Effect of *Sclerotinia sclerotiorum* on the plant defense response in *Brassica napus* and *Arabidopsis thaliana*

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ABSTRACT

The fungal pathogen *S. sclerotiorum* (*Sclerotinia sclerotiorum*) impacts production and yield in one of Canada's number one crops, canola (*Brassica napus*). Unfortunately, few cultivars show any tolerance to this devastating fungal pathogen. Thus, understanding how the plant responds to this aggressive fungus at the cellular level will facilitate the identification of genes and gene products responsible for improved plant performance. While our understanding of the host pathogen interaction is becoming clearer, there is remarkably little information available for Sclerotinia, especially its pathogenicity in canola. Moreover, we know nothing about how this interaction is specified at the cellular, physiological or molecular level directly at the site of infection in mature leaves following petal inoculation. Thus, we compared differences in plant structure, antioxidant response, and genes involved in the salicylic acid, jasmonic acid and ethylene defense pathways in a susceptible cultivar, Westar, and a previously described tolerant cultivar, Zhongyou821 (ZY821). Our data showed that at the cellular level, ZY821 was able to suppress the Sclerotinia penetration. The ascorbate-glutathione pathway and resistant signaling pathways were all associated with the canola defense response to *S. sclerotiorum*, while stronger antioxidant and signaling pathways responses were observed in ZY821 leaves at the site of infection. Also, transcriptional regulators not previously associated with plant defense in the Arabidopsis- *S. sclerotiorum* pathosystem were identified through bioinformatics approaches. By comparing plant susceptibility to *S. sclerotiorum* between Arabidopsis wild type and seven loss-of-function mutants, I found transcription factor *JAM2* might be involved in plant tolerance to *S. sclerotiorum.*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 Literature Review

1. 1. General introduction to *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary (*S. sclerotiorum*) causes economically important plant diseases worldwide (Rioux et al., 2013). This fungus is capable of infecting at least 450 species of dicotyledonous plants in 75 families, including many important cash crops such as soybean (*Glycine max*), peanut (*Arachis hypogaea*), sunflower (*Helianthus annuus*) and canola (*Brassica napus*) (Garg and Li, 2010). Diseases caused by *S. sclerotiorum* have proven to be difficult to manage either through crop rotation or application of various fungicides and many plants exhibit inherent genetic resistance to the pathogen (Williams et al., 2011).

S. sclerotiorum is a necrotrophic fungal pathogen that requires dead host tissue in order to obtain nourishment (Boland and Hall, 1994). Necrotrophic pathogens infect hosts by secreting toxins (non-host-selective toxins or/and host-selective toxins), plant cell wall-degrading enzymes and proteinases to facilitate host cell death and pathogen entry (Zhu et al., 2013). Like other necrotrophs, *S. sclerotiorum* produces a wide range of lytic enzymes including pectinases, cellulases, hemicellulases and proteases to penetrate host tissue and access host cellular contents for continued fungal growth (Marciano et al., 1983; Riou et al., 1992). The mechanistic details of the complicated interaction between *S. sclerotiorum* and its host plants are still unknown (Amselem et al., 2011). Studies with oxalic acid (OA)-deficient *S. sclerotiorum* mutants have demonstrated that OA is essential for *S. sclerotiorum* pathogenicity and plays a key role in infecting a broad-spectrum of hosts (Cessna et al., 2000; Kim et al., 2008; Riou et al., 1992). As the most

important determinant in the *S. sclerotiorum* pathogenic strategy, OA is remarkably multifunctional and contributes to numerous physiological processes such as: (i) acidifying the environment, (ii) inducing acidity-activated enzymes, (iii) elevating Ca^{2+}, and (iv) regulating plant guard cells to keep stomata open (Williams et al., 2011). Additionally, researchers find OA can act as a fungal elicitor that induces host cell death and is also able to suppress plant resistance by functioning as a signaling molecule (Zhu et al., 2013).

1. 2. Canola stem rot

Brassica napus L. (canola) is an important commercial oilseed rape grown across Canada, Australia, Europe, and China (Hayward et al. 2012). An estimated 60.4 million metric tons of oil rapeseed was produced worldwide in 2010-2011, accounting for 14% of all oilseed production (Hayward et al. 2012). Since its development in Saskatchewan and Manitoba (Casseus, 2009), the growth and export of canola has become extremely important to the Canadian economy. In fact, 14 million tons of canola seeds were harvested in Canada in 2011, and canola farming and export represents a 19.3 billion dollar industry to the Canadian economy (Canola council of Canada, 2011; 2013). However, despite its growing prominence around the world, canola is still susceptible to fungal diseases that can affect crop production, yield, and overall seed quality.

Canola stem rot (also known as white mold) is caused by *S. sclerotiorum* and is responsible for devastating canola yield losses in North America, Europe, India, Australia, New Zealand, China and many other countries (Abdullah and Ali, 2008; Hegedus and Rimmer, 2005; Bolton et al., 2006; Garg et al., 2010; Baharlouei et al., 2011). In

connection with the increase in canola production and wetter climate trends, the disease incidence of canola stem rot has increased in recent years (Canola Council of Canada, 2011), highlighting the necessity of a complete biological understanding of *S. sclerotiorum* -plant interactions.

1. 3. Lifecycle of *S. sclerotiorum* in canola

The life cycle of *S. sclerotiorum* involves a number of steps. Figure 1.1 illustrates the life cycle of *S. sclerotiorum* using canola as a host plant. First, *S. sclerotiorum* overwinters in the form of sclerotia. Sclerotia are hyphal aggregates that form a resting body with hard black walls and can survive in soil for over ten years with little loss of viability (Backhouse and Stewart, 1987; Bolton et al., 2006). In spring or early summer, when the environmental conditions (light, moisture, nutrient availability) become suitable, sclerotia germinate either myceliogenically (producing hyphae directly), or carpogenically (producing apothecia to release ascospores) (Liang et al., 2013). In the canola- *S. sclerotiorum* pathosystem, the carpogenically-produced airborne ascosporeof *S. sclerotiorum* are the primary source of inoculum. The sclerotia require prolonged periods of moist soil to germinate and form the fruiting body apothecia, which release large amounts of ascospore(Yang et al., 2007). While *S. sclerotiorum* ascosporecan germinate in water alone, they are unable to infect plant tissue directly, and thus require external nutrients for the adherence of the hyphae to the plant surface and eventual penetration of host tissues (Lumsden, 1979; Huang et al., 2008). In the field, *S. sclerotiorum* ascosporecan land on the senescing petals of canola, germinate on the leaf surface or lodge between the main stem and side branches (Huang et al., 2008). After the

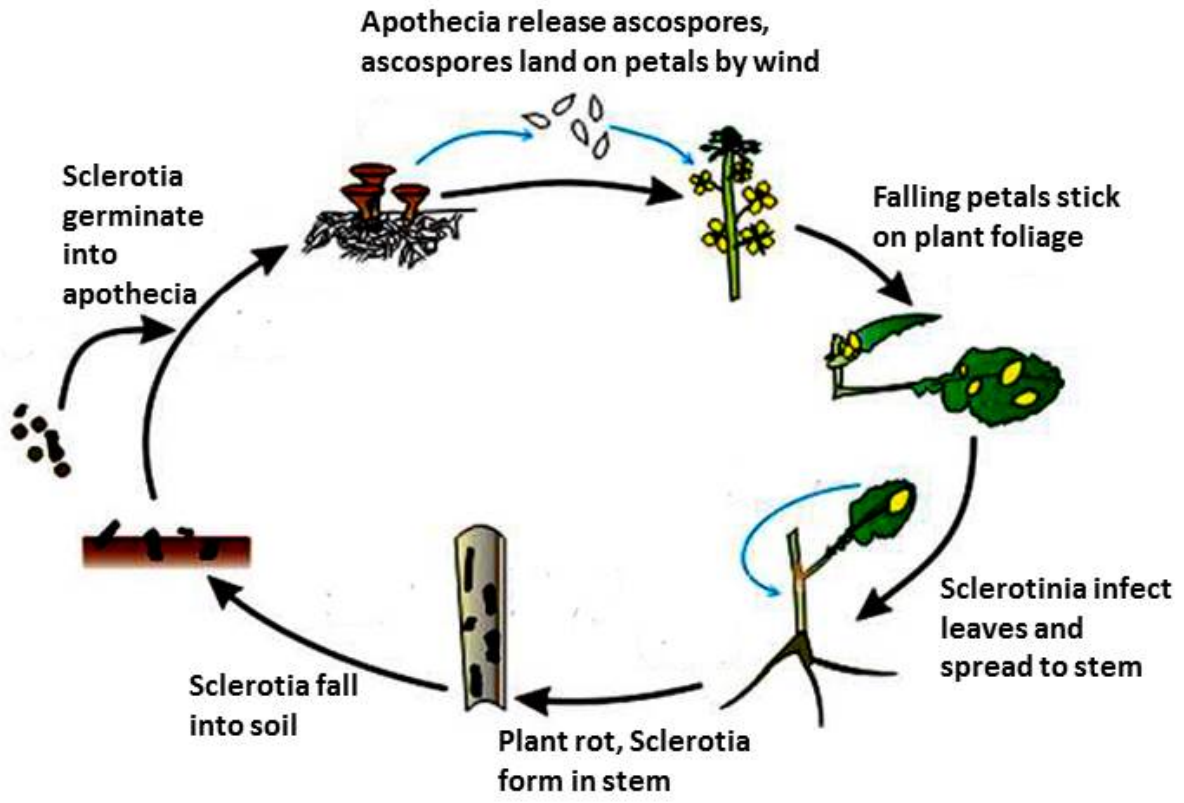

Figure 1.1: Life cycle of *Sclerotinia sclerotiorum* stem rot in *Brassica napus*.

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infected petals land on the surface of plants, the germinated hyphae produce toxins coupled with cell-wall degrading enzymes to directly penetrate the plant tissue and progress rapidly through the internal tissues (Cessna et al., 2000; Yang et al., 2007). Accompanied with wet weather, initial symptoms appear as water-soaked, light-brown lesions on leaves or stems that expand and become greyish-white (Hegedus and Rimmer, 2005). After the lesion completely girdles the main stem, the plant quickly wilts and dies while the *S. sclerotiorum* hyphae aggregate and form Sclerotia inside the stems and sometimes on the surface of the stem which are later released into the ground during harvesting (Iriarte et al., 2010).

The plant-pathogen interaction is called the infection process (Huang et al., 2008). The canola- *S. sclerotiorum* infection process can be divided into three stages (i) pre-penetration, in which the ascosporegerminate and adhere to the plant surface; (ii) penetration, the process by which *S. sclerotiorum* hyphae grow into the host internal tissues; and (iii) colonization, in which the pathogen expands in the host tissues (Lumsden, 1979). Among these steps, penetration is the most critical step, as disease pathogenicity is directly dependent upon success of penetration (Zheng et al., 2011). Studies on other *S. sclerotiorum* host plants, including bean, lettuce, pea and tomato, indicate that senescing petals are a favorable nutrient source for *S. sclerotiorum* ascospore germination and subsequent penetration of host tissues (Jamaux et al., 1995). However, no literature currently exists that describes how senescing petals are essential for the canola – *S. sclerotiorum* pathosystem.

1. 4. Methods of controlling canola stem rot

The most common methods to control canola stem rot include crop rotation, fungicide application, biological control methods and cultivating resistant crop cultivars (Fernando et al., 2007; Tollenaere et al., 2012). Crop rotation is used to reduce *S. sclerotiorum* presence in soil by letting it germinate on non-host crops in the hopes of reducing soil inoculum. However, since sclerotia can survive for more than ten years in the soil and has a wide host range, crop rotation as a means to mitigate infection rates is unreliable (Backhouse and Stewart, 1987; Garg et al., 2010). Application of fungicides, the most common method of *S. sclerotiorum* control, may have negative environmental effects and adverse effects on non-target organisms (Fernando et al., 2007; Zhao et al., 2003). Biological control has been considered as an alternative strategy to prevent *S. sclerotiorum* from infecting valuable crops (Fernando et al., 2007). Although many biocontrol agents display excellent disease control in greenhouses, due to the variable expression of disease suppressive factors, the production of antifungal compounds can be dramatically impacted by environmental conditions, making their performance in the field unstable (Fernando et al., 2007). Overall, the most effective and sustainable control method of controlling canola stem rot is identifying resistance genes in canola and breeding resistant cultivars (Zhao and Meng, 2003).

To date only a few tolerant cultivars to *S. sclerotiorum* have been developed. Zhongyou821 (ZY821) is one of the successful moderately tolerant cultivars (Li et al., 2006). Unfortunately, ZY821 is a crossbreed of a wide range of *B. napus* and *B. campestris* lines in China, and thus exhibits high seed glucosinolate and erucic acid

content, making the seeds less suitable for human consumption (Li et al., 1999). Despite showing tolerance against *S. sclerotiorum*, we still do not understand how this variety slows the infection process. This thesis compares the defense response of ZY821 and Westar (a susceptible cultivar) to *S. sclerotiorum* directly at the site of infection at the cellular, physiological, and molecular levels. Finally, through bioinformatics approaches, I have identified a possible transcriptional regulator of host pathogen interactions using the Arabidopsis – *S. sclerotiorum* system.

1. 5. General introduction to plant resistance

Plants have evolved various complicated and efficient defense mechanisms to recognize and combat pathogens when attacked, such as modifications to tissues and cell structure (Freeman and Beattie, 2008), physiological resistance (De Pinto et al., 2012), and the activation of resistant genes (Yang et al., 2007). Microarray technology has been used to examine the canola response to *S. sclerotiorum*, revealing an overexpression of a large number of transcripts that are related to cell wall structure and function, the plant antioxidative system and genes involved in salicylic acid (SA), jasmonic acid (JA), ethylene (ET) signaling pathways at the whole plant level (Yang et al., 2007). However we still know nothing about how the plant responds to Sclerotinia infection directly at the site of infection on the mature leaf.

1. 5. 1. Plant structural resistance

Anatomical structures that are already present in the plant before pathogen infection, such as the cuticle, closed stomata and thickened cell walls are the first line of

plant defense (Freeman and Beattie, 2008). These features can physically inhibit the advancement of fungal pathogens through plant tissues to some extent (Freeman and Beattie, 2008). The cuticle, the first barrier to infection, is comprised of several distinct layers: (i) an outer layer composed of intracuticular wax or cutin, and (ii) the epicuticular wax, composed of lipids linked by ester bonds covered with a film of soluble lipids (Bashi et al., 2011). The second barrier to infection is the plant cell wall, which not only provides mechanical support, but also protects the plant against pathogen attack (Steinwand and Kieber, 2010). A previous study showed that the thicker plant cell walls could reduce the cellular damage caused by pathogen infection (Hématy et al., 2007).

1. 5. 2. Plant antioxidant systems

Microarray experiments based on a whole plant canola- *S. sclerotiorum* pathosystem revealed that genes associated with the plant antioxidant system are involved in canola response to *S. sclerotiorum* infection (Yang et al., 2007). However, physiological levels of the enzymes responsible for the cycling of these metabolites were not measured. The heart of the plant antioxidant system is the ascorbic acid-glutathione pathway (Fig 1.2) (Foyer and Noctor, 2011). By reducing oxidative damage, the plant antioxidant system is able to ensure accurate activation of signaling pathways to prevent damage to on cellular components like membranes (De Pinto et al., 2012). In addition to the crucial roles of antioxidant systems in influencing plant growth and development by modulating processes from mitosis and cell elongation to senescence and death, plant antioxidant systems are also involved in plant defense (Foyer and Noctor, 2005). During the plant defense response, the antioxidant systems are not only able to

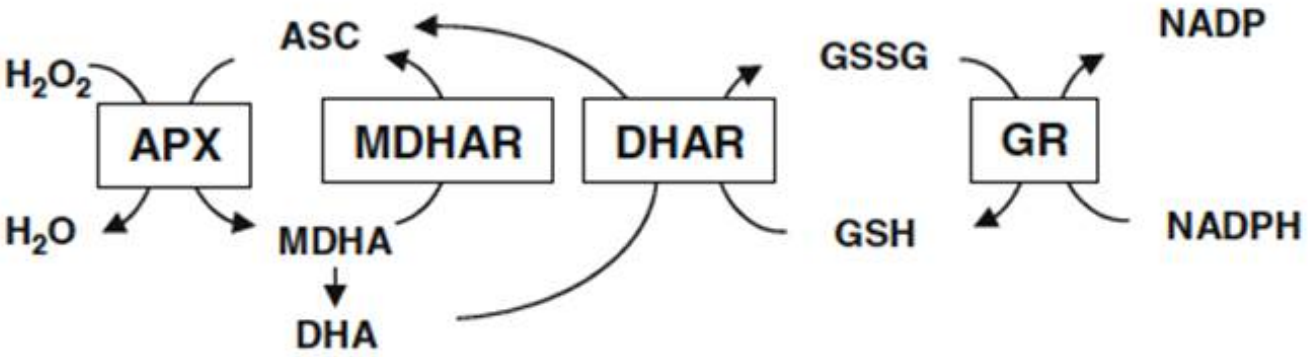

Figure 1.2: Schematic diagram of the ascorbate-glutathione pathway. APX, ascorbate peroxidase; ASC, reduced ascorbate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O, water; H_2O_2, hydrogen peroxide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; NADP, nicotinamide adenine dinucleotide phosphate oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form.

counteract the oxidative stress caused by pathogens, but can also scavenge the deleterious effects caused by plant reactive oxygen species (ROS) (Li et al., 2012). Plant ROS are forms of oxygen that are more reactive than molecular oxygen (O_2), including oxygen ions, peroxides and hydrogen peroxide (De Gara et al., 2003). Although the production of ROS is an important part of triggering resistant pathways and inhibiting the development of biotrophic pathogens, it can also affect many plant cellular functions that can cause plant cell death, which might benefit necrotrophic pathogens (Kuz´niak and Urbanek, 2000; Yang et al, 2010). In plant cells, increased ROS can cause peroxidation of essential membrane lipids, damage carbohydrates, proteins, nucleic acids and pigments within the plant (Lu and Higgins, 1999; Philosoph-Hadas et al, 1994). Therefore, the plant antioxidant system is essential in the plant defense response as an antioxidant protective system to manage the oxidative levels in cells (De Pinto et al., 2012).

One of the most effective mechanisms for scavenging free radicals caused by oxidative stress is the production of antioxidative enzymes and reducing agents like ascorbic acid and glutathione (Li et al., 2012). Enzymes that are involved in the ascorbic acid-glutathione pathway include ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Foyer and Noctor, 2011). Figure 1.2 illustrates the ascorbate-glutathione pathway. In this pathway, hydrogen peroxide (H_2O_2) is initially reduced by APX using ascorbate as the electron donor; the reduced ascorbate (AsC) is oxidized to form monodehydroascorbate (MDHA). MDHA is regenerated into AsC by MDHAR. However, MDHA is unstable and tends to convert to dehydroascorbate (DHA) instead. DHA is reduced into AsC by glutathione-dependent dehydroascorbate reductase (DHAR)

and the resulting oxidized glutathione (GSSG) is reduced by glutathione reductase (GR) using NADPH as an electron donor. These enzymes are crucial to maintain a balanced redox status in plant cells (Li et al., 2012; Foyer and Noctor, 2005). Among all the enzymes that participate in the ascorbate-glutathione system, as the enzyme that directly reduces hydrogen peroxide in water, APX is one of the most powerful antioxidant enzymes in plants (Dabrowska et al., 2007).

Besides antioxidant enzymes, another key component of the plant antioxidant system is the metabolite pool (Foyer and Noctor, 2005), including small soluble molecular antioxidants such as ascorbate and glutathione (Colville and Smirnoff, 2008). As a major antioxidant, ascorbic acid protects plant cells from oxidative stress. It plays a crucial role in many environmental stress responses (drought, salinity, and cold) and it regulates maturation and dormancy during late stages of seed development (Foyer and Noctor, 2005; Yang et al., 2007). Beyond its role in the ascorbate-glutathione pathway, ascorbate is also capable of reducing superoxide, singlet oxygen and hydroxyl radicals directly (Colville and Smirnoff, 2008).

Glutathione is another important cellular antioxidant that has been shown to increase following pathogen attack (Dempsey et al., 2012). In plant cells, glutathione represents the most abundant non-protein thiol. As an antioxidant, glutathione not only can reduce the oxidative damage from ROS, but can also participate in the detoxification of xenobiotics, herbicides, and heavy metals (Zechmann and Müller, 2010), and is involved in stress signaling and defense gene expression (Foyer and Noctor, 2009).

Ascorbate and glutathione act as redox buffers that interact with numerous cellular components and influence gene expression associated with the plant biotic and

abiotic stress response (Foyer and Noctor, 2005). Previous studies have shown that accumulation of cellular ascorbate acts as an environmental sensor and mediator between changes in environmental conditions and plant developmental processes (Barth et al., 2006). Although increased cellular levels of ascorbate is not essential in plant defense, interestingly, the low levels of cytoplasmic ascorbate might be necessary for the initiation of signals eliciting downstream defense responses (Foyer and Noctor, 2005). Conversely, accumulation of glutathione is required for the plant antioxidant system and resistance response (Foyer and Noctor, 2011). It is reported that increases in cellular levels of glutathione help to mitigate oxidative stress in tobacco (Luis et al, 2006) and to inhibit the development of the fungal pathogen *Colletotrichum gloeosporioides* in Arabidopsis (Hiruma et al., 2013).

In healthy leaves, approximately 90% of the ascorbate pool is present in the reduced form. However, under environmental stresses, the ascorbate pool can become more oxidized as the electron donor (Foyer and Noctor, 2011; Gillespie and Ainsworth, 2007). As an indicator of the general cellular redox balance, the reduced glutathione (GSH)-oxidized glutathione (GSSG) couple acts as an important redox sensor (Pavet et al., 2005). Like ascorbate, glutathione maintains a low oxidized concentration under optimal conditions in order to confer high sensitivity in signal transduction (Foyer and Noctor, 2011). Most data suggest that the oxidized ascorbate ratio is less impacted in the antioxidant system than the redox status of glutathione; the ascorbate pools could remain highly reduced if there are sufficient amounts of glutathione present to scavenge ROS and free radicals (Foyer and Noctor, 2011). Therefore the ascorbate-glutathione metabolite pool would be able to sense the ROS burst produced by the plant in response

to pathogenic invasion, potentially triggering the expression of PR genes and enhancing plant resistance (Foyer and Noctor, 2005). Canola has been shown to produce an ROS burst in response to *S. sclerotiorum* infection (Dong et al, 2008; Lamb and Dixon, 1997; Yang et al., 2007). However, we still do not know how levels of ascorbate and glutathione and specifically the redox ratios of these metabolites react to pathogen attack and certainly know nothing about how Westar and ZY821 respond at the physiological level to infection.

1. 5. 3. Qualitative (gene-for-gene) and quantitative genetic resistance

While plant structural resistance and antioxidant systems are important components of the plant defense response and plant resistance, the majority of plant defense processes take place at the molecular level. Researchers have divided plant resistance into two categories: i) qualitative and ii) quantitative resistance (Ansan-Melayah et al., 1998). Qualitative resistance is also referred to as gene-for-gene resistance, which is considered to be race-specific while quantitative resistance is race-nonspecific and is mediated by multiple genes that may only confer partial resistance to the pathogen (Ansan-Melayah et al., 1998; Tollenaere et al., 2012). In qualitative resistance, the product of a resistance gene in the host is specifically triggered by the corresponding *AVIRULENCE* (*Avr*) gene product of the pathogen, thus initiating a plant resistance response (van der Biezen and Jones, 1998). This model was central to the breeding of resistant cultivars in most modern crops (Crute and Pink, 1996). However, this model is based on pathogens *Avr* genes. Once this single gene has been changed in pathogens, the plant cannot respond to pathogen attack and thus becomes susceptible. It

is thought that certain pathogens can breakdown plant qualitative resistance easily (Brun et al., 2010).

Moreover, not all pathogens have *Avr* genes (Wang et al., 2009). In this case, the plant requires a number of genes to act in synergy to inhibit pathogen attack and development (Tollenaere et al., 2012). However, although quantitative resistance only confer a partial resistance to pathogens, it is harder for pathogens to breakdown. That is because a gigantic network manipulates quantitative resistance to which it is thus hard for the pathogens to adapt (Boyd, 2006). These multiple genetic factors are called quantitative trait loci (QTL) (Brun et al., 2010). Since most QTLs are complex traits, it is difficult to study the genetics and functions of QTLs (Wang et al., 2009). Some scientists have considered identifying QTLs as the greatest challenge for genetics in this century (Luo et al., 2002).

In the canola- *S. sclerotiorum* pathosystem, no *R* or *Avr* genes have been identified; instead, a number of QTL have been considered as providing partial tolerance to *S. sclerotiorum* stem rot in *B. napus* (Yin et al., 2010; Zhao and Meng, 2003; Zhao et al., 2006). Therefore it is believed that canola resistance against *S. sclerotiorum* stem rot should be primarily via quantitative resistance. However, very little information about the genes involved in canola response to *S. sclerotiorum* infection has been reported since the identification of such genes is a difficult process in plants with a complex genome like *B. napus* (Tollenaere et al., 2012). Moreover, studies have identified many regions in the *B. napus* genomes involved in quantitative resistance yet the number of QTLs reported varies considerably (Zhao and Meng, 2003; Zhao et al., 2006; Yin et al., 2010; Raman et al., 2012).

Unfortunately, there is remarkably little information about canola's response to *S. sclerotiorum* infection. The interaction between canola and *S. sclerotiorum* at the cellular, physiological and molecular levels remain unclear, especially at the site of infection in the leaf. However, microarray studies of whole plant canola and *S. sclerotiorum* interaction indicated that the signaling pathways such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) might be involved in the canola defense response to *S. sclerotiorum* (Yang et al., 2007). Thus, to identify whether those signaling pathways also contributed to canola tolerance against *S. sclerotiorum* infection, the expression of the marked genes in SA, JA and ET pathways were tested and compared between mature leaves of Westar and ZY821 at the early stages of the infection.

1. 5. 4. Signaling pathways involved in the plant defense response

The activation of pathogen-induced plant defense is mediated by different cross-communicating signaling pathways, including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and mitogen-activated protein kinase (MAPK) signaling pathways (Bari and Jones, 2009). Among all the signaling pathways mentioned, SA is most often associated with plant resistance to biotrophic pathogens (Perchepied et al, 2010). JA and ET pathways tend to be involved in the plant resistance to herbivory and necrotrophic pathogen attack (Nakagami et al., 2005). While the MAPK pathway is co-expressed with the SA, JA and ET pathways (van Verk et al., 2011). Figure 1.3 illustrates the co-expression of SA, ET/JA and MAPK pathways. However, each of these pathways acts independent of each other or together to mount a stronger defense response (Bari and Jones, 2009; Perchepied et al., 2010). Therefore, it is possible that effective canola

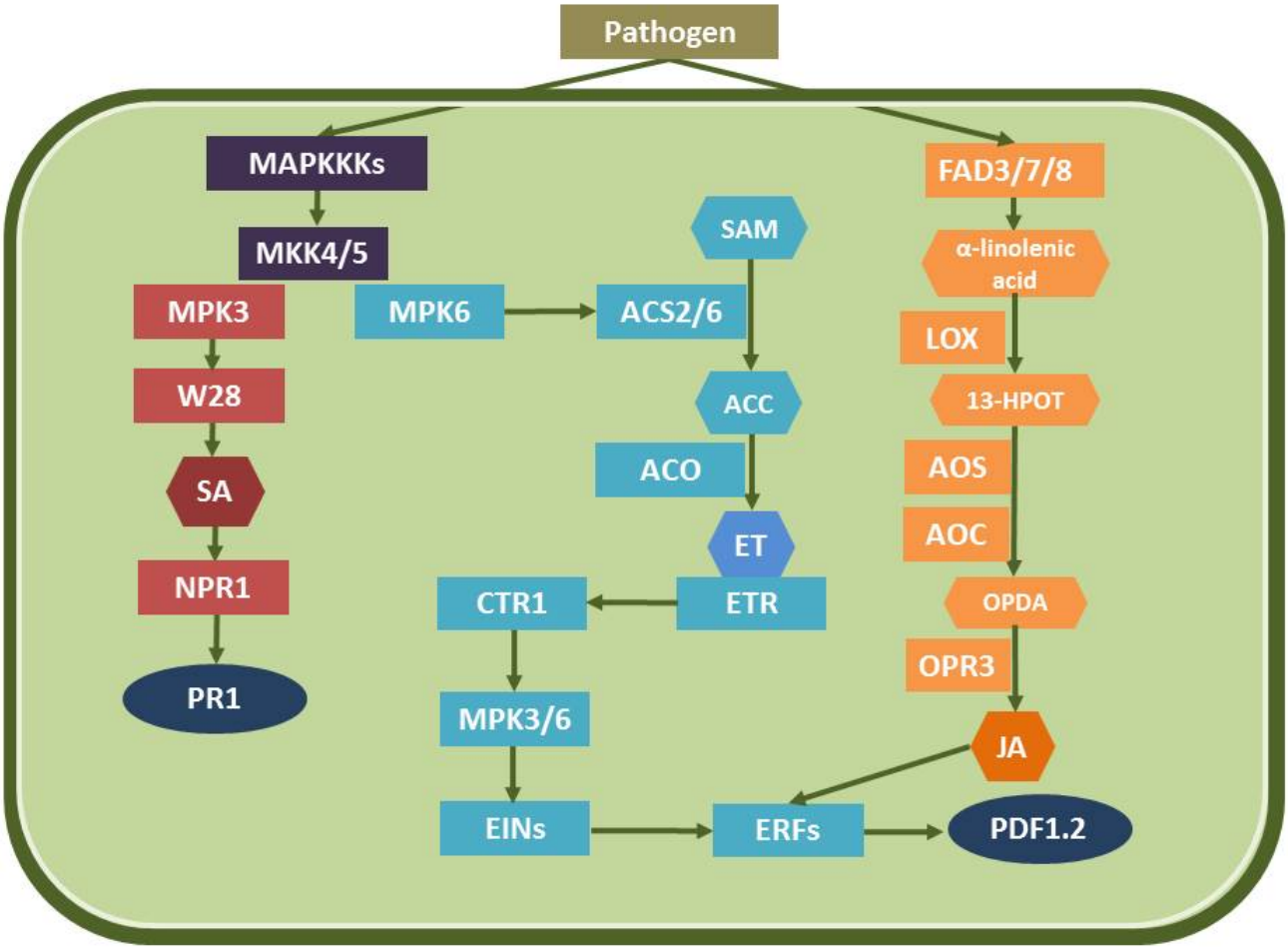
Pathogen
MAPKKKs
MKK4/5
MPK3
W28
SA
NPR1
PR1
MPK6
ACS2/6
SAM
ACC
ACO
ET
ETR
CTR1
MPK3/6
EINs
ERFs
PDF1.2
FAD3/7/8
α-linolenic acid
LOX
13-HPOT
AOS
AOC
OPDA
OPR3
JA

Figure 1.3 Overview of plant salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) signaling pathways under pathogen attack. Purple boxes: *MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE* (*MAPKKKs*) genes that lead to the induction of SA and ET signaling pathways: *MKK4/5*: *MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4/5.* Red boxes: genes within SA pathway: *MPK3*: *MITOGEN-ACTIVATED PROTEIN KINASE 3; W28: WRKY DNA-BINDING PROTEIN 28; NPR1: NONEXPRESSER OF PR GENES 1.* Dark blue ellipses: plant antifungal genes: *PR1: PATHOGENESIS-RELATED GENE 1*. Light Blue boxes: genes within ET pathway: *MPK6: MITOGEN-ACTIVATED PROTEIN KINASE 6; ACS2/6: 1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2/6; ACO: 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE; ETR: ETHYLENE RESPONSE; MPK3/6: MITOGEN-ACTIVATED PROTEIN KINASE 3/6; CTR1: CONSTITUTIVE TRIPLE RESPONSE 1; EINs: ETHYLENE INSENSITIVE; ERFs: ETHYLENE RESPONSE FACTORs*. Light blue hexagon: chemical compounds within ET signaling pathway: SAM: S-adenosylmethionine; ACC: 1-aminocyclopropane-1-carboxylate. Orange boxes: genes within JA pathway: *FAD3/7/8: FATTY ACID DESATURASE 3/7/8; LOX: LIPOXYGENASE; AOS: ALLENE OXIDE SYNTHASE; AOC: ALLENE OXIDE CYCLASE; OPR3: OXOPHYTODIENOATE-REDUCTASE 3.* Orange hexagon: chemical compounds within JA signaling pathway: α-linolenic acid: alpha Linolenic Acid; 13-HPOT: (9Z, 11E, 15Z)-(13S)-hydroperoxyoctadeca-9, 11, 15-trienoate; OPDA: o- (2'-Pyridyldithio) benzyldiazoacetate. Dark blue ellipses: plant antifungal genes: *PDF1.2: PLANT DEFENSIN 1.2.*

tolerance to *S. sclerotiorum* pathogenic attack involves the co-ordination of gene activity of multiple signaling pathways. A study in Arabidopsis has shown that the defense to *S. sclerotiorum* is dependent of the JA/ET, and SA signaling pathways (Guo and Stotz, 2007). Similarly, an ABA-deficient tomato mutant that displays an SA-dependent defense response is more tolerant to *S. sclerotiorum* infection (Asselbergh et al., 2008). However, there is no information about canola defense mechanisms against *S. sclerotiorum*. Thus understanding whether those signaling pathways also involved in canola tolerance to *S. sclerotiorum* would be benefitted for exploring potential defense genes.

1. 5. 4. 1. SA signaling pathway

Salicylic acid (SA) is an important plant hormone that regulates various aspects of plant growth and development, including regulation of photosynthesis and cell expansion (Kästner et al., 2014). In plant defense, SA is synthesized by plants in response to a diverse range of pathogens and is essential to establishment of the defence response (Loake and Grand, 2007). Application of SA is able to trigger the accumulation of pathogenesis-related (PR) proteins though the SA signaling pathway, and thus induce plant resistance (Yang et al., 2010). PR proteins can be classified into 17 families, some of which exhibit antimicrobial properties, function as oxidase enzymes, or act as signals that spread to nearby cells (Sels et al., 2008). Among all those PR proteins, PR1, which is strongly conserved and appears to be represented in every plant species investigated to date, is one of the most important fungicidal defense proteins (Sels et al., 2008; van Loon et al., 2006).

WRKY transcription factors (TFs) are important regulators of defense gene

expression and are strongly regulated by SA (Eulgem & Somssich, 2007). It has been demonstrated that the network of *WRKY* TFs both positively (Asai et al., 2002) and negatively (Zheng et al., 2007) regulates defense gene expression as well as transcription of other *WRKY* genes. Also, it is reported that a large amount of *WRKY* TFs are triggered by necrotrophic fungal pathogens in Arabidopsis (Zheng et al., 2006). In *Brassica napus*, *BnWRKY25*, *28, 33, 53* and *75* might potentially activate downstream JA/ET signaling pathways or cell death (Yang et al., 2009). Among those mentioned, *WRKY28* is rapidly induced by SA treatment and is co-regulated with other important genes in SA biosynthesis (van Verk et al., 2011).

1. 5. 4. 2. JA and ET signaling pathway

In the JA and ET signaling pathways, JA and ET are the signaling hormones that initiate and maintain the defense response to broad-spectrum pathogens in a wide variety of plants (Bari and Jones, 2009; Yang et al., 2010). Corporately, they act in synergy to induce the accumulation of PR proteins and plant defensin 1.2 (PDF1.2), an antifungal small, basic peptide (Thomma et al., 2002; van Loon et al., 2006).

Jasmonic acid is an important regulator that rapidly accumulates in response to stresses such as ozone exposure, wounding, water deficit, and pathogen and pest attack (Devoto and Turner, 2003; Xu et al., 2005). Under pathogen attack, the transcription factors can reprogram JA biosynthesis and lead to increased JA levels in plant cells (Sasaki-Sekimoto et al., 2013). Large numbers of genes are involved in JA biosynthesis (Sasaki et al., 2001). Among those genes, *LIPOXYGENASE* (*LOX2*), *ALLENE OXIDE SYNTHASE* (*AOS*), *ALLENE OXIDE CYCLASE* (*AOC*) and *12-OXOPHYTODIENOATE*

REDUCTASE (*OPR3*) directly participate in JA biosynthesis while *AOC* and *AOS* are key enzymes in the biosynthesis of JA (Browse, 2009; Park et al., 2012; Schaller et al, 2008). In the JA pathway, transcription factors like *JASMONATE INSENSITIVE 1* (*JIN1*/*MYC2*) play an important role in the transcriptional regulation of Arabidopsis defense against pathogen and insect attack (Bari and Jones, 2009). Activation of *MYC2* is able to enhance plant resistance in Arabidopsis against bacterial pathogen (Pozo et al., 2008). The sequences of transcription factors in *JASMONATE ASSOCIATED MYC2 LIKE* family (*JAM*s) are similar to *MYC2*, for example, *JAM1* shares 30% amino acid identities with *JIN1* while *JAM2* is 47% identical to *JAM1* at the amino acid level (Sasaki-Sekimoto et al., 2013). However, as JA-responsive genes (Sasaki-Sekimoto et al., 2013), the homologs in *JAM*s have not been well investigated, and there is no information about *JAM2* participating in plant defense in canola.

Ethylene, another important plant hormone, is associated with many plant developmental processes such as seed germination, fruit ripening, abscission and senescence (Pirrello et al., 2006). The last step of the ethylene-signaling pathway involves genes in the *ERF* (*ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR*) family (Hao et al., 1998). The *ERF* transcription factors contain a highly conserved DNA binding domain that binds to the AP2/GCC box motif; these are involved in various developmental and physiological processes in plants (Hao et al., 1998; Yang et al., 2007). Previous reports have shown that several *ERF*s are directly involved in the regulation of Arabidopsis defense response against necrotrophic fungal pathogens (Berrocal-Loba and Molina, 2007). Among all the *ERF*s, *AtERF1*, *AtERF2*, *AtERF5* act as transcriptional activators, whereas *AtERF3* and *AtERF4* are transcriptional repressors (Fujimoto, 2000).

The previous studies proved that the *AtERF1*, *AtERF2* gain-of-function mutant can overexpress *PDF1.2* and *PR*, and thus enhance plant resistance against multiple necrotrophic fungi such as *Fusarium oxysporum* (Berrocal-Loba and Molina, 2008), *Botrytis cinerea* and *Plectosphaerella cucumerina* (Berrocal-Loba and Molina, 2004). By contrast, *AtERF4* does not respond to ET/ JA, and negatively regulates the JA-responsive expression of *PDF1.2* (McGrath et al., 2005). Interestingly, *ERF*s are not only induced by JA and ET (Zhang et al, 2004), but are also triggered by abscisic acid (ABA), NaCl (Zhang et al, 2004), SA (Gu et al, 2000), wounding (Tournier et al., 2003), and pathogen attack (Onate-Sanchez and Singh, 2002; Pirrello et al., 2006). This illustrates the high degree of crosstalk between signalling pathways and defense responses.

1. 5. 4. 3. MAPK signaling pathway

The MAPK signaling pathway shows a high degree of co-expression with large numbers of genes involved in the SA, JA and ET pathways in response to external stimuli such as biotic and abiotic stress (Nakagami et al., 2005; van Verk et al., 2011). This pathway consists of three types of protein kinases, MAPK, MAPK kinase (MKK), and MAPKK kinase (MKKK) (Chen et al., 2001). Previous sequencing results revealed that 20 MAPK, 10 MKK and more than 60 MKKK genes are expressed in *Arabidopsis thaliana* (Ichimura et al., 2002). However, only a few genes involved in the MAPK pathway, such as *MPK3*, *MPK4*, and *MPK6*, have been well studied so far (Nakagami et al., 2005). For example, *MPK4* is a negative regulator of the SA signalling pathway and a positive regulator of *PDF1.2* expression (Brodersen et al., 2006) while *MPK3* and *MPK6* are required for plant abiotic stress response (Miles et al., 2005). In Arabidopsis MAPK

pathway, four *MKKs*: *MKK2*, *MKK4*, *MKK5*, and *MKK3* are able to activate *MPK6*. These *MKKs*-*MPK6* pathways respond to different elicitors and exhibit crosstalk with each other (Takahashi et al., 2007). The *MKK3*-*MPK6* pathway may be involved mainly in JA signaling. By contrast, the *MKK4*/*MKK5*–*MPK6* and *MKK2*–*MPK6* pathways are involved in ET signaling (Liu and Zhang, 2004) and cold/salt stress signaling (Teige et al., 2004), respectively. Therefore, testing the expression of genes involved in signalling pathways associated with the defense response can help us to further identify whether JA/ET pathways are specifically involved in the canola mature leaf response to *S. sclerotiorum*.

In contrast to numerous biotrophic pathogens, there are no products of major plant resistance (*R*) genes that have been identified to specifically recognize necrotrophic pathogens (Birkenbihl et al., 2012). Moreover, there is little information about the complex molecular control involved in plant response to necrotrophic pathogens, especially to *S. sclerotiorum*. Therefore, it is important to study the interaction between canola and *S. sclerotiorum* at the cellular, physiological and molecular level directly at the site of infection in mature leaves. To date, no *S. sclerotiorum* resistant canola cultivar to has been developed, while Zhongyou821 (ZY821) is one of the successful moderately tolerant cultivars (Li et al., 2004). By comparing the differences in plant defense response mechanisms between Westar (the susceptible cultivar) and ZY821, we can identify the possible molecular players that participate in canola tolerance to *S. sclerotiorum* infection.

1. 6. Identification of novel regulators in Arabidopsis defense response to *S. sclerotiorum* infection

While it is important to identify possible transcriptional regulatory mechanisms underlying the defense response to *S. sclerotiorum* infection in canola**,** we still have to functionally characterize those predicted regulatory mechanisms in the laboratory. One of the best ways to functionally characterize possible candidates is through mutant analysis (Melo-Oliveira et al., 1996). Comparing disease incidence in wild type and mutants lacking specific transcription factor activity, we can identify whether the target genes are indeed involved in the plant-defense response (Mészáros et al., 2008).

Because of its short generation time and completely sequenced genome, Arabidopsis is the longest standing model plant for genetic analysis (Meinke et al., 1998). Since Arabidopsis also belongs to the *Brassicaceae* family and is a close relative to canola *(Brassica napus)* (Guo and Stotz, 2007; Meinke et al., 1998)*,* it has been used successfully to study molecular interactions with *S. sclerotiorum* in a number of studies (Guo and Stotz, 2007; Stotz et al., 2011; Subramanian et al., 2011; Zhou et al., 2013). More importantly, there are a large number of publicly available loss-of-function mutant resources (http://www.arabidopsis.org/), which saves the researchers a large amount of time and money from creating mutations themselves. Thus, in Chapter 3, loss-of-function mutations in genes encoding transcription factors that were identified through gene regulatory network analysis were challenged with *S. sclerotiorum.* This enabled us to observe whether those regulators were important in the plant defense response against *S. sclerotiorum*. Transcription factors (TFs) were targeted first because they control gene expression by binding to the promoter region of DNA adjacent to the genes that they are

regulated (Narlikar et al., 2002). Further, TFs govern the way genes are transcribed and result in the incredible number of plant genetic responses to pathogen attack (Narlikar et al., 2002; Phillips and Hoopes, 2008; Yang et al., 2007).

1. 7. Objectives

The aim of this thesis is (i) to gain insight into the canola- *S. sclerotiorum* interaction at the site of infection on mature leaves, (ii) to compare the plant defense response to *S. sclerotiorum* between a susceptible cultivar, Westar, and a moderately tolerant ZY821 variety at the cellular, physiological and molecular level, and (iii) to identify novel transcriptional regulators involved in the defense response to *S. sclerotiorum* by performing in depth bioinformatics analyses on publically available data in Arabidopsis.

To satisfy these objectives I will attempt to answer the following questions:

1). Are senescing petals essential in the canola- *S. sclerotiorum* pathosystem? (Chapter 2)

2). Are there differences in mature leaf anatomy between Westar and ZY821 that contribute to differences in the plant response to *S. sclerotiorum*? (Chapter 2)

3). What are the differences in the ascorbic acid-glutathione pathway in Westar and ZY821 mature leaves in response to *S. sclerotiorum* infection? (Chapter 2)

4). What are the differences in SA, ET, JA and MAPK signaling pathways at the mRNA level in Westar and ZY821 mature leaves in response to *S. sclerotiorum* infection? (Chapter 2)

5). What regulators that have not been associated in plant defense response can be identified in Arabidopsis - *S. sclerotiorum* pathosystem using bioinformatic analyses? (Chapter 3)

To examine how mature canola leaves respond to *S. sclerotiorum* infection at the cellular level, I used light microscopy, scanning electron microscopy and transmission electron microscopy to compare the *S. sclerotiorum* infection processes at the cellular level in Westar and ZY821. Differences in cellular architecture in mature leaves are described in Chapter 2. In summary, I found ZY821 could suppress *S. sclerotiorum* penetration, which may contribute to its tolerance against this pathogen.

To test the physiological response in canola leaves to pathogenic attack, I studied how the ascorbic acid-glutathione pathway responds to *S. sclerotiorum* infection. The changes of the amount of ascorbate, reduced ascorbate redox ratio, glutathione, reduced glutathione redox ratio, and the APX, MDHAR, DHAR, GR enzyme activities in Westar and ZY821 before and during the infection are reported in Chapter 2. Specifically, I found that the plant antioxidant system was rapidly induced in both cultivars, but especially in ZY821, during the infection process.

To better understand the role of signaling pathways in plant defense in the canola-*S. sclerotiorum* pathosystem, the expression of the SA, ET and JA marker genes before and during the *S. sclerotiorum* infection in Westar and ZY821 are compared in Chapter 2. The results show that genes involved in the JA/ET and SA signaling pathways are rapidly activated in ZY821 within hours of the initial infection and provide insight into the regulatory mechanisms governing plant pathogen interactions.

To identify novel transcriptional regulators in the Arabidopsis – *S. sclerotiorum* pathosystem, mRNA profiling data was processed to identify transcriptional circuits and regulators of those circuits that may play a role in the plant defense response. Since Arabidopsis is a close relative of canola, we can use this model system to identify Arabidopsis mutants defective in selected transcription factors that respond to *S. sclerotiorum* infection. Overall, I found that the mutant defective in *JASMONATE ASSOCIATED MYC2 LIKE 2* (*JAM2*), involved in JA signaling, was more susceptible to *S. sclerotiorum* than the wild type and other six mutants (*myb hypocotyl elongation-related* (*mybh*)*, ocs-element binding factor 5* (*obf5*)*, udp-glucosyl transferase 85a3* (*ugt85a3*)*, unfertilized embryo sac 10* (*une10*)*, at2g22760* and *at2g43140*). This result indicates that *JAM2* is likely involved in the Arabidopsis defense response to *S. sclerotiorum* and can be considered as a possible regulator in canola resistance against *S. sclerotiorum* infection.

Chapter 2: Comparison of plant defense response at the site of infection in a susceptible and moderately tolerant cultivar of *Brassica napus* to the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*

2. 1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a global fungal pathogen with a wide host range: it can infect at least 450 plant species in 75 families, including many important cash crops (Garg and Li, 2010). Canola stem rot, also known as white mold, caused by *S. sclerotiorum*, has been reported globally including Canada (Hegedus and Rimmer, 2005, Bolton et al., 2006, Garg et al., 2010, Baharlouei et al., 2011). Increased incidence of canola stem rot has been reported in recent years because of increased canola production and wetter climate trends (Canola Council of Canada, 2012).

In the field, *S. sclerotiorum* survives during the winter by means of a long-term survival structure called sclerotia (Backhouse and Stewart, 1987; Bolton et al., 2006). Sclerotia germination can be myceliogenic by producing hyphae directly or cargenic by producing apothecia to release ascospore(Liang et al., 2013). In the canola- *S. sclerotiorum* pathosystem, *S. sclerotiorum* infects plants cargenically (Yang et al., 2007). *S. sclerotiorum* ascospore can germinate in water alone, but they require external nutrients in order to penetrate the host surface (Lumsden, 1979; Huang et al., 2008). Studies on bean, lettuce, pea and tomato indicate that senescing petals are a suitable nutrient source for *S. sclerotiorum* ascosporegermination and subsequent penetration of host tissues (Jamaux et al., 1995). It is also believed that senescing petals are required for lesion formation and the advancement of the disease in canola (Bom and Boland, 2000).

As far as we know, there is no data to support this hypothesis in the canola- *S. sclerotiorum* pathosystem.

To date, no fully resistant cultivar of canola has been published and most studies have used a moderate tolerant variety called Zhongyou821 (ZY821) to gain insight into the possible mechanisms of plant tolerance against *S. sclerotiorum* at the whole plant level (Li et al., 2004). However, no studies have been carried out examining how the plant responds to the infection processes using the petal inoculation method that recapitulates field conditions directly at the site of infection. Furthermore, it is still unclear how these two varieties respond to pathogen attack at the cellular, physiological and molecular levels.

Plants have evolved sophisticated and effective defense mechanisms against pathogen attack (Dominguez et al., 2011). The first line of defense is the anatomical architecture already present in the plant prior to the initial plant-pathogen interaction. Pre-existing structural defenses in the plant include the cuticle, closed stomata and thickened cell walls, which can inhibit the advance of fungal pathogens through plant tissues to some extent (Freeman and Beattie, 2008). The cuticle is a non-cellular covering on epidermal cells that can serve to prevent injury caused by microorganisms and pests (Dominguez et al., 2011). Meanwhile it has been reported that plant cell walls can decrease the cellular damage caused by pathogen ingression (Hematy et al., 2007).

Microarray experiments revealed that the canola antioxidant response had been induced during *S. sclerotiorum* infection (Yang et al., 2007). The plant antioxidant system is essential for the plant resistance response by ensuring the execution of resistant signaling functions and by preventing toxicity (De Pinto et al., 2012). The ascorbate-

glutathione antioxidative pathway is the heart of the plant antioxidant system (Foyer and Noctor, 2011). This pathway can reduce the H_2O_2 caused plant cell death, which can benefit necrotrophic pathogens since they obtain nutrients for growth and development from senescing cells (Kuz´niak and Urbanek, 2000). Therefore this study focused on the ascorbic acid-glutathione antioxidative pathway response differences between Westar and ZY821 before and during infection. In this pathway, ascorbate and glutathione are two major soluble small molecule antioxidants (Colville and Smirnoff, 2008). Ascorbate peroxidase (APX) reduces H_2O_2 into water using ascorbate as the electron donor, which oxidizes into monodehydroascorbate (MDHA). MDHA is unstable and tends to turn into dehydroascorbate (DHA) while monodehydroascorbate reductase (MDHAR) and glutathione-dependent dehydroascorbate reductase (DHAR) regenerates MDHA and DHA into reduced ascorbate (AsC). Finally, the resulting oxidized glutathione (GSSG) is reduced by glutathione reductase (GR) (Foyer and Noctor, 2011). This effective mechanism neutralizes the deleterious effects caused by H_2O_2 (Li et al., 2012).

Changes in gene activity associated with the plant defense response have also been shown to mitigate progression of the disease (Perchepied et al., 2010; van Verk et al., 2011). For example, the initiation and activation of the jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) signaling pathways have all been shown to respond to pathogen attack (Bari and Jones, 2009). In the JA/ET signaling pathway, both phytohormones act as signaling molecules that initiate and maintain the broad-spectrum defense response (Yang et al., 2010). They cooperate synergistically to activate pathogenesis-related (PR) proteins and *PLANT DEFENSIN 1.2* (*PDF1.2*) (Takahashi et al., 2007). *PDF1.2* is an antimicrobial small, basic peptide, and is often used as a marker

for the induction of the JA and ET-dependent signaling pathway (Thomma et al., 2002; van Loon et al., 2006). To date, 41 genes were found to be involved in JA biosynthesis in Arabidopsis (Sasaki et al., 2001). Among these genes, *ALLENE OXIDE SYNTHASE* (*AOS*) and *ALLENE OXIDE CYCLASE* (*AOC*) are considered to be rate limiting enzymes in the biosynthesis of JA (Schaller et al, 2008) and mutants defective in these genes lose the ability to protect themselves against pathogen invasion (Browse, 2009; Park et al., 2002; Von Malek et al., 2002). In the ET pathway, ethylene responsive genes like *ETHYLENE RESPONSE FACTOR1* (*AtERF1)*, *AtERF2* and *AtERF5* act as transcriptional activators, whereas *AtERF3* and *AtERF4* are transcriptional repressors (Fujimoto et al., 2000). For example, the overexpression of either *AtERF1* or *AtERF2* can up regulate the expression of *PDF1.2* and other PR proteins thus enhancing plant resistance against necrotrophic fungi such as *Fusarium oxysporum* (Berrocal-Loba and Molina, 2008), *Botrytis cinerea* and *Plectosphaerella cucumerina* (Berrocal-Loba and Molina, 2004). The SA signaling pathway participates in SA accumulation, *PATHOGENESIS-RELATED* (*PR*) gene expression, and induction of local and systemic acquired resistance (Yang et al., 2010). The increased SA levels can cause the plant redox change and activate SA-responsive gene expression such as *PR1* (Lai and Menqiste, 2013), one of the most important fungicidal defense proteins in plants (Lai and Menqiste, 2013; van Loon et al., 2006). In plants, the mitogen-activated protein kinase (MAPK) pathway is activated in response to external stimuli such as biotic and abiotic stress and is highly co-expressed with a large number of genes involved in the SA, JA and ET pathways (Nakagami et al., 2005; van Verk et al., 2011). For example, *MAP KINASE KINASE 4* (*MKK4*) and *MAP KINASE 6 (MPK6)* are bonded to JA and ET signaling

pathways and are able to enhance ET biosynthesis (Liu and Zhang, 2004; Nakagami et al, 2005; Takahashi et al, 2007) while *WRKY TRANSCRIPTION FACTOR 28* (*WRKY28*) can induce SA biosynthesis and is co-regulated with other important genes in the SA pathway (van Verk et al., 2011).

Among all the signaling pathways mentioned, the JA and ET pathway tend to be most involved in resistance to insects and necrotrophic pathogens (Perchepied et al., 2010). However, in Arabidopsis, Guo and Stotz (2007) reported that defense against *S. sclerotiorum* is dependent of the JA/ET, and SA signaling pathways. Likewise, an ABA-deficient tomato line, which displays an SA-dependent defense response, was reported to be less susceptible to *S. sclerotiorum* infection (Asselbergh et al., 2008). While each of these pathways may act independent of one another or together to mount a stronger defense response (Perchepied et al., 2010), it is still unclear how these pathways are activated within hours of infection in canola following inoculation with *S. sclerotiorum* ascospores.

In the current study we set out to investigate the cellular, physiological and molecular defense response to *S. sclerotiorum* directly at the site of infection using the petal inoculation method. To accomplish this goal we used a susceptible and moderately tolerant variety of *B. napus*. My results show that ZY821 is more tolerant than Westar to *S. sclerotiorum* infection. Specifically, we show differences in cellular architecture in mature leaves in addition to rapid changes in the ascorbate-glutathione antioxidant cycle. At the molecular level, it was shown that genes involved in the JA/ET and SA signaling pathways are rapidly activated in ZY821 within hours of the initial infection, providing insight into the regulatory mechanisms governing plant-pathogen interactions.

2. 2. Materials and Methods

2. 2. 1. Plant material and growth conditions

A susceptible *Brassica napus* cultivar (Westar) and a partially resistant cultivar (ZY821) were grown in Sunshine Mix #1 and placed in growth chambers with the following specified conditions: 22°C, 50-70% relative humidity and long day conditions (16 hours light, 8 hours dark -100-150 $\mu E/m^2/s$). Plants were grown until the 30-50% bloom stage before *S. sclerotiorum* inoculation. Plants were inoculated in a high humidity chamber with the following conditions: 22°C, 80-90% relative humidity and long day conditions (16 hours light, 8 hours dark -100-150 $\mu E/m^2/s$).

2. 2. 2. Petal and leaf inoculation

S. sclerotiorum ascosporewas stored at 4°C. A solution of *S. sclerotiorum* ascospore($8 \times 10^{-1} 4$ ml^{-1}) were suspended in water with 0.02% Tween 80 (Fernando et al., 2007).

The ascosporeof *S.sclerotiorum* were purchased from Dr. M.G. Boosalis, Department of Plant Pathology, University of Nebraska, Lincoln, NE, USA. Whatman™ filter paper fragments, young petals and senescing petals were inoculated with 30µl of the *S. sclerotiorum* ascosporesolution on Petri plates and sealed with Parafilm (Fig 2.1). Seven treatments were designed to identify whether senescing petals were essential for lesion formation in the canola-*S. sclerotiorum* pathosystem: i) treatment of canola leaves with the *S. sclerotiorum* ascospore solution in the absence of petals; ii) treatment of canola leaves with the *S. sclerotiorum* ascospore solution that was allowed to germinate in the absence of petals; iii) treatment of canola leaves with the *S. sclerotiorum* ascospore

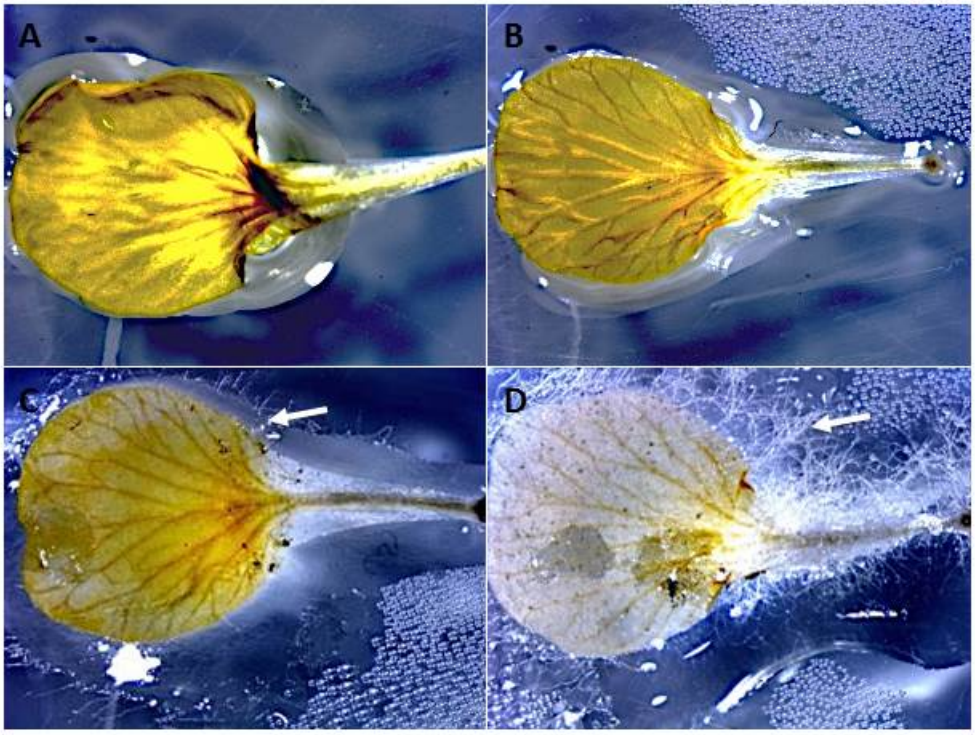

Figure 2.1 Canola senescing petals inoculated in 30µl of *S. sclerotiorum* ascospore solution ($8x10^4$ ascospores/ml) on a petri dish. A: Petal inoculated 0 hour; B: Petal inoculated and symptoms after 24 hours; C: Petal inoculated and symptoms after 48 hours, the arrow points to the *S. sclerotiorum* hyphae; D: Petal inoculated and symptoms after 72 hours, the arrow points to the *S. sclerotiorum* hyphae.

solution inoculated on Whatman™ filter paper fragments that were trimmed to resemble the approximate size and shape of the petal; iv) treatment of canola leaves with young leaves inoculated in the *S. sclerotiorum* ascospore solution; v) treatment of canola leaves with senescing leaves; vi) treatment of canola leaves with young petals inoculated in the *S. sclerotiorum* ascospore solution; vii) treatment of canola leaves with senescing petals (identified as such when the sepals of the flower turned white) inoculated in the *S. sclerotiorum* ascospore solutions. All seven treatments were incubated in petri dishes for 72 hours in a growth chamber at 22°C, 16 hours light. After 72 hours post inoculation (hpi), plants were inoculated with each of the seven treatments in the high humidity chamber.

The senescing petals inoculated with the *S. sclerotiorum* ascospore solution for 72 hours were used to infect Westar and ZY821. The lesion sizes in the Westar and ZY821 plants were measured at 24, 48, 72, 96, and 110 hpi.

2. 2. 3. Light Microscopy

2. 2. 3 .1. Tissue processing

Light microscopy was performed exactly as described in Chan and Belmonte (2013). Healthy leaves and infected leaves 1 cm around the lesion at 24, 48, 72 hpi 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 1X phosphate buffered saline. The samples were dehydrated in methyl cellosolve for one day followed by a day of dehydration in 100% ethanol, and another day with a fresh change 100% ethanol. Fixation and dehydration occurred at 4ºC. After dehydration, the tissues were infiltrated

with a graded series of Historesin (Leica Microsystems)/100% ethanol solution from 1/3 to 2/3 to 100% Historesin, remaining in each solution for a day in a rotary mixer at room temperature. Two additional days of infiltration with a fresh change of 100% Historesin each day followed. Following infiltration, the tissues were embedded in a liquid medium of 91.5% Historesin, 2.4% polyethylene glycol 400 and 6.1% Historesin Hardener (Leica Microsystems), and left undisturbed overnight at room temperature to polymerize.

2. 2. 3. 2. Sectioning and staining

The solid blocks 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 stained with 0.1% periodic acid, then Schiff's reagent for 15 minutes each, and then counterstained with toluidine blue O (TBO) for 1 minute.

2. 2. 3. 3. Imaging

Glass coverslips were applied to the slides using Cytoseal^{TM} 60. Slides were viewed using a Leica DM2500 brightfield light microscope 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.

2. 2. 4. Scanning Electron Microscopy

At 6, 12, 24 and 48 hpi leaves, 1 cm around the lesion were collected. 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. Images were collected using TM-1000 software.

2. 2. 5. Transmission Electron Microscopy

2. 2. 5. 1. Tissue processing

Electron microscopy was performed exactly as outlined in Chan and Belmonte (2013). A 0.5 cm range of tissue was collected around the lesion of the infected leaves at 24, 48 hpi as well as healthy leaves. Tissues were fixed in 3% glutaraldehyde in 0.025 mol/L cacodylate buffer supplemented with 5 mmol/L $CaCl_2$ (pH 7.0) overnight at 4˚C. The tissues were rinsed with cacodylate buffer before being postfixed overnight at 4 ˚C in a solution of 2% OsO4 and 0.8% $KFe(CN)_6$ in cacodylate buffer. The next day, the tissues were rinsed with distilled water and then stained overnight with a 0.5% uranyl acetate solution at 4 ˚C. The following day, the tissues were rinsed several times with distilled water then dehydrated in a graded ethanol series [(35%, 50%, 70%, 95% (2 changes), then 100% ethanol (3 changes)], remaining in each solution in the series for approximately 30 min at 4 ˚C. The tissues were then further dehydrated in a 1:1 (v/v) ratio of 100% ethanol–propylene oxide, followed by 3 changes of 100% propylene oxide, remaining in each rinse for 10–15 min before being infiltrated with and embedded in Spurr's epoxy resin.

2. 2. 5. 2. Sectioning and imaging

The blocks were trimmed and sectioned at approximately 70nm using a diamond knife on a Reichert-Jung Stereo Star Zoom ultramicrotome. Then the sections were

mounted on copper mesh grids and examined with a Hitachi H-7000 transmission electron microscope at 75 V. The pictures were taken by AMT Image Capture Engine version 601.384 software and modified (cropped, addition of scale bars, contrast and brightness adjusted, color balanced) using Adobe Photoshop CS2 version 9.0 software.

2. 2. 6. Ascorbate-glutathione redox assays

Determination of reduced and oxidized forms of ascorbate and glutathione was carried out as reported by Zhang and Kirkham (1996). Enzymatic activity was examined following the methods of Arrigoni et al (1992) as modified by Belmonte et al. (2005). Protein content was determined following the Bradford (1976) method using BSA as a standard. Each extraction was repeated at least three times.

Healthy leaf tissues and 1cm range of tissues around the lesion of infected leaves at 24, 48 and 72 hpi were collected and frozen in liquid nitrogen before being ground to a fine powder. Approximately 50mg of each sample was weighed out for each analysis. Analyses of ascorbate and glutathione were carried out exactly as in Zhang and Kirkham (1996). Samples were ground in 5% metaphosphoric acid and centrifuged at 4^{o}C for 20 minutes at 15 000X *g* before analysis. The total ascorbate pool was measured by reducing the oxidized ascorbate to the reduced form using dithiothreitol (DDT), while the amount of oxidized ascorbate was calculated by the difference between the total ascorbate pool and the reduced ascorbate pool. In the case of glutathione, its amount and GSH redox ratio was determined using the DTNB-GSSG reductase assay. GSH amount was calculated as the difference between the total glutathione pool and GSSG, the oxidized glutathione pool.

The activities of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were analyzed following their homogenization in 50mM phosphate buffer containing 1mM EDTA, 1mM PMSF and 1% (w/v) PVP-40. The homogenate was then centrifuged at 4^{o}C for 20 minutes at 17 000X *g*. Ascorbate peroxidase (APX) measurement began with homogenization in 50mM phosphate buffer containing 10mM ascorbic acid and 1% (w/v) PVP-40 followed by the centrifugation at 4^{o}C for 20 minutes at 15 000X *g*. Analysis of enzyme activity was performed exactly as reported by Arrigoni et al. (1992). Protein content for all enzyme assays was measured using the Coomassie Plus (Bradford) Assay Kit from Thermo Scientific.

APX activity was estimated by measuring the hydrogen peroxide dependent oxidation of ASC (extinction coefficient 2.8 mM^{-1}/cm^{-1}) following the decrease in absorbance at 265 nm. Only the cytosolic component of APX activity was measured since no ASC was added to the grinding medium. The reaction mixture contained 0.5 mM ASC, 0.15mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0). The reaction mixture in the absence of the enzyme extract was used as the blank. The ratio changes were recorded after 1min.

MDHAR activity was determined by following the NADH, H^+ oxidation dependent production of ascorbate at 340 nm (extinction coefficient 6.2 $mM^{-1}cm^{-1}$). The reaction mixture contained 0.125% Triton X-100, 0.2mM NADH, 2.5 mM ascorbate in 50mM Tris-HCl buffer (pH 7.6). The reaction mixture in the absence of the enzyme extract was used as the blank. The reaction was initiated by 5µg ascorbate oxidase. The ratio changes were recorded at 1min.

DHAR activity was determined by following the GSH-dependent production of ascorbate at 265 nm (extinction coefficient 14 $mM^{-1}cm^{-1}$). The reaction mixture contained 3.5 mM GSH, 0.4mM dehydroascorbate in 25mM potassium phosphate buffer (pH 7.0). The reaction mixture in the absent of the enzyme extract was used as the blank. The ratio changes were recorded at 1min.

GR activity was determined following the NADPH-dependent oxidation of GSSG at 340 nm (extinction coefficient 6.22 $mM^{-1}cm^{-1}$). The reaction mixture contained 2 nM EDTA and 0.5 mM GSSG in 0.1 M Tris-HCl buffer (pH 7.8). The reaction was initiated with the addition of 50 µM NADPH. One unit of activity was defined as the amount of enzme that oxidizes I pmol of substrate per minute under standard conditions. The reaction mixture in the absence of the enzyme extract was used as the blank. The ratio changes were recorded at 1min.

2. 2. 7. RNA Extraction and q-RT-PCR

2. 2. 7. 1. RNA isolation

Healthy leaf tissues and 1cm range of tissues around the lesion of infected leaves at 12, 24, 48 hpi were collected and frozen in liquid nitrogen before being ground to a fine powder. Samples that were not used immediately were stored at -80ºC. Approximately 50mg of each sample was ground in PureLink® Plant RNA Reagent (Invitrogen). The RNA extraction process was followed exactly as per the manufacturer's instructions for small-scale RNA extractions. Tissue was ground in 500 µl of Invitrogen plant RNA reagent and incubated for 5 minutes at room temperature. The solution was clarified via centrifugation at 12,000g for 2 minutes. A 100 µl of 5M NaCl was added to

the supernatant, followed by 300 µl of chloroform. Samples were centrifuged at 12,000g and 4°C for ten minutes. The top aqueous layer was collected and added to an equal volume of isopropanol (~400 µl) and then left at room temperature for 10 minutes. After centrifugation at 12,000g and 4°C for 10 minutes, the pellet was washed with 75% ethanol. The pellet was then resuspended in 20 µl of RNase-free water.

2. 2. 7. 2. DNase treatment

Samples were DNase treated to remove contaminating DNA using the Ambion Turbo DNA-Free Kit as per the manufacturer's instructions. Concentration and quality of the DNase-treated RNA was assessed via Nanodrop.

2. 2. 7. 3. cDNA synthesis

A 1µg of DNase-treated RNA was converted to cDNA using the Fermentas Maxima First Strand cDNA synthesis kit as per the manufacturer's instructions. The total 20 µL reaction volume of each sample was synthesized into cDNA using a Bio Rad My Cycler^{TM} Thermal Cycler.

2. 2. 7. 4. q-RT-PCR

Primer sets used for q-RT-PCR can be found in the supplementary data. Primers were run at 0.25µM concentrations with 1/10 dilutions of cDNA. Biorad IQ SYBR Green Supermix was used according to the manufacturer's instructions. The reactions were processed using Bio Rad CFX Connect^{TM} Real-Time System; the PCR parameters were set as an initial holding at 95°C for 3 minutes, and then 40 cycles of: 95°C for 30s, 60°C

for 30s, 72°C for 30s, melting at 60°C for 1 min, followed by 99.9°C for 15s**.** Data was collected by Bio Rad Manager and analyzed using the delta-delta-Ct algorithm. Actin was used as the housekeeping gene. The primers used are listed in Table 2. 1.

2. 8. Statistical analysis

All experiments were performed using at least three biological replicates. ANOVA was employed to determine significant differences in lesion size, antioxidant levels, and gene activity between the control group (0 hpi) and the treatment groups (infected tissues) in Westar and ZY821. A *p* value less than 0.05 was considered statistically significant. The raw data were inputted into JMP version 8.0.1 software.

2. 3. Results

2. 3. 1. Senescing petals are essential in the canola-*S. sclerotiorum* patho-system

S. sclerotiorum ascosporewere inoculated in one of the following: water, filter paper, young leaves, senescing leaves, young petals or senescing petals. Results showed no lesion formation when the leaves were treated with the *S. sclerotiorum* ascospore solution in the absence of petals. Germinated *S. sclerotiorum* hyphae in water, on filter paper and on leaves did not cause lesions to form nor cause symptoms associated with stem rot. While the *S. sclerotiorum* ascosporeinoculated on young petals caused lesion formation in the leaves 20% of the time, those inoculated on sensing petals caused a 100% infection rate (Table 2.2).

Table 2.1. Sequences of the gene-specific primer pairs used in quantitative real-time polymerase chain reaction experiments.

Gene	**Accession Number**	**Forward and Reverse Primers**	**Amplicon Size (bp)**
Actin	AF111812.1	5'-ACGAGCTACCTGACGGACAAG-3' 5'-GAGCGACGGCTGGAAGAGTA-3'	80
Allene Oxide Cyclase	FJ788939.1	5'-CCAGATTTCTCCTCCCAATCA-3' 5'-GACTTTCCCCAGTTCCAGAAGA-3'	80
Allene Oxide Synthase	CD828070	5'-CAAGCAAAAACCCGAGGAGTT-3' 5'-CTGGTGGCATATTGACTCGAAA-3'	80
Ethylene Response Factor2	CN737061.1	5'-TGTTGAATTTCCCGTTGAGA-3' 5'-TGTTTTCTGACGAAGACGAT-3'	102
Plant Defensin1.2	AY884023.1	5'-TTGTGCGAGAGGTCAAGTGG-3' 5'-ACACTTGTGAGCAGGGAACA-3'	129
MAPKinase kinase4	JF268686.3	5'-TGCAACTCCTCTGTCGGAAC-3' 5'-TATATCTCCCGCGTAGCCGT-3'	96
MAPKinase6	HQ156228.1	5'-AGATCAAGCTCCTTCGCCAC-3' 5'-TGAAAGCGGTTCTTAGCGGT-3'	84
WRKY DNA-binding Protein 28	EU912399.1	5'-ATCCACCGTCCGATTCATGG-3' 5'-CTTCCTTGCCGGACATCCTT-3'	116
Pathogenesis-related Protein1	U64806.1	5'-TGTGGCAAAGCAAGGTGTAA-3' 5'-TTCCCCGAGGATCATAGTTG-3'	64

Table 2.2. The lesion formation rates in the *S. sclerotiorum* -canola pathosystem by inoculating *S. sclerotiorum* ascosporeon different tissues. Treatments involved inoculating with ascospore for 72hours, and then each treatment was put on the canola leaf surface to infect the plant. The data were collected after the leaves had been infected 48hours.

	Treatments	**Disease Incidence**	**Total Number of Replicates**
1	*S. sclerotiorum* ascospore solution in water	0%	80
2	The germinated hyphae in *S. sclerotiorum* spore solution in water	0%	80
3	*S. sclerotiorum* ascospore solution inoculated on filter paper	0%	80
4	*S. sclerotiorum* ascospore solution inoculated on healthy leaves	0%	80
5	*S. sclerotiorum* ascospore solution inoculated on senescing leaves	0%	80
6	Young petals inoculated in *S. sclerotiorum* ascospore solution	20%	80
7	Senescing petals inoculated in *S. sclerotiorum* ascospore solution	100%	>1000

2. 3. 2. ZY821 is more tolerant than Westar to *S. sclerotiorum* infection

Leaf inoculation experiments were performed in both Westar and ZY821. In both cultivars, lesions appeared after 24hpi, and expanded over time during the infection period (Fig.2.2). However, the expansion of the lesion occurred more rapidly in Westar than in ZY821. Specifically, compared to ZY821, the lesion size in Westar was 250% larger at 72 and 96 hpi, and 449% larger at 110hpi (Fig.2.3).

2. 3. 3. Differences in Westar and ZY821 mature leaf anatomy during the *S. sclerotiorum* infection

The infection process of *S. sclerotiorum* on canola included pre-penetration, penetration of the epidermal cell layer, spreading in the mesophyll, colonization and symptom development (Lumsden, 1979). Scanning electron microscopy observations showed that the infection hyphae developed from the senescing petals over the leaf surface within 6 hpi in both Westar and ZY821 (Fig.2.4.A). Oxalic acid crystals were visible on senescing petals (Fig.2.4.A). Besides the oxalic acid crystals, *S. sclerotiorum* secreted some substances above the plant cuticle in both cultivars within 6 hpi (Fig.2.4.A). Interestingly, unlike most fungal pathogens, which gain access to internal leaf tissues by growing through stomatal openings (Zhang and Wang, 2011), *S. sclerotiorum* hyphae appeared to bypass the stomata on both the upper and lower epidermis in both cultivars (Fig.2.4.B; Fig.2.4.C). After spreading over the leaf surface, the pathogen formed appressorium on the Westar upper epidermis to help penetration (Fig.2.4.C). At 48 hpi, through SEM, we observed that the *S. sclerotiorum* hyphae had penetrated through the cuticle and formed a dense subcuticular hyphal network and subsequently colonized

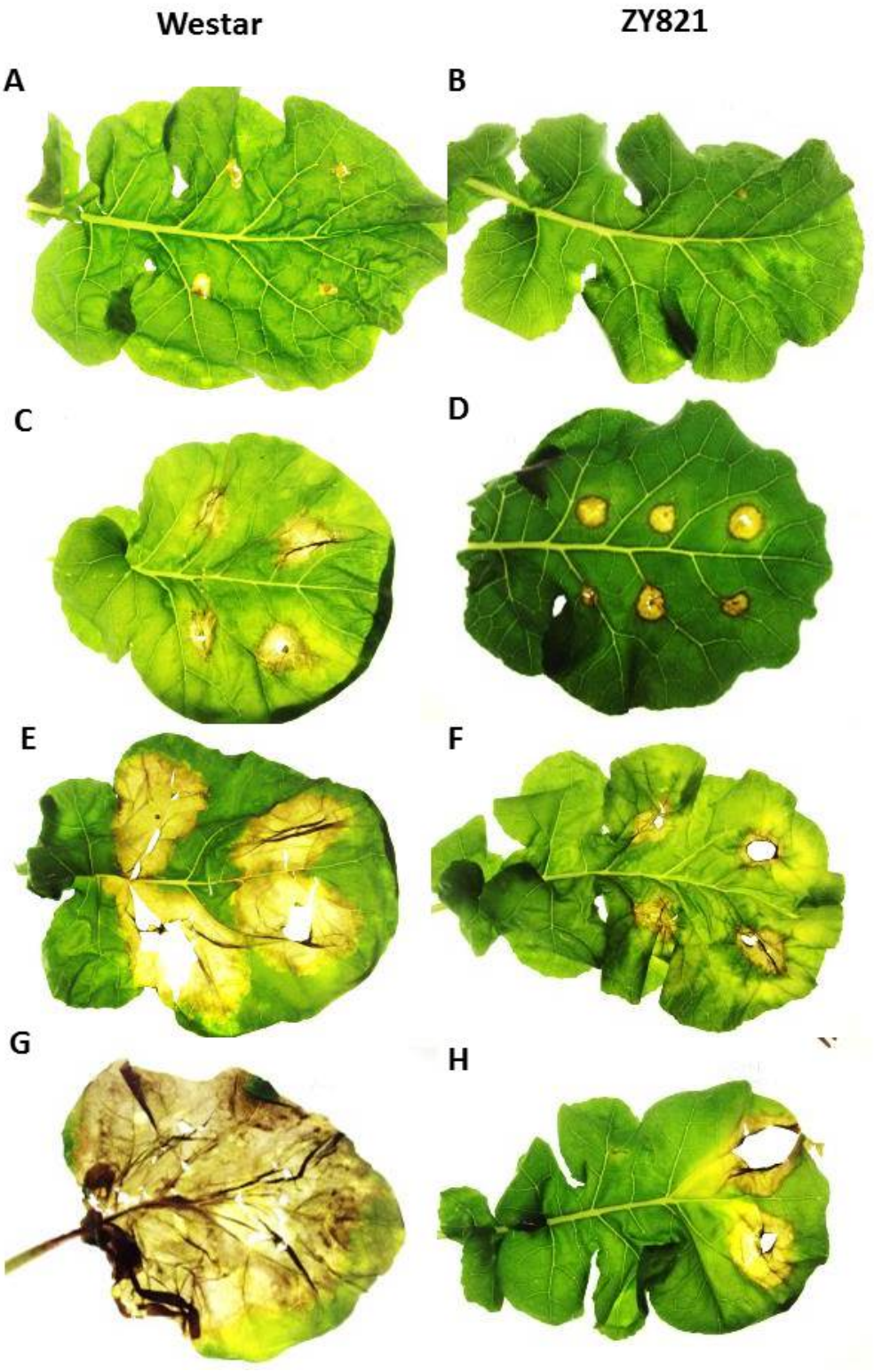
Westar
ZY821
A
B
C
D
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Figure 2.2: Differences in susceptibility of mature leaves of a *B. napus* susceptible cultivar Westar and a moderate tolerant cultivar ZY821 to *S. sclerotiorum* infection. Mature leaves were infected at 30% bloom stage by senescing canola petals that had been inoculated for 72 hours in a 30µl volume *S. sclerotiorum* ascospore solution (8x10^4 ascospores/ml). A: Westar infected leaf at 24 hours post inoculation (hpi); B: ZY821 infected leaf at 24 hpi; C: Westar infected leaf at 48 hpi; D: ZY821 infected leaf at 48 hpi; E: Westar infected leaf at 72 hpi; F: ZY821 infected leaf at 72 hpi; G: Westar infected leaf at 96 hpi; H: ZY821infected leaf at 96 hpi.

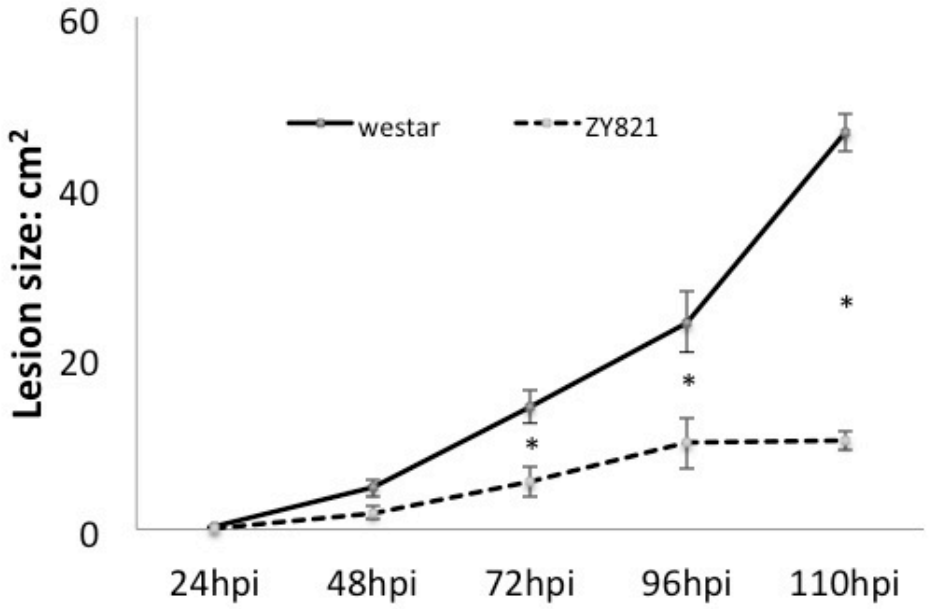
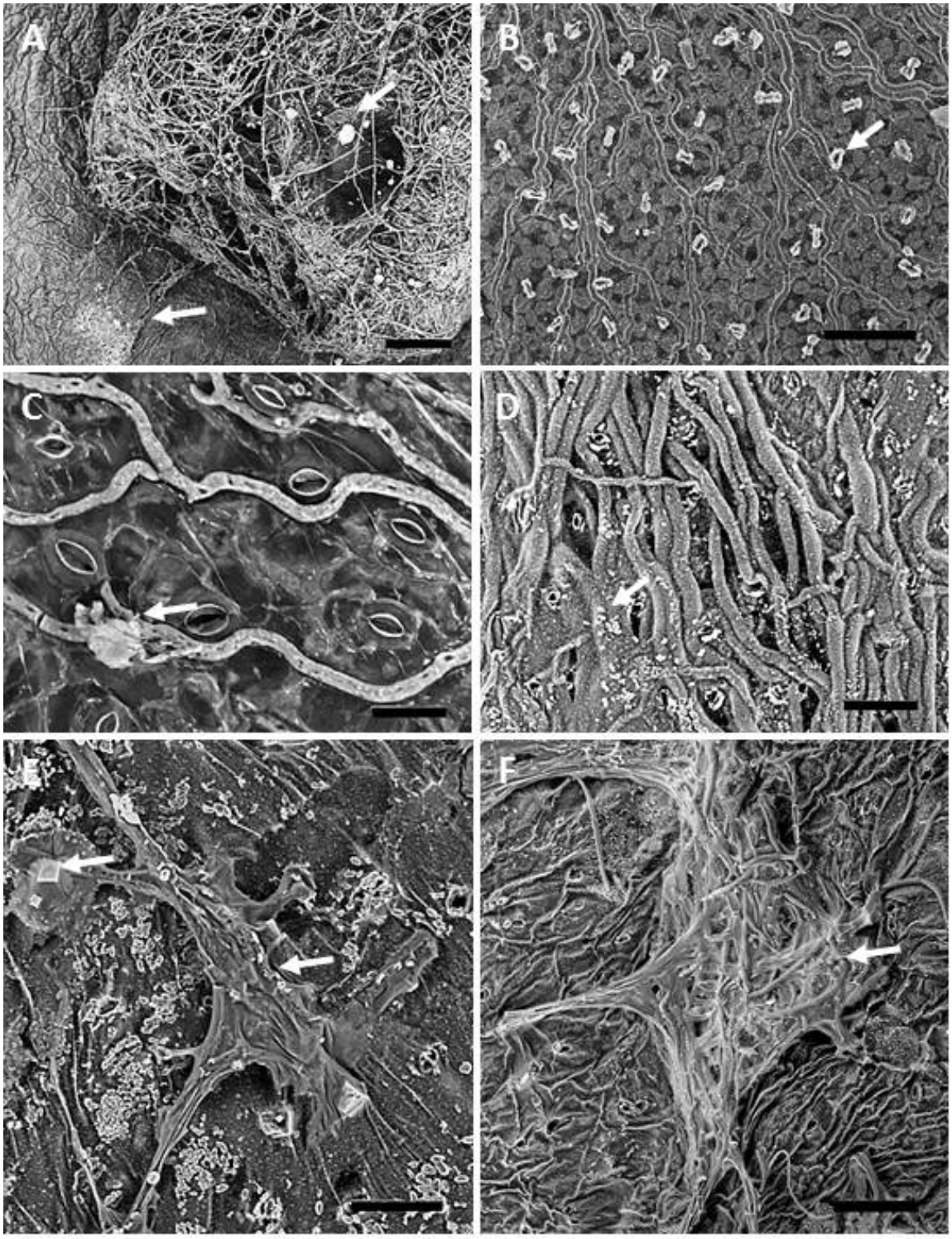


Figure 2.3: Lesion size (cm^2) growth over time (24hpi, 48hpi, 72hpi, 96hpi and 110hpi) on leaves of a *B. napus* susceptible cultivar Westar and a moderate tolerant cultivar ZY821 after *S. sclerotiorum* inoculation. Mature leaves were infected at 30% bloom stage by senescing canola petals previously inoculated for 72 hours in a 30µl volume of *S. sclerotiorum* ascospore solution (8x10^4 ascospores/ml). * indicates there is a significant difference between the two cultivars ($p < 0.05$).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E
F

Figure 2.4: Scanning Electron Micrographs showing the *S. sclerotiorum* infection processes following *S. sclerotiorum* inoculation on mature leaves of a *B. napus* susceptible cultivar Westar and a moderately tolerant cultivar ZY821. The plant mature leaves were infected at 30% bloom stage by canola senescing petals that presiously inoculated for 72 hours in a 30µl volume *S. sclerotiorum* ascospore solution (8x10^4 ascospores/ml). A: Canola senescing petal covered with *S. sclerotiorum* hyphae on Westar upper epidermis at 6 hpi. The arrow at the top is pointing to the oxalic acid crystal on the senescing petal. The arrow at the bottom shows the *S. sclerotiorum* hyphae germinated from the senescing petal developing on the Westar leaf upper epidermis; the *S. sclerotiorum* hyphae produced secreted material that covering the leaf surface. B: *S. sclerotiorum* hyphae spreading on the ZY821 upper epidermis at 24 hpi and avoiding the stomata. The arrow points to the stomata. C: *S. sclerotiorum* hyphae spreading on the Westar upper epidermis at 24 hpi and avoiding the stomata. The appressorium forms on Westar upper epidermis at 24 hpi. The arrow shows the appressorium. D: The subcuticle hyphae network on ZY821 lower epidermis at 48 hpi. E: Infection cushion on Westar lower epidermis at 48hpi. The arrows point to the oxalic acid crystals. F: Infection cushion on ZY821 lower epidermis at 48hpi. Scale bars: A: 300 µm. B: 90 µm. C: 80 D: 60 µm. E: 60 µm. F: 120 µm.

epidermal tissue subcuticular in both cultivars, those hyphae were oriented parallel to each other on the lower epidermis of ZY821 leaves (Fig.2.4.D). The infection cushions, complex appressoria that increase the adhesion of the pathogen to the host surface (Nicholson and Epstein, 1991), were observed after 48 hpi in both Westar and ZY821, and surprisingly, were larger and more commonly found in ZY821 (Fig.2.4.E; Fig.2.4.F). Also, more oxalic acid crystals were observed on the Westar leaf surface (Fig.2.4.E; Fig.2.4.F).

Light microscopy pictures revealed *S. sclerotiorum* growth inside the Westar epidermal cells that caused the mesophyll cell death, which was evidenced by the membrane shrinkage in advance of the infection hyphae (Fig.2.5.A). Besides extending between the cuticle and the epidermal cell wall, the *S. sclerotiorum* hyphae penetrated directly through the epidermis and spread into the mesophyll tissues in both cultivars (Fig.2.5.B). After the penetration, *S. sclerotiorum* hyphae expanded into the Westar vasculatures (Fig.2.5.C). In ZY821 infected leaves, *S. sclerotiorum* formed subcuticular hyphae oriented parallel to each other on the lower epidermis at 48 hpi (Fig.2.5.D). Interestingly, unlike Westar, only the cells surrounding the hyphae show anatomical features of cellular degradation in ZY821 (Fig.2.5.D). On the ZY821 upper epidermis, the infection cushions were observed helping the pathogen to penetrate (Fig.2.5.E). Compared with Westar, where *S. sclerotiorum* hyphae were observed in vascular bundles in partially degraded leaf tissues (Fig.2.5.C), even when the mesophyll cells were highly degraded, *S. sclerotiorum* in ZY821 did not invade the vascular bundles (Fig.2.5.E).

The transmission electron micrographs showed that in Westar palisade mesophyll,

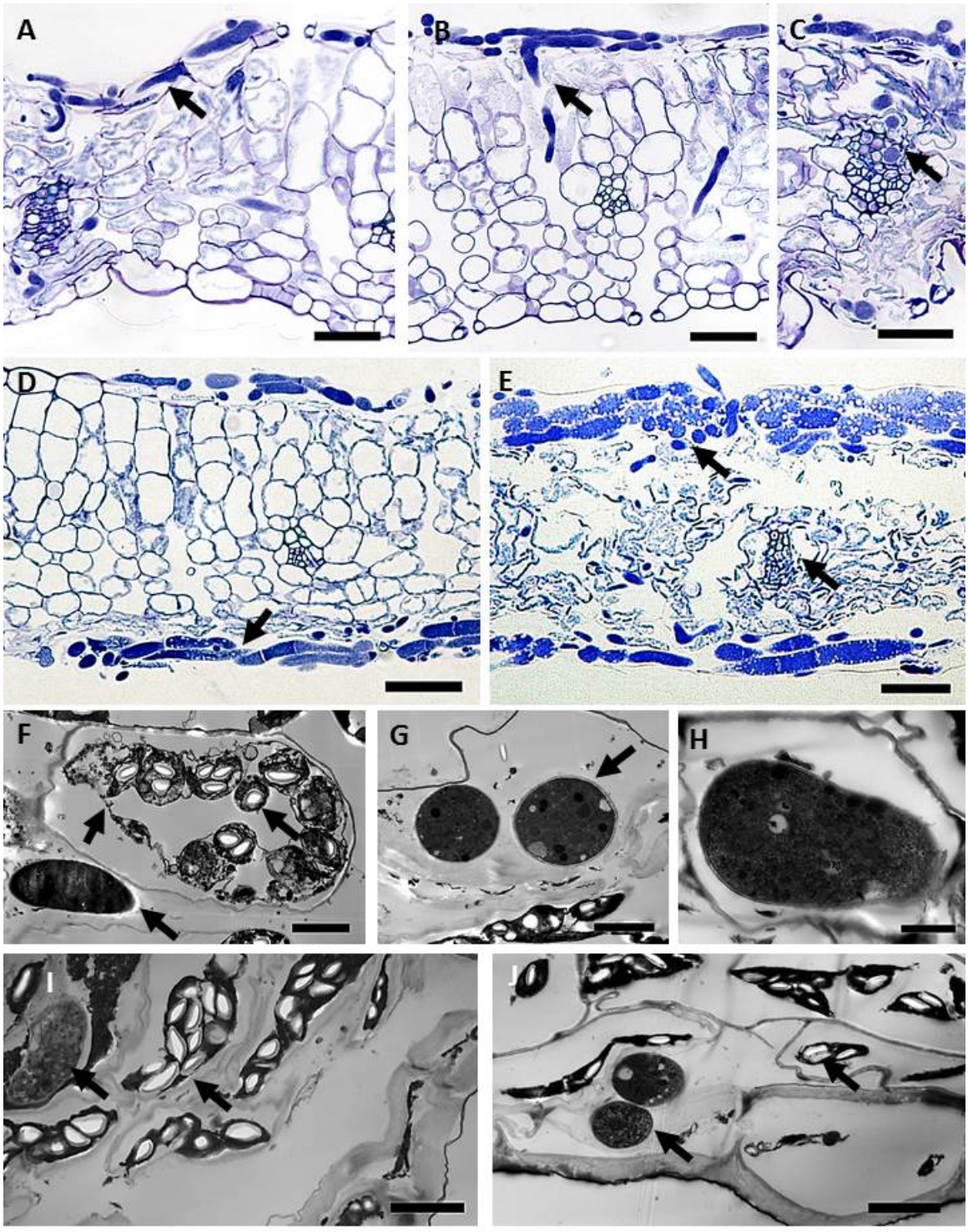
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Figure 2.5: Light Micrographs and Transmission Electron Micrographs show a *B. napus* susceptible cultivar Westar and a moderate tolerant cultivar ZY821 leaf cellular response to the *S. sclerotiorum* at the site of the infection. Mature leaves were infected at 30% bloom stage by canola senescing petals that had been inoculated for 72 hours in 30µl *S. sclerotiorum* ascospore solution (8x10^4 ascospores/ml). A: Light Micrograph of *S. sclerotiorum* hyphae spreading inside the Westar upper epidermal cells at 48 hpi and avoiding the stomata. Cell degradation appears in advance of the hyphae. The arrow points to the *S. sclerotiorum* hyphae. B: Light Micrograph of *S. sclerotiorum* hyphae directly penetrating the cuticle and upper epidermis without the help of an appressorium or infection cushion at 48 hpi in Westar. The cell degradation appears in advance of the hyphae. The arrow points to the *S. sclerotiorum* hyphae. C: Light Micrograph of *S. sclerotiorum* hyphae invading the Westar vasculature at 48 hpi. The cell degradation appears in advance of the hyphae. The arrow points to the *S. sclerotiorum* hyphae in vasculature. D: Light Micrograph of *S. sclerotiorum* hyphae oriented parallel to each other on the ZY821 lower epidermis at 48 hpi. The cell degradation appears in the cells surrounding the *S. sclerotiorum* hyphae. The arrow points to the parallel hyphae. E: Light Micrograph of the infection cushion aimed *S. sclerotiorum* penetration on ZY821 upper epidermis at 48 hpi. The upper arrow points to the infection cushion on the ZY821 upper epidermis. The lower arrow shows the ZY821 vasculature had not been infected. F: Transmission Electron Micrograph of Westar mesophyll cell death at 24 hpi and intracellular hyphae. This picture shows the rupture of chloroplasts and the plasma membrane as well as the shrinkage of the protoplast. The arrow points to the *S. sclerotiorum* intracellular hyphae. G: Transmission Electron Micrograph of *S.* the cell

sclerotiorum subcuticle hyphae in Westar at 24 hpi. H: Transmission Electron Micrograph of intercellular hyphae in Westar mesophyll cell at 24 hpi. I: Transmission Electron Micrograph of starches in Westar mesophyll cells at 24 hpi and the *S. sclerotiorum* intercellular hyphae. The left arrow shows the *S. sclerotiorum* intercellular hyphae, while the right arrow points to the starches in Westar mesophyll cells. J: Transmission Electron Micrograph of starch in ZY821 mesophyll cells at 24 hpi and the *S. sclerotiorum* intercellular hyphae. The left arrow shows the *S. sclerotiorum* intercellular hyphae, while the right arrow points to the starches in ZY821 mesophyll cells. Scale bars: A: 50 µm. B: 50 µm. C: 40 µm. D: 80 µm. E: 25 µm. F: 8 µm. G: 6 µm. H: 2500 nm. I: 8 µm. J: 8 µm.

death happened by the side of the *S. sclerotiorum* hyphae, which was evidenced by the rupture of the chloroplasts, plasma membrane and general membrane shrinkage (Fig.2.5.F). The TEM pictures further proved that *S. sclerotiorum* hyphae spread between the cuticle and outer epidermal cell walls, and caused cell wall degradation in both cultivars (Fig.2.5.G). The intracellular hyphae were observed by TEM in Westar mesophyll cell (Fig.2.5.H). Furthermore, the TEM pictures revealed starches exhibited in chloroplasts of both cultivars, but bigger starch size was observed in Westar mesophyll cells (Fig.2.5.I; Fig.2.5.J).

2. 3. 4. Activity of the ascorbate-glutathione redox cycle in response to *S. sclerotiorum* infection

Cellular concentrations of total ascorbate were higher in ZY821 than in Westar before and during *S. sclerotiorum* infection. In Westar, the reduced ascorbate content did not change over the course of the infection; however, in ZY821, endogenous levels of reduced ascorbate were 148% higher when compare to the uninfected control (Fig.2.6.B). Interestingly, although the ascorbate redox ratio (AsC/AsC+DHA+MDHA) was 20% higher in healthy Westar leaf tissues, it decreased to levels comparable to those of ZY821 at the start of the inoculation and showed a similar pattern to ZY821 ascorbate redox ratios throughout the infection (Fig.2.6.C).

Endogenous glutathione levels (Fig.2.6.D) and the glutathione redox ratio (GSH/ GSH+GSSG; Fig.2.6.E) were similar in both Westar and ZY821 in healthy leaf tissues. After inoculation with *S. sclerotiorum*, the glutathione content sharply increased in

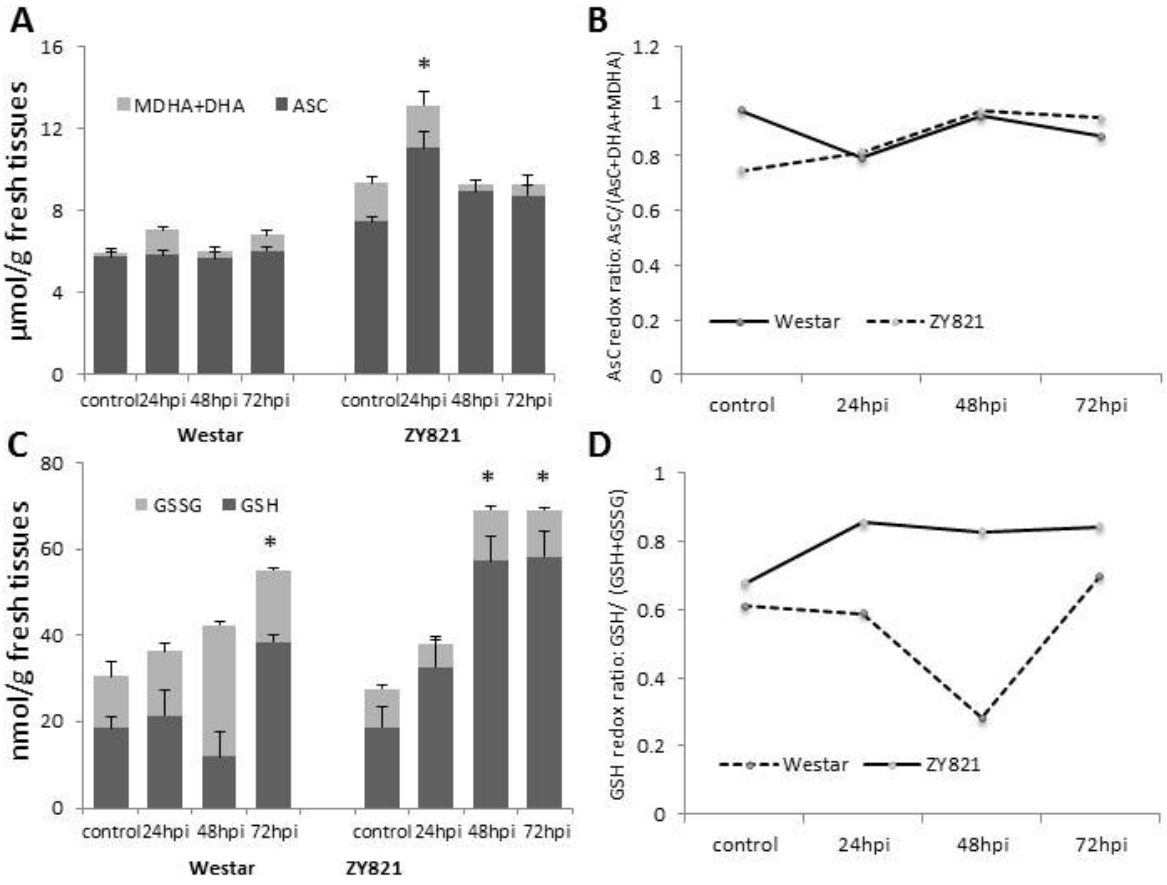
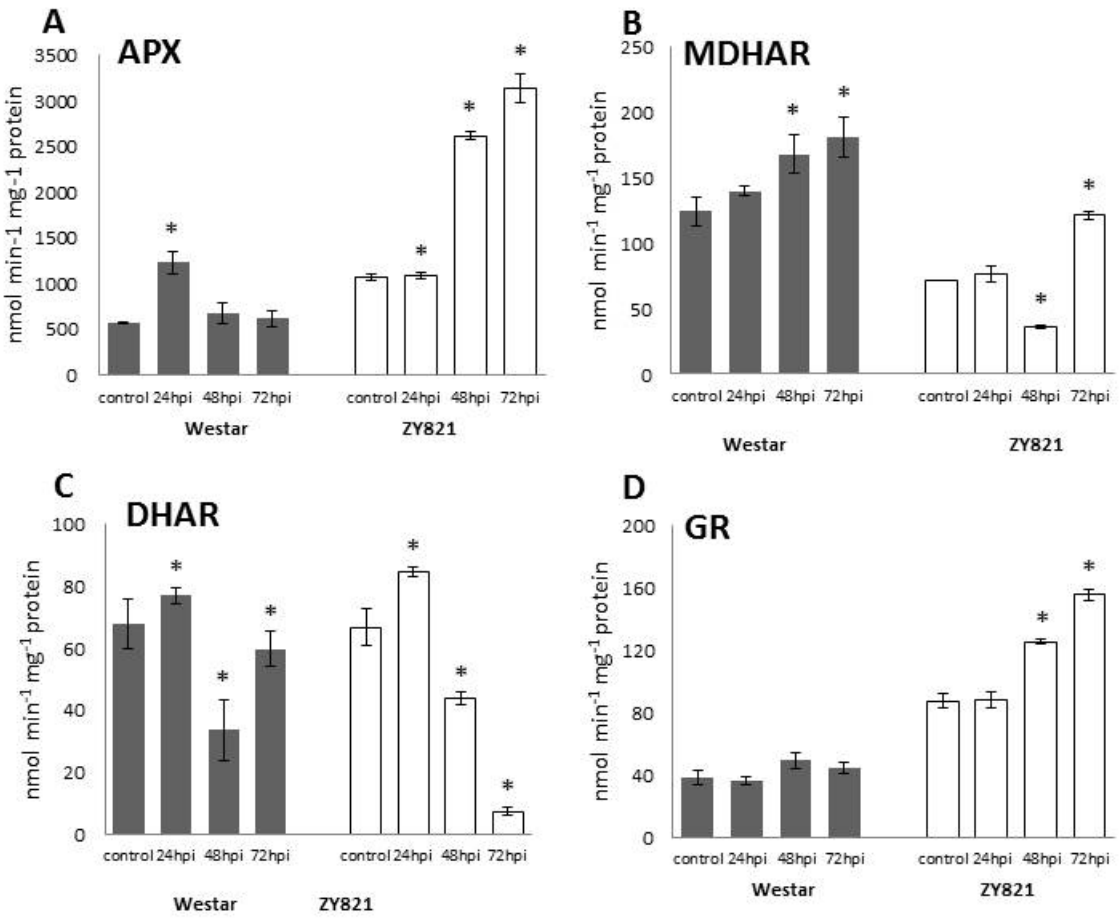

A
µmol/g fresh tissues
MDHA+DHA
ASC
control 24hpi 48hpi 72hpi
Westar
ZY821
B
AsC redox ratio: AsC/(AsC+DHA+MDHA)
Westar
ZY821
control
24hpi
48hpi
72hpi
C
nmol/g fresh tissues
GSSG
GSH
D
GSH redox ratio: GSH/ (GSH+GSSG)

Figure 2.6: The changes of ascorbate and glutathione endogenous levels and redox ratios in a *B. napus* susceptible cultivar Westar and a moderately tolerant cultivar ZY821 mature leaves before (control group) and during the *S. sclerotiorum* infection (24, 48 and 72 hpi). The plant mature leaves were infected at 30% bloom stage by canola senescing petals that had been inoculated for 72 hours in a 30µl volume *S. sclerotiorum* ascospore solution ($8x10^4$ ascospores/ml). * indicates there is a significant difference compare to the control group ($p < 0.05$).

A: Endogenous levels of reduced (ASC) and oxidized ascorbate (MDHA+DHA) in Westar and ZY821 mature leaf tissues before and during the *S. sclerotiorum* infection; B: Reduced ascorbate redox ratios [ASC/(ASC+MDHA+DHA)] in Westar and ZY821 mature leaf tissues before and during the infection ; C: Endogenous level of reduced (GSH) and oxidized glutathione (GSSG) pool in Westar and ZY821 mature leaf tissues before and during the infection; D: Reduced glutathione redox ratios [GSH/(GSH+GSSG)] in Westar and ZY821 mature leaf tissues before and during the infection.

Westar and ZY821. At 72 hpi, the total glutathione content increased 180% and 250%, respectively (Fig.2.6.D). In Westar, the glutathione redox state shifted towards its oxidized form, GSSG. Compared to the control group, a significant decline (57%) of the GSH redox ratio was observed in Westar at 48hpi. This is in contrast to ZY821 where the glutathione redox ratio increased over the course of infection (Fig.2.6.E).

The presence of *S. sclerotiorum* on the leaf surface resulted in a general increase in antioxidant enzyme activities in both Westar and ZY821. In non-infected tissues of Westar and ZY821, ascorbate peroxidase (APX) contents were twice the amount in ZY821 than in Westar (Fig.2.7.A) while levels of monodehydroascorbate reductase (MDHAR) content in Westar were double that of ZY821 (Fig.2.7.B). The dehydroascorbate reductase (DHAR) contents were similar in both cultivars (Fig.2.7.C), however, glutathione reductase (GR) contents were twice the amount in ZY821 compare with Westar (Fig.2.7.D). Over the course of the infection process, the greatest APX activity in Westar was observed at 48hpi, which showed a 210% increase compared to the control group while the activities of APX in ZY821 increased 247% and 296% by 48 hpi and 72hpi respectively (Fig.2.7.A). MDHAR activity increased gradually in Westar after the *S. sclerotiorum* infection and increase 145% at 72hpi, while in ZY821, it declined by 48hpi and increased 170% at 72 hpi (Fig.2.7.B). DHAR activity reached a peak at 24hpi in both cultivars and deceased thereafter (Fig.2.7.C). The activity of GR slightly increased in Westar during the *S. sclerotiorum* inoculation, it was significantly lower than ZY821, which increased 143% and 176% by 48 hpi and 72hpi respectively (Fig.2.7.D).


A
APX
nmol min-1 mg-1 protein
3500
3000
2500
2000
1500
1000
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control 24hpi 48hpi 72hpi
control 24hpi 48hpi 72hpi
Westar
ZY821
B
MDHAR
nmol min-1 mg-1 protein
250
200
150
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50
0
control 24hpi 48hpi 72hpi
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Westar
ZY821
C
DHAR
nmol min-1 mg-1 protein
100
80
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control 24hpi 48hpi 72hpi
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Westar
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GR
nmol min-1 mg-1 protein
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0
control 24hpi 48hpi 72hpi
control 24hpi 48hpi 72hpi
Westar
ZY821

Figure 2.7: Activity of Enzymes within the ascorbate-glutathione pathway in a *B. napus* susceptible cultivar Westar and a moderately tolerant cultivar ZY821 mature leaves before (control group) and during the *S. sclerotiorum* infection (24, 48 and 72 hpi). The plant mature leaves were infected at 30% bloom stage by canola senescing petals that had been inoculated for 72 hours in a 30µl volume *S. sclerotiorum* ascospore solution ($8x10^4$ ascospores/ml). * indicates there is a significant difference compare to the control group ($p < 0.05$).

A: Enzyme activities of ascorbate peroxidase (APX) in Westar and ZY821 mature leaves before and during the *S. sclerotiorum* infection; B: Enzyme activities of monodehydroascorbate reductase (MDHAR) in Westar and ZY821 mature leaves before and during the *S. sclerotiorum* infection; C: Enzyme activities of dehydroascorbate reductase (DHAR) in Westar and ZY821 mature leaves before and during the *S. sclerotiorum* infection; D: Enzyme activities of glutathione reductase (GR) in Westar and ZY821 mature leaves before and during the *S. sclerotiorum* infection.

2. 3. 5. Changes in gene activity of plant defense response pathways to *S. sclerotiorum* infection

Relative mRNA abundance of *ALLENE OXIDE CYCLASE* (*AOC*) and *ALLENE OXIDE SYNTHASE* (*AOS*) were observed to be significantly different between the two plant cultivars (Fig.2.8.A; Fig.2.8.B). In Westar, *AOC* levels gradually increased throughout the infection and reached its maximum value at 48hpi (80-fold), whereas expression of *AOS* peaked at 24hpi (15-fold). The expression of *AOC* in ZY821 increased almost 180 fold at 24hpi and 48hpi, while expression of *AOS* significantly increased at 48hpi (107-fold). Expression of *ETHYLENE RESPONSE FACTOR 2* (*ERF2*) did not change significantly in ZY821, while the relative abundance of this transcript increased 43-fold by 24hpi in Westar (Fig.2.8.C). Relative mRNA abundance of *PLANT DEFENSIN 1.2 (PDF1.2)* was induced by 12 hpi in both Westar and ZY821 (15-fold and 60-fold, respectively) (Fig.2.8.D). For genes involved in the MPK pathways, in both cultivars, the expression of *MAPKinase kinase4 (MKK4)* and *MAPKinase6 (MPK6)* increased at 12hpi but decreased afterwards (Fig.2.8.E; Fig.2.8.F). Similarly, the activity of *WRKY28* significantly increased at 12hpi: 50-fold and 83-fold in Westar and ZY821, respectively (Fig.2.8.G). Compared to control tissues, the expression of *PATHOGENESIS-RELATED PROTEIN1 (PR1)* significantly increased following *S. sclerotiorum* infection in Westar and ZY821 (Fig.2.8.H). At 12hpi, the relative abundance of *PR1* transcripts was much higher in ZY821 (17-fold) than in Westar and the expression of this gene was similar at the later stages of infection (24hpi and 48hpi) between both cultivars.

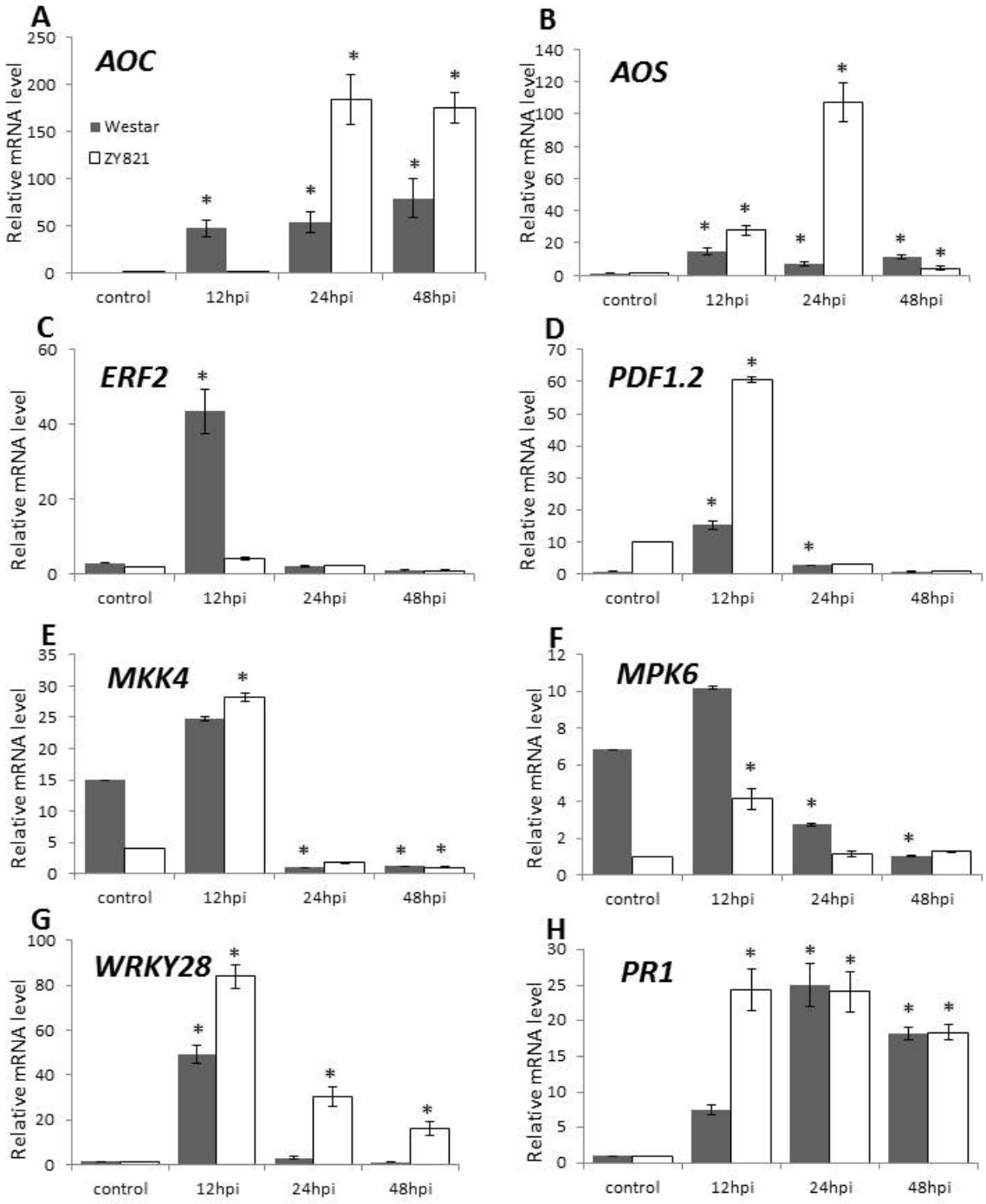
A
AOC
Westar
ZY821
Relative mRNA level
control
12hpi
24hpi
48hpi
B
AOS
C
ERF2
D
PDF1.2
E
MKK4
F
MPK6
G
WRKY28
H
PR1

Figure 2.8: Activity of genes within jasmonic acid (JA), ethylene (ET), salicylic acid (SA), and mitogen-activated protein kinase (MAPK) pathways in a *B. napus* susceptible cultivar Westar and a moderately tolerant cultivar ZY821 mature leaves before (control group) and during the *S. sclerotiorum* infection (12, 24 and 48 hpi). The plant mature leaves were infected at 30% bloom stage by canola senescing petals previously inoculated for 72 hours in a 30µl volume *S. sclerotiorum* ascospore solution ($8x10^4$ ascospores/ml). Actin was used as the internal control. * indicates there is a significant difference compare to the control group ($p < 0.05$).

A: *ALLENE OXIDE CYCLASE* (*AOC*) expression change in Westar and ZY821 mature leaves before and during the infection; B: *ALLENE OXIDE SYNTHASE* (*AOS*) expression change in Westar and ZY821 mature leaves before and during the infection; C: *ETHYLENE RESPONSE FACTOR 2* (*ERF2*) expression change in Westar and ZY821 mature leaves before and during the infection; D: *PLANT DEFENSIN 1.2* (*PDF1.2*) expression change in Westar and ZY821 mature leaves before and during the infection; E: *MAP KINASE KINASE 4* (*MKK4*) expression change in Westar and ZY821 mature leaves before and during the infection; F: *MAP KINASE 6* (*MPK6*) expression change in Westar and ZY821 mature leaves before and during the infection; E: *WRKY DNA-BINDING PROTEIN 28* (*WRKY28*) expression change in Westar and ZY821 mature leaves before and during the infection; F: *PATHOGENESIS-RELATED PROTEIN1* (*PR1*) expression change in Westar and ZY821 mature leaves before and during the infection.

2. 4. Discussion

2. 4. 1. Senescing petals are essential in the canola-*S. sclerotiorum* patho-system

Results demonstrated that senescing petals are essential for *S. sclerotiorum* pathogenicity in canola. The 20% infection rate caused by young petals might be due to the young petals producing a small, but insufficient food source for *S. sclerotiorum* ascospores. These results agreed with the previous assumption that senescing petals are essential in canola- *S. sclerotiorum* pathosystem (Lumsden, 1979; Cessna et al., 2000). In the field, the canola petals turn senescent at the 30% bloom stage while the *S. sclerotiorum* ascospore germinate on those petals, then the infected petals lodge on the leaf or at the axis between the stem and branches (Bashi et al., 2011). After the infected petals land on the surface of plants, the germinated hyphae produce toxins coupled with cell-wall degrading enzymes to directly penetrate the plant surface and progress rapidly though the internal tissues (Cessna et al., 2000; Yang et al., 2007). In order to resemble the field conditions, we used the inoculated senescing petals to infect the plant mature leaves at 30% bloom stage in this study.

2. 4. 2. Anatomical differences in Westar and ZY821 mature leaves during S. *sclerotiorum* infection might contribute to ZY821 tolerance to *S. sclerotiorum*

No previous detailed anatomical studies of the interactions between canola and *S. sclerotiorum* during the infection process had been completed in mature leaves inoculated with *S. sclerotiorum* -infected petals. The interaction processes between canola and *S. sclerotiorum* include pre-penetration where ascospore germinate and hyphae elongate on the leaf surface; penetration where hyphae break through the leaf surface and into the

underlying cell structures; colonization and development of the pathogen within tissues of the leaf; and finally the appearance of visible lesions. Among all these steps, penetration, which determined whether the pathogen could cause the plant disease, was found to be the most critical (Zhang and Wang, 2011). Adhesion of fungal pathogens to the host surface was considered an essential quality for pathogen germination, penetration and colonization (Jamaux et al, 1995). At the pre-penetration stage, the senescing petals were able to adhere to the leaf surface, whereas the *S. sclerotiorum* hyphae that germinated in the absence of petals failed to germinate and colonize the leaf. At the penetration stage, some pathogens can form specialized infection structures like appressoria and infection cushions, while others enter through wounds or natural openings like stomata (Zhang and Wang, 2011). In the case of *S. sclerotiorum*, for most host species, the penetration is directly through the cuticle, not through the stomata (Hegedus and Rimmer, 2005). Results from this study were consistent with this conclusion: instead of growing through the stomata my results clearly showed the penetration of *S. sclerotiorum* hyphae through the cuticle of the upper and lower epidermal surface (Fig.2.4.B; Fig.2.4.C). The current study found that *S. sclerotiorum* form appressoria and infection cushions which penetrate the host tissues (Fig.2.4.C; Fig.2.4.E; Fig.2.4.F). Infection cushions are complex appressoria that increase the adhesion of the pathogen to the host surface. These structures enhance physical contact between fungal toxins or enzymes and the plant in order to effectively penetrate the cuticle and epidermal cells (Nicholson and Epstein, 1991). The greater frequency and larger infection cushions found on the ZY821 leaf surface might be due to this tolerant cultivar being able to suppress the production of *S. sclerotiorum* toxins and enzymes.

Oxalic acid is largely considered to be responsible for the broad infectious capabilities of *S. sclerotiorum* (Hegedus and Rimmer, 2005; Williams et al., 2011; Zhu et al., 2013). It can acidify the environment, disrupt the host redox pathway, and inhibit other defense responses (Williams et al., 2011; Zhu et al., 2013). While numerous crystals were present on the leaf surface in the presence of *S. sclerotiorum*, we cannot say for certain whether these crystals contain oxalic acid, which has been shown to enhance pathogenicity (Heller and Witt-Geiges, 2013). However, this result was consistent with former studies conducted in other oilseeds plants (Huang et al., 2008), bean (Lumsden and Dow, 1973), and sunflower (Smith et al., 1986) in which oxalate crystals were observed on the host surface. Based on our results it can be hypothesized that ZY821 might be able to inhibit the *S. sclerotiorum* oxalic acid production or secretion. This might explain why there were more hyphae on the epidermis in ZY821 that are necessary for fungal penetration and colonization.

The fact that cell death happened ahead of the *S. sclerotiorum* hyphae in both cultivars might support the growth of this necrotrophic pathogen, which obtains nutrients from necrotic host tissues (William et al., 2011). Similar observations have also been reported for other plant-necrotrophic fungal pathosystems such as *Nicotiana tabacum* (tobacco)- *S. sclerotiorum* (Kim et al., 2008; Dickman et al., 2001), tobacco- *Botrytis cinerea*, tobacco- *Cercospora nicotianae* (Dickman et al., 2001), and Arabidopsis- *Botrytis cinerea* (Govrin and Levine, 2000) pathosystems. Additionally, in the *B. napus*- *S. sclerotiorum* pathosystem, cell death was more prevalent in susceptible cultivar. A similar result had also been observed in the *B. napus* susceptible cultivar RQ001-02M2 cotyledons compared with another *B. napus* tolerant cultivar Charlton (Garg et al., 2010).

The cells in Westar leaf tissues were more degraded in advance of the *S. sclerotiorum* hyphae suggesting that Westar leaf cells are more sensitive to the *S. sclerotiorum* invasion.

2. 4. 3. Plant antioxidant system response to *S. sclerotiorum* infection

In the ascorbate-glutathione cycle, ascorbate and glutathione play important roles in maintaining a balanced cellular redox state while their redox status is considered to act as sensors and mediators under pathogen stresses (Foyer and Noctor, 2011). In un-stressed leaves, the ascorbate and glutathione pools are highly reduced and they shift towards oxidized states as the electron donors when the oxidative load increases in the cells (Gillespie and Ainsworth, 2007). In the pea- *S. sclerotiorum* pathosystem, an increase of ascorbate acid was observed in pathogen-challenged plants initially, but levels declined thereafter (Jian et al., 2013). A low concentration of ascorbate, as a result of defense responses, was also observed in Arabidopsis plants inoculated with virulent pathogens (Barth et al., 2004). The decrease in ascorbate and increase in glutathione were also reported in both susceptible and resistant cultivars of barley leaves infected with biotrophic pathogens (El-Zahaby et al., 1995). Interestingly, the low levels of cytoplasmic ascorbate might be necessary for the initiation of signals eliciting downstream defense responses (Foyer and Noctor, 2005). APX, which reduces H_2O_2 to water, was found to be the most important enzyme in H_2O_2 detoxification in plant cells (Omidi, 2010; Ishikawa and Shigeoka, 2008). The elevated APX activity in both cultivars, especially in ZY821, was more pronounced, indicating that ZY821 is highly efficient in the plant leaf antioxidant response (Fig.2.7.A). Also, the marked decline of ascorbic acid

in ZY821 at 48 and 72hpi can be explained by the strong induction of APX activity. Taken together, it is possible that *S. sclerotiorum* tolerance observed in ZY821 may be medicated, in part, be through changes in cellular ascorbate levels that create the necessary signals responsible for inhibiting lesion formation.

Conversely, the accumulation of glutathione is required for plant defense (Foyer and Noctor, 2011), for example, glutathione was found to be essential in Arabidopsis resistance against the hemibiotroph fungal pathogen *Colletotrichum gloeosporioides*, which can use living and dead cells as nutrients (Hiruma et al., 2013). Therefore, the significant increases in glutathione in Westar and ZY821 leaves during the infection prove that the accumulation of glutathione plays an important role in the canola response to *S. sclerotiorum*. Moreover, changes in the glutathione redox ration have been shown to control processes like transcriptional activation (Foyer and Noctor, 2011). A significant decrease in the GSH redox ratio in susceptible cultivars was reported in the *Eucalyptus-Phytophthora cinnamomi* patho-system (Dempsey et al., 2012). Also, glutathione has clearly been implicated in signaling through SA and JA pathways, and the addition of GSH but not GSSG is sufficient to induce the expression of genes responsible for the plant defense response like *PR1* (Gomez et al., 2004). Therefore, GR activity is also critical in the plant antioxidant response by maintaining the reduced ratio of glutathione (Foyer and Noctor, 2011). The pronounced rise of GR activity in ZY821 resulted in the increase in the reduced glutathione ratio during the *S. sclerotiorum* infection. As the only route for GSH oxidation in the ascorbate-glutathione pathway, remarkably declined activity of DHAR in ZY821 further explains the increase in reduced glutathione levels

during the infection. Thus it can be suggested that the increased GSH redox ratio facilitated the suppression of *S. sclerotiorum* development in ZY821.

Overall, the ascorbate-glutathione pathway was quickly triggered in Westar and ZY821 mature leaves at the site of the infection. Since this antioxidant pathway was more active in ZY821, the moderately tolerant cultivar, it may be responsible for delayed lesion formation. Furthermore, the plant antioxidant system can reduce the oxidative cell death, which benefits the necrotrophic fungal pathogen *S. sclerotiorum* (Kuz´niak and Urbanek, 2000; Yang et al., 2010). The stronger antioxidant response in ZY821 might inhibit *S. sclerotiorum* development by providing fewer nutrients. It also explained the anatomy results in which cells were less degraded in advance of the pathogen hyphae in ZY821 infected leaves.

2. 4. 4. Rapid activation of the plant defense response pathway following *S. sclerotiorum* infection

The JA/ET signaling pathways have been shown to be essential for the expression of *PLANT DEFENSIN 1.2* (*PDF1.2*); the pathogen-induced PDF1.2 gene expression is tightly correlated with the rise in endogenous JA levels (van Loon et al., 2006). Expression of genes encoding the rate limiting steps of JA biosynthesis and *PDF1.2* were triggered in both Westar and ZY821 at the early stage of the infection (Fig. 2.8.D). However, more pronounced responses were observed in ZY821 providing evidence that the JA plant defense response pathway was activated in mature leaves following *S. sclerotiorum* infection. Interestingly, the expression of *AOC* and *AOS*, the most important JA biosynthesis genes, consistently increased at the later stage of the infection (24hpi and

48hpi) in both cultivars (Fig.2.8.A; Fig.2.8.B). However, after its significantly increased expression at 12 hpi, *PDF1.2*, the genes responsive to JA, was down-regulated at the later infection stage (Fig.2.8.D). Although those changes in gene expression do not necessarily reflect changes in metabolite levels (Yang et al., 2007), these results indicate that *S. sclerotiorum* might not inhibit JA biosynthesis, but suppress the JA signaling pathway downstream at the later infection stages. The findings are in agreement with other studies that showed JA plays an important role in defense against *S. sclerotiorum* infection (Liang et al., 2008; Yang et al., 2007; Zhao et al., 2007).

The *ERF* family is involved in various developmental and physiological processes in plants. Previous reports demonstrated several *ERF*s to be directly involved in the activation or inhibition of Arabidopsis defense molecules against necrotrophic fungal pathogens, while the overexpression of the *ERF2* gene lead to greater resistance to the fungal pathogen *Fusarium oxysporum* in Arabidopsis (Berrocal-Loba and Molina, 2008). Interestingly, in our results, *ERF2* appeared to significantly increase early during infection (12 hpi) in the susceptible cultivar Westar, however no such increase was observed in the tolerant cultivar ZY821 at any of the investigated time points (Fig.2.8.C). While there was a difference in *ERF2* expression in Westar, it was still possible that other *ERF*s that were not tested in this experiment may be operative to facilitate the plants response to *S. sclerotiorum*. Conversely, the ethylene response pathway may not be responsible for the canola- *S. sclerotiorum* pathosystem interaction. Global RNA profiling experiments will help to provide further insight into this hypothesis.

While *ERF2* may not be responsible for conferring moderate tolerance to *B. napus,* other genes may facilitate the integration of plant defense signal transduction

pathways like *MKK4* and *MPK6* (Takahashi et al, 2007). *MPK6*, one of the best-characterized MAPKs, is activated by a diversity of stimuli including pathogen attack and oxidative stress, and acts as positive mediator in plant defense response. *MKK4* is also involved in plant response to fungal pathogens (Ren et al., 2008). The expression patterns of these genes in Westar and ZY821 are consistent with *PDF1.2* expression in both cultivars and with Westar *ERF2* expression. These results suggest that *MKK4* and *MPK6* are bonded to the JA and ET signaling pathways and this conclusion is supported by previous studies that *MPK6* is required for stress induced ethylene production in Arabidopsis (Liu and Zhang, 2004). Moreover, this ET production enhancing *MPK6* was activated by *MKK4* (Nakagami et al, 2005). Researchers also found that in Arabidopsis, JA can induce the expression of *MPK6* in response to abiotic and biotic stresses (Takahashi et al, 2007).

We next studied the cellular levels of *PR1*, a SA-responsive gene that had been shown to confer plant resistance against fungal pathogens (Lai and Menqiste, 2013). *PR1* is an important fungicidal defense protein, which is strongly conserved and appears to be represented in every plant species investigated to date (van Loon et al., 2006). PR1 is operated through the SA plant defense pathway in plant local and systemic acquired resistance (Yang et al., 2010). Our work showed rapid activation of *PR1* within 12 hour of *S. sclerotiorum* infection in ZY821 and to a lesser extent in Westar (Fig.2.8.H). This result indicated that *PR1* is associated with the canola leaf response to *S. sclerotiorum* infection, which agrees with former studies showing that the *PR1* overexpress Arabidopsis line was more tolerant to *S. sclerotiorum* infection (Chen et al., 2006). Therefore, the SA pathway might be required in canola defense against *S. sclerotiorum*

infection. This assumption is consistent with Guo and Stotz's (2007) conclusion that the SA pathway is required for Arabidopsis defense against *S. sclerotiorum*. The increase in *WRKY28* expression further proves that the SA pathway is involved in the canola response to *S. sclerotiorum*. *WRKY28* is a transcriptional factor that was rapidly induced by SA and is co-regulated with other important genes in SA biosynthesis (van Verk et al., 2011). It has been shown to contribute to plant resistance in Arabidopsis against the necrotrophic fungal pathogen *Botrytis cinerea* (Wu et al., 2011). Activation and expression of *WRKY28* in ZY821 through to 48 hpi might contribute to delayed lesion formation since levels of *WRKY28* were undetectable in the susceptible Westar variety. *WRKY28* has also been shown to integrate with the plants antioxidant system (Wu et al., 2011) supporting my current results of a stronger antioxidant response in ZY821 infected leaves.

The coordination of the plant defense response to *S. sclerotiorum* infection under replicated field conditions was complex. JA, ET and SA resistant signaling pathways all responsive to the *S. sclerotiorum* infection in Westar and ZY821 mature leaves at the site of the infection. Rapid activation of components of the JA, ET and SA plant defense pathways in ZY821 may be responsible for the delayed lesion formation observed under the growth conditions employed. While conditions were optimal for both plant and fungal growth, it was unclear whether other signaling pathways involved in cellular response to necrotrophic pathogens were activated and to what levels they would affect canola – *S. sclerotiorum* interaction and infection.

Chapter 3: Identifying novel regulators in the *Arabidopsis thaliana-Sclerotinia sclerotiorum pathosystem* using bioinformatic analyses

3. 1. Introduction:

Transcriptional networks help to regulate the plant's defense response to pathogen attack (Singh et al., 2002). To understand these complex networks, fine observations of gene activity must be made in plants before and during the infection process (Llorca et al., 2014). Genome-wide high-throughput experiments, such as microarrays, often result in thousands of identified genomic regions and genes that respond to the pathogen (Welch et al., 2014). Therefore, interpreting the high-throughput gene expression profiles and gaining insights into biological mechanisms becomes a new challenge (Subramanian et al., 2005).

The ChipEnrich program can identify predicted transcriptional regulatory mechanisms called transcriptional modules. A transcriptional module is anchored on a set of co-expressed genes that encode any number of biological process or cellular functions. These genes may also be enriched for DNA sequence motifs that lie within the promoter of a gene or a set of genes that serves as the binding site for a transcription factor (Belmonte et al., 2013). Using information from the Arabidopsis ATH1 GeneChip, ChipEnrich is able to identify GO terms (Gene Ontology terms), DNA Sequence Motifs (which regulate transcription factor binding within the promoter of a gene), metabolic pathways and transcription factor (TF) families in coexpressed gene sets (Belmonte et al., 2013). Based on published GeneChip data, this program can link the DNA sequence motifs, which are located in the region 1 kb upstream of the enriched genes' transcription start site (TAIR9, www.arabidopsis.org), with the TFs that are known or predicted to

interact with these motifs (Arabidopsis Gene Regulation Information Server, http://arabidopsis.med.ohio-state.edu/, August, 2009). The enriched genes are characterized into GO terms according to their identified function in plant development and defense (Welch et al., 2014). The predicted transcriptional modules are visualized using Cytoscape (Brady et al., 2007; Davuluri et al., 2003). Previous studies have used ChipEnrich to identify regulators and predicted regulatory processes in large sets of genes associated with seed development (Belmonte et al., 2013, Khan et al., 2014).

Although incredibly useful for the analyses of large genomic datasets and construction of gene regulatory networks, ChipEnrich is currently limited to data obtained from the Arabidopsis ATH1 GeneChip. As a result, microarray datasets obtained for canola- *S. sclerotiorum* cannot be introduced into ChipEnrich since the experiments did not used this technology. Fortunately, *Arabidopsis thaliana*, a model plant organism, is a close relative of canola and has been used as a host model to study the plant defense response to *S. sclerotiorum* (Guo and Stotz, 2007; Stotz et al., 2011; Subramanian et al., 2011; Zhou et al., 2013). However, to date, there is no ATH1 microarray data available on the Arabidopsis- *S. sclerotiorum* pathosystem. Although we could not predict the gene regulatory networks based on Arabidopsis- *S. sclerotiorum* interaction, we could use the microarray datasets from the Arabidopsis-*Botrytis cinerea* pathosystem (Birkenbihl et al., 2012; Ferrari et al., 2007). *B. cinerea* and *S. sclerotiorum* are closely related necrotrophic fungal pathogens notable for their wide host ranges and environmental persistence (Amselem et al. 2011). Analyses of the *S. sclerotiorum* and *B. cinerea* genomes show high collinearity, and moreover, the genes associated with the necrotrophic infection process are similar between them, including genes involved in

plant cell wall degradation and oxalic acid production (Amselem et al. 2011). Thus, it is possible that the Arabidopsis- *B. cinerea* pathosystem could be used to help predict novel regulators of host pathogen interactions that could eventually be translated into canola.

Although the transcriptome analyses published for the Arabidopsis – *B. cinera* system were useful, it was still unclear how large sets of genes were activated and no information about the predicted gene regulatory networks was presented. Furthermore, functional characterization of the host pathogen interaction was lacking. One of the most straightforward methods for accomplishing this is through mutant analysis (Melo-Oliveira et al., 1996).

In this study, loss-of-function Arabidopsis mutants of selected transcription factor genes (Table 3.1) identified from ChipEnrich were established in Arabidopsis and infected with *S. sclerotiorum*. By comparing the susceptibility to *S. sclerotiorum* between wild type Arabidopsis and the loss-of-function mutants, I expect that mutants' defective in TFs that are involved in plant tolerance to be more susceptible to pathogen attack. This will enable identification of possible transcriptional regulators that can ultimately be used to enhance canola tolerance to *S. sclerotiorum.*

3. 2. Materials and Methods:

3. 2. 1. Identification and visualization of predicted transcriptional modules

Publicly available genomic datasets of host-pathogen interactions were interrogated to find lists of genes that responded to necrotrophic fungal infection in the model plant Arabidopsis using the Affymetrix ATH1 GeneChip (http://www.pathoplant.de/documentation_microarrays.php). Studies based on the

Arabidopsis- *B. cinerea* pathosystem were used for further data analysis (Birkenbihl et al., 2012; Ferrari et al., 2007). *B. cinerea* is a necrotrophic fungal pathogen that is closely related to *S. sclerotiorum* and these two pathogens share similar penetration strategies (Amselem et al. 2011). Since there is no available ATH1 datasets based on the Arabidopsis- *S. sclerotiorum* pathosystem, we used Arabidopsis- *B. cinerea* to represent the Arabidopsis- *S. sclerotiorum* interaction. In these two studies, the global gene activity was compared between healthy wild type leaves and *B. cinerea* challenged wild type leaves; healthy *wrky33* mutant leaves and *B. cinerea* challenged *wrky33* mutant leaves; healthy wild type leaves and healthy *wrky33* mutant leaves; *B. cinerea* challenged wild type leaves and *B. cinerea* challenged *wrky33* mutant leaves (Birkenbihl et al., 2012; Ferrari et al., 2007).

We divided those four groups into eight groups based on whether genes were up regulated or down regulated and uploaded each group into ChipEnrich separately. By using ChipEnrich, we identified the transcription factors that associate with DNA sequence motifs that are found upstream of the transcription start site. Cytoscape (version 3.0.1, http://www.cytoscape.org) was used to visualize the association of the enriched GO terms, DNA sequence motifs and TFs. ChipEnrich was used exactly as reported in Belmonte et al. (2013). Briefly, the 'Analysis' tool in ChipEnrich generate four txt files, including <node.txt>, <significant.txt>. The <significant.txt> file represents the network file. In this file, each unique enriched category was separated in different row. Lastly, the <significant.txt> file was introduced into Cytoscape to visualize the network while <node.txt> file was used as the attributes file, which identifies whether a node (first column of the <significant. txt> file) is a pattern, GO term, DNA motif, TF family, or TF.

TFs identified through ChipEnrich were selected for functional characterization based on two criteria: (i) the gene must be enriched in plant response to pathogen attack, (ii) the gene must be novel, which means has not been studied in plant-pathogen interactions. The SALK or CS Mutant lines corresponding to TF-knockouts of interest were ordered from TAIR (http://www.arabidopsis.org/), and characterized as described in section 3.3. SALK and CS Mutant lines are available Transfer-DNA (T-DNA) insertion mutants, and most of them are loss-of-function mutants.

3. 2. 2. Plant materials and growth

Wild type *Arabidopsis thaliana (L.)* Heynh, ecotype Columbia (col) plants and the Columbia background mutants *jasmonate associated myc2-like2* (*jam2*), *myb hypocotyl elongation-related* (*mybh*), *ocs-element binding factor 5* (*obf5*), *udp-glucosyl transferase 85a3* (*ugt85a3*), *unfertilized embryo sac 10* (*une10*), *at2g22760* and *at2g43140* were grown in Sunshine Mix #1 with 50–70% humidity and constant light (100-150 uE/m^2 s^{-1}) at 22˚C. The AGI accession number of mutated TFs and their seed stock identifiers can be found in Table 3.1.

3. 2. 3. Plant inoculation

The ascosporeof *S. sclerotiorum* were purchased from Dr. M.G. Boosalis, Department of Plant Pathology, University of Nebraska, Lincoln, NE, USA. The *S. sclerotiorum* ascosporewere inoculated on canola (*Brassica napus*) senescing petals for 72 hours at room temperature, and then the infected petals were mounted on canola branches. The harvested Sclerotia from canola stems were cultured on PDA medium. An agar plug

Table 3.1: Selected transcription factors from gene regulatory networks for investigation of the Arabidopsis defense response to *S. sclerotiorum*.

Common Name	AGI Name	Seed Stock Name
JAM2	AT1G01260	SALK_150281
MYBH	AT5G47390	CS_807614
OBF5	AT5G06960	SALK_130355
UGT85A3	AT1G22380	SALK_070258
UNE10	AT4G00050	CS_25591
N/A	AT2G22760	CS_355930
N/A	AT2G43140	SALK_123812

(2.5 mm in diameter) containing the advancing edge of *S. sclerotiorum* was removed for Arabidopsis leaf inoculation. One single rosette leaf from each 4-6 week-old plant was inoculated while all inoculations were expected between 2:00 PM to 3:00 PM to exclude variation from circadian rhythm. The lesion sizes and infection rate were recorded at 1-day post inoculation (dpi), 2 dpi, 3 dpi and 4 dpi. Lesion dimensions were measured and recorded before disease progression beyond inoculated leaves. Decay percentage (the number of infected leaves / the number of total leaves) of individual plants was scored after infection expanded beyond the inoculated leaf to assess disease development.

3. 2. 4. Statistical analysis

All experiments were performed using at least fifteen biological replicates. ANOVA tests were used to determine significant differences in lesion sizes and decay percentages were compared between wild type (control group) and mutants after *S. sclerotiorum* inoculation. A *p* value less than 0.05 was considered statistically significant. The raw data were analyzed using JMP version 8.0.1 software.

3. 3. Results

3. 1. Identification and visualization of predicted transcriptional modules

In total, four transcriptional modules that include TFs were identified from the enrichment analysis. Over 100 TFs were found from these networks and predicted to be active during the infection. In order to test the regulators that have not been previously associated with plant defense. An exhaustive online search of these TFs was undertaken.

The majority of identified TFs were previously characterized in plant defense response, such as the *BZIP* family TFs (Kim and Delaney, 2002; Lee et al., 2006; Schütze et al., 2008), the *TGA* family TFs (Kesarwani et al., 2007; Zhang et al., 2003; Zhou et al., 2000) and the *MYC* family TFs (Dombrecht et al., 2007; Zhou et al., 2000).

Figure 3.1 shows the predicted transcriptional module for a set of up-regulated genes in Arabidopsis *wrky33* leaves following *B. cinerea* infection. This module linked a large number of TFs, GO terms with G-box promoter motif. In total 64 GO terms were identified as being enriched (including response to fungus, defense response to fungus, response to reactive oxygen species, leaf senescence, cell wall, membrane, salicylic acid biosynthetic process, defense response, response to oxidative stress, ethylene mediated signaling pathway and defense response to bacterium). I also identified 30 TFs that interact with the G-box promoter motif. The enriched TFs in this module included known regulators involved in the plant defense response, such as *BZIP, TGA* and *MYC* family TFs, as well as regulators that had not been investigated in plant defense, such as *JAM2, JAM3* and *OBF5,* and some uncharacterized regulators, including *AT2G43140.*

Of the identified TFs that have not been studied in plant-pathogen interactions, seven were selected for functional characterization: *JASMONATE ASSOCIATED MYC2-LIKE2* (*JAM2*)*, MYB HYPOCOTYL ELONGATION-RELATED* (*MYBH*)*, OCS-ELEMENT BINDING FACTOR 5* (*OBF5*)*, UDP-GLUCOSYL TRANSFERASE 85A3* (*UGT85A3*)*, UNFERTILIZED EMBRYO SAC 10* (*UNE10*)*, AT2G22760* and *AT2G43140*.

Among the TFs we selected, *JAM2* and *OBF5* were up regulated in *wrky33* infected leaves compared to wild type infected leaves and *wrky33* healthy leaves. *MYBH,*

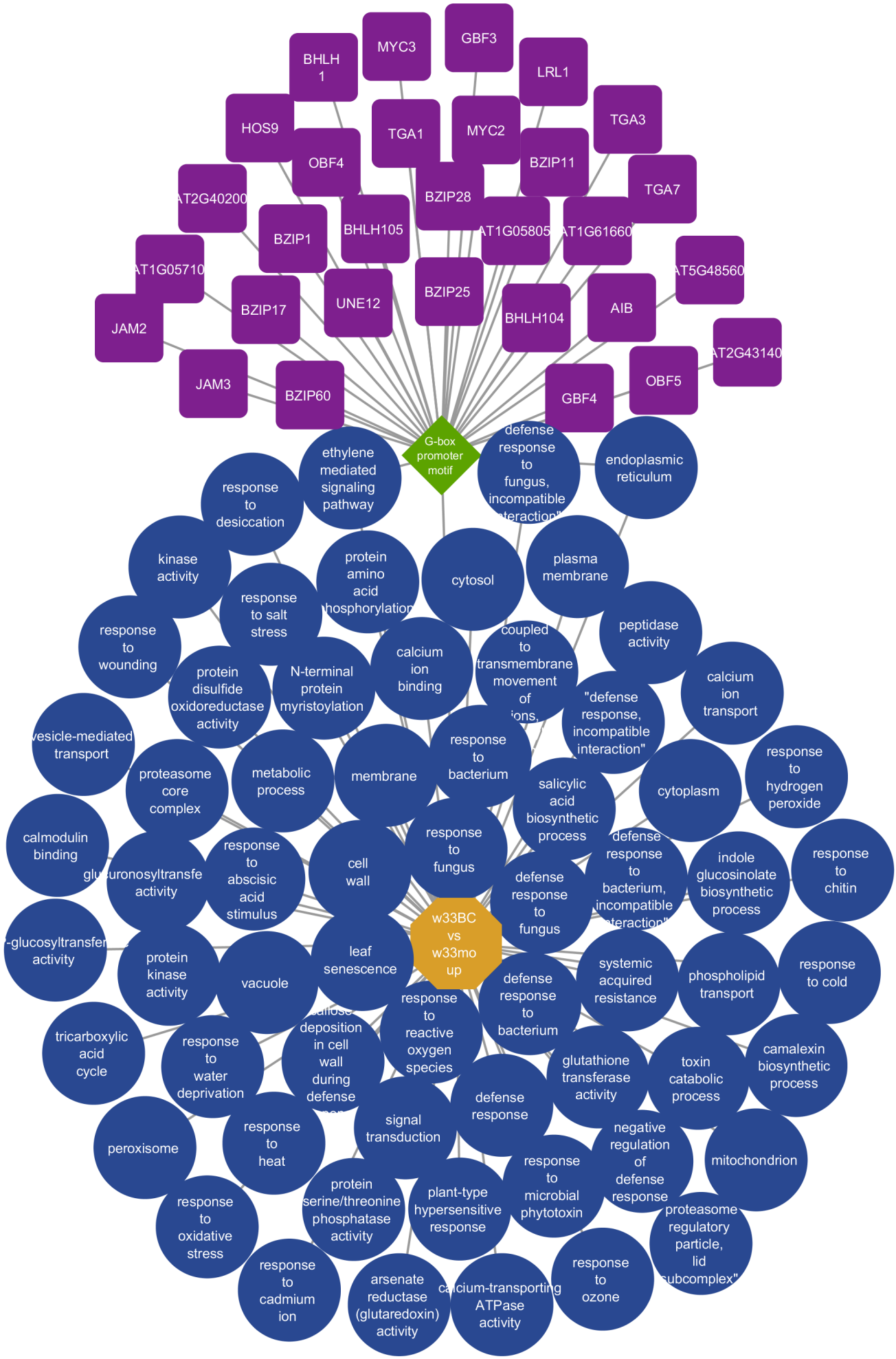
BHLH 1
MYC3
GBF3
LRL1
HOS9
TGA1
MYC2
TGA3
OBF4
BZIP11
T2G40200
BZIP28
TGA7
BZIP1
BHLH105
T1G05805
T1G61660
T1G05710
T5G48560
JAM2
BZIP17
UNE12
BZIP25
BHLH104
AIB
T2G43140
JAM3
BZIP60
GBF4
OBF5
G-box promoter motif
ethylene mediated signaling pathway
defense response to fungus, incompatible
endoplasmic reticulum
response to desiccation
kinase activity
protein amino acid phosphorylation
cytosol
plasma membrane
response to salt stress
response to wounding
peptidase activity
protein disulfide oxidoreductase activity
N-terminal protein myristoylation
calcium ion binding
coupled to transmembrane movement of
"defense response, incompatible interaction"
calcium ion transport
vesicle-mediated transport
proteasome core complex
metabolic process
membrane
response to bacterium
salicylic acid biosynthetic process
cytoplasm
response to hydrogen peroxide
calmodulin binding
response to abscisic acid stimulus
cell wall
response to fungus
defense response to bacterium, incompatible
indole glucosinolate biosynthetic process
response to chitin
activity
w33BC vs w33mo up
defense response to fungus
protein kinase activity
vacuole
leaf senescence
defense response to bacterium
systemic acquired resistance
phospholipid transport
response to cold
tricarboxylic acid cycle
response to water deprivation
deposition in cell wall during defense
response to reactive oxygen species
glutathione transferase activity
toxin catabolic process
camalexin biosynthetic process
peroxisome
response to heat
signal transduction
defense response
negative regulation of defense response
mitochondrion
response to oxidative stress
protein serine/threonine phosphatase activity
plant-type hypersensitive response
response to microbial phytotoxin
proteasome regulatory particle, lid subcomplex"
response to cadmium ion
arsenate reductase (glutaredoxin) activity
ATPase activity
response to ozone

Figure 3.1: Predicted transcriptional module for the up-regulated genes in Arabidopsis *wrky33* leaves after *Botrytis cinerea* infection. Orange octagon: the pattern of co-expressed genes; Blue circle: enriched Gene Ontology (GO) terms; Green diamond: enriched DNA sequence motif; Purple squircle: transcription factors. The enrichment level of GO terms, DNA motifs and transcription factors is $P< 0.001$ (hypergeometric distribution).

wild-type healthy leaves. *UNE10* was up regulated in *wrky33* healthy leaves compared with wild-type healthy leaves, *AT2G43140* was identified from *wrky33* infected leaves compare with *wrky33* healthy leaves (Table 3.2).

3. 3. 2. Differences in plant susceptibility to *S. sclerotiorum* between Arabidopsis wide type and loss-of-function mutants

For all seven mutants (*jam2*, *mybh*, *obf5*, *ugt85a3*, *une10, at2g22760* and *at2g43140*) tested in this experiment, *jam2* showed significant susceptibility to *S. sclerotiorum* infection compared to the wild-type (Fig 3.2). At 1-day post inoculation (dpi), a soft-rotting necrosis occurred in all cultivars, however, the disease symptoms had not expanded beyond the inoculated leaves while the inoculated leaves were partially alive. At this stage, the average lesion size on wild-type plants was 0.88 cm^2 whereas *jam2* leaves have 1.31 times bigger lesion size compared to the control group. The lesion sizes on *mybh* and *at2g22760* were similar to the wild-type. Interestingly, the lesions were significantly smaller on all the other mutant leaves; the average lesion sizes were 0.28, 0.29, 0.43 and 0.24 cm^2 in *obf5, ug85a3, une10* and *at2g43140* respectively (Fig.3.3.A).

At 2 dpi, the infection expanded beyond the inoculated leaf to the stem and nearby leaves. Once the entire leaf was consumed, the *S. sclerotiorum* hyphae developed on other leaf tissues, and as a result, the lesion size could no longer be determined. Instead, the percentage of whole-plant decay was estimated. In wild-type plants, 7.5 % of the recorded plant tissues were infected at this stage. The decay percentage of *jam2* was 3.9 times higher than the control group (29.6%). Besides *mybh* and *obf5* which shared

Table 3.2: Presence of identified transcription factors in co-expression gene sets of Arabidopsis challenged with *Botrytis cinerea.*

Transcription factors	Arabidopsis wild-type before and after the *Botrytis cinerea* infection	*wrky33* before and after the *Botrytis cinerea* infection	Healthy *wrky33* compare to healthy wild - type	*Botrytis cinerea* infected *wrky33* compare to infected wild - type
JAM2	+	+		+
MYBH				
OBF5		+		+
UGT85A3	+			
UNE10			+	
AT2G22760	+			
AT2G43140		+		

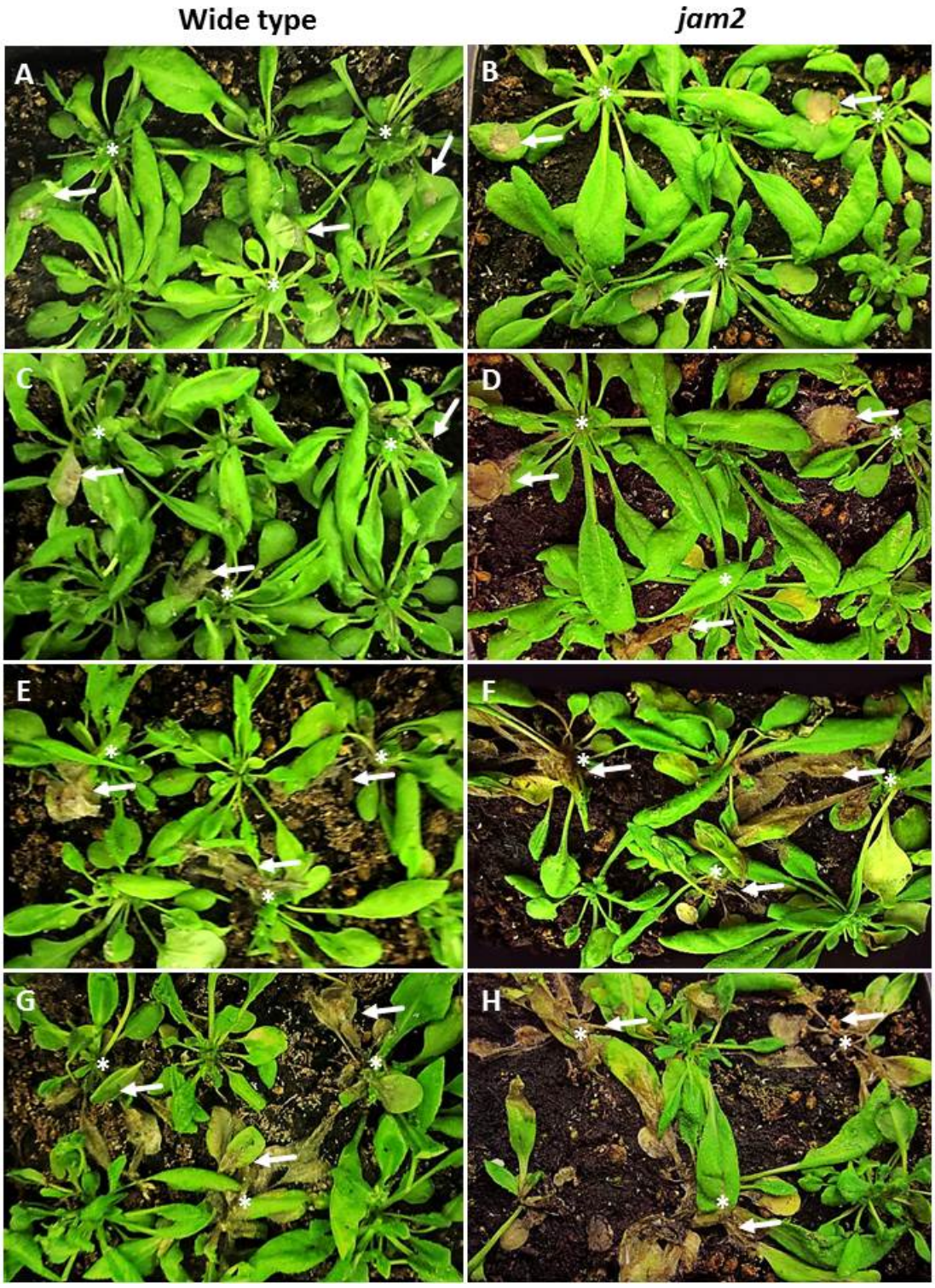
Wide type
jam2
A
B
C
D
E
F
G
H

Figure 3.2: Differences between Arabidopsis wild type (wt) and *jam2* mutant susceptibility to *S. sclerotiorum* mycelial plug inoculation over time. For all seven mutants (*jam2*, *mybh*, *obf5*, *ugt85a3*, *une10, at2g22760* and *at2g43140*) that were used in this experiment, *jam2* shows significant susceptibility to *S. sclerotiorum* infection compared to the wild-type. The plants infected with *S. sclerotiorum* are labeled with *, while the control plants (uninfected plants) are unlabeled. A: wt at 1-day post inoculation (dpi), the arrows point to the lesion; B: *jam2* at 1 dpi, the arrows point to the lesion; C: wt at 2 dpi, the arrows point to the lesion; D: *jam2* at 2 dpi, the arrows point to the lesion; E: wt at 3 dpi, the arrows point to the lesion; F: *jam2* at 3 dpi, the arrows point to the lesion; G: wt at 4 dpi; H: *jam2* at 4 dpi, the arrows point to the lesion.

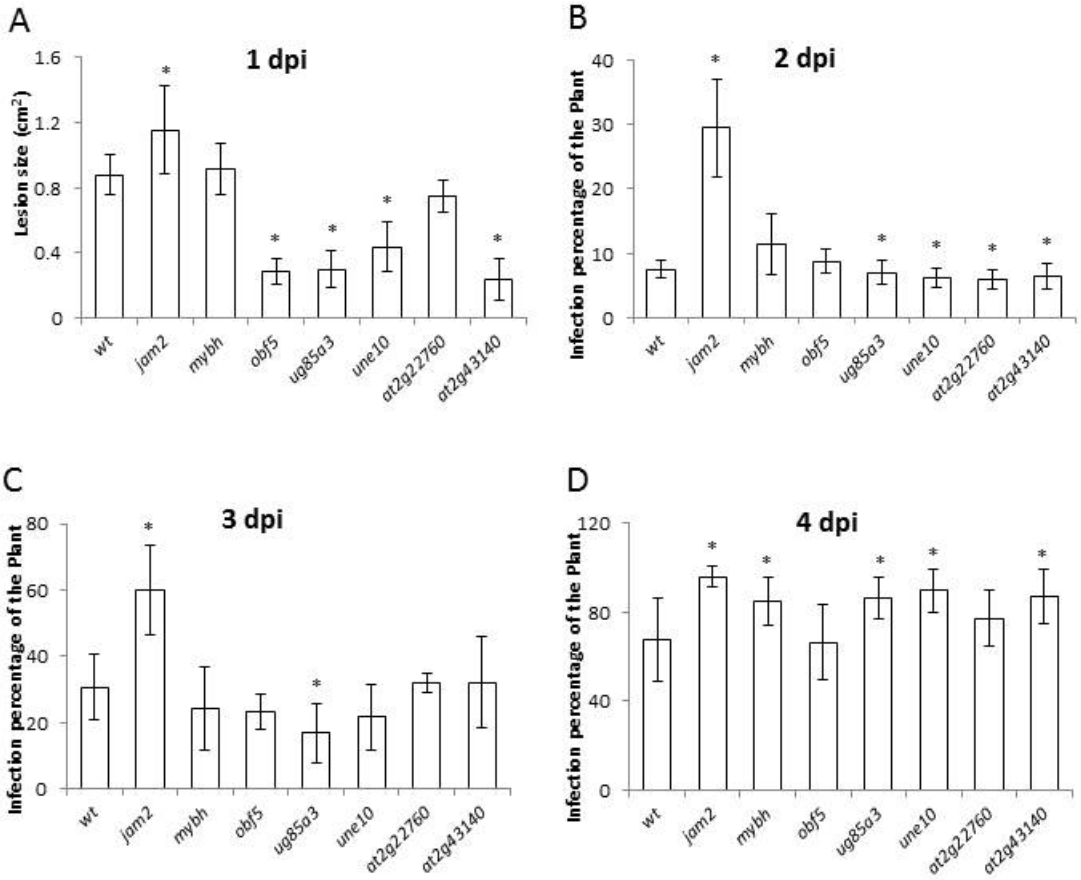
A
1 dpi
Lesion size (cm²)
1.6
1.2
0.8
0.4
0
wt
jam2
mybh
obf5
ug85a3
une10
at2g22760
at2g43140
B
2 dpi
Infection percentage of the Plant
40
30
20
10
0
C
3 dpi
Infection percentage of the Plant
80
60
40
20
0
D
4 dpi
Infection percentage of the Plant
120
80
40
0

Figure 3.3: Lesion sizes and decay percentages on Arabidopsis wild-type (control group), *jasmonate associated myc2-like2* (*jam2*), *myb hypocotyl elongation-related* (*mybh*), *ocs-element binding factor 5* (*obf5*), *udp-glucosyl transferase 85a3* (*ugt85a3*), *unfertilized embryo sac 10* (*une10*), *at2g22760* and *at2g43140* mutants leaves after *S. sclerotiorum* mycelia plug inoculation. A: The lesion sizes (cm^2) on Arabidopsis wild type and mutants infected leaves at 1-day post inoculation (dpi); B: The decay percentages on Arabidopsis wild-type and mutant infected leaves at 2 dpi; C: The decay percentages on Arabidopsis wild-type and mutant infected leaves at 3 dpi; D: The decay percentages on Arabidopsis wild-type and mutant infected leaves at 4 dpi. * indicates there is a significant difference compare to the control group ($p < 0.05$).

similar susceptibility with the wild-type, all the other mutants had significantly less decay symptoms, the average decay percentages were 7%, 6.12%, 6% and 6.35% in *ug85a3, une10, at2g22760* and *at2g43140* respectively (Fig.3.3.B).

At 3 dpi, *S. sclerotiorum* consumed 31% leaves in wild type plants, whereas the decay percentage of *jam2* was 60%. However, for the other mutants, only the decay percentage in *ugt85a3* was significantly smaller (16.8%) than wild type; all the other mutants shared the same infection percentages with the control group (Fig.3.3.C).

At 4 dpi, *S. sclerotiorum* infection damaged 67.5% of tissues in wild type Arabidopsis while the decay percentages for the *jam2* mutant reached 96%. Interestingly, only *obf5* showed very similar infection percentage with the control group, whereas all the other mutants were significantly more decayed than the wild type plants. The average decay percentages were 85%, 86.5%, 89.7%, 77.3% and 87.2% in *mybh, ug85a3, une10, at2g22760* and *at2g43140* respectively (Fig.3.3.D).

After 4 dpi, all infected plants were completely damaged by *S. sclerotiorum*. Therefore the data were no longer recorded and compared.

4. Discussion

This study demonstrated that the published datasets could be used to predict transcriptional regulators and identify sets of genes that are coexpressed in the plant defense response to pathogen attack. Previous microarray studies showed large and coordinated changes in canola and Arabidopsis gene activity in response to *S. sclerotiorum* (Liu et al., 2005; Yang et al., 2007). One key to understand the plant defense response is to define the regulatory circuitry that governs those diverse

coexpressed gene sets. Thus we could use ChipEnrich to uncover new and significant information that underlies the plant defense response to necrotrophic fungal pathogens.

As the major model plant with a whole sequenced genome, there are a large number of publicly available Arabidopsis mutant resources online (http://www.arabidopsis.org/), which saves researchers a large amount of time and money. The most readily available Arabidopsis mutants, including the Salk and CS lines, are Transfer-DNA (T-DNA) insertion mutants (O'Malley and Ecker, 2010; Wang, 2008). T-DNA insertion is a highly effective mutagen for producing mutants (Wang, 2008), it can be used for insertional mutagenesis (loss-of-function mutants) and it can also serve as an efficient vehicle for delivering target genes into plant cells (gain-of-function mutants) (Gao and Zhao, 2013). Since most available T-DNA mutants are loss-of-function lines, our experiments can start with the Arabidopsis loss-of-function mutants (Wang, 2008). The T-DNA could be probed by PCR with a T-DNA specific primer pair to search for inserts in the genes of interest. The primers are also available online (http://signal.salk.edu/tdnaprimers.2.html). The confirmed knock out mutants can then be challenged with *S. sclerotiorum* to test whether mutants are more susceptible to *S. sclerotiorum*.

In this study, when compared with wild type, the percent infection was consistently pronounced in the *jam2* mutant from 1 dpi to 4 dpi and almost reached 100% decay percentage at 4 dpi when the wild-type was not even 70% decayed. This result indicated that *JAM2* is an important regulator in the Arabidopsis defense response to *S. sclerotiorum*. *JAMs* are JA-responsive genes, while jasmonic acid (JA) regulates a wide spectrum of plant processes such as growth and development as well as defense systems

against biotic and abiotic stresses (Yang et al., 2010). Under pathogen attack, transcription factors can reprogram JA biosynthesis, which is leads to increased physiological concentrations of JA in the plant (Sasaki-Sekimoto et al., 2013). *JAM1, 2, 3* belong to the *JAM* family and are closely related to *JASMONATE INSENSITIVE 1* (*JIN1*/*MYC2*) (Sasaki-Sekimoto et al., 2013), an important regulator in the JA pathway (Bari and Jones, 2009; Pozo et al., 2008). *MYC2* has been reported to positively regulate JA-mediated resistance to insect pests, such as *Helicoverpa armigera*, and tolerance to oxidative stress, via enhanced ascorbate-glutathione redox cycling (Dombrecht et al., 2007). In Arabidopsis, *JAM1* shares 30% amino acid identity with *MYC2*, while *JAM2* is 47% identical to *JAM1* at the amino acid level (Sasaki-Sekimoto et al., 2013). Under optimized conditions, the deficient mutant *jam1jam2jam3* shares similar levels of expression of *PDF1.2* with the wild-type (Sasaki-Sekimoto et al., 2013). *PDF1.2* is an antifungal peptide is often used as a marker gene for the induction of the JA and ET-dependent signaling pathway (Thomma et al., 2002; van Loon et al., 2006). In the wild-type, *JAM1* and *JAM2* expression are induced under stress conditions including wounding and drought stress, while *JAM3* expression is almost unchanged under any conditions (Sasaki-Sekimoto et al., 2013). However, there is no information about how *JAMs* participates in the plant defense response to pathogen attack. Our results indicate that *JAM2* is also associated with Arabidopsis tolerance to *S. sclerotiorum* and might up-regulate genes associated with the JA signaling pathway.

The MYB family protein is large, functionally diverse and represented in all eukaryotes. Most MYB proteins function as transcription factors that control gene expression by binding to DNA via MYB domain repeat sequences (Dubos et al., 2010).

In Arabidopsis, they play important roles in many developmental processes and are involved in the regulation of primary and secondary metabolisms, such as cell wall biosynthesis, lignin biosynthesis and the secondary wall deposition in anthers (Dubos et al., 2010). Although more than 100 MYBs have been identified in Arabidopsis, the function of many MYB proteins remains unknown (Kwon et al., 2013). MYBH is a MYB-like transcription factor protein that is involved in hypocotyl elongation (Kwon et al., 2013). However, no literature describes whether *MYBH* is involved in the plant defense response. In our experiments, the decay percentages were very similar between *mybh* mutant and wild type at 1 dpi, 2dpi and 3dpi, but significantly higher in *mybh* mutant at 4 dpi. This result indicates that the *MYBH* might participate in the plant tolerance at later infection stages.

In this study, the fact that *obf5, ugt85a3, une10, at2g22760* and *at2g43140* had significantly smaller decay rates at 1 dpi and/or 2 dpi but had significantly larger infection percentages at 4 dpi is also interesting. OBF5 is a bZIP (basic region-leucine zipper) protein that binds to the promoter region of *GST6,* which is induced by auxin, salicylic acid (SA) and H_2O_2 and responds to oxidative stress (Suzuki et al., 2001). Like *JAM2* and *MYBH*, there is no information about how *OBF5* is directly involved in the plant defense response to pathogens. Unfortunately, there is little information about *UGT85A3*, *UNE10, AT2G22760* and *AT2G43140* available. Our results suggest that these regulators might negatively regulate the plant defense response at the early infection stages but play a positive role at later infection stages.

Overall, all TF mutants showed varied susceptibility to fungal pathogen attack, but one mutant, *jam2*, in particular stood out from the rest. The disease incidence was

much higher in *jam2* compared to the wild-type and the other mutants. Since *JAM2* is a JA-responsive gene, this result supports previous data that the JA signaling pathway is associated with the canola defense response to *S. sclerotiorum*. Therefore if the *jam2* gain-of-function mutant shows increased fungal tolerance, the possibility of utilizing *JAM2* to enhance plant tolerance to necrotrophic fungal pathogens in field cultivars should be explored by overexpressing this gene in canola.

Chapter 4: General discussion and Future direction

In this study, we clearly demonstrated the requirement of senescing petals for infection of mature canola leaves with *S. sclerotiorum* ascospores. To assess how a susceptible and moderately tolerant variety of canola responds to *S. sclerotiorum*, we tested the plant cellular, physiological and molecular responses. We found the moderately tolerant variety, ZY821, was able to slow the penetration of *S. sclerotiorum* hyphae and that the antioxidant, JA, ET and SA signaling pathways were rapidly induced in Westar and ZY821 leaves at the site of the infection.

The information generated from studying the plant-pathogen interaction at the cellular, physiological and molecular levels could aid in further understanding the plant physiological, biochemical and molecular response to pathogen interactions. The research reported in this thesis successfully revealed that the plant structural response, antioxidant system and SA, JA, ET signalling pathways are all involved in the canola defense response to *S. sclerotiorum*, and likely contributed to ZY821 tolerance to *S. sclerotiorum* infection. At the cellular level, ZY821 can suppress the penetration and colonization of *S. sclerotiorum* hyphae at the epidermal, mesophyll and vasculature tissues. Thus, it is necessary to understand how ZY821 responds to *S. sclerotiorum* at the spatial level. In order to achieve this objective, we can use Laser Micro Dissection (LMD) technology to isolate the epidermis, mesophyll and vasculature tissues separately. By using a laser coupled with a microscope that focuses onto the tissues of interest, we can collect specific sets of cells and profile their gene expression, which will give us a spatial resolution of the molecular responses to *S. sclerotiorum* in canola (Chandran et al., 2010). Also, the SEM results suggested that ZY821 might be able to inhibit the production of *S.*

sclerotiorum oxalic acid, the most important toxin involved in *S. sclerotiorum* pathogenicity. Therefore, we should test oxalic acid concentrations on Westar and ZY821 leaf tissues in the future.

qPCR results revealed the induction of the SA, JA and ET signalling pathways in canola during *S. sclerotiorum* infection. Although JA biosynthesis genes were induced, genes downstream of the JA and ET pathways such as *PDF1.2* (the final antifungal product of the JA and ET pathways) were suppressed at later stages of infection. However, there are a lot of genes that were not tested and may involve in canola defense response to *S. sclerotiorum*. In future studies, global RNA profiling experiment (RNA sequencing) will help to provide further insight into how *S. sclerotiorum* suppresses the canola defense response and to identify genes that may be operative to facilitate canola tolerance to *S. sclerotiorum.* RNA sequencing technology is a quick, relatively inexpensive and comprehensive tool to sequence complex populations of RNAs (Brautigam and Gowik, 2010). It can provide a greater scope to identify candidate genes in the canola tolerant response to *S. sclerotiorum* (Stapley et al., 2010). Moreover, by combining RNA sequencing technology with LMD, we can investigate gene expression changes in specific cells at different infection stages. Therefore, we can obtain datasets with extremely high spatial and temporal resolution.

We were also interested in identifying regulators of host pathogen interactions that have yet to be characterized in canola. However, since the functional characterization of these genes takes years in the lab, we decided to test for novel regulators of the plant defense response in Arabidopsis. In Arabidopsis, a number of mutants defective in gene function are available and the quick lifecycle of this plant also makes it an excellent

model to study. In this study, we used published datasets to predict and identify possible regulators of the plant defense response using bioinformatics approaches. In these approaches, I identified a series of transcriptional modules, which consist of enriched GO terms, DNA sequence motifs of the enriched genes and the transcriptional factors that interact with those motifs. By analyzing these coexpressed genes, researchers can accurately predict the biological function of sets of genes and provide fresh insight into host-pathogen interactions. Host-pathogen interactions involve changes in the expression of larges sets of genes (Liu et al., 2005; Yang et al., 2007). By defining the transcriptional regulators that govern these gene sets we can help researchers to better understand the plant defense response.

Bioinformatics of publicly available data sets involving the Arabidopsis – Botrytis pathosystem identified a suite of TFs thought to play a role in the plant defense response. We then challenged the Arabidopsis mutants defective in these TFs with Sclerotinia. We found the percent infection was consistently pronounced in the *jam2* mutant from 1 dpi to 4 dpi compared with wild type. Interestingly, our qPCR results from Chapter 2 suggested that the jasmonic acid (JA) signal pathway is involved in the plant defense response to *S. sclerotiorum,* Thus, *JAM2* might be an important regulator in the Arabidopsis defense response to *S. sclerotiorum*. To further test this hypothesis, we can use qPCR technology to compare the activity of *PDF1.2* in healthy and infected tissues between *jam2* mutants and the wild-type. *MYC2,* the gene closely related to *JAM2,* is able to enhance the plant antioxidant system (Dombrecht et al., 2007; Sasaki-Sekimoto et al., 2013). Since we found the ascorbate glutathione antioxidant system might contribute to canola tolerance to *S. sclerotiorum* infection, we should also compare the differences of the ascorbate-

glutathione pathway in response to *S. sclerotiorum* between *jam2* and wild type plants. For homologs of the *JAM* family, loss-of-function mutants of *jam1* and *jam3* could also be challenged with *S. sclerotiorum* to identify whether they are involved in Arabidopsis tolerance to *S. sclerotiorum.*

Gain-of-function studies are another tool to identify possible gene functions in the plant and are particularly important when studying large gene families (Kuromori et al., 2009). In gain-of-function mutant analysis, a member of a gene family can produce a mutant phenotype without interference from other members in this family (Nakazawa et al., 2001). Often the mutant phenotypes induced by loss-of-function and gain-of-function approaches are complementary to each other (Bolle et al., 2011). Driven by a strong promoter, such as CaMV35S that causes gene expression throughout the life cycle of the plant in most tissues (Wang et al., 2012), we can overexpress a gene that may result in gain-of-function phenotypes (Bolle et al., 2011). Confirmed transgenics will then be tested using quantitative PCR to assess activity of the transgene, and plants will be challenged with *S. sclerotiorum.* For example, if the *jam2* gain-of-function mutant shows more tolerance to *S. sclerotiorum* infection, we can confidently conclude that *JAM2* is involved in Arabidopsis tolerance to *S. sclerotiorum.* However, such differential responses to pathogen attack may be the result of the induced gene expression, the changes in cellular redox system or through specific signaling pathways, such as the SA, JA and ET pathways. In order to understand how *JAM2* regulates the plant defense response to *S. sclerotiorum*, we can compare the *jam2* gain-of-function mutant response to *S. sclerotiorum* at the cellular, physiological and molecular levels to that of the wild-type.

Since our primary results showed promise in combining the ChipEnrich data analysis and loss-of-function mutant assay in plant-pathogen interactions, in future studies we can analyze more publically available datasets though ChipEnrich to identify additional candidate genes. These data should reveal both conserved regulators across many plant pathosystems or regulators specific to a given plant pathogen interaction.

Genes that are able to enhance plant tolerance will be introduced and overexpressed in canola to confirm the role of these regulators in the plant defense response against *S. sclerotiorum*. For canola mutants that appear more tolerant to *S. sclerotiorum*, we will compare their robustness and productivity to other commonly used canola cultivars under field conditions and *S. sclerotiorum* attack. Different *S. sclerotiorum* isolates will be collected from the field to test whether our tolerant varieties show consistent protection to other *S. sclerotiorum* isolates. This type of information would be invaluable to increasing plant tolerance to *S. sclerotiorum* and possibly other necrotrophic fungal pathogens present in the field.

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