* 2017).

1.4.3 INSECT UPTAKE: ENDOCYTOSIS

Clathrin-mediated endocytosis has been demonstrated to be the primary mode of uptake in several insect species. The involvement of endocytosis was first described in S2 Drosophila cells, which lack any SID-like proteins. through experiments providing both direct and indirect evidence of this process (Saleh *et al.* 2006, Ulvila *et al.* 2006). Accumulation of fluorescently labelled dsRNA within distinct cytoplasmic vesicles of S2 cells provided direct evidence of uptake, and pretreatment of cells with inhibitors of endocytic processes such as chlorpromazine and bafilomycin A1 indicated that clathrin-mediated endocytosis was facilitating the process (Saleh *et al.* 2006, Ulvila *et al.* 2006). RNAi-mediated knockdown of genes encoding proteins involved in clathrin dependent endocytosis, resulted in impaired RNAi of a secondary reporter gene, confirming that uptake of dsRNA was occurring by this process (Saleh *et al.* 2006, Ulvila *et al.* 2006). The proteins identified in the RNAi knockdown screens encompassed the entire endocytic pathway, from early vesicle formation (clathrin, *AP50*) to late endosomal release (*RAB7* and VH^{+} *ATPase*), indicating that the dsRNA was traversing through the endosomal pathway, only to be released to the cytoplasm before being degraded within the lysosomes.

Knockdown of these same endocytic components in *C. elegans* also impaired subsequent RNAi, providing additional evidence that endocytosis is an important component of the dsRNA uptake process even in species that rely on SID proteins to support uptake (Saleh *et al.* 2006). Clathrin-mediated endocytosis has been demonstrated to be involved in uptake of exogenous dsRNA in several more insect species, using labeled dsRNA to track cellular entry. In *T. castaneum,* fluorescent labeling and endocytic inhibitors provided direct evidence of clathrin mediated endocytosis in uptake (Xiao *et al.* 2015). In *B. dorsalis* and *D. virgifera* clathrin related genes were identified through an RNAi mediated knockdown approach (Figure 1) (Li *et al.* 2015, Pinheiro *et al.* 2018). Interestingly, in some species such as *L. decemlineata*, both SIL channels and endocytosis appear to be involved (Figure 1.1) (Cappelle *et al.* 2016). Vacuolar H^+ ATPase and the clathrin heavy chain were identified through RNAi -mediated knockdown experiments, in addition to *SIL-A* and *SIL-C*, however, the relative contribution of each uptake mechanism remains uncertain. The endocytic process encompasses dsRNA binding to a receptor, inducing the invagination of the membrane. Clathrin and its adaptors are recruited and a vesicle forms and releases from the membrane. The endosomal vesicle matures and via pH shifts from proton pumps the dsRNA is released into the cytoplasm. It is still unknown when the dsRNA is released and how it is moved throughout the cells. It is interesting to note that in lepidopteran cells, which are generally more recalcitrant to RNAi, the dsRNA can enter the cells but remains trapped in the endosomes (Yoon *et al.* 2016). It is unclear what factors are preventing the dsRNA escape from the endosomal pathway, but for these species, efforts to improve RNAi efficiency have focused on alternative delivery molecules to help the dsRNA reach the cytoplasm (see dsRNA Formulation section).

Two Drosophila scavenger receptors, *SR-CI* and Eater were also identified in Ulvila's (2006) screen and were found to account for over 90% of dsRNA uptake in Drosophila S2 cells. These receptors are involved in receptor mediated phagocytosis of gram negative and positive bacteria in Drosophila. In the desert locust (*Schistocerca gregaria*) *SR-CI* and Eater were also identified to be important to uptake through chemical inhibition of the receptor (Kim *et al.* 2017). However, only a weak response of knocking down another scavenger receptor, *SC-R2*, was observed in Colorado potato beetle cell lines (Yoon *et al.* 2016), and in the *Aedes aegypti* mosquito cells, where clathrin-mediated endocytosis facilitated uptake, chemical inhibition of

scavenger receptors had no impact on dsRNA uptake (Abbasi *et al.* 2020). Based on these findings, it seems likely that dsRNAs can bind to different, and perhaps multiple receptors in each species. Determining the identity, structure, and selectivity of these receptors will prove valuable in the design of dsRNAs with improved binding affinities and uptake capabilities.

1.4.4 FUNGAL UPTAKE

Without orthologs to SID proteins, fungi also appear to rely on endocytosis to facilitate uptake of dsRNA. Uptake of fluorescently-labelled dsRNAs in fungi was first reported in *Botrytis cinerea* by Wang *et al.* (2016), although the uptake mechanism was not identified. A study by Wytinck *et al.* (2020) demonstrated that uptake of dsRNA in *Sclerotinia sclerotiorium* occurs through clathrin-mediated endocytosis, analogous to insect systems that do not rely on SIL channels (Figure 1.1). While a dsRNA specific receptor was not identified in the study, endocytic proteins *CHC, Arf72A, AP2, FCHO1*, amphiphysin and VH^{+} *ATPase* were shown to be involved in dsRNA uptake and processing through RNAi mediated knockdown experiments. They demonstrated that uptake is localized to the hyphal tip in younger, more actively growing hyphae through live cell imaging. Endocytosis has also been shown to localize at the hyphal tip of the developing fungus (Commer and Shaw 2020).

Fungi lack homologues to the insect scavenger receptors, and therefore genome wide screens may be necessary in order to identify potential candidate dsRNA receptors. The list of candidates could then be narrowed to those that specifically bind dsRNA through affinity capture techniques and then role in uptake could be assessed through RNAi mediated knockdown. Within the fungal kingdom, most fungi assessed are ammenable to RNAi, however a couple such as *U. maydis* and *S. cerevisiae* lack the core RNAi components such as Dicer or Argonaute (Drinnenberg *et al.* 2011). One notable exception is *Zymoseptoria tritici* which encodes the core components however is still insensitive to dsRNA (Kettles *et al.* 2019). Through live cell imaging, the authors showed that conidospores of *Z. tritici* were unable to uptake dsRNA, suggesting that there may be not be a dsRNA receptor encoded or a there is a defect in the uptake pathway. Research into uptake mechanisms fungi that have shown to be sensitive to RNAi is needed to gain a more complete understanding of the mode(s) of uptake. Overall, there is a dearth of information

relating to uptake in fungi and this may be a result of fewer studies demonstrating the efficacy of SIGS against phytopathogenic fungi.

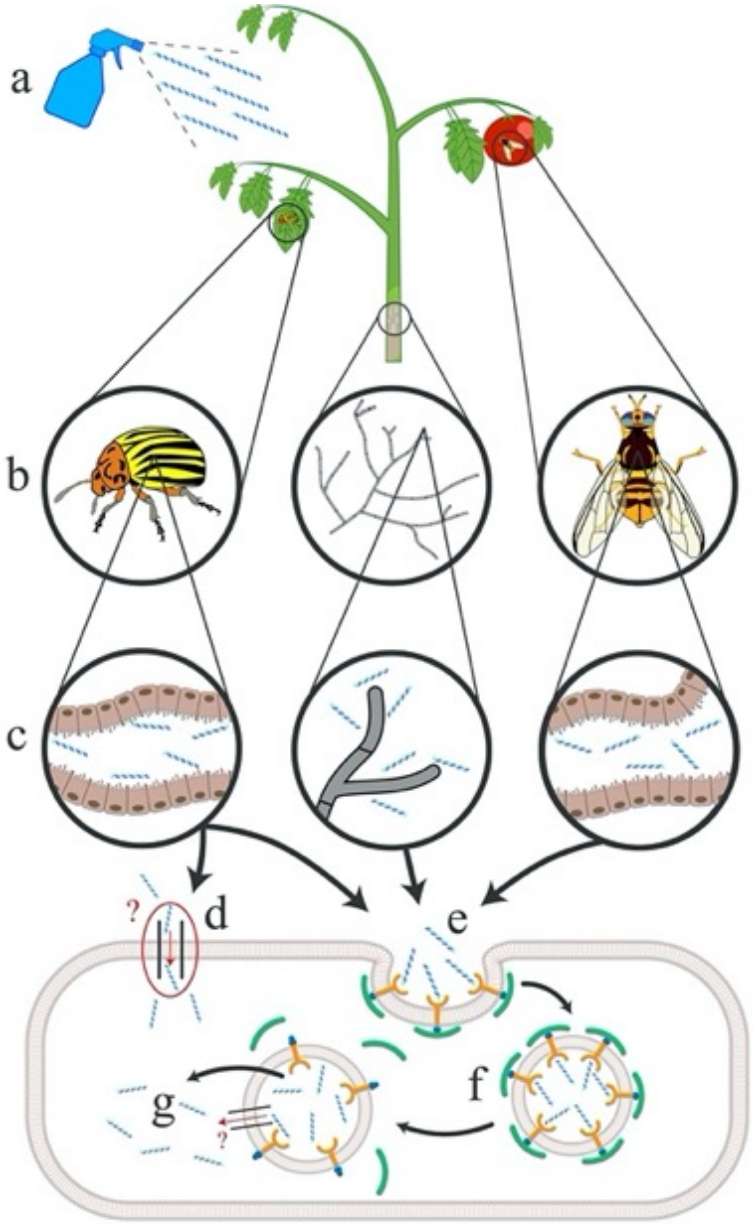

Figure 1.1 Overview of described modes of dsRNA uptake within insect pests and fungal pathogens using *L. decemlineata, S. sclerotiorum and B. dorsalis* as representatives. (a) Exogenous dsRNA can be applied to host plants through spray applications, and (b) the dsRNA can be transferred to the pest or pathogen upon (c) ingestion of plant material by the gut of the insect or absorption by fungi hyphal tips. DsRNA can be internalized by SIL proteins (d) in *L. decemlineata* or through clathrin mediated endocytosis (e) in *L. decemlineata, S. sclerotiorum and B. dorsalis*. Following endosomal release and maturation (f), dsRNA is released into the cytoplasm from the endosome (g), potentially facilitated by SIL proteins in *L. decemlineata.*

1.5: IMPORTANCE OF UNDERSTANDING DSRNA UPTAKE

1.5.1 RESISTANCE

Historically speaking, following the deployment of a novel control measure, strains resistant to the technology eventually emerge (Hawkins *et al.* 2019). Given that RNAi is a sequence-specific process, changes in a target gene's sequence could potentially render the pest or pathogen resistant, but this type of resistance can be easily and quickly overcome by changing gene targets or sequences. A more problematic resistance to overcome would be a change in the uptake mechanism. While it seems unlikely that an organism would tolerate substantive changes to essential proteins such as clathrin, it is possible that dsRNA-specific receptors or channels could be modified to no longer function in promoting uptake (Zotti and Smagghe 2015). Reduced dsRNA uptake resistance has already been demonstrated in a laboratory strain of corn rootworm (*D. virgifera*) that was exposed to progressively higher doses of dsRNA through several generations (Khajuria *et al.* 2018). Within eleven generations, insects had greater than a 130-fold increase in resistance. The nature of the resistance was attributed to significantly reduced gut luminal uptake, and the molecular mode behind the mutations was linked to a single recessive locus. The mechanism of which these mutations altered uptake is unknown. Although this resistant strain was created in a laboratory, it highlights that resistance through reduced uptake can indeed occur. Researchers are attempting to minimize this risk by investigating multiple modes in which dsRNA can be delivered to the cells by different vehicles or carriers. If different modes of uptake can be achieved that are not reliant on a single dsRNA specific receptor, then the risk of RNAi insensitivity will be reduced. While much remains to be understood about many of these different carriers, they show considerable promise in using dsRNA-based control methods for wide range of pests and pathogens.

1.5.2 DSRNA FORMULATIONS

SIGS using naked dsRNA has been shown to be an effective control method against both insect pests and fungal pathogens (Killiny *et al.* 2014, Wang *et al.* 2016, San Miguel and Scott 2016). Despite these advances, topically applied dsRNA molecules are vulnerable to degradation. Environmental factors such as water, sunlight/UV light, and interactions with soil microbes all contribute to a reduced availability of dsRNA on plant surfaces and possible absorption into plant tissues (Li *et al.* 2015, Fischer *et al.* 2016, Parker *et al.* 2019). Upon ingestion by an insect,

dsRNA also must contend with dsRNA-degrading enzymes and an unfavorable pH level within the gut (Song *et al.* 2019). To solve these issues, carrier molecules can be utilized to protect the dsRNA, increasing bioavailability and improving control of pathogens and pests. For most carrier particles, a primary goal is to increase stability and persistence of the dsRNA before and after ingestion, to protect the dsRNA from abiotic environmental factors and biotic factors within the insect gut (Gillet *et al.* 2017, Cagliari *et al.* 2019). While the dsRNA carriers are often providing some degree of protection from the gut environment of insects, it is important to understand how these carriers interact at the cellular level of uptake, and whether they also play a role in translocating dsRNA into cellular compartments where they can take effect.

While most investigations of dsRNA carriers for plant protection have focused on insects, the enhanced stability and penetrability of some formulations will also be applicable to phytopathogenic fungi. While the barriers of gut pH and nucleases are not an issue for fungi, stability from environmental conditions is still critical. It is difficult to predict when a fungal outbreak is going to occur, and therefore the longer the preventative antifungal treatment can remain intact on the plant surface, the more likely it will be effective when the infection emerges. Additionally, several necrotrophic pathogens, such as *S. sclerotiorum*, can become systemic within the plant within a matter of days (Girard *et al.* 2017). This underlies the importance of getting the maximal load of dsRNA into the fungus as quickly as possible, and carriers enhancing penetrability have the potential to do this.

1.5.3 NANOPARTICLES

When dsRNA is ingested by an insect, degradation by gut nucleases continues until absorption into the cells occurs. The RNAi effect diminishes the longer the naked dsRNA is exposed to this harsh environment (Joga *et al.* 2016). One of the most commonly used polymers to generate nanoparticles to protect and deliver dsRNA and siRNA to target cells is chitosan (Lichtenberg *et al.* 2020). Chitosan is a biodegradable and nontoxic polymer, prepared through the deacetylation of the highly abundant biopolymer chitin (Dass and Choong 2008). Electrostatic binding between the dsRNA and the chitosan occurs through the negative charges on the phosphate backbone of the dsRNA binding to the positively charged amino group of the chitosan (Zhang *et al.* 2010). Chitosan-based formulations have been shown to improve stability from

endonucleases and uptake in a number of insect species including *Anopheles gambiae, Aedes aegypti, Chilo suppressalis,* and *S. frugipera* (Zhang *et al.* 2010, Ramesh-Kumar *et al.* 2016, Wang *et al.* 2020, Gurusamy *et al.* 2020). Interestingly, chitosan appears to improve uptake in lepidopteran insects such as *S. frugipera,* which are normally fairly inefficient in terms of RNAi response (Gurusamy *et al.* 2020). Naked formulations of dsRNA appear to get trapped in endosomes and are unable to induce silencing, however dsRNA complexed with chitosan showed reduced accumulation within these endosomes. While it is unknown if the dsRNA-chitosan conjugate enters through endocytic mechanisms similar to naked dsRNA or through alternative mechanisms, it is highly encouraging that this formulation can improve efficiency in species that were previously insensitive to RNAi.

Layered double hydroxide clay nanosheets are another nanoparticle delivery system that shows promise in increasing RNAi efficiency in pest control. Stacks of positively charged nanosheets are able to electrostatically bind the negative charges of the dsRNA and provide improved protection against environmental elements and nuclease activity. Mitter *et al.* (2017) showed that atmospheric conditions slowly break down the clay nanosheets on the plant surface releasing the dsRNA. In their study, the clay nanosheet provided protection against pepper mild mottle virus up to 20 days post spraying, providing a much longer window of protection compared to the naked dsRNA treatment. This technology also holds potential to be of utility in insect and fungal protection due to this increased length of bioactivity. Interestingly, this formulation also appears to encourage uptake and systemic spread within the host plant that was sprayed. Mitter *et al.* (2017) were able to detect dsRNA in distal, unsprayed regions of the plant indicating that whole plant protection can be achieved after spraying only a portion of the plant.

1.5.4 RIBONUCLEOTIDE PROTEIN DSRNA CARRIERS

The plasma membrane is the primary barrier of uptake, and dsRNA's negative charge prevents passive transport through the negatively charged membrane. To remedy this, cell penetrating peptides (CPPs) can facilitate uptake into the epithelial gut cells and provide protection from nucleases. CPPs are a class of peptides able to cross cellular membranes and can function as a carrier for siRNAs, proteins, additional peptides, and other small molecules (Milletti 2012). More specifically, the cationic, arginine-rich Tat peptides have been shown to successfully

internalize plasmid DNA and hormones within insect cells (Chen *et al.* 2012, Zhou *et al.* 2015). An improved version of this Tat peptide is the peptide transduction domain (PTD), which unloads the carrier's cargo by destabilizing the vesicle membrane following endocytosis (Erazo-Oliveras *et al.* 2012). By pairing the PTD with a dsRNA binding domain (DRBD), ribonucleoprotein particles (RNP) can be formed to carry dsRNA past the plasma membrane, escape the endosome, and induce silencing. Gillet *et al.* (2017) demonstrated that RNPs can improve oral delivery of dsRNA and enhance RNAi effects.

PTD-eGFP applied to a cotton boll weevil (*Anthonomus grandis*) gut cell suspension showed PTD-eGFP clustered at the membrane of gut cells within 2 minutes and showed colocalization with FM4-64 endosomal stain within vesicles. When *A. grandis* midgut was incubated with Cy3-labeled dsRNA complexed with PTD-DRBD, the particles were shown to associate with the plasma membrane, within endovesicles, and ultimately into the cytoplasm. Reduced expression of a chitin synthase gene was improved with oral delivery of the RNPs compared to naked dsRNA, demonstrating the utility of CPPs in delivering dsRNAs to the cytoplasm of insect cells. The PTD peptide used in this study was optimized to carry short dsRNAs or siRNAs (Eguchi *et al.* 2009, Geoghegan *et al.* 2012), and may need to be modified to ensure effective delivery of long dsRNAs typically used in insect RNAi applications. With the research being conducted to overcome negatively charged membranes using cell-penetrating peptides, there may be discoveries that could transfer to fungal-based systems since CPPs have been shown to be effective nanocarriers of other antifungal compounds in fungal systems (Jain *et al.* 2015, Vedadghavami *et al.* 2020).

1.5.5 CATIONIC LIPOSOMES DSRNA CARRIERS

In pharmacological studies where efficient drug delivery is highly dependent on absorption into cells, lipid carrier molecules have been observed to facilitate gene delivery. Cationic liposomes consist of cationic and neutrally charged lipids that encapsulate nucleic acid to form lipoplexes (Simoes *et al.* 2005). The positively charged outer lipid coating allows association with the plasma membrane and isolates the negative charges to inside the liposome. One of the first instances of using a liposome-based delivery method was to encapsulate an antiviral, immunomodulating dsRNA to protect against influenza in mice (Wong *et al.* 1999). Since then,

this technology has been applied in insects, fungi, nematodes, and crustacean viruses (Sanitt *et al.* 2016, Lin *et al.* 2017, Nami *et al.* 2017, Adams *et al.* 2019) with successes in lowering gene expression and/or mortality.

In the limited number of studies conducted so far, interactions between insect gut epithelial cells and lipoplexes have yet to be understood. However, insights from recent papers in other cell types may provide a better understanding of the role lipoplexes have in associating with the plasma membrane and subsequent release into the cytosol. Sarker *et al.* (2020) reported that cationic liposomes containing a fluorescently marked phallotoxin were indeed associated with the plasma membrane of HeLa cancer cells and were translocated via an endocytic pathway. Interestingly the phallotoxin was not released within 30 minutes of incubation but was released into the periphery of the cell by 24 hours. To confirm liposomes were entering via endocytosis, HeLA cells were treated with chemical inhibitors of clathrin-mediated endocytosis (chlorpromazine), caveolae-mediated endocytosis (nystatin), and micropinocytosis (cytochalasin D). Fluorescent confocal imaging appeared to show caveolae-mediated endocytosis is a primary pathway for lipoplexes, but the other two endocytic pathways also had some role in uptake. A similar study (Norris *et al.* 2020) in mosquitoes (*A. aegypti*) found caveolae-mediated endocytosis is essential for nanoparticle (anhydride) internalization into epithelial cells. This is especially true for smaller particles (<100 nm) but could also allow larger particles such as lipoplexes which can easily exceed that size. Pharmacological studies have also confirmed that dsRNA lipoplexes are an effective mode of treatment against the human fungal pathogen *Aspergillus flavus,* however no mode of uptake was described (Nami *et al.* 2017). Chavan *et al.* (2012) showed that liposomes have an affinity for the *β*-glucan in *Aspergillus* cell walls, which is primarily exposed at the septa of the hyphae during growth and division of the cells. Given that lipoplexes appear to deliver dsRNA by a mechanism other than clathrin-mediated endocytosis, they could potentially prove effective in administering dsRNAs to RNAi insensitive organisms such as the lepidopterans, as well as strains resistant to clathrin-mediated uptake.

1.6 CONCLUDING REMARKS

RNAi technologies hold the potential to generate a novel class of pesticides to provide growers with additional tools to overcome unpredictable changes and guidelines to traditional

chemistries. While modes of uptake have been described in many insect species, there remains much to be discovered. Relatively little is known about uptake in various fungal species and dsRNA formulations add another level of complexity to this. How different formulations and delivery methods interact with these various forms of uptake in the pest or pathogen as well as how the host plant is affected are questions that need further investigation. As with any crop protection strategy, resistance is always a concern. Uptake mechanisms will shine light on potential areas where resistance may develop. Ultimately, the answers to these questions will be crucial for the implementation of RNAi in agriculture. The development of successful dsRNA formulations that protect, facilitate delivery, and discourage resistance buildup will guarantee RNAi as a next generation crop protection tool.

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RESEARCH OBJECTIVES

My research aims to explore the *B. napus – S. sclerotiorum* pathosystem and to develop novel methods of control using our gained knowledge of the biology of this interaction. To accomplish this, the research will be divided into four main biological questions:

Question: What is the uptake mechanism of dsRNA within *S. sclerotiorum?* (This question is addressed in Chapter 2)

Hypothesis: Clathrin mediated endocytosis is involved in dsRNA uptake.

Through the alteration of secondary structure of the dsRNA molecules, as well as the addition of chemical adjuvants to promote uptake I hypothesize that we can improve the efficiency of the dsRNA uptake process enabling the cell to acquire enough dsRNA to disrupt essential, biological processes. However, it is first imperative to gain a better understanding of the molecular biology underpinning the mechanism by which dsRNA enters the fungal cell.

Question: Can we improve *S. sclerotiorum* protection in *B. napus* through the creation of RNAi-based transgenic plants*?* (This question is addressed in Chapter 3)

Hypothesis: Canola plants expressing dsRNA hairpins will tolerate *S. sclerotiorum* infection more effectively than susceptible lines.

To analyze RNAi applications through a host induced gene silencing approach, we will transform canola with RNAi silencing constructs. Genetic engineering of *B. napus* offers durable protection throughout the crop's lifecycle, as compared to a dsRNA foliar spray. Transformed explants generated using tissue culture methods are induced to form shoots, roots and leaves before grown to seed. Plant protection can be assessed upon the ability to overcome *S. sclerotiorum* infection through leaf, stem and whole plant inoculation assays.

Question: How do plant overexpressing a dsRNA construct respond to *S. sclerotiorum* at the transcriptome level? (This question is addressed in Chapter 3)

Hypothesis: *B. napus* overexpressing the RNAi constructs will activate genes associated with defense.

The lack of transcriptome data on fungal response to host induced gene silencing and dsRNA molecules could offer an opportunity to explore the response of the pathogen to this RNAi

technology. Additionally, differences in the response of the transgenic plant compared to that of the wild-type cultivar will offer important insights into how a higher level of tolerance is achieved at a transcriptomic level. Dual RNA sequencing from tissue collected from the site of infection in both the wild-type and transgenic lines provides the opportunity to study gene expression in both the host plant and fungal pathogen simultaneously. Functional characterization of this interaction provides evidence into the anatomical and genetic mechanisms underlying plant protection.

Question: How does the small RNA landscape in *B. napus* and *S. sclerotiorum* change during infection in both susceptible *B. napus* and cultivars overexpressing an RNAi construct? (I begin to address this question in Appendix 1)

Hypothesis: There will be large changes in small RNAs populations produced before and during infection.

Changes in the small RNA landscape play an important role in plant-pathogen interactions and studying how they regulate gene expression provides insight into novel strategies for pathogen management. Cross-kingdom RNAi trafficking involves plants sending small RNA molecules to target pathogenicity genes of invading fungi and fungal species transferring small RNA signals to the plant to weaken host defense. Identification the targets of bidirectional small RNA trafficking may prove invaluable to further development of RNAi targets. Small RNA sequencing performed on tissue collected from the site of infection from susceptible and RNAi lines allows us to identify molecules produced both by the plant and fungus. Potential targets of these small RNA molecules can then be predicted within the *B. napus* and *S. sclerotiorum* genomes.

CHAPTER 2: CLATHRIN MEDIATED ENDOCYTOSIS IS INVOLVED IN THE UPTAKE OF EXOGENOUS DOUBLE-STRANDED RNA IN THE WHITE MOLD PHYTOPATHOGEN *SCLEROTINIA SCLEROTIORUM*

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2.1 ABSTRACT

RNA interference (RNAi) technologies have recently been developed to control a growing number of agronomically significant fungal phytopathogens, including the white mold pathogen, *Sclerotinia sclerotiorum*. Exposure of this fungus to exogenous double-stranded RNA (dsRNA) results in potent RNAi-mediated knockdown of target genes' transcripts, but it is unclear how the dsRNA can enter the fungal cells. In nematodes, specialized dsRNA transport proteins such as *SID-1* facilitate dsRNA uptake, but for many other eukaryotes in which the dsRNA uptake mechanisms have been examined, endocytosis appears to mediate the uptake process. In this study, using live cell imaging, transgenic fungal cultures and endocytic inhibitors, we determined that the uptake mechanism in *S. sclerotiorum* occurs through clathrin-mediated endocytosis. RNAi-based knockdown of several clathrin-mediated endocytic genes' transcripts confirmed the involvement of this cellular uptake process in facilitating RNAi in this fungus. Understanding the mode of dsRNA entry into the fungus will prove useful in designing and optimizing future

dsRNA-based control methods and in anticipating possible mechanisms by which phytopathogens may develop resistance to this novel category of fungicides.

2.2 INTRODUCTION

Fungal phytopathogens cause billions of dollars of crop losses each year, despite our efforts to control them using a spectrum of chemical fungicides (Dietzel *et al*. 2019). With continued use of these chemicals, there are increasing concerns of their negative impacts on off-target species. The fungicide vinclozolin, for example, was recently banned due to concerns of threats to human health (Anway *et al*. 2012), while strobilurin fungicides have been observed to adversely affect non-targeted soil microorganisms and aquatic animals if runoff occurs after spraying (Liu *et al*. 2013). The frequent use of various fungicides has also resulted in the development of resistant fungi for multiple classes of these chemicals (Wang *et al*. 2014, Sierotzki 2015, Derbyshire and Denton-Gilles 2016). New, more species-specific fungal control strategies are clearly needed. RNA interference (RNAi)-based technologies are emerging as viable and promising alternative control strategies for a growing number of fungal phytopathogens. Due to RNAi's sequence specificity, double-stranded RNA-based fungicides could provide species-limited control of pathogenic fungi without adversely affecting non-target organisms (Sang and Kim 2020).

RNAi-based fungal pathogen protection in plants can be achieved through a transgenic approach, where the host plant produces hairpin (hp)RNAs that can induce transcript knockdown in an invading pathogen. This so-called host-induced gene silencing (HIGS) has been observed to provide effective protection in tobacco (Andrande *et al*. 2016, Mamta Reddy *et al*. 2016), cotton (Zhang *et al*. 2016), banana (Ghag *et al*. 2014), barley and wheat (Koch *et al*. 2013, Panwar *et al*. 2013). While HIGS can provide a consistent level of resistance for these plants, the requirement for the development of transformation protocols for each plant species, as well as the public's perception of genetically modified organisms may limit its application in many commercial crops (Wang *et al*. 2018).

A more versatile dsRNA delivery system that could be applied to any plant is spray-induced gene silencing (SIGS), where dsRNAs are applied topically. The application of SIGS was first demonstrated as a viable method of fungal control for the cereal pathogen *Fusarium*

graminearum (Koch *et al*. 2016). In that study, SIGS was used to deliver to barley plants a dsRNA molecule that simultaneously targeted three *Fusarium* cytochrome P450 lanosterol C-14-α demethylase genes' transcripts . This dsRNA was effective at knocking down the three mRNA targets and significantly reduced pathogen growth on the host plant. Interestingly, the topically-applied dsRNA appeared to spread over and through the barley leaves, the molecules were detected within the plant's vasculature and as such, *Fusarium*'s conidial germination was also inhibited on unsprayed distal portions of the leaves. SIGS was also found to be effective in the control of *Botrytis cinerea,* a necrotrophic fungus that is particularly destructive to horticultural crops. Wang *et al*. (2016) designed dsRNA molecules to target the Dicer-like (*DCL) 1/2* transcripts in this fungus, which reduced production of small RNAs that inhibit host plant defense genes (Weiberg *et al*. 2013). By interfering with the fungus' ability to produce these pathogenicity factors, Wang and colleagues demonstrated reduced infection of a wide range of hosts including strawberries, grapes, lettuce and tomatoes.

With these initial studies demonstrating the potential of this technology to control fungal pathogens, increased efforts to identify novel gene targets in other phytopathogen species followed. McLoughlin *et al*. (2018) developed an RNAi target identification pipeline using the *Brassica napus-Sclerotinia sclerotiorum* pathosystem as a template. A suite of novel gene targets effective at limiting the pathogenicity of this aggressive necrotroph was established through the interrogation of transcriptomic data and functional bioassays, which screened putative dsRNA targets using mature leaf infection assays with *Sclerotinia*-inoculated petals. The applicability of this target identification system in other species was further demonstrated by effectively targeting homologous genes' transcripts in *B. cinerea*. In each of these fungal species for which SIGS-induced RNAi has been observed, the dsRNA evidently entered the fungal cells to initiate the RNAi-mediated knockdown of target transcripts. How the dsRNA enters the fungal cells was unknown, but understanding the mechanism(s) could prove invaluable in future efforts to improve dsRNA uptake for enhanced RNAi efficacy, as well as to identify any possible barriers to dsRNA uptake for applications in a broader range of fungi (Wang *et al*. 2018). For example, in different insect orders, there is a gradient in the efficiency of RNAi responses, with coleopterans (beetles) generally showing high susceptibility and lepidopterans (moths) being much more refractory to dsRNA. In the lepidopterans, reduced sensitivity was attributed to both nuclease-

mediated degradation of dsRNA as well as entrapment of dsRNA within the cells' endosomes, resulting in failure to enter the cytoplasm to reach the mRNA targets (Shukla *et al*. 2016). Failure of cells to take up dsRNA has also been observed in a lab-selected dsRNA-resistant strain of corn rootworm *Diabrotica virgifera,* although the mechanisms restricting uptake have not yet been identified. (Khajuria *et al*. 2018).

Uptake of dsRNA into cells has been more thoroughly studied in invertebrates. The nematode *Caenorhabditis elegans* was the first organism to have RNAi described, and in these worms, several dsRNA transmembrane proteins, known as systemic RNA interference defective (SID) proteins, have been observed to facilitate dsRNA uptake (Winston *et al*. 2002, Winston *et al*. 2007, Jose *et al*. 2009, Jose *et al*. 2012, Hinas *et al*. 2012). RNAi has since been applied widely in many species including various pest insects, where dsRNAs are being developed as novel biocidal molecules that target essential genes, like those in the pathogenic fungi described above. In most insect control applications, the dsRNAs are intended to be incorporated into or sprayed on their food source, and hence, uptake of the dsRNA will occur through the intestinal cells (Terenius *et al*. 2011). In some species of beetles, SID1-like (SIL) proteins have been identified, but their role in dsRNA uptake is unclear, as knockouts of these genes in some species had no impact in RNAi efficacy (Tomoyasu *et al*. 2008). In the fruit fly, *Drosophila melanogaster,* orthologs for *SID1* or SIL proteins do not exist (Huvenne *et al*. 2010). Instead, genetic screening of S2 cells in *Drosophila* identified clathrin-mediated endocytosis (CME) as a likely route for dsRNA uptake (Saleh *et al*. 2006, Ulvila *et al*. 2006). CME has since been found to be the primary mode of uptake in various other insect species, including *Tribolium castaneum, Bactrocera dorsalis* and *D. virgifera* (Xiao *et al*. 2015, Li *et al*. 2015, Pinheiro *et al*. 2018). In this process, dsRNA binds to an as yet unidentified receptor, induces the formation of a clathrin-coated pit that invaginates to form a vesicle around the dsRNA. The vesicle is then transported through the endosomal compartment, and eventually is released from the endosome via pH shifts generated by proton pumps. While, insect species such as *Leptinotarsa decemlineata* use both SIL channels and CME to take up dsRNA, although the relative contributions of each remain unclear (Cappelle *et al*. 2016).

Despite these advances in insect systems, the dsRNA uptake mechanisms have not been resolved in fungal species. Understanding how exogenously-applied dsRNAs enter fungal cells is of critical importance in developing the technology for commercial applications. For example, understanding uptake mechanisms can help identify potential rate-limiting barriers to RNAi, either in dsRNA-insensitive strains or species, or in strains that might develop resistance due to changes in uptake mechanisms. Secondly, understanding uptake will help in identifying suitable formulations for SIGS, to maximize retention of dsRNA on the plant while still facilitating dsRNA uptake by the fungus. In this study, we explored the role of dsRNA uptake via CME in the fungal pathogen *S. sclerotiorum*. Using both chemical and RNAi-based methods of inhibiting endocytic processes, we evaluated the uptake of dsRNA into growing *S. sclerotiorum* hyphae. With the aid of live cell imaging and confocal microscopy, coupled with measurements of RNAi-mediated knockdown of reporter genes within the fungal cells, we show that proteins associated with CME are essential for dsRNA uptake. Taken together, our findings provide insight into the mode of action of exogenously-applied dsRNAs in fungal cells through SIGS, and how RNAi may provide effective control of agronomically important fungal pathogens.

2.3: MATERIALS AND METHODS:

2.3.1 *SCLEROTINIA* CULTURE

S. sclerotiorum cultures were obtained from the Morden Research and Development Centre, Agriculture and Agri-Food Canada, Morden, MB, Canada. Ascospore-bearing apothecia were generated from sclerotia germinated carpogenically on specialized germination medium (54 g cornmeal, 3.5 g vermiculite, 37.5 mL of 1% casamino acids and 1% yeast extract) and incubated on wet sand at 20°C (Nelson *et al*. 1988). After isolation, ascospores were stored in aluminum foil packets at 4°C. Ascospores (1×10^5 mL^{-1}) were plated on potato dextrose agar (PDA) to germinate. After 2 days' growth, mycelia were harvested from the plates using a P1000 pipette tip to collect agar plugs containing actively growing fungus for use in *in vitro* liquid culture growth experiments, as described below.

2.3.2 DESIGN AND *IN VITRO* SYNTHESIS OF DSRNAS

In vitro synthesis of *Sclerotinia* dsRNAs were designed according to the methods of McLoughlin *et al*. (2018). Reference genes targeted to monitor RNAi-mediated knockdown in this study were selected from a subset of genes from McLoughlin *et al*. (2018) that showed only minimal dsRNA-mediated death after 3 days' exposure to dsRNA, but displayed moderate (>60%) transcript knockdown. Genes encoding proteins associated with endocytosis were identified from the *S. sclerotiorum* transcriptome based on >60% amino acid conservation to other annotated fungal species such as *Aspergillus clavatus*, using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Gene function and percent identity to an annotated fungal species can be found in Table S2.1. Primers (Table S2.2) were designed using Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to create PCR gene fragments between 200-500 bp in length. Target sequences were amplified using Phusion Taq (Thermo Scientific, Waltham, MA, US) from Sclerotinia cDNA synthesized using the RNeasy Plant Extraction kit (Qiagen, Germantown, MD, US), RNase Free DNase set (Qiagen, Germantown, MD, US) and qScript cDNA synthesis kit (Quantabio, Beverly, MA, US)). PCR products were digested using FastDigest KpnI and XhoI (Thermo Scientific, Waltham, MA, US) and ligated into the similarly digested pL4440 vector using T4 Ligase (Invitrogen, Carlsbad, CA, US). Primers (F: CAACCTGGCTTATCGAA, R: TAAAACGACGGCCAGTGA) were

designed to amplify the T7 promotors flanking the insert and dsRNA was synthesized via T7 reverse transcription using the MEGAscript RNAi kit (Thermo Scientific, Waltham, MA, US).

2.3.3 *IN VITRO* DSRNA FLUORESCENCE LABELLING FOR LIVE CELL IMAGING AND CONFOCAL MICROSCOPY

Ss-ThioR dsRNA was labeled with 12-UTP fluorescein and synthesized using the Fluorescein RNA Labeling Mix (Sigma-Aldrich, Oakville, ON, CA). For whole organism labelling, live cell imaging using the ImageXpress plate system (Molecular Devices, San Jose, CA, US) was used. Ascospores (10 µL of 10^6 ascospores/mL solution in potato dextrose broth (PDB)) were applied to the center of each well of 12-well culture plates containing 400 µL of ¼ x PDA. Imaging of the fungal growth began 14 hours post germination. Fluorescein-labelled Ss-ThioR dsRNA (200 ng) was pipetted directly onto the site of ascospore inoculation immediately prior to imaging. Images were captured in a four by four grid in each well at an excitation/emission of 488 nm and 525 nm wavelengths every 20 minutes for 24 hours to examine fluorescein-labelled dsRNA uptake. A Zeiss LSM 700 confocal microscope (Jena, DEU) was used to study dsRNA distribution within *Sclerotinia* hyphae. One mm agar plugs from the edge of 3-day old PDA cultures were incubated for 4 hours to examine the earlier stages of uptake and 8 hours for the later stages in PDB plus F-Ss-ThioR (200 ng). To correct for possible artificial emitted fluorescence, laser gain settings were adjusted to 650 and 350 for fluorescein and T-PMT respectively, in the Zen Black software using untreated controls as the baseline. The same settings were applied to all samples. All microscopic analyses were repeated at least three times, with similar results.

2.3.4 DSRNA UPTAKE INHIBITION USING CHEMICAL INHIBITION AND RNAI OF RNAI EXPERIMENTS

To examine the role of endocytosis in dsRNA uptake, *in vitro* liquid cultures of *Sclerotinia* were used. Three 1 mm agar plugs of *Sclerotinia* were incubated in 3 mL potato dextrose broth plus 100 µg mL^{-1} ampicillin (Sigma-Aldrich, Oakville, ON, CA) in 15 mL culture tubes. Next, 20 µM chlorpromazine (dissolved in ddH_2O), 0.2 µM bafilomycin A1 (dissolved in DMSO) or 2 mM methyl-beta-cyclodextrin (dissolved in ddH_2O) was added to each tube. Two hours after inhibitor

inoculation, cultures were treated with 500 ng mL^{-1} Ss-ThioR or Ss-TIM44 dsRNAs and allowed to incubate for 48 hours at 22°C on a shaking incubator, wrapped in aluminum foil. Negative controls were treated with a corresponding volume of water in place of the dsRNA treatment. We and others have shown previously that there is no statistical difference when using controls either treated with water or a non-target dsRNA molecule such as GFP (Killiny *et al*. 2014, McLoughlin *et al*. 2018). Cultures were then thoroughly rinsed with ddH_2O to remove liquid media, and the solid hyphal mass was flash frozen in liquid nitrogen and RNA extracted and cDNA synthesized as described above. Transcript levels of these two genes were determined using qRT-PCR on the Bio-Rad CFX96 Connect Real-Time system using PerfeCTa SYBR (Quantabio, Beverly, MA, US) according to the manufacturer's protocol. Relative mRNA abundance was calculated using the ΔΔCq method using *Sac7* (SS1G_12350) as the housekeeping control. As the efficiencies of all the primers used in this study ranged between 90 and 110% (Table S2.3), a single control gene was considered sufficient. Melt-curve analyses confirmed that only a single product was produced in all RT-PCR reactions. To identify specific genes involved in the endocytosis process, an RNAi-of-RNAi approach was taken, whereby *Sclerotinia* was first treated with dsRNA targeting genes associated with endocytosis, and later treated with Ss-ThioR dsRNA. Liquid *in vitro* cultures of *Sclerotinia*, as described above, were treated with 500 ng mL^{-1} of each endocytosis target dsRNA, and after four days growth in a shaking incubator at 22°C, 500 ng mL^{-1} Ss-ThioR dsRNA was added. After an additional 48 hours, RNA was extracted and both the endocytic gene and the reference genes' transcript accumulations were measured as described above.

2.3.5 *SCLEROTINIA* TRANSFORMATION

Transformation was performed with the pCT74 plasmid (Nova Lifetech Ltd, HK) using polyelthylene glycol (PEG)-mediated transformation of protoplasts according to the methods of de Silva *et al*. (2009). Briefly, approximately 1 g of hyphae was harvested from *in vitro* cultures and incubated with 200 mg driselase (Sigma-Aldrich, Oakville, ON, CA) that had been resuspended in 0.7 M NaCl. After 3.5 hr incubation at 24 °C in driselase to produce protoplasts, the mixture was filtered through sterile cheesecloth and the protoplasts were collected by centrifugation at 400 x g and resuspended in STC (20 % sucrose, 10 mM Tris-HCl pH 8.0, and

50 mM $CaCl_2$). A total of 10 μg of plasmid DNA was added to the protoplasts and they were incubated on ice for 30 min. PEG-4000 (60% w/v) in STC buffer was added to the protoplast mixture and incubated on ice for an additional 5 minutes. Protoplasts were then re-isolated via centrifugation and plated onto PDA plates. After 2 hours, the plates were overlaid with molten PDA plus 100 μg/mL hygromycin B (Thermo Scientific, Waltham, MA, US). As the pCT74 plasmid contains a GFP reporter gene, putative transformants were examined for their ability to fluoresce at excitation/emission wavelengths of 488 nm and 510 nm, respectively, and successful transformants were re-cultured on fresh hygromycin-augmented PDA several times to ensure purity of the culture. One mm plugs from successful transformants were grown in PBD plus 100 μg/mL hygromycin B inoculated with 1000 ng mL^{-1} of Ss-eGFP dsRNA with or without the addition of 20 μM chlorpromazine for four days. Mycelia were imaged using the Zeiss Axioscope A1 (Jena, DEU) and the extent of GFP transcript knockdown was quantified using qRT-PCR as described above using primers designed around the eGFP mRNA and using Sac7 as the housekeeping control.

2.3.6 DATA ANALYSIS

To analyze the data, JASP (jasp-stats.org) software was used to compute hypothesis testing (JASP Team 2020). To determine whether dsRNA-induced gene knock down significantly differed from the control, *student's t-tests* were performed with a Bonferroni correction to the level of significance of each comparison.

2.4: RESULTS

2.4.1 VISUALIZING REGIONS OF DSRNA UPTAKE

Liquid cultures of *Sclerotinia* hyphae, when incubated with dsRNAs targeting transcripts of either thioredoxin reductase (Ss-ThioR) or Ss-TIM44, a gene encoding a mitochondrial inner membrane transporter, showed reduction of either gene's transcripts by approximately 50% after 48 h (Figure 2.1A). Clearly, both dsRNAs could readily enter the fungal cells to induce RNAi and there does not seem to be significant differences related to extent of knockdown for the two different dsRNA targets.

To visualize the uptake of exogenous dsRNA in *Sclerotinia*, ascospores were germinated and then inoculated and allowed to grow in the presence of fluorescein-labeled Ss-ThioR dsRNA. The distribution of labeled RNA was examined over an 8 h time period. The fluorescently-labeled dsRNA was detectable on or inside the fungal cells within two hours of application of the dsRNA (Figure 2.1B). Interestingly, the fluorescence accumulated at specific sites, initially localized on the newly budding branches, and over the next several hours, the fluorescence was concentrated at the tips of these branches and at the junction points of the branches and the main trunk of the hyphae. Examination of the focal stacks of the images revealed fluorescence throughout the hyphal tips and branch points, suggesting that the fluorescently-labeled dsRNA was not on the surface, but was localizing within the cytoplasm of the cells at these locations (Figure S2.1).

While newly-germinated spores showed rather localized concentration of the fluorescently-labeled dsRNA, the distribution of the fluorescence in 3-day old hyphae that were exposed to dsRNA for 8 h showed more widespread distribution of the dsRNA. Confocal microscopy revealed that the fluorescence was not evenly dispersed throughout all hyphae, as thinner, younger hyphae had higher levels of fluorescence than the thicker, more mature and vacuolated counterparts (Figure 2.1C).

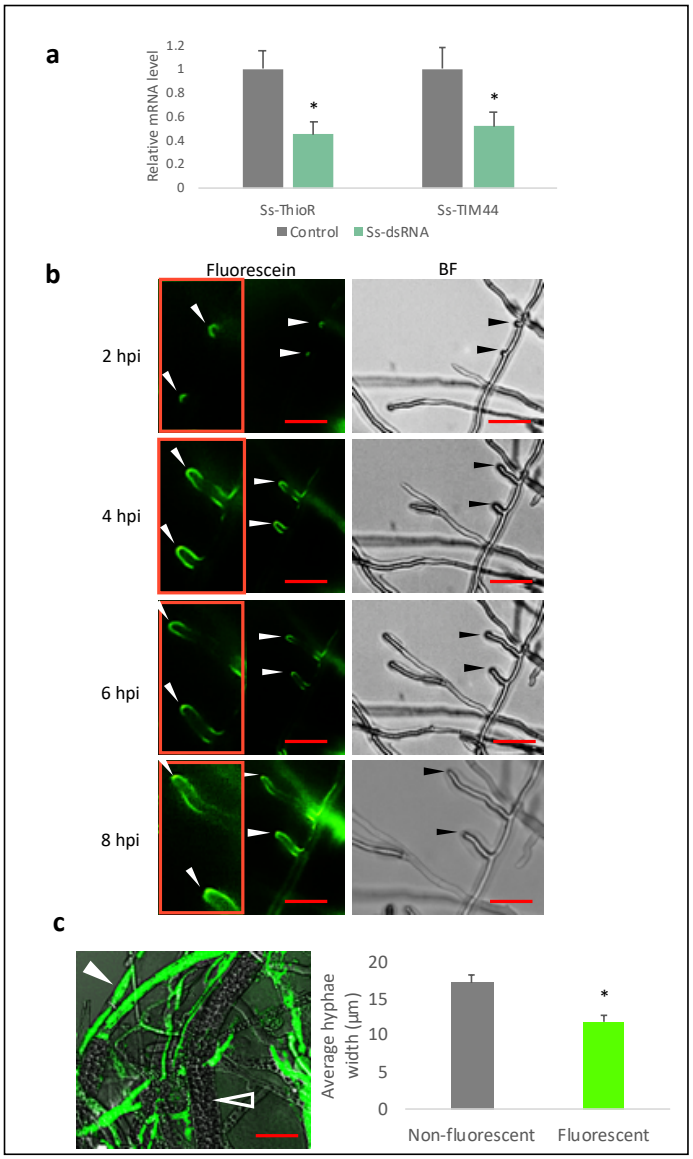

Figure 2.1. Uptake of dsRNA by *Sclerotinia*. a) *In vitro* cultures were treated with 500 ng/mL of dsRNA targeting two *Sclerotinia* genes, Ss-ThioR and Ss-TIM44. Transcript abundance was measured after 48 hours. * denotes significant reduction in transcripts, relative to the controls (one-tailed t-test, $p<0.05$ from 3 independent biological replicates) b) Detection of fluorescein-tagged Ss-ThioR dsRNA in newly germinated hyphae from ascospores. Growing hyphae were imaged using the ImageXpress platform. At each time point, representative bright-field (BF) images (right) reveal growing hyphae (arrow heads), and their fluorescence is visualized in the middle panel. Enlarged images of the hyphal tips are shown in the left panel (Scale bars: 40μm). c) Three day-old *Sclerotinia* inoculated with fluorescein-labelled dsRNA show different levels of fluorescence 8 h later. (Hollow arrow with white border: Mature, vacuolated hyphae, White arrow: Younger hyphae, more prone to uptake). The widths of all hyphae were measured at their midpoints, and were scored for absence or presence of fluorescence (Scale bars: 20μm). * denotes significant difference in the width of fluorescent hyphae, relative to non-fluorescing hyphae (one-tailed t-test, $p<0.05$, n = 40 hyphae of each category). All error bars represent standard error.

2.4.2 CHEMICAL INHIBITION OF ENDOCYTIC PROCESSES

To examine the possible role of clathrin mediated endocytosis (CME) or clathrin-independent (e.g. caveolae-mediated endocytosis) in dsRNA uptake in *Sclerotinia*, hyphae were grown in liquid cultures dosed with different chemical inhibitors of endocytosis and then treated with dsRNAs targeting Ss-ThioR or Ss-TIM44. Hyphae treated with chlorpromazine (CPZ), which inhibits the recruitment of clathrin to the plasma membrane and thus inhibits the formation of vesicles, or bafilomycin A1 (BafA), which impedes the function of vacuolar ATPases and therefore prevents the release of vesicular cargo into the cytoplasm, failed to induce RNAi-mediated knockdown of either Ss-ThioR or Ss-TIM44 (Figure 2.2 AB). This lack of transcript knockdown suggests that CME is playing a significant role in dsRNA uptake in the *Sclerotinia* hyphae. A third inhibitor, methyl-beta-cyclodextrin (MBCD), which inhibits clathrin-independent processes such as caveolae mediated endocytosis and lipid raft formation, was also tested. Interestingly, both target genes' transcript levels were reduced when treated with their respective dsRNAs after this inhibitor treatment (Figure 2.2 C). This suggests that clathrin-independent uptake processes are not playing a major role in dsRNA uptake in the fungus.

Confocal microscopy was used to detect the fluorescently-labeled dsRNAs after treatment with the different inhibitors. Hyphae from liquid cultures treated only with fluorescently-labeled dsRNAs were readily able to internalize the molecules. The labeled dsRNA remained cytoplasmic, with no fluorescent signal appearing within the vacuole structures (Figure 2.2 D). CPZ treatment completely prevented the labeled dsRNAs from entering the cells, as all fluorescence was external, with the strongest signal appearing around the outside of newly budded branches (Figure 2.2 E). Bafilomycin-treated cells were able to internalize the fluorescence, however, as evidenced by the lack of transcript knockdown, the dsRNA was not released from the internal vesicles (Figure 2.2 F). It is interesting that there was no obvious cell-to-cell spread of the dsRNA over the 4 h experiment, and the fluorescent signal appears quite punctate. The distribution of the fluorescence in the MBCD-treated cells did not appear to differ greatly from that of the untreated cultures (Figure 2.2 G). These cell images therefore support the premise that CME is a major contributor to dsRNA uptake.

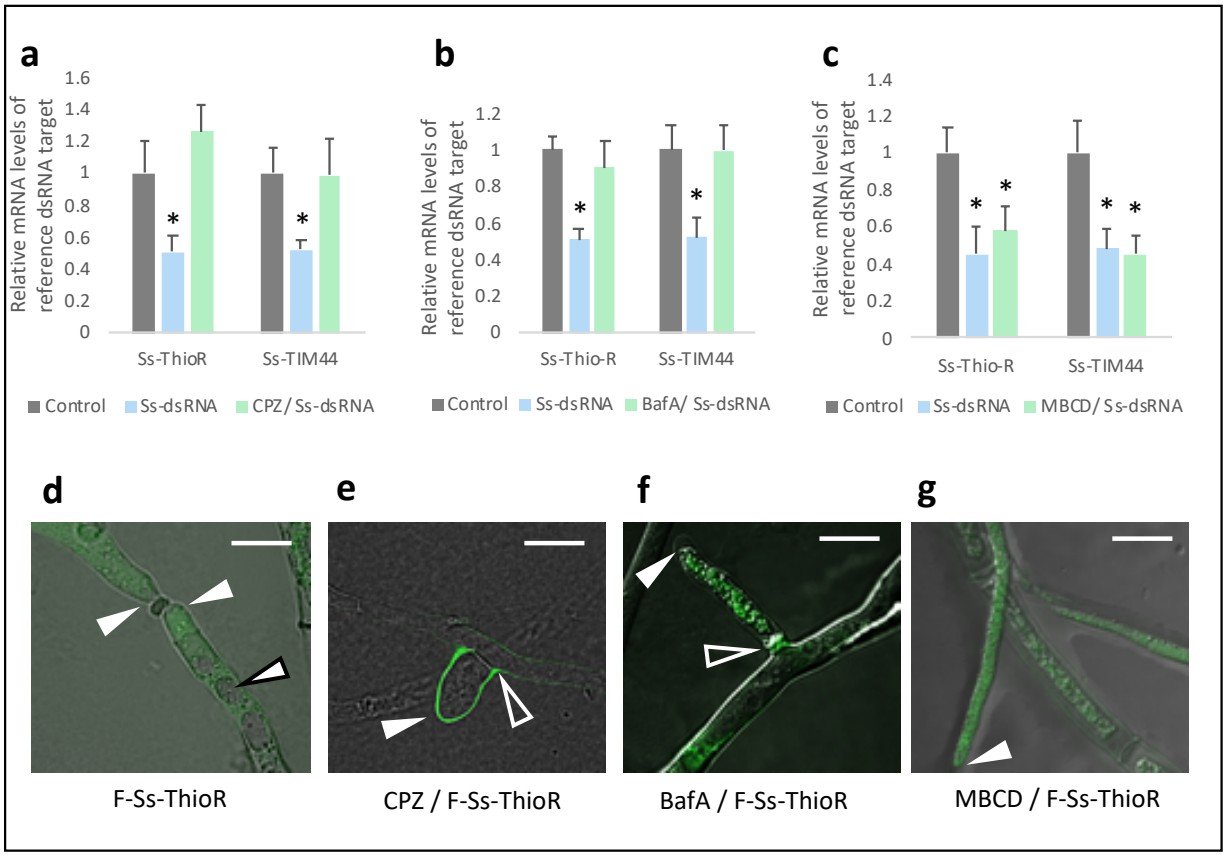

Figure 2.2. Chemical inhibition of clathrin mediated endocytosis inhibits dsRNA uptake. a) *In vitro* cultures were inoculated with 20 μM chlorpromazine (CPZ), b) 0.2 μM bafilomycin A1 (BafA), or c) 2 mM methyl-beta-cyclodextrin (MBCD). Two hours later, hyphae were treated with 500 ng/mL Ss-ThioR or Ss-TIM44 dsRNA and transcript levels of these two genes were measured two days later. * denote significantly different values relative to the negative control (one-tailed t-test, $p<0.05$, from 3 independent biological replicates). d-g) Confocal microscopy reveals distribution of fluorescein-labelled dsRNA in hyphae, 4 h post-treatment, treated with or without inhibitors. d) no inhibitors, e) 20 μM CPZ, f) 0.2 μM BafA, or g) 2 mM MBCD. Cultures were imaged three separate times with similar results. (White arrow with black border: fungal vacuole, White arrow with no border: hyphal tip, Hollow arrow with white border: branch point of a hyphal bud). (Scale bars: 10μm). All error bars represent standard error.

2.4.3 RNAI OF RNAI EXPERIMENT TO ELUCIDATE CANDIDATE UPTAKE GENES

To validate that CME is facilitating dsRNA uptake, an 'RNAi of RNAi' experiment was conducted (Figure 2.3 A). In these experiments, cultured cells were first treated with a dsRNA targeting a gene putatively involved in dsRNA uptake. After sufficient time had passed to permit RNAi-mediated knockdown and reduction of the targeted gene's protein, cultures were treated

with a secondary, reference dsRNA. If the reference dsRNA did not knock down its target transcript to the same extent as untreated or negative controls, it is evidence that the second dsRNA failed to enter the cells, and thus confirms the involvement of the initial dsRNA target in uptake.

Six *Sclerotinia* genes with high sequence identity to genes encoding proteins annotated to function in clathrin mediated endocytosis in *Aspergillus clavatus* were identified and dsRNAs were prepared to target each. *Sclerotinia* liquid hyphae cultures pretreated for 4 days with these each of these dsRNAs (clathrin heavy chain (Ss-CHC), vacuolar H^+ ATPase 16kDa subunit (Ss-VATPase), ADP ribolysation factor 72A (Ss-Arf72A), Fes/CIP4 homology domain only 1 (Ss-FCHO1), amphiphysin (Ss-Amph), and clathrin adaptor protein 2 subunit A2 (Ss-AP2) showed significant knockdown of each of the six targets (Figure 2.3 B). Subsequent treatments with the reference dsRNA, Ss-ThioR, failed to induce gene knockdown (Figure 2.3 C). This provides strong evidence in favour of the involvement of these genes, and therefore CME in dsRNA uptake in *Sclerotinia*.

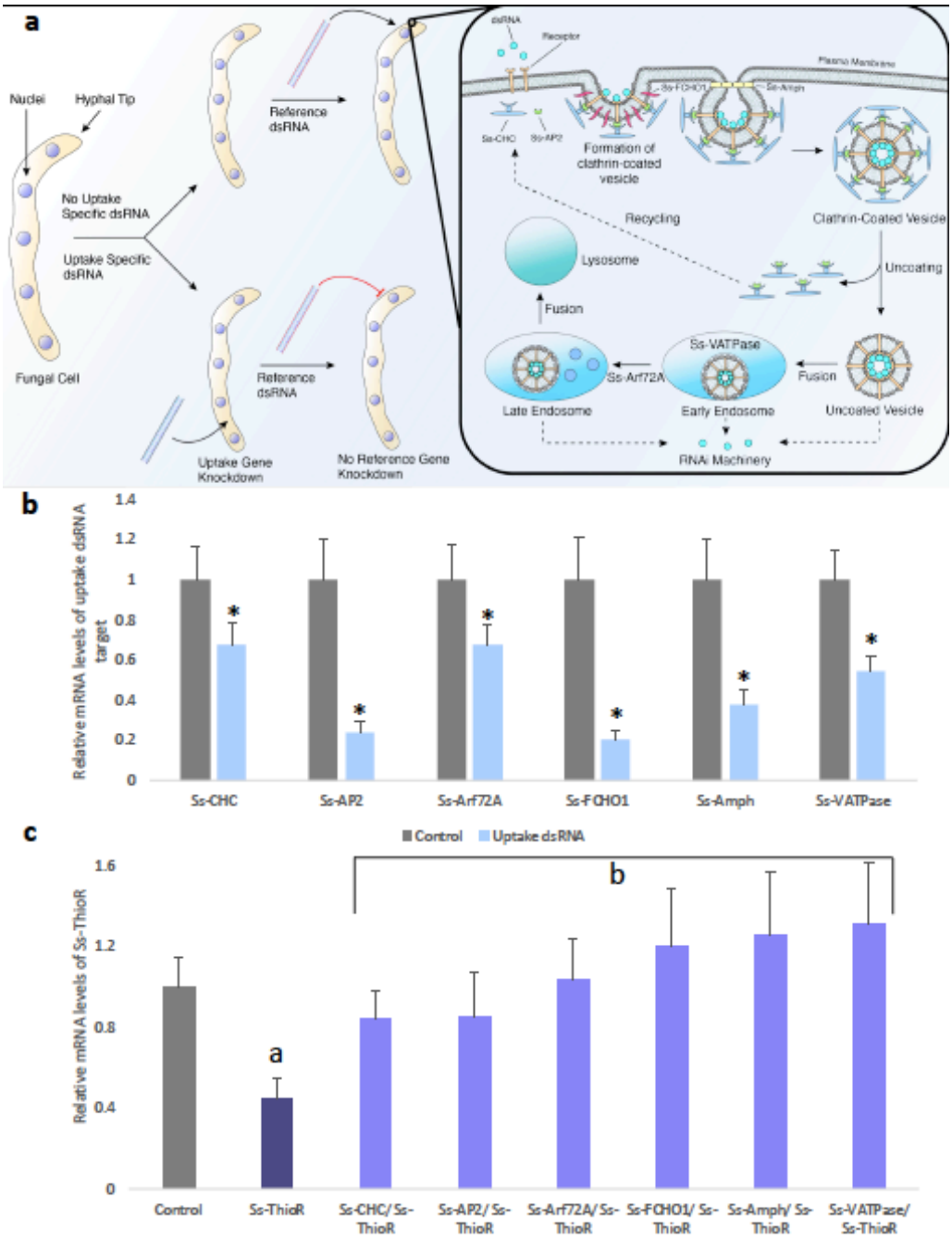

Figure 2.3. An RNAi of RNAi experiment to identify proteins involved in dsRNA uptake. a) The knockdown of genes involved in uptake will inhibit further uptake of reference dsRNA and thus prevent its knockdown. Whereas in cultures untreated with uptake dsRNA, reference dsRNA will be taken up via our proposed uptake mechanism. The binding of exogenous dsRNA to its receptor induces the localization of clathrin and its adaptor to the membrane. FCHO contributes to the invagination of the vesicle and amphiphysin to its release from the membrane. Arf72A aids in the transition from early to late endosome, and vesicle acidification via VH+ ATPase induces cytoplasmic dsRNA release. b) *In vitro* cultures were first treated with 500 ng/mL of dsRNA targeting putative uptake genes (Ss-CHC, Ss-AP2, Ss-Arf72A, Ss-FCHO1, Ss-

Amph and Ss-VATPase) and transcript levels of each gene was assessed by quantitative real-time PCR 4 days later. * denotes significant knockdown of the targeted gene (one-tailed t-test, with a Bonferroni correction $p<0.05$ from 3 independent biological replicates). c) At day 4, a second dsRNA, targeting Ss-ThioR, was applied, and Ss-ThioR transcript levels were measured after an additional two days. Levene's test was used to assess the homogeneity of the variance of the data and ANOVA was performed using the Games-Howell post-hoc test ($p < 0.05$ from 3 independent biological replicates). Note that only the hyphae treated with Ss-ThioR dsRNA alone resulted in significant difference from the controls. All error bars represent standard error.

2.4.4 TRANSGENIC *SCLEROTINIA* ENCODING EGFP TO VALIDATE CME AS THE PRIMARY MODE OF UPTAKE

Further evidence that CME is a primary means of dsRNA uptake was demonstrated using transgenic *Sclerotinia* that constitutively expressed eGFP (Figure 2.4 A). Liquid cultures of the eGFP strain, when treated with dsRNA targeting the eGFP gene's transcripts, had reduced fluorescence. Intriguingly, the region behind hyphal tip, where fluorescein-labeled dsRNA had previously been observed to localize, showed the strongest reduction in eGFP fluorescence, which suggests that this region could be the point of entry of dsRNA, or alternatively, is a site where the dsRNA preferentially localizes as it induces knock down (Figure 2.4 B). Cultures treated with eGFP dsRNA also showed significantly reduced eGFP mRNA levels (Figure 2.4 D). In order to demonstrate that the eGFP dsRNA entered via clathrin mediated endocytosis, the liquid cultures were treated with chlorpromazine. Hyphae treated with CPZ prior to eGFP dsRNA addition did not display a reduction in fluorescence, indicating that the dsRNA was unable to enter the hyphae (Figure 2.4 C). Transcript reductions of eGFP mRNA also were not observed in these CPZ-treated cultures (Figure 2.4 D).

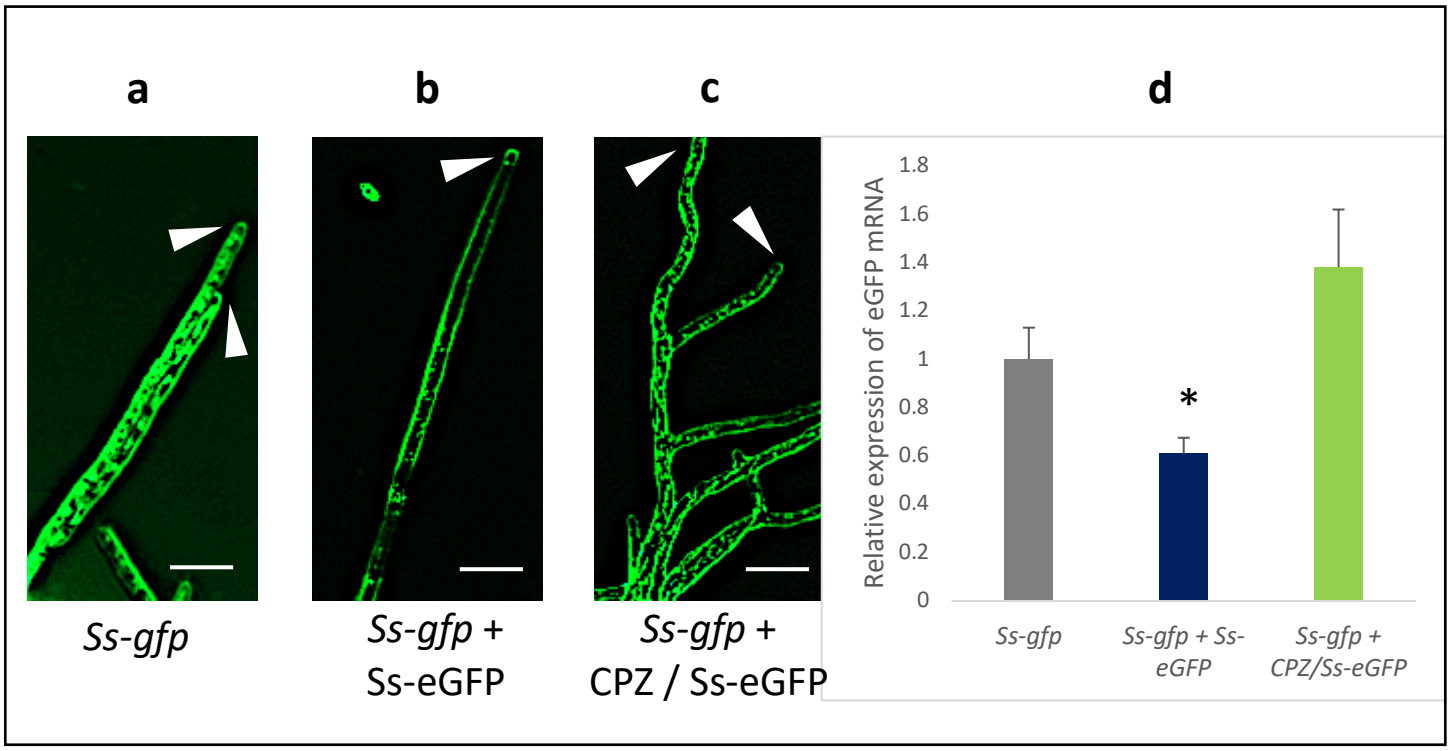

Figure 2.4. EGFP-transgenic *Sclerotinia* show reduced fluorescence when treated with dsRNA targeting eGFP, but not when first treated with chlorpromazine (CPZ). *In vitro* cultures of the eGFP-*Sclerotinia* were treated with 1000 ng/mL of dsRNA targeting eGFP with or without 20 μM CPZ. After four days, hyphae were imaged. a) Untreated eGFP-*Sclerotinia* showing normal fluorescence (White arrows with no border: hyphal tips) b) eGFP-*Sclerotinia* treated with eGFP dsRNA. Note stronger loss of fluorescence at hyphal tips. c) CPZ-treated eGFP-*Sclerotinia* treated with eGFP dsRNA (Scale bars: 20μm). d) eGFP transcript levels in each of the treatments. * denotes significant difference from the negative control (one-tailed t-test, $p < 0.05$ from 3 independent biological replicates). Error bars represent standard error.

2.5 DISCUSSION

RNAi-based fungicides offer great potential for managing some of our crops' most damaging pathogens. For the technology to reach its fullest potential, we will need to have a good grasp of the molecular processes involved in dsRNA uptake in fungi, to achieve effective dsRNA delivery and potent RNAi. Our findings demonstrate how exogenously-applied dsRNAs can enter fungal cells to initiate RNAi-based gene knockdown, which we and others are considering for use in both gene validation studies and control of this pathogenic fungus in the field. In this study, we used chemical inhibitors, targeted gene knockdown, and live cell imaging to demonstrate that clathrin-mediated endocytosis (CME) plays a major role in the uptake of exogenously-applied dsRNAs in the white mold pathogen *S. sclerotiorum*.

Chemical inhibitors have been widely used to examine dsRNA uptake in a variety of insect species (Saleh *et al*. 2006, Xiao *et al*. 2015, Cappelle *et al*. 2016), and in this study, two inhibitors of CME, chlorpromazine (CPZ) and bafilomycin A (BafA), confirmed a role for CME in dsRNA uptake through the reduction of the cell's RNAi response to the two reference genes. CPZ is a cationic amphiphilic compound that prevents the formation of clathrin-coated pits at the site of vesicle invagination by translocation of clathrin from the plasma membrane to intracellular vesicles (Vercauteren *et al*. 2010). Visualization of the CPZ-treated hyphae using confocal microscopy indicated that the fluorescein-labeled dsRNA failed to enter the cells, but instead was concentrated on the cell's exterior, possibly binding to the positively charged chitin in the cell wall (Riquelme and Martinez-Nunez 2016). The use of the fluorescein dye to label the dsRNA did not likely impair dsRNA uptake, as fluorescently-labeled dsRNAs have been demonstrated to enter other cell types readily, including *Fusarium* (Koch *et al*. 2016) and gut cells of the fall army worm, *Spodoptera frugiperda* (Yoon *et al*. 2017).

BafA inhibits vacuolar H^+ATPase (VATPase), a proton pump responsible for the acidification of endosomal vesicles to facilitate the release of endocytosed contents into the cytoplasm (Dettmer *et al*. 2006). BafA has been used to confirm the involvement of clathrin-mediated endocytosis in the uptake of dsRNA in the midgut of the Colorado potato beetle, *L. decemlineata* as well as the hepatopancreas of the whiteleg shrimp, *Litopenaeus vannamei* (Cappelle *et al*. 2016, Maruekawong *et al*. 2019). The BafA-treated hyphal cultures also showed a lack of RNAi, but

these cells displayed a different dsRNA distribution than that of the CPZ-treated hyphae. The inhibition of VATPase would prevent acidification of endosomes and release of their contents to the cytoplasm. Hence, in the BafA-treated hyphae, we observed intracellular fluorescence of the fluorescein-labeled dsRNA in a punctate pattern, as the dsRNA remained within endosomal vesicles, and failed to induce RNAi.

In contrast, MBCD did not alter the distribution of fluorescein-labeled dsRNA within the cells, relative to the negative control, and nor did it have a negative effect on RNAi of our reference genes. MBCD is known to affect caveolae-dependent endocytosis (Perez *et al*. 2011), and as we observed no significant reduction of RNAi in the MBCD-treated hyphae, caveolae-dependent endocytosis appears to play no major role in dsRNA uptake. While we cannot rule out that other clathrin-independent processes, such as RhoA-regulated or CDC42-regulated endocytosis (Mayor *et al*. 2007) may have minor roles in dsRNA uptake, they have not previously been implicated in dsRNA uptake in other organisms. Given the strong response observed using CME inhibitors, CME is clearly a dominant player in the uptake process.

To validate CME's involvement in dsRNA uptake in *Sclerotinia*, we also used an RNAi-of-RNAi approach to identify key players in this endocytosis pathway. By knocking down transcripts of genes encoding CME pathway proteins, we significantly reduced or completely abolished the cells' abilities to subsequently initiate RNAi of the reference gene. Although we did not survey all possible CME-associated genes, we identified several components of this endocytosis pathway, from early vesicle formation to late endosomes, that are required for dsRNA uptake. The receptor(s) that bind the dsRNA have yet to be identified, but we confirmed that clathrin (or more specifically, the heavy chain of the clathrin complex, CHC) and a component of the clathrin adaptor protein 2 (AP2) complex are essential for dsRNA uptake. Similarly, FCHO1, a protein with membrane binding and bending activity, and amphiphysin (Amph), a protein that recruits dynamin to facilitate the formation and budding process of the endocytic vesicle, respectively, were also observed to be essential for dsRNA uptake, as their knockdown abolished RNAi of the reference genes. Proteins associated with assembly and functioning of late endosomes, including an ADP ribosylation factor-like 1 protein (Arf72A) and

a Vacuolar H+ ATPase, were also all confirmed as essential to effective RNAi, which suggests that the release of the dsRNA to the cytoplasm occurs late in the endocytic pathway.

The live cell *Sclerotinia* imaging in the presence of fluorescently-labeled dsRNA suggests that uptake does not occur evenly along the fungal hyphae, but instead occurs predominantly at the hyphal tip of actively growing branches of hyphae. Similarly, the localized reduction of eGFP fluorescence in our transgenic strain of *Sclerotinia* near the tips of the growing hyphae is supporting evidence that this region is a site of dsRNA entry, facilitating localized gene knockdown effects, followed by a gradual reduction of eGFP fluorescence over the length of hyphae, as the silencing signals spread. The fungal hyphal tip is the site of polarized cell growth, supported by tip-directed transport of secretary vesicles containing a variety of lytic enzymes used to facilitate invasion into a host plant's tissues (Rodrigues *et al*. 2011). The tip of the hyphae is seen as a site of relatively rapid membrane deposition as vesicles continually fuse with the plasma membrane to exocytose their contents (Echauri -Espinosa *et al*. 2012). An endocytic collar, located directly behind the hyphal tip functions to recycle the excess membrane produced by exocytosis. Since the chitinous cell wall must continually expand as the hypha grows, it is thinner at the tip, enabling extracellular materials to easily reach the endocytic collar, which is considered to be the site where the majority of endocytosis occurs in filamentous fungi (Steinberg 2014). Once entering the cell, dsRNA can be transported short distances between fungal cells through septal pores (Wang and Dean 2020). It is also worth noting that *Sclerotinia* contains sequences highly similar to RNA-dependent RNA polymerases (RdRps) found in *Verticillium nonalfalfae*. RdRps have been implicated in generating secondary small interfering RNAs (siRNAs) by using the primary siRNAs of an RNAi response as primers to generate more dsRNA using the target mRNA as the template (Jesenicnik *et al*. 2019). It will be of interest to determine whether RdRp from *Sclerotinia* can contribute to amplification of the RNAi signal, which could help reduce the amount of dsRNA needed to control this fungus effectively.

We observed significant, but not complete knockdown of the targeted genes with the doses applied in this study. If CME is insufficient to deliver effective doses of dsRNA to all cells, and certainly the live cell imaging suggests that the uptake process is not uniform across all hyphae, alternative dsRNA delivery methods may help complement or even circumvent the CME

process. In other systems, combining dsRNA with carrier molecules has been shown to enhance uptake. Lipid-based delivery methods have been investigated extensively in human therapeutics, but have also been tested in the fungus *Aspergillus flavus* (Schroeder *et al*. 2010, Nami *et al*. 2017). Increasing the stability of the molecule is also important. In plants, dsRNA bound to clay nanosheets remained intact for up to 30 days *in planta* in addition to increasing uptake (Mitter *et al*. 2017). An approach such as this could be applicable for *Sclerotinia* and other antifungal dsRNA applications.

In this study, and in one of our previous studies (McLoughlin *et al*. 2018), we demonstrated effective RNAi-mediated knockdown of transcripts in *Sclerotinia* through exogenous dsRNA applications. Other research groups have similarly observed RNAi in other fungal species such as *F. graminearum* (Koch *et al*. 2016) and *B. cinerea* (Wang *et al*. 2016), where transcript reductions resulted in reductions in pathogenicity following dsRNA treatments. In these two other species, along with *S. sclerotiorum* studied here, the dsRNA can obviously enter the fungal cells, but in some fungi, dsRNA does not appear to readily enter the cells. For example, *Zymoseptoria tritici* lacks the ability to take up externally applied fluorescent dsRNAs *in vitro* (Kettles *et al*. 2019). A BLAST search of its genome suggests that this fungus has the key genes necessary for clathrin-mediated endocytosis and RNAi, but it may lack the necessary, but still unidentified, dsRNA receptors needed to trigger CME-mediated uptake of dsRNA. In contrast, *Ustilago maydis* appears to have lost the genes encoding key components of the canonical RNAi pathway such as DCL, AGOs and RdRps (Billmyre *et al*. 2013). These species, however, may be the exception, as the majority of fungi examined to date contain functional dsRNA uptake and RNAi pathways. Indeed, a steadily growing number of species have been shown to be susceptible to either SIGS or host-induced gene silencing (HIGS) -based control methods (Sang and Kim 2020).

The ability to deliver dsRNA to various pathogenic fungi has driven many research groups to consider the development of novel dsRNA-based fungicides. The prospect of developing fungicides with high specificity for target species, without adversely affecting non-target species, is a strong motivator to develop this technology. However, with every new pesticide developed, there is the inevitable development of resistance to the pesticide that will arise over time. Due to

its sequence-specificity, overcoming small nucleotide mutations is relatively easy to overcome with dsRNA-based pesticides, as there are potentially many different genes that could be targeted to control the pathogenic fungus (McLoughlin *et al*. 2018). However, of greater concern is the possibility that the fungus could develop resistance to many different dsRNAs. Zotti and Smagghe (2015) suggested that one such problematic mode of resistance is alteration in dsRNA uptake mechanisms. This type of resistance has already been identified in a laboratory-selected population of corn rootworms (*D. virgifera*). Within 11 generations of selection on progressively higher doses of dsRNA, Khajuria *et al*. (2018) produced insects with >130-fold resistance levels. The resistance was attributed to reduced gut luminal uptake, although the nature of the resistance mutations has yet to be identified. Clearly, resistance due to impaired dsRNA uptake can occur, and if dsRNAs are to be used as biocides, understanding uptake mechanisms will aid in avoiding rapid development of resistance in the field.

Taken together, our results indicate that clathrin-dependent endocytosis is likely responsible for the uptake of dsRNAs in *S. sclerotiorum*. CME has been known to be an integral player in the pathway for the internalization of extra membrane, lipids and receptor-bound macromolecules within eukaryotic organisms and is critical for numerous biological processes such as nutrient acquisition and cell signalling (Conner and Schmid 2003). In Figure 3A, we adapted the general CME type mechanism to display how we believe dsRNA is internalized within *Sclerotinia*. The process includes dsRNA molecules first binding to unknown receptor proteins on the cell surface. It has been shown in some insect species, including S2 cells in *Drosophila*, that scavenger- like receptors are responsible for the binding of dsRNA (Ulvila *et al*. 2006, Wynant *et al*. 2014). In humans, Toll-like receptor 3, in addition to class-A type scavenger receptors, specifically *SR-AI/II* and *SCARA 3/4/5* are all able to bind dsRNA (Dewitte-Orr *et al*. 2010, Watanabe *et al*. 2011). However, to our knowledge, no homologues of these receptors exists within *Sclerotinia*. Once bound, the dsRNA and its receptor interact with clathrin and its adaptor protein complex (AP2) to form a coated pit, which invaginates to form a vesicle. The clathrin sheath uncoats and the vesicle fuses with an early endosome, which finally matures into a late endosome. In this schematic, the dsRNA molecules must release from the endosomes before they mature into functional lysosomes and into the cytoplasm to trigger RNAi machinery to perform dsRNA processing and targeted transcript knockdown. While we have shown that CME is the

predominant mode of uptake, there are several questions that remain unanswered as we continue to develop species-specific molecular fungicides. We now know how the dsRNA enters the cell but know little about the fate of the molecule once inside, how it is dispersed through the fungus, and whether barriers exist that limit its movements. Identifying other components of the uptake machinery, including the dsRNA receptors, and a closer examination of what dsRNA uptake or dispersal machinery is missing in RNAi-refractory fungal species is a good first step to understanding how resistance to dsRNA might arise, and could help prolong the utility of the technology in phytopathogen control programs.

2.6 CONCLUSIONS

RNAi technologies offer the promise to provide a new generation of species-specific, environmentally safe fungicides. To maximize this potential, we need to understand how dsRNAs can be delivered to the pathogenic fungi in sufficient quantities to induce strong knockdown of the target genes. In this study, we demonstrated that CME is an effective dsRNA uptake mechanism in the phytopathogen *Sclerotinia*. Understanding the cellular machinery involved in dsRNA uptake in fungi will help in the design of more efficient dsRNA delivery formulations and in the development of effective strategies to overcome the possible development of fungal strains that are resistant to dsRNA uptake.

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COMPETING INTERESTS

The authors declare no competing interests.

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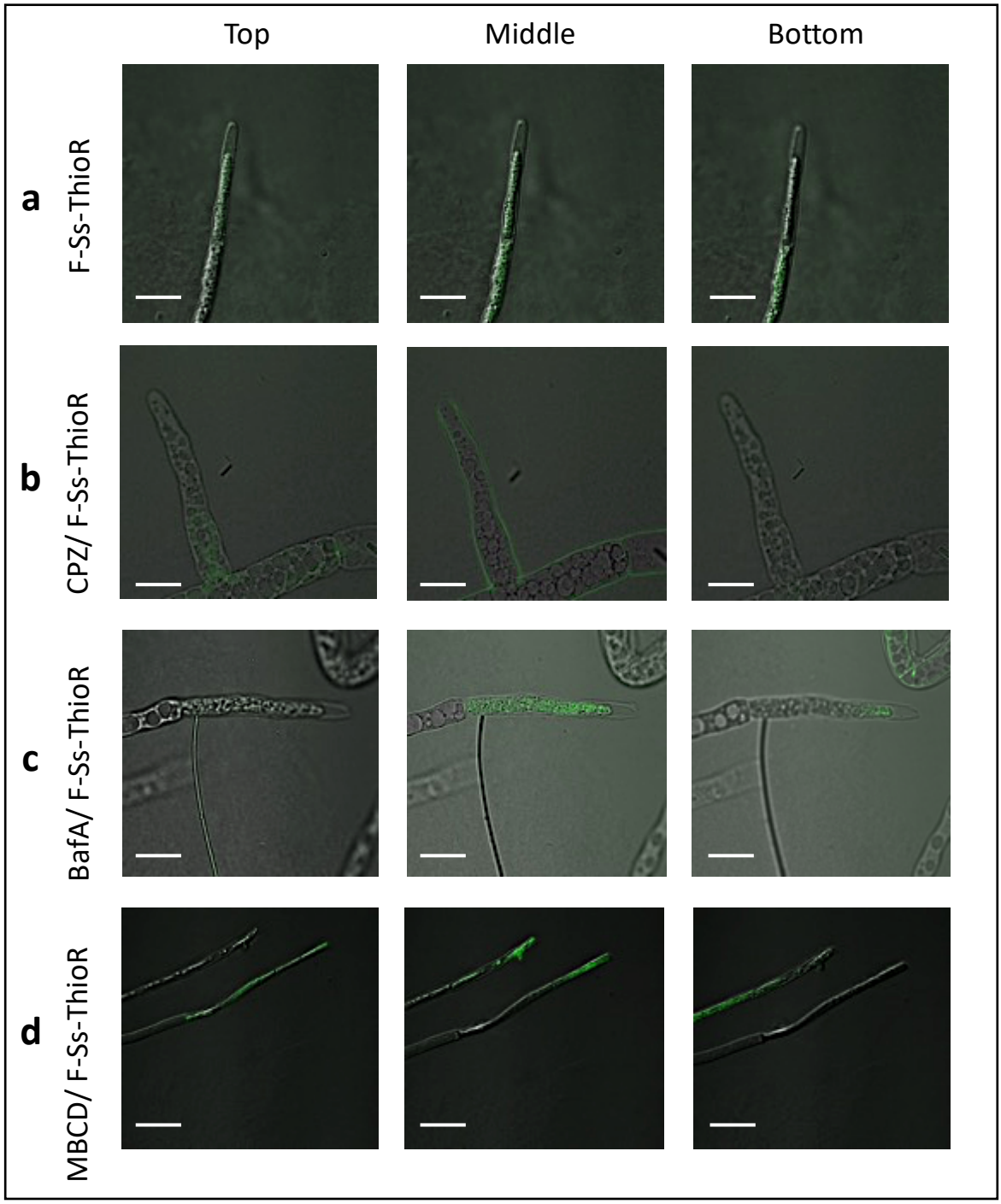

Figure S2.1 . a) *In vitro* cultures were inoculated with 20 μM chlorpromazine, b) 0.2 μM Bafilomycin A1, or c) 2 mM methyl-beta-cyclodextrin and two hours later, with 500 ng/mL Ss-ThioR dsRNA. Confocal microscopy was used to view above and below the focal plane of the specimen to demonstrate that the dsRNA was intracellular and not only bound to the outer surface of the hyphae.

Table S2.1. Gene identifications and functions for each of the gene targets used in this study. In order to identify the Sclerotinia copy of each of these genes, amino acid homology to an annotated fungal species, *Aspergillus clavatus*, was used.

Ss Gene name	**Gene Function**	**Ss gene ID**	**Orthologous *Aspergillus* gene ID**	**Amino acid percent identity**
Ss-ThioR	Thioredoxin reductase	SS1G_05899	ACLA_051990	67%
Ss-TIM44	Mitochondrial inner membrane translocase subunit TIM44	SS1G_06487	ACLA_025660	77%
Ss-CHC	Clathrin heavy chain	SS1G_12840	ACLA_047060	78%
Ss-AP2	Clathrin adaptor protein 2	SS1G_06066	ACLA_054080	68%
Ss-Arf72A	ADP ribosylation factor 72A	SS1G_06730	ACLA_023910	92%
Ss-FCHO1	FES-CIP4 homology domain only 1	SS1G_06645	ACLA_038110	68%
Ss-Amph	Amphiphysin	SS1G_00939	ACLA_041720	64%
Ss-VATPase	Vacuolar H+ ATPase 16 kDa subunit	SS1G_10240	ACLA_040240	87%

Table S2.2. Cloning primer sequences as well as dsRNA fragment lengths used in this study.

Ss gene ID	Forward Primer	Reverse Primer	dsRNA length (bp)
Ss-ThioR	GCTCACACTGCTGCCGTATA	CTCCGCAGCACTATCTCCAC	435
Ss-TIM44	CGTAGCATATCCGACCGAGT	AGACGGAGAATTGAGCTGCT	211
Ss-CHC	TTGACCCAAATCAGGCAGCT	ATGGCGACTGGGAAATCGTT	397
Ss-AP2	GCGATGACCCTGTTCCTTCA	GACCGAGAGAGCAACACCAA	333
Ss-Arf72A	GGTTGAGGCTCGTGAGGAAT	GCGAGCCATTCCAATCCTTC	222
Ss-FCHO1	AGACGAAAGAGCACCAAGGG	TTTCTCAGGTGCTGCGACAT	261
Ss-Amph	AGCGCCTCAAACGTTCAAAG	TTCTTCACACGCCCTGATCC	280
Ss-VATPase	CACTTGTTTCGGTGCTGCAT	AGACGTGGTTGTTGAGCTGT	306
Ss-eGFP	CTGACCTACGGCGTGCAG	GACCATGTGATCGCGCTTCT	468

Table S2.3. qRT PCR primer sequences used in this study.

Ss gene ID	**Forward Primer**	**Reverse Primer**	**Efficiency**
Ss-ThioR	ACACGGTTGGAGGCGAAATT	TCCTCTCCAGTAACGACGTTC	96%
Ss-TIM44	TACTTGACAAGGCGACGCAA	ACCCACCCTCCGTACCTC	108%
Ss-CHC	GCCTTCCTTAGCGCTGATCT	GCAAGCTCTCATTGTCGCTG	98%
SS-AP2	AAATGCGGTTTTGTTTGAGG	TTGGATGAACTTCCCGAGTC	93%
Ss-Arf72A	TACAACTATCCCAACCATCGGC	CACCAACATCCCACACGGTA	95%
Ss-FCHO1	TCGAAGTCAATGCAGCAAAC	CTGTGAATCTGGCCTTGGTT	92%
Ss-Amph	TGATGAACCACCAGATCGAA	CCCTTCTTGAACCAGGGAAT	98%
Ss-VATPase	GTATACGCGCCATTCTTCGG	GCAGCACCGAAACAAGTGAA	102%
Ss-eGFP	AACGAGAAGCGCGATCACAT	TTGTACAGCTCGTCCATGCC	91%

CHAPTER 3: HOST INDUCED GENE SILENCING OF THE *SCLEROTINIA SCLEROTIORUM ABHYDROLASE-3* GENE REDUCES DISEASE SEVERITY IN *BRASSICA NAPUS*

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3.1 ABSTRACT

Sclerotinia sclerotiorum is a pathogenic fungus that infects hundreds of crop species, causing extensive yield loss every year. Chemical fungicides are used to control this phytopathogen, but with concerns about increasing resistance and impacts on non-target species, there is a need to develop alternative control measures. In the present study, we engineered *Brassica napus* to constitutively express a hairpin (hp)RNA molecule to silence *ABHYRDOLASE-3* in *S. sclerotiorum*. We demonstrate the potential for Host Induced Gene Silencing (HIGS) to protect *B. napus* from *S. sclerotiorum* using leaf, stem and whole plant infection assays. The interaction between the transgenic host plant and invading pathogen was further characterized at the molecular level using dual-RNA sequencing and at the anatomical level through microscopy to understand the processes and possible mechanisms leading to increased tolerance to this damaging necrotroph. We observed significant shifts in the expression of genes relating to plant defense as well as cellular differences in the form of structural barriers around the site of infection in the HIGS-protected plants. Our results provide proof-of-concept that HIGS is an effective means of limiting damage caused by *S. sclerotiorum* to the plant and demonstrates the utility of this biotechnology in the development of resistance against fungal pathogens.

3.2 INTRODUCTION

Fungal pathogens represent a worldwide threat to food security and cost producers billions of dollars in lost yield (Fisher *et al*. 2020). Traditionally, broad-spectrum agrochemicals have been used to limit the effects of these pathogens, but due to growing concerns of the impacts of these products on the agroecological environment as well as increasing incidences of fungicide resistance, there is a need to find more effective and sustainable solutions (Liu *et al*. 2013, Aoki *et al*. 2015, Derbyshire and Denton-Gilles 2016). Breeding for genetic resistance to fungal pathogens is one approach at reducing our dependence on traditional fungal control strategies, but for necrotrophic fungi, genetic resistance is difficult to achieve (Birkenbihl and Somssich 2011). Sclerotinia stem rot, caused by the fungal pathogen *Sclerotinia sclerotiorum,* is a disease that affects over 600 hosts, including *Brassica napus* (Liang and Rollins 2018). The complex lifecycle, which involves several years of dormancy within the soil, a broad host range and multifaceted infection of all phyllospheric components of the plant, provides numerous challenges for effective control (Laluk and Mengiste 2010, Liang and Rollins 2018). Since no truly resistant cultivars of *B. napus* have been identified against *S. sclerotiorum,* there is an immediate need to develop novel crop protection technologies to control this fungal pathogen (Wang *et al*. 2014, Neik *et al*. 2017, Ding *et al*. 2021).

S. sclerotiorum requires a senescing nutrient source in order to germinate ascospores. In *B. napus* infection, it is the flower petal that facilitates germination. The infection can spread to leaf tissue as the flowers fall off, where the fungus is able to penetrate and colonize the vasculature of the plant (Girard *et al*. 2017). Once inside the vasculature, the fungus travels laterally into the stem, penetrating the interior, and degrading the pith tissue, and ultimately causing lodging (Derbyshire and Denton-Gilles 2016). Due to the highly aggressive nature and speed of this infection, innate plant defense mechanisms are generally insufficient, and the host plant becomes overwhelmed by the fungus (Derbyshire and Denton-Gilles 2016). During their coevolutionary arms race, plants have developed a basal immune system that first detects pathogen-specific cell surface components through pattern recognition receptors (inducing pattern triggered immunity) and secreted pathogen-derived effectors by nucleotide-binding leucine rich repeat proteins (inducing effector triggered immunity) (Song *et al*. 2021). Detection of the fungal pathogen elicits downstream cellular responses including changes in hormone signalling, transcriptional

reprogramming, and defense gene activation (Pruitt *et al*. 2021, Yuan *et al*. 2021). In *B. napus*, pattern triggered immunity induces the synthesis of reactive oxygen species, changes in ion fluxes across cellular membranes, and increases in calcium (Ca^{2+}) concentrations within cellular spaces (Torres 2010, Meng and Zhang 2013, Seybold *et al*. 2014, Girard *et al*. 2017). Calcium ions act as messengers triggering several cellular responses including the synthesis of phytoalexins, pathogen-related (PR) proteins, and programmed cell death of neighbouring cells (Zurbriggen *et al*. 2010). Endogenous hormone changes further induce cell wall reinforcement through polysaccharide deposition such as callose around phloem sieve pores, activation of downstream defense genes and accumulation of secondary metabolites (Peltier *et al*. 2009, Chowdhury *et al*. 2014, Mattern *et al*. 2019). *S. sclerotiorum* does not exhibit a gene-for-gene response during interactions with the host unlike other *B. napus* pathogens such as *Leptosphaeria maculans* or *Plasmodiophora brassicae*, and therefore despite advances made in the understanding of the *B. napus – S. sclerotiorum* interaction, few control strategies using genetic tools have proven successful.

RNA interference (RNAi)-based technologies have emerged as a promising alternative control strategy for a growing number of fungal phytopathogens (Wang *et al*. 2016, Wytinck *et al*. 2020, Sang and Kim 2020). Given the sequence specificity of RNAi, application of these technologies could limit off-target effects (Christaens *et al*. 2018). RNAi-based crop protection technologies can be designed in two ways. First, through a topical approach where the double stranded RNA is applied as a spray (spray induced gene silencing, SIGS). Second, through genetic modification of the host plant to produce hairpin dsRNA molecules targeting the pathogen (host induced gene silencing, HIGS) (Sang and Kim 2020). Both methods have proven effective at limiting infections and disease symptoms of various fungal pathogens. SIGS is considered to be a more versatile technology, as the molecules can be applied to any host plant, they can be synthesized and produced in large quantities, and the technology may be more readily accepted in countries where genetically modified technologies are banned (Wang *et al*. 2018, McLoughlin *et al*. 2018). HIGS on the other hand, is considered a more durable technology that provides constitutive protection against the pathogen and limits the amount of fungicide applied to the crop (Ghag *et al*. 2017). HIGS can be tailored to provide a novel source of genetic control that specifically

targets the virulence of the pathogen, unlike many traditional engineering technologies, which instead aim to enhance the defense response of the plant (Qi *et al*. 2019, Niu *et al*. 2021).

HIGS was first demonstrated by Tinoco *et al*. (2010) by engineering tobacco to express a β-glucuronidase (GUS) hairpin to specifically silence GUS transcripts in a GUS-expressing strain of *Fusarium verticilloides*. HIGS has also proven effective in protecting plants against biotrophic fungi such as *Blumeria graminis* (Nowara *et al*. 2010), *Puccinia triticina* (Panware *et al*. 2013), *Fusarium oxysporum* (Ghag *et al*. 2014), hemibiotrophs such as *F. graminearum* (Koch *et al*. 2013) and necrotrophs such as *Botrytis cinerea* (Xiong *et al*. 2019) and *S. sclerotiorum* (Andrande *et al*. 2016). The mechanism behind HIGS, called cross-kingdom RNAi has been observed in plant-fungal interactions, where native small RNA molecules from the host plant are produced to minimize the virulence of the pathogen, or conversely, the pathogen can produce RNAs that reduce the ability of the plant to activate a defense response (Niu *et al*. 2021). For example, Weiberg *et al*. (2013) observed that *Botrytis cinerea* produces small RNA effectors that target and suppress host cellular immune machinery, and this transfer of small RNAs to the host could be inhibited by knocking down fungal *DICER-LIKE 1/2*. Cross-kingdom RNAi has also been observed in the *S. sclerotiorum-B. napus* pathosystem, where the host genes *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 2* and *SNAKIN-LIKE CYSTEIN RICH PROTEIN 2* are specific targets of *S. sclerotiorum* small RNAs (Derbyshire *et al*. 2019). Cai *et al*. (2018) demonstrated that *Arabidopsis thaliana* plants could, in contrast, protect themselves from *B. cinerea* by transferring small RNA molecules within extracellular vesicles to the fungus to silence virulence gene expression. Koch *et al*. (2020) confirmed that small RNAs derived from HIGS-based transgenes are also shuttled to invading fungi in this manner within the *A. thaliana-Fusarium graminearum* pathosystem. Thus, HIGS can exploit cross-kingdom RNAi to complement the other defenses within the plant against the pathogen.

While effective HIGS solutions have been developed for many economically significant pathosystems (Koch *et al*. 2013, Ghag *et al*. 2014, Xiong *et al*. 2019), one system that remains unreported is the *B. napus-S. sclerotiorum* interaction. The dsRNA-mediated knockdown of a predicted aflatoxin biosynthesis gene and pathogenicity factor, SS1G_01703 (*ABHYDROLASE-3*) proved to be highly effective at conferring plant protection using SIGS (McLoughin *et al*.

2018). Here, we hypothesize that this gene target would be a good candidate to develop a HIGS-based control system in *B. napus*. In the current study, we demonstrate that *B. napus* expressing hairpin (hp) RNAs targeting the *S. sclerotiorum ABHYDROLASE-3* gene (SS1G_01703) produce transgene-derived small RNAs and reduced the transcript levels of SS1G_01703. Restriction of *S. sclerotiorum* infection through knockdown of *ABHYDROLASE-3* together with global changes in plant defense resulted in improved plant health and seed set. HIGS provides a novel tool that works with the innate defense response of the host plant to reduce *S. sclerotiorum* infection in *B. napus* through a targeted RNAi approach.

3.3: MATERIALS AND METHODS

3.3.1 *B. NAPUS* GROWTH CONDITIONS

Seeds of *B. napus* cv. Westar and the resultant transgenic lines were germinated in germination pouches (Phyto AB Inc., San Jose, CA, USA) and transplanted at the cotyledon stage into Sunshine Mix No. 1 (SunGro Horticulture, Agawam, MA, USA) at 22 °C and 50–70% humidity within greenhouse conditions. The plants were grown until the 30% flowering stage for use in infection experiments.

3.3.2 PREPARATION OF *AGROBACTERIUM TUMEFACIENS*

Primers for cloning SS1G_01703 can be found in Table S3.1. 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 pENTR4 vector (Invitrogen, Carlsbad, CA, US) using T4 ligase (Invitrogen, Carlsbad, CA, US) according to the manufacturer's protocol. Prepared plasmids were used to transform *Escherichia coli* MachI cells (Thermo Scientific, Waltham, MA, US), selected using 50 mg/L chloroamphenicol (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 pHellsgate8 (Invitrogen, Carlsbad, CA, US). 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.

3.3.3 *B. NAPUS* TRANSFORMATIONS

Cotyledons were excised at the petiole of five-day old *B. napus* cv. Westar seedlings. We found that the petiole method of transformation, as described by Bhalla and Singh (2008) produced fewer false postitive transformants as compared to the hypocotyl transformation method (Jonoubi *et al*. 2005). The cut petioles were dipped into a solution of transformed *Agrobacterium tumefaciens* (O.D. of 0.7 at 600 nm). Generation of calli, leaves, shoots and roots on the explants was induced through media transfers. Briefly, infected explant petioles were placed into cocultivation media (1 × MS major salts, 1 ml MS minor salts, 2.9 ml CaCl2 stock, 1 ml KI stock, 1 ml vitamin stock and 20 g sucrose; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 100 µl AgNO3, 75 µl BAP (stock 1, 10 mg ml−1), 20 µl NAA and 5µl GA3) in the dark for two days. Explants were then transferred to callus induction media (1 × major salts, 1 ml minor salts, 2.9 ml CaCl2 stock, 1 ml KI stock, 1 ml vitamin stock and 20 g sucrose; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 100 µl AgNO3, 75 µl BAP (stock 1, 10 mg ml−1), 20 µl NAA, 5 µl GA3 and 500 mg carbenicillin) for one week in 16 hour daylight cycles. Explants were then transferred to shoot initiation media (1 × major salts, 1 ml minor salts, 2.9 ml CaCl2 stock, 1 ml KI stock, 1 ml vitamin stock and 20 g sucrose; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 100 µl AgNO3, 300 µl BAP (stock 1, 10 mg ml−1), 20 µl NAA, 5 µl GA3, 500 mg carbenicillin and 25 mg kanamycin) for four weeks with fresh media transfers every two weeks. Successful transformants (phenotypically green leaves and shoots) were then transferred to shoot outgrowth media (1 × major salts, 1 ml minor salts, 2.9 ml CaCl2 stock, 1 ml KI stock, 1 ml vitamin stock, 20 g sucrose, 40 mg adenine hemisulfate and 500 mg PVP 40,000; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 5 µl BAP (stock 2, 0.25 mg ml−1), 500 mg carbenicillin and 25 mg kanamycin) for two weeks. After two weeks, transformants were transferred to shoot outgrowth selection media containing higher kanamycin and lowered sucrose (1 × major salts, 1 ml minor salts, 2.9 ml CaCl2 stock, 1 ml KI stock, 1 ml

vitamin stock, 10 g sucrose, 40 mg adenine hemisulfate and 500 mg PVP 40,000; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 5 µl BAP (stock 2, 0.25 mg ml−1), carbenicillin 500 mg and kanamycin 50 mg). Transformants were then transferred to root initiation media (0.5 × major salts, 0.5 ml minor salts, 1.95 ml CaCl2 stock, 0.5 ml KI stock and 10 g sucrose; pH 5.8. Add 4 g phytagel, autoclave and add 100 µl filter-sterilized IBA stock solution) for four weeks. After root regeneration, the explants were transplanted to soil and allowed to grow and mature to produce T1 seed.

3.3.4 DNA EXTRACTION AND GENOTYPING

DNA was extracted from leaf samples of 7-day-old *B. napus* 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). DNA was precipitated with isopropanol, washed with 75% ethanol, and suspended in water.

PCR was performed using GoTaq® Green Master Mix (Promega, Madison WI, USA) according to the manufacturer's instructions using SS1G_01703 specific primers (Table S3.1) . Thermocycler conditions were as follows: 94°C for 2 min., 35 cycles of: 94°C for 1 min., 56°C for 30s., 72°C for 35s., final extension of 72°C for 5 minutes. PCR products were loaded on a 1% agarose gel containing ethidium bromide. 2µL of sample was loaded per well with FastRuler Middle Range DNA Ladder (Thermo Fisher) as reference to compare band size. Gels were run for 18 minutes at 100 volts and 200 mAh. Gels were visualized under UV light using an Axygen® Gel Documentation System (Axygen, Corning, NY, US).

3.3.5 COPY NUMBER DETERMINATION

The number of transgene copies present within T1 explants was determined via quantitative RT PCR using BNHMG I/Y as the endogenous reference gene as described in Weng *et al*. (2004) (Table S2). The cycle threshold values of the DNA of our transgene were compared to that of the endogenous reference gene through the formula:

$$X_{\circ}/R_{\circ} = 10^{((CT,X - IX)/SX) - ((CT,R - IR)/SR)}$$

Where X is the transgene, R is the endogenous control, I is the intercept of a standard curve dilution series for each primer set and S is the slope of the standard curve.

3.3.6 RNA ISOLATIONS

RNA was extracted from infected and uninfected plant tissue. For infected leaves, a circular punch (30 mm diameter) was taken to collect the necrotic lesion and surrounding leaf tissue. For infected stems, cross-section disks (10 mm wide) were taken from the site of infection. The tissue was ground in an RNase-free mortar and RNA extracted using the Qiagen RNeasy Plant Mini kit (Toronto, ON, Canada). RNA was treated with Qiagen RNAse free DNAse (Toronto, ON, Canada), then cDNA synthesized using Quantabio qScript cDNA (Beverly, MA, USA). Quantity and purity were assessed spectrophotometrically and the quality of RNA samples verified using electropherogram profiles and RNA Integrity Numbers (RIN) (All samples had a RIN greater than 8.5) with an Agilent 2100 Bioanalyzer and RNA Pico Chips (Agilent, Santa Clara, CA, USA).

3.3.7 RT qPCR

cDNA was diluted 1:40 in DNAse-free water for all qPCR reactions unless otherwise specified. *B. napus* and *S. sclerotiorum* primers were designed using Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) to PCR amplify gene fragments ranging between 180 and 200 bp in length and Primer3 (http://bioinfo.ut.ee/primer3/) was used to evaluate potential primers (Untergasser *et al*. 2012). A complete list of primers and their efficiencies can be found in Table S3.2. mRNA transcript abundance was 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. Relative accumulation was calculated using the ΔΔCq method, relative to Sac7 (SS1G_12350) for *S. sclerotiorum* and BNATGP4 (*BnaC08g11930D*) for *B. napus*.

3.3.8 LINEAR HAIRPIN VARIABLE RT-qPCR

Linear-hairpin primers were designed to target select small RNAs as described by Lan *et al*. (2019). SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, US) was used for

qRT PCR with conditions of 94 °C for 4 min, followed by 50 cycles of 94 °C for 30 s and 56 °C for 30 s. Primer sequences can be found in Tables S1 and S2.

3.3.9 LEAF INFECTION ASSAYS

S 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. Plants were infected when they reached the 30% flowering stage. *S. sclerotiorum* ascospores (1 x10^6 mL^{-1}) were suspended in a 0.02% Tween80 (Sigma-Aldrich, St. Louis, MO, USA) solution. 10 μL 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. The infected petal was placed onto the surface of a host leaf and sealed in a zip-lock bag to maintain humid conditions. After two days, lesion sizes were quantified using ImageJ software (imagej.nih.gov).

3.3.10 STEM INOCULATIONS

A 3 x 3 mm cube of actively growing *S. sclerotiorum* from the leading edge of hyphae on a potato dextrose agar plate was collected using a syringe tip and placed onto the stem of a mature *B. napus* plant 30-50 mm above the soil. The fungus was sealed to the stem using a 30 x 20 mm strip of parafilm and lesion length was measured seven days post inoculation.

3.3.11 WHOLE PLANT SPRAY INOCULATIONS

Plants were infected when they reached the 30% flowering stage. *S. sclerotiorum* ascospores (1 x10^6 mL^{-1}) were suspended in a 0.02% Tween80 (Sigma-Aldrich, St. Louis, MO, USA) solution. 25 mL of spore solution was sprayed onto the whole surface of each plant using a spray bottle. A 4x6 ft humidity chamber was constructed and covered with vinyl plastic sheeting. Once sprayed, the plants were placed within the humidity chamber and the chamber was heavily misted to maintain high humidity. The plants remained in the chamber for a seven-day period, at which point they were removed and the infection was allowed to continue to completion on the greenhouse benchtop.

3.3.12 mRNA LIBRARY SYNTHESIS, SEQUENCING AND BIOINFORMATIC ANALYSIS

RNA was extracted as described above and cDNA libraries were constructed by Genome Québec according to their mRNA stranded library protocol. The Illumina HISeq4000 was used to generate 100 bp PE reads. 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 40 nucleotides were also removed. The high sensitivity mapping program HISAT2 (Kim *et al*. 2019) was then used to align trimmed reads to the *B*. *napus* and *S*. *sclerotiorum* genomes (Derbyshire *et al*. 2017, Song *et al*. 2020). The mapped reads were sorted with Samtools (Li *et al*. 2009) and assigned to genes with featureCounts (Liao *et al*. 2014). EdgeR (Robinson and Oshlack 2010) was used to perform differential gene expression analysis on raw counts using a log2 fold change and FDR cutoff of $|2|$ and <0.01, respectively. All RNA-seq data exploration were performed in R studio. The Principal Component Analysis was carried out using plotPCA function in DESEq2 (Love *et al*. 2014) package. Stacked bar and Upset plots were generated in R (R Core Team, 2020, Conway *et al*. 2017). GO term enrichment was carried out using SeqEnrich to identify enriched processes and the genes associated with them (Becker *et al*. 2017).

All data have been deposited at the Gene Expression Omnibus (GEO): GSE 184812.

3.3.13 SMALL RNA LIBRARY SYNTHESIS, SEQUENCING AND BIOINFORMATIC ANALYSIS

Total RNA was extracted as described above and libraries were constructed by Genome Quebec. Briefly, the Illumina (San Diego, USA) TruSeq Small RNA Library Preparation kit was used to generate small RNA libraries from total RNA. The Illumina HISeq4000 was used to generate 100 bp PE reads. FastQ files were trimmed using Trimmomatic 0.36 (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 or under 18 bp removed during the trimming process. Bowtie was then used to align the SS1G_01703 transgene

to the small RNA dataset (Langmead *et al*. 2009) and Geneious Prime (2022) was used to map each aligned read to its location within the transgene sequence.

All data have been deposited at the Gene Expression Omnibus (GEO): GSE 192379

3.3.14 LIGHT MICROSCOPY

Tissues were embedded in historesin (Leica, Wetzlar, GER). Briefly, tissue was vacuum infiltrated in 2.5% glutaraldehyde/1.6% paraformaldehyde fixative. Methylcellosolve (Sigma-Aldrich, St. Louis, MO, USA) was used to remove pigment and three transfers of 100% ethanol every 24 hrs for three days was used to completely decolourize and dehydrate the tissue. Historesin was then infiltrated into the tissue during a three-day period of increasing concentrations of the resin. Day one was 1:3 activated historesin:100% alcohol, day 2 was 2:3 activated historesin:100% alcohol and day 3 was 100% historesin. The tissue was then embedded into blocks with an embedding solution of activated historesin (Leica, Wetzlar, GER), hardener (barbituric acid) and polyethylene glycol. Sections were cut at 3 μM and stained with lactophenol 'cotton' blue (to detect fungal chitin) and counterstained with 0.1% Safranin O for 20 minutes and 5 seconds respectively, aniline blue (to detect callose) and Periodic-Acid Schiff's (to detect polysaccharides) for 10 minutes and 15 minutes for both Periodic-Acid and Schiff's base respectively or 0.1% toluidine blue O (to detect lignin) and Periodic-Acid Schiff's for 15 minutes and 15 minutes for both Periodic-Acid and Schiff's base respectively. Hand sections of tissue were also cut and stained with Sudan IV (Sigma-Aldrich, St. Louis, MO, USA) (to detect suberin) for 20 minutes and cleared in deionized water. Slides were imaged using a Leica DFC450C camera. Three replicates (stems from individual plants) were viewed of both RNAi and untransformed lines and a representative image was published.

3.3.15 SCANNING ELECTRON MICROSCOPY

Stem cross-sections from 7-day old infections were collected and imaged immediately on the Hitachi TM1000 tabletop system using environmental SEM. Images were taken quickly after sealing the chamber, as to not allow the samples to dry out. Three replicates (stems from individual plants) were viewed of both RNAi line and untransformed and a representative image was published.

3.4: RESULTS

3.4.1 CONFIRMATION OF TRANSGENE EXPRESSION

B. napus cv. Westar was transformed to express a hpRNA targeting *S. sclerotiorum ABHYDROLASE-3* (SS1G_01703) under the control of a CaMV 35S promotor (Bn35S::SS1G_01703RNAi hereby referred to as BN1703) (Figure 3.1 A, Figure S3.1). *S. sclerotiorum ABHYDROLASE-3* is predicted to be involved in aflatoxin synthesis due to amino acid similarity to aflatoxin biosynthesis genes in related fungi (Table S3.3). Five different independent insertion lines were propagated for three generations. The transgene copy number in these lines, determined by qPCR of the transgene relative to the single copy gene, High Mobility Group protein *HMG-I/HMG-Y* (BNHMG I/Y, *BnaA06g33780D*) (Weng *et al*. 2003), ranged between two and three copies in each genome (Figure 3.1 B). We also examined the expression levels of the gene encoding the endonuclease *DICER-LIKE 2,* which has been shown to process hpRNAs derived from transgenes (Mlotshwa *et al*. 2008). qRT-PCR of *B. napus DICER-LIKE 2* (*BnaA05g32530D*) was 150 to 350 times more abundant in the transgenic lines compared to their untransformed counterparts (Figure 3.1 C). BN1703.2 demonstrated the highest abundance of DCL2A and transgene copies. To confirm that the hpRNAs were processed into small RNAs in BN1703.2, we sequenced small RNA populations genome-wide in uninfected and infected stems. Small RNAs specific to the SS1G_01703 transgene were indeed present prior to and during infection, none of these small RNAs were detected in untransformed plants. Within the pool of small RNAs aligning to the transgene, sequence reads aligning to specific regions of the transgene were found to be in greater abundance than others (Figure S3.2). Using linear-hairpin variable primer qRT PCR (Figure 3.1 D), the levels of one of these abundant siRNAs was quantified, and observed to be more abundant in the BN1703.2 lines, relative to the other lines (two times more abundant than in BN1703.1), and not detectable in the untransformed control (Figure 3.1 E). qRT-PCR analyses of SS1G_01703 transcript levels in both untransformed and BN1703.2 stem tissues revealed a strong and persistent reduction of SS1G_01703 transcripts in the BN1703.2 plants over the course of 7-days infection, compared to the untransformed plants (Figure 3.1 F). Thus, the successful expression of a hpRNAi transgene construct resulted in gene silencing of the *S. sclerotiorum ABHYDROLASE-3* transcript.

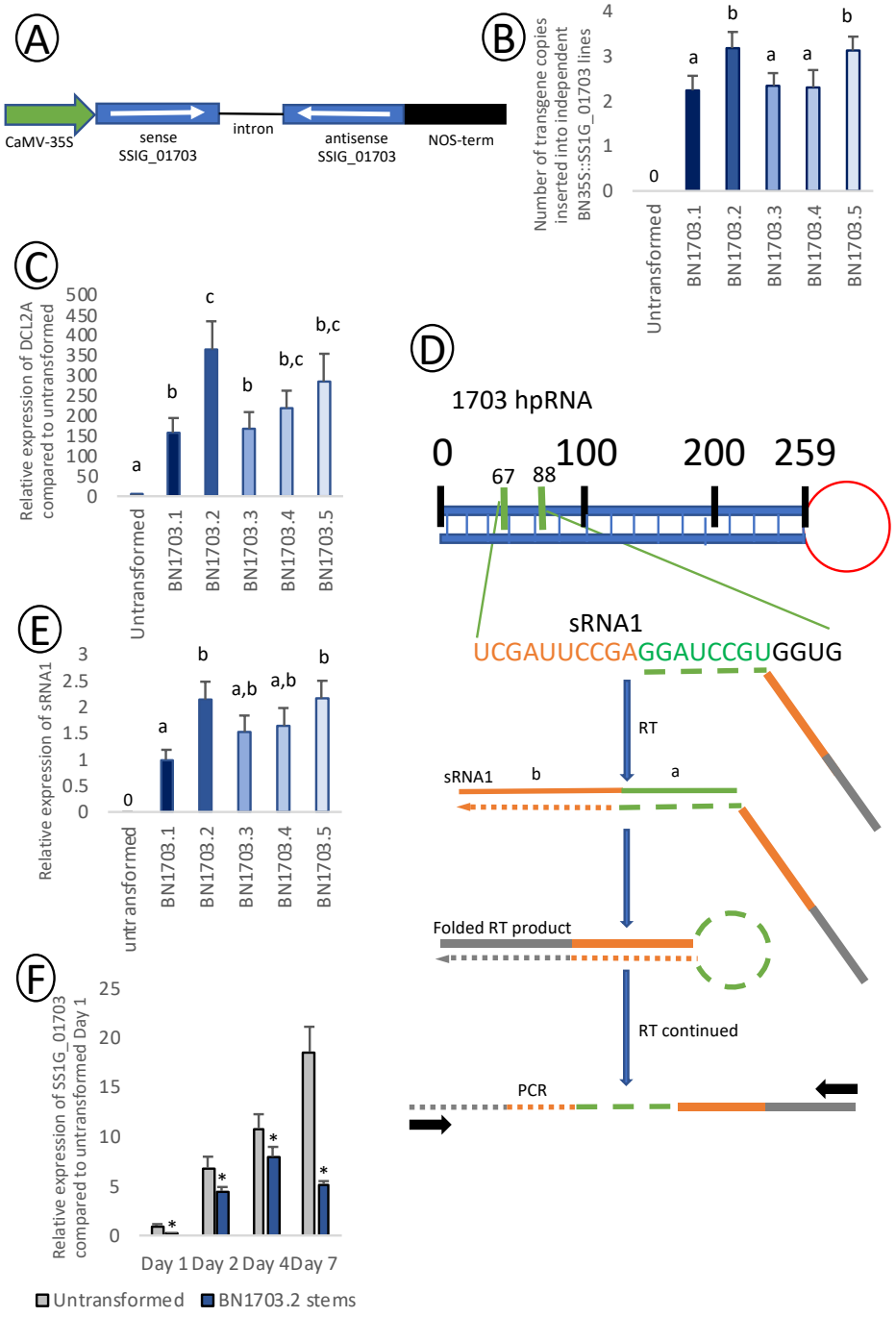

Figure 3.1. (A) Schematic map of the BN1703 RNAi cassettes within the pHELLSGATE8 vector. The intron-hairpin interfering cassette is under the control of the 35S promotor of Cauliflower mosaic virus. The 259 bp fragment of the *ABHYDROLASE-3* gene coding sequence from *S. sclerotiorum* (SS1G_01703) was directionally cloned to generate sense and antisense arms flanking an intronic spacer. NOS (nopaline synthase) provides selection against kanamycin. (B) Estimated number of BN1703 copies inserted into *B. napus* transformants relative to single

copy gene BNHMG I/Y. (C) Relative expression of BNDCL2A in *B. napus* transformants relative to untransformed plants and normalized to the housekeeping reference BNATGP4. (D) Schematic diagram of the amplification of sRNA1 from 1703 hpRNA using linear hairpin variable qPCR through the reverse transcribed extension of a hairpin primer. (E) Transcript abundance of sRNA1 using linear variable hairpin qPCR of *B. napus* transformants. Data for nucleic acid quantifications represents three biological replicates with error bars representing standard error. Levene's test was used to assessed the homogeneity of the variance of the data. Statistical differences calculated using ANOVA using the Bonferroni post-hoc test (with significance of $p<0.05$). (F) Relative expression of SS1G_01703 in infected untransformed and BN1703.2 *B. napus* stems 1, 2, 4, and 7 days post-inoculation demonstrating transcript knockdown within BN1703.2 stems. Asterisks represent statistical differences from the untransformed control (one-tailed t-test with Bonferroni correction, $p<0.05$ from three biological replicates).

3.4.2 HpRNA PRODUCTION IN *B. NAPUS* HOST TARGETING SS1G_01703 SIGNIFICANTLY REDUCES *S. SCLEROTIORUM* INFECTION

Next, we challenged BN1703 lines with *S. sclerotiorum* using a petal inoculation method on leaf tissues (Girard *et al*. 2017) and the mycelial plug method on stems (Roy and Mendoza 2021) (Figure 3.2 A). Within the T2 and T3 generations, reductions in lesion sizes were observed in both stem and leaf infection assays (Figure S3.3 A). In addition to highest copy number (Figure 3.1 B), DCL2A (Figure 3.1 C) and sRNA1 abundance (Figure 3.1 D), BN1703.2 also showed the greatest reduction in fungal lesion size (Figure 3.2 B). Line BN1703.2 also accumulated significantly less fungal rDNA in the stem over the course of infection compared to the untransformed control (Figure 3.2 C). Lactophenol 'cotton' blue staining, which detects fungal chitin (Shamly *et al*. 2014), was used to examine plant stem infections of *S. sclerotiorum*. Transverse sections of untransformed plant stems demonstrated that the fungus successfully colonized the interior pith region 7 days post inoculation. Lactophenol staining within the secondary xylem indicated radial infiltration of the fungus along medullary rays in the untransformed plants (Figure 3.2 D). Conversely, the *S. sclerotiorum* infiltration within the BN1703.2 plants does not proceed into the secondary xylem at 7dpi as observed in untransformed plants, and instead is observed no further inward than the cortex and secondary phloem. (Figure 3.2 E).

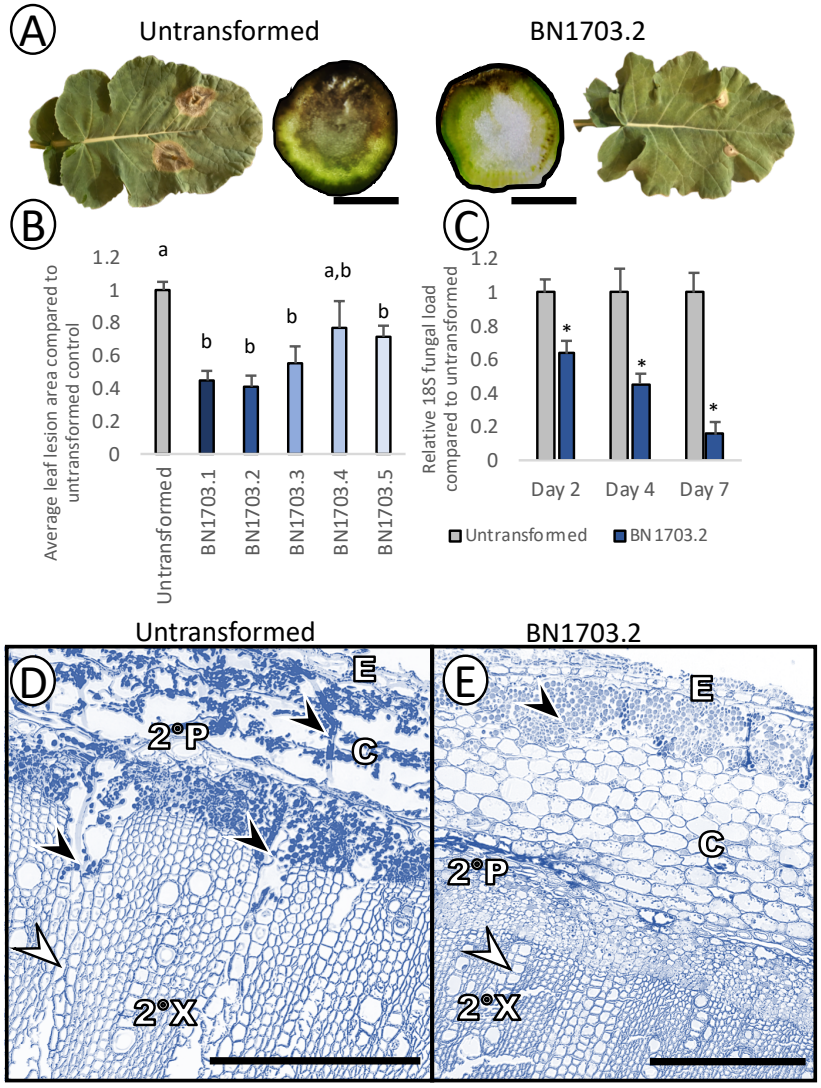

Figure 3.2. (A) Representative images of infected untransformed and BN1703.2 *B. napus* leaves and stem cross-sections 3 and 7 days post-inoculation respectively. Scale bars represent 1 cm for stem cross sections. (B) Relative leaf lesion area of infected *B. napus* transformants compared to untransformed leaf lesions. Data represents at least ten leaf lesions per line with error bars representing standard error. Levene's test was used to assessed the homogeneity of the variance of the data. Statistical differences were tested with a one-way ANOVA with a Bonferroni post-hoc test (with significance of $p<0.05$), where significant differences are denoted with differing letters. (C) Relative fungal load of infected stem lesions 2, 4, and 7 days post inoculation. 18S rDNA abundance was quantified for untransformed and T2 BN1703.2 stem lesions. Asterisks represent statistical differences from the untransformed control (one-tailed t-test with Bonferroni correction, $p<0.05$ from 3 biological replicates). Lactophenol blue staining of stem transverse-sections 7 days post-inoculation of untransformed (D) and BN1703.2 (E) stems. Untransformed stems show extensive colonization and degradation of the epidermis (E), cortex (C) and secondary phloem (2°P) and penetration into the secondary xylem (2°X) through medullary rays.

BN1703.2 stems show colonization of the epidermis (E) and cortex (C), although they remain intact. Secondary phloem (2°P) and xylem (2°X) remain uncolonized. Black arrowheads indicate *S. sclerotiorum* hyphae highlighted by lactophenol blue stain and white arrowheads indicate medullary rays. Scale bars represent 250 μm and micrographs are representative images of at least three biological replicate stems.

3.4.3 HOST INDUCED GENE SILENCING OF SS1G_01703 CONFERS TOLERANCE TO *S. SCLEROTIORUM* ACROSS THE PLANT LIFECYCLE

Quantification of the SS1G_01703-specific siRNAs confirmed that the transgene was expressed and processed into the hairpin siRNA in flowers, leaves and stems in uninfected BN1703.2 plants, the three prominent locations of *S. sclerotiorum* infection on *B. napus* (Figure 3.3 A). *B. napus* plants at the 30% flowering stage were sprayed with a concentrated ($1x10^6$ spores/mL) solution of *S. sclerotiorum* ascospores, coating the whole plant, and disease progression was surveyed over 7 days under 90-100% humidity. After 7 days, untransformed plants were more affected by *S. sclerotiorum*. Petal forming lesions and total sclerotia were reduced by 70 and 60 percent respectively in infected BN1703.2 plants compared to the untransformed counterparts. Finally, total seed mass of the plants after maturation increased three-fold in line BN1703.2 compared to that in untransformed (Figure 3.3 B). In untransformed plants, most leaves became necrotic and the stem was weakened by fungal infection at several sites (Figure 3.3 C). In contrast, while lesions were present on leaves and stems of line BN1703.2, these plants showed less necrosis and were able to continue through to maturation (Figure 3.3 C).

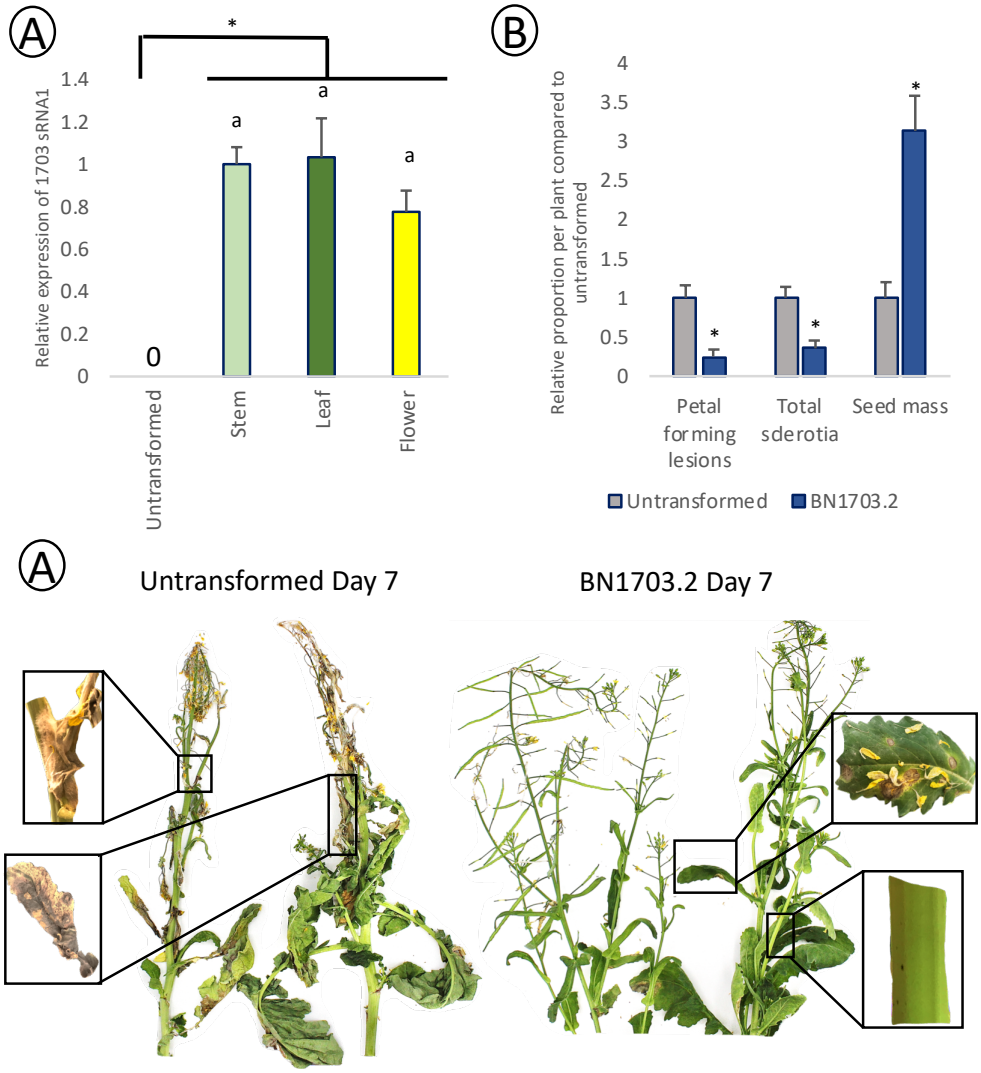

Figure 3.3. (A) Relative abundance of sRNA1 in BN1703.2 uninfected stems, leaves and flowers to demonstrate transgene expression in these organs. Levene's test was used to assessed the homogeneity of the variance of the data. Statistical differences were tested with a one-way ANOVA with a Games-Howell post-hoc test (with significance of $p<0.05$), where significant differences are denoted with differing letters. Error bars represent standard error. (B) Total petal forming lesions per plant were quantified after 7 days of incubation in a humidity chamber after spraying. Plants were then allowed to mature fully before total sclerotia and seed mass were quantified per plant. Data represents at least ten plants per cultivar and asterisks represent statistical differences from the untransformed control (one-tailed t-test with Bonferroni correction, $p<0.05$). (C) Representative images of ascospore spray infected untransformed and transgenic BN1703.2 plants after seven days of incubation in a humidity chamber after spraying. Untransformed plants show extensive infection on flowers and leaves and several sites of lesion progression into the stem. BN1703.2 plants show infection of flowers and leaves however, minimal lesion progression upon the leaves and stem.

3.3.4 RNA SEQUENCING OF HIGS-BASED PLANT PROTECTION

To better understand how both transformed and untransformed *B. napus* respond to *S. sclerotiorum*, we profiled global gene activity in the stem at the plant-pathogen interface (Figure 3.4 A). RNA sequence reads were mapped to the reference genomes of *B. napus* cv. Westar (Song *et al*. 2020) and *S. sclerotiorum* (Derbyshire *et al*. 2017) and differentially expressed genes (DEGs) compared to day 0 of the uninfected stems. We identified 37778 *B. napus* DEGs throughout the experiment (21405 upregulated and 16373 downregulated). The most substantial transcriptional response in terms of differential expression occurred at 1 dpi in BN1703.2 (6654 upregulated and 5692 downregulated) and the most minor transcriptional response occurred at 7 dpi also within BN1703.2 (3787 upregulated and 2388 downregulated). In total, we identified 1594 shared DEGs upregulated within all four comparisons (darkest shaded bar), indicating a common, coordinated response regardless of host genotype or stage of infection.

Principal component analysis of detected transcripts in *B. napus* grouped samples based on the timing of infection and genotype suggesting both genotypes underwent significant shifts in transcript abundance in response to infection (Figure S3.4 A). *S. sclerotiorum* reads clustered based on timing of infection (Figure S3.4 B). To determine whether gene sets were induced specifically by either genotype or time of infection, we identified *B. napus* DEGs induced by all potential combinations of patterns (Figure 3.4 B). This analysis demonstrated that coordinated DEG sets upregulated between both genotypes at 1 dpi and common DEG shared between both genotypes at both 1 dpi and 7 dpi were most abundant of all tested combinations. However, specific DEGs to BN1703.2 at 1 dpi were the next most abundant, and therefore encompassed the most unique DEGs of any individual comparison. Upregulated DEGs shared between untransformed and BN1703.2 at 7 dpi were the least abundant of all possible combinations, thus indicating that the responses of these genotypes are vastly different later in infection. Taken together, BN1703.2 had the strongest transcriptional response to *S. sclerotiorum* at 1 dpi.

Next, to investigate biological processes, molecular functions, and cellular components contributing to the *B. napus* defense response, we performed Gene Ontology (GO) term enrichment on all upregulated differentially expressed gene sets (Becker *et al*. 2017) (Figure 3.4 C). GO terms were considered significantly enriched if the hypergeometric P-value was <0.001.

We aimed to uncover GO terms that were uniquely enriched within each genotype and timepoint in addition to those terms that were shared between all timepoints and genotypes. For example, biological processes such as toxin catabolism, systemic acquired resistance through salicylic and jasmonic acid signalling, production of glucosinolates and phytoalexins, callose and suberin deposition in addition to fungal cell wall degradation were all enriched within the BN1703.2 and untransformed plant gene sets relative to uninfected plants (Figure 3.4 C). We then compared the transcript abundance values for each of the individual genes encompassed by these biological processes GO terms between untransformed and BN1703.2. Interestingly, while many of these genes are differentially expressed during both untransformed and BN1703.2 infections, we see large quantitative differences in gene activity, especially at 1 dpi (Fig. 3.4 D). In BN1703.2, there was greater transcript accumulation of pathogenesis related proteins (*PR1-PR5)*, 1.5-4-fold increase in abundance, PR proteins are induced as part of the systemic acquired resistance response. Additionally, a suite of chitinases and endogluconases responsible for fungal cell wall degradation, in particular *CHB4* and *BETA 1,3 ENDOGLUCONASE,* were overrepresented within the transgenic plants (2.5-4.5-fold increase). SA and SA-mediated signalling has been shown to play a significant role in early defense responses against *S. sclerotiorum* in *B. napus* (Novakova *et al*. 2014). Genes associated with the generation of SA-activated defense molecules such as camalexin and scopoletin (*FERULOYL COA ORTHO-HYDROXYLASE*, *WRKY TRANSCRIPTION FACTOR 70*), two excreted secondary metabolites with antifungal properties, also showed increased activity in BN1703.2 plants at 1 dpi (1.5-2.5-fold increase). By 7 dpi, this trend had reversed, with untransformed plants showing higher levels of defense gene transcripts as the infection progressed. The pathogenesis-related protein, *PR1*, (*BnaC03g45470D*), is another common marker for salicylic acid signalling. The increases in transcript levels of *PR1* in the RNA-seq dataset reflect closely to the qRT-PCR analyses which showed a 1.5 to 2-fold increase in transcript accumulation of this gene 1-4 dpi in BN1703.2 plants compared to untransformed plants (Figure S3.3 B). In uninfected stems, we observed no difference in *PR1* expression between BN1703.2 and untransformed plants through qRT-PCR providing evidence for no premature transcriptional activation of plant defense responses to the dsRNA transgene insertion (Figure S3.3 B).

Next, we examined global gene activity in *S. sclerotiorum* on both transformed and untransformed *B. napus* (Figure S3.5). At day 1 post infection, significantly fewer transcripts encoding plant cell wall degrading enzymes, including cellulases, endo-glucanases, exo-glucanases and beta-glucanases were 50-80% less abundant while hemi-cellulases such as endo-beta-xylanases, beta-mannosidases, alpha-xylosidases, alpha-galactosidases and alpha-I-arabinofuranosidases were 90% less abundant in BN1703.2 compared to their untransformed counterparts. The expression of specific proteases such as *CALPAIN FAMILY CYSTEINE PROTEASE* and *SUBTILISIN-LIKE SERINE PROTEASE,* involved in the degradation of pathogenesis related proteins and the suppression of host plant immunity, were 2-3 fold higher in BN1703.2 at 1 dpi compared to the same point in the untransformed control stems of *B. napus*. Genes involved in detoxification, particularly glutathione S-transferases, were also enriched 1.5-2-fold within transgenic plants compared to untransformed plants at 1 dpi. We observe a similar trend at 7 dpi with the gene activity of *S. sclerotiorum* within BN1703.2 infections as we did with the gene activity of *B. napus*. As the infection progresses, there is a reduction in the quantitative differences observed between BN1703.2 and untransformed infections.

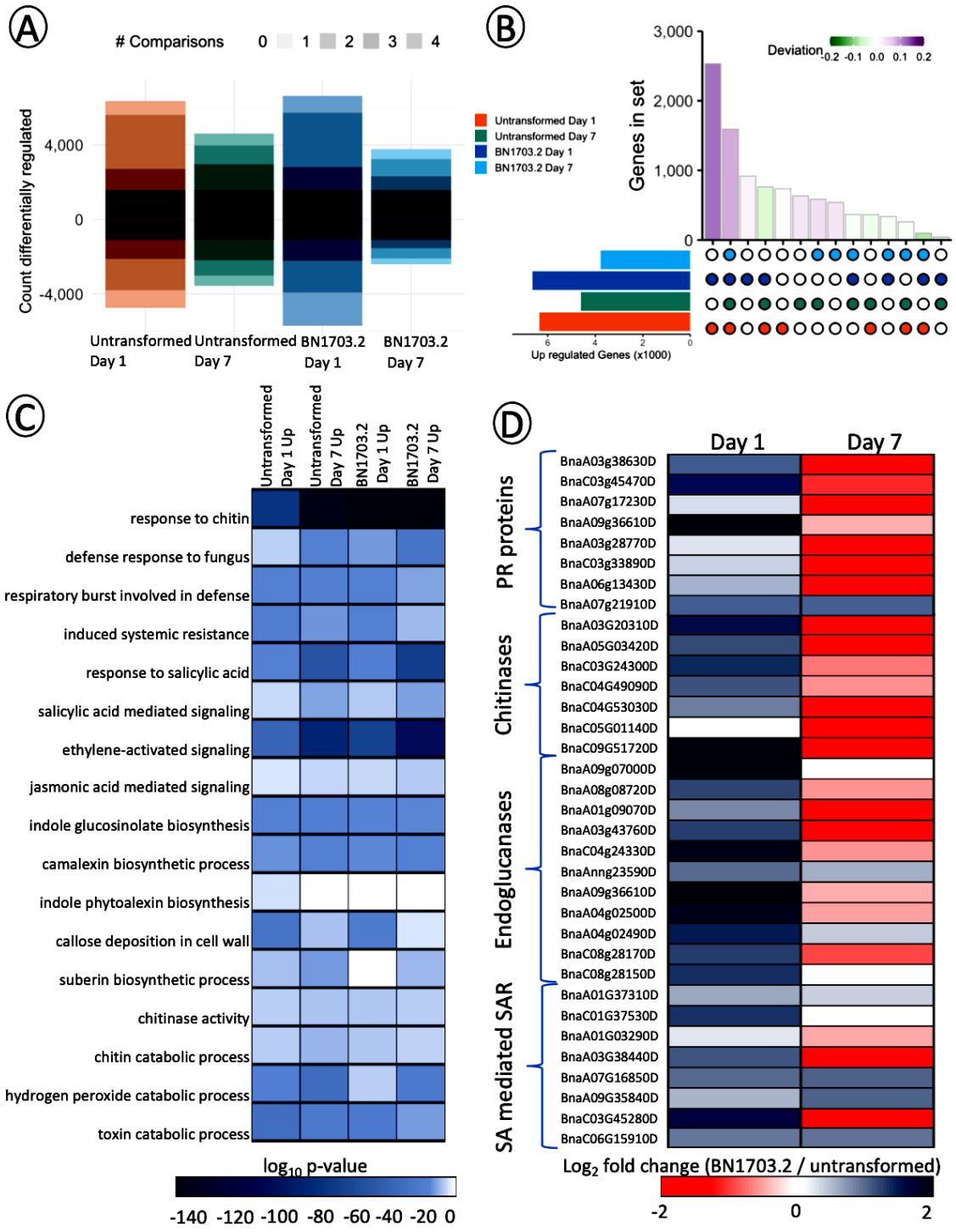

Figure 3.4.(A) Stacked bar plot displaying the number of *B. napus* genes differentially expressed in untransformed and transgenic BN1703.2 infected stems 1 and 7 days post-inoculation and compared to the uninfected control (false discovery rate<0.01). Darker shades of color represent higher numbers of patterns shared between treatments. (B) Upset diagram showing the sizes of gene sets induced by each combination of patterns. The bars for the set sizes are coloured by the deviation from the size predicted by random mixing. (C) Heatmap of significantly enriched GO

terms of untransformed and BN1703.2 days 1 and 7 compared to uninfected from total DEG. GO terms are considered statistically significant if the hypergeometric P-value<0.001. (D) Heatmap of a subset of genes encompassing significantly enriched GO terms within BN1703.2 during infection. GO terms are considered statistically significant if the hypergeometric P-value<0.001.

3.4.5 ACTIVATION OF STRUCTURAL DEFENSE PATHWAYS IN BN1703.2 IN RESPONSE TO *S. SCLEROTIORUM* INFECTION

Transcripts of each of the ten *B. napus* genes encoding suberin biosynthesis enzymes were in low abundance in BN1703.2 plants at 1 dpi and increased between 2-7-fold higher than their untransformed counterparts by 7 dpi (Figure 3.5 A, Table S3.4). Suberin is an extracellular polymer component of the so-called vascular coating within stems and roots, providing strength, structural support and a possible barrier to pathogen entry (Figure 3.5 B). Scanning electron microscopy of infected BN1703.2 plant stems revealed a band running parallel to the length of the infection site on the stem surface within the secondary xylem (Figure 3.5 D). In BN1703.2 plants, the fungus did not appear to cross this band, whereas in untransformed plants, the fungus had heavily colonized the pith of the stems (Figure 3.5 C). Interestingly, we were also able to visualize a similar structure within infected stem cross-sections of the semi-resistant cultivar, *B. napus* cv. ZhongYou 821 (Figure S3.6 A). To gain insight into the composition of this vascular coating, we used a lipophilic stain (Sudan IV) to detect the presence of lipids such as suberin within infected stems of BN1703.2 and untransformed plants. In untransformed transverse hand-sections, there is sparse accumulation of the stain within the secondary xylem (Figure 3.5 E). In contrast, within BN1703.2 stems, we detected heavy deposits of Sudan IV stain localized within the xylem in a similar location to the electron dense band pattern observed using SEM (Figure 3.5 F). Staining transverse sections of BN1703.2 plants with Periodic Acid-Shiffs and toluidine blue did not, however, highlight the electron-dense ring observed in the SEM sections, which suggests that the ring observed is not composed predominantly of polysaccharides or lignins (Figure S3.6 B).

Six genes associated with callose production (*CALLOSE SYNTHASES 3* and *7)* showed a 1.5-2-fold increase in transcript accumulation in BN1703.2 (Figure 3.5 A). Aniline blue was used to detect callose deposition within the secondary phloem. Within the BN1703.2 line, there appears to be abundant deposition of callose within the secondary phloem, particularly around the

medullary ray regions proximal to the vascular cambium (Figure 3.5 H). Untransformed stems did not show the same pattern of deposition (Figure 3.5 G). Further, lumens of the sieve tube members proximal to the vascular cambium accumulate callose in BN1703.2 stems, which is not seen in the untransformed stems. Suberin and callose deposition appears to be a specific response to infection, as uninfected plants, whether transgenic or untransformed, showed no differences in the accumulation of these components (Figure S3.6 C-H).

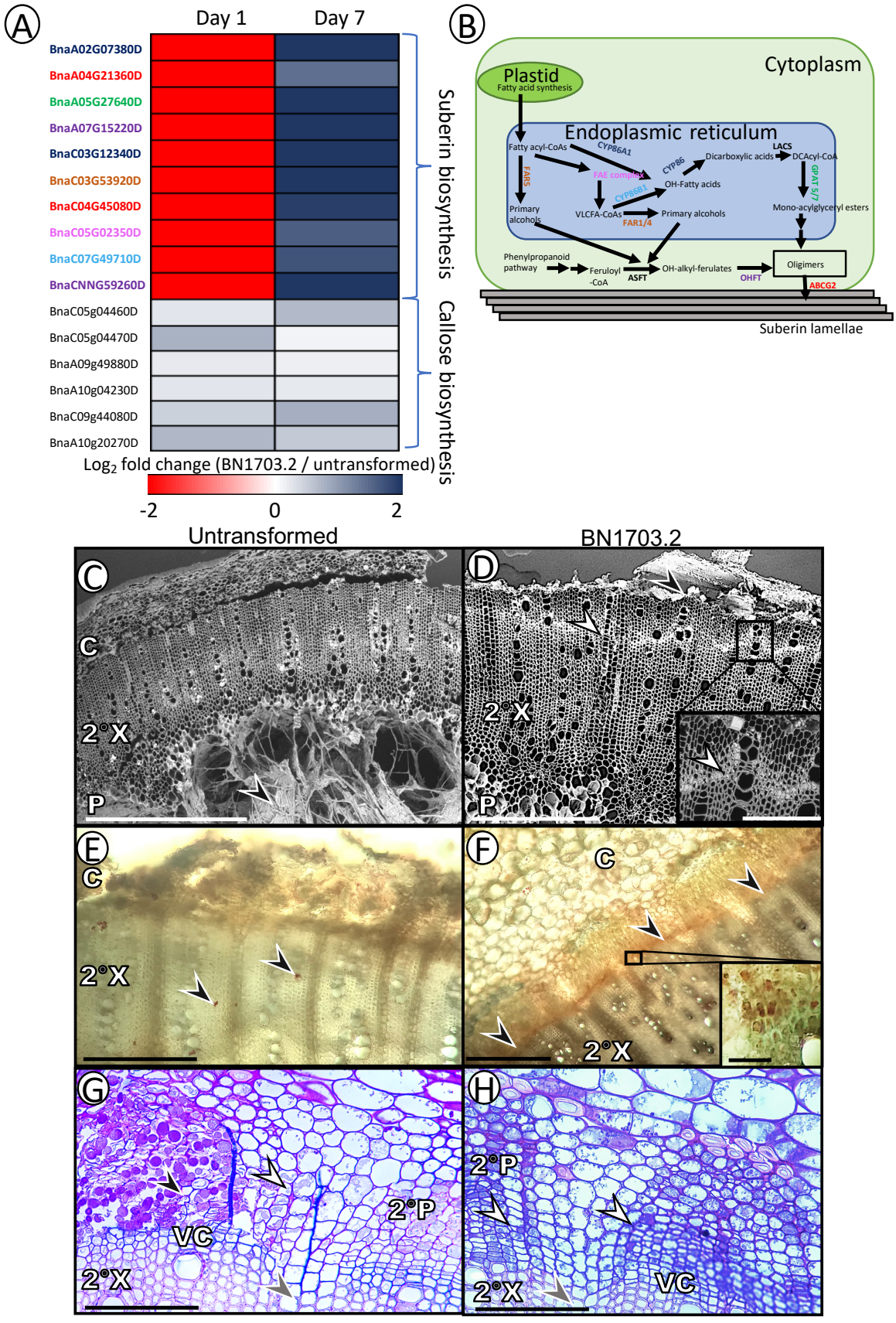
A
Day 1
Day 7
BnaA02G07380D
BnaA04G21360D
BnaA05G27640D
BnaA07G15220D
BnaC03G12340D
BnaC03G53920D
BnaC04G45080D
BnaC05G02350D
BnaC07G49710D
BnaCNNG59260D
BnaC05g04460D
BnaC05g04470D
BnaA09g49880D
BnaA10g04230D
BnaC09g44080D
BnaA10g20270D
Suberin biosynthesis
Callose biosynthesis
Log2 fold change (BN1703.2 / untransformed)
-2
0
2
B
Cytoplasm
Plastid
Fatty acid synthesis
Endoplasmic reticulum
Fatty acyl-CoAs
CYP86A1
FAE complex
CYP86
Dicarboxylic acids
LACS
DCAcyl-CoA
FAR5
CYP86B1
OH-Fatty acids
GPAT 5/7
Primary alcohols
VLCFA-CoAs
FAR1/4
Primary alcohols
Mono-acylglycerol esters
Phenylpropanoid pathway
Feruloyl -CoA
ASFT
OH-alkyl-ferulates
OHFT
Oligimers
ABCG2
Suberin lamellae
Untransformed
BN1703.2
C
2°X
P
D
2°X
P
E
C
2°X
F
C
2°X
G
2°P
VC
2°X
H
2°P
2°X
VC

Figure 3.5. (A) Heatmap of genes involved in suberin and callose biosynthesis, which were enriched GO terms for BN1703.2. GO terms are considered statistically significant if the hypergeometric P-value<0.001. (B) A diagram of the biosynthesis pathway of suberin in plant cells. The enzyme font color corresponds to the *B. napus* gene ID in the heatmap in (A). Scanning electron micrographs of infected *B. napus* stems 7 days post-inoculation in untransformed (C) and BN1703.2 (D) genotypes. The untransformed stem shows extensive colonization throughout, particularly the pith (P) region. The BN1703.2 stem shows no colonization of the pith and a vascular coating within the secondary xylem (2°X). Scale bars represent 1 mm for untransformed and 0.5 mm and 50 μm for BN1703.2. Black arrowheads indicate Sclerotinia hyphae and white arrowheads indicate the vascular coating. Hand cut stem transverse sections stained with Sudan IV of untransformed (E) and BN1703.2 (F) stems 7 days post-inoculation. Red staining indicates deposition of suberin. The untransformed stem shows sparse Sudan staining while BN1703.2 has extensive deposition within the 2°X. Black arrowheads indicate Sudan IV stain and scale bars represent 150 and 50 μm for the lower and higher magnification respectively. Micrographs of stem transverse sections 7 days post-inoculation of untransformed (G) and BN1703.2 (H) stems stained with aniline blue. Aniline blue stains callose depositions dark blue-purple with the Periodic Acid-Schiff's reagent stain staining plant and fungal cell walls bright pink. Untransformed stems show low levels of aniline blue stain in the secondary phloem (2°P) while BN1703.2 stems show heavy staining. Black arrowheads indicate Sclerotinia hyphae, white indicate callose, empty arrowheads indicate the vascular cambium and grey indicate the medullary ray. Scale bars represent 50 μm. All micrographs are representative of at least three biological replicates showing similar phenotypes.

3.5: DISCUSSION

Fungal control using RNAi-based strategies, and in particular, those using HIGS, provides a genetic tool to control fungal disease in crop plants (McLoughlin *et al*. 2018, Sang and Kim 2020). Here, we report HIGS control for *S. sclerotiorum* in *B. napus*. The *ABHYDROLASE-3* gene (SS1G_01703) was selected as a target for HIGS based on the previous work of McLoughlin *et al*. (2018), which showed no orthologous gene targets in *B. napus* and knockdown of this target through foliar applications reduced *S. sclerotiorum* disease severity. While foliar applications of dsRNAs have shown promise in this pathosystem, a HIGS approach was hypothesized to confer more durable protection across the lifecycle of *B. napus*.

For HIGS to be effective, cross-kingdom trafficking must occur early in the infection, before it becomes systemic and overwhelms the plant (Koch *et al*. 2021, Secic and Kogel 2021). Ghag *et al*. (2014) postulate that host-derived mobile signals such as small RNAs can gain entry into the fungal cytosol and induce gene silencing as cellular components between the host and pathogen are exchanged during the preceding biotrophic phase. The multistep lifecycle of *S. sclerotiorum* on *B. napus* provides opportunities for transgene-derived siRNA to be absorbed prior to necrotrophy. Wang *et al*. (2016) and Wytinck *et al*. (2020) have shown that fungal spores and emerging germ tubes in *B. cinerea* and *S. sclerotiorum* respectively, readily take in dsRNAs and siRNAs. Therefore, transgene-derived siRNAs may enter the fungal cell during the earliest stages of its lifecycle in planta. This early knockdown may lead to enhanced protection as the fungus transitions to necrotrophy and the infection cycle progresses. Our gene expression data for *S. sclerotiorum* support that HIGS took effect within the first 24 hours of infection. We observe large shifts in gene expression between fungus within the untransformed and BN1703.2 lines, specifically with genes relating to necrotrophy. Overexpression of cell wall degrading enzymes is characteristic of the fungal necrotrophic phase (Seifbarghi *et al*. 2017) and we observe significant reductions in the expression of genes responsible for cell wall degradation in BN1703.2 infections at 24 hpi. We also observe heightened expression of genes relating to the suppression of plant host defenses within BN1703.2 infections, which a characteristic of biotrophy (Seifbarghi *et al*. 2017). This may indicate that the transition to necrotrophy has been delayed or the strength of necrotrophy has been reduced. In our study, we did not observe a visible phenotypic effect induced by the knockdown of the pathogenicity factor,

ABHRDROLASE-3, in *S. sclerotiorum*. However, Song *et al*. (2018) observed strong morphological changes in fungal hyphae induced by the application of exogenous dsRNA targeting *MYOSIN5* in *F. asiaticum, F. graminearum, F. tricinctum* and *F. oxysporum*. These changes may be attributed to the essential role *MYOSIN5* plays in hyphal growth and function (Zheng *et al*. 2015), compared to *ABHYDROLASE-3*, which only is involved in infection. This does not preclude a potential molecular phenotypic effect upon interacting protein partners or downstream targets and this is currently being investigated.

While cross-kingdom transfers of processed small RNAs between host plants and fungal pathogens have been described (Cai *et al*. 2018, Koch *et al*. 2020), there exists the possibility that unprocessed long hpRNAs are being taken up by the fungus in a yet to be described mechanism. In transgenic maize expressing DvSnf7, sufficient quantities of un-diced, long dsRNA were sufficient to control the western corn rootworm, *Diabrotica virgifera* (Bolognesi *et al*. 2012). In that study, only long dsRNAs greater than 60 base pairs were shown to enter the gut cells of the insects to induce RNAi, while 21 bp siRNA molecules did not show knockdown of the target mRNA. In fungi, multiple studies have shown that both long dsRNAs and siRNAs can induce target mRNA knockdown. For example, in *B. cinerea*, both exogenous *DCL1, DCL2* dsRNA and siRNA caused similar levels of transcript knockdown in vitro, while siRNAs designed against *CHITIN SYNTHASE* in *Macrophomina phaseolina* were effective at suppressing fungal growth and inducing significant gene knockdown *in vitro* (Wang *et al*. 2016, Forster and Shuai 2020). However, while reduction in accumulation of target gene transcript provides an indication of RNAi-based silencing, examination of the degradome would be required to provide direct evidence for target cleavage.

While our initial hypothesis was that the constitutive knockdown of *ABHYDROLASE-3* would be sufficient to cause a reduction in *S. sclerotiorum* disease progression and infection in *B. napus*, our RNA-seq analyses and histological examination of the infected tissues suggested that other cellular defense systems were also playing a role in the observed phenotype in BN1703.2 plants. BN1703.2 host defense pathways appear to play significant roles in host plant protection through quantitative upregulation of PR proteins, salicylic acid-mediated signalling and defense, fungal cell wall degrading enzymes and the production of secondary metabolites, compared to

untransformed plants early in infection. Accumulation of defense-related mRNAs appears to be a common response within tolerant or resistant genotypes of *B. napus* in response to different pathogens. For example, the *P. brassicae* resistant *B. napus* cultivar, Laurentian, activates salicylic acid-mediated defense responses, thaumatins, and PR proteins (Galindo-Gonzalez *et al.* 2020). Additionally, the over-expression of *MITOGEN ACTIVATED PROTEIN KINASE 4 SUBSTRATE 1* in *B. napus* confers tolerance to *L. maculans* and accumulates transcripts associated with salicylic acid mediated defense (Zou *et al.* 2021). Taken together, initial knockdown of a fungal mRNA facilitated by HIGS enhances innate defense of the host plant and provides additional levels of protection against pathogen infection over time.

Inducible anatomical barriers formed at or around the xylem of the host plant provide an additional level of protection that prevents the movement of the advancing fungus (Kashyap *et al.* 2021). Traditionally, these barriers have been observed in plants infected by vascular wilt pathogens such as *Fusarium sp.* and *Verticillium sp.* (Xu *et al.* 2011, Bu *et al.* 2014, Seo *et al.* 2017, Novo *et al.* 2017). Here, we observed both horizontal and vertical restrictions within cross-sections of *S. sclerotiorum* infected stems seven days post infection. However, horizontal restrictions through the formation of the vascular coating and deposition of callose are a structural barrier more likely to prevent radial mycelial movement towards the pith. Callose has been described as both a structural barrier against fungal pathogens and a cellular matrix that can accumulate antimicrobial compounds (Luna *et al.* 2011). The deposition of callose in BN1703.2 like slows progression of *S. sclerotiorum* into medullary rays. Furthermore, the electron dense band running parallel to the site of infection likely serves as a second horizontal restriction coating cells of the secondary stem tissues. Vascular coatings occur at vessel walls, parenchyma cells and pit membranes to reinforce and create a dense, amorphous layer (Kashyap *et al.* 2021). The composition of the coating varies, depending on the host and pathogen, but phenolics are the primary compounds, and phenolic polymers like lignin or suberin are the principal multi-molecular components (Eynck *et al.* 2009). Upregulation of the entire suberin biosynthesis pathway within BN1703.2 stems together with positively staining Sudan IV regions in the xylem, suggests that the cellular coating is at least partially suberized. Suberin is a complex polyester biopolymer consisting of polyphenolic and polyaliphatic domains, and it is not normally produced within the stem (Pouzoulet *et al.* 2017). However, in many plant species, it

has been shown that suberin production is induced during pathogen attack (Pouzoulet *et al*. 2017). The deposition of suberin likely acts as a cellular obstruction to pathogen-derived metabolites such as pathogenicity factors and cell wall degrading enzymes, while still allowing plant-derived defense factors to be secreted (Araujo *et al*. 2014). A vascular coating was also observed within *B. napus* cv. ZY821, a moderately tolerant cultivar to *S. sclerotiorum*. Its tolerance is controlled through the activation of glucosinolate biosynthesis, which slows pathogen progression. It is possible that the formation of this vascular coating in *B. napus* in response to *S. sclerotiorum* infection is facilitated by the diminished pathogen advancement induced by the hpRNA or glucosinolate production.

Taken together, this study provides evidence that HIGS-based control of *S. sclerotiorum* in *B. napus* contributes to and complements innate cellular defense mechanisms of the host plant to reduce disease symptoms. Our results show that the reduction in disease symptoms was not solely the result of a hpRNA targeting *ABHYDROLASE-3* of *S. sclerotiorium*. Instead, activity of the hpRNA appears to weaken the ability of *S. sclerotiorum* to infect the host and provides *B. napus* with the necessary time required to activate innate global defense gene activity. While this approach was successful in protecting *B. napus* against *S. sclerotiorum*, a number of unanswered questions remain. For example, how is the transgene-derived RNA processed, sorted and taken up by the pathogen? Are long hpRNAs trafficked to the fungus in a similar mechanism as siRNAs and how can this dsRNA/siRNA transfer system be enhanced to confer additional levels of durability in economically important crops? Answering these questions along with others will ensure further development of this species-specific alternative to traditional fungal control and provide a path towards a more environmentally sustainable agriculture system.

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COMPETING INTERESTS

The authors declare no competing interests.

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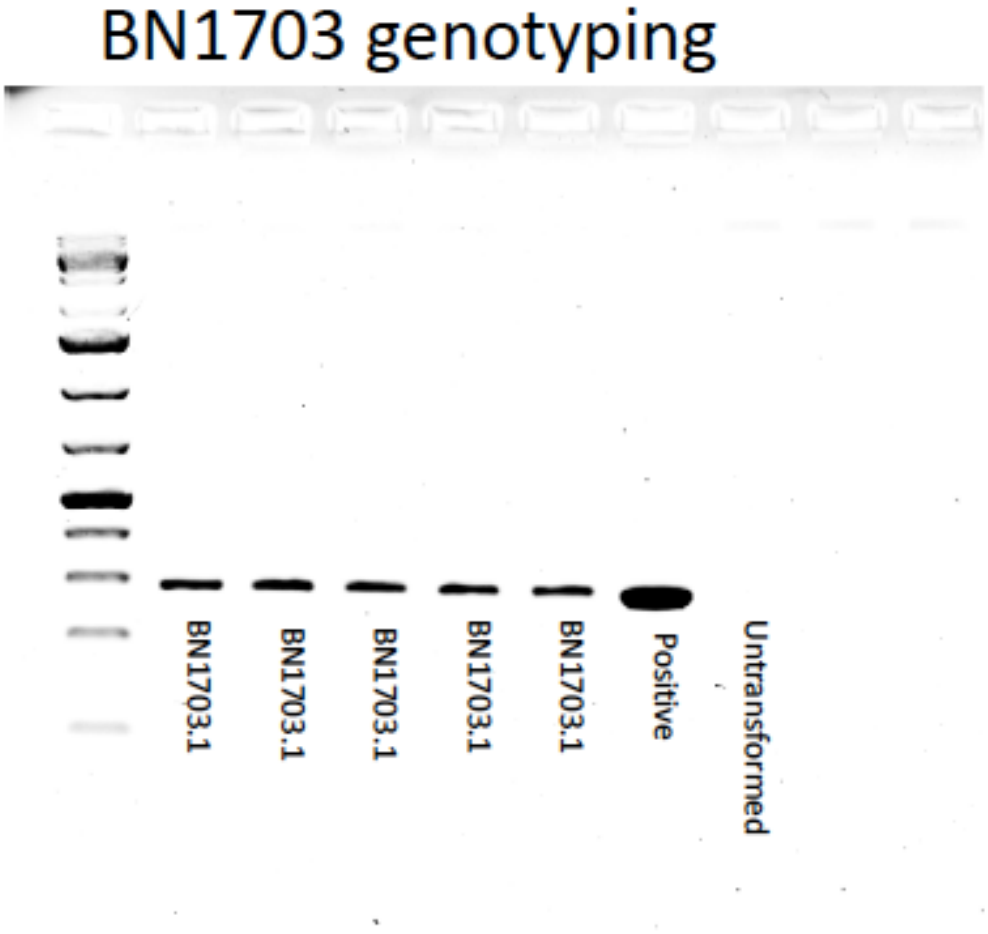

Figure S3.1. Agarose gel images of the 1703 transgene fragment (a) and the linear hairpin variable PCR amplification product of 1703 sRNA1 (b) in BN1703 lines. Positive control uses the 1703:pHELLSGATE8 construct.

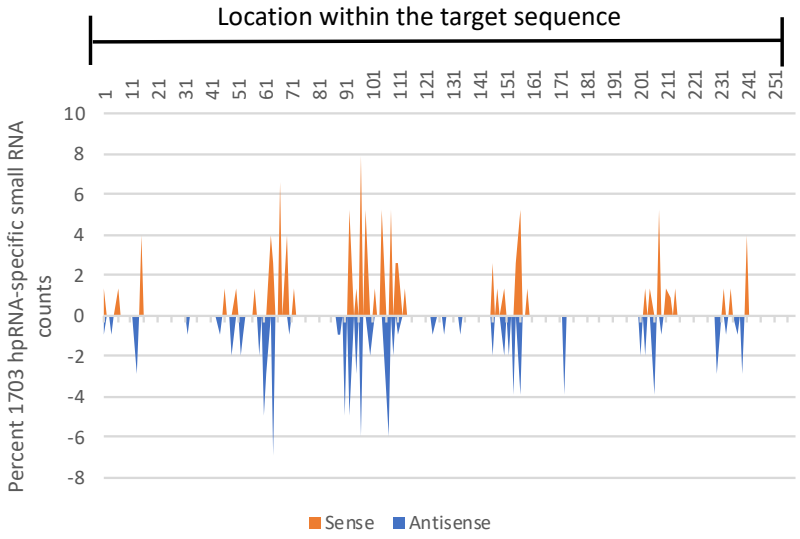

Figure S3.2. Small RNA sequence reads aligning to the 1703 transgene were plotted to their respective location upon the transgene sequence using Geneious Prime software (https://www.geneious.com) to identify highly abundant molecules.

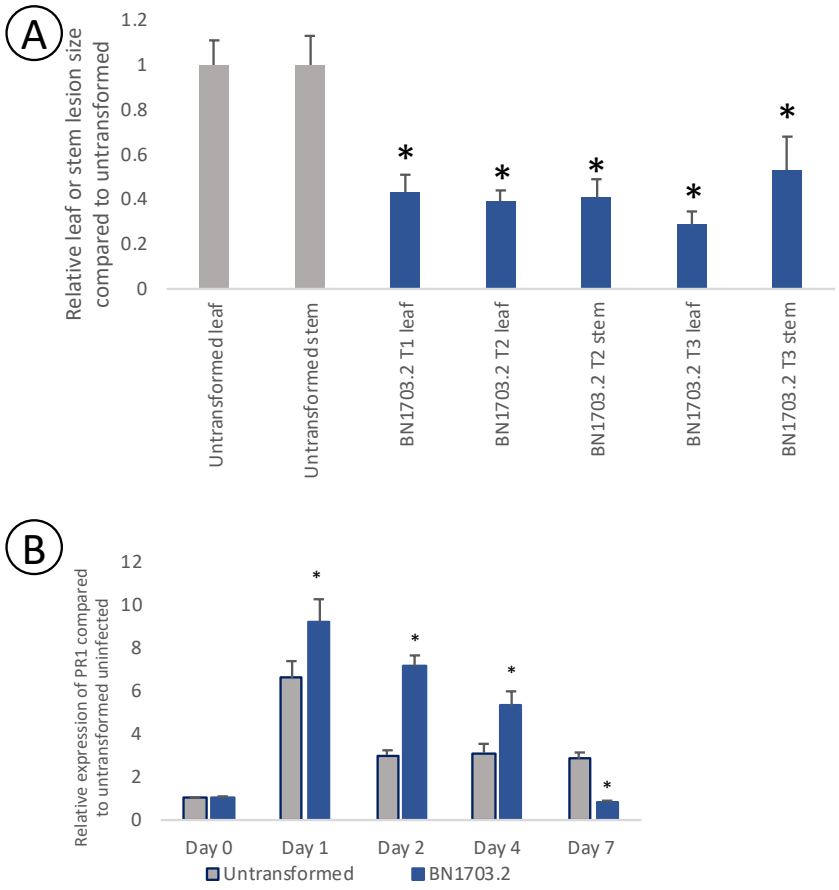

Figure S3.3. (a) Relative leaf and stem lesion sizes of BN1703.2 infections in T1-T3 generations compared to untransformed leaves and stems. Asterisks represent statistical differences from the untransformed control (one-tailed t-test with Bonferroni correction, $p<0.05$). (b) Relative expression of BNPR1 within *B. napus* BN1703.2 stems relative to untransformed plants and normalized to the housekeeping reference BNATGP4. Data represents 3 biological replicates with error bars representing standard error. Asterisks represent statistical differences from the untransformed control (one-tailed t-test with Bonferroni correction, $p<0.05$).

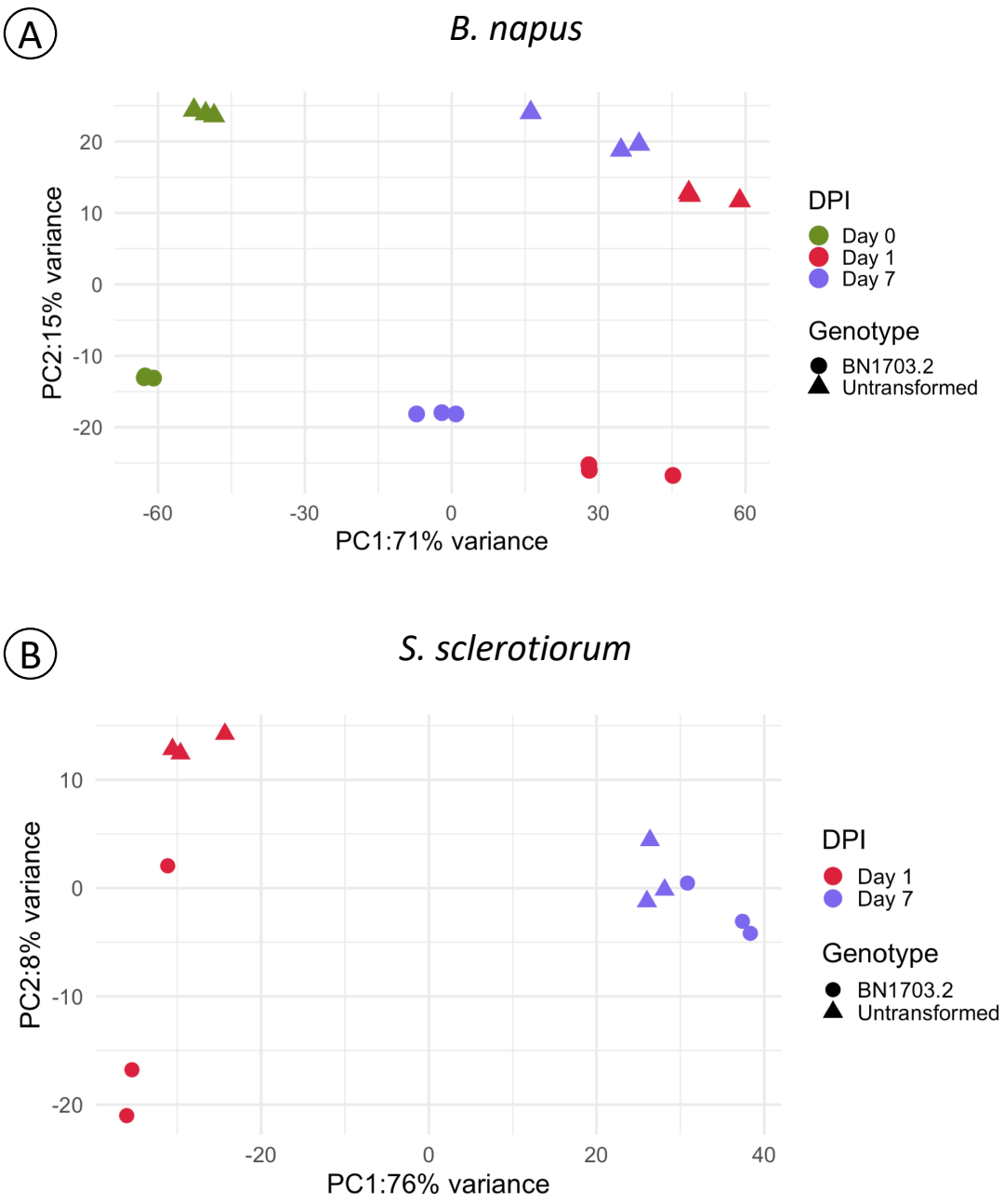

Figure S3.4. PCA clustering analysis of *B. napus* (A) and *S. sclerotiorum* (B) aligned reads of infected untransformed and BN1703.2 stems 0, 1 and 7 days post-inoculation.

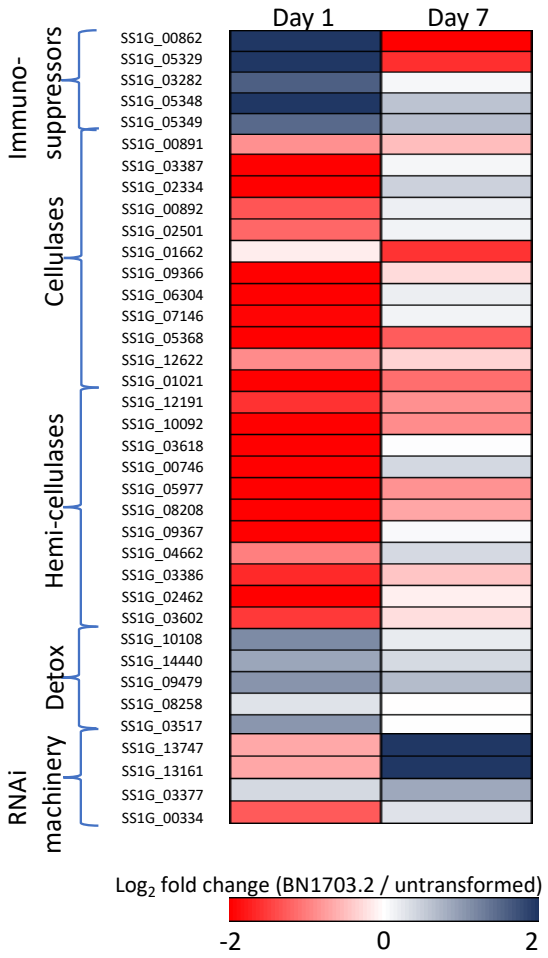

Figure S3.5. Heatmap of a subset of differentially expressed genes in untransformed and transgenic BN1703.2 infected stems 7 days post-inoculation compared to 1 dpi post infection stems (false discovery rate<0.05) involved in *S. sclerotiorum* infection common to the biotrophic and necrotrophic stages of infection.

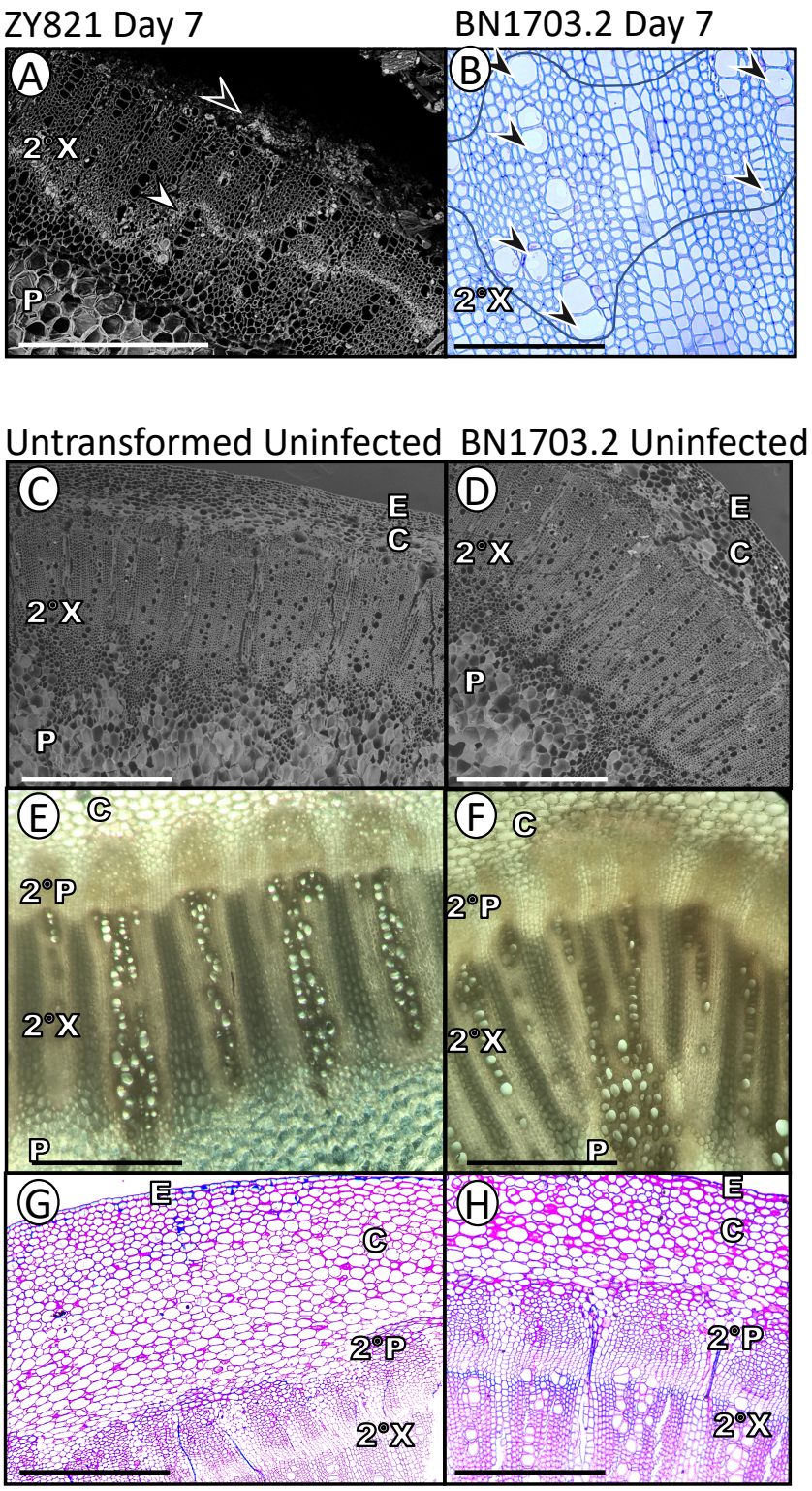

Figure S3.6. (A) Scanning electron micrograph of a stem cross-section of the semi-resistant cultivar of *Brassica napus* cv. ZhongYou 821 7 days post-inoculation. Black arrowheads indicate *S. sclerotiorum* hyphae and white indicates the vascular coating. Scale bar represents 0.5 mm. (B) Toludine-blue and Periodic-Acid Schiff's staining of BN1703.2 infected stem cross-sections

7 days post inoculation. Black arrowheads indicate negative detection to the vascular coating deposits within the secondary xylem by these stains. Scale bar represents 50 μm. Scanning electron micrographs of uninfected untransformed (C) and BN1703.2 (D) stem cross-sections. Scale bars represent 0.5 mm. Hand-cut stem transverse sections stained with Sudan IV of uninfected untransformed (E) and BN1703.2 (F) stems. Uninfected stems exhibit low affinity for Sudan IV in both. Scale bars represent 250 μm. Uninfected untransformed (G) and BN1703.2 (H) stem cross-sections stained with aniline blue and periodic acid-Schiff's reagent. Aniline blue staining is scant in both cultivars, indicating low callose deposition. Scale bars represent 250 μm.

Table S3.1. Cloning primer sequences used in this study.

Name	Fwd Primer	Rev Primer
SS1G_01703 cloning (KpnI/XhoI)	GTATAGGTACCTTCTGCCGGAAACCCTCTTC	GTATACTCGAGACCGCCGATTGTGAAGACTT
SS1G_01703 genotyping	TTCTGCCGGAAACCCTCTTC	ACCGCCGATTGTGAAGACTT
sRNA1 hairpin	ACGACATGATGAGTTCTGAAGGCCTTTCGATTCCGAACGGATCC	

Table S3.2. qRT PCR primer sequences used in this study.

Name	Fwd Primer	Rev. Primer	Efficiency (%)
SS1G_12350	CGATACTGTGCCTGTGACCA	CCTCTCCTCAAGCGCCATAG	102
SS1G_01703	CTTCCATCCCTGCCGCTTAC	CCATAGCGGCTCGATCTAGAATC	99
SS18S rDNA	AGCCGATGGAAGTTTGAGGC	CTCGTTGGCTCTGTCAGTGT	103
BNATGP4	CGTCTTCCTCTTCCCTCACC	ACAGTTGGAATAGAATAGTAGGCTC	100
BNHMG I/Y	GGTCGTCCTCCTAAGGCGAAAG	CTTCTTCGGCGGTCGTCCAC	95
BNPR1	TGTGGCAAAGCAAGGTGTAA	TTCCCCGAGGATCATAGTTG	98
BNDCL2A	TGAGAAACGGCATGAGGTTCA	GTGAAGGTTGTTATGCAGCGT	92
sRNA1	ACGACATGATGAGTTCTG	AGAGGTAGTAGGTTGT	96

Table S3.3. Gene homologues to SS1G_01703 in related fungal species.

Organism	Gene ID	Name	Percent amino acid identity to SS1G_01703
Botrytis fragariae	Bfra_002567	alpha beta hydrolase fold protein	70%
Botrytis cinerea	BofuT4_P121410	lipase/esterase	70%
Mollisia scopiformis	LY89DRAFT_594986	alpha/beta-hydrolase	61%
Acephala macrosclerotiorum	BDZ45DRAFT_40961	alpha/beta-hydrolase	58%
Phialocephala subalpina	PAC_04011	Putative sterigmatocystin biosynthesis lipase/esterase STCI	57%
Rhynchosporium commune	RCO7_04596	Putative sterigmatocystin biosynthesis lipase/esterase STCI	57%
Rhynchosporium agropyri	RAG0_00765	Putative sterigmatocystin biosynthesis lipase/esterase STCI	57%

Table S3.4. Gene IDs and annotation of differentially expressed genes identified within this study.

Gene ID	Name
BnaA03g38630D	PR1
BnaC03g45470D	PR1
BnaA07g17230D	PR2
BnaA09g36610D	PR2
BnaA03g28770D	PR4
BnaC03g33890D	PR4
BnaA06g13430D	PR5
BnaA07g21910D	PR5
BnaA03G20310D	Basic endochitinase CHB4
BnaA05G03420D	CHI
BnaC03G24300D	Chitinase family protein
BnaC04G49090D	Endochitinase

BnaC04G53030D BnaC05G01140D	CHI Chitinase family protein
BnaC09G51720D	Chitinase family protein
BnaA09g07000D	Beta-1,3-glucanase
BnaA08g08720D	Beta-1,3-glucanase-like protein
BnaA01g09070D	Beta-1,3-glucanase-like protein
BnaA03g43760D	Beta-1,3-glucanase-like protein
BnaC04g24330D	Beta-1,3-glucanase 2
BnaAnng23590D	Beta-1,3-glucanase 2
BnaA09g36610D	Beta-1,3-glucanase 2
BnaA04g02500D	Beta-1,3-glucanase 2
BnaA04g02490D	Beta-1,3-glucanase 2
BnaC08g28170D	Beta-1,3-glucanase 2
BnaC08g28150D	Beta-1,3-glucanase 2
BnaA01G37310D	Feruloyl CoA ortho-hydroxylase 1
BnaC01G37530D	Feruloyl CoA ortho-hydroxylase 1
BnaA07G16850D	Probable WRKY transcription factor 70
BnaA09G35840D	Probable WRKY transcription factor 70
BnaC06G15910D	Probable WRKY transcription factor 70
BnaA01G03290D	L,L-diaminopimelate aminotransferase
BnaA03G38440D	EDTS5
BnaC03G45280D	EDTS5
BnaA02G07380D	Cytochrome P450 86
BnaA04G21360D	ABCG2
BnaA05G27640D	Glycerol-3-phosphate acyltransferase 5
BnaA07G15220D	Omega-hydroxypalmitate O-feruloyl transferase
BnaC03G12340D	Cytochrome P450 86
BnaC03G53920D	Fatty acyl-CoA reductase
BnaC04G45080D	ABCG2
BnaC05G02350D	3-ketoacyl-CoA synthase
BnaC07G49710D	Cytochrome P450 86B
BnaCNNG59260D	Omega-hydroxypalmitate O-feruloyl transferase
BnaC05g04460D	CALS7
BnaC05g04470D	CALS7
BnaA09g49880D	CALS7
BnaA10g04230D	CALS7
BnaC09g44080D	CALS3
BnaA10g20270D	CALS3
SS1G_00862 (EDN91459)	cysteine protease (calpain family)
SS1G_05329 (EDO02852)	aspartyl protease

SS1G_03282 (EDO00808) SS1G_05348 (EDO02871)	serine protease (subtilisin-like) metalloprotease
SS1G_05349 (EDO02872)	metalloprotease
SS1G_00891 (EDN91488)	beta-1,4-endo-glucanase
SS1G_03387 (EDO00913)	endo-glucanase
SS1G_02334 (EDN99480)	exo-glucanase (cellobiohydrolyase)
SS1G_00892 (EDN91489)	exo-glucanase
SS1G_02501 (EDN99643)	Concanavalin A-like lectin/glucanase
SS1G_01662 (EDN96736)	beta-glucosidase
SS1G_09366 (EDN93499)	beta-glucosidase
SS1G_06304 (EDO03823)	beta-glucosidase
SS1G_07146 (EDO04663)	beta-glucosidase
SS1G_05368 (EDO02891)	beta-glucosidase
SS1G_12622 (EDN97768)	beta-glucosidase
SS1G_01021 (EDN91618)	beta-glucosidase
SS1G_12191 (EDN95985)	endo-1,4-beta-xylanase
SS1G_10092 (EDN94219)	endo-beta-xylanase
SS1G_03618 (EDO01144)	endo-beta-xylanase
SS1G_00746 (EDN91343)	beta-mannosidase
SS1G_05977 (EDO03496)	beta-mannosidase
SS1G_08208 (EDN92345)	endo-1,4-beta-mannosidase
SS1G_09367 (EDN93500)	alpha-xylosidase
SS1G_04662 (EDO02186)	alpha-galactosidase
SS1G_03386 (EDO00912)	alpha-galactosidase
SS1G_02462 (EDN99605)	alpha-l-arabinofuranosidase
SS1G_03602 (EDO01128)	alpha-l-arabinofuranosidase
SS1G_13747 (EDN98888)	DCL1
SS1G_13161(EDN98303)	RDRP2
SS1G_03377 (EDO00903)	RDRP 3
SS1G_00334 (EDN90934)	AGO2

APPENDIX 1: THE INFLUENCE OF HIGS ON THE SMALL RNA LANDSCAPE WITHIN THE *BRASSICA NAPUS – SCLEROTINIA SCLEROTIORUM* PATHOSYSTEM

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4.1 ABSTRACT

Small RNAs are key regulators of gene expression that control development, adaptation to abiotic stressors and play an important role in plant-pathogen interactions. During host plant infection, cross-kingdom RNAi involves the trafficking of small RNA molecules between plant and fungal cells that induce the silencing of fungal virulence genes. Host induced gene silencing (HIGS) exploits this cross-kingdom phenomena by trafficking engineered small RNAs to inhibit fungal virulence. In this study, I examined how the small RNA landscape between the pathogenic fungus, *Sclerotinia sclerotiorum*, and the highly susceptible oilseed, *Brassica napus*, is altered by HIGS within the stem. HIGS does not appear to affect the production of native small RNA species specific to infection. However, several previously uncharacterized small RNA species and their corresponding gene targets that may play a biological role in the *B. napus*-*S. sclerotiorum* interaction within the stem were identified. These results provide insight into the impact of HIGS on small RNA production and provide new evidence of the importance of small RNA molecules during plant pathogenesis.

4.2 INTRODUCTION

Small RNAs are a class of endogenous, regulatory RNA molecules that can modulate gene expression through post transcriptional gene silencing mechanisms (Lee *et al*. 1993). These small RNAs can be classified in two groups: i) micro RNAs (miRNAs) and ii) small interfering RNAs (siRNAs) (Bartel 2004). miRNAs and siRNAs are differentiated by their origin of biogenesis and involve different precursor molecules. For example, miRNAs are generally 20-24 nucleotides in length, have hairpin precursor molecules, and are encoded by loci proximal to protein coding regions throughout the genome. miRNAs are specifically involved in post transcriptional gene silencing (Axtell and Meyers 2018). siRNAs are highly diverse molecules that can originate from anywhere in the genome including intergenic loci but also transposable elements and heterochromatic regions. The functions of siRNAs are more complex and include epigenetic restructuring, methylation cascades, as well as post-transcriptional gene silencing (Iwakawa *et al*. 2021).

In plants, miRNAs are part of complex gene regulatory networks and play critical roles in processes such as development, stress responses and hormone signalling (Vaucheret *et al*. 2004, Sun *et al*. 2015, Liu and El-Kassaby 2017). There are also numerous studies that provide support for the importance of miRNAs in response to biotic stressors like plant pathogens (Cao *et al*. 2016). For example, miR393 levels increase when bacterial *flg22,* a known elicitor of plant pattern triggered immunity, is detected on the plant (Navarro *et al*. 2006). This miRNA negatively regulates F-box auxin receptor proteins *TIR1*, *AFB2* and *AFB3* which repress auxin signalling and confers anti-bacterial defense in *Arabidopsis thaliana* against *Pseudomonas syringae pv*. *tomato*. The bacterium in turn produces effector proteins to prevent miR393 accumulation (Navarro *et al*. 2008). miR393-mediated auxin repression was also shown to play an important role in virus-aphid transmission (Vat)-mediated resistance against *Aphis gossypii* in melon, *Cucumis melo* (Sattar and Thompson 2016). It was later shown that miR393 is also involved in the redirection of secondary metabolite pathways away from camalexin production and towards glucosinolate biosynthesis, providing enhanced protection against biotrophic pathogens in Brassica species (Robert-Seilaniantz *et al*. 2011). Several other miRNA families such as miR1507, miR2109, miR1885 and miR482 target genes encoding nucleotide binding site leucine-rich repeat (NBS-LRR) immune receptors. Thus, these molecules regulate the

accumulation of NBS-LRR transcripts and may guide effector triggered immunity (ETI) (Shivaprasad *et al*. 2012, Zhu *et al*. 2013). In particular, miR482 has been shown to be involved in potato (*Solanum tuberosum*) and cotton (*Gossypium hirsutum*) resistance to *Verticillium dahlia* (Zhu *et al*. 2013, Yang *et al*. 2015). In *B. napus*, three miRNAs, ath-miR168a_1ss21AC, bna-miR403 and aly-miR403a-3p_L+1, targeting *AGO1* and *AGO2,* two players involved in post transcriptional gene silencing, were found to be differentially downregulated in response to *S. sclerotiorum* infection in the leaf (Cao *et al*. 2016). The production of reactive oxygen species (ROS) is also an important defense reaction to combat biotic stressors and miRNAs play a role in the regulation of their production. For example, in rice, miR398b has been shown to involved in the promotion of superoxide dismutase (SOD) activity in response to *Magnaporthe oryzae* infection (Li *et al*. 2019).

In addition to miRNAs functioning to strengthen host defense through the modulation of native plant genes, there are a growing number of studies describing the phenomena known as bidirectional cross-kingdom RNAi. Cross-kingdom RNAi occurs when host plants respond to infection by exporting specific small RNAs to induce silencing of genes in the invading pathogen or vice versa (Wang *et al*. 2016). miRNAs produced by the plant can target and affect the abundance of fungal mRNAs involved in pathogenicity (Zhao *et al*. 2021). For example, *Phytophthora* infection induces the production of several siRNAs from *A. thaliana* that can be trafficked to the pathogen through extracellular vesicles (Hou *et al*. 2019). *A. thaliana* miR166 has also been identified to be trafficked in response to *V. dahliae* (Cai *et al*. 2018). In cotton (*G. hirsutum*), both miR166 and miR159 were induced by infection and were shown to target the genes *Clp-1* and *HiC-15* respectively (Zhang *et al*. 2013). *A. thaliana* shuttles miR166 as well other sRNAs into *Botrytis cinerea* using this same exosomal trafficking (Cai *et al*. 2018). In turn, fungal small RNAs can influence the transcript abundance of plant mRNAs that dictate plant defense and immunity (Weiberg *et al*. 2013). Interestingly, one identified target of *B. cinerea* sRNAs is plant *AGO1*, which is involved in post transcriptional gene silencing (Weiberg *et al*. 2013). By silencing *AGO1*, post transcriptional gene silencing mechanisms are inhibited and the plant can no longer use cross-kingdom RNAi as a means of defense. Another example of a fungal sRNA targeting plant immunity is in *Puccinia striiformis f. sp. tritici*. The miRNA produced by this pathogen was demonstrated to silence wheat *PR2* transcripts (Wang *et al*.

2017). Cross-kingdom RNAi has also been observed in the *S. sclerotiorum-B. napus* pathosystem, where the host genes *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 2* and *SNAKIN-LIKE CYSTEIN RICH PROTEIN 2* are specific targets of *S. sclerotiorum* small RNAs (Derbyshire *et al*. 2019). This bidirectional arms race occurs naturally between plant hosts and fungal pathogens, however it is unclear how the constitutive production of dsRNAs by HIGS plants may affect this mechanism of defense

Here, I analyzed the accumulation of small RNA populations in *B. napus* stems in response to *S. sclerotiorum*. Small RNA populations have yet to be described and characterized from *S. sclerotiorum* infections within *B. napus* stem and thus provides the opportunity to identify small RNA sequences that may play a role in this pathosystem. I performed this experiment on the universally susceptible B. napus line, Westar, in addition to the HIGS line BN1703.2 described in the previous chapter. BN1703.2 confers enhanced resistance through host induced gene silencing of *S. sclerotiorum ABHYDROLASE-3* transcripts. In the current chapter, I identified both known and uncharacterized plant sRNA sequences that are predicted to target specific genes within this pathosystem. In addition, novel *S. sclerotiorum* sRNAs were identified that have predicted targets within the *B. napus* genome. Taken together, this study provides insight into the small RNA landscape within HIGS engineered plants and identifies novel small RNA sequences that may contribute to further development of pathogen resistance.

4.3: MATERIALS AND METHODS

4.3.1 *B. NAPUS* GROWTH CONDITIONS

B. napus was growth as described in Chapter 3.

4.3.2 PREPARATION OF *AGROBACTERIUM TUMEFACIENS*

Agrobacterium was engineered and transformed as described in Chapter 3.

4.3.3 *B. NAPUS* TRANSFORMATIONS

B. napus was transformed as described in Chapter 3.

4.3.4 DNA EXTRACTION AND GENOTYPING

DNA extractions and genotyping were performed as described in Chapter 3.

4.3.5 STEM INOCULATIONS

Plant infection experiments were carried out as described in Chapter 3.

4.3.6 SMALL RNA LIBRARY SYNTHESIS, SEQUENCING

Small RNA libraries were constructed and sequenced as described in Chapter 3.

4.3.7 BIOINFORMATIC ANALYSIS

FastQ files were trimmed using Trimmomatic 0.36 (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 or under 18 bp were removed during the trimming process. Bowtie (Langmead *et al*. 2009) was then used to align the reads to either the *B. napus* (Song *et al*. 2020) or *S. sclerotiorum* genomes (Derbyshire *et al*. 2017). *B. napus* FASTQ files were converted to FASTA and miRDeepP2 (Kuang *et al*. 2019) on default settings was used to identify and predict miRNAs. Next, for *S. sclerotiorum*, we used Bowtie to remove all 0 dpi reads from 1 and 7 dpi and then collected all reads between 18-24 bp. For both *B. napus* and *S. sclerotiorum*, Venny 2.1.0 (Oliveros 2015) was used to identify small RNA species specific to each treatment and we moved forward only with small RNA species that were present in at least two out of the three biological replicates for each treatment. psRNATarget (Dai *et al*. 2018) was then used to identify putative mRNA targets from these

small RNA sequences, with parameters of E-value less than or equal to one and seed region 2-13 nucleotides.

4.4 RESULTS

4.4.1 SMALL RNA SEQUENCING OF HIGS PLANTS INFECTED WITH *S. SCLEROTIORUM*

B. napus cv. Westar was transformed to express a hpRNA targeting *S. sclerotiorum ABHYDROLASE-3* (SS1G_01703) under the control of a CaMV 35S promotor (Bn35S::SS1G_01703RNAi hereby referred to as BN1703; as described in chapter 3). Untransformed and BN1703 plants at the 30% flowering stage were inoculated with *S. sclerotiorum* using the stem inoculation method described in chapter 3 and tissue was collected from the site of infection at 1 and 7 dpi. Small RNA populations were sequenced to study how the small RNA landscape is influenced by the constitutive production of BN1703 dsRNA. Small RNA sequence reads were mapped to the reference genomes of *B. napus* cv. Westar (Song *et al*. 2020) and *S. sclerotiorum* (Derbyshire *et al*. 2017). For reads aligning to *B. napus*, I used the plant miRNA analysis and prediction software miRDeepP2 (Kuang *et al*. 2019) to identify known and predicted miRNA sequences from our dataset. For the reads aligning to *S. sclerotiorum*, I followed the methodology of Derbyshire *et al*. (2019) to identify small RNAs. Repetitive RNA sequences were removed in addition to reads within 1 and 7 dpi samples that were also present within 0 dpi samples and considered reads only between 18 and 24 nucleotides in length. The length distribution of each putative small RNA species generated through these analyses was then plotted (Figure 4.1). In *B. napus*, the majority of species appear to fall in the 18-21 nucleotide range in both BN1703 and untransformed lines at all timepoints (Figure 4.1 A, B). In RNA species aligning to *S. sclerotiorum*, there are differences in RNA length between the BN1703 and untransformed samples. For example, there are 45% more small RNA species in the 22-24 nucleotide length range in the untransformed infections than in BN1703 (Figure 4.1 C, D).

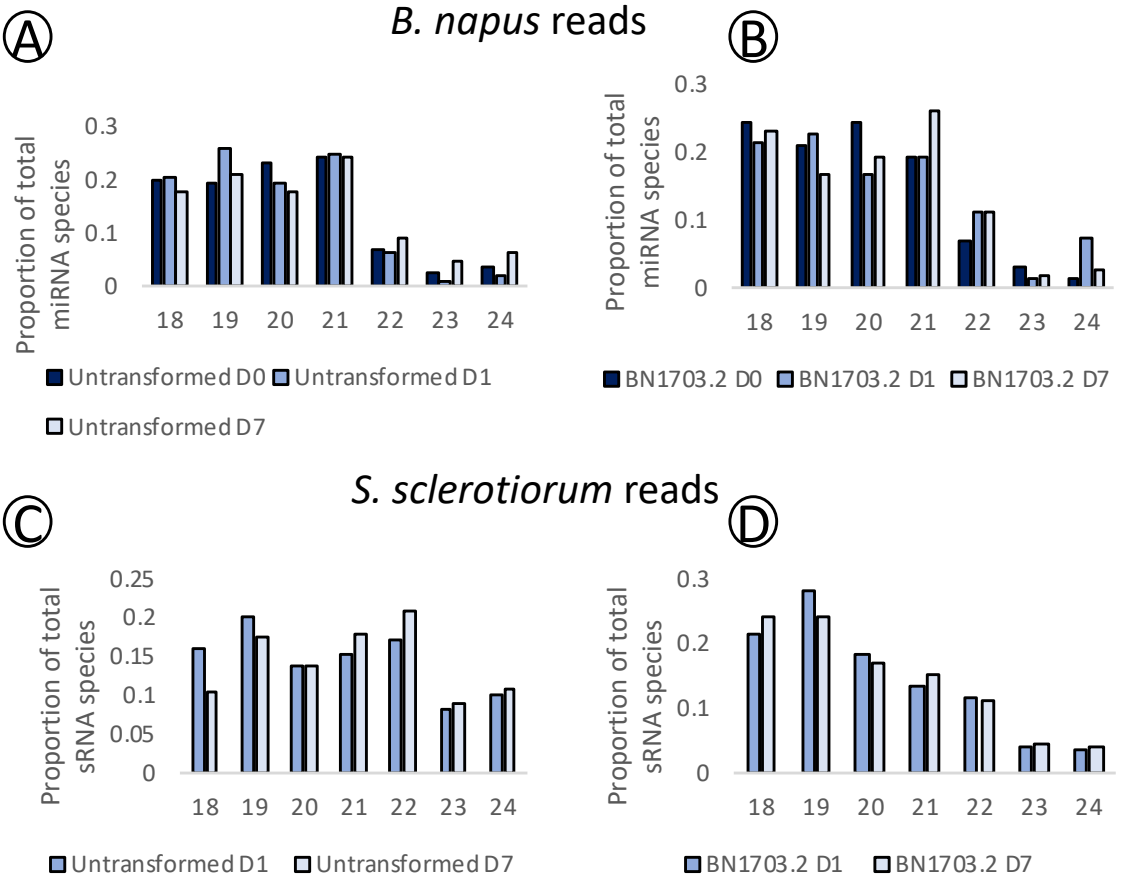

Figure 4.1. Length distributions of small RNA species identified from *S. sclerotiorum* infected *B. napus* stems at 0, 1 and 7 dpi. Reads were aligned to the *B. napus* and *S. sclerotiorum* genomes and the length of each species was plotted.

4.4.2 IDENTIFICATION OF SMALL RNA SPECIES INVOLVED IN PLANT DEFENSE AND FUNGAL PATHOGENICITY

Next, I aimed to identify miRNA species that were either induced or repressed during infection conditions in both untransformed and BN1703 lines. In the untransformed stems, 1 dpi accumulated the most unique miRNA species (132 at 1 dpi versus 65 and 53 at 0 and 7 dpi respectively), while in BN1703, 7 dpi accumulated the most (88 at 7 dpi versus 59 and 60 at 0 and 1 dpi respectively) unique miRNAs. I then compared the unique miRNAs produced at each timepoint between the untransformed and BN1703.2 (Figure S4.1). A similar trend continued where at 1 dpi, untransformed contained more unique species than BN1703 (123 versus 51) and

at 7 dpi, BN1703 contained more unique species (83 versus 48). I also examined small RNA populations produced by *S. sclerotiorum* during infection. Data show *S. sclerotiorum* infecting untransformed stems accumulated 3-4 times as many miRNAs compared to BN1703. Interestingly, there appears to be little overlap in the miRNAs detected in both the untransformed or BN1703 lines and may be a reflection of the lack of annotation and family groupings of small RNA species within this fungus.

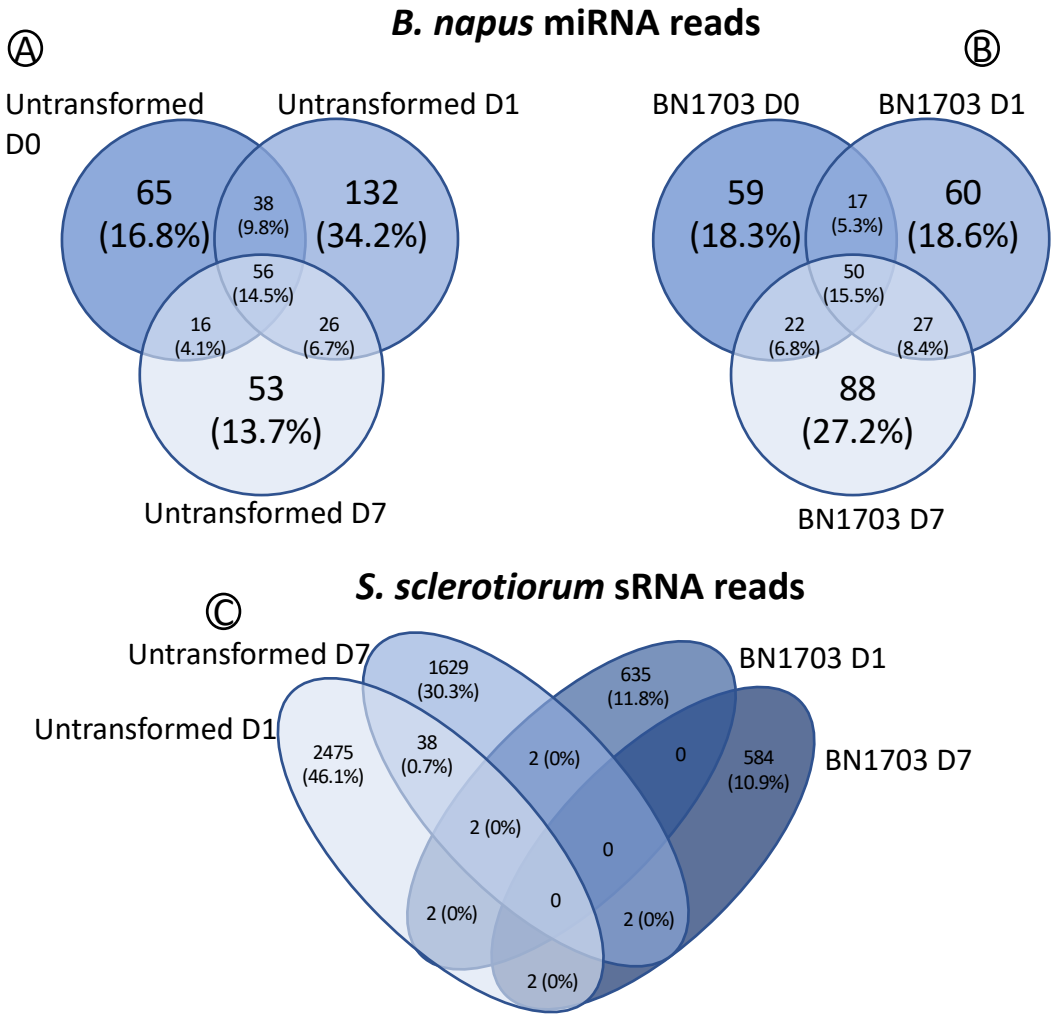

Figure 4.2. Venn diagrams displaying unique and shared small RNA species between BN1703 and untransformed 0, 1, and 7 dpi for *B. napus* and BN1703 and untransformed 1 and 7 dpi for *S. sclerotiorum*.

4.4.3 mRNA TARGETS OF IDENTIFIED SMALL RNA SPECIES IN UNTRANSFORMED AND BN1703 LINES

I then studied miRNA species that were specific to any given time of the infection process in both the untransformed and BN1703 lines (Table 4.1). With the predicted small RNA species, cross-kingdom mRNA targets can be examined using psRNATarget. The small RNA analysis server, psRNATarget, allows for identification of potential mRNA targets of predicted miRNA species (Dai *et al*. 2018). Data show *S. sclerotiorum* produce sRNA molecules that target *B. napus* mRNAs. At 1 dpi, two small RNA species were identified in tissue samples collected from untransformed stems that are predicted to target *INDOLE-3-ACETIC ACID AMIDO SYNTHETASE* involved in the suppression of auxin signalling (Ding *et al*. 2008) and *YDG DOMAIN CONTAINING PROTEIN* involved in DNA methylation (Ding *et al*. 2007). In untransformed tissues at day 7, a small RNA was identified and is predicted to target *DIHYDROCERAMIDE FATTY ACYL 2-HYDROXYLASE FAH1-LIKE,* involved in the biosynthesis of very long chain fatty acids and resistance to oxidative stress (Nagano *et al*. 2012) (Table 4.1). Additionally, data revealed a *B. napus* miRNA that is predicted to target *S. sclerotiorum* SS1G_07343. This gene is predicted to encode a mannose-6-phosphate receptor, based on BLAST homology to other fungal species. This miRNA species was present in both untransformed and BN1703 infections at 1 dpi (Table 4.1).

I then examined known and predicted plant miRNA species unique for each treatment and timepoint that had mRNA targets of native host genes. miR1885 was only present at D0 in both the untransformed and BN1703.2 lines and absent at D1 and D7 in response to *S. sclerotiorum*. miR1885 has been shown to downregulate the expression of TIR-NBS disease resistance protein in Brassica species (Paul *et al*. 2021). At D0, data show miRNAs such as miR162, miR166 that target transcription factors, homeobox leucine zipper proteins for example. Additionally, miRDeepP2 also predicted candidate miRNAs that were predicted to target octanoyl transferase, involved in lipoic acid metabolism at D0. At D1, detection of miR162, miR164, and miR166 were predicted to target transcription factors, as well as a candidate miRNA predicted to target a mitogen activated protein kinase kinase kinase in untransformed D1. miR393 which has been

shown to be elevated during infection by a variety of pathogens and suppress auxin signalling (Navarro *et al*. 2006) was identified at 7 dpi in untransformed infections (Table 4.1).

For *S. sclerotiorum* small RNA production, the main target of many of the small RNA species produced in untransformed infections appears to be retrotransposons based on the psRNATarget predictions (Table 4.1). No targets of the small RNA molecules produced in BN1703 samples were identified by psRNATarget using our stringent E-value cut-off.

Table 4.1. Small RNA species identified in this study and their predicted mRNA target. Small RNA sequences were only considered if they were present in at least two out of three biological replicates and mRNA targets were considered only if the E-value was less than or equal to 1. E-values correspond to the number of mismatches between a small RNA and its target, where an E-value of zero represents no mismatches.

Sample	Origin	sRNA sequence and family (if known)	Target organism	Target gene ID	Target gene function	E-value
Untransformed D1	*S. sclerotiorum*	TTTATGACCAGGGACCCATCGG (uncharacterized)	*B. napus*	BnaC07g40810D, BnaA03g48550D	Indole-3-acetic acid-amido synthetase	0
Untransformed D1	*S. sclerotiorum*	TGATTTCTTGAAAGGCTTGC (uncharacterized)	*B. napus*	BnaC02g33260D	YDG domain-containing protein	1
Untransformed D7	*S. sclerotiorum*	CTTCACCAAGATGACAAACCGTA (uncharacterized)	*B. napus*	BnaC03g50400D, BnaA06g23040D, BnaA04g20360D, BnaA03g16020D, BnaA04g20340D	Dihydroceramide fatty acyl 2-hydroxylase FAH1-like	0.5
BN1703.2 D1	*B. napus*	GAGCGATGATGAAAATATCTTGAG (uncharacterized)	*S. sclerotiorum*	SS1G_07343	Mannose 6 phosphate receptor	1
Untransformed D1	*B. napus*	GAGCGATGATGAAAATATCTTGA (uncharacterized)	*S. sclerotiorum*	SS1G_07343	Mannose 6 phosphate receptor	1
BN1703.2 D0	*B. napus*	CTGGTTGATGAATCATGGCCGT (uncharacterized)	*B. napus*	BnaC03g77400D	Octanoyltransferase LIP2p	0
BN1703.2 D0	*B. napus*	TACATCTTCTCCGCGGAAGC (miR1885)	*B. napus*	BnaAnng10090D, BnaC07g18010D	UDP-arabinose 4-epimerase 4	0
BN1703.2 D0	*B. napus*	TCGATAAACCTCTGCATCCAG (miR162)	*B. napus*	BnaC02g01260D	NF-YB12	0
BN1703.2 D0	*B. napus*	TACATCTTCTCCGCGGAAGC (miR1885)	*B. napus*	BnaC03g05380D	Disease resistance protein (TIR-NBS-LRR class)	0.5
BN1703.2 D0	*B. napus*	TCGGACCAGGCTTCATTCCCCA (miR166)	*B. napus*	BnaC03g69040D, BnaA08g01600D, BnaA06g01940D, BnaC06g05240D	Homeobox-leucine zipper protein ATHB-15-like	0.5
BN1703.2 D0	*B. napus*	TACATCTTCTCCGCGGAAGC (uncharacterized)	*B. napus*	BnaC02g07640D, BnaA02g03790D, BnaA03g03260D	Cysteine/Histidine-rich C1 domain family protein	1
BN1703.2 D0	*B. napus*	TCGGACCAGGCTTCATTCCCCA (miR1885)	*B. napus*	BnaAnng30670D, BnaC03g66860D, BnaA02g06170D,	Homeobox-leucine zipper protein ATHB-8	1

				BnaA08g11980D, BnaA04g20300D, BnaC05g23470D, BnaA09g26050D, BnaC09g54340D		
Untransformed D0	*B. napus*	ACAGGGAACAAGCAGAGCAT (miR408)	*B. napus*	BnaC04g00470D	Peptide chain release factor 1	0
Untransformed D0	*B. napus*	ATCTTCTCCGCGGAAGCTC (miR1885)	*B. napus*	BnaAnng10090D, BnaC07g18010D	UDP-arabinose 4-epimerase 4	0
Untransformed D0	*B. napus*	ATGATAATGATGATGATGA (miR5658)	*B. napus*	BnaA08g18450D, BnaA07g14030D, BnaCnng37160D, BnaA01g32510D, BnaC07g33810D	SMG7L	0
Untransformed D0	*B. napus*	ATGCACTGCCTCTTCCCTGGT (miR408)	*B. napus*	BnaC04g00470D	Peptide chain release factor 1	0
Untransformed D0	*B. napus*	CTGGTTGATGAATCATGGC (uncharacterized)	*B. napus*	BnaC03g77400D	Octanoyltransferase LIP2p	0
Untransformed D0	*B. napus*	TGCACTGCCTCTTCCCTGGC (miR408)	*B. napus*	BnaC04g00470D	Peptide chain release factor 1	0
Untransformed D0	*B. napus*	TGTGTTCTCAGGTCACCCCAG (miR398)	*B. napus*	BnaC09g42920D	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase	0
Untransformed D0	*B. napus*	ATGATAATGATGATGATGA (miR5658)	*B. napus*	BnaC07g33810D, BnaC09g42220D, BnaC09g05870D, BnaA09g06300D, BnaC06g05030D, BnaA10g04530D	Cysteine-rich receptor-like protein kinase 13	0.5
BN1703.2 D1	*B. napus*	TGTGTTCTCAGGTCACCCCAG (miR398)	*B. napus*	BnaC09g42920D	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase	0
BN1703.2 D1	*B. napus*	TTTGGATTGAAGGGAGCTCA (uncharacterized)	*B. napus*	BnaCnng51960D, BnaAnng27960D	Transcription factor MYB101	1
BN1703.2 D1	*B. napus*	TGGAATGATGATTACTCTTATTCT (uncharacterized)	*B. napus*	BnaA07g31870D, BnaA01g28990D, BnaC01g36350D	FCS-Like Zinc finger 13	1
Untransformed D1	*B. napus*	CTGGTTGATGAATCATGGCCGT (uncharacterized)	*B. napus*	BnaC03g77400D	Octanoyltransferase LIP2p	0
Untransformed D1	*B. napus*	GAGCTGTGATGATATTGGCT (uncharacterized)	*B. napus*	BnaC03g16930D	Mitogen-activated protein kinase kinase kinase ANP1-like	0
Untransformed D1	*B. napus*	TCGATAAACCTCTGCATCC (miR162)	*B. napus*	BnaC02g01260D	NF-YB12	0
Untransformed D1	*B. napus*	TCGGACCAGGCTTCATTCCCC (miR166)	*B. napus*	BnaC03g69040D, BnaA08g01600D, BnaA06g01940D, BnaC06g05240D	Homeobox-leucine zipper protein ATHB-15-like	0.5
Untransformed D1	*B. napus*	TGGAGAAGCAGGGCACGTG (miR164)	*B. napus*	BnaAnng07000D, BnaAnng06380D	NAC domain-containing protein 100	0.5

Untransformed D1	*B. napus*	TACATCTTCTCCGCGGAAGC (uncharacterized)	*B. napus*	BnaC02g07640D, BnaA02g03790D, BnaA03g03260D	Cysteine/Histidine-rich C1 domain family protein	1
Untransformed D7	*B. napus*	TCGGACCAGGCTTCATTCCAA (miR166)	*B. napus*	BnaC03g69040D, BnaA08g01600D, BnaA06g01940D, BnaC06g05240D	Homeobox-leucine zipper protein ATHB-15-like	0.5
Untransformed D7	*B. napus*	TCGGACCAGGCTTCATTCCAA (miR166)	*B. napus*	BnaA04g20300D, BnaC05g23470D, BnaA09g26050D	Homeobox-leucine zipper protein ATHB-14	1
Untransformed D7	*B. napus*	ATCATGCGATCTCTTCGGATT (miR393)	*B. napus*	BnaC06g43000D	TIR1	1
BN1703.2 D7	*B. napus*	ACAGGGAACAAGCAGAGCAGG (miR408)	*B. napus*	BnaC04g00470D	Peptide chain release factor 1	0
BN1703.2 D7	*B. napus*	CTGGTTGATGAATCATGGC (uncharacterized)	*B. napus*	BnaC03g77400D	Octanoyltransferase LIP2p	0
BN1703.2 D7	*B. napus*	GAGCTGTGATGATATTGGCT (uncharacterized)	*B. napus*	BnaC03g16930D	Mitogen-activated protein kinase kinase kinase ANP1-like	0
BN1703.2 D7	*B. napus*	TCGATAAACCTCTGCATCC (miR162)	*B. napus*	BnaC02g01260D	NF-YB12	0
BN1703.2 D7	*B. napus*	TGCACTGCCTCTTCCCTGGC (miR408)	*B. napus*	BnaC04g00470D	Peptide chain release factor 1	0
BN1703.2 D7	*B. napus*	TGGAGAAGCAGGGCACGTG (miR164)	*B. napus*	BnaC04g34520D, BnaC05g38460D	CUC1	0.5
BN1703.2 D1	*S. sclerotiorum*	AATAAAATAACTCTTTGAAAA (uncharacterized)	*S. sclerotiorum*	SS1G_12797	Integral membrane protein	1
Untransformed D1	*S. sclerotiorum*	CTGGCCTCCTTCTTGGTACCCATA (uncharacterized)	*S. sclerotiorum*	SS1G_06460	Snf2 family helicase	0
Untransformed D1	*S. sclerotiorum*	GACCCATCGCCCTACTCTCA (uncharacterized)	*S. sclerotiorum*	SS1G_09525	Kinesin	0
Untransformed D1	*S. sclerotiorum*	TACGGGCTCCACATCTGCAA (uncharacterized)	*S. sclerotiorum*	SS1G_04940, SS1G_12417, SS1G_11082, SS1G_06563	Reverse transcriptase	0
Untransformed D1	*S. sclerotiorum*	TATATTCTCCACCTCGTACTCG (uncharacterized)	*S. sclerotiorum*	SS1G_00932	Retrotransposon polyprotein	0
Untransformed D1	*S. sclerotiorum*	TCGGAAGGACCAGTTTGTTCTA (uncharacterized)	*S. sclerotiorum*	SS1G_07080	Unknown	0
Untransformed D1	*S. sclerotiorum*	TCTCTATCTCCCCCAGGTT (uncharacterized)	*S. sclerotiorum*	SS1G_02323	Unknown	0
Untransformed D1	*S. sclerotiorum*	TGAGAGTAGGGCGATGGGTC (uncharacterized)	*S. sclerotiorum*	SS1G_04940, SS1G_12417, SS1G_11082, SS1G_06563	Reverse transcriptase	0

4.5 DISCUSSION

In this study, I identified a wide range of small RNA molecules induced or repressed during the infection of *B. napus* with *S. sclerotiorum* that may play an important role in the outcome of this interaction. One of the questions that I hoped to address whether constitutive dsRNA production had any impacts on small RNA production in both the host plant and the infecting pathogen. I previously characterized the BN1703 HIGS line using global RNA sequencing (Wytinck *et al.* 2021), and identified differences in the transcript abundance of the RNAi machinery pathway. Despite these advances, the production of native small RNA species was unresolved. Small RNA species play an important role in the regulation of developmental processes as well as response to abiotic and biotic stressors (Axtell and Meyers 2018). The analyses show 386 miRNAs species in untransformed *B. napus* stems across all timepoints and 323 miRNA species in BN1703. Early in the infection process, 2.5-fold more miRNAs were detected in the untransformed line compared to BN1703, while at day 7, we identified 1.75-fold more BN1703 miRNAs compared to the control. This is likely a reflection of the level and stage of infection observed between the control and transgenic line, where infection was more robust in untransformed plants. I observed larger differences in the total number of small RNAs produced by *S. sclerotiorum* in the untransformed and BN1703 samples; a four and a three-fold increase in species in the untransformed at 1 and 7 dpi respectively. Jian *et al.* (2018) and Regmi *et al.* 2021 have previously examined *B. napus* small RNA responses to infection and Derbyshire et al. (2019), examined *S. sclerotiorum* sRNA production during pathogenesis, however to my knowledge, this is the first example of small RNA-seq on the *B. napus* stem in addition to our examination of the effect of HIGS. I was then interested in identifying mRNA targets of some of the small RNAs within the dataset. Since mRNA targets of most of the small RNA species produced between the untransformed and BN1703 infections are conserved (Table 4.1), the difference in small RNA species numbers may be attributed to the different levels of infection observed in the stem and not due to differing levels in RNAi machinery.

I identified miRNAs produced by *B. napus* that are predicted to regulate the expression of native genes in addition to those predicted target mRNAs in *S. sclerotiorum*. For miRNAs predicted to regulate host gene expression, I identified miRNAs that were repressed during infection and

those that were induced. For example, Paul *et al*. (2021) demonstrated that small RNAs repressed during *Plasmodiophora brassicae* infection on *Brassica rapa*, including miR1885, may regulate mRNA targets that play a role in host defense, such as LRR-NBS. miR1885 is also identified in my dataset at 0 dpi. miRNAs induced during infection may also negatively regulate suppressors of host defense, such as the suppression of auxin signalling by miR393 (Navarro *et al*. 2006). This miRNA was identified at 7 dpi in untransformed samples. A level of conservation in the regulation of basal host defense through small RNAs between plant species and pathosystems appears to exist, given the overlap in induced and repressed reads between my study and others in the literature,

I was also interested in the phenomenon of cross-kingdom RNAi, first described by Weiberg *et al*. (2013). In the current study, we observed the production of two uncharacterized, independent *B. napus* miRNAs, in both untransformed and BN1703 samples, predicted to target a fungal mannose-6-phosphate receptor (MPR). MPRs are well characterized cargo proteins that are involved in the sorting of late, clathrin coated endosomes that are leaving the *trans*-Golgi network and are destined for lysosomes (Rohn *et al*. 2000). Mannose-6-phosphate residues are present specifically on acid hydrolases that function within activated lysosomes and therefore MPRs concentrate these proteins into clathrin coated vesicles destined for lysosomes (Nicoziani *et al*. 2000). Lysosomes are acidic organelles that contain hydrolytic enzymes functioning in the digestion of damaged organelles, material from the extracellular environment as well as aiding in the process of apoptosis (Luzio *et al*. 2007). Thus, data suggest the host plant is producing miRNAs to silence *S. sclerotiorum* MPRs in an attempt to interfere with the ability of *S. sclerotiorum* to digest endocytosed nutrients. Despite these observations, functional characterization of these uncharacterized miRNA species in cell sorting between the host plant and fungal pathogen is required.

Small RNAs produced by *S. sclerotiorum* also had predicted mRNA targets in *B. napus*. For example, mRNA transcripts of *DIHYDROCERAMIDE FATTY ACYL-2 HYDROXYLASE* have perfect complementarity to an uncharacterized *S. sclerotiorum* small RNA molecule produced during infection. *DIHYDROCERAMIDE FATTY ACYL-2 HYDROXYLASE* functions in the biosynthesis pathway of very long chain fatty acids (Nagano *et al*. 2012). Interestingly, very long

chain fatty acids are one of the building-blocks of the lipid-based polymer, suberin (Batsale *et al*. 2021). Suberin is a component of an inducible, anatomical barrier present in the plant vasculature in response to pathogen infection (Kashyap *et al*. 2021). Recently, a vascular coating composed of suberin has been shown to play a role in *B. napus* defense to *S. sclerotiorum* stem infections, presented in Chapter 3 (Wytinck *et al*. 2021). This uncharacterized, small RNA molecule was identified only in untransformed infections, in which I observed no vascular coating formation in response to infection (Chapter 3). Furthermore, in BN1703 in addition to the moderately tolerant commercial line, cv. ZhongYou821, formation of the vascular coating was always present in response to *S. sclerotiorum* infection. A second target of uncharacterized *S. sclerotiorum* small RNAs was *INDOLE-3-ACETIC ACID AMIDO SYNTHETASE,* which is involved in auxin signalling repression through the prevention of indole acetic acid accumulation (Ding *et al*. 2008). Auxin induces the loosening of the cell wall to promote growth, however this facilitates infection from pathogens and therefore during infection, auxin signalling is not conducive to host plant defense (Wang *et al*. 2007). In *A. thaliana*, a close relative of *B. napus*, miR393, has been shown to negatively regulate the F-box auxin receptor proteins *TIR1*, *AFB2* and *AFB3* during infection (Navarro *et al*. 2006). In the current study, the same miR393 was also identified in the *B. napus* sRNA dataset. Thus, it appears as if the accumulation of small RNAs present in *S. sclerotiorum* during the infection process may function to repress the negative regulation of auxin signalling that may lead to more profound infection.

Taken together, this study serves as a foundation for further research in bidirectional small RNA production and crosstalk in the *B. napus -S. sclerotiorum* pathosystem. However, functional characterization of gene knockouts targeting the RNAi machinery is required to validate the predictive findings of this study. Genome editing technologies can also be used to control the activity of miRNA populations that may play a role in susceptibility to enhance resistance against pathogen attack. The understanding of this complex biological interaction, especially at the RNA level, provides an important next step in the protection of oilseed crops like canola.

4.6 REFERENCES

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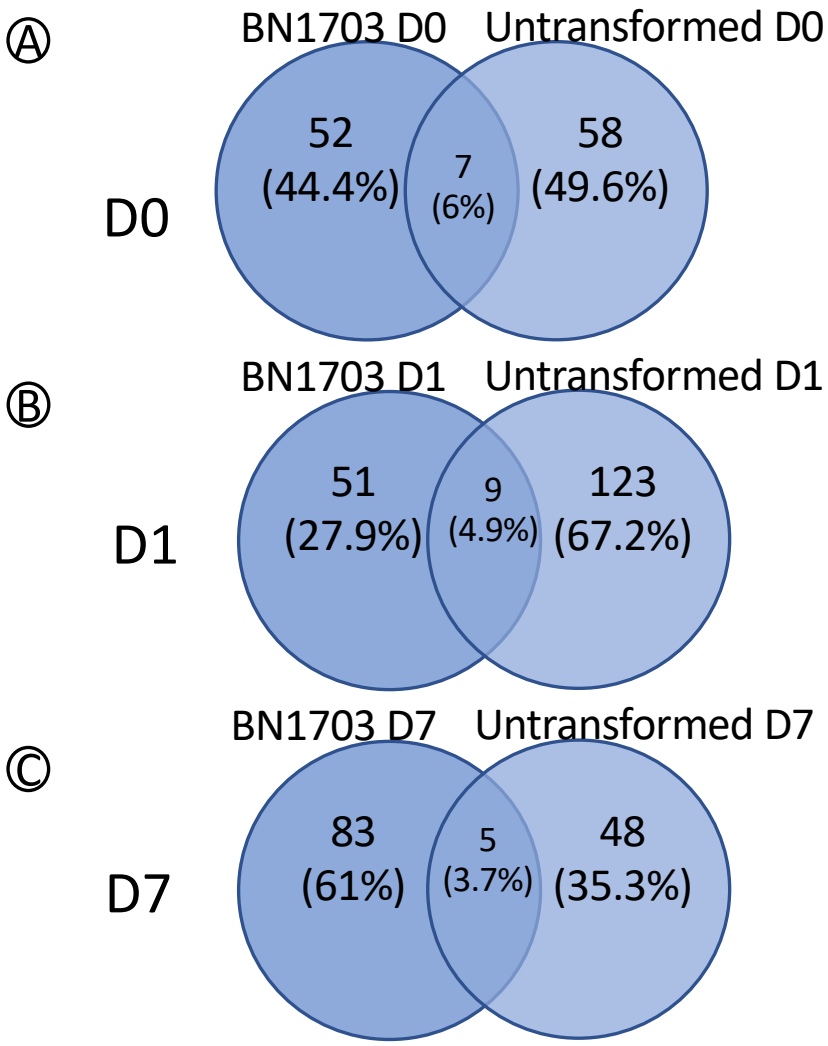

Figure S4.1. Venn diagrams displaying unique and shared small RNA species between BN1703 and untransformed at each timepoint for *B. napus*.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Overview of impact

Sclerotinia scleriorum is a devastating pathogen of many important agronomic crops, not only in Canada but around the world. Traditional approaches to mitigate pathogen damage, such as chemical application, farming or agronomic practices, or genetic modification have so far had meager or decreasing levels of success, which underlines the need to investigate new and more innovative approaches to reduce the virulence of the pathogen or increase the resilience of the host in this pathological system. Throughout my thesis, I have built a strong body of evidence for the utilization of RNA interference-based technologies and management strategies against fungal phytopathogens in *Brassica napus*. In particular, I have worked on developing and optimizing two successful implementations of this technology, through a topical application-based approach (SIGS) and through a transgenic approach (HIGS) and worked to better understand the molecular mechanisms underlying these systems.

In Chapter 2, I provided the first description of the mechanism of dsRNA uptake in a fungal species through my description of uptake facilitated by clathrin-mediated endocytosis. It is my hope that this knowledge will be critical in the further development of SIGS-based applications through optimization of dsRNA lengths, structures and formulations to promote uptake. The experiments I performed in Chapter 3 represent a deep exploration into HIGS as a stable technology for fungal pathogen control. We demonstrated not only the effectiveness of this technology at promoting increased protection, but also teased apart the the interplay between this genetic engineering strategy and host defense pathways that were unlocked through the attenuation of the pathogen. This work combines transcriptomics with phenotypic work done at the cellular level to validate these enriched bioprocesses. This work also bleeds into Appendix 1, which is an exploratory chapter on the small RNA landscape within the *B. napus – S. sclerotiorum* pathosystem. These experiments are preliminary analyses of this interaction and provide a predictive framework into the cross-kingdom communication between host plants and fungal pathogens at the small RNA level, but may also help to explain how *S. sclerotiorum* is such a successful pathogen.

5.2 Implications and Future Directions

The implications of this work are wide reaching. First, this RNA interference technology has the potential to be a safe, sustainable and highly effective option for producers when approved by regulatory bodies. While much of my research focused on the more applied side of science and technological development, the uncovering of the mechanism of dsRNA uptake represents a more fundamental and discovery focus in the hope of appealing and benefitting a wide audience of researchers working to optimize SIGS-based systems. Future research is required to investigate the modes of uptake of differing dsRNA structures and whether they utilize mechanisms alternative to clathrin mediated endocytosis. However, the methods to investigate this in other fungal species have been laid out in this study. My study was also unable to identify a dsRNA specific receptor that initiates uptake and this will be a critical finding to understand and circumvent possible modes of resistance to RNAi. Identification of this receptor will likely require genome-wide screening combined with affinity capture techniques such as chromatin-immunoprecipitation (ChIP) to identify receptors with dsRNA binding potential.

Plant resistance is a complex and refined process involving multiple layers of immune signaling to assess the appropriate response to a pathogen. Understanding the mechanisms involved is of the upmost importance for pathologists and has a direct impact on food safety. Through our use of transcriptomics we were able to resolve mechanisms by which *B. napus* was able to increase its tolerance in the HIGS line compared to untransformed. This has a direct application to further resistance breeding efforts where we may be able to select for these traits that confer a host advantage. A next step would be to combine our understanding of how tolerance is promoted at the gene expression level with what we have learned about small RNA production and bidirectional transfer. This may involve further examination of sRNA populations and performing degradome sequencing to gain evidence into predicted mRNA targets of the small RNAs produced by either *B. napus* or *S. sclerotiorum*. Furthermore, it would be insightful to characterize the genes that are predicted targets of this cross-kingdom RNAi that may lead to a susceptible or resistant phenotype.

My research has produced resources that will be valuable to the scientific community. This includes large RNA and small RNA sequencing datasets that have been all been made publicly available through GEO. The utilization of my data into larger metanalyses should provide valuable information for plant pathologists specifically studying HIGS or general plant defense.

Off-target effects of RNAi remain a concern for those interested in the specificity of the technology. For example, infecting HIGS plants with a variety of closely and distantly related fungal pathogens and investigating potential off-target effects at the molecular and phenotypic level is a worthwhile endeavor. Questions also remain regarding the level of dsRNA processing, mechanism of dsRNA sorting and exporting of the hpRNA molecule within the plant. . Additionally, are other gene targets or dsRNA structures more effective at controlling *S. sclerotiorum* infection? Can we target multiple *S. sclerotiorum* genes at one time for enhanced protection and can we engineer *B. napus* to express hairpin RNAs that target multiple crop pests and pathogens. The combination of global transcriptomic analysis together with cellular visualization tools will provide additional information into the underlying mechanisms of host induced gene silencing.

While there remains unanswered questions concerning the broader impacts of exogenous dsRNA and constitutive dsRNA production in plants, my thesis nevertheless provides predictive and functional evidence that a new generation of sustainable technologies has the potential to overcome food production challenges and provide alternative tools to traditional chemistries. Experimental validation of these questions and considerations would add important data to conclude a compelling investigation of the potential of RNAi-based strategies for phytopathogenic management.