Global RNA profiling of susceptible and tolerant genotypes of *Brassica napus* infected with *Sclerotinia sclerotiorum* and prediction and functional characterization of novel regulators of plant defense

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ABSTRACT

Brassica napus (L.) contributes over $19 billion dollars each year to the Canadian economy. However, yields are constantly threatened by *Sclerotinia sclerotiorum* (Lib) de Bary, the fungus responsible for Sclerotinia stem rot. To date, there are no global RNA profiling data or gene regulatory analyses of plant tissues directly at the main site of foliar infection in the *B. napus*-*S. sclerotiorum* pathosystem. Using RNA sequencing and a gene regulatory analysis, I discovered putative transcriptional regulators of biological processes associated with the tolerant phenotype of *B. napus* cv. Zhougyou821 including subcellular localization of proteins, pathogen detection, and redox homeostasis. Functional characterization of Arabidopsis mutants identified a number of genes that contribute directly to plant defense to *S. sclerotiorum*. Together this research amounts to the expansion of our understanding of the *B. napus*-*S. sclerotiorum* pathosystem and a valuable resource to help protect *B. napus* crops from virulent pathogens such as *S. sclerotiorum*.

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NON-COMMON ABBREVIATIONS USED

OA	Oxalic acid
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
MAPK	Mitogen activated protein kinase
DAMP	Damage associated molecular pattern
SA	Salicylic acid
ET	Ethylene
JA	Jasmonic acid
PTI	PAMP-triggered immunity
ETI	Effector-triggered immunity
RNA-seq	RNA sequencing
LMD	Laser microdissection
TF	Transcription factor
RNAi	RNA interference
dsRNA	double-stranded RNA
ROS	Reactive oxygen species
OG	Oligogalacturonides
FPKM	Fragments Per kilobase of gene per million reads mapped
GO	Gene ontology
DP	Dominant pattern
RT-qPCR	Reverse transcribed quantitative polymerase chain reactio

CHAPTER ONE: BACKGROUND AND RELEVANCE

To meet the demands of a rapidly growing global population that is expected to reach 10 billion people by the year 2050 (United Nations, 2015), collective agricultural output will need to increase from 60-110% of current production without significant increases in arable land (Ray et al., 2013; Tilman et al., 2011). Over the same time frame, abiotic stresses brought on by anthropogenic climate change may independently lead to global crop losses of 17% (Nelson et al., 2014). Furthermore, fungal pathogens have the capacity to destroy up to 60% of all crops in a severe epidemic, and can to lead social and economic problems (Fisher et al., 2012). It is therefore critical for plant scientists, researchers and agronomists to understand the underlying biology behind plant defense processes to limit fungal pathogenesis and protect the world's food supply.

1.1 BRASSICA NAPUS IS A MAJOR OILSEED CROP AND ESSENTIAL PART OF THE CANADIAN ECONOMY

Production, processing and use of canola (*Brassica napus* (L.)) are major parts of the agricultural economy, with of approximately 20 million acres planted in Canada, canola contributes an estimated 19.3 billion dollars and providing nearly 250,000 jobs across Canada alone (Canola Council of Canada 2014). The high oil and protein content of *B. napus* with low glucosinolate (<30 moles per gram) and erucic acid (<2% in oil) make canola a valuable crop with diverse uses as components of edible oil, feed, and industrial applications (Rahman et al.,

2013). However, as with many widely cultivated species, it is constantly under attack by a number of pathogens that can devastate yields and productivity. Sclerotinia stem rot, caused by the versatile necrotrophic fungus *Sclerotinia sclerotiorum* (Lib) de Bary is one of the widely occurring diseases of canola in Canada (McLaren et al., 2004) as well as around the world in India, China, and Australia (Barbetti et al., 2015).

1.2 SCLEROTINIA BIOLOGY

Sclerotinia is a versatile and devastating necrotrophic fungal pathogen capable of infecting over 450 plant species in a variety of plant families (Boland and Hall, 1994). Fungal inoculum persists in the soil as hardened and melanised hyphal structures termed sclerotia. When environmental conditions are appropriate, sclerotia can germinate either myceliogenically to directly produce infectious hyphae, or carpogenically, which yields ascospore-producing apothecia (Bolton, 2006). In the *B. napus*-*S. sclerotiorum* pathosystem infection process, wind-borne ascospores produced by apothecia during the flowering stages of plant development land on flower petals. During suitably humid or wet environmental conditions, the infecting ascospores utilize nutrients released from the senescing petals to germinate and finance a hyphal attack on leaf and stem structures of the canola plant (Figure 1.1, Hegedus and Rimmer, 2005). Sclerotinia uses a number of different cell wall degrading enzymes, production of oxalic acid, as well as other effectors to maintain its virulence (Bolton, 2006). In order to overcome the physical barriers of the plant, *S. sclerotiorum* uses specialized complex appressoria termed 'infection cushions' and infection pegs (Jamaux et al., 1995). Oxalic acid is the most important factor, as OA production mutants fail to infect plant hosts (Cessna et al., 2000). OA has a number of functions including acidification of the cell wall, sequestering calcium, and disrupting redox homeostasis (Kim et al., 2008). Once the physical barriers of the plant have been penetrated, the

infection progresses through plant tissues, eventually resulting in lodging and death of the plant and production of more sclerotia (Figure 1.1).

1.3 PLANT DEFENSE

The first layer of plant defense to pathogens encompasses structural features such as the plant cuticle and stomata, and together these prevent the majority of attacks from opportunistic pathogens (Łaźniewska et al., 2012). However effective pathogens can easily overcome structural resistance and all plants have a multilayered signalling network to detect pathogens and divert resources from growth and reproduction to defense processes (Huot et al., 2014). Artificially induced pre-emptive or constitutive defense response activation drains energy from growth and development, whereas insufficient activation of defenses leaves plants vulnerable (Malinovsky et al., 2014). Thus balancing defense and developmental signals is a critical component of crop species viability.

1.3.1 Detecting and transducing pathogen signals

The first step in a defence response is the detection of pathogens and plants have receptor proteins, capable of detecting conserved signatures of pathogens collectively called PAMPs (Pathogen Associated Molecular Patterns). Some major PAMPs that have been well characterized and studied are chitin oligomers, bacterial flagellin, and bacterial elongation factors (Wu et al., 2014). PAMPs are typically detected by well-conserved plasma membrane-localized pattern recognition receptors (PRRs) and upon activation, lead to an array of defense processes and cellular reprogramming. For example, the Arabidopsis plasma membrane bound atCERK1 is the canonical example of a chitin receptor (Liu et al., 2012). Homodimerization of the receptor with a chitin oligomer activates the first round of defense signalling including

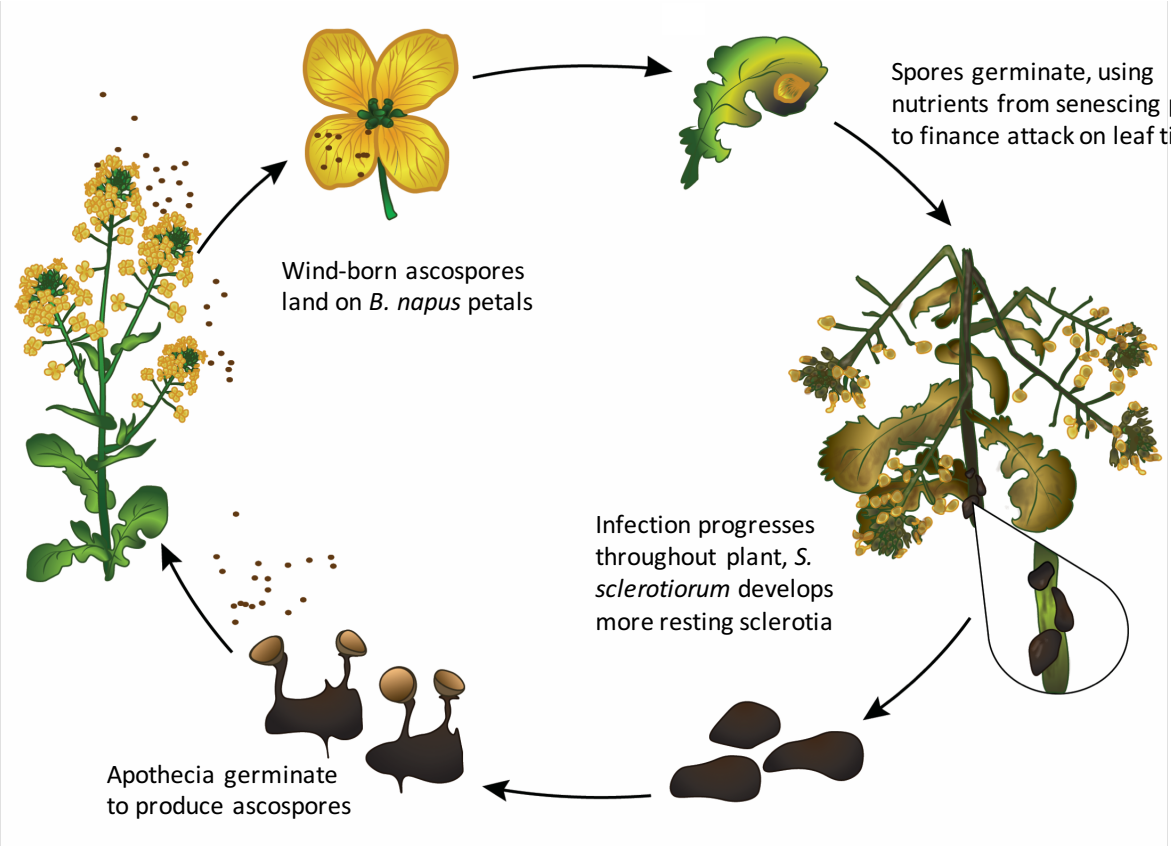

Figure 1.1 Life cycle of *Sclerotinia sclerotiorum* causing stem rot in *Brassica napus*. Resting sclerotia in the soil germinate spore-producing apothecia under the canopy of canola in suitable environmental conditions. Wind-borne ascospores land on senescing petals which land on leaf and shoot tissues. The ascospores may then use the released nutrients from the petal to infect healthy plant tissue. Hyphal growth progresses through the plant eventually resulting in plant death and production of more sclerotia.

reactive oxygen production as well as mitogen activated protein kinase (MAPK) activation (Faulkner et al., 2013).

In addition to a detection system for other organisms, plants also have an innate ability to sense damage to self. Damage associate molecular patterns (DAMPs) function in a very similar way to PAMPs and many of the responses overlap. Pattern recognition receptors detect plant breakdown products such as oligogalacturonides, cutin monomers, and certain peptides (Heil and Land, 2014). Much less is known about the perception mechanisms of DAMPs, however the recent discovery of an extracellular ATP receptor in Arabidopsis, DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1), points to a possible molecular mechanism for detecting damaged cells (Choi et al., 2014). DORN1 is a plasma membrane-localized lectin receptor kinase shown to bind extracellular ATP and trigger Ca^{2+} influxes. Ca^{2+} influxes trigger ROS production (Beneloujaephajri et al., 2013), which leads to activation of Mitogen activated protein kinase (MAPK) cascade genes leading to transcriptional responses (Smékalová et al., 2014). MAPK cascades are a complex signal transduction mechanism conserved across euakyotes involving three types of protein kinases: MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs). In plants, MAPK signalling is responsible for coordinating a wide variety of responses from both biotic and abiotic stresses (Nakagami et al., 2005). In *B. napus* overexpression of *MITOGEN ACTIVATED PROTEIN KINASE 4*, has been shown to confer resistance to *S. sclerotiorum* (Wang et al., 2009b), highlighting the importance of MAPK signalling in response to necrotrophic pathogens.

Plant defense hormones are small molecules essential to effective and timely defense responses. In general, biotrophic pathogens tend to elicit stronger activation of the salicylic acid pathway (SA), which elicits *PATHOGENESIS-RELATED GENE* family member expression and

is required for systemic immunity (Kumar, 2014). Herbivores and necrotrophic pathogens generally activate the ethylene (ET)/jasmonic acid (JA) pathway more strongly, which activates *PLANT DEFENSIN* genes and *ETHYLENE RESPONSE FACTOR* family transcription factors (Pieterse et al., 2012). Our current understanding of hormones and plant defense show that there is a complex interaction between the different pathways required for a strong defence response (Perchepied et al., 2010).

1.3.2 PAMP-triggered immunity and pathogen effectors

The successful activation of plant defense in response to PAMPs is termed PAMP-Trigger Immunity (PTI). However, effective pathogens find ways to skirt PTI and the first level of plant defense mechanisms. Effector triggered immunity (ETI) refers to the defense pathways activated in response to the detection of particular pathogen effector molecules, virulence gene products designed to interfere with plant PTI (Cui et al., 2015). These pathogenic effectors antagonize particular steps in either the detection processes or downstream plant signal transduction, with many conserved targets (Mukhtar et al., 2011; Weßling et al., 2014). To combat this, plants have evolved to produce molecules that identify and flag pathogen effector molecules. Plant detection of these effectors elicits signals for an even stronger defense response including cell death through the hypersensitivity response (Cui et al., 2015). ETI is generally associated with plant defense to biotrophic and hemi-biotrophic pathosystems (Wang et al., 2014) and no true resistance genes have been found in the *B. napus*-*S. sclerotiorum* pathosystem.

1.3.3 The Brassica napus-Sclerotinia sclerotiorum *pathosystem*

Considering the economic importance of the *B. napus*-*S. sclerotiorum* pathosystem, it follows that a great deal of insight into the structural and molecular aspects of the infection process have been described. For example Garg et al. (2013) identified proteins differentially

expressed in a tolerant cultivar to sclerotinia inoculation of susceptible and resistant cotyledons, and other work has found a correlation between field resistance and optimal stem diameter of both *B. napus* and *B. juncea* (Li et al., 2006). At the molecular level, it has been nearly a decade since the first array-based RNA profiling experiments investigating the *B. napus*-*S. sclerotiorum* pathosystem were presented. Arabidopsis microarrays used to detect changes in RNA following infection have provided lots of information about how *B. napus* hormone pathways and metabolism changes in response to infection with *S. sclerotiorum* (Yang et al., 2007; Zhao et al., 2007). More recently, a microarray specifically developed for *B. napus* was also later used to uncover differences in gene expression patterns between susceptible and tolerant lines of canola using an artificial stem inoculation method (Zhao et al., 2009). One of the major limitations of these studies has been the limited effectiveness of microarray technologies. Since *B. napus* is an allopolyploid (Chalhoub et al., 2014), cross hybridization between homologues may obscure results and as well as the limited number of probsets, constitute a major gap in their capacity to evaluate global changes in gene activity following infection. High-throughput RNA sequencing (RNA-seq) experiments have many advantages over microarray technology, including higher sensitivity, no requisite *a priori* information required, and the ability to profile all RNAs (Metzker, 2009). Recently, Wu et al. (2016) used RNA-seq and differential gene expression to investigate defense responses in infected stem tissues of a susceptible and resistant line of *B. napus* and found differences in signal transduction networks and glucosinolate biosynthesis. However, this study does not contribute to our understanding of how susceptible or tolerant phenotypes are specified at the main foliar site of infection. We also have little to no information on how defence processes leading to positive disease outcomes are transcriptionally controlled.

1.4 RESEARCH OBJECTIVES

Brassica napus is an important part of the agricultural economy in Canada and around the world. Considering that yield losses caused by *S. sclerotiorum* can be devastating, a comprehensive understating of the genetic programs underlying tolerance to sclerotinia is critical to building better crops. Previous work has uncovered much about the pathosystem but no analysis of the global transcriptional changes undergone directly at the main site of infection using a petal inoculation technique has been described. The objective of my research is to discover how global biological processes shift in response to infection with *S. sclerotiorum* in susceptible *B. napus* cv. Westar, and tolerant cv. Zhongyou821 (ZY821). I will discover how defense processes are specified at the site of infection. Additionally, I will identify putative regulators of the defense processes and explore their functional role as regulators of plant defense.

1.5 RESEARCH QUESTIONS AND HYPOTHESISES

1.5.1 Global transcriptional profiling of *Brassica napus* response to *Sclerotinia sclerotiorum*

Question: How do global transcriptional programs change in response to infection with sclerotinia?

Hypothesis:

I hypothesize that both susceptible and tolerant genotypes of *B. napus* will undergo large shifts in transcriptional programming following infection with *S. sclerotiorum*. I hypothesize that Westar and ZY821 will both activate genes involved in the plant response to pathogens but I expect there will be differences at the RNA level that underpin the tolerant phenotype. I expect there will be large sets of significantly differentially expressed genes following infection in both

cultivars, and that co-expression analysis will identify sets of genes with diverse transcript accumulation patterns. Using gene ontology enrichment to uncover biological processes active within these gene sets, I hypothesize that I will discover differences in signal transduction, protein turnover, and cytostructural responses that underpin the tolerant and susceptible phenotypes.

Relevance:

There is currently little known about how global transcriptional change in *B. napus* leaves following infection with *S. sclerotiorum*. Profiling susceptible and tolerant cultivars using RNA-sequencing and gene ontology analyses to discover what types of processes are activated directly at the site of infection will greatly contribute to our understanding of how infection outcomes are specified in this pathosystem. This will provide invaluable information about the biological processes that underlie *B. napus* tolerance to *S. sclerotiorum*.

1.5.2 Functional characterization of predicted regulators in Arabidopsis

Question: What molecules are responsible for regulating defense processes in B. napus? How do loss-of-function mutants of predicted plant defense regulators affect the plant response to Sclerotinia in Arabidopsis?

Hypothesis:

I hypothesize gene analysis will identify putative regulators of plant defense processes active in ZY821 following sclerotinia infection. I also hypothesize that knocking down expression of predicted regulators of plant defense with T-DNA insertion lines will induce susceptibly to *Sclerotinia* in arabidopsis. Given that the timely activation of defense processes is essential for plant protection against fungal pathogens, I hypothesize that mutants will have

aberrant signal transduction cascades and will be more susceptible to *S. sclerotiorum*. I also expect to detect differences in how defense genes are activated using quantitative reverse-transcribed PCR.

Relevance:

The activation of defense processes in plants is complex and requires the activation of genes and gene regulatory networks controlling biological processes responsible for plant protection in space and time. While there remain gaps in our scientific understanding of how infection outcomes are regulated at the RNA level, the use of novel molecular biology and computational tools should provide clues into the underpinnings of plant tolerance to fungal pathogens like *S. sclerotiorum* directly at the site of infection. Identifying putative regulators through bioinformatic analyses should identify candidate molecules for functional validation, thus reducing the time and costs associated with broad-scale screening efforts. Taken together, this body of work will contribute to a better understanding of the genes responsible for mediating positive disease outcomes in *Sclerotinia*-infected crops.

1.6 LITERATURE CITED

Barbetti, M. J., Li, C. X., Banga, S. S., Banga, S. K., Singh, D., Sandhu, P. S., et al. (2015). New host resistances in *Brassica napus* and *Brassica juncea* from Australia, China and India: Key to managing Sclerotinia stem rot (*Sclerotinia sclerotiorum*) without fungicides. *Crop Prot.* 78, 127–130. doi:10.1016/j.cropro.2015.09.004.

Baum, J. a, Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007). Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–6. doi:10.1038/nbt1359.

Belmonte, M. F., Kirkbride, R. C., Stone, S. L., Pelletier, J. M., Bui, A. Q., Yeung, E. C., et al. (2013). Comprehensive developmental profiles of gene activity in regions and subregions of the Arabidopsis seed. *Proc. Natl. Acad. Sci. U. S. A.* 110, E435–44. doi:10.1073/pnas.1222061110.

Berardini, T. Z., Reiser, L., Li, D., Mezheritsky, Y., Muller, R., Strait, E., et al. (2015). The arabidopsis information resource: Making and mining the "gold standard" annotated reference plant genome. *Genesis* 53, 474–485. doi:10.1002/dvg.22877.

Boland, G. J., and Hall, R. (1994). Index of plant hosts of *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol.* 16, 93–108. doi:10.1080/07060669409500766.

Bolton, M. (2006). *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol.* 7, 1–16. doi:10.1111/J.1364-3703.2005.00316.X.

Cessna, S. G., Sears, V. E., Dickman, M. B., Low, P. S., Cessna, S. G., Sears, V. E., et al. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *Plant Cell* 12, 2191–2199.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I. a. P., Tang, H., Wang, X., et al. (2014). Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science (80-.).* 345, 950–953. doi:10.1126/science.1253435.

Chandran, D., Inada, N., Hather, G., Kleindt, C. K., and Wildermuth, M. C. (2010). Laser microdissection of Arabidopsis cells at the powdery mildew infection site reveals site-specific processes and regulators. *Proc. Natl. Acad. Sci. U. S. A.* 107, 460–5. doi:10.1073/pnas.0912492107.

Choi, J., Tanaka, K., Cao, Y., Qi, Y., Qiu, J., Liang, Y., et al. (2014). Identification of a plant receptor for extracellular ATP. *Science* 343, 290–4. doi:10.1126/science.343.6168.290.

Cui, H., Tsuda, K., and Parker, J. E. (2015). Effector-Triggered Immunity: From pathogen perception to robust defense. *Annu. Rev. Plant Biol.* 66, 287–511. doi:10.1146/annurev-arplant-050213-040012.

Day, R. C., Grossniklaus, U., and Macknight, R. C. (2005). Be more specific! Laser-assisted microdissection of plant cells. *Trends Plant Sci.* 10, 397–406. doi:10.1016/j.tplants.2005.06.006.

Deal, R. B., and Henikoff, S. (2011). The INTACT method for cell type-specific gene expression and chromatin profiling in *Arabidopsis thaliana*. *Nat. Protoc.* 6, 56–68. doi:10.1038/nprot.2010.175.

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., et al. (2014). Environmental fate of double-stranded RNA in agricultural soils. *PLoS One* 9, 1–7. doi:10.1371/journal.pone.0093155.

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., Mccraw, S. L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484, 186–

194. doi:10.1038/nature10947.

Garg, H., Li, H., Sivasithamparam, K., and Barbetti, M. J. (2013). Differentially expressed proteins and associated histological and disease progression changes in cotyledon tissue of a resistant and susceptible genotype of *Brassica napus* infected with *Sclerotinia sclerotiorum*. *PLoS One* 8, e65205. doi:10.1371/journal.pone.0065205.

Gautam, V., and Sarkar, A. K. (2014). Laser assisted microdissection, an efficient technique to understand tissue specific gene expression patterns and functional genomics in Plants. *Mol. Biotechnol.* 57, 299–308. doi:10.1007/s12033-014-9824-3.

Ghag, S. B., Shekhawat, U. K. S., and Ganapathi, T. R. (2014). Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against Fusarium wilt in banana. *Plant Biotechnol. J.* 12, 541–553. doi:10.1111/pbi.12158.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29. doi:10.1038/nbt.1883.

Grant, D., Nelson, R. T., Cannon, S. B., and Shoemaker, R. C. (2010). SoyBase , the USDA-ARS soybean genetics and genomics database. *Nucleic Acids Res.* 38, 843–846. doi:10.1093/nar/gkp798.

Hacquard, S., Delaruelle, C., Legué, V., Tisserant, E., Kohler, A., Frey, P., et al. (2010). Laser capture microdissection of uredinia formed by *Melampsora larici-populina* revealed a transcriptional switch between biotrophy and sporulation. *Mol. Plant. Microbe. Interact.* 23, 1275–1286. doi:10.1094/MPMI-05-10-0111.

Hayden, K. J., Garbelotto, M., Knaus, B. J., Cronn, R. C., Rai, H., and Wright, J. W. (2014). Dual RNA-seq of the plant pathogen *Phytophthora ramorum* and its tanoak host. *Tree*

Genet. Genomes 10, 489–502. doi:10.1007/s11295-014-0698-0.

Hegedus, D. D., and Rimmer, S. R. (2005). *Sclerotinia sclerotiorum*: When "to be or not to be" a pathogen? *FEMS Microbiol. Lett.* 251, 177–184. doi:10.1016/j.femsle.2005.07.040.

Heil, M., and Land, W. G. (2014). Danger signals - damaged-self recognition across the tree of life. *Front. Plant Sci.* 5, 578. doi:10.3389/fpls.2014.00578.

Hirooka, T., and Ishii, H. (2013). Chemical control of plant diseases. *J. Gen. Plant Pathol.* 79, 390–401. doi:10.1007/s10327-013-0470-6.

Huot, B., Yao, J., Montgomery, B. L., and He, S. Y. (2014). Growth-defense tradeoffs in plants: a balancing act to optimize fitness. *Mol. Plant* 7, 1267–87. doi:10.1093/mp/ssu049.

Inada, N., and Wildermuth, M. C. (2005). Novel tissue preparation method and cell-specific marker for laser microdissection of Arabidopsis mature leaf. *Planta* 221, 9–16. doi:10.1007/s00425-004-1427-y.

Jamaux, I., Gelie, B., and Lamarque, C. (1995). Early stages of infection of rapeseed petals and leaves by *Sclerotinia sclerotiorum* revealed by scanning electron microscopy. *Plant Pathol.* 44, 22–30. doi:10.1111/j.1365-3059.1995.tb02712.x.

Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T., and Minami, E. (2012). Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. *PLoS One* 7, e49423. doi:10.1371/journal.pone.0049423.

Khan, D., Chan, A., Millar, J. L., Girard, I. J., and Belmonte, M. F. (2014a). Predicting transcriptional circuitry underlying seed coat development. *Plant Sci.* 223, 146–152. doi:10.1016/j.plantsci.2014.03.016.

Khan, D., Millar, J., Girard, I., and Belmonte, M. (2014b). Transcriptional circuitry underlying seed coat development in Arabidopsis. *Plant Sci.* 219-220, 51–60.

doi:doi:10.1016/j.plantsci.2014.01.004.

Kim, K. S., Min, J.-Y., and Dickman, M. B. (2008). Oxalic acid is an elicitor of plant programmed cell death during. *Mol. Plant. Microbe. Interact.* 21, 605–612. doi:10.1094/MPMI-21-5-0605.

Klink, V. P., Hosseini, P., Matsye, P., Alkharouf, N. W., and Matthews, B. F. (2009). A gene expression analysis of syncytia laser microdissected from the roots of the *Glycine max* (soybean) genotype PI 548402 (Peking) undergoing a resistant reaction after infection by *Heterodera glycines* (soybean cyst nematode). *Plant Mol. Biol.* 71, 525–567. doi:10.1007/s11103-009-9539-1.

Klink, V. P., and Thibaudeau, G. (2014). Laser microdissection of semi-thin sections from plastic-embedded tissue for studying plant–organism developmental processes at single-cell resolution. *J. Plant Interact.* 9, 610–617. doi:10.1080/17429145.2013.879677.

Kumar, D. (2014). Salicylic acid signaling in disease resistance. *Plant Sci.* 228, 127–134. doi:10.1016/j.plantsci.2014.04.014.

Łaźniewska, J., Macioszek, V. K., and Kononowicz, A. K. (2012). Plant-fungus interface: The role of surface structures in plant resistance and susceptibility to pathogenic fungi. *Physiol. Mol. Plant Pathol.* 78, 24–30. doi:10.1016/j.pmpp.2012.01.004.

Li, C. X., Li, H., Sivasithamparam, K., Fu, T. D., Li, Y. C., Liu, S. Y., et al. (2006). Expression of field resistance under Western Australian conditions to *Sclerotinia sclerotiorum* in Chinese and Australian *Brassica napus* and *Brassica juncea* germplasm and its relation with stem diameter. *Aust. J. Agric. Res.* 57, 1131–1135. doi:10.1071/AR06066.

Lucht, J. (2015). Public acceptance of plant biotechnology and GM Crops. *Viruses* 7, 4254–4281. doi:10.3390/v7082819.

Malinovsky, F. G., Batoux, M., Schwessinger, B., Youn, J. H., Stransfeld, L., Win, J., et al. (2014). Antagonistic regulation of growth and immunity by the arabidopsis basic helix-loop-helix transcription factor HOMOLOG OF BRASSINOSTEROID ENHANCED EXPRESSION2 INTERACTING WITH INCREASED LEAF INCLINATION1 BINDING bHLH1. *Plant Physiol.* 164, 1443–1455. doi:10.1104/pp.113.234625.

Martin, J. A., and Wang, Z. (2011). Next-generation transcriptome assembly. *Nat. Publ. Gr.* 12, 671–682. doi:10.1038/nrg3068.

McLaren, D. L., Conner, R. L., Platford, R. G., Lamb, J. L., Lamey, H. a, and Kutcher, H. R. (2004). Predicting diseases caused by *Sclerotinia sclerotiorum* on canola and bean - a western Canadian perspective. *Can. J. Plant Pathol. Can. Phytopathol.* 26, 489–497. doi:10.1080/07060660409507169.

Metzker, M. L. (2009). Sequencing technologies — the next generation. *Nat. Rev. Genet.* 11, 31–46. doi:10.1038/nrg2626.

Mukhtar, M. S., Carvunis, A.-R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., et al. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333, 596–601. doi:10.1126/science.1203659.

Nakayashiki, H. (2005). RNA silencing in fungi: Mechanisms and applications. *FEBS Lett.* 579, 5950–5957. doi:10.1016/j.febslet.2005.08.016.

Nations, U. (2015). Key findings and advanced tables.

Nelson, G. C., Valin, H., Sands, R. D., Havlík, P., Ahammad, H., Deryng, D., et al. (2014). Climate change effects on agriculture: economic responses to biophysical shocks. *Proc. Natl. Acad. Sci. U. S. A.* 111, 3274–9. doi:10.1073/pnas.1222465110.

Palli, S. R. (2014). RNA interference in Colorado potato beetle: steps toward development of

dsRNA as a commercial insecticide. *Curr. Opin. Insect Sci.* 6, 1–8. doi:10.1016/j.cois.2014.09.011.

Perchepied, L., Balagué, C., Riou, C., Claudel-Renard, C., Rivière, N., Grezes-Besset, B., et al. (2010). Nitric oxide participates in the complex interplay of defense-related signaling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *Mol. Plant. Microbe. Interact.* 23, 846–860. doi:10.1094/MPMI-23-7-0846.

Pieterse, M. J., Does, D. Van Der, Zamioudis, C., Leon-reyes, A., and Wees, S. C. M. Van (2012). Hormonal modulation of plant immunity. doi:10.1146/annurev-cellbio-092910-154055.

Podio, N. S., Guzmán, C. a, and Meriles, J. M. (2008). Microbial community structure in a silty clay loam soil after fumigation with three broad spectrum fungicides. *J. Environ. Sci. Health. B.* 43, 333–340. doi:10.1080/03601230801941675.

Priebe, S., Kreisel, C., Horn, F., Guthke, R., and Linde, J. (2015). FungiFun2: A comprehensive online resource for systematic analysis of gene lists from fungal species. *Bioinformatics* 31, 445–446. doi:10.1093/bioinformatics/btu627.

Rahman, H., Harwood, J., and Weselake, R. (2013). Increasing seed oil content in *Brassica* species through breeding and biotechnology. *Lipid Technol.* 25, 182–185. doi:10.1002/lite.201300291.

Ramsay, K., Wang, Z., and Jones, M. G. K. (2004). Using laser capture microdissection to study gene expression in early stages of giant cells induced by root-knot nematodes. *Mol. Plant Pathol.* 5, 587–592. doi:10.1111/J.1364-3703.2004.00255.X.

Ray, D. K., Mueller, N. D., West, P. C., and Foley, J. A. (2013). Yield trends are insufficient to double global crop production by 2050. *PLoS One* 8. doi:10.1371/journal.pone.0066428.

Reuter, J. A., Spacek, D. V, and Snyder, M. P. (2015). High-throughput sequencing technologies. *Mol. Cell* 58, 586–597. doi:10.1016/j.molcel.2015.05.004.

Robinson, K. E., Worrall, E. A., and Mitter, N. (2014). Double stranded RNA expression and its topical application for non-transgenic resistance to plant viruses. *J. Plant Biochem. Biotechnol.* 23, 231–237. doi:10.1007/s13562-014-0260-z.

Rudd, J. J., Kanyuka, K., Hassani-Pak, K., Derbyshire, M., Andongabo, A., Devonshire, J., et al. (2015). Transcriptome and metabolite profiling of the infection cycle of *Zymoseptoria tritici* on wheat reveals a biphasic interaction with plant immunity involving differential pathogen chromosomal contributions and a variation on the hemibiotrophic lifestyle def. *Plant Physiol.* 167, 1158–85. doi:10.1104/pp.114.255927.

San Miguel, K., and Scott, J. G. (2015). The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Manag. Sci.* 72, 801–809. doi:10.1002/ps.4056.

Santi, S., Grisan, S., Pierasco, A., DE Marco, F., and Musetti, R. (2013). Laser microdissection of grapevine leaf phloem infected by stolbur reveals site-specific gene responses associated to sucrose transport and metabolism. *Plant. Cell Environ.* 36, 343–55. doi:10.1111/j.1365-3040.2012.02577.x.

Schiebold, S., Tschiersch, H., Borisjuk, L., Heinzel, N., Radchuk, R., and Rolletschek, H. (2011). A novel procedure for the quantitative analysis of metabolites, storage products and transcripts of laser microdissected seed tissues of *Brassica napus*. *Plant Methods* 7, 19. doi:10.1186/1746-4811-7-19.

Schulze, S., Henkel, S. G., Driesch, D., Guthke, R., and Linde, J. (2015). Computational prediction of molecular pathogen-host interactions based on dual transcriptome data. *Front. Microbiol.* 6, 1–11. doi:10.3389/fmicb.2015.00065.

Schulze, S., Schleicher, J., Guthke, R., and Linde, J. (2016). How to predict molecular interactions between species? *Front. Microbiol.* 7, 1–13. doi:10.3389/fmicb.2016.00442.

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., et al. (2013). Towards the elements of successful insect RNAi. *J. Insect Physiol.* 59, 1212–1221. doi:10.1016/j.jinsphys.2013.08.014.

Senthil-kumar, M., and Mysore, K. S. (2010). RNAi in plants: recent developments and applications in agriculture. *Gene Silenc. Theory, Tech. Appl.*, 183–199.

Sierotzki, H. (2015). "Fungicide Resistance in Plant Pathogens," in, eds. H. Ishii and D. W. Hollomon (Tokyo: Springer Japan), 119–145. doi:10.1007/978-4-431-55642-8.

Stein, J., Naithani, S., Wei, S., Dharmawardhana, P., Kumari, S., Amarasinghe, V., et al. (2014). Gramene 2013: comparative plant genomics resources. *Nucleic Acids Res.* 42, 1193–1199. doi:10.1093/nar/gkt1110.

Tierney, L., Linde, J., M??ller, S., Brunke, S., Molina, J. C., Hube, B., et al. (2012). An interspecies regulatory network inferred from simultaneous RNA-seq of *Candida albicans* invading innate immune cells. *Front. Microbiol.* 3, 1–14. doi:10.3389/fmicb.2012.00085.

Tilman, D., Balzer, C., Hill, J., and Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proc. Natl. Acad. Sci. U. S. A.* 108, 20260–4. doi:10.1073/pnas.1116437108.

Tsuda, K., and Somssich, I. E. (2015). Transcriptional networks in plant immunity. *New Phytol.* 206, 932–947. doi:10.1111/nph.13286.

Wang, X., Jiang, N., Liu, J., Liu, W., and Wang, G.-L. (2014). The role of effectors and host immunity in plant-necrotrophic fungal interactions. *Virulence* 5, 722–32. doi:10.4161/viru.29798.

Wang, Z., Gerstein, M., and Snyder, M. (2009a). RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63.

Wang, Z., Mao, H., Dong, C., Ji, R., Cai, L., Fu, H., et al. (2009b). Overexpression of *Brassica napus* MPK4 Enhances Resistance to *Sclerotinia sclerotiorum* in Oilseed Rape. 22, 235–244.

Ward, J. A., Ponnala, L., and Weber, C. A. (2012). Strategies for transcriptome analysis in nonmodel plants. *Am. J. Bot.* 99, 267–276. doi:10.3732/ajb.1100334.

Weßling, R., Epple, P., Altmann, S., He, Y., Yang, L., Henz, S. R., et al. (2014). Convergent targeting of a common host protein-network by pathogen effectors from three kingdoms of life. *Cell Host Microbe* 16, 364–375. doi:10.1016/j.chom.2014.08.004.

Westermann, A. J., Gorski, S. a., and Vogel, J. (2012). Dual RNA-seq of pathogen and host. *Nat. Rev. Microbiol.* 10, 618–630. doi:10.1038/nrmicro2852.

Whyard, S., Singh, A. D., and Wong, S. (2009). Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* 39, 824–832. doi:10.1016/j.ibmb.2009.09.007.

Wu, J., Zhao, Q., Yang, Q., Liu, H., Li, Q., Yi, X., et al. (2016). Comparative transcriptomic analysis uncovers the complex genetic network for resistance to *Sclerotinia sclerotiorum* in *Brassica napus*. *Sci. Rep.* 6, 19007. doi:10.1038/srep19007.

Yang, B., Srivastava, S., Deyholos, M. K., and Kav, N. N. V. (2007). Transcriptional profiling of canola (*Brassica napus* L.) responses to the fungal pathogen *Sclerotinia sclerotiorum*. *Plant Sci.* 173, 156–171. doi:10.1016/j.plantsci.2007.04.012.

Yang, F., Li, W., and Jørgensen, H. J. L. (2013). Transcriptional reprogramming of wheat and the hemibiotrophic pathogen *Septoria tritici* during two phases of the compatible

interaction. *PLoS One* 8, 1–15. doi:10.1371/journal.pone.0081606.

Yazawa, T., Kawahigashi, H., Matsumoto, T., and Mizuno, H. (2013). Simultaneous transcriptome analysis of Sorghum and *Bipolaris sorghicola* by Using RNA-seq in combination with de novo transcriptome assembly. *PLoS One* 8. doi:10.1371/journal.pone.0062460.

Yu, H., Zhang, F., Wang, G., Liu, Y., and Liu, D. (2013). Partial deficiency of isoleucine impairs root development and alters transcript levels of the genes involved in branched-chain amino acid and glucosinolate metabolism in Arabidopsis. *J. Exp. Bot.* 64, 599–612. doi:10.1093/jxb/ers352.

Zhang, C., Barthelson, R. A., Lambert, G. M., and Galbraith, D. W. (2008). Global characterization of cell-specific gene expression through fluorescence-activated sorting of nuclei. *Plant Physiol.* 147, 30–40. doi:10.1104/pp.107.115246.

Zhao, J., Buchwaldt, L., Rimmer, S. R., Sharpe, A., McGregor, L., Bekkaoui, D., et al. (2009). Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotiorum*. *Mol. Plant Pathol.* 10, 635–649. doi:10.1111/J.1364-3703.2009.00558.X.

Zhao, J., Wang, J., An, L., Doerge, R. W., Chen, Z. J., Grau, C. R., et al. (2007). Analysis of gene expression profiles in response to *Sclerotinia sclerotiorum* in *Brassica napus*. *Planta* 227, 13–24. doi:10.1007/s00425-007-0586-z.

CHAPTER TWO: INTEGRATING LARGE-SCALE DATA AND RNA TECHNOLOGY TO PROTECT CROPS FROM FUNGAL PATHOGENS

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2.1 ABSTRACT

With a rapidly growing human population it is expected that plant science researchers and the agricultural community will need to increase food productivity using less arable land. This challenge is complicated by fungal pathogens and diseases, many of which can severely impact crop yield. Current measures to control fungal pathogens are either ineffective or have adverse effects on the agricultural enterprise. Thus, developing new strategies through research innovation to protect plants from pathogenic fungi is necessary to overcome these hurdles. RNA sequencing technologies are increasing our understanding of the underlying genes and gene regulatory networks mediating disease outcomes. The application of invigorating next generation sequencing strategies to study plant-pathogen interactions has and will provide unprecedented insight into the complex patterns of gene activity responsible for crop protection. However, questions remain about how biological processes in both the pathogen and the host are specified in space directly at the site of infection and over the infection period. The integration of cutting edge molecular and computational tools will provide plant scientists with the arsenal required to identify genes and molecules that play a role in plant protection. Large scale RNA sequence data can then be used to protect plants by targeting genes essential for pathogen viability in the production of stably transformed lines expressing RNA interference molecules, or through foliar applications of double stranded RNA.

2.2 INTRODUCTION

The world's population is expected to increase to nearly 10 billion people in the next 35 years (United Nations, 2015). To meet the demands of a growing population, it is estimated we will need to increase the production of safe, healthy and just food by 60-110% over current rates without an increase in arable land (Ray et al., 2013; Tilman et al., 2011). With fungal pathogens capable of destroying 60% of all crops in a sever epidemic (Fisher et al., 2012), it is an immediate concern and a priority for plant science researchers, breeders and growers to find new, innovative and translatable solutions to protect global food systems. Protecting crops from major fungal outbreaks is traditionally done by employing either lengthy crop rotation times, undesirable for many cash crop producers, or by the application of broad spectrum fungicides that can have adverse consequences for the environment (Podio et al., 2008), and limited usefulness due to development of resistance (Hirooka and Ishii, 2013). To overcome the negative impacts of disease on food production, plant science researchers are turning to modern cutting edge molecular techniques to uncover and understand the underlying genes and gene regulatory networks in host pathogen interactions. A deep understanding of the biology behind the processes that drive either plant tolerance and resistance or susceptibility, is required for breeding new crops and implementing the next generation of pathogen control measures.

For example, global RNA profiling experiments are used to understand gene activity and can evaluate implicit changes in biological processes following the plant-fungal interaction. High throughput sequencing technologies have been available to the scientific community for over a decade now (Metzker, 2009), and more specialized techniques are being developed to investigate

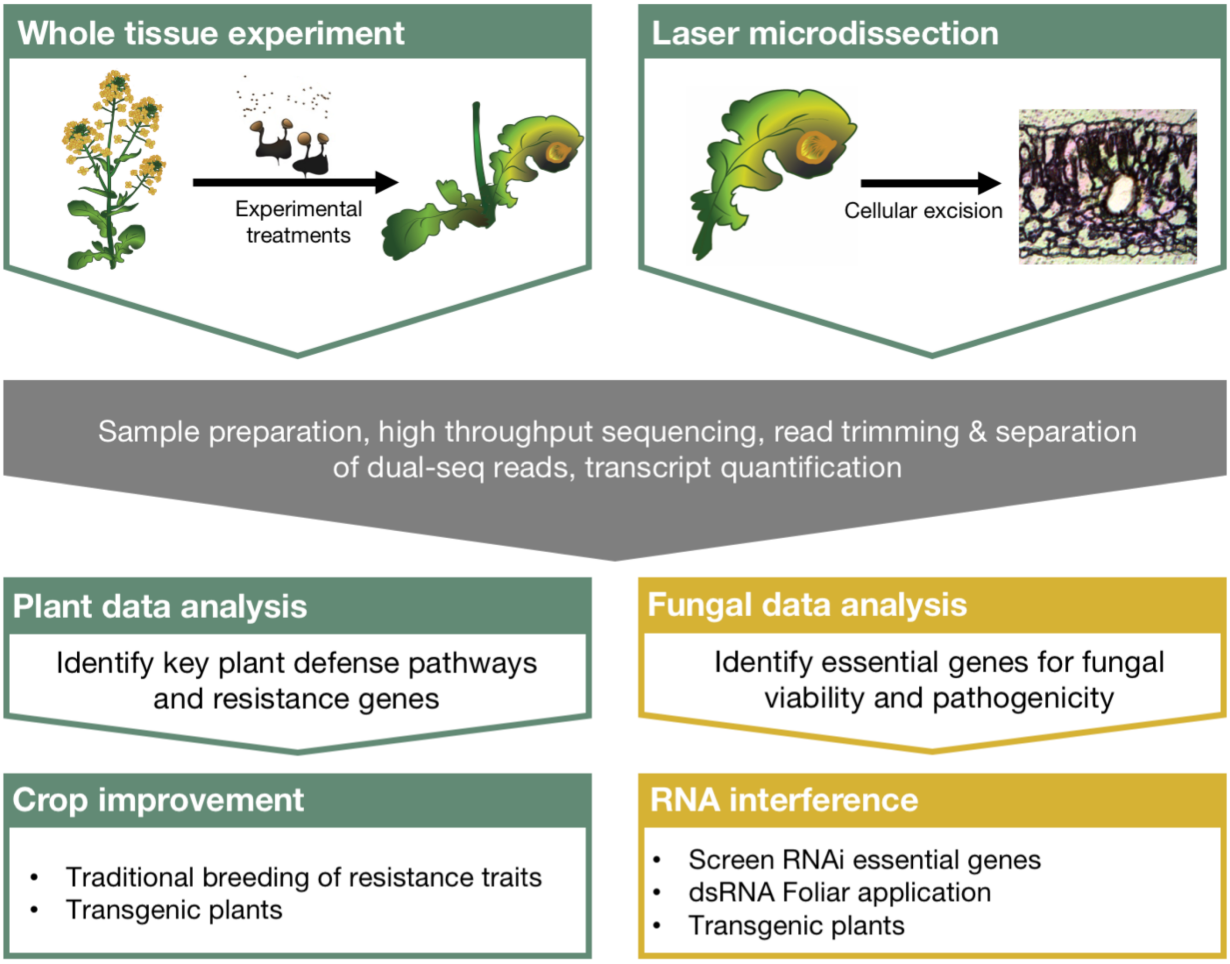

Figure 2.1 Comprehensive strategy linking large scale RNA sequencing experiments with crop protection using RNA interference. Dual sequencing and laser microdissection can overcome many of the limitations of traditional sequencing experiments and greatly improve our understanding of plant-fungal pathosystems. Robust bioinformatics strategies to identify critical regulators of plant defense and fungal pathogenesis can then be directly integrated with crop improvement strategies as well as RNA interference applications for crop protection from pathogenic fungi.

chromatin modifications, microRNAs, and RNA-protein interactions (Reuter et al., 2015). More recently, dual sequencing experiments, those that profile RNA from both the host and the pathogen, have been used to further our understanding of complex molecular interactions. Despite these advancements and the abundance of 'big data' generated to understand host pathogen interactions, the bottleneck in understanding its genetic and biological relevance lies in the distillation process. Additionally, no clear link has been described between the scientific insights taken from these experiments and meaningful ways to protect crops. Here we discuss the integration of RNA sequencing to plant-fungal pathogen interaction studies, the technologies that will increase our resolution and understanding of the complex transcriptional circuitry regulating these interactions, and describe a direct path leading from these experiments to the protection of crops in the field (outlined in Figure 2.1).

2.3 DUAL SEQUENCING OF HOST PATHOGEN INTERACTIONS

Thus the resulting sequence reads from dual-sequencing experiments, regardless of origin, contain a snapshot of the underlying transcriptional programs from both the host and pathogen. The reads that successfully map to the respective genomes can then be used to assess gene activity in the two species. A general dual sequencing experimental outline is described by Westermann et al. (2012), however since its publication, the cost of sequencing experiments has gone down further, supporting the the accessibility of RNA-seq and opening new opportunities for the development of dual sequencing study systems.

Despite the advantages of dual-sequencing, relatively few studies investigate fungal-plant interactions using this approach. For example, *Septoria tritici*, one of the most economically important wheat pathogens, was shown to alter gene activity to marginalize wheat defenses

during its biotrophic phase before transitioning to a necrotic lifestyle and causing plant disease (Yang et al., 2013). However, this study omits an investigation into the transcriptional changes during the infection process with an incompatible host, thus forgoing critical insights to plant immunity. Using this strategy, Kawahara et al. (2012) discovered a number of rice transcripts highly upregulated specifically during an incompatible interaction with the blast fungus *Magnaporthe oryzae*. Comparing the differences between resistant and tolerant lines of crop systems should help researchers discover key attenuations of the plant defense response, and provide answers into the genetic and molecular mechanisms underlying plant immunity.

Because of the diverse nature of economically important fungal plant pathogens, researchers may not have access to reference genomes or transcriptome assemblies to aid in pathogen gene expression analysis. Previous RNA profiling techniques such as microarrays, are expensive to develop and require a priori knowledge of the organism (Wang et al., 2009a). This limitation is easily overcome with RNA-seq experiments, wherein there exists well-established computational tools to generate transcriptome profiles from raw sequencing data (Grabherr et al., 2011; Martin and Wang, 2011; Ward et al., 2012). With this approach, Yazawa et al. (2013) identified putative transcription factors and cell wall degrading enzymes expressed by *Bipolaris sorghicola* while a resistant line of its host, sorghum, activates putative WRKY transcription factors along with other defense-related genes. Likewise, this approach can be effectively used to study host-pathogen interactions in non-model hosts. For example, researchers studying sudden oak death used the available *Phytophthora ramorum* reference annotation to separate dual RNA-seq reads, and generated a reference transcriptome of its host (Hayden et al., 2014). These studies exemplify how effectively RNA-seq technology can be directly applied to translate model system data to help understand critically important fungal pathosystems.

In most cases, annotation tools developed from model systems to assign biological information to transcriptomic data are designed to be used independently on plants and fungi. Many tools and databases exist for plants, for example, araport.org for arabidopsis, soybase.org for soybean, gramene.org for rice, maize and many other grasses (Grant et al., 2010; Stein et al., 2014; Krishnakumar et al., 2015). However only recently have comprehensive tools been available for fungi, with development of FungiFun2 (elbe.hki-jena.de/fungifun/, Priebe et al., 2015). Network inference is another powerful tool to predict molecular interactions between hosts and pathogens by analyzing co-expressed gene sets and has been successfully used to discover interactions between immune cells and fungi in a mouse system (Tierney et al., 2012). Tools built specifically for dual RNA-seq experiments are in their infancy but promise to serve unique roles in unraveling gene regulatory networks in host-pathogen interactions (Schulze et al., 2015, 2016). Future expansion and development of these programs into general bioinformatics tools such as the Galaxy Project (galaxyproject.org), should proceed with a focus on building user-friendly interfaces in an open-access forum in order to maximize their utility within the plant science community.

2.4 LASER DISSECTION OF HOST PATHOGEN INTERACTIONS

One of the limitations of traditional RNA-seq experiments is that it evaluates the collective population of mRNAs from a complex multicellular tissue or organ system. This is particularly problematic for investigating early stages of infection where limited fungal biomass means few sequence reads relative to the host can be detected using traditional technology. In a recent dual sequencing experiment, Rudd et al. (2015) demonstrated that *Zymoseptoria tritici* modifies defense gene activity without significant nutrient acquisition from the host during early stages of infection before degrading and consuming host-derived nutrients during necrosis. However, less

than 2% sequence reads from *Z. tritici* infected wheat mapped to the fungal genome at four days post inoculation, which increases to 40% at 14 days post inoculation. The limited sequencing depth directly results in limited quality of the RNA-seq data for early stages of infection, potentially obfuscating early events critical to pathogenesis. The result of any interactions between a fungal pathogen and its plant host is specified at the cellular level, directly at the site of infection. Thus, the microscopic scale of these interactions is a major limiting factor on the quality of sequencing experiments as traditional protocols may dilute early signaling events and molecular responses will have faded beyond detection limits by the majority of transcripts originating distal to the infection site. Taken together, understanding how plant defense molecules are controlled at the cellular level requires new technological approaches.

Over the past decade, laser microdissection (LMD) has emerged as a robust way to isolate individual cells and tissues from complex organs and tissue systems (Day et al., 2005; Gautam and Sarkar, 2014; Khan et al., 2014a). Other technologies including fluorescently-activated cell sorting and the isolation of nuclei tagged in specific cell types (INTACT) are limited in their applications due to the need for protoplasting or transformations with cell type-specific markers (Zhang et al., 2008; Deal and Henikoff, 2011). While there are variations in LMD design from different manufacturers, tissues are generally fixed and sectioned using common histological techniques and placed on specialized microscopy slides or plates. Depending on the system of study, wax or plastic can be used to embed tissues and preserve RNA (Inada and Wildermuth, 2005; Klink and Thibaudeau, 2014). Once fixed, the samples are then visualized using light or fluorescence microscopy and individual cells and tissues are selected and excised with a laser and collected for downstream molecular analysis (Gautam and Sarkar, 2014; Schiebold et al., 2011). The cellular-level resolution provided by LMD based tissue collection is therefore

uniquely suited to overcome the often low coverage of pathogen transcripts and the signal dilution of pathogen-specific RNAs inherent to traditional RNA-seq experiments.

Laser microdissection has been used in model systems, and as a tool to discover how transcription factors are modulated in Arabidopsis leaves following infection by the biotrophic powdery mildew causing *Golovinomyces orontii* (Chandran et al., 2010). This seminal paper provided new insights into plant defense, however, the transcriptomic data were quantified using microarrays, a technology that relies on *a priori* knowledge of the system. In a similar array-based experiment, LMD was used to discover how different molecular processes occur in the spatially distinct infection regions of colonized poplar leaves (Hacquard et al., 2010) further supporting the application of the technique to fungal biology. Thus, LMD coupled with next generation RNA sequencing should detect a broader and more dynamic range of gene activity in addition to resolving new transcripts with essential roles in the regulation and integration of the plant defense process.

In the case of the complex tissue systems of the leaf, it is likely that each tissue or cell type plays a different or overlapping role in general cell function and plant defense. For example, when *Sclerotinia sclerotiorum* interacts with the canola leaf, the fungal hyphae first grow laterally along the leaf surface under the cuticle before penetrating the epidermis and moving through the mesophyll and finally infiltrating the vasculature leading to systemic colonization of the plant body. Therefore, the fungus is in direct contact with each type of tissue and investigating tissue-specific roles in defense will strengthen our understanding of plant defense systems.

Surprisingly, few plant mRNA profiling studies have used LMD to better understand the genetic response to pathogen interactions and none have combined this technology with a dual

sequencing strategy, thus providing unprecedented opportunity for future research. However, these technologies have already provided insights into plant responses to nematodes in both tomato (Ramsay et al., 2004) and soybean (Klink et al., 2009), and pointed to modifications in sugar metabolism as a result of grapevine infection with a phytoplasma (Santi et al., 2013). The precision LMD adds to these experiments and makes it an essential tool for understanding the specific molecular events that influence the outcome of a host-pathogen interaction, and complement a broader strategy that utilizes genetic information to best overcome major crop pathogens.

2.5 COMPUTATIONAL PREDICTION OF BIOLOGICAL REGULATORS

The majority of cellular reprogramming during the plant defense response is transcriptionally controlled through complex networks of transcription factors (TFs) and their DNA binding sites (see Tsuda and Somssich, 2015 for review). The addition of dual sequencing and LMD to the plant pathologist's tool kit will increase the resolution of these interactions, but does nothing to discover the transcriptional regulators of the genetic and molecular processes involved. Elucidating the complex regulatory network of transcription factors with their DNA binding sites and the sets of genes and biological processes they control will provide the foundation for building crops that are more resistant or tolerant to fungal pathogens. This complex task requires multiple resources encompassing data on experimentally and computationally derived TF – DNA binding site motif interactions in addition to annotated gene lists from co-expressed or differentially expressed gene sets (Belmonte et al., 2013; Khan et al., 2014b).

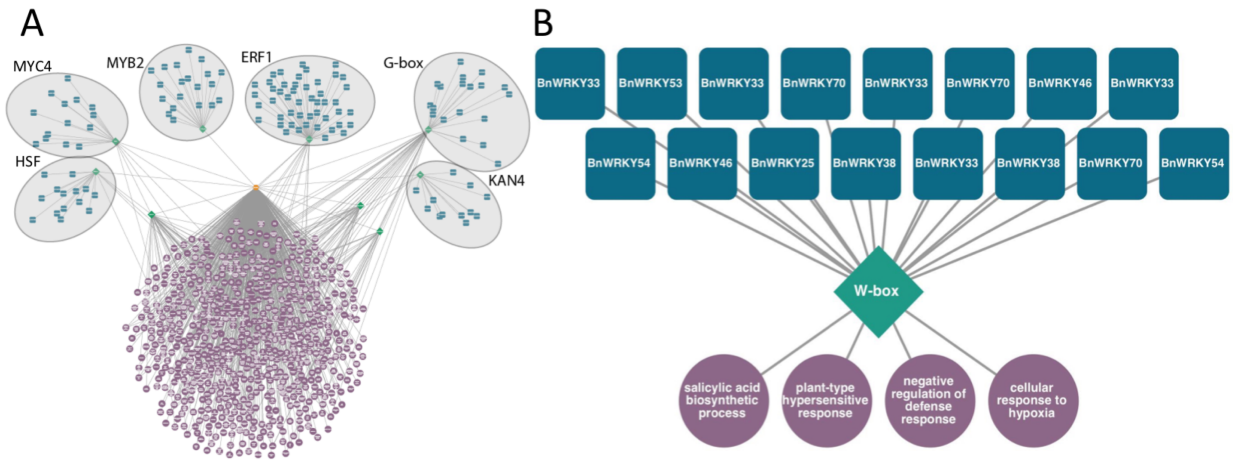

Figure 2.2 Predicted transcriptional modules from sets of genes differentially expressed following fungal infection of *Brassica napus*. Transcription factors (blue squircles) predicted to bind to DNA sequence motifs (green diamonds) located 1 kb upstream of transcription start sites of a set of genes (orange octagon) enriched in gene ontology terms (purple circles, P<0.0001 hypergeometric distribution). (A) Transcriptional module within differentially expressed genes of *B. napus* in response to *Sclerotinia sclerotiorum*, Heatshock factor (HSF), MYC, MYB, Ethylene Response Factor (ERF), G-box, and KAN4 motifs are predicted to control defense related biological processes. (B) Predicted WRKY transcriptional circuit from genes specifically differentially expressed in *B. napus* resistant to *Leptospheria maculens* 3 days post-inoculation.

This type of tool can be used to discover potential transcriptional regulators in large sets of genes differentially expressed in response to fungal infection. For example, in *Sclerotinia*-infected canola leaves, six overrepresented DNA sequence promoter motifs, HSF, MYC4, MYB2, ERF1, G-box, and KAN4 are predicted to regulate genes associated with signaling, defense and translation (Figure 2.2A). Likewise, smaller subsets of co-expressed gene sets can be analyzed (Figure 2.2B). These modules consisting of transcription factors predicted to bind to these DNA motifs found within gene sets are therefore potential regulators of these processes. Of genes exclusively differentially expressed in a resistant line of canola infected with *Leptospheria maculens*, sixteen WRKY homologues are predicted to control genes associated with SA biosynthesis and the hypersensitive response (publicly available dataset available on NCBI's Gene Expression Omnibus, GSE77723).

An opportunity also exists for the development of a similar prediction tool based on fungal sequencing data to better understand the regulation of processes involved in pathogenesis as well as an avenue to identify putative targets for functional applications. These bioinformatics tools can serve as a valuable resource to the scientific community through mining existing and previously published large scale genes expression data sets. Predicting transcriptional regulators in economically important crop pathogens using this targeted approach should allow researchers to identify genes essential for growth and pathogenesis quickly using functional tests.

2.6 PROTECTING CROPS WITH RNA TECHNOLOGY

Researchers are now able to apply transcriptomic data in the development of innovative crop protection technologies. RNA interference (RNAi), promises to best the current control broad spectrum measures, eliminate negative consequences of current disease control, and

combat the alarming rise of fungicide resistant phytopathogens (Ishii and Holloman, 2015). RNAi specifically knocks down genes using an intrinsic cellular defense phenomenon. Through the detection and processing of double stranded RNA (dsRNA) or hairpin RNA (hpRNA) by fungal cells, transcripts are targeted using sequence homology leading to degradation or silencing (Nakayashiki, 2005). The application of cell specific and dual RNA sequencing data should provide the information to identify novel fungal targets. Hairpin RNA or dsRNA molecules can then be tailored for a specific transcript and upon delivery, can directly limit fungal pathogenesis.

Both dsRNA and hpRNA have the potential to protect cropping systems through topical applications or using a transgenic approach. Ghag et al. (2014) demonstrated the utility of transgenic plants expressing anti-pathogenicity RNA molecules against *Fusarium oxysporum,* the causative agent of Fusarium wilt. Banana plants were engineered to express intron hpRNA constructs for VELVET or FUSARIUM TRANSCRIPTION FACTOR1 and maintained some level of resistance for at least eight months. Despite the demonstrated success of RNAi technology against fungal pathogens, foliar applications have not yet come to fruition. However, they offer many benefits over transgenics including: the ability to explore a greater variety of novel targets compared to the production of stably transformed plants, a more rapid response to emerging pathogens and races, and wider public acceptance since host plant genomic changes have not occurred (Lucht, 2015; Senthil-kumar and Mysore, 2010). Fortunately, foliar application of RNAi technology has been successfully used as an insecticide in both lab and field studies (Baum et al., 2007; Whyard et al., 2009; Yu et al., 2013). In particular, San Miguel and Scott (2015) demonstrated the viability of a foliar application of actin dsRNA molecules to protect potato plants from Colorado potato beetles (*Leptinotarsa decemlineata)*. The molecules

were remarkably stable, showing bioactivity for over 28 days. With all the benefits and the proven viability of a topical application, future work should invest in the development of effective anti-fungal RNAi application methods.

In spite of the successes, some environmentalists are concerned with RNAi technology introducing large quantities of persistent molecules into the environment. Early results show dsRNA molecules will not persist or accumulate in soil (Dubelman et al., 2014). However, without a robust body of research on the environmental fate of RNA molecules, caution must be taken to prevent deleterious effects. Due to conserved sequences, molecules must be designed to have no more than 20 bases of homology to other transcripts, followed by thoroughly performing *in vitro* assays on various types of organisms. With meticulous molecular design, RNAi technology holds the promise to revolutionize agricultural disease management. While the cost to produce enough dsRNA molecules to protect broad acre crops is high, the expense to produce these molecules continues to decrease with the implementation of bacterial production systems (Palli, 2014; Robinson et al., 2014). The use of dsRNA molecules to protect against major crop pathogens will provide a targeted response for producers, and promises to be more effective while evading negative environmental consequences associated with broad spectrum fungicides.

2.7 OUTLOOK

The development of innovative research technologies to protect the agricultural landscape should provide the necessary tools to sustain global food demand. Through these technologies we have developed a deep understanding of host pathogen interactions at the RNA level. However, there are still many gaps in our knowledge that surround emerging crop systems where genetic information is lacking. Fundamental details remain to be resolved on how plant defense,

and fungal pathogenic processes are specified at the cellular level at the site of infection and the contribution of transcriptional circuits controlling these processes. The application of RNA sequencing technologies coupled with cutting edge laser microdissection methods should provide plant science researchers with answers to protect our food systems. While the analysis of large scale datasets still remains a challenge, new and user friendly computational pipelines and programs will allow for broader access to and the potential for innovative product development. These strategies will also provide information essential for implementing the next generation of thorough, effective, and responsible RNAi-based fungal control measures in plant crop systems.

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2.9 REFERENCES

Baum, J. A, Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007). Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–6. doi:10.1038/nbt1359.

Belmonte, M. F., Kirkbride, R. C., Stone, S. L., Pelletier, J. M., Bui, A. Q., Yeung, E. C., et al. (2013). Comprehensive developmental profiles of gene activity in regions and subregions of the Arabidopsis seed. *Proc. Natl. Acad. Sci. U. S. A.* 110, E435–44. doi:10.1073/pnas.1222061110.

Chandran, D., Inada, N., Hather, G., Kleindt, C. K., and Wildermuth, M. C. (2010). Laser microdissection of Arabidopsis cells at the powdery mildew infection site reveals site-specific processes and regulators. *Proc. Natl. Acad. Sci. U. S. A.* 107, 460–5. doi:10.1073/pnas.0912492107.

Day, R. C., Grossniklaus, U., and Macknight, R. C. (2005). Be more specific! Laser-assisted microdissection of plant cells. *Trends Plant Sci.* 10, 397–406. doi:10.1016/j.tplants.2005.06.006.

Deal, R. B., and Henikoff, S. (2011). The INTACT method for cell type-specific gene expression and chromatin profiling in Arabidopsis thaliana. *Nat. Protoc.* 6, 56–68. doi:10.1038/nprot.2010.175.

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., et al. (2014). Environmental fate of double-stranded RNA in agricultural soils. *PLoS One* 9, 1–7. doi:10.1371/journal.pone.0093155.

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., Mccraw, S. L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484, 186–

194. doi:10.1038/nature10947.

Gautam, V., and Sarkar, A. K. (2015). Laser assisted microdissection, an efficient technique to understand tissue specific gene expression patterns and functional genomics in plants. *Mol. Biotechnol.* 57, 299–308. doi:10.1007/s12033-014-9824-3.

Ghag, S. B., Shekhawat, U. K. S., and Ganapathi, T. R. (2014). Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against Fusarium wilt in banana. *Plant Biotechnol. J.* 12, 541–553. doi:10.1111/pbi.12158.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29. doi:10.1038/nbt.1883.

Grant, D., Nelson, R. T., Cannon, S. B., and Shoemaker, R. C. (2010). SoyBase, the USDA-ARS soybean genetics and genomics database. *Nucleic Acids Res.* 38, 843–846. doi:10.1093/nar/gkp798.

Hacquard, S., Delaruelle, C., Legué, V., Tisserant, E., Kohler, A., Frey, P., et al. (2010). Laser capture microdissection of uredinia formed by Melampsora larici-populina revealed a transcriptional switch between biotrophy and sporulation. Mol. Plant. Microbe. Interact. 23, 1275–1286. doi:10.1094/MPMI-05-10-0111.

Hayden, K. J., Garbelotto, M., Knaus, B. J., Cronn, R. C., Rai, H., and Wright, J. W. (2014). Dual RNA-seq of the plant pathogen Phytophthora ramorum and its tanoak host. Tree Genet. Genomes 10, 489–502. doi:10.1007/s11295-014-0698-0.

Hirooka, T., and Ishii, H. (2013). Chemical control of plant diseases. *J. Gen. Plant Pathol.* 79, 390–401. doi:10.1007/s10327-013-0470-6.

Inada, N., and Wildermuth, M. C. (2005). Novel tissue preparation method and cell-specific

marker for laser microdissection of Arabidopsis mature leaf. *Planta* 221, 9–16. doi:10.1007/s00425-004-1427-y.

Ishii, H., and Holloman, D. W. (2015). *Fungicide resistance in plant pathogens*. Tokyo: Springer Japan doi:10.1007/978-4-431-55642-8.

Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T., and Minami, E. (2012). Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. PLoS One 7, e49423. doi:10.1371/journal.pone.0049423.

Khan, D., Chan, A., Millar, J. L., Girard, I. J., and Belmonte, M. F. (2014a). Predicting transcriptional circuitry underlying seed coat development. *Plant Sci.* 223, 146–52. doi:10.1016/j.plantsci.2014.03.016.

Khan, D., Millar, J., Girard, I., and Belmonte, M. (2014b). Transcriptional circuitry underlying seed coat development in Arabidopsis. *Plant Sci.* 219-220, 51–60. doi:10.1016/j.plantsci.2014.01.004.

Klink, V. P., Hosseini, P., Matsye, P., Alkharouf, N. W., and Matthews, B. F. (2009). A gene expression analysis of syncytia laser microdissected from the roots of the Glycine max (soybean) genotype PI 548402 (Peking) undergoing a resistant reaction after infection by Heterodera glycines (soybean cyst nematode). *Plant Mol. Biol.* 71, 525–567. doi:10.1007/s11103-009-9539-1.

Klink, V. P., and Thibaudeau, G. (2014). Laser microdissection of semi-thin sections from plastic-embedded tissue for studying plant–organism developmental processes at single-cell resolution. *J. Plant Interact.* 9, 610–617. doi:10.1080/17429145.2013.879677.

Krishnakumar, V., Hanlon, M. R., Contrino, S., Ferlanti, E. S., Karamycheva, S., Kim, M., et al. (2015). Araport: The Arabidopsis Information Portal. Nucleic Acids Res. 43, D1003–

D1009. doi:10.1093/nar/gku1200.

Lucht, J. (2015). Public Acceptance of Plant Biotechnology and GM Crops. *Viruses* 7, 4254–4281. doi:10.3390/v7082819.

Martin, J. A., and Wang, Z. (2011). Next-generation transcriptome assembly. *Nat. Publ. Gr.* 12, 671–682. doi:10.1038/nrg3068.

Metzker, M. L. (2009). Sequencing technologies — the next generation. *Nat. Rev. Genet.* 11, 31–46. doi:10.1038/nrg2626.

Nakayashiki, H. (2005). RNA silencing in fungi: Mechanisms and applications. *FEBS Lett.* 579, 5950–5957. doi:10.1016/j.febslet.2005.08.016.

Palli, S. R. (2014). RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide. *Curr. Opin. Insect Sci.* 6, 1–8. doi:10.1016/j.cois.2014.09.011.

Podio, N. S., Guzmán, C. a, and Meriles, J. M. (2008). Microbial community structure in a silty clay loam soil after fumigation with three broad spectrum fungicides. *J. Environ. Sci. Health. B.* 43, 333–340. doi:10.1080/03601230801941675.

Priebe, S., Kreisel, C., Horn, F., Guthke, R., and Linde, J. (2014). FungiFun2: A comprehensive online resource for systematic analysis of gene lists from fungal species. *Bioinformatics* 31, 445–446. doi:10.1093/bioinformatics/btu627.

Ramsay, K., Wang, Z., and Jones, M. G. K. (2004). Using laser capture microdissection to study gene expression in early stages of giant cells induced by root-knot nematodes. *Mol. Plant Pathol.* 5, 587–592. doi:10.1111/J.1364-3703.2004.00255.X.

Ray, D. K., Mueller, N. D., West, P. C., and Foley, J. A. (2013). Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PLoS One* 8. doi:10.1371/journal.pone.0066428.

Reuter, J. A., Spacek, D. V, and Snyder, M. P. (2015). High-Throughput Sequencing Technologies. *Mol. Cell* 58, 586–597. doi:10.1016/j.molcel.2015.05.004.

Robinson, K. E., Worrall, E. A., and Mitter, N. (2014). Double stranded RNA expression and its topical application for non-transgenic resistance to plant viruses. *J. Plant Biochem. Biotechnol.* 23, 231–237. doi:10.1007/s13562-014-0260-z.

Rudd, J. J., Kanyuka, K., Hassani-pak, K., Derbyshire, M., Andongabo, A., Devonshire, J., et al. (2015). Transcriptome and metabolite profiling of the infection cycle of Zymoseptoria tritici on wheat reveals a biphasic interaction with plant immunity involving differential pathogen chromosomal contributions and a variation on the hemibiotrophic lifestyle definition. *Plant Physiol.* 167, 1158–1185. doi:10.1104/pp.114.255927.

San Miguel, K., and Scott, J. G. (2015). The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Manag. Sci.*, n/a–n/a. doi:10.1002/ps.4056.

Santi, S., Grisan, S., Pierasco, A., DE Marco, F., and Musetti, R. (2013). Laser microdissection of grapevine leaf phloem infected by stolbur reveals site-specific gene responses associated to sucrose transport and metabolism. *Plant. Cell Environ.* 36, 343–55. doi:10.1111/j.1365-3040.2012.02577.x.

Schiebold, S., Tschiersch, H., Borisjuk, L., Heinzel, N., Radchuk, R., and Rolletschek, H. (2011). A novel procedure for the quantitative analysis of metabolites, storage products and transcripts of laser microdissected seed tissues of Brassica napus. *Plant Methods* 7, 19. doi:10.1186/1746-4811-7-19.

Schulze, S., Henkel, S. G., Driesch, D., Guthke, R., and Linde, J. (2015). Computational prediction of molecular pathogen-host interactions based on dual transcriptome data. *Front. Microbiol.* 6, 1–11. doi:10.3389/fmicb.2015.00065.

Schulze, S., Schleicher, J., Guthke, R., and Linde, J. (2016). How to Predict Molecular Interactions between Species? Front. Microbiol. 7, 1–13. doi:10.3389/fmicb.2016.00442.

Senthil-kumar, M., and Mysore, K. S. (2010). RNAi in plants : recent developments and applications in agriculture. *Gene Silenc. Theory, Tech. Appl.*, 183–199.

Stein, J., Naithani, S., Wei, S., Dharmawardhana, P., Kumari, S., Amarasinghe, V., et al. (2014). Gramene 2013: comparative plant genomics resources. *Nucleic Acids Res.* 42, 1193–1199. doi:10.1093/nar/gkt1110.

Tierney, L., Linde, J., M??ller, S., Brunke, S., Molina, J. C., Hube, B., et al. (2012). An interspecies regulatory network inferred from simultaneous RNA-seq of Candida albicans invading innate immune cells. Front. Microbiol. 3, 1–14. doi:10.3389/fmicb.2012.00085.

Tilman, D., Balzer, C., Hill, J., and Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. *Proc. Natl. Acad. Sci. U. S. A.* 108, 20260–4. doi:10.1073/pnas.1116437108.

Tsuda, E., and Somssich, I. E. (2015). Transcriptional networks in plant immunity. *New Phytol.* 206, 932-947. doi:10.1111/nph.13286.

United Nations, Department of Economic and Social Affairs, Population Division (2015). World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. Working Paper No. ESA/P/WP.241.

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63.

Ward, J. A., Ponnala, L., and Weber, C. A. (2012). Strategies for transcriptome analysis in nonmodel plants. *Am. J. Bot.* 99, 267–276. doi:10.3732/ajb.1100334.

Westermann, A. J., Gorski, S. A., and Vogel, J. (2012). Dual RNA-seq of pathogen and host.

Nat. Rev. Microbiol. 10, 618–630. doi:10.1038/nrmicro2852.

Whyard, S., Singh, A. D., and Wong, S. (2009). Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* 39, 824–832. doi:10.1016/j.ibmb.2009.09.007.

Yang, F., Li, W., and Jørgensen, H. J. L. (2013). Transcriptional reprogramming of wheat and the hemibiotrophic pathogen septoria tritici during two phases of the compatible interaction. *PLoS One* 8, 1–15. doi:10.1371/journal.pone.0081606.

Yazawa, T., Kawahigashi, H., Matsumoto, T., and Mizuno, H. (2013). Simultaneous transcriptome analysis of sorghum and bipolaris sorghicola by using rna-seq in combination with de novo transcriptome assembly. *PLoS One* 8. doi:10.1371/journal.pone.0062460.

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., et al. (2013). Delivery of dsRNA for RNAi in insects: An overview and future directions. *Insect Sci.* 20, 4–14. doi:10.1111/j.1744-7917.2012.01534.x

Zhang, C., Barthelson, R. A., Lambert, G. M., and Galbraith, D. W. (2008). Global characterization of cell-specific gene expression through fluorescence-activated sorting of nuclei. *Plant Physiol.* 147, 30–40. doi:10.1104/pp.107.115246.

CHAPTER THREE: GLOBAL RNA PROFILING OF THE INITIAL FOLIAR *SCLEROTINIA SCLEROTIORUM-BRASSICA NAPUS* INFECTION PROCESS REVEALS CROSS TALK BETWEEN REDOX HORMONE AND PATHWAYS

This chapter has been submitted for publications with some modifications to Molecular Plant.

3.1 ABSTRACT

Brassica napus is one of the world's most valuable oilseeds and is under constant pressure by the necrotrophic fungal pathogen, *Sclerotinia sclerotiorum*, the causal agent of white stem rot. Despite our growing understanding of host pathogen interactions at the molecular level, we have yet to fully understand the biological processes underlying the initial interaction of this pathosystem on the foliar surface and certainly nothing about the genes and gene regulatory networks responsible for host tolerance and susceptibility. We profiled gene activity genome-wide at the host pathogen interface in susceptible (*B. napus* cv. Westar) and tolerant (*B. napus* cv. Zhongyou 821) plants on the foliar surface during the first 24 hours of the infection process using global RNA sequencing. The resulting dataset offers a timely description of the biological processes responsible for host tolerance to a necrotrophic fungal pathogen. We discovered the activation of a putative ethylene response factor transcriptional circuit controlling biological processes associated with fungal recognition, subcellular organization and redox homeostasis in tolerant genotype of *B. napus*. Physiological investigation into the role of redox homeostasis was further studied by quantifying cellular levels of the glutathione and ascorbate redox pathway and

the cycling enzymes associated with host tolerance to *S. sclerotiorum*. Functional characterization of redox mutants of Arabidopsis challenged with fungus provides compelling evidence into the role of the ascorbate – glutathione redox hub in the maintenance of and enhancement of host tolerance against fungal pathogens.

3.2 INTRODUCTION

Canola (*Brassica napus*) is the second most valuable oilseed crop in the world and is vulnerable to white stem rot caused by the necrotrophic ascomycete *Sclerotinia sclerotiorum*, one of the most devastating fungal crop pathogens (Bolton, 2006; Hegedus and Rimmer, 2005). Currently, the majority of *S. sclerotiorum* protection strategies for *B. napus* are based on the application of broad-spectrum fungicides (Bradley et al., 2006), as few tolerant cultivars have been developed and biocontrol methods, although promising, have yet to be commercially proven (Fernando et al., 2007; Khot et al., 2011). Thus, a robust crop security strategy to combat yield losses in canola requires a thorough understanding of the genes and gene regulatory networks underlying the plant defense response (Garg et al., 2010a).

S. sclerotiorum survives in the soil as sclerotia, long-lived melanised hyphal resting structures that germinate under suitable environmental conditions. These sclerotia can infect plants directly through myceliogenic germination, or carpogenically via the production of ascospore-producing apothecia (Bolton, 2006). *B. napus* is vulnerable to carpogenic attack from *S. sclerotiorum*; however, in order for the fungus to penetrate the host plant, the ascospores require an external source of energy (Garg et al., 2010b; Hegedus and Rimmer, 2005; Mclean, 1958). Field-based evidence has shown that fungal colonization of petals may provide a source of nutrients for producing the infection cushions required for penetrating mature leaf tissues (Jamaux et al., 1995; Huang et al. 2008). Unfortunately, all recent work on understanding the pathosystem uses either an artificial sugar-phosphate based ascospore assay (Garg et al., 2013) or a myceliogenic infection strategy using sclerotia germinated on growth media (Wu et al., 2016; Zhao et al., 2009). Given the first line of defense likely occurs at the petal inoculum-leaf

interface, there is a need to understand global transcriptional responses directly at the site of foliar infection immediately following the plant pathogen interaction.

Plants have elegant and dynamic signalling networks to carefully balance growth and defence processes and maximize fitness (Huot et al., 2014). Pathogen perception in the host is orchestrated in part through pathogen associated molecular pattern (PAMP) detection via pattern recognition receptors (PRRs) and the subsequent transduction signals to the nucleus to transcriptionally reprogram cellular processes (Park and Ronald, 2012; Zipfel, 2014). PAMP detection elicits a signalling cascade through Ca^{2+} channel activation, nitric oxide signalling, reactive oxygen species (ROS) bursts, and mitogen activated protein kinase (MAPK) signalling (Boller and Felix, 2009; Meng and Zhang, 2013).

Damage associated molecular patterns (DAMPs) act similar to PAMPs and are generally defined as molecules associated damaged plant molecules or cell wall components. The most well characterized class of DAMPs are oligogalacturonides (OGs). OGs form a major component of plant pectins, one of the components of primary cell wall chemistry. Their degradation by pathogen polygalacturonases releases OG oligomers of varying length- with the 'eggbox' configuration of 10-15 polymerized OGs as being the most important for signalling purposes. OGs have been the primary focus of DAMP-based research and a possible receptor for OGs in Arabidopsis has been identified as *WALL ASSOCIATED KINASE 1* (Brutus et al., 2010). Wound induced local priming of Arabidopsis defense against the necrotroph *Botrytis cinerea* has been shown to be independent of SA, JA & ET signalling (Chassot et al., 2008), but compromised in *pad3* mutants. Similar to OGs, the breakdown products of cutin, the main constituent of plant cuticle, can elicit a priming effect against necrotrophic fungi as well. For example, both fungal cutinase producing Arabidopsis plants and plants treated with exogenous cutinase, showed near

complete resistance to attack by *B. cinerea* and, similar to OGs, the effect was independent of SA, JA, and ET signalling (Chassot et al., 2007).

Extracellular ATP concentrations have long been known to directly effect a number of growth and defense related processes in plant cells, however only recently has the perception mechanism been identified. Choi et al. (2014) identified the lectin receptor kinase *DOES NOT RESPOND TO NUCLEOTIDES 1* (*DORN 1)* as a plasmamembrane localized receptor for extra cellular ATP. Extracellular ATP elicits ROS production via Ca^{2+} influxes, triggering the activation of MAPK3 in Arabidopsis roots (Demidchik et al., 2009).

Effective signal transduction leads to activation of transcription factors (TFs) with well-established roles in shifting biological and cellular processes towards defence (Schluttenhofer and Yuan, 2015; Tsuda and Somssich, 2015). Defense to necrotrophic fungi is partially controlled via jasmonic acid (JA)/ethylene (ET) hormone signalling (Denancé et al., 2013). These hormones activate defense genes and the production of antimicrobial compounds including camalexins and glucosinolates (Wu et al., 2014). Successful pathogen evasion via PAMP signalling leads to PAMP-triggered immunity (PTI), and is generally synonymous with basal or non-host plant defense. A second layer of plant defense, effector-triggered immunity (ETI), involves the detection of specific pathogen effector molecules via *Avr*- and *R*-gene interactions, typically resulting in the hypersensitive response. Although common in plant defense to biotrophic and hemibiotrophic pathogens, ETI is exceedingly rare in necrotrophic fungal pathosystems (Wang et al., 2014). Salicylic acid (SA) is another important defense hormone generally associated with plant responses to biotrophic pathogens, and acts antagonistically to the JA/ET pathway (Van der Does et al., 2013; Vlot et al., 2009). However, SA has also been implicated in defense to necrotrophic fungi, as more recent work suggests the

JA/ET and SA hormone pathways are not exclusive, and effective cross-talk between them is essential for successful pathogen evasion (Mur et al., 2013; Tsuda et al., 2009).

Sclerotinia sclerotiorum produces a suite of digestive enzymes to degrade host tissues, however its main pathogenicity factor is oxalic acid (OA, Cessna et al., 2000). OA has a dual role of initially suppressing host cell ROS signalling, then subsequently eliciting ROS and plant cell death (Horbach et al., 2011; Kim et al., 2008; Williams et al., 2011). The ascorbate-glutathione (ASC-GSH) pathway is at the core of the plant antioxidant system as it protects ROS signalling functions and reduces H_2O_2 toxicity and maintains cellular redox homeostasis (Foyer and Noctor, 2011; de Pinto et al., 2012). Previous microarray-based experiments have implicated the antioxidant response with defense against *S. sclerotiorum* infection in *B. napus* (Yang et al., 2007); however, the physiological changes required for immunity and their transcriptional regulation have yet to be studied at the global RNA level.

RNA sequencing (RNA-seq) experiments with a concomitant burgeoning of computational tools, are quickly becoming the method of choice for researchers to profile plant defense processes and identify the gene regulatory networks that control them (Schulze et al., 2016; Girard et al. 2016). These tools have been applied to explore ETI in *B. napus* infected by the hemibiotroph *Leptospheria maculans* (Lowe et al., 2014; Becker et al. 2016?). Recent work profiling *S. sclerotiorum*-infected stem tissues of *B. napus* has provided insight into the molecular processes that underlie plant defense to *S. sclerotiorum* in stem tissues (Wu et al., 2016). For example, glucosinolate biosynthesis and chitinases activities were more strongly up-regulated in a resistant line of *B. napus* at earlier time points than in a susceptible line. In *B. napus* cv. Zhongyou821 (ZY821), a moderately tolerant cultivar, a putative resistance-associated quantitative trait locus has been identified as INDOLE GLUCOSINOLATE METHYL

TRANSFERASE 5 (Li et al., 2006; Wu et al., 2013). However, glucosinolate biosynthesis and modification are downstream of an activated defense response transcriptionally controlled through gene regulatory networks (Schweizer et al., 2013; Sønderby et al., 2010). Thus, a thorough analysis of the transcriptional circuits that determine plant defense outcomes directly at the foliar site of infection should provide evidence into the genetic control of disease progression directly at the host pathogen interface.

In this study, we present a structural, molecular, and physiological investigation of the biological processes that underlie *S. sclerotiorum* infection outcomes in susceptible and tolerant cultivars of *B. napus* using a petal inoculation method. Global transcriptome profiling coupled with a comprehensive bioinformatic analysis revealed distinct biological processes not previously described in this system including a putative ethylene response factor-controlled transcriptional circuit regulating redox state homeostasis to limit lesion spread in plant tissues. We provide compelling evidence of the complex and diverse aspects of plant defense processes required for plant defense directly at the site of infection of the *B. napus*-*S. sclerotiorum* pathosystem.

3.3 RESULTS

3.3.1 Petal inoculation is essential for *S. sclerotiorum* infection

We developed a *S. sclerotiorum* petal inoculation method for *B. napus* to identify how canola responds to fungal attack at the earliest stage of the infection process. This method was used to test disease progression and plant responses in universally su sceptible (cv. Westar) and tolerant (cv. ZY821) genotypes of *Brassica napus.* The petal inoculation method replicates the infection process that naturally occurs in the field (Jamaux et al., 1995; Huang et al. 2008). When

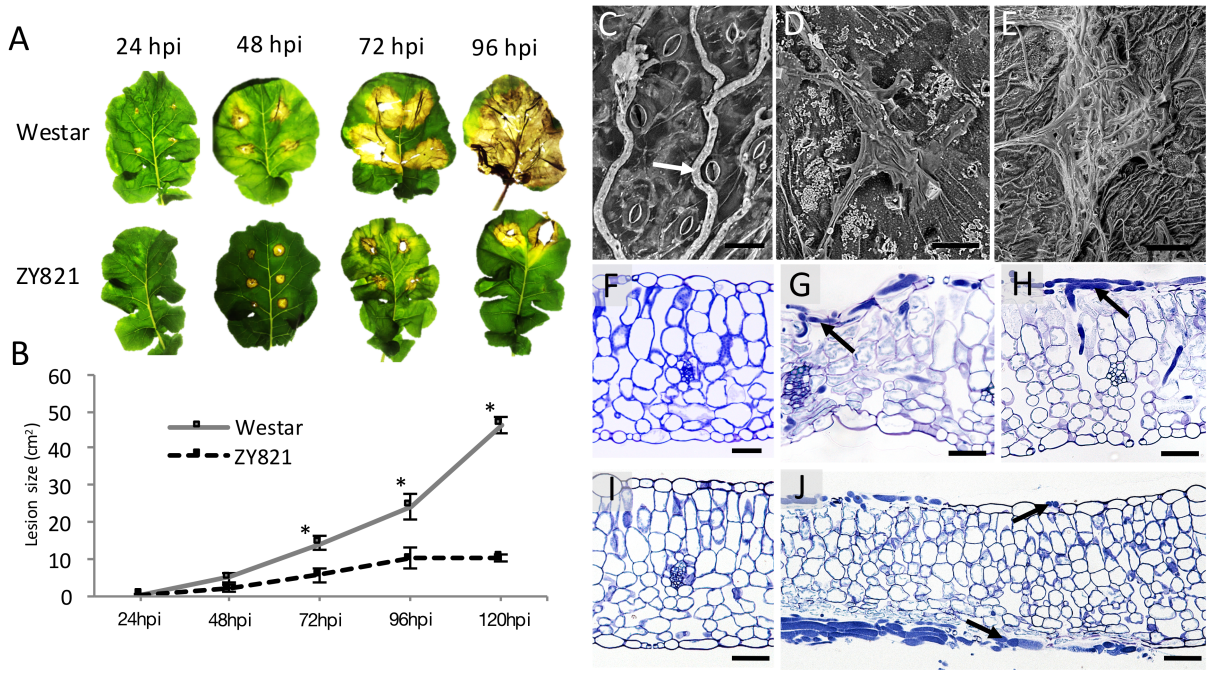

Figure 3.1 Profiling *Brassica napus-Sclerotinia sclerotiorum* pathosystem using the petal inoculation technique. (A) S. sclerotiorum lesion progression in susceptible (cv. Westar) and tolerant (cv. ZY821) cultivars of *B. napus*. (B) Lesion size measurements from 24-120 hours post-inoculation (hpi) in Westar and ZY821, significant differences between the two cultivars using ANOVA p-value < 0.05 indicated by *. (C) Scanning electron micrograph (SEM) of S. sclerotiorum hyphae (arrow) avoiding stomata on the adaxial epidermis of Westar 24hpi. Representative S. slcerotiorum infection cushions on abaxial epidermis of (D) Westar and (E) ZY821. (F-J) Light microscopy of transvers leaf sections stained with Toluidine Blue O. (F) Un-inoculated section of healthy Westar tissue. (G) Westar leaf 48hpi showing extensive membrane shrinkage caused by S. sclerotiorum, and darkly staining hyphae (arrow). (H) Infection cushion (arrow) on adaxial epidermis of Westar 48 hpi. (I) Un-inoculated healthy section of ZY821. (J) Cross-section of infected ZY821 leaves showing limited cellular degradation in advance of sclerotinia hyphae (arrows). Scale bars, C: 80µm; D: 60µm; E: 120µm; F-J: 50µm

leaf tissues were infected with a senescing petal, we observed 100% infection compared to 20% for a fresh petal. Profound differences in disease progression were observed 48hpi, with little green tissue remaining by 96 hpi in the susceptible line (Figure 3.1A). We then quantified lesion size and observed statistically significant differences at 72 hpi with a 78% increase between the two cultivars by 120 hpi (Figure 3.1B). Scanning electron microscopy revealed topographic features of the infection process directly at the infection site. *S. sclerotiorum* hyphae were not observed to penetrate host tissue via stomata (Figure 3.1C). Infection cushions were observed in both cultivars; however, they were qualitatively larger and more abundant in ZY821 (Figures 3.1D and 3.1E).

We then used light microscopy to better understand the infection process at the cellular level directly at the site of infection in susceptible (Figure 3.1 F-H) and tolerant (Figure 3.1 I-J) cultivars. Data revealed *S. sclerotiorum* growth inside susceptible epidermal cells at 48hpi and membrane shrinkage in advance of the infecting hyphae (Figure 3.1G). Cellular degradation indicated by membrane shrinkage was only observed in the cells surrounding the hyphae in the tolerant cultivar. Likewise, fungal hyphae were more readily able to penetrate the mesophyll in susceptible leaf tissues (Figure 3.1H) than in tolerant leaves (Figure 3.1J).

3.3.2 Global patterns of gene activity in response to *S. sclerotiorum*

To interrogate global changes in gene activity directly at the site of infection and elucidate potential transcriptional regulation of biological process contributing to the tolerant phenotype observed in ZY821, we performed high-throughput RNA-seq on both cultivars 24 hpi using ascospore- and mock-inoculated petals (Figure 3.2). Illumina sequencing reads were mapped to the reference assembly of *B. napus* v5.0 (Chalhoub et al., 2014). Total numbers of reads per biological replicate are presented in Table S1. We considered a gene ‘detected’ if the

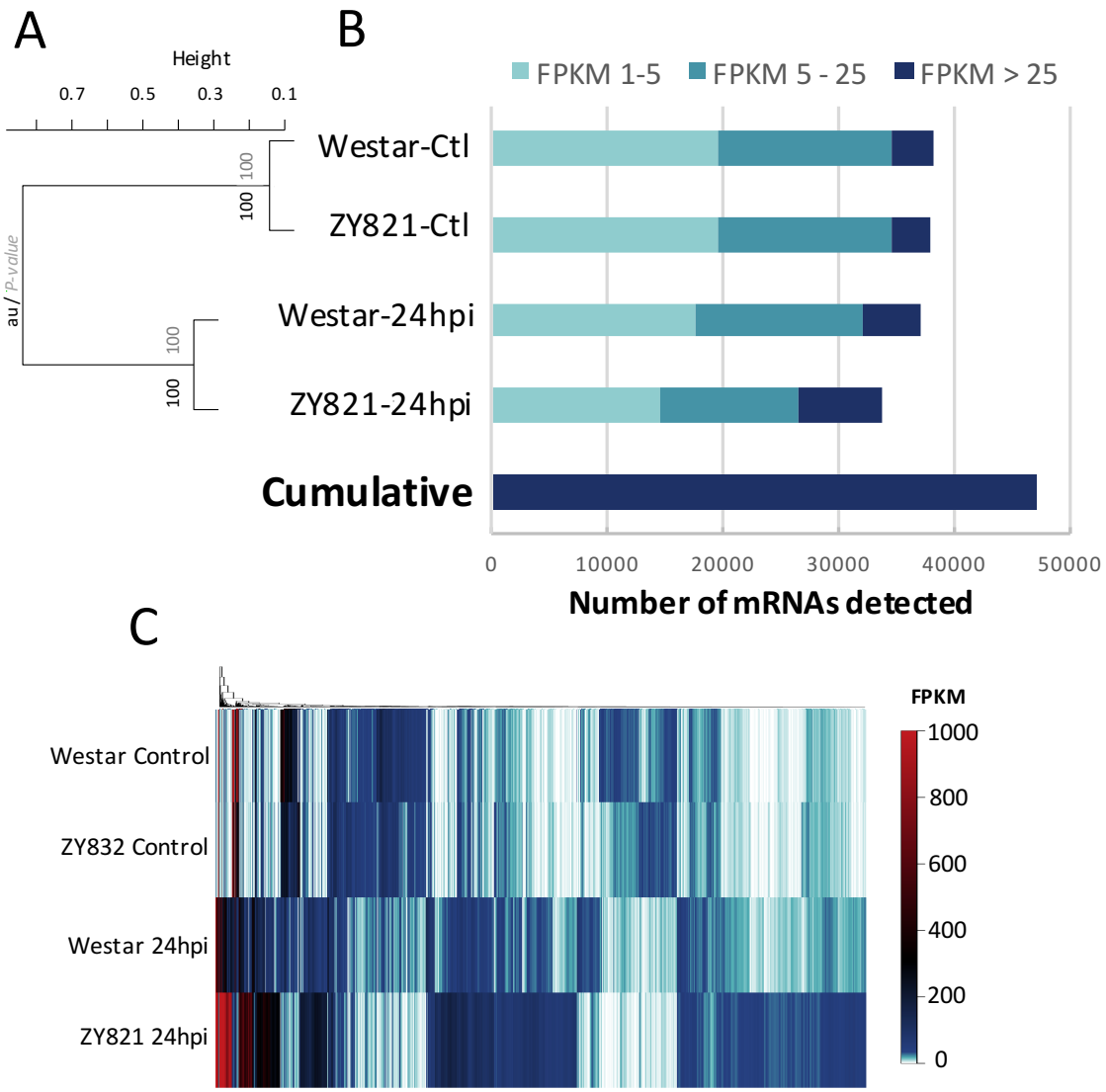

Figure 3.2 RNA Sequencing of susceptible (Westar) and tolerant (ZY821) genotypes of *Brassica napus* infected with *Sclerotinia sclerotiorum* using the petal inoculation technique. (A) Hierarchical clustering of genes detected with a minimum of 1 Fragment Per Kilobase of transcript per Million mapped reads (FPKM), with approximately unbiased (au) values in black and bootstrap P-values in grey. (B) Distribution of transcript abundances in FPKM of the four treatments. (C) Clustered heatmap of top 10,000 most highly abundant mRNAs.

transcript level in Fragments Per Kilobase of gene per Million reads mapped (FPKM) level was ≥ 1 (Bhardwaj et al., 2015; Chan et al., 2016). Hierarchical clustering of detected genes grouped samples together based on treatment, indicating both cultivars undergo a broad shift in gene activity in response to *S. sclerotiorum* infection (Figure 3.2A). Transcripts were then divided into those with low (FPKM ≥1, <5), moderate (FPKM ≥5, <25), or high (FPKM ≥ 25) accumulation levels. Mock-inoculated samples in both cultivars had similar numbers of low, moderate and highly accumulating mRNAs. We detected a total of 38,254 genes in Westar and 37,977 in ZY821. When leaf tissues were infected with *S. sclerotiorum*, 33,720 genes were detected in ZY821, 9% fewer than those detected in the susceptible cultivar (36,986). Of the genes detected in infected leaf tissue of the tolerant genotype, a much higher percentage was found to be highly abundant (21.1%) than in the susceptible Westar (13.4%). In total 47,154 genes were detected across all treatments, with the top 10,000 showing distinct patterns of accumulation between the two cultivars (Figure 3.2C).

3.3.3 Identification of un-annotated genes with roles in plant defence

To identify whether previously un-annotated genes play a role in *B. napus* defense to *S. sclerotiorum*, the sequence reads of individual samples were assembled, merged, and compared with the existing *B. napus* transcriptome annotation with the Cufflinks (v2.2.1) set of tools (Chalhoub et al., 2014; Trapnell et al., 2012). To strengthen predictive power, only transcripts not overlapping in existing gene models, having predicted protein blast hits to *A. thaliana*, and a minimum FPKM ≥ 1 in one of the treatments were retained for further analysis. A total of 1,233 previously unannotated transcripts were detected (Dataset S1). The 20 most highly accumulating novel transcripts are presented in Table S2, and include potential homologues of genes with characterized roles in Arabidopsis including lipid transfer proteins, genes involved in sulfur

assimilation (*ATP SULFURYLASE 1),* protein turnover (*POLYUBIQUITIN 10),* and signalling hubs like *MAP KINASE 4 (MPK4)* genes.

3.3.4 Differential gene activity in response to *S. sclerotiorum* infection

We were then interested in identifying gene sets activated in *B. napus* in response to *S. sclerotiorum* in both susceptible and resistant cultivars. We found 53% of genes detected were differentially expressed (false discovery rate < 0.05) in susceptible leaves with total of 11,726 mRNAs being differentially up and 10,936 differentially down regulated. Overall, 65% genes are differentially expressed in the tolerant cultivar, with 13,782 up- and 14,375 down-regulated. Although a large number of differentially expressed genes were shared between the two cultivars, we found relatively large number of genes specifically differentially expressed in each cultivar (Figure 3.3A), suggesting large and coordinated shifts in cultivar-specific biological processes at the earliest stages of the infection process.

3.3.5 Gene ontology analysis reveals biological processes shared between tolerant and susceptible *B. napus* cultivars

To query the biological processes encoded within sets of differentially expressed genes at the earliest stage of the infection process, we used the custom gene ontology (GO) enrichment function of ChipEnrich (Belmonte et al., 2013). GO terms were considered significantly enriched if the hypergeometric *P*-value < 0.001 (Figure 3.3B, Table S3.2). We predicted plant defense hormones to be active in both susceptible and tolerant leaf tissues directly at the site of infection in response to *S. sclerotiorum*. Salicylic acid (*P* = 2.72E-79), abscisic acid (*P* = 1.85E-73), and jasmonic acid (*P* = 2.73E-71) signalling processes, as well as response to ethylene (*P* = 1.89E-69), are all significantly enriched in mRNAs differentially accumulating in response to the fungus in both treatments (Table S3).

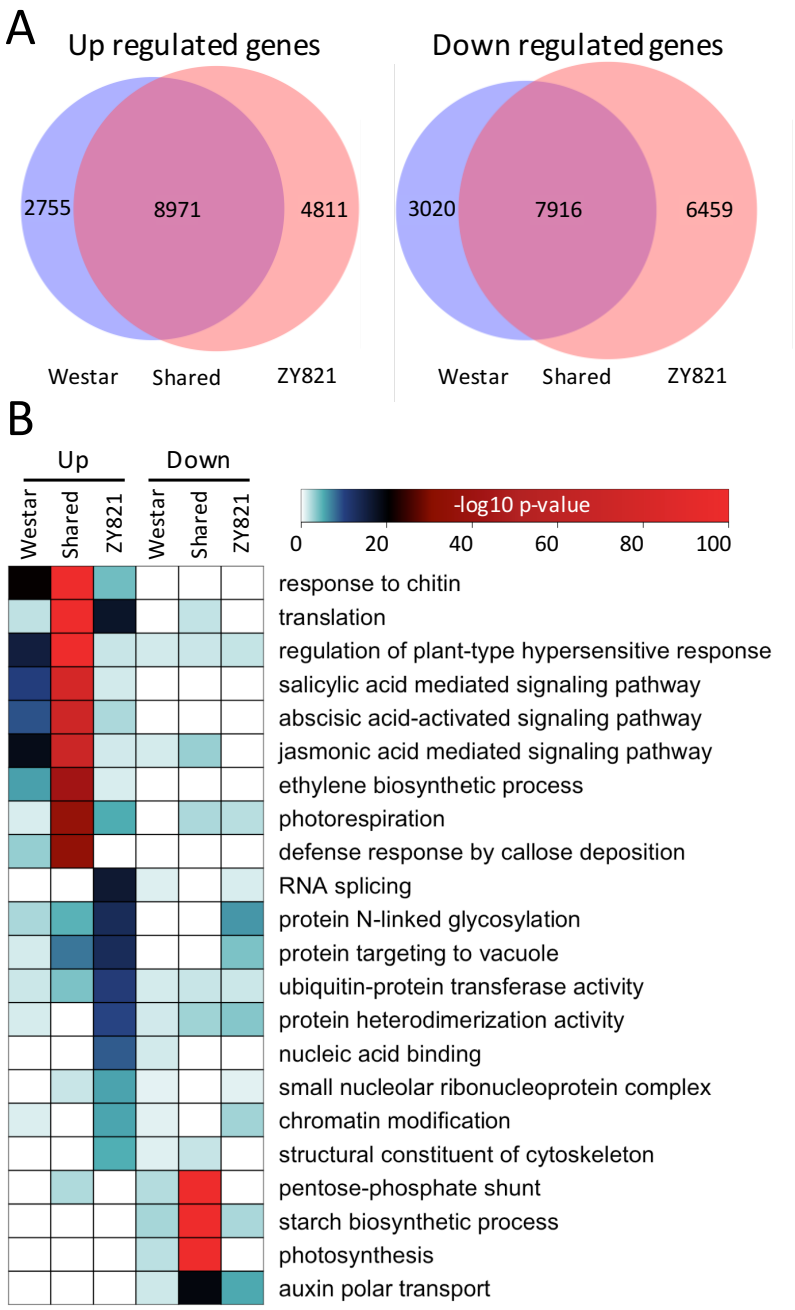

Figure 3.3 Differential gene expression and gene ontology (GO) analysis performed using the Cuffdiff software package (v. 2.2.1). (A) Euler diagrams showing number of *Brassica napus* genes differentially upregulated and down regulated following infection with Sclerotinia sclerotiorum (false discovery rate < 0.05). (B) Heatmap of a subset of significantly enriched GO terms. GO terms are considered statistically significant if the hypergeometric p-value < 0.001.

Enrichment of photorespiration in the upregulated shared genes (P = 2.67E-36) and a concomitant down-regulation of photosynthesis and starch biosynthetic processes, as indicated by their enrichment in genes down regulated in response to *S. sclerotiorum* (P = 5.06E-137 and P = 1.27E-180 respectively) suggests both tolerant and susceptible cultivars effectively down-regulate growth related biological processes following infection. Likewise, we observed a shift in auxin polar transport (P= 4.69E-06) and auxin-activated signaling pathways (P= 3.27E-06) enriched in down-regulated genes, indicative of large-scale transcriptional reprogramming following infection of leaf tissues with *S. sclerotiorum*.

3.3.6 Identification of tolerant-specific biological processes in *B. napus* against *S. sclerotiorum*

Next, we were interested in exploring the biological processes active in the 4,811 genes exclusively differentially up-regulated in response to the fungus that may contribute to the tolerant phenotype (Figure 3.3B). Biological processes with previously uncharacterized roles in the *B. napus*-*S. sclerotiorum* pathosystem were enriched in this set of genes including RNA-splicing (P = 3.49E-17) and chromatin modification (P = 3.43E-06). GO terms associated with protein sorting and trafficking, including protein N-linked glycosylation (P = 4.26E-14) and protein targeting to vacuole (P = 5.52E-14), in which *VACUOLAR SORTING RECEPTOR 1*, vSNARE protein family members, as well as *BZIP53* transcripts are also differentially up-regulated. Interestingly the data also point to a role of the structural component of the cytoskeleton (P = 8.60E-06), with both *ACTIN 2* and *TUBULIN BETA CHAIN 4* differentially up-regulated in ZY821 (Figure S3.5, Table S3.4).

To identify potential regulatory networks controlling these biological processes in this set of genes, we used the custom genome function in ChipEnrich (Belmonte et al., 2013) to predict the association of TFs with DNA sequence motifs overrepresented ($P < 0.001$) within the promoter of genes contributing to biological processes (GO terms). Two sub-modules were identified in genes specifically up-regulated in tolerant leaf tissues within 24 hpi (Table S5). TFs including *C-TERMINAL DOMAIN PHOSPHATASE-LIKE 3* (*BnaC02G27760D*), and MYB family genes were predicted to bind to the CCA1 and KAN DNA promoter motifs found upstream of genes enriched for histone methylation and protein modification (Figure 3.4A). The same analysis also identified four WRKY DNA-BINDING PROTEINS (WRKYs) predicted to control the plant response to chitin ($P = 3.44E-05$, Figure 3.4B). The association of the CCA1 DNA motif with genes specifically upregulated in the tolerant cultivar lead us to investigate whether circadian rhythm-regulated genes are globally affected at the RNA level in the two lines following infected with *S. sclerotiorum*. We plotted the corresponding FPKMs of homologous genes shown to be circadian regulated in Arabidopsis (Covington et al., 2008), and show clear differences in how circadian clock associated gene sets respond to *S. sclerotiorum* in the two *B. napus* cultivars (Figure S3.2). Both homologues of the master clock regulator *CCA1* shared similar expression profiles in both cultivars, however *LHY* homologues accumulate at an average of 4.5 times higher in ZY821 control samples (Table S3.4).

3.3.7 Co-expression analysis reveals distinct patterns of gene activity and regulatory networks between susceptible and tolerant genotypes

To identify co-expressed transcripts with finer patterns of gene activity than detectable with traditional differential expression, we used a modified fuzzy *K*-means clustering algorithm to identify groups of genes with similar expression profiles across all samples and treatments

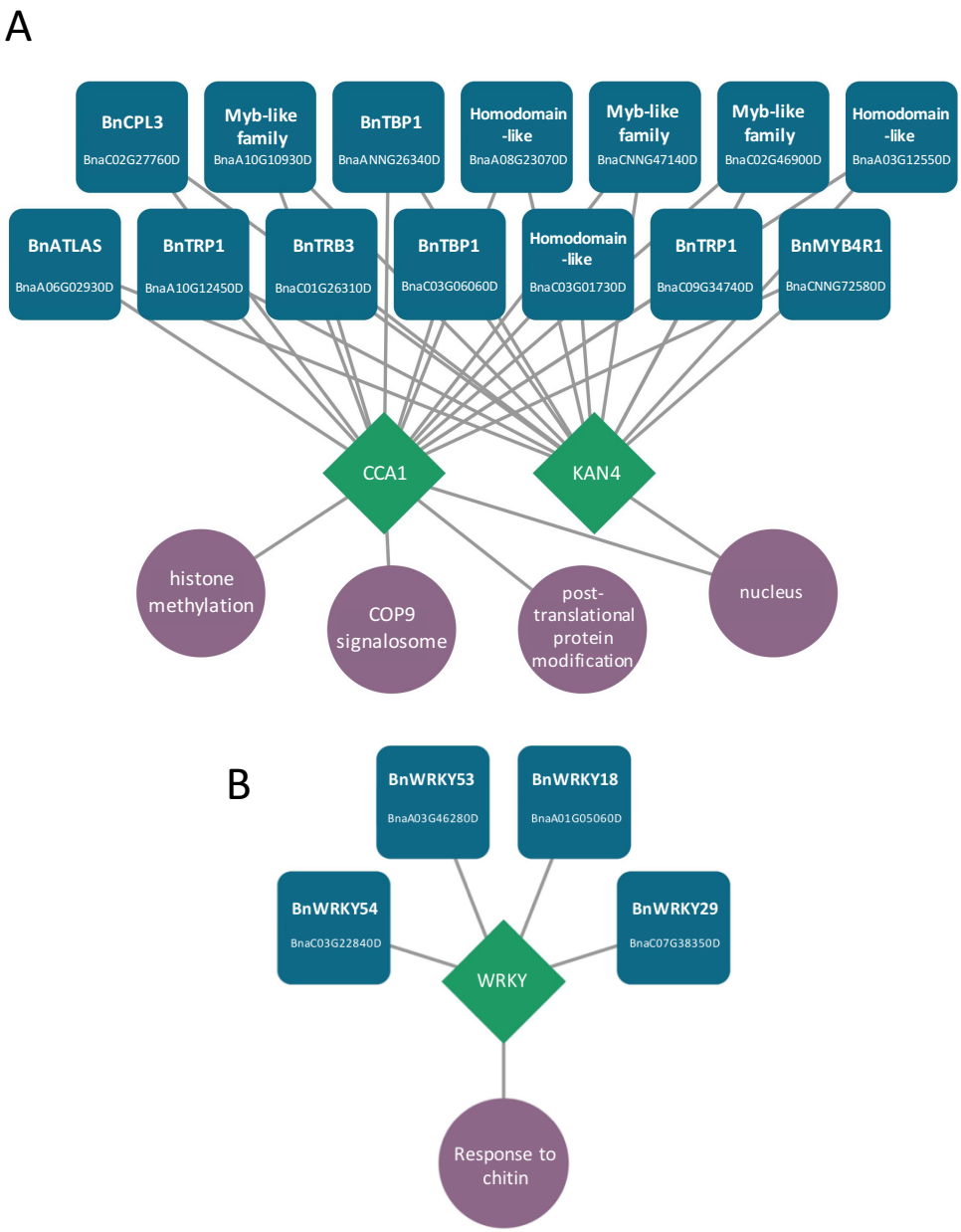

Figure 3.4 Putative transcriptional modules identified with modified ChipEnrich software from genes specifically differentially up-regulated in infected ZY821. (A) Putative circuit controlled by CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and KAN4 binding sites. Transcription factors (blue squircles) are predicted to bind to the overrepresented ($p<0.001$) DNA motifs (green diamonds) to control GO terms (purple circles). (B) Putative transcriptional circuit regulated by WRKY DNA binding motif.

(Belmonte et al., 2013). Ten dominant patterns of gene activity (DPs) were discovered, each containing anywhere from 599 to 9536 genes with a median of 1284 (Table S3.6). Our analysis identified patterns with increased mRNA relative abundance in infected samples in either Westar (DP4) or ZY821 (DP2), and DP9 represents a set of mRNAs increasing in relative abundance in both cultivars following infection (Figure 3.5A). We also discovered sets of mRNAs that accumulate largely based on cultivar –DP8 accumulating primarily in Westar and DP3 accumulating primarily in ZY821 (Figure S3.3).

Predicted TF-promoter interactions identified a putative transcriptional circuit in a set of mRNAs accumulating in tolerant leaf tissues within 24 hpi (DP2). HEATSHOCK TRANSCRIPTION FACTORS (HSPs), ETHYLENE RESPONSIVE BINDING FACTORS (ERFs), and MYC-family TFs (MYCs) were predicted to control a large suite of biological processes including defense signaling processes, protein biosynthesis and trafficking, secondary metabolite production and redox regulation (Figure 3.5B, Table S5). The implication of ERFs as regulators of defense related processes in the tolerant cultivar is concordant with their canonical role in the plant defense response to necrotrophic fungi. We next examined accumulation levels of all ERF transcripts, the majority of which were up-regulated following infection in both cultivars, but accumulated at much higher levels in the tolerant line (Figure 3.5C).

3.3.8 Activation of the cellular redox system in tolerant *B. napus* leaf tissues

Enrichment of redox-related GO terms in DP2 (Table S3.3) lead us to investigate whether there were differences in the ascorbate-glutathione redox cycle between the two cultivars at the earliest stages of the infection process (Figure 3.6A). Transcript levels for homologues of major enzymes in the ascorbate-glutathione pathway including cytosolic *ASCORBATE PEROXIDASE* (*APX*) and *GLUTATHION REDUCTASE* (*GR*), accumulated up to 15- and 33-fold higher

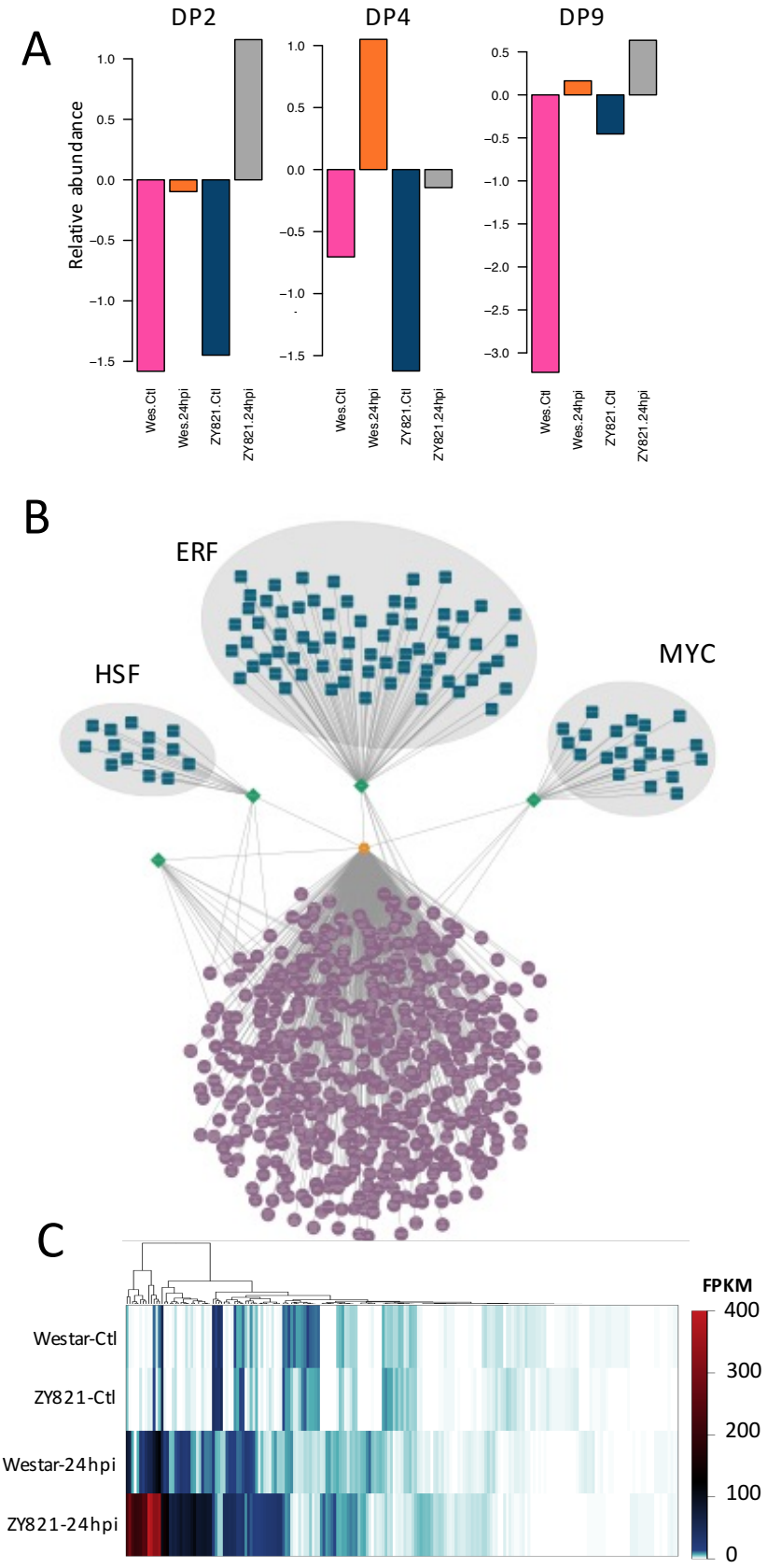
A
DP2
DP4
DP9
Relative abundance
Wes.Ctl
Wes.24hpi
ZY821.Ctl
ZY821.24hpi
B
ERF
HSF
MYC
C
Westar-Ctl
ZY821-Ctl
Westar-24hpi
ZY821-24hpi
FPKM
400
300
200
100
0

Figure 3.5 Dominant patterns (DPs) of gene activity discovered using Fuzzy k-means clustering and enrichment analysis. (A) Bar plots showing relative abundance of mRNAs assigned to three DPs. (B) Predicted transcriptional module identified in DP2 (gold hexagon) accumulating primarily in *Brassica napus* cv. ZY821 24hpi. Sets of Heatshock factors (HSF), Ethylene response factors (ERF) and MYC transcription factors (blue squircles) are predicted to bind to their overrepresented DNA motifs ($p<0.001$) up stream of genes enriched for gene ontology terms (purple circles). (C) Heatmap of all putative ERFs with a minimum FPKM of 5 in at least one of the samples.

respectively in the *S. sclerotiorum*-infected tolerant leaf tissues of ZY821 than in Westar (Figure 3.6B). We next measured enzyme activity levels to study whether they correlated to the observed transcript levels from our RNA-seq data. APX activity was between 41-80% higher across each time point in ZY821 compared to Westar, and in the susceptible cultivar, GR activity did not significantly change after *S. sclerotiorum* infection, whereas in ZY821, GR activity had approximately doubled by 72hpi (Figure 3.6C & 3.6D). We then tested whether endogenous levels of ascorbate and glutathione in both susceptible and resistant leaf tissues changes in response to infection. Total cellular ascorbate content in ZY821 was higher than in Westar for all time points, with only the ZY821 24hpi treatment showing a significant deviation from the endogenous levels in the control (Figure S3.4). Total glutathione levels as well as the glutathione redox ratio (GSH/(GSH+GSSG)) were similar in control treatments of the two cultivars. However, in ZY821, the glutathione redox ratio remained relatively constant following infection and the total glutathione pool increased 60% by 48hpi. Whereas in Westar, the glutathione redox ratio fell from 0.6 to 0.3 over the same period and the total pool increased only by 28% at 48hpi (Figure 3.6E).

3.3.9 Redox homeostasis mutants in Arabidopsis are hyper-susceptible to *S. sclerotiorum*

To further validate the role of redox homeostasis in the plant defense response to *S. sclerotiorum*, we used Arabidopsis loss-of-function mutants to assess the role of redox-related genes in host-pathogen interactions. We focused our attention on *vtc2* knockdown lines of the rate-limiting enzyme of ascorbate biosynthesis in Arabidopsis, VITAMIN C DEFECTIVE 2 (VTC2, Figure 3.7A). Loss-of-function mutants produces 10-25% of wild-type ascorbate levels (Becker et al., 2014; Pavet et al., 2005), limiting their capacity to buffer redox changes through

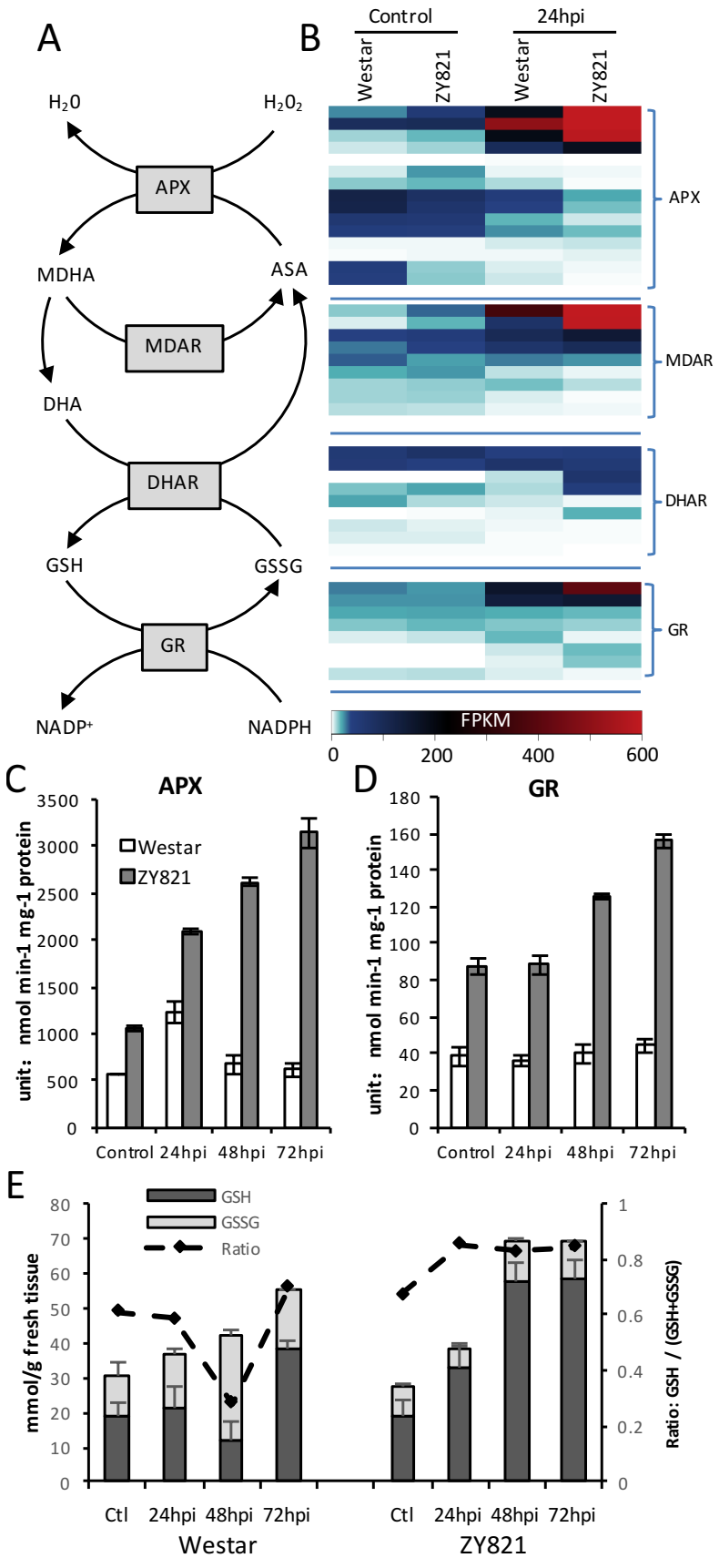
A
B
Control
24hpi
Westar
ZY821
Westar
ZY821
H2O
H2O2
APX
MDHA
ASA
MDAR
DHA
DHAR
GSH
GSSG
GR
NADP+
NADPH
APX
MDAR
DHAR
GR
FPKM
0
200
400
600
C
APX
unit: nmol min-1 mg-1 protein
Westar
ZY821
Control 24hpi 48hpi 72hpi
D
GR
unit: nmol min-1 mg-1 protein
Control 24hpi 48hpi 72hpi
E
GSH
GSSG
Ratio
mmol/g fresh tissue
Ratio: GSH / (GSH+GSSG)
Ctl 24hpi 48hpi 72hpi
Westar
Ctl 24hpi 48hpi 72hpi
ZY821

Figure 3.6 Redox and ascorbate transcripts and physiological characteristics associated with tolerances. (A) Overview of main enzymes and products involved in the ascorbate-glutathione redox homeostasis. (B) Accumulation of transcripts from all homologues of enzymes involved in pathway represented as heatmap. (C) Ascorbate peroxidase enzymatic activity measured from infected leaves of *Brassica napus* (D) Glutathione reductase enzymatic activity of infected leaves of *B. napus*. (E) Levels and ratios of reduced and oxidized glutathione. Abbreviations: APX, ascorbate peroxidase; ASC, reduced ascorbate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; NADP, nicotinamide adenine dinucleotide phosphate oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form.

the ascorbate-glutathione cycle. In *B. napus*, all *VTC2* homologue FPKMs are down regulated in response to infection at 24hpi (Figure 3.7B), similar to what was observed in Arabidopsis, as measured by quantitative RT-PCR (Figure 3.7C). Early in the Arabidopsis infection assay, *vtc2* plants show delayed lesion growth; however, at 72hpi, the infection is twice as severe (Figures 3.7A & 3.7D). A similar hyper-susceptible phenotype was observed in plants with a knockdown of *GLUTATHIONE SYNTHETASE 2*.

3.3.10 DAMP receptor mutants of Arabidopsis are deficient in defense activation

To test for functional roles of predicted regulators of defense, I next conducted a screen of Arabidopsis T-DNA insertion lines to test for significant phenotypes in the lesion progression at 24hpi and 72hpi using rosette leaves inoculated with PDA plugs with growing *S. sclerotiorum* hyphae (Table 2). Two of the gene knockdown lines with susceptible phenotypes selected for further analysis were *DOES NOT RESPOND TO NUCLEOTIDES 1* (*DORN1*) and *AT1G05340*, an uncharacterized protein. Clear differences in lesion progression between Wild Type and *dorn1* plant lines were visible at 24hpi (Figure 3.8A & 3.8B). Although quantitative differences were not observed at 24hpi, lesion growth was significantly more advanced in *dorn1* at 72 hpi (Figure 3.8C). The mutant *at1g05340* plants were more susceptible to *S. sclerotiorum* at both 24 and 72hpi time points, however, due to the recent characterization of *DORN1* as the primary means of detecting extracellular ATP (Choi et al., 2014), I proceeded to test defense responses at the molecular level in *dorn1* plants. Using RT-qPCR to validate the *DORN1* knockdown (Figure 3.8D), we found that *dorn1* mutants only accumulated 60% of Wild Type levels of *DORN1* in mock-inoculated samples, and 11% of Wild Type levels in *S. sclerotiorum* infected leaves. Testing for relative changes in transcript accumulation of the JA- and ET-activated defense gene *PLANT DEFNESIN 1.2* found a 90% difference in fold change between the two lines.

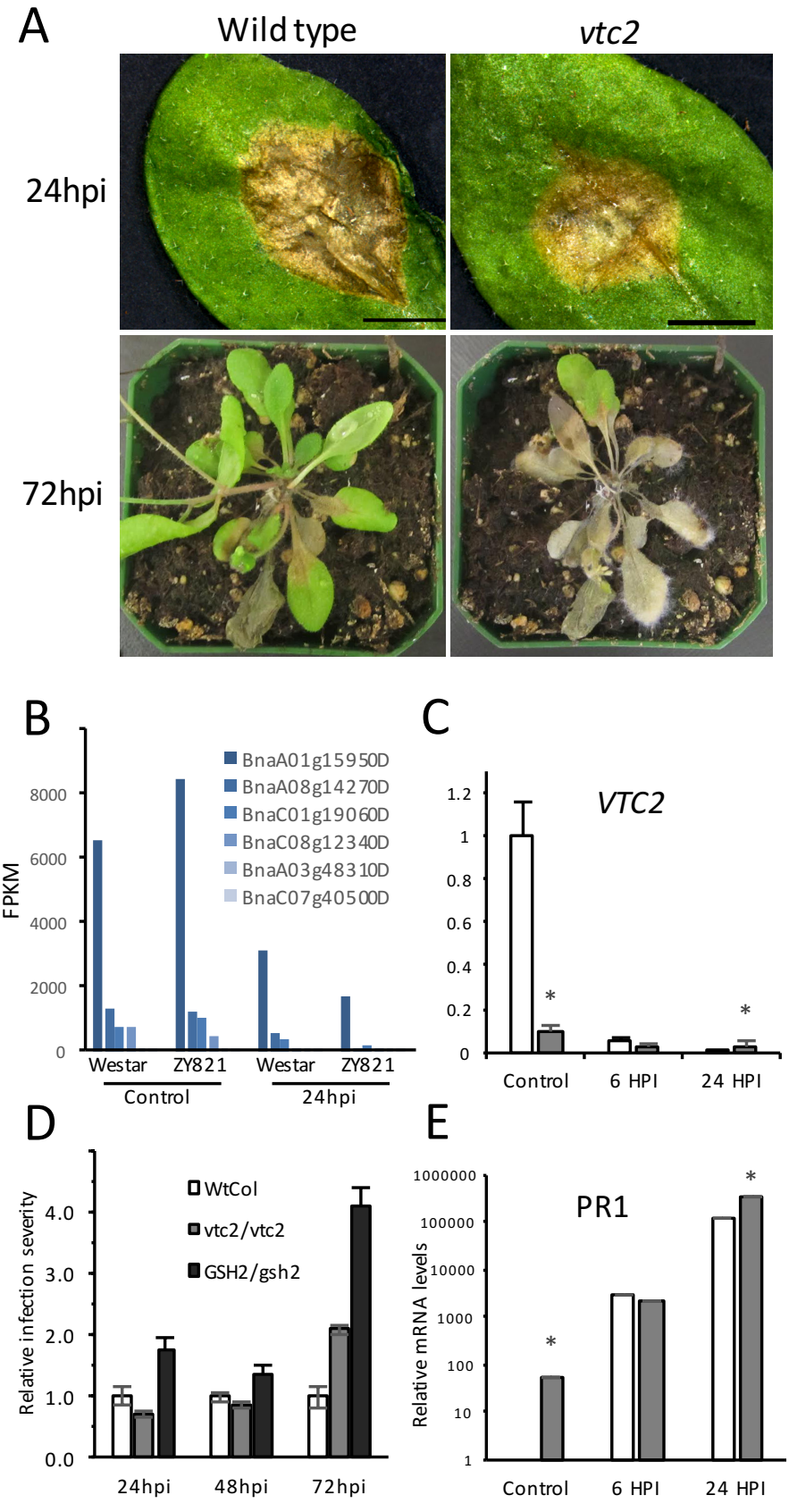
A
Wild type
vtc2
24hpi
72hpi
B
FPKM
8000
6000
4000
2000
0
BnaA01g15950D
BnaA08g14270D
BnaC01g19060D
BnaC08g12340D
BnaA03g48310D
BnaC07g40500D
Westar
ZY821
Westar
ZY821
Control
24hpi
C
VTC2
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
6 HPI
24 HPI
D
Relative infection severity
4.0
3.0
2.0
1.0
0.0
WtCol
vtc2/vtc2
GSH2/gsh2
24hpi
48hpi
72hpi
E
PR1
Relative mRNA levels
1000000
100000
10000
1000
100
10
1
*
*
Control
6 HPI
24 HPI

Figure 3.7 Arabidopsis-*Sclerotinia sclerotiorum* pathosystem. Plugs of potato dextrose agar containing growing sclerotinia hyphae were placed on leaves of Wild-type Columbia (Wild Type) and knock down line of *VITAMIN C DEFECTIVE 2*, *vtc2*. (B) FPKM levels of all detected homologues of *VTC2* in *S. sclerotiorum* infected leaves of B. napus. (C) Relative mRNA levels of VTC2 6 and 24hpi in infected leaves measured by quantitative RT-PCR. (D) Lesion progression measured in 4-week old plants at 24 and 48hpi was measured by calculating percent of total rosette leaf covered. Results were normalized to wild type at each time point. 72-hour infection progress was evaluated as the proportion of leaves infected in the mutant compared to wild type. (E) Quantitative RT-PCR was used to determine relative mRNA levels in infected plants for *PATHOGENESIS-RELATED GENE 1* (*PR1*). Statistical significance was determined using a student's t-test with $p < 0.05$ as statistically significant. Scale bars in (A) are 3mm.

Table 3.1 List of Arabidopsis T-DNA insertion line mutants challenged with *Sclerotinia sclerotiorum* using a myceliogenic assay. Phenotypes were qualitatively measured as susceptible or no difference (ND) compared to Wild Type.

AGI	T-DNA insertion line	Gene name	Phenotype	
			24hpi	72hpi
AT5G39020	SALK_125986	Malectin/receptor-like protein kinase family protein	Susceptible	Susceptible
AT1G66340	SALK_072009C	ETHYLENE RESPONSIVE 1	ND	Susceptible
AT5G60300	SALK_024581	DOES NOT RESPOND TO NUCLEOTIDES 1	ND	Susceptible
AT2G39660	SALK_066010	BOTRYTIS-INDUCED KINASE 1	ND	Susceptible
AT5G13220	CS879864	JASMONATE-ASSOCIATED 1	Susceptible	Susceptible
AT5G60900	SALK_053703C	RECEPTOR-LIKE PROTEIN KINASE 1	ND	Susceptible
AT5G65300	SALK_069313C	Unknown protein	ND	ND
AT5G25930	CS346871	Protein kinase family protein with leucine-rich repeat domain	ND	ND
AT1G05340	SALK_110092	Unknown protein	Suceptible	Susceptible
AT5G13490	CS367218	ADP/ATP CARRIER 2	ND	ND
AT5G14040	SALK_017261	MITOCHONDRIAL PHOSPHATE TRANSPORTER 3	ND	ND
AT5G24150	SALK_082809C	SQUALENE MONOOXYGENASE GENE HOMOLOGUE 1	Susceptible	ND
AT5G63820	SALK_039200C	DUF626	ND	ND
AT5G23540	CS874614	Mov34/MPN/PAD-1 family protein	Susceptible	ND

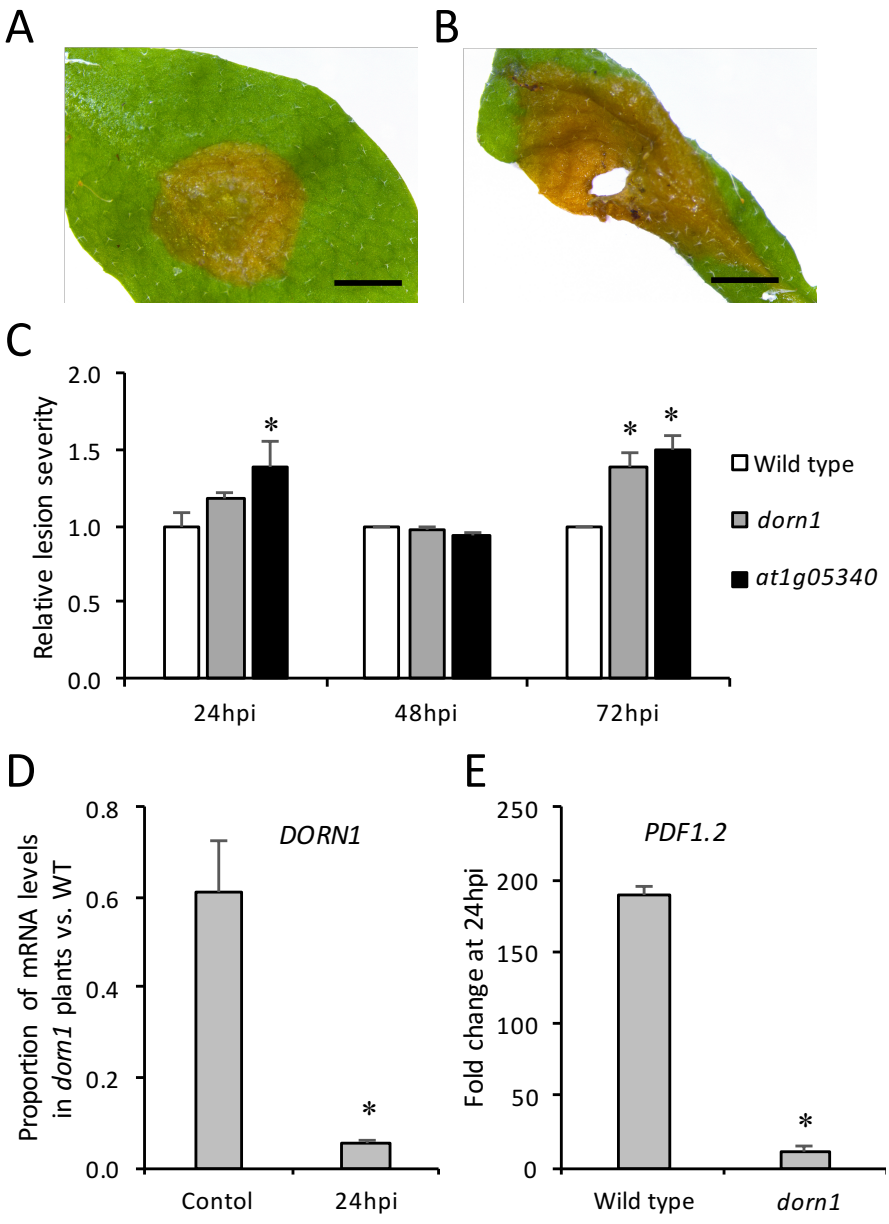
A
B
C
Relative lesion severity
2.0
1.5
1.0
0.5
0.0
*
*
*
24hpi
48hpi
72hpi
Wild type
dorn1
at1g05340
D
Proportion of mRNA levels in dorn1 plants vs. WT
0.8
0.6
0.4
0.2
0.0
DORN1
*
Contol
24hpi
E
Fold change at 24hpi
250
200
150
100
50
0
PDF1.2
*
Wild type
dorn1

Figure 3.8 Assessing plant defense to *Sclerotinia sclerotiorum* in T-DNA insertion lines of Arabidopsis. Macrographs of lesions on leaves of 4 week old plants in (A) Wild type Columbia, and (B) T-DNA insertion line of *DOES NOT RESPOND TO NUCLEOTIDES 1* (*DORN1*) 24 hours post inoculation (hpi) with and agar plug of growing *S. sclerotiorum* hyphae. (C) Relative infection severity in three plant lines is measured at 24 and 48hpi by calculating percent of total rosette leaf covered compared to Wild Type. 72-hour infection progress was evaluated as the proportion of leaves infected in the mutant compared to wild type. (D) Confirmation of *DORN1* knockdown by measuring fold change between *dorn1* and Wild Type in control and 24hpi samples. (E) Fold change of *PLANT DEFENSIN 1.2* (*PDF1.2*) 24hpi in leaf tissues of Wild Type and *dorn1* plants. Statistical significance ($P<0.05$, students t-test) indicated by asterisk, scale bars in (A) and (B) are 3mm.

3.4 DISCUSSION

Analysis of the *B. napus*-*S. sclerotiorum* interaction directly at the host pathogen interface, provides novel insight into the structural, genetic, and physiological changes in the canola leaf at the earliest stages of the infection process. We identified putative transcriptional circuits controlling biological processes and defense genes associated with the plant response to *S. sclerotiorum* that may regulate lesion spread and disease progression. A comprehensive investigation of redox homeostasis following *S. sclerotiorum* infection revealed major genotypic differences between our two experimental cultivars, providing a physiological explanation for the tolerant phenotype of ZY821.

Previous studies investigating this pathosystem towards the later stages of the infection process in stem tissues used artificial nutrient sources to promote infection and does not replicate what is observed in the field (Wu et al., 2016; Zhao et al., 2009). Our method using senescing petals more closely simulates field conditions as senescing petals likely serve as a nutrient source required for *S. sclerotiorum* to effectively finance the production of toxins, cell wall degrading enzymes, and appressoria to penetrate host tissues (Bolton, 2006). Interestingly, we did not observe stomatal penetration of host tissue in either cultivar, even though these leaf structures are directly regulated via OA (Stotz and Guimaraes, 2004), and serve as ideal entry points for a number of pathogenic fungi (Kim et al., 2011). Instead, SEM and light microscopy data revealed advancing hyphae penetrating the physical barriers of the plant via infection cushions, the occurrence of which is correlated to pathogenicity in *S. sclerotiorum* (Jurick and Rollins, 2007). Thus, the abundance of complex appressoria on the epidermis of tolerant *B. napus* suggests *S. sclerotiorum* requires additional energetic resources to colonize the plant. The cellular differences observed between the susceptible and tolerant cultivars provide additional structural

evidence for the colonization strategy of *S. sclerotiorum* on the leaf surface. Li et al., (2006) showed structural differences in stem tissues correlated with *S. sclerotiorum* pathogenicity in susceptible and resistant lines of *B. napus*. Together, these findings suggest cellular organization of both leaf and stem tissues, including cell density and cell wall thickness, may serve as important structural boundaries limiting infection in *Brassica* plant species.

Global RNA profiling of the *B. napus* infection site within the first 24 hours of the host pathogen interaction revealed large and coordinated shifts in gene activity. While large numbers of differentially expressed genes have been reported in stem tissues of *S. sclerotiorum*-resistant plants (Wu et al., 2016), here, ZY821 is able to control transcriptional activation within hours of the infection process to limit *S. sclerotiorum* penetration and colonization. Biological processes associated with differential expression of up- and down-regulated gene sets provide detailed insight into the molecular underpinnings of the plant response.

RNA data show both cultivars effectively down-regulated transcripts associated with growth-related processes such as photosynthesis and carbon fixation, which is largely consistent with cross-kingdom studies on photosynthesis deregulation following biotic stress (Bilgin et al., 2010). Our gene ontology analysis shows defense related processes and all major hormone signalling pathways were up-regulated in leaf tissues of both genotypes following infection. However, we discovered many differences in the amplitudes and attenuations of genes fundamental to the defense process, providing a new understanding of the molecular foundations of tolerance to *S. sclerotiorum*. For example, activation of both the SA and JA/Ethylene hormone signal transduction networks in leaf tissue 24 hpi supports a conserved defense response in both stem and leaf tissues following *S. sclerotiorum*infection (Wu et al., 2016; Zhao et al., 2009). Interestingly, transcripts for SA biosynthesis, including *ISOCHORISMATE SYNTHASE 1*

homologues accumulated up to six times more in the susceptible cultivar than in ZY821 (Table S4). This somewhat contradicts previous work of Zhao et al. (2009) who found *ICS1* homologues down-regulated in stem tissues the same two genotypes 24 hpi using a *B. napus* microarray. These differences may be explained by differential activation of homologues not included in the array used by Zhao et al. (2009), however, Wu et al. (2016) also found similar down-regulation of *ICS1* homologue accumulation levels in both susceptible and resistant cultivar stem tissues. Although transcript levels do not explicitly describe enzyme activities, together this suggests differential defense hormone responses to *S. sclerotiorum* in different organs of *B. napus*. Whereas SA biosynthesis transcripts were upregulated in the susceptible genotype of *B. napus*, mRNAs of genes associated with ethylene biosynthesis, and ERFs were much higher in the tolerant cultivar (Figure 3.5C). Given that SA accumulation directly represses one of the major JA/ET pathway TFs, *OCTADECANOID-RESPONSIVE ARABIDOPSIS 59* (Caarls et al., 2015), the accumulation of SA in the susceptible cultivar may explain how *ORA56* copy levels accumulate at an average of 6.5 fold greater in the tolerant cultivar than in Westar. This suggests upstream restriction of SA signalling, limiting SA-mediated JA antagonism, may be required for *S. sclerotiorum* tolerance in *B. napus* leaf tissues (Van der Does et al., 2013).

The MAPK signalling cascade forms an essential part of the plant defense signal transduction flow and transduces JA signals to transcriptional reprogramming (Meng and Zhang, 2013; Takahashi et al., 2007). Not only did we discover differential activation of MAPK genes (Table S4), but in our novel transcript detection analysis, an *MPK4* homologue, *CUFF.20400.1*, was exclusively and abundantly detected in infected tissues of the tolerant cultivar (Table S2, Figure S3.4). In Arabidopsis, *MPK4* has recently been shown to be a positive regulator of PTI

and an activator of *WRKY33* (Qiu et al., 2008; Zhang et al., 2012); thus, *CUFF.20400.1* may contribute to the signalling events responsible for *S. sclerotiorum* tolerance in ZY821.

Effective suppression of fungal attack also relies on the timely activation of TFs controlling defense gene networks. We identified a suite of WRKY TFs potentially controlling the plant response to chitin within the first 24 hours of the host-pathogen interaction. Specifically, *WRKY 18,* which has been shown to play a role in Arabidopsis defense against the necrotroph *Botrytis cinerea* (Xu et al., 2006), was also expressed following Sclerotinia interaction with the *B. napus* leaf. This suggests that *WRKY18*, along with additional WRKY TFs that form the module including *WRKY 29*, *53* and *54*, may have evolved to play a regulatory role in mitigating attack from necrotrophic fungi. Thus, our predictive transcriptional module activated specifically in the tolerant cultivar provides a platform for future studies to uncover the role of WRKY TFs as master regulators of necrotrophic defense in canola and other crop species.

Our data have uncovered complex TF-DNA motif binding interactions required for *S. sclerotiorum* tolerance including HSFs, ERFs, and MYC TFs. Both ERFs and MYC TFs are downstream of JA signalling with antagonistic properties (Lorenzo et al., 2004; Niu et al., 2011). Thus suggesting the activation and coordination of both ERF and MYC streams of JA signalling is an esstial part of plant tolerance to *S. sclerotiorum*. For example, among the TFs predicted to bind to the MYC4 DNA motif (Figure 3.5B), are homologues of *MYC3*, a bHLH TF shown to interact with MYB TFs activating glucosinolate biosynthesis in Arabidopsis (Schweizer et al., 2013). All three homologues of *INDOLE GLUCOSINOLATE METHYLTRANSFERASE 5* detected in this study (*BnaA07G33060D, BnaC06G37610D*, and *BnaC06G21620D*) are found in DP2, and predicted to be controlled through network interactions, offering insight into how glucosinolate production is transcriptionally controlled in response to *S. sclerotiorum*. Although

the interactions remain putative, our network analyses provide an elegant and direct avenue for further investigation using functional characterization pipelines.

Defense related gene expression and PTI activation is tied to natural circadian rhythms within the plant (Wang et al., 2011; Zhang et al., 2013) and integrate hormone, redox, sugar, and other genetic regulatory signals (Bass and Takahashi, 2011; Cui et al., 2014). Although we observed similar transcript levels of the master circadian regulators *CCA1* and *LHY* following *S. sclerotiorum* infection between the two cultivars (Table S3.4), the strongly divergent responses of circadian-regulated genes provides insight into an additional layer of complexity to this pathosystem and suggests either downstream or post-translational control of clock-regulated genes are required for defense against *S. sclerotiorum*.

Global transcriptional reprogramming observed in tolerant leaf tissues, which expressed fewer genes following infection than the susceptible cultivar, suggests broad genomic control of the genetic regulatory machinery following interaction with *S. sclerotiorum*. Enrichment of DNA methylation GO terms controlled by *TELEMORIC BINDING PROTEIN* homologues in genes specifically expressed in ZY821 (Figure 3.4A), and higher accumulation levels of *SET* homologues (Table S3.4) hint at differences in how the two cultivars adjust chromatin and chemical modifications of genetic material in response to fungal infection. Since DNA methylation is an essential part of plant defense response (Dowen et al., 2012), and full activation and transcriptional regulation of JA/ET activated genes requires the histone methyltransferase *SET DOMAIN GROUP8* in Arabidopsis (Berr et al., 2010), epigenetic control mechanisms may directly contribute to tolerance to *S. sclerotiorum* thus limiting disease symptoms.

Reprogramming of the plant cytoskeleton in response to fungal interaction provides a cellular mechanism by which defense compounds and proteins are targeted to the cell wall through rapid activation of vesicle trafficking (Assaad et al., 2004; Wang et al., 2005). The enrichment of biological processes associated with protein transport (Table S3) and increased abundance of qSNAREs critical to plant immunity such as *SYNTAXIN OF PLANTS 121* and *122* at the infection site in tolerant host leaf tissues suggest that up-regulation of intracellular transport process is at least partly responsible for mitigating disease (Collins et al., 2003; Pajonk et al., 2008). Vesicle transport depends on the underlying cytoskeleton and the differentially regulated, but functionally redundant actin isoforms. Global RNA sequencing identified differences in cytoskeleton reorganization in the two cultivars response to *S. sclerotiorum*. For example, *ACTIN 7* transcript levels have been shown to respond to external stimuli, such as wounding and hormone treatment (Kandasamy et al., 2009; Mcdowell et al., 1996), and the majority of homologues are much more highly abundant in ZY821 (Table S4). Transcript levels of *BnaA03G34950D*, a homologue of another major vegetative actin (*ACT2*), doubles following infection in Westar and is differentially expressed over five times higher in ZY821 (Figure S3.5). This may be explained by the presence of the ETHYLENE RESPONSE FACTOR BINDING SITE 'GCCGCC' (Mathelier et al., 2015) present in the promoter of the *ACTIN2* homologue *BnaA03G34950D,* but not *ACT7 (BnaC09G46850D)*. Increased transcript levels of actin isoforms may contribute to a larger pool of actin required for rapid accumulation of actin filaments that assembles in response to invasion attempts (Henty-Ridilla et al., 2013; Shimada et al., 2006). Theses cytostructural responses to pathogens are a critical component of an effective PTI response that our RNA data analysis links to the tolerant phenotype of ZY821.

Reactive oxygen species produced during the host pathogen interaction serve as important cellular defense signalling molecules, but can also damage the host's molecular machinery, thus affecting the defense response (Scheler et al., 2013; Tripathy and Oelmüller, 2012). Global RNA profiling identified elevated redox buffering capacity in tolerant leaf tissues of ZY821 activated at the first point of infection through genes contributing to GO terms such as response to oxidative stress and glutathione metabolic process (Table S3). Our GO enrichment analysis was further validated by APX and GR enzyme activity assays and the quantification of their small molecule substrates, ascorbate and glutathione, both of which are critical components of plant defense (Foyer and Noctor, 2011; Ishikawa and Shigeoka, 2008). Interestingly, elevated levels of ASC 24 hpi in tolerant leaf tissues do not correspond with transcript levels of the rate-limiting enzyme in the ascorbic acid biosynthesis pathway, VTC2, of which all homologues were down-regulated following infection in both cultivars (Figure 3.7B). However, transcript levels of *ERF98*, a positive transcriptional regulator of ascorbic acid biosynthesis (Wang et al., 2013; Zhang et al., 2012a), were considerably higher in tolerant leaf tissues (20-fold in Westar and 1600-fold in ZY821, Table S4). These data further support our transcriptional circuit that places an *ERF98* homologue, *BnaA07G06750D,* as a regulator of redox related defense processes, and further provides novel insight into the transcriptional regulation of the complex networks underlying tolerance to *S. sclerotiorum*.

Cellular redox buffering through the ascorbate-glutathione pathway may provide a physiological means to overcome or at least prevent the spread of necrotrophic fungal infections. Excess ROS can trigger cell death (Breusegem and Dat, 2013; Chaouch et al., 2010) and the ASC-GSH pathway activation in tolerant leaves of ZY821 likely contributes to the lack of cellular degredation observed in advance of fungal hyphae (Figure 3.1J), thus limiting nutrient

availability from OA-induced cell death (Kim et al., 2008). The importance of redox regulation and homeostasis in defence against *S. sclerotiorum* is further reinforced by the hyper susceptible phenotype of the Arabidopsis *vtc2* and *gsh2* mutants (Figure 3.7A& 3.7D). The delayed lesion growth at the early stages of infection in *vtc2* plants is likely caused by the constitutive expression of *PR1* through redox state-derived activation of the TF *NONEXPRESSER OF PR GENES 1* (Tada et al., 2008). Thus, while the *vtc2* mutant has elevated defense capacities within the first 24 hours of infection, the necrotrophic nature of *S. sclerotiorum* is too powerful for sustained protection over time. Taken together, the coordination and functioning of cellular redox buffering systems including enzyme activation and the biosynthesis of their small-molecule substrates serves a major role in plant defense response pathways and management of tissue damage.

The preliminary screen of T-DNA insertion knockdowns of putative plant defense regulator genes narrowed the list of candidates, allowing a more thorough functional analysis of those showing a susceptible phenotype. Interestingly, *AT1G05340* is an uncharacterized gene, and its susceptible phenotype in the lesion size assay, identifies it as playing a role in conferring plant defense processes, and an excellent candidate for future study. Since DORN1 has been established as the primary means of sensing extracellular ATP in Arabidopsis (Choi et al., 2014), our data clearly showing the hyper susceptible phenotype of *dorn1* plants, suggests extracellular ATP detection as a critical part of plant defense to *S. sclerotiorum*. The lower levels of the JA-induced *PDF1.2* in infected *dorn1* plants suggests that extracellular ATP detection may contribute an additive effect to JA defense signalling, and act as an important signalling molecule for plant defense against *S. sclerotiorum*.

We present a comprehensive investigation into the transcriptional and physiological changes contributing to *S. sclerotiorum* tolerance in ZY821. Although we have yet to fully understand the molecular underpinnings of *S. sclerotiorum* infection severity in *B. napu*s or the full list of genes required for genetic resistance, we have uncovered new molecular and physiological processes associated with tolerance at the first point of infection. The wholesale transcriptional reprogramming undergone by plant cells infected with fungal pathogens is an intricately regulated processes controlled through the activity of transcriptional circuits. This regulatory analysis highlighted the transcriptional control of redox response and highlights the critical nature of redox homeostasis for *B. napus* tolerance to *S. sclerotiorum*. We also uncovered additional areas of study yet to be explored in this pathosystem including cytosolic structural changes associated with the tolerant phenotype and uncovered the potential role of higher-level genome control in regulating plant responses. Together this dataset, and our analysis thereof, provides an important resource for breeding more tolerant crops to devastating fungal pathogens like *S. sclerotiorum*.

3.5 METHODS FOR CANOLA EXPERIMENTS

3.5.1 *Brassica napus* growth conditions

The two *Brassica napus* cultivars used for all experiments are the susceptible *B. napus* cv. *Westar* (Westar) and *B. napus* cv. *Zhongyou821* (ZY821). Westar seeds stored at 4˚C were planted in Sunshine Mix No.1 soil and grown at 22˚C with 50-70% humidity with long day conditions (16 hours light, 8 hours dark 150-200 μE/m^2/s). ZY821 plants were treated similarly, however 1 month after planting they were subjected to a four-week vernalization treatment (8 hours light, 16 hours dark, 40% humidity, 8-10˚C and 100 μE/m^2/s), before being transferred back to long day conditions. Both cultivars were inoculated at 30-50% bloom stage.

3.5.2 *Sclerotinia sclerotiorum* inoculum preparation

Sclerotinia sclerotiorum field-collected ascospores were purchased from Dr Khalid Rashid, Morden Research and Development Centre – Agriculture and Agri-Food Canada, Morden, MB, Canada, and stored at 4˚C in desiccant in the dark. Inoculum was made by suspending ascospores at a concentration of 8 x10^4 spores per mL in a 0.02% Tween80 (http://www.sigmaaldrich.com/) solution. 30μL of the solution was pipetted onto senescing petals placed in a petri plate and sealed with parafilm. The Tween solution without ascospores was used as a control and applied to petals used for mock inoculation. Ascospores were allowed to germinate into hyphae and grow for 72 hours prior to being used in the leaf inoculation experiments.

3.5.3 Leaf inoculation and tissue collection

At approximately 1PM, either mock-inoculated petals or infected petals and *S. Sclerotiorum* hyphae were transferred onto healthy leaves and covered with a clear plastic bag in order to maintain the high level of humidity required for *S. sclerotiorum*infection. Plants were

then grown under normal conditions, and lesion sizes were measured at 24, 48, 72, 96, and 120 hours post-inoculation (hpi). For RNA collection and redox assays, lesions and surrounding 1cm of healthy leaf tissue were excised and immediately frozen in liquid nitrogen. Lesions from a minimum of 3 different plants were combined and ground to a powder in liquid nitrogen for each biological replicate.

3.5.4 Light Microscopy

Healthy leaves and infected leaves 1 cm around the lesion site were collected by hand using a double-sided razor blade and then fixed in a solution of 2.5% glutaraldehyde and 1.6% paraformaldehyde in phosphate buffered saline. Tissue processing was carried out exactly as described in Chan and Belmonte (2013). The solid blocks of tissue imbedded in historesin were then sectioned at a thickness of 3 μm using disposable steel blades mounted on a Leica RM2245 microtome. Sections were placed on glass slides, and the slides were treated with 0.1% periodic acid, then Schiff's reagent for 15 minutes each, and then counterstained with toluidine blue O (TBO) for 1 minute to visualize cellular features. A Leica DM2500 bright field light microscope was used for imaging and micrographs were taken with Leica Application Suite (version 3.7) software. Images were modified (cropped, scale bars added, and contrast, brightness and color balance adjusted) using Adobe Photoshop CS2 (version 9.0) software.

3.5.5 Scanning Electron Microscopy

Similar tissue collection techniques to the light microscopy methods, collecting 1 cm around the lesion were collected in addition to uninfected leaf tissue for controls. The fresh leaf tissues were mounted on the aluminum stubs using double stick carbon tape. The samples were introduced into the chamber of a Hitachi TM-1000 Tabletop microscope, and images were collected using TM-1000 software.

3.5.6 RNA Isolation and cDNA sequencing library synthesis

RNA was isolated using Invitrogen Plant RNA Purification Reagent, and subsequently treated with the Ambion Turbo DNA-free DNase kit according to the manufacturer's protocol (https://www.thermofisher.com). Quantity and purity of RNA samples were assessed spectrophotometrically and quality of RNA samples was verified with electropherogram profiles and RNA Integrity Numbers (RIN) using an Agilent 2100 Bioanalyzer and RNA Nano Chip (http://www.genomics.agilent.com/). Sequencing cDNA libraries were prepared from 5µg of total RNA using a modified version of the alternative Ravi protocol (Kumar et al., 2012). Briefly, mRNA was isolated from total pool of RNA using NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, https://www.neb.ca/) according to instructions with the following modifications: all reaction volumes were halved, only 7.5µL of Oligo $d(T)_{25}$ beads were used per sample. The remaining preparation steps were performed according to the Ravi alternative HTR protocol (C2), starting with the first strand cDNA synthesis. NEXTflex™ ChIP-Seq Barcodes (Bioo Scientific, http://www.biooscientific.com/) were used as adaptors for the adapter ligations and NEXTflex™ PCR Primer Mix was used for the library enrichment PCR step. Individual libraries were verified using a High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer and pooled, then size selected using E-Gel® SizeSelect™ 2% agarose gel (Life Technologies, www.thermofisher.com) to target fragments from 250-500 base pairs in length. Fragments were sequenced with 100bp single-end reads using the Illumina HiSeq2000 platform at McGill University and Génome Québec Innovation Centre (Montreal, Canada, http://gqinnovationcenter.com/).

3.5.7 Bioinformatics Pipeline

Fastq files were trimmed using Trimmomatic (Bolger et al., 2014), trimming adapter sequences, headcrop of 6 bases, bases with an average quality score of less than 30 in a sliding window of 4 bases, and dropping remaining reads shorter than 50nt. The splice junction mapping software TopHat (v2.0.13, Trapnell et al., 2012, http://ccb.jhu.edu/software/tophat/index.shtml) was used to align and map the trimmed reads to the published *B. napus* genome v5.0 (Chalhoub et al., 2014, http://www.genoscope.cns.fr/brassicanapus/). Transcripts from each sample were assembled with Cufflinks (v2.2.1, https://github.com/cole-trapnell-lab/cufflinks) using the published transcript annotation file to guide assembly. Individual samples' transcript assemblies were merged with the reference. For a conservative and robust prediction of novel transcripts, the merged assembly was used to predict open reading frames with Transdecoder (v2.0.1, https://transdecoder.github.io/), and newly predicted genes were only kept if they were located within intergenic regions and the largest ORF had a translated protein blast hit using BLAST+ (v2.2.30, http://blast.ncbi.nlm.nih.gov/) to Arabidopsis TAIR10 protein annotation (http://arabidopsis.org), with an E-value cut off < $1x10^{-10}$. Quantitation of mapped reads was done with the Cufflinks suite of tools (v2.2.1, http://cole-trapnell-lab.github.io/cufflinks). This included differential expression analyses, and normalization of transcript abundances across samples in FPKM.

3.5.8 Quantitative RT-PCR analysis

One microgram of RNA was used to construct cDNA libraries for real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) using Maxima First Strand Synthesis Kit (https://www.thermofisher.com/). RT-qPCR was carried out using Sso Fast Evagreen Supermix (http://www.bio-rad.com/), following manufacturer's instructions but proportionally

adjusting to a 10µL reaction volume. Reaction steps were performed as follows: 95˚C for 30 seconds, followed by 45 cycles of 95˚C for 2 seconds and 5 seconds of 60˚C. A melt curve ran from 65˚C to 90˚C to evaluate product sizes and detect primer dimers. The ΔΔCt method, using the ubiquitin family protein *BnaC08g11930D* as a housekeeping gene in *B. napus*, was used to calculate fold changes between mock and *S. sclerotiorum* inoculated treatments in both cultivars. For Arabidopsis experiments, *EF1ALFA* (*AT5G60390*) was used as a housekeeping gene. Statistical significance was calculated using students t-test and a cut-off of $p < 0.05$, and all primer sequences used are presented in Table 3.5.

3.5.9 Enzyme assays

S. sclerotiorum inoculation and tissue collection was carried out as described above, quantification of reduced and oxidized forms of the small molecules ascorbate and glutathione were carried out as described by Zhang and Kirkham, (1996). Enzyme activities of ASCORBATE PEROXIDASE (APX), MONODEHYDROASCORBATE REDUCTASE (MDAR), DEHYDROASCORBATE REDUCTASE (DHAR), and GLUTATHIONE REDUCTASE (GR) were carried out exactly as described in Belmonte and Stasolla (2009).

3.5.9 Arabidopsis pathogenicity experiments

Arabidopsis thaliana and T-DNA insertion lines were obtained from The Arabidopsis Biological Resource Center (Ohio State University, https://abrc.osu.edu/). Seeds were surface sterilized using 75% ethanol and grown on Murashige and Skrooge medium (Phytotechnology Labratories) with 1% phytagel at pH 5.7. Seeds were vernalized for 3 days at 4°C before being transferred to an incubation chamber 2 weeks. Seedlings were then transplanted into Sunshine Mix #1 soil and kept at 22°C a 16-h photoperiod. T-DNA insertion presence was verified with PCR and plants were inoculated at four weeks old.

3.5.10 Leaf inoculation with *S. sclerotiorum* and lesion size assays

Sclerotia harvested from carpogenically infected canola plants were sterilized sing bleach, halved, plated on potato dextrose agar, and incubated for 4 days at room temperature in the dark. Four-week old plants were inoculated with 4.5mm diameter agar plugs containing growing fungal hyphae placed on one rosette leaf of inoculated plants. Pots containing 6 plants were then enclosed in a plastic bag to maintain humidity. Progression of infection at 24 and 48hpi was determined by measuring the leaf length and width and calculating elliptical area of infection. Progression of infection at 72 hpi was determined by dividing total number of infected rosette leaves by total number of rosette leaves. RNA isolation, cDNA synthesis, and qPCR reactions were carried out as described above.

3.5.11 Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number GSE81545

3.6 AUTHOR CONTRIBUTIONS

I.J.G., C.T., X.M, T.d.K., W.G.D.F., S.L., and M.F.B. designed research; I.J.G., C.T., X.M, M.G.B., P.L., D.A.D. performed research; I.J.G., C.T., S.L., and M.F.B. wrote the paper.

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The authors declare no conflict of interest.

3.8 REFERENCES

Assaad, F., Qiu, J., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., et al. (2004). The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. *Mol. Biol. Cell* 15, 5118–5129. doi:doi/10.1091/mbc.E04–02–0140.

Bass, J., and Takahashi, J. S. (2011). Circadian rhythms: Redox redux. *Nature* 469, 476–478. doi:10.1038/469476a.

Becker, M. G., Chan, A., Mao, X., Girard, I. J., Lee, S., Mohamed, E., et al. (2014). Vitamin C deficiency improves somatic embryo development through distinct gene regulatory networks in Arabidopsis. *J. Exp. Bot.* doi:10.1093/jxb/eru330.

Belmonte, M. F., Kirkbride, R. C., Stone, S. L., Pelletier, J. M., Bui, A. Q., Yeung, E. C., et al. (2013). Comprehensive developmental profiles of gene activity in regions and subregions of the Arabidopsis seed. *Proc. Natl. Acad. Sci. U. S. A.* 110, E435–44. doi:10.1073/pnas.1222061110.

Belmonte, M. F., and Stasolla, C. (2009). Altered HBK3 expression affects glutathione and ascorbate metabolism during the early phases of Norway spruce (Picea abies) somatic embryogenesis. *Plant Physiol. Biochem.* 47, 904–911. doi:10.1016/j.plaphy.2009.05.011.

Berr, A., McCallum, E. J., Alioua, A., Heintz, D., Heitz, T., and Shen, W.-H. (2010). Arabidopsis histone methyltransferase SET DOMAIN GROUP8 mediates induction of the jasmonate/ethylene pathway genes in plant defense response to necrotrophic fungi. *Plant Physiol.* 154, 1403–1414. doi:10.1104/pp.110.161497.

Bhardwaj, A. R., Joshi, G., Kukreja, B., Malik, V., Arora, P., Pandey, R., et al. (2015). Global

insights into high temperature and drought stress regulated genes by RNA-Seq in economically important oilseed crop Brassica juncea. *BMC Plant Biol.* 15, 9. doi:10.1186/s12870-014-0405-1.

Bilgin, D. D., Zavala, J. a., Zhu, J., Clough, S. J., Ort, D. R., and DeLUCIA, E. H. (2010). Biotic stress globally downregulates photosynthesis genes. *Plant. Cell Environ.* 33, 1597–1613. doi:10.1111/j.1365-3040.2010.02167.x.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi:10.1093/bioinformatics/btu170.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi:10.1146/annurev.arplant.57.032905.105346.

Bolton, M. (2006). Sclerotinia sclerotiorum (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol.* 7, 1–16. doi:10.1111/J.1364-3703.2005.00316.X.

Bradley, C. a., Lamey, H. a., Endres, G. J., Henson, R. a., Hanson, B. K., McKay, K. R., et al. (2006). Efficacy of Fungicides for Control of Sclerotinia Stem Rot of Canola. *Plant Dis.* 90, 1129–1134. doi:10.1094/PD-90-1129.

Breusegem, F. Van, and Dat, J. F. (2013). Reactive Oxygen Species in Plant Cell Death. *Am. Soc. Plant Biol.* 141, 384–390. doi:10.1104/pp.106.078295.384.

Brutus, A., Sicilia, F., Macone, A., Cervone, F., De Lorenzo, G., and Lorenzo, G. De (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U. S. A.* 107, 9452–7. doi:10.1073/pnas.1000675107.

Caarls, L., Pieterse, C. M. J., and Van Wees, S. C. M. (2015). How salicylic acid takes transcriptional control over jasmonic acid signaling. *Front. Plant Sci.* 6, 170. doi:10.3389/fpls.2015.00170.

Cessna, S. G., Sears, V. E., Dickman, M. B., Low, P. S., Cessna, S. G., Sears, V. E., et al. (2000). Oxalic acid, a pathogenicity factor for Sclerotinia sclerotiorum, suppresses the oxidative burst of the host plant. *Plant Cell* 12, 2191–2199.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I. a. P., Tang, H., Wang, X., et al. (2014). Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. *Science (80-.).* 345, 950–953. doi:10.1126/science.1253435.

Chan, A. C., Khan, D., Girard, I. J., Becker, M. G., Millar, J. L., Sytnik, D., et al. (2016). Tissue-specific laser microdissection of the *Brassica napus* funiculus improves gene discovery and spatial identification of biological processes. *J. Exp. Bot.*, erw179. doi:10.1093/jxb/erw179.

Chaouch, S., Queval, G., Vanderauwera, S., Mhamdi, A., Vandorpe, M., Langlois-Meurinne, M., et al. (2010). Peroxisomal hydrogen peroxide is coupled to biotic defense responses by ISOCHORISMATE SYNTHASE1 in a daylength-related manner. *Plant Physiol.* 153, 1692–1705. doi:10.1104/pp.110.153957.

Chassot, C., Buchala, A., Schoonbeek, H. J., Métraux, J. P., and Lamotte, O. (2008). Wounding of Arabidopsis leaves causes a powerful but transient protection against Botrytis infection. *Plant J.* 55, 555–567. doi:10.1111/j.1365-313X.2008.03540.x.

Chassot, C., Nawrath, C., and Métraux, J. P. (2007). Cuticular defects lead to full immunity to a major plant pathogen. *Plant J.* 49, 972–980. doi:10.1111/j.1365-313X.2006.03017.x.

Choi, J., Tanaka, K., Cao, Y., Qi, Y., Qiu, J., Liang, Y., et al. (2014). Identification of a plant receptor for extracellular ATP. *Science* 343, 290–4. doi:10.1126/science.343.6168.290.

Collins, N. C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J.-L., et al. (2003). SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* 425, 973–977. doi:10.1038/nature02076.

Covington, M. F., Maloof, J. N., Straume, M., Kay, S. A., and Harmer, S. L. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biol.* 9, R130. doi:10.1186/gb-2008-9-8-r130.

Cui, Z., Xu, Q., and Wang, X. (2014). Regulation of the circadian clock through pre-mRNA splicing in Arabidopsis. *J. Exp. Bot.* 65, 1973–80. doi:10.1093/jxb/eru085.

Demidchik, V., Shang, Z., Shin, R., Thompson, E., Rubio, L., Laohavisit, A., et al. (2009). Plant extracellular ATP signalling by plasma membrane NADPH oxidase and Ca2+ channels. *Plant J.* 58, 903–913. doi:10.1111/j.1365-313X.2009.03830.x.

Denancé, N., Sánchez-Vallet, A., Goffner, D., and Molina, A. (2013). Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. *Front. Plant Sci.* 4, 155. doi:10.3389/fpls.2013.00155.

Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M. C., Rodenburg, N., Pauwels, L., et al. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *Plant Cell* 25, 744–61. doi:10.1105/tpc.112.108548.

Dowen, R. H., Pelizzola, M., Schmitz, R. J., Lister, R., Dowen, J. M., Nery, J. R., et al. (2012). Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl. Acad. Sci. U. S. A.* 109, E2183–91. doi:10.1073/pnas.1209329109.

Fernando, W. G. D., Nakkeeran, S., Zhang, Y., and Savchuk, S. (2007). Biological control of Sclerotinia sclerotiorum (Lib.) de Bary by Pseudomonas and Bacillus species on canola

petals. *Crop Prot.* 26, 100–107. doi:10.1016/j.cropro.2006.04.007.

Foyer, C. H., and Noctor, G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* 155, 2–18. doi:10.1104/pp.110.167569.

Garg, H., Atri, C., Sandhu, P. S., Kaur, B., Renton, M., Banga, S. K., et al. (2010a). High level of resistance to Sclerotinia sclerotiorum in introgression lines derived from hybridization between wild crucifers and the crop Brassica species B. napus and B. juncea. *F. Crop. Res.* 117, 51–58. doi:10.1016/j.fcr.2010.01.013.

Garg, H., Li, H., Sivasithamparam, K., and Barbetti, M. J. (2013). Differentially expressed proteins and associated histological and disease progression changes in cotyledon tissue of a resistant and susceptible genotype of brassica napus infected with Sclerotinia sclerotiorum. *PLoS One* 8, e65205. doi:10.1371/journal.pone.0065205.

Garg, H., Li, H., Sivasithamparam, K., Kuo, J., and Barbetti, M. J. (2010b). The infection processes of Sclerotinia sclerotiorum in cotyledon tissue of a resistant and a susceptible genotype of Brassica napus. *Ann. Bot.* 106, 897–908. doi:10.1093/aob/mcq196.

Hegedus, D. D., and Rimmer, S. R. (2005). Sclerotinia sclerotiorum: When "to be or not to be" a pathogen? *FEMS Microbiol. Lett.* 251, 177–184. doi:10.1016/j.femsle.2005.07.040.

Henty-Ridilla, J. L., Shimono, M., Li, J., Chang, J. H., Day, B., and Staiger, C. J. (2013). The Plant Actin Cytoskeleton Responds to Signals from Microbe-Associated Molecular Patterns. *PLoS Pathog.* 9. doi:10.1371/journal.ppat.1003290.

Horbach, R., Navarro-quesada, A. R., Knogge, W., and Deising, H. B. (2011). When and how to kill a plant cell : Infection strategies of plant pathogenic fungi. *J. Plant Physiol.* 168, 51–62. doi:10.1016/j.jplph.2010.06.014.

Huot, B., Yao, J., Montgomery, B. L., and He, S. Y. (2014). Growth-defense tradeoffs in plants:

a balancing act to optimize fitness. *Mol. Plant* 7, 1267–87. doi:10.1093/mp/ssu049.

Ishikawa, T., and Shigeoka, S. (2008). Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms. *Biosci. Biotechnol. Biochem.* 72, 1143–1154. doi:10.1271/bbb.80062.

Jamaux, I., Gelie, B., and Lamarque, C. (1995). Early stages of infection of rapeseed petals and leaves by Sclerotinia sclerotiorum revealed by scanning electron microscopy. *Plant Pathol.* 44, 22–30. doi:10.1111/j.1365-3059.1995.tb02712.x.

Jurick, W. M., and Rollins, J. A. (2007). Deletion of the adenylate cyclase (sac1) gene affects multiple developmental pathways and pathogenicity in Sclerotinia sclerotiorum. *Fungal Genet. Biol.* 44, 521–530. doi:10.1016/j.fgb.2006.11.005.

Kandasamy, M. K., McKinney, E. C., and Meagher, R. B. (2009). A single vegetative actin isovariant overexpressed under the control of multiple regulatory sequences is sufficient for normal Arabidopsis development. *Plant Cell* 21, 701–18. doi:10.1105/tpc.108.061960.

Khot, S. D., Bilgi, V. N., del Río, L. E., and Bradley, C. A. (2011). Identification of Brassica napus Lines with Partial Resistance to Sclerotinia sclerotiorum. *Plant Heal. Prog.* Online. doi:10.1094/PHP-2010-0422-01-RS.

Kim, H., Ridenour, J. B., Dunkle, L. D., and Bluhm, B. H. (2011). Regulation of stomatal tropism and infection by light in cercospora zeae-maydis: Evidence for coordinated host/pathogen responses to photoperiod? *PLoS Pathog.* 7. doi:10.1371/journal.ppat.1002113.

Kim, K. S., Min, J.-Y., and Dickman, M. B. (2008). Oxalic Acid Is an Elicitor of Plant Programmed Cell Death during. *Mol. Plant. Microbe. Interact.* 21, 605–612. doi:10.1094/MPMI-21-5-0605.

Kumar, R., Ichihashi, Y., Kimura, S., Chitwood, D. H., Headland, L. R., Peng, J., et al. (2012). A High-Throughput Method for Illumina RNA-Seq Library Preparation. *Front. Plant Sci.* 3, 1–10. doi:10.3389/fpls.2012.00202.

Li, C. X., Li, H., Sivasithamparam, K., Fu, T. D., Li, Y. C., Liu, S. Y., et al. (2006). Expression of field resistance under Western Australian conditions to Sclerotinia sclerotiorum in Chinese and Australian Brassica napus and Brassica juncea germplasm and its relation with stem diameter. *Aust. J. Agric. Res.* 57, 1131–1135. doi:10.1071/AR06066.

Lorenzo, O., Chico, J. M., Sánchez-Serrano, J. J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell* 16, 1938–50. doi:10.1105/tpc.022319.

Lowe, R. G. T., Cassin, A., Grandaubert, J., Clark, B. L., Van De Wouw, A. P., Rouxel, T., et al. (2014). Genomes and transcriptomes of partners in plant-fungal- interactions between canola (Brassica napus) and two Leptosphaeria species. *PLoS One* 9. doi:10.1371/journal.pone.0103098.

Mathelier, A., Fornes, O., Arenillas, D. J., Chen, C., Denay, G., Lee, J., et al. (2015). JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res.* 44, gkv1176. doi:10.1093/nar/gkv1176.

Mcdowell, J. M., An, Y., Huang, S., Mckinney, E. C., and Meagher, R. B. (1996). The Arabidopsis ACT7 Actin Gene Is Expressed in Rapidly Developing Tissues and Responds to Several External Stimuli. *Plant Physiol.* 111, 699–711.

Mclean, D. . (1958). Role of dead flower parts in infection of certain crucifers by Sclerotinia sclerotiorum (Lib.) de Bary. *Plant Dis. Rep.* 42, 663–666.

Meng, X., and Zhang, S. (2013). MAPK cascades in plant disease resistance signaling. *Annu. Rev. Phytopathol.* 51, 245–66. doi:10.1146/annurev-phyto-082712-102314.

Mur, L. a J., Prats, E., Pierre, S., Hall, M. a, and Hebelstrup, K. H. (2013). Integrating nitric oxide into salicylic acid and jasmonic acid/ ethylene plant defense pathways. *Front. Plant Sci.* 4, 215. doi:10.3389/fpls.2013.00215.

Niu, Y., Figueroa, P., and Browse, J. (2011). Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. *J. Exp. Bot.* 62, 2143–2154. doi:10.1093/jxb/erq408.

Pajonk, S., Kwon, C., Clemens, N., Panstruga, R., and Schulze-Lefert, P. (2008). Activity determinants and functional specialization of Arabidopsis PEN1 syntaxin in innate immunity. *J. Biol. Chem.* 283, 26974–26984. doi:10.1074/jbc.M805236200.

Park, C., and Ronald, P. C. (2012). immune receptor. *Nat. Commun.* 3, 1–6. doi:10.1038/ncomms1932.

Pavet, V., Olmos, E., Kiddle, G., Mowla, S., Kumar, S., Antoniw, J., et al. (2005). Ascorbic acid deficiency activates cell death and disease resistance responses in Arabidopsis. *Plant Physiol.* 139, 1291–1303. doi:pp.105.067686 [pii]\r10.1104/pp.105.067686.

de Pinto, M. C., Locato, V., and de Gara, L. (2012). Redox regulation in plant programmed cell death. *Plant, Cell Environ.* 35, 234–244. doi:10.1111/j.1365-3040.2011.02387.x.

Qiu, J.-L., Fiil, B. K., Petersen, K., Nielsen, H. B. B., Botanga, C. J., Thorgrimsen, S., et al. (2008). Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *EMBO J.* 27, 2214–2221. doi:doi:10.1038/emboj.2008.147.

Scheler, C., Durner, J., and Astier, J. (2013). Nitric oxide and reactive oxygen species in plant biotic interactions. *Curr. Opin. Plant Biol.* 16, 534–539. doi:10.1016/j.pbi.2013.06.020.

Schluttenhofer, C., and Yuan, L. (2015). Regulation of Specialized Metabolism by WRKY Transcription Factors. *Plant Physiol.* 167, 295–306. doi:10.1104/pp.114.251769.

Schulze, S., Schleicher, J., Guthke, R., and Linde, J. (2016). How to Predict Molecular Interactions between Species? *Front. Microbiol.* 7, 1–13. doi:10.3389/fmicb.2016.00442.

Schweizer, F., Fernandez-Calvo, P., Zander, M., Diez-Diaz, M., Fonseca, S., Glauser, G., et al. (2013). Arabidopsis basic helix-loop-helix transcription factors MYC2, MYC3, and MYC4 regulate glucosinolate biosynthesis, insect performance, and feeding behavior. *Plant Cell* 25, 3117–32. doi:10.1105/tpc.113.115139.

Shimada, C., Lipka, V., O'Connell, R., Okuno, T., Schulze-Lefert, P., and Takano, Y. (2006). Nonhost resistance in Arabidopsis-Colletotrichum interactions acts at the cell periphery and requires actin filament function. *Mol. Plant. Microbe. Interact.* 19, 270–279. doi:10.1094/MPMI-19-0270.

Sønderby, I. E., Burow, M., Rowe, H. C., Kliebenstein, D. J., and Halkier, B. A. (2010). A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in Arabidopsis. *Plant Physiol.* 153, 348–363. doi:10.1104/pp.109.149286.

Stotz, H. U., and Guimaraes, R. L. (2004). Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant Physiol.* 136, 3703–3711. doi:10.1104/pp.104.049650.into.

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi:10.1126/science.1156970.

Takahashi, F., Yoshida, R., Ichimura, K., Mizoguchi, T., Seo, S., Yonezawa, M., et al. (2007).

The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. *Plant Cell* 19, 805–818. doi:10.1105/tpc.106.046581.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* 7, 562–78. doi:10.1038/nprot.2012.016.

Tripathy, B. C., and Oelmüller, R. (2012). Reactive oxygen species generation and signaling in plants. *Plant Signal. Behav.* 7, 1621–33. doi:10.4161/psb.22455.

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., and Katagiri, F. (2009). Network properties of robust immunity in plants. *PLoS Genet.* 5. doi:10.1371/journal.pgen.1000772.

Tsuda, K., and Somssich, I. E. (2015). Transcriptional networks in plant immunity. *New Phytol.* 206, 932–947. doi:10.1111/nph.13286.

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic Acid, a Multifaceted Hormone to Combat Disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi:10.1146/annurev.phyto.050908.135202.

Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. (2005). Induction of Protein Secretory Pathway Is Required for Systemic Acquired Resistance. *Science (80-.).* 308, 1036–1040. doi:http://www.jstor.org/stable/3842056.

Wang, W., Barnaby, J. Y., Tada, Y., Li, H., Tör, M., Caldelari, D., et al. (2011). Timing of plant immune responses by a central circadian regulator. *Nature* 470, 110–114. doi:10.1038/nature09766.

Wang, X., Jiang, N., Liu, J., Liu, W., and Wang, G.-L. (2014). The role of effectors and host immunity in plant-necrotrophic fungal interactions. *Virulence* 5, 722–32.

doi:10.4161/viru.29798.

Williams, B., Kabbage, M., Kim, H. J., Britt, R., and Dickman, M. B. (2011). Tipping the balance: Sclerotinia sclerotiorum secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLoS Pathog.* 7. doi:10.1371/journal.ppat.1002107.

Wu, J., Zhao, Q., Yang, Q., Liu, H., Li, Q., Yi, X., et al. (2016). Comparative transcriptomic analysis uncovers the complex genetic network for resistance to Sclerotinia sclerotiorum in Brassica napus. *Sci. Rep.* 6, 19007. doi:10.1038/srep19007.

Wu, S., Shan, L., and He, P. (2014). Microbial signature-triggered plant defense responses and early signaling mechanisms. *Plant Sci.* 228, 118–126. doi:10.1016/j.plantsci.2014.03.001.

Yang, B., Srivastava, S., Deyholos, M. K., and Kav, N. N. V. (2007). Transcriptional profiling of canola (Brassica napus L.) responses to the fungal pathogen Sclerotinia sclerotiorum. *Plant Sci.* 173, 156–171. doi:10.1016/j.plantsci.2007.04.012.

Zhang, C., Xie, Q., Anderson, R. G., Ng, G., Seitz, N. C., Peterson, T., et al. (2013). Crosstalk between the Circadian Clock and Innate Immunity in Arabidopsis. *PLoS Pathog.* 9. doi:10.1371/journal.ppat.1003370.

Zhang, J., and Kirkham, M. B. (1996). Antioxidant responses to drought in sunflower and sorghum seedlings. *New Phytol.* 132, 361–373. doi:10.1111/j.1469-8137.1996.tb01856.x.

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., et al. (2012). Disruption of PAMP-induced MAP kinase cascade by a pseudomonas syringae effector activates plant immunity mediated by the NB-LRR protein SUMM2. *Cell Host Microbe* 11, 253–263. doi:10.1016/j.chom.2012.01.015.

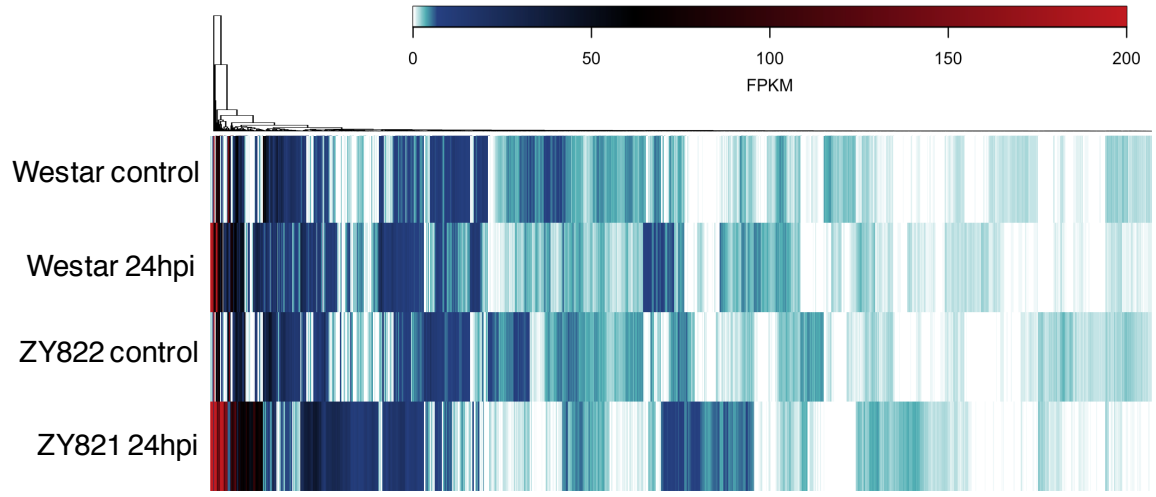
Zhao, J., Buchwaldt, L., Rimmer, S. R., Sharpe, A., McGregor, L., Bekkaoui, D., et al. (2009).

Patterns of differential gene expression in Brassica napus cultivars infected with Sclerotinia sclerotiorum. *Mol. Plant Pathol.* 10, 635–649. doi:10.1111/J.1364-3703.2009.00558.X.

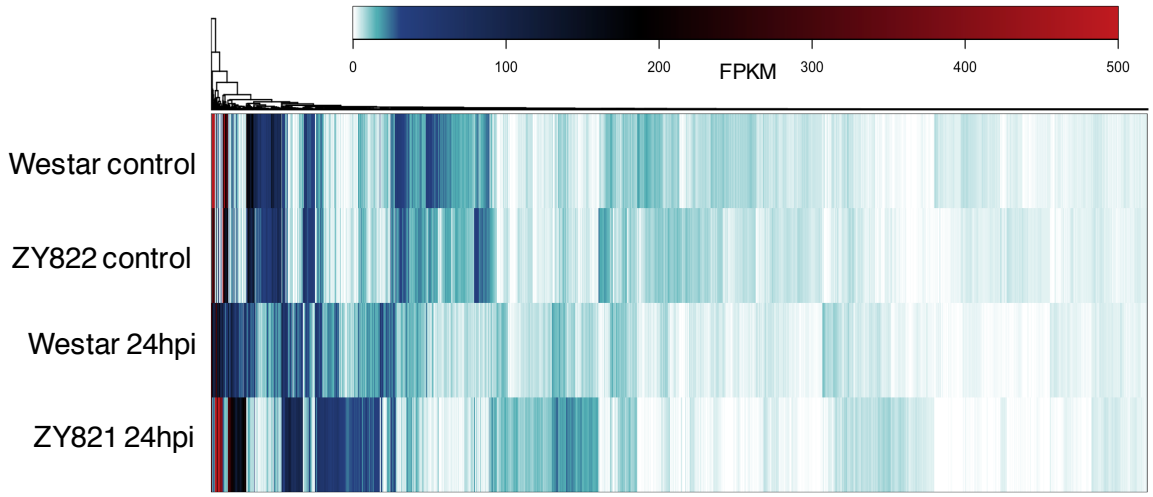
Zipfel, C. (2014). Plant pattern-recognition receptors. *Trends Immunol.* 35, 345–51. doi:10.1016/j.it.2014.05.004.

3.9 SUPPLEMENTAL INFORMATION

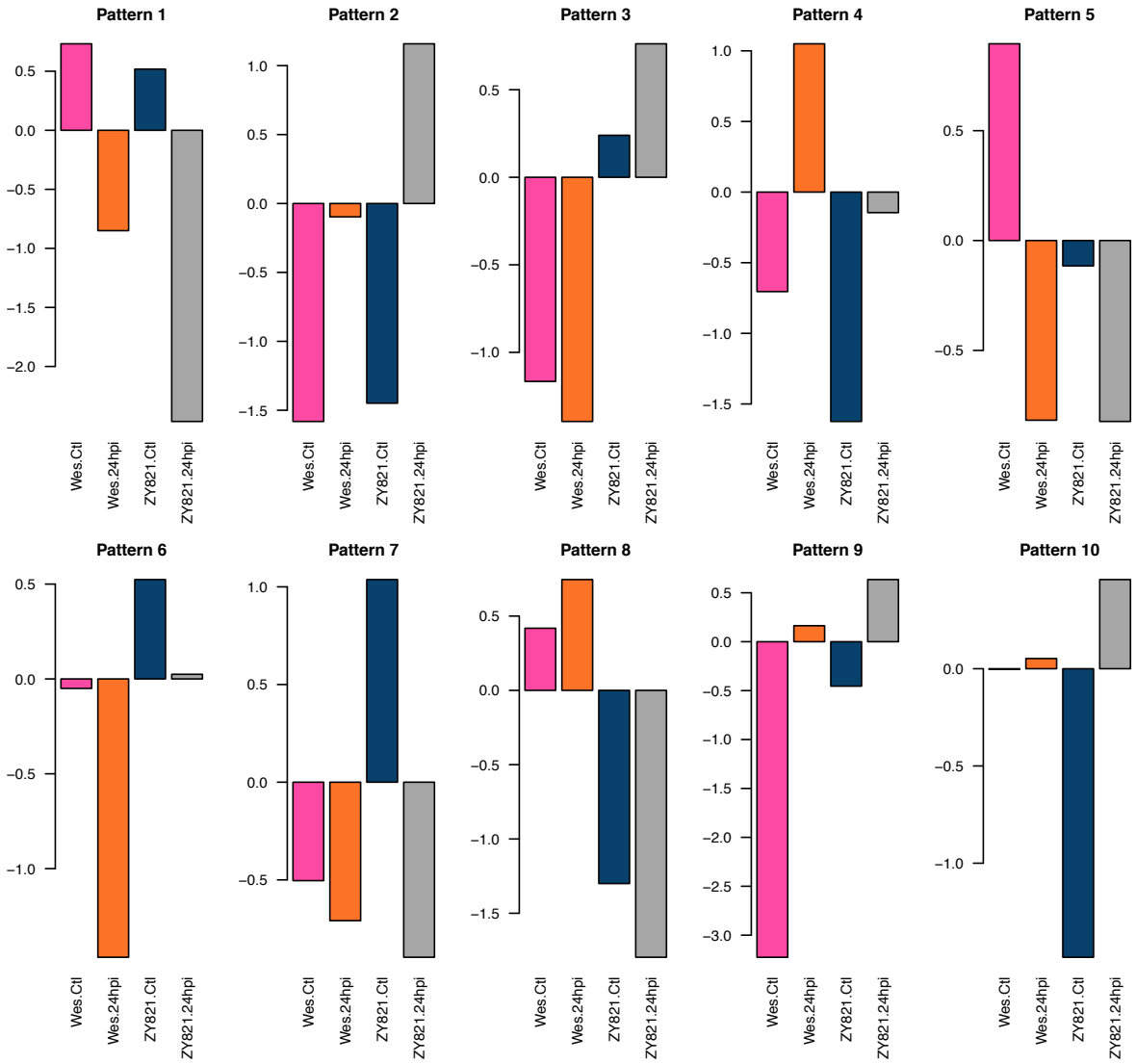
3.9.1 Supplemental Figures



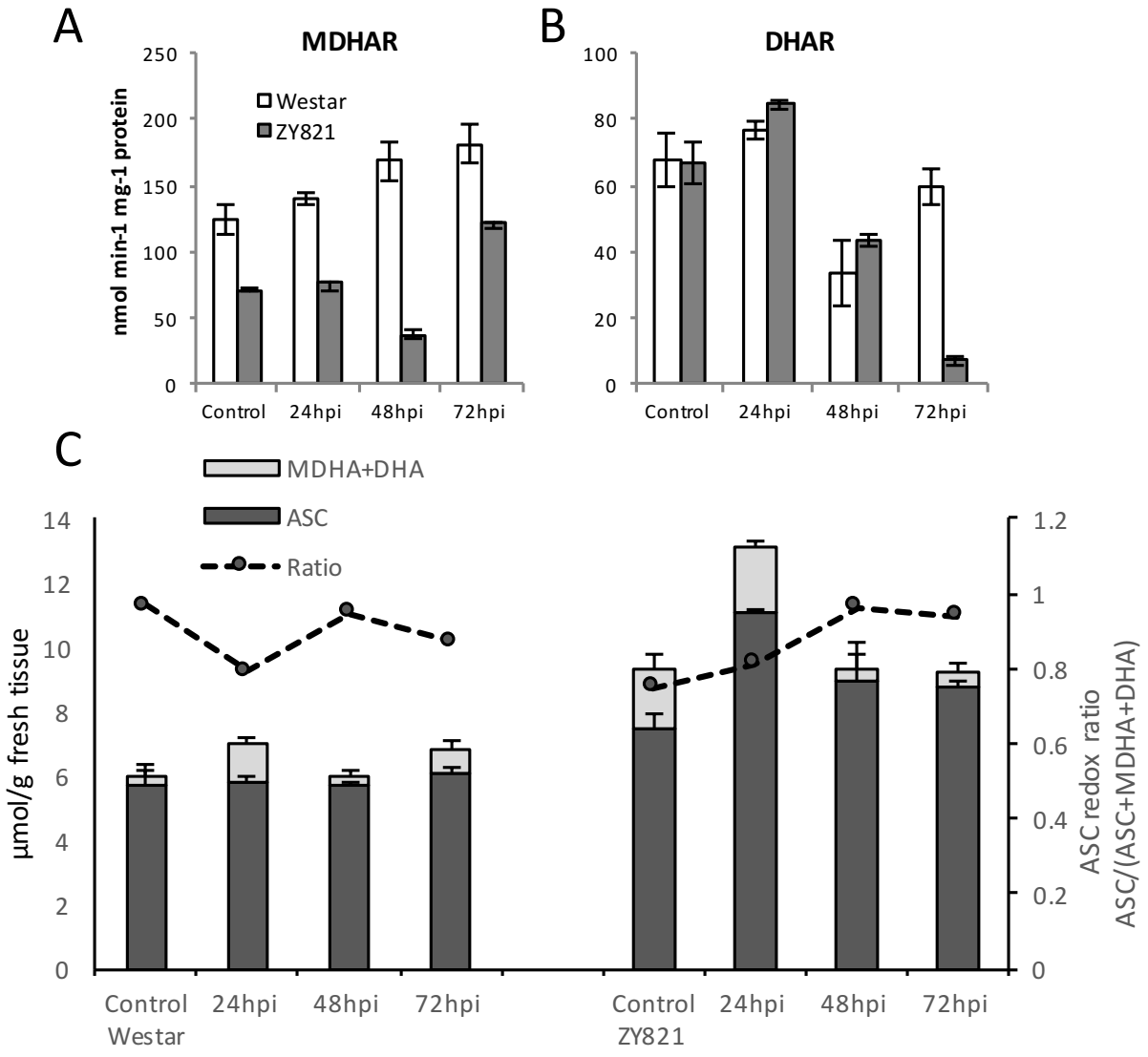
Supplemental Figure 3.1 Heat map of all 1233 novel transcript



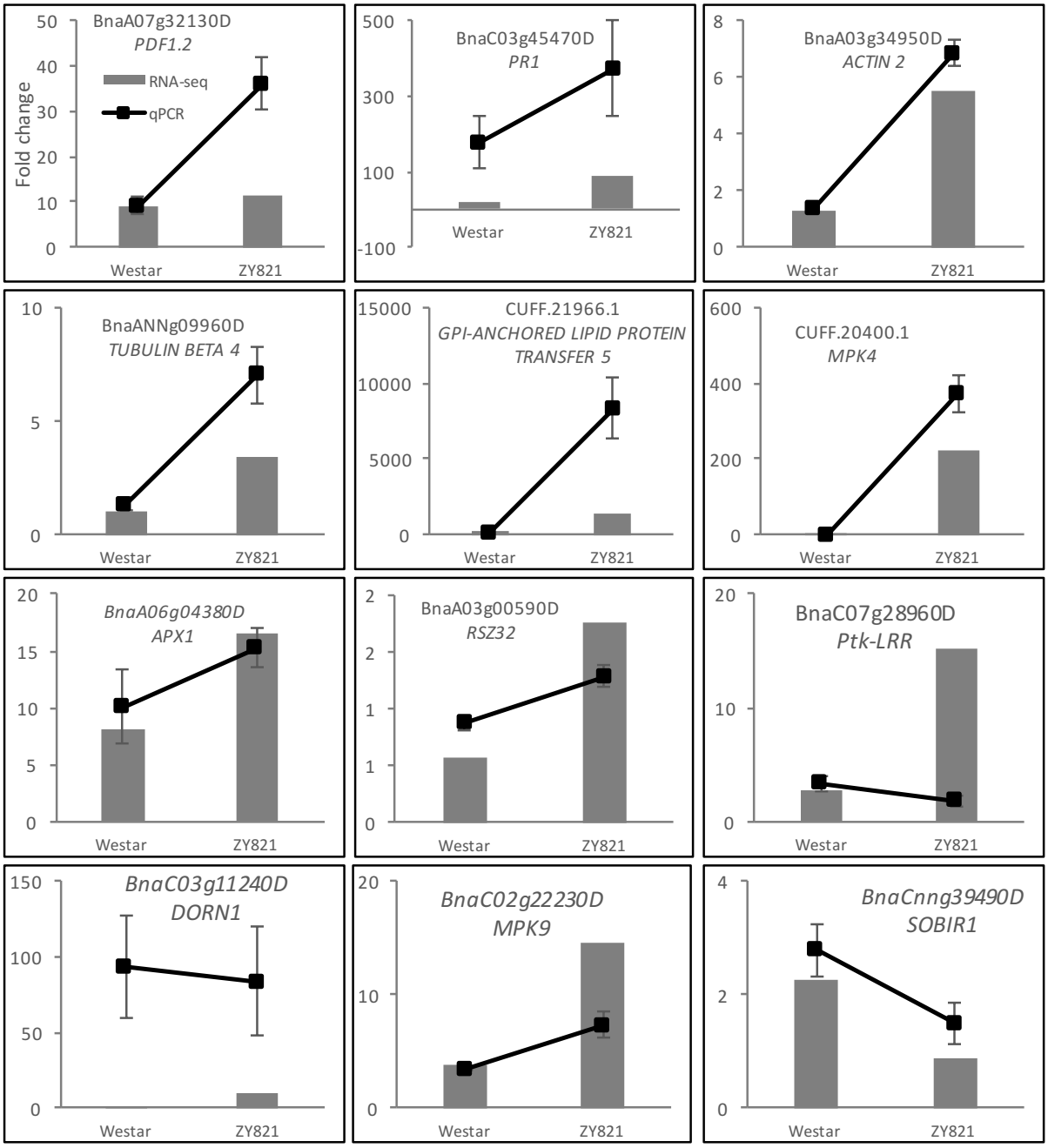
Supplemental Figure 3.2 Heatmap of clustered FPKMs of all homologues of genes determined to be circadian-regulated in Covington et al. (2008).



Supplemental Figure 3.3 Dominant patterns of gene activity revealed by fuzzy k-means clustering. Bar plots represent relative accumulation of mRNAs assigned to each pattern across the four samples.



Supplemental Figure 3.4 Enzyme activity levels of MONODEHYDROASCORBATE REDUCTASE (MDHAR) and DEHYDROASCORBATE REDUCTASE (DHAR) , and levels of reduced (ASC) and oxidized (MDHA+DHA) ascorbic acid.



Supplemental Figure 3.5 Quantitative reverse transcribed PCR of selected transcripts. Gray bars represent fold change calculated by Cuffdiff software (v2.2.1), and

3.9.2 Supplemental Tables

Supplemental Table 3.1 Number and rate of Illumina sequence reads that mapping to the Brassica napus genome v5.0 with TopHat software (v2.0.13).

Cultivar & Treatment	Replicate	Trimmed reads (millions)	Mapped reads (millions)	overall mapping %	% uniquely mapping
Westar Control	1	12.4	9.4	75.5	83.6
	2	13.2	10.4	79	82.8
	3	33.1	26.3	79.3	83.3
Westar 24hpi	1	34.1	21.3	62.5	83.5
	2	35.6	22.6	63.5	84.3
	3	44.0	29.9	68	83
ZY821 Control	1	48.2	38.1	79.1	82.6
	2	2.4	1.9	79.1	83.0
	3	30.4	24.8	81.4	83.9
ZY821 24hpi	1	19.7	12.6	63.7	84.1
	2	30.4	18.4	60.6	84.0
	3	43.0	28.4	66.1	83.9

Supplemental Table 3.2 Top 25 most highly abundant transcripts uncovered in the novel transcript discovery analysis. Closest Arabidopsis match determined using BLAST+ protein alignment to TAIR 10 release.

Brassica napus ID	FPKM				Closest Arabidopsis match	Putative annotation
	Westar Mock	Westar 24hpi	ZY821 Mock	ZY821 24hpi		
CUFF.21966.1	2.13	439.75	2.06	3002.7	AT3G22600	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
CUFF.3931.1	1.08	294.36	1.70	1697.0	AT3G22600	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
CUFF.4914.2	0.65	171.24	1.00	905.75	AT3G60120	beta glucosidase 27
CUFF.26852.1	9.84	242.26	12.87	898.50	AT3G22890	ATP sulfurylase 1
CUFF.21152.2	0.44	81.63	0.16	367.73	AT2G39200	Seven transmembrane MLO family protein
CUFF.37984.1	359.03	73.62	202.18	231.46	AT2G41430	dehydration-induced protein (ERD15)
CUFF.16712.3	348.21	65.92	270.58	22.97	AT2G39730	rubisco activase
CUFF.22197.12	64.66	158.84	66.36	323.13	AT4G05320	polyubiquitin 10
CUFF.8326.4	95.38	228.50	58.22	181.04	AT4G05320	polyubiquitin 10
CUFF.24223.1	10.36	74.74	9.63	223.57	AT3G62830	NAD(P)-binding Rossmann-fold superfamily protein
CUFF.26562.1	1.21	44.40	0.83	220.17	AT1G30700	FAD-binding Berberine family protein
CUFF.28512.1	8.81	68.41	9.98	208.08	AT1G59870	ABC-2 and Plant PDR ABC-type transporter family protein
CUFF.20400.1	0.35	0.03	0.85	193.79	AT4G01370	MAP kinase 4
CUFF.26855.1	3.31	43.77	3.17	184.54	AT3G22890	ATP sulfurylase 1
CUFF.28029.1	168.87	34.16	51.73	5.91	AT5G09810	actin 7
CUFF.23944.1	83.84	14.48	160.19	5.91	AT2G13770	CONTAINS InterPro DOMAIN/s: Putative harbinger transposase-derived nuclease (InterPro:IPR006912)
CUFF.29327.1	0.28	22.44	0.58	159.48	ATMG00710	Polynucleotidyl transferase, ribonuclease H-like superfamily protein
CUFF.37867.1	1.67	43.79	0.84	148.10	AT3G52400	syntaxin of plants 122
CUFF.4883.1	5.80	50.06	5.11	147.95	AT3G62830	NAD(P)-binding Rossmann-fold superfamily protein
CUFF.35598.2	61.91	63.54	68.60	142.21	AT5G03240	Polyubiquitin 3

Supplemental Table 3.3 Summary of gene ontology enrichment analysis

Supplemental Table 4.4 Transcript levels in FPKM of selected genes. Arabidopsis Gene Identifiers were matched to *Brassica napus* genes by using BLAST+ protein alignment to the TAIR 10 release.

***Brassica napus* ID**	**FPKM**				**Arabidopsis Gene Identifier**	**Gene name**
	Westar Control	Westar 24hpi	ZY821 Control	ZY821 24hpi		
BnaCnng08300D	7.7	95.0	3.5	15.6	AT1G74710	*ISOCHORISMATE SYNTHETASE 1*
BnaC06g22820D	18.4	61.3	12.6	7.0		
BnaA07g22090D	14.0	23.6	11.8	3.4		
BnaC06g35540D	3.8	20.4	1.9	3.1		
BnaCnng23220D	3.6	0.9	2.8	0.5		
BnaC04g00590D	13.4	6.8	18.3	9.3	AT2G46830	*CIRCADIAN CLOCK ASSOCIATED 1*
BnaA05g01050D	58.1	25.6	57.2	29.9		
BnaA10g00780D	9.5	7.8	35.4	8.8	AT1G01060	*LATE ELONGATED HYPOCOTYL*
BnaC05g00840D	7.8	8.7	30.8	12.4		
BnaAnng17890D	2.8	6.7	15.1	9.3		
BnaC03g00040D	0.9	0.0	12.0	2.8		
BnaA07g15790D	21.6	23.2	12.7	22.0	AT3G52850	*VACUOLAR SORTING RECEPTOR 1*
BnaC06g18530D	58.9	87.0	30.7	284.4	AT3G62420	*BASIC LEUCINE ZIPPER 53*
BnaC08g32220D	42.4	51.1	47.8	218.8		
BnaA09g39870D	60.6	62.8	56.2	135.4		
BnaA07g19330D	5.7	39.5	1.7	5.1		
BnaA07g06750D	0.7	13.0	0.1	236.6	AT3G23230	*ETHYLENE RESPONSE FACROR 98*
BnaA07g33060D	1.2	84.2	2.2	908.7	AT1G76790	*INDOLE GLUCOSINOLATE METHYLTRANSFERASE 5*
BnaC06g37610D	1.6	180.7	1.8	769.5		
BnaC06g21620D	0.9	145.0	1.1	536.9		
BnaC08g44670D	0.5	73.6	2.6	470.3	AT1G06160	*OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59*
BnaA09g50010D	0.6	78.7	2.3	378.6		
BnaC05g04210D	0.0	4.2	0.1	37.0		
BnaA10g04090D	0.0	3.5	0.1	22.2		

Supplemental Table 3.5 Summary of transcriptional module analysis.

Supplemental Table 3.6 List of genes assigned to dominant expression patterns

Supplemental Table 3.7 Primer sequences used for quantitative reverse transcription PCR

Species	Gene	Gene Identifier	Direction	Sequence (5' to 3')
Brassica napus	ATGP4	BnaC08g11930D	Forward	CGTCTTCCTCTTCCCTCACC
			Reverse	ACAGTTGGAATAGAATAGTAGGCTC
	LTPG5	CUFF.21966.1	Forward	CCCGAGATATGAACTGAAAGACCT
			Reverse	TGTGCACCTATAAAATTCATACGGC
	MPK4	CUFF.20400.1	Forward	AGATGCTAACCAAGAACACAGA
			Reverse	ACCAGAAAGCTAAACCAAAATGCA
	ACT2	BnaA03g34950D	Forward	TCGAATCTTTCTCGTCCAAGCT
			Reverse	CCAGCGAAACCAGCCTTGA
	TUB4	BnaAnng09960D	Forward	GTTTCGCTCCGTTGACATCG
			Reverse	CGCACATCATGTTCTTCGCA
	APX1	BnaA06g04380D	Forward	CTGCTGACGAGGAAGCATTT
			Reverse	CACACAAGGAACGACACAGC
	RSZ32	BnaA03g00590D	Forward	AGAACAGTCCCAAGAAGCTCAA
			Reverse	ACTAGGACGAGGAGGTGACC
	DORN1	BnaC03g11240D	Forward	TGTGCAAGTACCAGAGAGCAG
			Reverse	GCATTTGTTTGACGAGGCGA
	PDF1.2	BnaA07g32130D	Forward	GCTGCTTTTGAAGCACCAAC
			Reverse	GTTGCAAGATCCATGTCGTG
	PR1	BnaC03g45470D	Forward	TCTCGTTGACCCAAAGGTTC
			Reverse	CAGCCTTCGCTCAAAGCTAC
	SOBIR1	BnaCnng39490D	Forward	CTTTCGTCTCCTCCGTTGAG
			Reverse	TCTCTCGCAGGAAACTCCTC
	MPK9	BnaC02g22230D	Forward	AACGGAAAGTGACCGACAAG
			Reverse	TGAGTCCGGAGAGGTTTGAC
Arabidopsis thaliana	EEF	AT1G30230	Forward	CTGGAGGTTTTGAGGCTGGTAT
			Reverse	CCAAGGGTGAAAGCAAGAAGA
	PR1	AT2G14610	Forward	CTCGGAGCTACGCAGAACAA
			Reverse	CGCTACCCCAGGCTAAGTTT
	PDF1.2	AT5G44420	Forward	CATCATGGCTAAGTTTGCTTCCA
			Reverse	ATTGCCGGTGCGTCGAAA
	VTC 2	AT4G26850	Forward	TCCGACCGTTGTTTCGAACT
			Reverse	CTAGCCCCGTTAAGGCAACA
	DORN1	AT5G60300	Forward	CTCACCCGCTGTCAAAATGG
			Reverse	AGGCTGGACTCTCTGACTGC

Dataset S1 Dataset of FPKM transcript levels of all genes tested across biological replicates and differential expression analysis.

CHAPTER FOUR: CONCLUSIONS AND FUTURE DIRECTIONS

In Chapter Three, I developed an RNA sequencing dataset profiling global changes in gene activity and identified putative regulators of biological processes associated with plant defense response to *S. sclerotiorum* in a tolerant genotype of *B. napus*. Although mRNA profiling experiments are an excellent way to monitor changes in gene activity following infection with fungal pathogens, they do invite further functional validation of biological processes predicted with gene ontology analyses. For example, I identified cytostructural changes in response to *S. sclerotiorum* in infected ZY821 at the mRNA level, however actual differences in cytoskeleton functionality can be definitely visualized. One way I would like to validate these results is by using fluorescent tags or antibodies specific to cytoskeleton components and imaging changes in cytostructural rearrangement following cellular contact with *S. sclerotiorum*. Likewise, the identification of histone methyltransferases and GO terms implicated in chromatin structural modifications, invites further functional analysis to test whether differential changes histone methylation states contribute to defense against *S. sclerotiorum*. Methylation sequencing experiments use high-throughput sequencing technologies to identify altered methylation states and would be an effective way to validate changes linked to the plant defense response to *S. sclerotiorum*.

The predicted TF-DNA binding motif interactions in the gene regulatory networks identified in Chapter Three are based off datasets of computationally and functionally identified in other species such as Arabidopsis. Thus I would like to functionally validate these interactions and expand the predictive power of my analysis of transcriptional circuitry. Yeast One hybrid assasy, which test for binding between DNA sequences and proteins are an effective test I would like to use in order to test the putative TF-DNA interactions *in vitro*. Specific transcriptional

regulators of interest can be investigated in chromatin immunoprecipitation sequencing (ChIP-seq) experiments to fully profile all of the genes targeted by TFs. Together, these experiments would be an excellent addition to the transcriptional profiling experiments described in Chapter Three.

Using the model organism Arabidopsis is an effective way to conduct preliminary functional characterization of defense genes identified in *B. napus*, due to the large number of pre-existing resources to study the plant, and their familial relationship as members of the Brassicaceae family. However, discovering how individual regulators affect defense processes in *B. napus* requires testing them in *B. napus* as well. From the candidates tested in Arabidopsis with interesting phenotypes, I would next like to design knockdown and overexpression lines in *B. napus*. The polyploid background and abundance of homologues with overlapping functions of *B. napus* presents a challenge for traditional molecular techniques, however, these can be overcome by using CRISPR/CAS9 editing systems to easily target multiple genes at once. Gain-of-function lines can also be generated in *B. napus* to test whether overexpression of specific regulators confers a more favourable infection outcome in plants challenged with *S. sclerotiorum*.

These additional areas of study will help contribute to a well-rounded understanding of how plant defenses are activated in response to *S. sclerotiorum*, and what biological processes and their regulating molecules are responsive for improving infection outcomes. Together with the findings in this thesis, they represent an important resource for plant scientists and crop breeders to overcome *S. sclerotiorum* disease and breed better crops.