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Characterization of the *Brassica napus*-fungal pathogen interaction

by

Bo Yang

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Examining Committee

Dr. Nat Kav (Supervisor)

Dr. Stephen Strelkov

Dr. Michael Deyholos

Dr. Walter Dixon

Dr. Mark Gijzen (External Examiner)

Abstract

Sclerotinia sclerotiorum is a devastating pathogen causing stem rot in *Brassica napus* (canola). Microarray analysis was performed to investigate pathogen-induced transcript profiling in *B. napus* responses to *S. sclerotiorum*. Several genes were identified, which included defensins, those encoding proteins involved in oxidative burst, the biosynthesis of jasmonic acid (JA), and several transcription factors (TFs), but not salicylic acid (SA)-related genes. To further characterize the roles of SA, JA and ethylene (ET) in the response of *B. napus* and *Brassica carinata* (tolerant species) to *S. sclerotiorum*, the expression of five genes known to respond to these phytohormones were investigated. We observed that *S. sclerotiorum* triggered JA/ET signaling in *B. napus*. Furthermore, the heterologous expression of 1-aminocyclopropane- 1-carboxylate (ACC) deaminase, which reduced ET levels, enhanced the susceptibility of *B. napus* to *S. sclerotiorum*. Our microarray analysis also revealed the importance of TFs in mediating responses of *B. napus* to *S. sclerotiorum*. To probe the involvement of one such TF family in *B. napus*, the WRKYs, public sequence databases were mined. Three groups of *B. napus* WRKYs were indentified from a phylogenetic tree and four selected ones were shown to localize to the nucleus using GFP fusions. *Sclerotinia sclerotiorum* and *Alternaria brassicae* (another necrotrophic pathogen affecting canola) and phytohormone-induced expression of representative WRKYs from each clade was also investigated using quantitative real-time PCR. In another aspect of our study, two recombinant single chain variable fragment (ScFvs) antibodies specific for a *S. sclerotiorum* endopoly-

galacturonase (SSPG1d) were isolated and characterized. Our results indicated that these antibodies may have utility in the detection of this pathogen when used with other *S. sclerotiorum*-specific antibodies in a panel format assay. Transgenic Arabidopsis expressing the ScFvs were evaluated for tolerance to *S. sclerotiorum* and it was observed that the heterologous expression of the cDNAs encoding these ScFvs did not enhance the tolerance of Arabidopsis. However, additional research aimed at stabilization of the ScFvs and/or their localization must be conducted together with research into their usefulness in imparting tolerance in *B. napus* to this pathogen, since the observed effects in this species may be different from *A. thaliana*.

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List of Abbreviations

AGI, Arabidopsis genome initiative

APX, ascorbate peroxidase

AOC, allene oxide cyclase

At, *Arabidopsis thaliana*

BAP, 6-benzylaminopurine

bp, base pairs

Bn, *Brassica napus*

BSA, bovine serum albumin

bZIP, basic leucine zipper

CaMV, cauliflower mosaic virus

CDS, coding region

CK, cytokinin

CWDE's, cell wall degrading enzymes

dNTP, deoxyribonucleotides

ERF, ethylene-responsive element binding factor

EST, expressed sequence tag

ET, ethylene

ETI, effector-triggered immunity

FDR, false discovery rate

Fwd, forward

GFP, green fluorescent protein;

GIP1, glucanase inhibitor protein-1

GST, glutathione S-transferase

IPTG, isopropyl-beta-D-thiogalactopyranoside

JA, jasmonic acid

LOX, lipoxygenase

LRR, leucine-rich repeat

MAPK, mitogen-activated protein kinase

MAPKK, mitogen-activated protein kinase kinase

MAPKKK/MAP3K, mitogen-activated protein kinase kinase kinase

MW, molecular weight

NLS, nuclear localization signal

NPR1, nonexpressor of pathogenesis-related genes 1

Os, *Oryza sativa*

OXO, oxalate oxidase

PAMP, pathogen-associated molecular patterns

PGIPs, Polygalacturonase-inhibiting proteins

PR, pathogenesis-related

PRX/PER, peroxidase

qRT-PCR, quantitative reverse transcription-polymerase chain reaction

RACE, rapid amplification of cDNA ends

ROS, reactive oxygen species

ROIs, reactive oxygen intermediates

RT-PCR, reverse transcription-polymerase chain reaction

Rvs, reverse

SA, salicylic acid

SAM, significance analysis of microarray

SAR, systemic acquired resistance

S.D., standard deviation

SDS, sodium dodecyl sulfate

SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SOD, superoxide dismutase

TAIR, the Arabidopsis Information Resource

TF, transcription factor

TIGR, the Institute of Genome Research

WT, wild-type

XTH, xyloglucan endotransglucosylase

ZIM, Zinc-finger protein expressed in inflorescence meristem

Chapter 1 Literature Review

Introduction

Using plant breeding techniques, Canadian scientists Keith Downey and Baldur Stefansson developed canola (*Brassica napus* L.) in the 1970s by lowering the two harmful substances, erucic acid and glucosinolates which were abundant in rapeseed oil (Snowden et al. 2007; Stefansson 1983). Canola oil contains only 0.5 to 1% erucic acid, which is well below the 2 percent limit set by the USDA (Snowdon et al. 2007). Erucic acid has been thought to be responsible for contributing to oil rancidity and is also believed to be carcinogenic, whereas glucosinalates cause the damage to the vital organs as well as interference with metabolic processes, which is a sulphur-containing secondary metabolites (β-D-thiolglucose group, a sulfonated oxime moiety and different sides chains of amino acids) (Beare et al. 1963; Tripathi and Mishra 2006; Graser et al. 2000). Canola is a major edible oilseed crop in Canada because of the size of the area of adaptation and the development of varieties with improved quality. Canola production contributes over $13 billion to the Canadian economy per year (http://www.canola-council.org/industry.html). In 2000, canola was the third major source of vegetable oil in the world after soybean and palm oil, and was the second leading source of protein meal following soybean (Snowdon et al. 2007). In 2008, harvested canola seed production was around 13 million tonnes in Canada (http://www.canola-council.org/acreageyields.aspx).

Canola yield is affected by both abiotic and biotic factors including temperature extremes, weeds and diseases (http://www.canola-council.org/contents10c.aspx). For example, canola diseases include bacterial diseases (bacterial black rot, bacterial leaf spot, bacterial soft rot and scab), fungal diseases (Alternaria black spot, blackleg, Sclerotinia stem rot and clubroot), viral diseases (cauliflower mosaic virus, beet Western yellows virus), and phytoplasmal disease (Aster yellows). Among the diseases affecting canola, those caused by fungal-like pathogen/fungal pathogens including clubroot, blackleg,

Sclerotinia stem rot, Alternaria black spot and clubroot have the potential to cause devastating crop losses (http://www.canola-council.org/chapter10c.aspx).

In general, fungal pathogens can cause significant crop losses in most commodities due to the unusual capability of breaking the intact surfaces of host (Egan and Talbot 2008). Fungal pathogens develop infection structures such as appressoria to penetrate plant cells and later to divert nutrients to their own growth and also to secrete effector proteins (Avr proteins) for the suppression of plant defense mechanisms (Egan and Talbot 2008). Fungal pathogens can be divided into three classes: necrotrophs, biotrophs and, more rarely, hemibiotrophs based on the mechanism of infection (Oliver and Ipcho 2004). In brief, those fungal pathogens that derive energy from living cells are biotrophs and those that extract energy from dead cells are referred to as necrotrophs (Lewis 1973). Hemibiotrophy is defined as an early period of biotrophy followed by growth of "necrotrophic hyphae" (Perfect and Green 2001).

Mechanisms of fungal infection

Studies on the strategies of fungal infection have been recently reviewed (Egan and Talbot 2008). Related functional studies on appressoria in the rice blast pathogen *Magnaporthe grisea* observed that generated reactive oxygen intermediates (ROIs), NADPH oxidase, the GTPase *MgRho3* and mitosis are necessary for the function and/or development of appressoria (Egan et al. 2007). Also, the role of cell wall degrading cutinases are well studied (Egan and Talbot 2008). For example, *CUT1*, a cutin-degrading enzyme, was observed to be required for the pathogenicity in the rice blast fungus (Sweigard, et al. 1992), whereas *CUT2* was upregulated during penetration *in vivo* (Skamnioti and Gurr 2007). Moreover, once fungus successfully penetrates, the multilayered plant defense response needs be overcomed for the fungus to colonize host tissue (Egan and Talbot 2008). For instance, the detoxification of H_2O_2 in *Ustilago maydis* was mediated by yeast AP-1-like (*Yap1*) (Molina and Kahmann 2007) while the induction of host defense was prevented though *SSD1*-mediated interference assembly of the fungal cell wall (Tanaka et al. 2007).

The mechanisms of host manipulation by fungal pathogens include: to perceive pit field sites at which the contact face of the two primary walls are unusually thin and plasmodesmata occur in high density (Kankanala et al. 2007); to constrict plasmodesmata by the fungal hypha (Kankanala et al. 2007); to manipulate host cytoskeleton and lead to the ingression of pathogens (Schmidt and Panstruga 2007); to secrete fungal effectors essential for the advance of fungi within host tissue (Chisholm et al. 2006). Effector proteins are delivered directly into plant cells were supported by the recognition of avirulence (Avr) proteins by the intracellular host resistance (R) proteins (Jones and Dangl 2006). For example, a P-type ATPase-encoding gene (*MgAPT2*) in *M. grisea*, has provided some insights into the mechanisms by which plant pathogenic fungi deliver effector proteins into plant cells (Gilbert et al. 2006). Analyzing the genome of pathogenic fungi for functional analysis of these genes have the potential to elucidate more effector proteins (Egan and Talbot 2008). So far, genomic, transcriptomic, proteomic, and metabolomic studies have been used to investigate the mechanisms by plant pathogens to infect and colonize hosts (Allwood et al. 2007; Cao et al. 2009; Li et al. 2004a; Tyler et al. 2006; Yajima et al. 2008). These studies of pathogenicity and virulence factors are important because they help to explain disease processes and the identified factors can be targeted for chemical control (Torto-Alalibo, et al. 2007).

Understanding the evolution of virulence and pathogenicity factors is a major goal of plant pathologists (Sacristan and Garcia-Arenal 2008). Virulence factor is the degree of damage caused in the host while pathogenecity is the ability of a pathogen to cause disease as defined by the American Phytopathological Society (APS) (Sacristan and Garcia-Arenal 2008 and reference therein). Both virulence factor and pathogenecity factors are thought to be negatively associated with host fitness (D'Arcy et al. 2001). To understand the evolution of virulence factors, it is necessary to explain the relationship between parasite fitness (multiplication of pathogens within host cells, transmission within hosts) and its virulence; the relationship between virulence and adaptation of parasites to hosts (Sacristan and Garcia-Arenal 2008). Based on the trade-off hypothesis, the traits

of parasite fitness and virulence of parasites are positively correlated to the evolution of the pathogens (Frank 1996). However, more traits should also be considered, including genotypes of host and pathogen (Lambrechts et al. 2006; Restif and Koella 2003), the diversity of pathogen life cycles, the specificities of host–pathogen interactions (Bull 1994; Ebert and Bull 2003), and saprophytic stages of the parasite (Abang et al. 2006). Of them, host range is predicted to be a major factor in the evolution of virulence (Frank 1996). Two models have been proposed to elucidate the evolution of pathogenicity: one is the gene-for-gene (GFG) model and the other is and the matching-allele (MA) model (Sacristan and Garcia-Arenal 2008). For the GFG model, co-evolution of host and pathogen will lead pathogens to alter their Avr factors by avoiding R-dependant recognition while correspondingly the hosts evolving new specific R proteins to identify the Avr factors (Sacristan and Garcia-Arenal 2008). As for the MA model, derived from the self-non-self recognition in inverterbrates, the infection requires a specific match between hosts and parasite genes (Sacristan and Garcia-Arenal 2008). It may be worthwhile to apply current knowledge on the mechanisms of plant–parasite interactions to test hypotheses and predictions on the evolution of virulence and pathogenicity (Sacristan and Garcia-Arenal 2008).

Plant defense systems

Upon pathogen attack, plants respond with a complex and integrated set of defense responses that are both constitutive and induced; the former includes constitutive barriers, including wax layers, pre-formed antimicrobial enzymes, secondary metabolites and toxic compounds and, the latter, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Dong 1998). The resistance of plants to pathogens has been classified into three categories: non-host resistance, also called species-level resistance; constitutive barriers; and race-specific resistance, which is better known as gene for gene resistance (Liu, et al. 2007).

In all three types of resistance, plant cell walls act as physical barriers against pathogens (Swain 1977). Hence, as the basal defense in plants, resistance to penetration of epidermal cells by pathogens is an important component of

defense reactions (McDowell and Dangl 2000). A recent study about the interaction between plants and pathogens observed that the central feature of the physical barriers-cell wall apoposition (CWA) can fortify cell walls at the sites of penetration, and this process involves H_2O_2, callose, phytoalexin, phenolic compounds, silicon, peroxidase and enzyme inhibitors (Hardham, et al. 2007). Both rapid reorganization of actin microfilaments and actin-dependent transport of secretory products also contribute to CWA (Opalski et al. 2005; Schutz, et al. 2006). Moreover, other proteins involved in penetration resistance such as soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins including AtSYP121/PEN1 were found to contribute CWA (Assaad, et al. 2004; Bhat, et al. 2005; Collins, et al. 2003). CWA can arrest the formation of the penetration peg if it is effective; otherwise, downstream defense response including hypersensitive response (HR) will be invoked to inhibit further infection (Aist 1976; An et al. 2006; Schmelzer 2002; Zeyen et al. 2002). Furthermore, in plant-pathogen interactions, programmed cell death (PCD) is a universal consequence of cell death (Glazebrook 2005; Greenberg and Yao 2004). To biotrophic pathogens, it may act as a formidable barrier while to necrotrophic organisms, PCD may accelerate disease (Gijzen and Nurnberger 2006; Greenberg and Yao 2004). Therefore, it is obvious that plant responses to pathogens are extremely complex and the important results from some of the research into the molecular events in plants are discussed below.

Molecular events triggered in plants during infection

Two generalized modes of detection of harmful microbes by plants have been proposed as following: the first one is the perception (at the cell surface) of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs), which then initiates basal resistance or PAMP-triggered immunity (PTI) in both non-host and host plants (Gomez-Gomez et al. 2001; Zipfel et al. 2006; Zipfel et al. 2004). The second one is effector-triggered immunity (ETI) involving the recognition of *Avr* by *R* gene products inside the cell if the first barrier is broken by the invading pathogen (Dangl and McDowell 2006; Hammond-Kosack and Parker 2003; Rosebrock et al. 2007). As we mentioned, race-specific resistance is better

known as gene for gene resistance (Liu et al. 2007). Plant defense responses could be triggered through the recognition of a host *R* gene product by a pathogen's *Avr* gene product, which includes defense signaling events, the expression of pathogenesis-related (*PR*) genes, SAR in plants and more importantly the incompatibility of host-pathogen interactions (between the avirulent pathogen and the resistant plant) (Durrant and Dong 2004; Flor 1971). However, absence of such a specific recognition leads to a compatible interaction, in which the pathogen is said to be virulent, the host susceptible, and disease ensues (Flor 1971). Significant progress in understanding *R* genes and *R* gene-mediated signaling pathways has been made during the past decades and they are the identification of structure, functional characterization, mechanism of *R-Avr* recognition and the evolutionary mechanism of *R* genes (Liu et al. 2007). Basal resistance induced by PAMPs is not as specific or rapid as those mediated by *R-Avr* gene recognition (Jones and Dangl 2006). However, they do share some common features: generation of ROIs, deposition of callose, activation of calcium-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs), and transcription of numerous defense genes (Navarro et al. 2004; Tsuda, et al. 2008) whereas the type of PCD may be different (Qutob, et al. 2006). For example, for necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs)-induced PCD needs active plant metabolism and light, which is also required in AVR effector-mediated hypersensitive response (HR) PCD, FB1 or ToxA toxin- or thaxtomin-induced PCD (Chivasa, et al. 2005; Duval, et al. 2005; Manning and Ciuffetti 2005; Qutob, et al. 2006). Unlike NLP-triggerred PCD, AVR effector or toxin FB1 requires plant defense-related hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Asai, et al. 2000; Pieterse and Van Loon 2004).

Signal transduction in plant defense

Whether pathogens are detected through avirulence determinants or PAMPs, the triggered signaling events converge into a limited number of interacting pathways or networks that rely on secondary messengers-SA and JA/ET (Hammond-Kosack and Parker 2003; Thomma, et al. 2001). However, the associated specific

signaling events in *Avr-R* gene or PAMP-mediated defense responses might differ (Zipfel et al. 2006; Zipfel et al. 2004).

SA, JA and ET are the main molecules signaling the activation of defense genes and the role of SA is well-known in mediating resistance to biotrophic pathogens whereas JA/ET is mainly associated with necrotrophic pathogens (Thomma et al. 2001). Downstream to these signaling pathways is the vital transcriptional control of the expression of stress-responsive genes as revealed by several large-scale transcription profiling studies (Chen and Chen 2002; Durrant et al. 2000; Maleck et al. 2000; Mysore et al. 2002). Of these stress-responsive genes, members of many transcription factors (TFs) including ethylene response factor (ERF)/APETALA2(AP2)-domain, homeodomain, basic leucine zipper (bZIP), Myb, WRKY families and many other zinc-finger factors were observed to be up-regulated during the multiple compatible and incompatible interactions (Rushton and Somssich, 1998; Riechmann and Ratcliffe, 2000; Singh et al. 2002). These defense-associated TFs can bind to promoters of defense-related genes and play a role in their regulation (Eulgem et al. 1999; Korfhage et al. 1994; Rushton et al. 1996; Zhou et al. 1997). Specific aspects of signaling processes in response to SA, JA and ET are discussed in the following sub-sections.

SA-dependent signaling

Several studies have demonstrated that SA is required for SAR and it is associated with the accumulation of PR proteins, contributing to resistance (Durrant and Dong 2004). Non-Expresser of PR genes1 (NPR1) was activated by SA and , moves to the cellular nucleus to interact with TGA transcription factors for further activating transcription of downstream defense genes leading to SAR (Durrant and Dong 2004). TGA factors are a class of bZIP TFs and they have a ability to bind to the SA-, JA-, and auxin-inducible a*ctivating* s*equence-1* (*as-1*) element found in the cauliflower mosaic virus 35S (CaMV 35S) promoter or the related *ocs* element in the octopine synthase promoter (Jakoby, et al. 2002). Some of the TGA factors have been identified in a recent study of *Arabidopsis* TGA TFs, which revealed that three related genes (*TGA2, TGA5,* and *TGA6*) was required

for *PR* gene expression and disease resistance (Zhang, et al. 2003). Other molecular events contributing to SAR also require the induction of SA as well as changes in redox status besides the above mentioned the induction of defense gene expression (Durrant and Dong 2004). Not only at the site of infection but also in remote tissues, the recognition of the invading pathogen causes these changes (Durrant and Dong 2004). Induction of SAR by application of BTH (benzothiadiazole S-methyl ester) or by overexpression of *NPR1* can protect crop plants from pathogens (Durrant and Dong 2004).

Biotic stresses are major external factors influencing the expression of *WRKY* genes (Eulgem et al. 2000; Ryu et al. 2006; Ulker and Somssich 2004). Also, WRKY transcription factors were reported to act as regulators of *NPR1* expression and as potential mediators of the NPR1-signaling pathway (Eulgem 2005; Pan 2004; Spoel et al. 2003; Yu et al. 2001). Arabidopsis WRKY70 was identified as a common regulatory component of SA- and JA- dependent defense responses, mediating cross-talk between antagonistic pathways downstream of NPR1 (Li et al. 2004). It has also been proposed that *WRKY* gene expression is activated by SA-induced protein kinase (SIPK)/wounding induced protein kinase (WIPK) and also that the W-box-binding activity is increased as well as further activation of downstream defense genes (Kim and Zhang 2004). Hence, WRKY transcription factors are known to be implicated in plant defense responses (Eulgem et al. 2000; Eulgem and Somssich 2007).

JA-dependent signaling

JA and MeJA are signaling molecules and they are important for initiating and/or maintaining developmental processes and defense responses in various plants (Wasternack and Hause 2002). The JA-responsive genes and genes involved in JA biosynthesis are described in Chapter 4. In addition, plant defensin 1.2 (*PDF1.2*) is also induced by JA and is often used as a marker for JA signaling in *Arabidopsis* (Fobert, 2007) even though it is known to be induced by ET as well (Chen and Bleecker 1995; Penninckx et al. 1996). Moreover, TFs involved in JA signaling have also been identified. For example, Arabidopsis WRKY70 was identified as a common regulatory component of both SA- and JA-dependent

defense responses (Li et al. 2004) and WRKY62 may be involved in the SA-mediated suppression of JA signaling (Mao et al. 2007). Hence, JA-dependent signaling and related up- or down-stream components play an important role in plant defense response to pathogens.

ET-dependent signaling

The role of ET in plant defense is well-known, however, ET plays a dual role in the interaction between plants and pathogens and the complex roles played by this hormone have been reviewed (van Loon et al. 2006). From the plant's perspective, increased production of ET, as an early, active reaction of plants to the presence of pathogens is coupled to the initiation of defense responses (Boller 1991). From the perspective of the pathogen, ET serves as a virulence factor to improve the colonization of plant tissue (Arshad and Frankenberger 1992). In addition, the timing of exposure to ET might decide whether resistance is induced or inhibited; the mechanisms used by the pathogen to overcome the influence of ET and the speed of the pathogen to colonize infected tissues may contribute to the dual roles of ET (van Loon et al. 2006).

Many ET-related genes have been characterized. For example, it has been observed that *Coronatine insensitive 1* (*COI1*) and *ethylene-insensitive 2* (*EIN2,* via JA and ET) control necrotrophic pathogens (Hammond-Kosack and Parker 2003). The ET-responsive genes and genes involved in ET signal tranduction are described in Chapter 4. Moreover, the rice ERF OsEREBP1 is a plant TF implicated in mediating defense gene expression that has been shown to be phosphorylated by a MAPK (Cheong et al. 2003).

One of strategies to study the role of ET is to modulate the production of ET and it includes the introduction of ACC-deaminase from bacteria into plants (Honma and Shimomura 1978). This enzyme competes with ACC oxidase to metabolize ACC into ketobutyrate and ammonia, and results in the reduction of ethylene (Honma and Shimomura 1978). Plants infected by plant growth-promoting rhizobacteria (PGPR) containing ACC deaminase can promote plant growth due to decreased levels of ET, and plants heterologously expressing ACC deaminase to modulate ET levels exhibited delayed fruit ripening (Klee et al.

1991) and tolerance to heavy metals (Grichko et al. 2000), diseases (Robison et al. 2001), flooding (Grichko and Glick 2001a and b) and salinity stresses (Sergeeva et al. 2006). In general, systematic testing of several phytosystems using different accessions and various lines of Arabidopsis infected with a necrotrophic or a hemi-necrotrophic pathogen revealed that ET was found to reduce symptoms, whereas for biotrophic pathogens, SA was found to combat the infection by pathogens (Thomma et al. 2001; Ton et al. 2002).

MAPK was observed to be involved in defense signaling pathways after the recognition of pathogens or elicitors (Asai et al. 2002; Ichimura et al. 2002; Jonak et al. 2002; Tena et al. 2001; Zhang and Liu 2001) and it is a conserved signal transduction molecule in eukaryotes (Chang and Karin 2001; Ichimura et al. 2002). MAPK cascades involves three protein kinases: a MAPK kinase kinase (MAPKKK) activates a cognate MAPK kinase (MAPKK) by phosphorylating specific serine/threonine residues; the MAPKK in turn activates a specific MAPK by phosphorylating specific tyrosine and threonine residues (Cobb et al. 1996; Marshall 1994). It has been reported that activation of SA-mediated defense was observed in an Arabidopsis *mapk4* mutant (Petersen et al. 2000). However, repression of JA/ET pathways, which enhances resistance toward the biotrophic pathogens, *Psudomonas syringae* and *Peronospora parasitica*, leads to greater susceptibility to the necrotrophic pathogen *A. brassicicola* (Brodersen et al. 2006; Petersen et al. 2000). A similar study in tobacco demonstrated that MPK4 is involved in JA signaling-mediated defense (Gomi et al. 2005; Wu et al. 2007). Other studies performed in oilseed rape also observed that overexpression of *Brassica napus* MPK4 resulted in enhanced resistance to another necrotrophic pathogen, *Sclerotinia sclerotiorum* (Wang et al. 2009). Unlike MPK4, other MAPKs can activate HR and SAR as well (Wang et al. 2009). For example, MPK3 and MPK6 were reported to be involved in bacteria-derived flagellin-triggered resistance activation (Asai et al. 2002; Menke et al. 2004; Romeis et al. 1999); the presence of MPK7 results in the accumulation of SA and constitutive expression of a PR gene, which increases the resistance to *P. syringae* and *Hyaloperonospora parasitica* (Zhang et al. 2007). MAPK cascades are also

observed to be induced by the phytohormones MeJA and ET (Menges et al. 2008). Two WRKY factors (WKRY22 and WRKY29) were identified as downstream components of a MAPK signaling cascade conferring basal resistance to bacterial and fungal pathogens in *Arabidopsis* (Asai et al. 2002). MAPK cascades involved in signal transduction play an important role in plants despite the differences between MAPK components exsiting in these cascades (Pedley and Martin 2005).

Other defense mechanisms of plants

The generation of reactive oxygen species (ROS), including H_2O_2, O_2^- and OH^- defends plants from pathogens by 1) acting as antimicrobial agents, 2) mediating the oxidative cross-linking of cell walls, and 3) acting as signaling molecules to induce defense genes and hypersensitive response (HR) (Custers et al. 2004). Chloroplasts, mitochondria or microbodies, and organelles with high metabolic activities or with intense rates of electron flow are major sources of ROS production in plant cells and of them, chloroplasts and peroxisomes are also thought to be two major contributors to the oxidative load in plant cells during abiotic stresses (Mittler et al. 2004). It has been suggested that in addition to the aforementioned roles in JA/ET and SA signaling, members of the WRKY transcription factor gene family play key roles in the response of plants to oxidative stress (Davletova et al. 2005; Eulgem and Somssich 2007; Gadjev et al. 2006; Lee et al. 2007; Li et al. 2004; Ulker and Somssich 2004; Vandenabeele et al. 2004; Vanderauwera et al. 2005). For example, Arabidopsis *WRKY6* and *WRKY75* were among the 27 transcripts elevated at least five-fold in six of the eight data sets from oxidative stress experiments (Gadjev et al. 2006) and *WRKY25* responds to oxidative stress as well as to wounding, heat and osmotic stresses (Miller et al. 2008). These studies illustrate the importance of the WRKY group of TFs in mediating plant responses to pathogen stress through various signaling pathways including ROS.

Enzyme-inhibitor interactions have also been observed to be important at plant-pathogen interfaces (Misas-Villamil and van der Hoorn 2008). Cell-wall-degrading enzymes and other hydrolases can help pathogen establish colonization

(Collmer and Keen 1986). Plants produce the inhibitors and PR protein to suppress these enzymes and also as a counter attack to pathogens and as a result, pathogens have also evolved to produce inhibitors against this counter attack (Collmer and Keen 1986). It is the co-evolution of two living organisms that make this whole process complicated (Misas-Villamil and van der Hoorn 2008). The structure and function of some of the plant inhibitors have been well-characterized and for example *Triticum aestivum* xylanase inhibitor (TAX1), xylanase inhibitor protein (XIP), polygalacturonase inhibiting protein (PGIP), glucanase and protease and they can be recognized and inactivated by pathogen enzymes glucanase inhibitor protein-1 (GIP1), Kazal-like inhibitors (EIP1) and AVR2 (Misas-Villamil and van der Hoorn 2008 and reference therein). Further studies through genomics, structural biology and advanced proteomics will facilitate the identification of more enzyme-inhibitor interactions (Misas-Villamil and van der Hoorn 2008).

Fungal pathogens of canola

Canola fungal like/fungal diseases include clubroot caused by *Plasmodiophora brassicae*, blackleg caused by *Leptosphaeria maculans*, Alternaria black spot caused by *Alternaria brassicae, Alternaria brassicicola, Alternaria japonica*, Fusarium wilt caused by *Fusarium oxysporum f.sp. conglutinans* and Sclerotinia stem rot caused by *Sclerotinia sclerotiorum*, among others (Kharbanda and Tewari 1996; Strelkov et al. 2006). Clubroot, blackleg, Sclerotinia stem rot, Alternaria black spot and clubroot are the major diseases affecting canola in Canada (http://www.canola-council.org/chapter10c.aspx). In this thesis project, we are focused on two fungal pathogens, *S. sclerotiorum* and *A. brassicae* and specific aspects of these two pathogens are discussed in the following sections.

Sclerotinia sclerotiorum

Kingdom: Fungi

Phylum: Ascomycota

Class: Discomycetes

Order: Helotiales

Family: Sclerotiniaceae

Genus: *Sclerotinia*

S. sclerotiorum (Lib.) de Bary is a necrotrophic and non-host-specific fungus capable of infecting at least 400 primarily dicotyledonous plant species, including canola, with a wide geographical distribution (Boland and Hall 1994; Bom and Boland 2000). *S. sclerotiorum* has the potential to significantly decrease canola yields (Bom and Boland 2000). Yield reduction for canola has been reported to be 5 to 100% in Western Canada (Manitoba Agriculture 2002) while an factor contributing to yield loss is the shattering of immature siliques caused by *S. sclerotiorum* (Morrall and Dueck 1983). Details about the origin and taxonomic history of this fungus have been reviewed in a relatively recent article (Bolton, et al. 2006).

This fungus can overwinter as sclerotia within infected tissues or in the soil, and as mycelium in dead or living plants (Agrios 1997). Sclerotia are hyphal aggregates and are long-term survival structures, which can survive in soil for at least 5 years (Lu 2003). When the environmental conditions are optimal in the spring or early summer, the sclerotia germinate and produce slender stalks terminating at a small, disk- or cup-shaped apothecium (5 to 15 mm in diameter), which produce asci (Agrios 1997). A large number of ascospores are released in the spring or early summer from these asci (Agrios 1997). The apothecium produces asci and a large number of ascospores over a period of 2-3 weeks (Agrios 1997). In addition to ascospores, sclerotia can also produce mycelia directly (Bolton, et al. 2006). Mycelia can initiate penetration under the help of enzymes and mechanical force through appressoria (Lumsden 1979; Lumsden and Dow 1973) and from there the fungus initiates development through the leaf tissue and proceeds to other parts of the plant (Bolton, et al. 2006). The ascospores favour senescent plant parts such as old blossoms to germinate and start infection (Hegedus and Rimmer 2005). In addition, sclerotia can germinate as mycelium to attack and infect young stems directly under moist conditions (Agrios 1997).

Two distinct types of disease symptoms are caused depending on the type of sclerotial germination (Bardin and Huang 2001). Ascospores can infect aboveground tissues later and can lead to stem blight, stalk rot, head rot (Huang 1983; Mundel, et al. 1985), pod rot (Huang and Kokko 1992), white mold and blossom blight of plants (Bardin and Huang 2001). Mycelia directly cause carrots crown rot/root rot (Finlayson et al. 1989) and sunflower basal stalk rot/wilt (Huang and Dueck 1980). For stem lesion, after the colonization by fungus, pale brown to gray-brown lesion appear and later on it became water-soaked lesion due to the severe degradation (Agrios 1997). Usually, as the lesion age, necrosis appears and subsequently tissue became bleached and shreddered (Bolton et al. 2006). Finally, sclerotia may form on either surface of tissues or inside infected tissue (Agrios 1997). Infection can be spreaded to plants nearby through direct contact part (Bolton et al. 2006).

Recent progress in the study of *S. sclerotiorum* (development and infection cycle)

The key components involved in the sclerotial development and infection cycle have been well studied. It was observed that cyclic AMP (cAMP) plays an important role in controlling the switch between mycelial growth and sclerotial differentiation and biosynthesis of oxalic acid (OA) (Rollins and Dickman 1998). Addition of cAMP inhibited both sclerotial development and the activation of SMK1, an ERK-type MAPK required for the development of sclerotia in S. sclerotiorum (Chen et al. 2004). Moreover, deleting the single copy adenylate cyclase (AC) sac1 gene from S. sclerotiorum leads to great reduction of cyclic AMP levels (Jurick and Rollins 2007) (Figure 1-1). Another refulatory cue is ambient pH involved in the events of development, virulence and pathogenicity (Rollins and Dickman 2001) (Figure 1-1). Pac1 levels paralleled increases in ambient pH, hence, it indicated that this gene

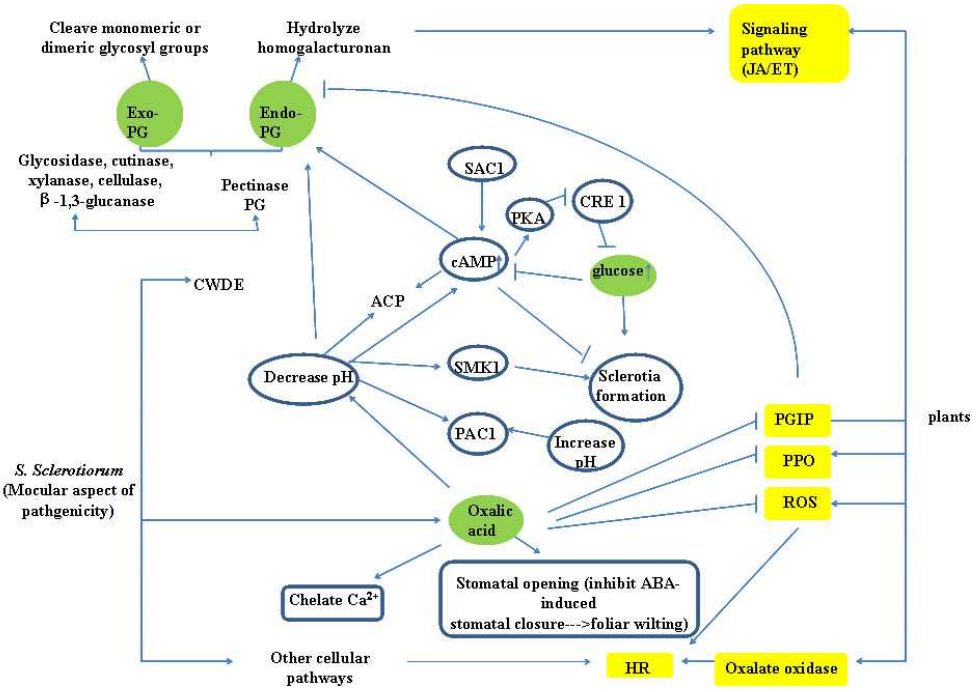

Figure 1-1 Interactions between *S. sclerotiorum* and host plants. Cell wall degrading enzymes (CWDEs) secreted by *S. sclerotiorum*, OA and other cellular products assist in the infection of plants. Plant defense systems including JA/ET signaling pathway, ROS and enzymes/proteins including PGIP or oxalate oxidase are activated to defend against the invading pathogen (Bolton, et al. 2006; Hegedus and Rimmer 2005). In the development and virulence of *S. sclerotiorum*, pH signaling mediated through PAC1, cAMP signaling through glucose, and PKA, CRE1, SAC and SMK signaling are very important.

might contribute in a molecular signaling pathway regulating the expression of gene in response to ambient pH (Rollins and Dickman 2001) (Figure 1-1). Fungal acid protease (ACP1) was observed to be dependent on a cyclic AMP/Protein kinase A (PKA) signalling pathway (Girard, et al. 2004) (Figure 1-1). Lowering the ambient pH by OA production would also indirectly favour the production of lytic enzymes such as Acp1 *in planta* (Girard, et al. 2004) (Figure 1-1). Low glucose levels facilate the accumulation of cAMP, hence, results in the de-repression of polygalacturonase (PG) and this events may be triggered by a protein kinase A (PKA)-dependent inactivation of CRE1 (Figure 1-1) (Hegedus and Rimmer 2005; Jurick et al. 2004; Vautard, et al. 1999). These factors essential for growth or survival of *S. sclerotiorum* could be targets for blocking disease development in this broad-host-range plant pathogen (Rollins and Dickman 2001) (Figure 1-1).

Virulence factors of *S. sclerotiorum* are essential for optimal infection of susceptible hosts and hence could also be targets of new strategies of blocking disease development (Bolton et al. 2006). Of them, OA secreted by this pathogen is very important (Godoy et al. 1990; Guimaraes and Stotz 2004). Firstly, it possibly chelate calcium ions bound to pectins and hence make the cell wall expoused to cell-wall degrading enzymes (Guimaraes and Stotz 2004). At the same time, the expression of the cell wall-degrading enzymes, including the family of PGs are activated because of the acidic ambient pH cause by by OA (Li et al. 2004b). In addition, Cessna et al. (2000) observed that OA can restrain the occurrence of defense-related oxidative burst in both soybean and tobacco cells (Cessna et al. 2000). Moreover, OA may cause wilting of plants through either increasing osmotic pressure because of starch breakdown and the increase of potassium ions, or by inhibiting abscisic acid (ABA)-mediated stomatal closure (Guimaraes and Stotz 2004). However, in a study investigating the production of phytotoxins by *S. sclerotiorum*, sclerin (a component of sclerotia) not OA was observed to cause severe necrosis and chlorosis on leaves of susceptible species: *B. napus, B. juncea*, and *S. alba* (Pedras and Ahiahonu 2004). In the same study, oleic acid also was found to be toxic to brine shrimp larvae (*A. salina*) and it is

one of the major fatty acid extracted from sclerotia of *S. sclerotiorum* (Pedras and Ahiahonu 2004). Besides, other dicarboxylic organic acids (succinic, malic, fumaric and glycolic) secreted by *S. sclerotiorum* may contribute to the toxcity of S. sclerotiorum however, further investigation is required to eluciate the exact roles (Vega et al. 1970).

Besides organic acids, *S. sclerotiorum* secretes multiple cell wall degrading enzymes, which facilitate pathogens to penetrate, colonize, and macerate plant tissues (Kasza et al. 2004; Li et al. 2004b; Yajima and Kav 2006). More importantly, the acidic conditions caused by OA activate a range of isoforms of endo-/exo-polygalacturonase (endoPG/exoPG) and pectin methyl esterase (PME), members of cell wall degrading enzymes (Li et al. 2004b). Moreover, PGs are encoded by multigene families and this grants the different specificities, which can confer the adaptative flexibility to pathogens when infecting different hosts or plant organs (De Lorenzo et al. 2001) (Figure 1-1). Besides, the multiplicity of PGs is further strengthened by base substitutions (Fraissinet-Tachet et al. 1995; Li et al. 2004b) and post translational modifications including glycosylation, proteolytic modifications (Carpita and Gibeaut 1993; Hegedus and Rimmer 2005). Polygalacturonases can result in the maceration of tissue because they degrade the middle lamella as well as primary cell wall homo-galacturonans through the hydrolyzation of internal α(1-4) glycosidic bonds (Zuppini et al. 2005). Li et al (2004b) identified four genes encoding endoPGs (*SSPG1d*, *SSPG3*, *SSPG5*, and *SSPG6*) and two genes encoding exopolygalacturonases (*SSXPG1* and *SSXPG2*). Among these PGs, SSPG1d was detected in the early stages of *S. sclerotiorum* pathogenesis (Li et al. 2004b). The function of PGs during infection was demonstrated by targeted mutagenesis in many fungal pathogens, including *Aspergillus flavus* (Shieh et al. 1997), *B. cinerea* (ten Have et al. 1998), *Penicillium alsonii* (Wagner et al. 2000) and *F. oxysporum* (Garcia-Maceira, et al. 2001). Furthermore, many studies observed that the PGs are activated by pectin or galacturonic acid while they are repressed simple sugars (Fraissinet-Tachet et al. 1995; Li et al. 2004b). However, the importance and the specific roles of these multiple PGs isoforms, as factors in

pathogen virulence, need to be elucidated later on (Li et al. 2004b). For example, the necrotizing activity of five endoPGs of *B. cinerea*, which is similar to *S. sclerotiorum* under the same genus, was investigated on tomato, broad bean and Arabidopsis (Kars et al. 2005). In this study, BcPG2 was observed to be an important virulence factor for *B. cinerea* (Kars et al. 2005). It has also been found that endoPG of *S. sclerotiorum* can induce calcium-mediated signaling as well as programmed cell death (PCD) in soybean (Zuppini et al. 2005), whereas this PCD response in host cells for necrotrophic pathogens actually help the infection of such pathogen (Dickman et al. 2001; Govrin and Levine 2000). Also, this PG-induced PCD may inhibit plant PGIP and hence interfere with plant resistance (Zuppini et al. 2005). PGs produced early during plant infection by *S. sclerotiorum* may also be targeted by recombinant antibody technology to neutralize their activities and lead to durable tolerance to this pathogen.

Alternaria brassicae (Berk.) Sacc

Kingdom: Fungi

Phylum: Ascomycota

Class: Ascomycetes

Sub-class: Dothideomycetes

Order: Pleosporales (anamorph Bipolaris, Curvularia)

Family: Isotomoidea

Genus: *Alternaria*

Dark leaf and pod spot, caused by *Alternaria brassicae* ((Berk.) Sacc. 1880), is an important disease of Brassica crops world-wide (Saharan 1993). *A. brassicae* can affect host species at all stages of growth, including seeds (Agrios 1997). In oilseed rape, dark leaf spot generally does not affect the yield, but when this disease spreads extensively onto pods leading to dark pod spot, it can lead to severe losses in yield and quality (Awasthi and Kolte 1989). The survival structures of *A. brassicae* consist of microsclerotia and chlamydospores exsisting on partially decayed infected leaves (Tripathi and Kaushik 1984). *Alternaria brassicae*, after sporulation, may produce up to ten conidia from conidiophores (Rangel 1945; Sharma et al. 2007).

After germination, *A. brassicae* produces at least one germ tube to penetrate stomata, cuticle and wounds (Rotem 1994). Seed production is affected by *A. brassicae* and two other *Alternaria* species (*A. brassicicola* and *A. raphani*) through shriveling of seeds within the pods and/or killing the pod stalks before seed formation, which also help bacteria to enter the stem and finally results in the death of plants (Chupp and Sherf 1960). Mycelia of this pathogen living within the seed or the spores existing on the seed coat can allow for the spread of the disease and lead to a loss of seedlings (Rangel 1945). When it causes leaf spot, it exists in the center of the lesion which is encircled by a chlorotic halo (Agrios 1997).

Recent progress on the study of *A. brassicae*

Athough our understanding of Alternaria pathogenesis mechanisms is limited (Lawrence, et al. 2008), Alternaria species possess two main features: one is the production of melanin and the other is the production of host-selective toxins (Thomma 2003). Melanins are pigments that protect fungi against harsh conditions like UV radiation and extreme temperatures (Kawamura et al. 1999; Lockwood 1960; Rehnstrom and Free 1996) and are very important in both conidial development and virulence (Kawamura et al. 1999). The toxins produced by *A. brassicae* include destruxin B and homodestruxin B (Ayer and Penarodriguez 1987; Bains and Tewari 1987; Buchwaldt and Jensen 1991; Tewari and Bains 1997). Destruxin B produced by *A. brassicae* has been well-studied (Cai, et al. 1998; Gupta, et al. 1989; Sharma and Tewari 1996). It was observed that the symptoms caused by *A. brassicae* and destruxin B are similar, which further confirmed the role for this toxin in the pathogenicity of this organism (Shivanna and Sawhney 1993). It opened a field for investigation into resistant/susceptible plants based on these toxins (Pedras et al. 2000). Destruxin B was first characterized as a host-specific toxin (Bains and Tewari 1987), while more recent studies observed that it is not a host-specific toxin even though toxin sensitivity decreases in non-host species (Buchwaldt and Green 1992; Parada et al. 2007). Like other necrotrophic pathogens, *Alternaria* species can also secrete CWDEs, which play important roles during the infection process (Eshel et al.

2002). In summary, even though the details regarding the mechanisms underlying infection and disease progression may be lacking, considerable progress has been made in understanding the roles of toxins and some CWDEs in this case (Lawrence et al. 2008). With the completion of the *A. brassicicola* genome sequencing project, it is only a matter of time before additional genes involved in pathogenesis are identified and characterized (Lawrence et al. 2008).

Traditional approaches to managing the two fungi

A combination of cultural and chemical means can be used to control *S. sclerotiorum* (Agrios 1997). Crop rotation is important practice to reduce the sclerotial population in the soil (Agrios 1997). Due to the long survival of sclerotia, the use of deep plowing is questioned (Agrios 1997). Also, drip irrigation was found to be able to dramatically reduce the incidence of sclerotinia disease (http://cesantabarbara.ucdavis.edu/ipm2.htm). Biological control has also been investigated over the last few decades because of increasing concerns over the use of chemical pesticides (Bardin and Huang 2001). Many biological agents are potential to control this disease and are listed in Table 1-1. The biocontrol agents most widely studied are mycoparasitic fungi, hypovirulent strains of *S. sclerotiorum*, bacteria and insects (Bardin and Huang 2001) (Table 1-1). Applying organic and inorganic materials or formulated compounds have also been shown to suppress *S. sclerotiorum* (Huang and Huang 1998).

Few genetic sources of resistance to the pathogen are available to breeders (Liu, et al. 2005) and this has resulted in the application of fungicides being a major control method for Sclerotinia disease (Bardin and Huang 2001; Steadman 1979). This method has been used successfully on a commercial scale with soybean, dry bean, oilseed rape and some vegetables (Bailey, et al. 2000; Budge and Whipps 2001; del Rio, et al. 2004; Twengstrom, et al. 1998). Benomyl, thiophanate-methyl, vinclozolin and Tebuconazole are some of the fungicides that

Table 1-1 Chemical and biological control of two fungi

	S. sclerotiorum	*A. brassicae*
fungicide	Benomyl, Thiophanate-methyl, Vinclozolin, Tebuconazole (Mueller, et al. 2002).	Dithane M-45 (Mancozeb), Dithane Z-78 (Zineb), Ziram, Difolatan-80, Blitox-50, Benlate (Ansari, et al. 1990); Iprodione, fenpropimorph (Maude, et al. 1984); Thiram (Valkonen and Koponen 1990)
biological control	*Gliocladium catenulatum* (Gilman and Abbott, 1927); *Trichoderma viride* Pers. Ex Fr (Bardin and Huang 2001) ; *Coniothyrium minitans* and *Talaromyces flavus* (Klöcker) Stolk and Samson (Huang and Erickson 2000).	Actinomycete fungus, *Streptomyces arabicus* (Sharma, et al. 1984; Sharma, et al. 1985).

were able to control Sclerotinia stem rot on soybean in the greenhouse (Mueller, et al. 2002). However, how to achieve a good spray coverage is interfered by the number of blossoms within canopy and density of plants and how to spray fungicide timely needs an effective a forecasting tool (Morton and Hall 1989). More importantly, the development of resistance to fungicides threatens the continued effectiveness of this mode of disease control (Morton and Hall 1989).

Different forecasting systems are employed besides routine chemical control, and include a petal testing method to help predict Sclerotinia in canola (Turkington, et al. 1991). This prediction method was developed based on the relationship between disease incidence and the level of infestation of petals by the pathogen at early bloom (Turkington, et al. 1991). A risk-point table was developed but the forecast based on risk map was not as accurate for different fields (Bom and Boland 2000a). Use both petal infestation and enzyme-linked immunosorbent assay (ELISA) can reduce some of the shortcomings of each forecast method as assessment tools for risk of stem rot on canola (Bom and Boland 2000b). Forecast maps of risk of *S. sclerotiorum* on canola are currently available to growers in both Manitoba for canola and the northern midwestern United States for white mold of bean (McLaren et al. 2004). However, it is region-based and is not accurate for individual area (McLaren et al. 2004). All of these methods have their own inherent shortcomings as alluded to earlier.

The detailed review for control of *A. brassicae* has been provided previously (Sharma 2008). Basically, rotation with non-cruciferous crops and eradication of cruciferous weed hosts can help control *A. brassicae* (Humpherson-Jones 1989). Since spores can survive on leaf tissue for 8 to 12 weeks and on stem tissue for up to 23 weeks, fields that are replanted soon after harvest often coincide with a large amount of inocula, which is likely to effect the crop's emergence and early growth stages (Humpherson-Jones 1989). To both minimize the risk of severe losses and the unnecessary use of fungicide, forecast systems for dark pod spot epidemics on oilseed rape are needed (Saharan 1992; Verma and Saharan 1994).

Current studies on plant responses to *S. sclerotiorum*

Activation of plant defense systems against necrotrophic pathogens is complex because HR-PCD triggered by the pathogen and host cell death pathway provides the necrotic tissue and nutrients that these fungi require to grow (Dickman et al. 2001; Govrin and Levine 2000). Although it has been reported that a JA/ET-dependent pathway is involved in the plant response to necrotrophic pathogens, the specific signal transduction pathways associated with *S. sclerotiorum* infection have not been reported. Additionally, three known phytoalexins have been isolated from leaves of *Erucastrum gallicum* (resistant to *S. sclerotiorum*) and these phytoalexins may become useful markers for resistance against *S. sclerotiorum* (Pedras, et al. 2004). Current approaches that may aid in the engineering of plants with tolerance/resistance to *S. sclerotiorum* are described below.

Interaction of OA and oxalate oxidase (OXO)

OA is a pathogenicity factor secreted by *S. sclerotiorum* (Godoy et al. 1990; Guimaraes and Stotz 2004) (Figure 1-1). Hence, degrading the secreted OA is an obvious approach to alleviate this disease (Lu 2003). Three catabolic enzymes can metabolize OA and they are OXO, oxalate decarboxylase and oxalyl-CoA decarboxylase (Lane et al. 1991; Mehta and Datta, 1991; Lung et al. 1994; Lu 2003). Of them, OXO has been well studied in this context (Lane et al. 1993; Kotsira and Clonis, 1997). This enzyme can release CO_2 and H_2O_2 from O_2 and OA without any toxic effects on plants. OXO was isolated firstly from barley and wheat (Kotsira and Clonis 1997; Lane et al. 1993) and is also named wheat germin (Dunwell et al. 2000; Lane 2000). Moreover, germins from monocot such as wheat, barley, maize, oat, rice, rye and pine exhibited OXO activity (Dunwell, et al. 2000; Lane 2000). Transgenic soybean with a wheat OXO gene was developed and showed greatly reduced disease progression and lesion length following cotyledon and stem inoculation with *S. sclerotiorum* (Donaldson et al. 2001). The heteologous expression of OXO in both soybean and sunflower exhibited enhanced resistance against *S. sclerotiorum* (Lu 2003; Hu et al. 2003). Recently, transgenic oilseed rape constitutively expressing wheat OXO has been

shown to have significantly greater OXO activity and higher resistance to OA and *S. sclerotiorum* compared to untransformed control plants (Liu et al. 2005). Beside its function of degrading OA, OXO also elicit the defense-inducing molecule H_2O_2, SA and defense gene expressing and these dual roles make combating Sclerotinia disease more promising (Lu 2003; Hu et al. 2003). Hence, the strategy to combat *S. sclerotiorum* through degrading the secreted OA shows promise as a means to generate *S. sclerotiorum* resistant canola.

Interaction of PGs and polygalacturonase-inhibiting proteins (PGIPs)

Biochemical evidence suggests that PGIPs are important for plant defense both *in vitro* (Lafitte et al. 1984) and *in vivo* (Bailey et al. 1992; Ferrari et al. 2003; Powell et al. 2000; Salvi et al. 1990). PGIPs are leucine-rich repeat (LRR) glycoproteins associated with the cell wall of both monocots and dicots (De Lorenzo et al. 2001; De Lorenzo and Ferrari 2002) and counteract the action of fungal PGs. It interfered the degradation of homogalacturonan by PGs through slowing the hydrolytic activity of endoPGs and leading to the increase of oligogalacturonides (OGs), which are elicitors of a variety of defense responses (D'Ovidio et al. 2004). But PGIPs have not shown inhibitory activities against other pectic enzymes either of microbial or plant origin (Cervone et al. 1990). There are distinct classes of *PGIP* genes based on differences in structure and in function and it is this multiplicity that confer the specific recognition between PGIPs and fungal secreted PGs (D'Ovidio et al. 2004). However, future studies about the interaction and specificity between PGIPs and PGs remain to be elucidated and may have a possible application in crop protection (D'Ovidio et al. 2004). More importantly, over-expression of PGIP has been considered as a possible strategy to increase plant resistance to diseases (Favaron et al. 2004).

Current studies on responses in plants to *A. brassicae*

Plants species such as *Cannabis sativa, Capsella bursapastoris,* and *Sinapis alba* are resistant to *A. brassicae* and their defense mechanisms are elucidated by Pedras (1998). In resistant lines of *C. sativa* and *C. bursapastoris*, camalexin (phytoalexin) can inhibit the production of destruxin B (Pedras 1998). Also, in

Sinapis alba, it is hydroxylation of destruxins that make the plants to detoxify the toxin from *A. brassicae* (Pedras et al. 2001). Hence, characterization of the gene encoding destruxin B hydroxylase may have potential for plant genetic engineering against *A. Brassicae* (Pedras et al. 2001).

A proteomic study on the mechanism underlying the incompatible/compatible interaction of the pathosystems has been reported (Sharma et al. 2007). They observed that plants derived from cross-hybridization of *B. napus* and *B. carinata* showed tolerance to *A. brassicae* and some proteins involved in ROS detoxification were induced in the tolerant lines together with Nucleoside diphosphate kinase, playing role in signal transduction pathway (NDPK; Sharma et al. 2007). The defense system of *A. brassicicola*-resistant Arabidopsis inoculated with *A. brassicicola* has also been well studied (Lawrence et al. 2008). *A. brassicicola*, which belongs to the same genus as *A. brassicae*, can also cause black spot on *B. napus* (Agrios 1997). A response similar to that of Arabidopsis to *A. brassicicola,* which involved JA-mediated defense but not SA-mediated defense, has also been observed in other plants when challenged with necrotrophic fungi such as a *Pythium* species and *Botrytis cinerea* (Staswick et al. 1998; Vijayan et al. 1998). It is believed that the JA-mediated defense response is important for resistance (Thomma et al. 1998). It is also known that the phytoalexin camalexin is important for the resistance of Arabidopsis to *A. brassicicola* based on the observation that the *pad-3* mutant which is deficient in camalexin biosynthesis, is more susceptible to *A. brassicicola* than wild-type plants (Thomma et al. 1999; Zhou et al. 1999). Moreover, some transcription factors such as R2R3-MYB (Mengiste, et al. 2003) and specific WRKY transcription factors (Li et al. 2006; Zheng et al. 2006) also appear to be important in Arabidopsis responses to *A. brassicicola*. This valuable pathosystem (Arabidopsis-*A. brassicicola*) may also contribute to our understanding of the canola-*A. brassicae* interaction (Lawrence et al. 2008). .

Recombinant antibodies and their application in plant pathology

Many kinds of antibody fragments, produced through recombinant technology, retain the antigen specificity of the whole immunoglobulins, which are including

variable fragments (Fv), disulfide-stabilized Fv fragments (dsFv), Fab fragments, and single chain variable fragments (ScFv) (Dubel and Kontermann 2001). Briefly, cDNA encoding these fragments are isolated from B-cells of either non-immunized or immunized animals and later on the genes are amplified through PCR using degenerate primers (Dubel and Kontermann 2001). The use of phage display techniques to isolate recombinant antibodies has the potential to reduce or eliminate the need for animals in antibody production and also to eliminate expensive hybridoma technology used in the production of monoclonal antibodies (Chen et al. 2006; Das et al. 2004; Padiolleau-Lefevre et al. 2007). Phage displaying antibodies on their coat proteins also contain the corresponding genetic information encoding the antibodies (McCafferty et al. 1990) and up to $1x10^9$ different antibodies clones can be isolated from phage display library (Dubel and Kontermann 2001).

The antibody-based strategy has benefited from the progress made in our understanding of plant diseases and the characterization of many proteins that are critical to pathogen infection, replication and spread (Schillberg et al. 2001). For example, the application of polyclonal antibodies targeting specific pathogenicity and virulence factors has been shown to protect banana, mango, and avocado from *Colletotrichum gloesporioides* (Wattad et al. 1997). Recombinant antibodies, especially of the single chain variable fragment (ScFv) type produced using phage display techniques, have also been used for the diagnosis of disease as well as for engineering disease tolerance (Cogotzi et al. 2009; Conrad and Fiedler 1998; Nolke et al. 2009; Schillberg et al. 2001). ScFvs consist of one variable heavy (V_H) and one variable light (V_L) chain of a whole antibody linked by a short polypeptide (Burmester and Plcükthun 2001). ScFv fragments have either equivalent or lower binding specificities because of the fact that both variable domains are present in a single polypeptide (Schillberg et al. 2001). Therefore, ScFv antibodies remain the most practical way to engineer a stable, minimal binding domain from a parental full-size antibody, making them very attractive for antibody-based plant genetic engineering (Nolke et al. 2006). ScFvs have been developed for applications in diagnostic plant pathology (Cogotzi et al.

2009; Yajima et al. 2008) as well as for other applications in plant science including for the neutralization of mycotoxins in animal feeds (Yuan et al. 2000), creating gibberellin-deficient plants (Suzuki, et al. 2008) and vaccines (McCormick, et al. 2008).

In the past, conventional breeding based on crossing, screening and backcrossing were used to generate plant lines resistant to viral, bacterial or fungal pathogens (Melchers and Stuiver 2000). Recent advances in molecular biotechnology have made it possible to obtain and modify genes that may be useful for generating disease tolerant/resistant crops and include the expression of pathogen-derived sequences or anti-pathogenic agents (Schillberg et al. 2001). One approach has involved the expression of antibodies or antibody fragments that target and inactivate plant pathogens or virulence factors produced by the pathogen (Tavladoraki et al. 1993). For example, transgenic plants expressing ScFvs targeted against the viral coat protein of the artichoke mottled crinkle virus (AMCV) demonstrated increased tolerance against the pathogen (Tavladoraki et al. 1993). Similarly, recombinant antibodies produced in plants have been demonstrated to interact with and inactivate invading fungal pathogens, thereby generating fungus-resistant plants (Boonrod et al. 2004; Nolke et al. 2004). An example of resistance to fungal pathogens mediated by recombinant antibodies linked to an antifungal peptide is the generation of transgenic Arabidopsis with high levels of resistance to the phytopathogenic fungus, *Fusarium oxysporum* f. sp. *matthiolae* (Peschen et al. 2004). However, due to the reducing conditions and the nature of the ScFv itself, these antibodies generally accumulate too low or undetectable levels in the cytosol (Schillberg et al. 2001). Many methods have been developed to stabilize the ScFv and hence lead to improved production of these antibodies in plants (Schouten et al. 1997; Schouten et al. 1996; Sunilkumar et al. 2008). Nevertheless, it is clear that studies on the applications of antibodies to engineer plants with tolerance/resistance to pathogens are producing promising results.

Objectives and significance of this research

Although a considerable amount of information on virulence factors, including OA, secreted by *S. sclerotiorum* is available, very little is known about the molecular events that occur in canola upon infection by this pathogen.

(1) Gene expression profiling has the potential to identify novel regulatory gene networks involved in mediating plant responses to pathogen infection and may contribute to an integrated understanding of complex plant-pathogen interactions (Maleck et al. 2000). Most studies are focused on the incompatible interactions on host and non-host resistance to avirulent pathogens, while the understanding of compatible interactions remains relatively limited (Kazan et al. 2001). In order to understand the molecular events in canola upon infection by *S. sclerotiorum*, we first used microarray technology to investigate the transcript changes of genes in canola infected by *S. sclerotiorum* at various time points.

(2) Based on the information gleaned from our microarray analysis and to further investigate the signaling pathways involved in two pathosystems (*B. napus-S. sclerotiorum* and *B. carinata-S. sclerotiorum*), we selected five orthologs as a set of representative genes of the JA/ET and SA signaling pathways for further study. It remains unknown whether decreased ET levels can influence the *Brassica-S. sclerotiorum*, hence, we characterized the response of transgenic aminocyclopropane-1-carboxylate (ACC)-deaminase canola to *S. sclerotiorum* to further elucidate the role of ET as a signaling molecule in plant defense against this pathogen.

(3) Despite the previously mentioned importance of WRKY TFs in pathogen responses and hormone signaling in Arabidopsis, rice and many other plants, there are no reports describing WRKY TFs in canola and their role(s) in mediating responses to the two fungal pathogens. In order to identify and characterize WRKY TFs involved in the interaction of two pathosystems (canola-*S. sclerotiorum* and canola-*A. brassicae*), we mined public databases to identify WRKY TF genes and investigated their evolutionary relationships with counterparts from Arabidopsis and rice. We examined the sub-cellular

localization of four BnWRKY proteins using green fluorescent protein (GFP). Subsequently, we studied the responses of representative members of distinct WRKY clades to two fungal pathogens (*S. sclerotiorum* and *A. brassicae*) as well as to five plant hormones in order to gain further insights into their roles in canola defense responses.

(4) To explore the use of ScFv antibody fragments against *S. sclerotiorum* infection, we isolated two ScFv antibodies against SSPG1d, which is one of the virulence factors of *S. sclerotiorum*, and characterized these two ScFvs. We hypothesized that by specific recognition of SSPG1d through ScFv-SSPG1d or ScFv-3796 together with other *S. sclerotiorum*-specific antibodies, it might be possible to develop a new diagnostic assay to accurately and quickly detect the presence of this fungus in canola fields.

(5) We hypothesized that by inhibiting selected virulence factors including endoPG by ScFv antibodies, we might be able to prevent or delay infection of canola by *S. sclerotiorum*. To test the hypothesis, one peptide from SSPG1d and whole SSPG1d (recombinant protein expressed and purified from *E. coli*) were used to isolate ScFv antibodies using phage display technology. The ScFv genes were introduced into *A. thaliana* and canola to test the ability of these ScFvs to protect plants against *S. sclerotiorum* infection. Hopefully, the economic losses in canola production caused by *S. sclerotiorum* may be alleviated through our efforts and this work will ultimately benefit farmers by avoiding the use of potentially environmentally unsafe chemicals and by helping breeders decrease the time needed to cultivate new varieties of fungal disease-resistant crops.

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Chapter 2 Transcriptional profiling of canola (*Brassica napus* L.) responses to the fungal pathogen *Sclerotinia sclerotiorum*

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary causes soft rot disease and is considered to be one of the most damaging pathogens, which affects at least 400 plant species including canola (*Brassica napus* L; (Bolton et al. 2006; Bom and Boland, 2000a)). Sclerotinia diseases cause estimated annual losses of US$ 200 million in the US alone (Bolton et al. 2006 and references therein). *B. napus* is an agriculturally and economically significant crop that adds over $11 billion to the Canadian economy (http://www.canola-council.org/industry. html) and Sclerotinia has the potential to cause significant reduction in its yield (Bom and Boland, 2000).

S. sclerotiorum over-winters as mycelia within plants or as sclerotia, which are hyphal aggregates and long-term survival structures (Lu, 2003). The sclerotia germinate and form apothecia, which produce asci and a large number of ascospores in the spring or early summer (Bolton et al. 2006). Either the ascospores or mycelium can invade plants through the stomata into the substomatal chamber, and from here, the fungus progresses rapidly through the leaf tissue (Bolton et al. 2006). During the infection of plant tissue, oxalic acid secreted by the pathogen is thought to be crucial in facilitating invasion and, for that reason, is considered a pathogenicity factor (Guimaraes and Stotz, 2004). Even though the role of oxalate is not completely clear, it has been proposed to remove calcium ions bound to pectins, exposing host cell walls to catabolic enzymes of fungal origin (Guimaraes and Stotz, 2004). In addition, oxalic acid also facilitates plant cell wall degradation by shifting the pH of infected plant

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tissues closer to the optimum of cell wall-degrading enzymes, such as polygalacturonases (Cotton et al. 2002). Apart from its role in weakening the plant cell wall and facilitating its degradation, oxalic acid has also been shown to suppress defense related oxidative burst in soybean and tobacco cells (Cessna et al. 2000) and alter guard cell movement by either increasing osmotic pressure via accumulation of potassium ions and starch breakdown or by inhibiting ABA induced stomatal closure (Guimaraes and Stotz, 2004).

The plant cell wall-degrading enzymes secreted by *S. sclerotiorum* during infection of plants include cellulolytic and pectinolytic enzymes, which have been studied for their roles in pathogenicity, including penetration, maceration, nutrient acquisition, plant defense induction and symptom expression (Li et al. 2004b; Yajima and Kav, 2006). As mentioned earlier, these enzymes are all optimally active under the acidic conditions provided by oxalic acid (Li et al. 2004b; Yajima and Kav, 2006).

In addition to their roles in reducing the integrity of the plant cell walls, the pectin methyl esterases, acid proteases and an aspartyl proteinase secreted by *S. sclerotiorum* are likely required for the degradation of cell wall proteins and the inactivation or inhibition of plant defense response proteins (Yajima and Kav, 2006). Even though a considerable amount of information on oxalic acid as well as the virulence factors secreted by the pathogen is available in the literature, very little is known about the molecular events that occur in the plant cell upon infection with this pathogen. One study reported an expressed sequence tag (EST) analysis of two cDNA libraries constructed using either fungal mycelia grown in pectin medium or tissues from infected *B. napus* stems and were used to identify genes involved in fungal development and pathogenesis (Li et al. 2004a). This study revealed a number of fungal genes including virulence factors such as exopolygalacturonases and several transporters; however, very little information on the changes in plant gene expression were reported (Li et al. 2004a). Transcriptional profiling using microarrays is a powerful tool that can be used to investigate gene expression in different tissues or organs under normal conditions or under abiotic (Jiang and Deyholos, 2006) and biotic stresses (Wan et al. 2002).

The wide application of microarray technology in *B. napus* is limited by the fact that canola microarrays are not commercially available. However, this limitation may be overcome by the use of readily available *A. thaliana* microarrays, given that protein coding sequences exhibit >86% similarity between *A. thaliana* and *B. napus* (Cavell et al. 1998). Indeed, *A. thaliana* cDNA arrays representing a small number (6120) of genes were previously used to investigate gene expression in canola following challenge with *S. sclerotiorum* (Liu et al. 2005).

In this study, we have used commercially available oligonucleotide (70 mers) arrays representing 26,090 *A. thaliana* genes to profile gene expression in canola leaves following challenge with *S. sclerotiorum*. Our study represents the first detailed investigation into gene expression during this host–pathogen interaction and has revealed many interesting genes responding to this pathogen.

Materials and methods

Fungal and plant materials

A strain of *S. sclerotiorum* was kindly provided by Dr. Stephen Strelkov, Plant BioSystems Group, Department of Agricultural, Food and Nutritional Science, University of Alberta. Sclerotia were subcultured on solid potato dextrose agar (PDA) media (Becton Dickinson, Sparks, MD, USA) under light (24 h/day). After 3 days, 0.2 cm agar plugs were removed with a sterile cork borer from the leading edge of the mycelium and were subcultured on PDA agar plate, following which 0.5 cm agar plugs were removed from the leading edge of the second two-day-old mycelia and used for inoculation. Wild type *B. napus* ('Westar') plants were grown in a greenhouse with a photoperiod of 16 h light (combination of natural light and T5 fluorescent tubes with a light intensity of 300 uE (umol) m^{-2} s^{-1} or mV)/8 h dark for 18 days and used in our experiments. PDA plugs prepared as described earlier were placed on the first and second true leaves, which were wounded using the tip of syringe. Leaves of uninoculated, control plants were treated similarly with PDA agar plugs without the mycelia. Plants, both control and inoculated, were placed in a humidity chamber for 24 h after which they were returned to the greenhouse. Leaves from control and inoculated plants were

harvested at 12, 24 and 48 h after inoculation, flash frozen in liquid nitrogen and stored at -80 ˚C until RNA extraction. Leaves from five plants out of three independent biological replicates were prepared for analysis.

RNA isolation, microarray slide preparation and hybridization conditions

Total RNA was isolated from both control and inoculated leaf tissue using the RNeasy Plant Mini kit (Qiagen, Mississauga, ON, Canada) with on-column DNA digestion. RNA was quantified with a NanoDrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, USA) and the integrity of the RNA was assessed on a 1% agarose gel. cDNA was synthesized from 5 mg of total RNA using SuperscriptII (Invitrogen, Burlington, ON, CA) with RT polyA capture primers (3D Array 900 kit; Genisphere, Hatfield, PA, USA). In these experiments, oligonucleotide (70 mer) arrays that contained a total of 26,090 probes (Array-Ready Oligo Set for Arabidopsis genome Version 1.0, Qiagen Operon, Alameda, CA, USA plus additional probes for quality control) spotted on superamine aminosilane-coated slides (Tele Chem International Inc., Sunnyvale, CA, USA) were used. The slides were covered with 24 X 60 mm Lifer Slips (Eric Scientific, Portsmouth, NH, USA) for all hybridizations which were performed in a two-step format at 55°C using the 3D Array 900 kit (Genisphere) as per manufacturer's instructions. Slides were scanned immediately using ArrayWoRx (Precision Scientific, Issaquah, WA, USA) and transformed into Tiff images. Two hybridizations were performed for each of the three independent biological replicates for each time point (total 18 hybridizations) which included dye swap hybridizations in order to avoid bias in the microarray evaluation as a consequence of dye (Cy3/Cy5)-related differences in labeling efficiency and/or differences in recording fluorescence signals.

Microarray data analysis

Microarray data were analyzed using TM4 suite (http://www.tm4.org (Saeed et al. 2003)). Briefly, spot intensities were measured using Spotfinder 3.11 software, normalized using MIDAS2.19 (LOWESS normalization method) and finally analyzed with TMeV4.0. The low intensity cut-off used was 1000 UNITS. Fold

changes of gene expression (inoculated/mock) were calculated based on normalized data in Microsoft Excel 2003 and, significance analysis of microarray (SAM, Tusher et al. 2001) was applied to find differentially expressed genes with fold changes differing significantly from 0 with a false discovery rate (FDR) of 5%. All the normalized, intensity-filtered data points from SAM analysis whose fold-changes differed significantly from 0 were analyzed using Short Time Expression Series Miner (STEM) in order to assign genes to temporal expression profiles. For STEM analysis, the maximum number of model profiles was set at 16 and maximum unit change was set to 4. Statistical analysis of the fold changes of the all genes in the SAM list was also performed using Student t-test and those exhibiting >two-fold ($P < 0.05$) up- or down-regulation were also identified.

Quantitative RT-PCR (qRT-PCR)

Genes of interest were validated using qRT-PCR. *B. napus* gene sequences with similarity to a given microarray probe sequence from *A. thaliana* were retrieved from the NCBI database and those with more than 80% similarity were used to design primers using PrimerExpress3.0 (Applied Biosystems, Foster, CA, USA) targeting an amplicon size of 80 bp. The list of genes that were validated as well as the primers used in these experiments is shown in Table 2-1. The same *B. napus* RNA used for our microarray experiments was also used for qRTPCR. Reverse transcription (RT) of total RNA (2.5 mg) was performed using SuperScript II (Invitrogen) and oligo(dT)18 (Fermentas, Burlington, ON, CA) in a 20 ml reaction volume. Each cDNA sample was diluted 1:12.5 in sterile deionized H_2O, and 2.5 ml of the diluted cDNA samples were used as templates. The qRT-PCR was performed with home-made 1x SYBR Green Master Mix (Tris (pH 8.3), KCl 50 mM, MgCl2 3 mM, glycerol 0.8%, Tween 20 0.01%, dimethyl sulfoxide (DMSO) 2%, dNTPs 0.2 mM, ROX 1xSYBR 1/40k or 0.25, Platinum Taq 0.03 units/ml (final concentration)) in 10 ml reactions using an ABI 7500 system (Applied Biosystems) according to the manufacturer's instructions. In addition to the cDNA, each PCR reaction contained each of the primers (0.4 mM) and 5 ml of 2_ SYBR Green I master mix. The initial denaturing time was 2 min at 95 °C, followed by 35 cycles of 95 °C for 15 s, 60 °C for 1 min. After

Table 2-1 Sequences of the gene-specific primer pairs used in real-time reverse-transcription polymerase chain reaction experiments

Gene	Accession number	Forward and reverse primers (amplicon size: 80bp)
Glyoxalase family protein	CD831005	5’-ATCCAGATGGCTTCATGATTGA-3’
		5’-GGACCGAGCCATTCCTCCTA-3’
Allene oxide synthase	CD828070	5’-CAAGCAAAAACCCGAGGAGTT-3’
		5’-CTGGTGGCATATTGACTCGAAA-3’
Nodulin / glutamate-ammonia ligase - like protein	CD832629	5’-TCAACCAAGCGGACAATACCA-3’
		5’-CATGCTTCACCAGGCTTTAGG-3’
Steroid sulfotransferase	AF000307	5’-GCCTCGAGAATCCGAACAAG-3’
		5’-GATTCGCTTAACCTCCACTTCAG-3’
Glutamate dehydrogenase	AB066298	5’-GGGTTTTGGGAATGTTGGAA-3’
		5’-TGATGTCGCTTACTGCAACCA-3’
Jacalin lectin family	AY337003	5’-CACGTGAATATGGGACAAAAGGT-3’
		5’-CACCGCTGTGATCTGTTCGT-3’
Allene oxide cyclase family	CD817484	5’-CCAGATTTCTCCTCCCAATCAA-3’
		5’-GACTTTCCCCAGTTCCAGAAGA-3’
Salt-tolerance zinc finger protein	AC189555	5’-AAGTCGTTCTCGTCTTACCAAGCT-3’
		5’-CCGCCGGATTGAGTCTGT-3’
Plant defensin protein	DY002971.1	5’-TGCTCTCGAAGCACCAACAA-3’
		5’-TTCCACAGACTCCTGACCATGT-3’
Plant defensin protein	CX193321	5’-GCCTGCAAGAATCAGTGCATT-3’
		5’-ATACACTTGTGAGCAGGGAACACA-3’

RD20 protein	DY024750.1	5’-CAACATTCACAAAGCCAAGCA-3’
		5’-TCTCGAGATTAACCGGGACGTA-3’
Xyloglucan		
endotransglycosylase	DY006021.1	5’-ATGGTGGATGAGACACCGATTC-3’
		5’-CCCATCGCTTGGTCTTTAGC-3’
2-cys peroxiredoxin-related		
protein	CD814109	5’-GAGGGCTCGGTGATCTGAAC-3’
		5’-AGGGATGAGCACACCAAAAGA-3’
Glutaredoxin protein family	CD820020.1	5’-AGGCTGCTCAAAGCCAGTTC-3’
		5’-GGTGCATGGACATGACTTCGT-3’
Starch synthase, putative	CD835340.1	5’-TGGGATTGCCTTCTGAATGG-3’
		5’-ACCGGTGTTTAGAGCATGTGTTC-3’
Actin	AF111812	5’-ACGAGCTACCTGACGGACAAG-3’
		5’-GAGCGACGGCTGGAAGAGTA-3’
GAPDH	DQ097338	5’-CCGAGGATGATGTTGTCTCAACT-3’
		5’-CAACGCGATTCCAGCCTTT-3’

completion of PCR amplification, a dissociation curve was run to examine the amplification specificity. For relative quantification of gene expression, amplification efficiencies (E) for each gene were determined from standard curves as follows: a portion of cDNAs transcribed from 2.5 mg of total RNA was diluted to 1/4, 1/16, 1/64, 1/256, 1/1024 and 1/4096, and 2.5 ml of each dilution was used to perform qRT-PCR as above. Amplification efficiencies (E) were calculated from the slopes of the standard curves using the equation: $E = 10^{-1/slope}$ (Pfaffl, 2001). Gene specific amplification efficiency was used to calculate the expression of target genes relative to the expression of the reference canola actin gene. qRT-PCR for each gene was performed in triplicate for each of the three biological replicates.

Results and discussion

Whole plant response to *S. sclerotiorum*

The appearance of control and inoculated leaves at various time points following challenge with *S. sclerotiorum* is shown in Figure 2-1. It is apparent that necrotic lesions appeared after 12 h and expanded rapidly with the lesions reaching leaf margins by 24 h and spread further by 48 h post-inoculation with the pathogen. From these experiments it appears that the critical time for rapid progression of the disease is between 12 and 24 h with further progression in the next 24 h. We therefore chose three different time points, 12, 24 and 48 h post-inoculation, for our transcriptional profiling experiments in order to identify those genes that are modulated in the plant as a result of pathogen invasion and disease progression.

Transcriptional profiling using microarrays

We used cDNA prepared from total canola leaf RNA isolated at 12, 24 and 48 h post-inoculation with *S. sclerotiorum* to perform our transcriptional profiling experiments. As indicated earlier, a total of 18 independent hybridizations were performed which included three biological replicates, three time points for each dye. Our slides contained 70-mer oligonucleotide probes representing 23,686

Figure 2-1 Appearance of *B. napus* leaves challenged with *S. sclerotiorum*. Photographs were taken at 12, 24 and 48 h following infection. The labels UN and IN refer to uninoculated and inoculated leaves, respectively.

unique genes identified by Arabidopsis genome initiative (AGI) locus identifiers. We used the SAM statistical package to identify transcripts that differed significantly between treated and untreated samples. This analysis identified 1675 genes whose expression ratio differed significantly ($P < 0.05$) from 0 (logscale) at various time points following pathogen challenge. Among these transcripts, 1134 decreased in abundance and 541 increased in abundance in the infected leaves compared to the uninoculated (but wounded) controls (Figure 2-2). Among the transcripts that decreased in abundance, 3 were detected at 12 h, 406 at 24 h, and 552 at 48 h post-inoculation with the pathogen. One transcript decreased in abundance at both 12 and 24 h; 171 transcripts decreased at both 24 and 48 h; no transcripts decreased in abundance at both 12 and 48 h, while 1 transcript decreased in abundance at all time points. Conversely, in the case of the transcripts that increased in abundance, 20 were observed at 12 h, 133 at 24 h and 279 at 48 h post-inoculation with the pathogen. Some of the genes with increased transcript abundance were also observed at more than one time-point, with 17 common to both 12 and 24 h; 71 at both 24 and 48 h; 6 at both 12 and 48 h and 15 transcripts with increased abundance at all three time points (Figure 2-2). Thus, in terms of the number of transcripts that differed in abundance compared to controls, the strongest responses to the pathogen were observed at 48 h, with the 24 and 48 h transcriptional profiles being most similar.

Temporal expression patterns of *Sclerotinia* responsive genes

In order to identify temporal patterns of expression within the Sclerotinia-responsive transcripts, we subjected the filtered, normalized microarray data points to STEM (Short Time-series Expression Miner) analysis which classifies the data into groups based on temporal expression patterns. The algorithms within the STEM package are designed specifically for the analysis of time series, such as ours, that sample a small number of time points. Expression profiles of these clusters are presented in Figure 2-3. Among these temporal expression profiles, 120 genes exhibited a gradual increase in transcript abundance between 12 and 24 h post-inoculation with a sudden increase in expression being between

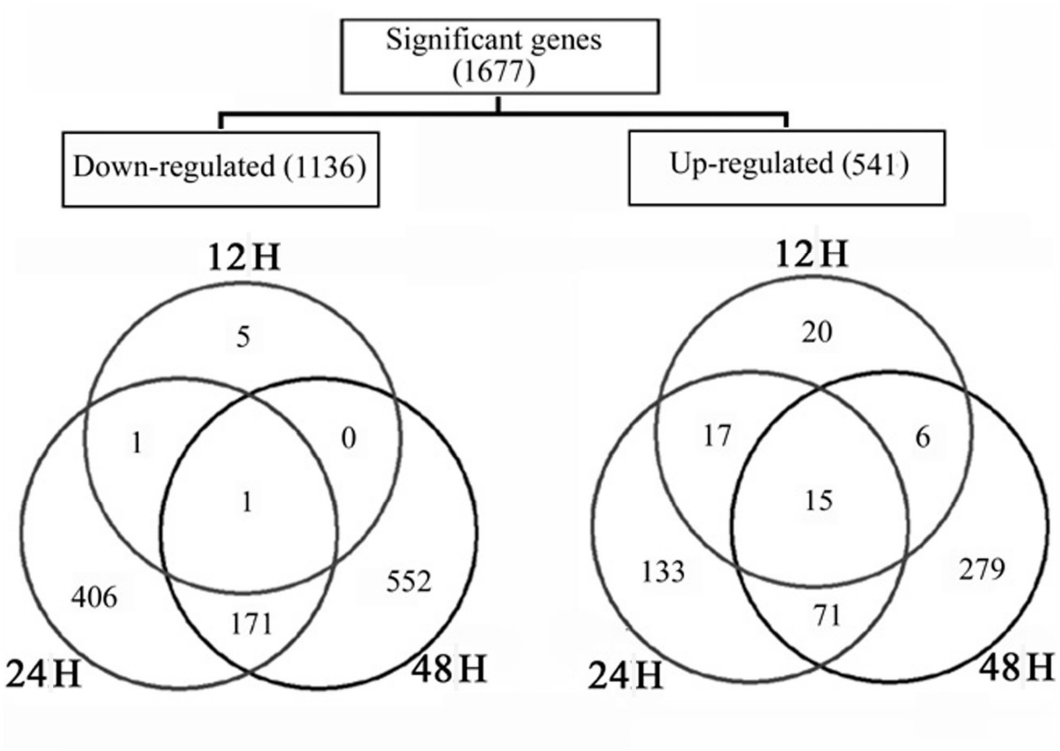

Figure 2-2 Pathogen-responsive transcripts detected by microarray analysis at each time point following challenge. Responsive transcripts are defined as those microarray probes for which the mean signal intensity differed significantly from control samples, according to SAM statistical analysis (FDR < 5%).

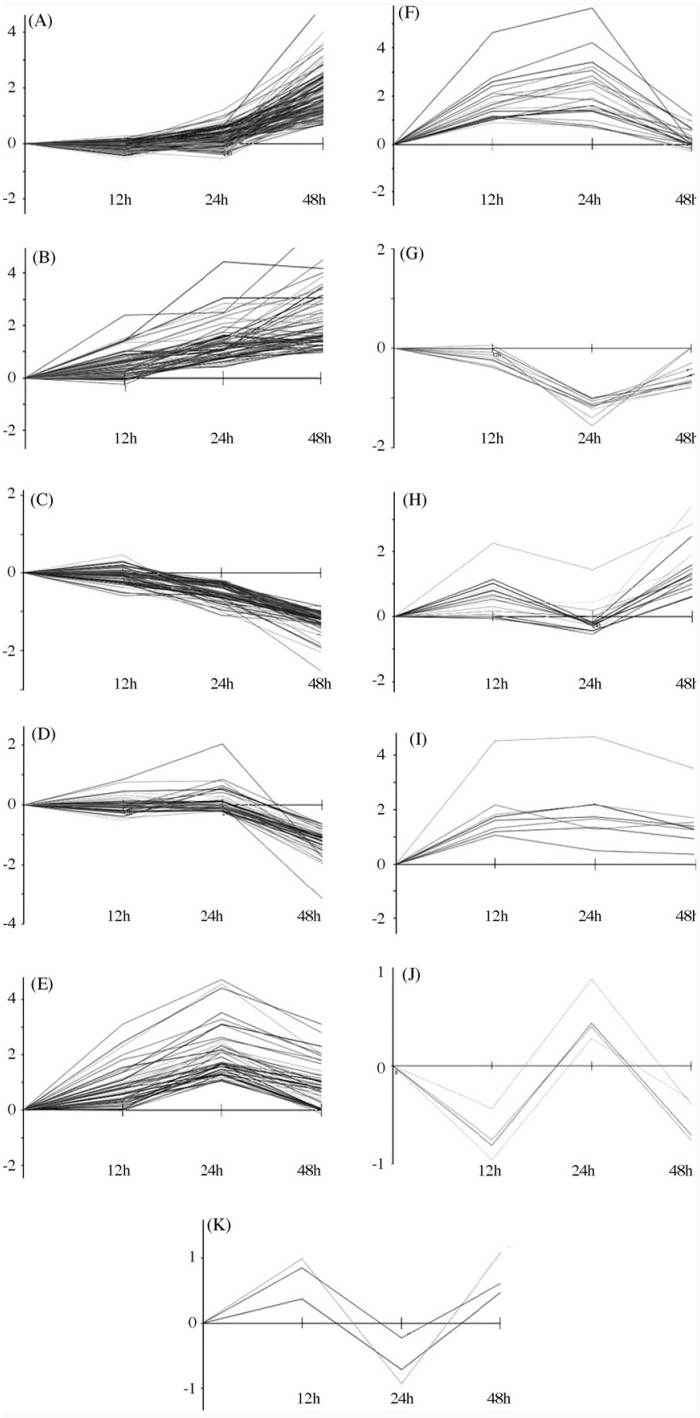

Figure 2-3 Temporal patterns of changes in transcript levels. Cluster profiles from STEM analysis are presented in 11 different groups and frequency of each cluster combination within the datasets at 12, 24 and 48 h time-points are indicated in panels A–K.

24 and 48 h (Figure 2-3A). Another group of genes (67) exhibited a more gradual increase in transcript abundance between 12 and 48 h with no discernable rapid increase between any given time points (Figure 2-3B). The profile of a cluster of 79 genes whose expression started to decline after 12 h post-inoculation exhibited a steeper decline between 24 and 48 h (Figure 2-3C). A group of 54 genes (Figure 2-3D) exhibited constant levels of transcript abundance up to 24 h post-inoculation, after which there was a rapid decrease. Another group of 77 genes (Figure 2-3E and F) exhibited a peak in transcript abundance at 24 h post-inoculation but it appeared that the abundance was less before and after the 24 h time-point (i.e. 12 and 48 h). Details of the genes identified in major clusters (Figure 2-3A-F) are presented in Table A-1. STEM analysis also indicated that the abundance of transcripts for 12 genes declined with a peak at 24 h (Figure 2-3G). The remaining groups of 17, 8, 4 and 3 genes belonging to groups H, I, J and K, respectively (Figure 2-3H, I, J and K) did not exhibit readily generalized patterns. Thus, it is evident that STEM analysis was able to group our data into distinct temporal patterns with the first six groups containing 397 genes and 44 in the remaining five groups. It is possible that the genes exhibiting similar temporal patterns of expression are functioning in concert to mediate plant responses to *S. sclerotiorum*.

Functional classification of *S. sclerotiorum*-responsive genes

Of the 1675 genes whose transcripts that exhibited statistically significant ($P < 0.05$) changes, 342 increased or decreased more than two-fold at any given time-point following pathogen challenge. These genes were further classified based on their putative functions, as is illustrated in Figure 2-4. Among the 258 genes identified as being more than two-fold increases in transcript abundance after pathogen-challenge, a large portion (26%; Figure 2-4A) could not be classified functionally based on existing information. The second largest group (18%) of genes belonged to the category of cell rescue/defense. Other major categories of genes that exhibited an increase in transcript abundance included those that were involved in either protein fate (8%) or those encoding transcription factors (6%; Figure 2-4A). Approximately 7% of genes whose transcript exhibited an increase

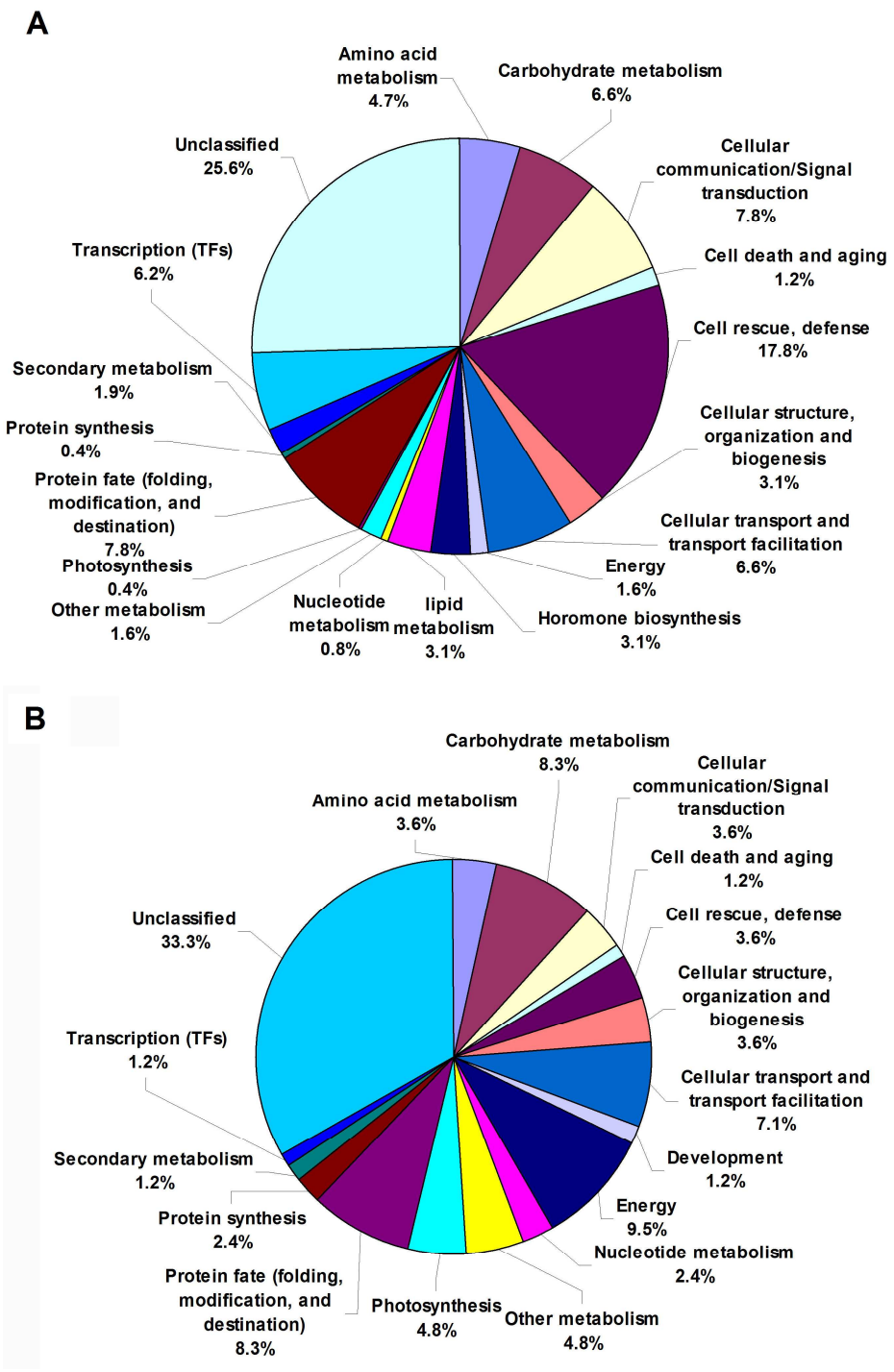

Figure 2-4 Functional classification of genes according to the biological processes in which they are known to participate. Genes exhibiting increase or decrease in transcript abundance following *S. sclerotiorum* challenge are presented in panels (A) and (B), respectively.

in abundance were involved in cellular transport, another 8% in cell signaling/communication, 2% in secondary metabolism, another 5% amino acid metabolism and 3% in hormone biosynthesis. Among the 84 genes that exhibited a decrease in transcript abundance, the biggest category (33%) was composed of those genes whose biological function(s) are not known (Figure 2-4B). Those genes involved in energy metabolism (10%), carbohydrate metabolism (8%), other metabolism (5%), protein fate (8%) and cellular transport (7%) were among the other main groups whose transcripts exhibited decreases in abundance (Figure 2-4B). The identity of those transcripts that exhibited a significant increase (>2-fold, all the time points) and decrease (<1-fold for 12 h and 0.5-fold for 24 and 48 h) are presented in Tables A-2 and A-3, respectively. We will focus the remainder of our report on five major functional categories: (1) Cell rescue and defense related transcripts, (2) transcription factors, (3) genes involved in jasmonate (JA) biosynthesis and signaling, (4) cell wall related transcripts and (5) other transcripts.

Cell rescue and defense related transcriptional

As indicated earlier, a major group of genes whose transcript abundance increased significantly following *S. sclerotiorum* challenge were those involved in cell rescue and/or defense (18%; Figure 2-4A). Specifically, at 12, 24 and 48 h post-challenge, we observed 19, 10 and 18% of transcripts in this category to be significantly more abundant in treated tissues as compared to controls.

Reactive oxygen responses

Reactive oxygen species (ROS) play a dual role in plant biology as both toxic byproducts of aerobic metabolism and key regulators of growth, development and defense pathways (Mittler et al. 2004). In response to pathogens, plants produce ROS, which serve as signaling molecules that lead to the induction of local and systemic resistance (Corpas et al. 2001). Enzymes in plants that are involved in the generation as well as detoxification of ROS include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR) (Mittler et al. 2004). In this study we observed

an increase in transcript abundance of those genes which are involved in ROS signaling including peroxidase (At4g08780, 3.6-fold and At3g49120, 2.5-fold) at 12 h; (At4g08780, 3.8-fold and At3g49120, 2.5-fold) at 24 h; (At5g05340, 2.4-fold and At3g49120, 2-fold) at 48 h; ascorbate peroxidase (At1g07890, 2-fold) at 48 h; glutathione peroxidase (At2g29460, 2.6-fold, At2g29420, 5.9-fold and At1g17170, 6.6- fold) at 48 h (Table S-2); peroxiredoxin (PrxR) (At5g06290, 0.4-fold) at 24 h (Table A-3); putative NADP-dependent oxidoreductase (P2) (At3g03080, 2.7-fold) at 48 h (Table S- 2); and members of glutaredoxin protein family (At1g28480, 2.2-fold and At5g40370, 2-fold) at 48 h (Table A-2). In addition, an increase in abundance of transcript for a related gene belonging to the amine oxidase family (At2g43020, 2.8-fold) was observed at 48 h post-pathogen challenge (Table A-2). The amine oxidase family includes copper-containing amine oxidases (CuAO) as well as flavin containing polyamine oxidases (PAO), which have crucial roles in ROS-mediated programmed cell death (PCD), cell wall reinforcement and in the production of secondary metabolite precursors involved in defense responses (Belenghi et al. 2004; Cona et al. 2005). Our findings are in agreement with previous reports indicating that an ROS burst occurs during the infection of canola with *S. sclerotiorum*, and several proteins with antioxidant functions were increased in abundance (Golkari et al. 2007; Lamb and Dixon, 1997; Zhou et al. 2005).

ABA plays a crucial role in many environmental stresses such as drought, salinity and cold, and it regulates dormancy and maturation during late stages of seed development (Leung and Giraudat, 1998; McCarty, 1995). Dehydrins and LEA proteins are homologous to ABA responsive proteins and they have been identified in different plant systems (Close et al. 1993; Skriver and Mundy, 1990). Our microarray experiments revealed an increase in transcript abundance of early-responsive to dehydration-stress protein (ERD6; At1g08930, 2.3-fold) at 12 h; dehydrin-related (At1g76180, 4.4-fold) at 48 h and RD20 protein (At2g33380, 3.6-fold) at 48 h; late embryogenesis abundant protein (LEA; At4g02380, 6-fold) at 12 h and (At5g06760, 11-fold and At4g02380, 12-fold) at 48 h (Table A-2). During later stages of embryogenesis, the ABA level increases and activates

accumulation of LEA genes. The expression of dehydration-inducible genes are very complex, however, ABA has been suggested to be an elicitor of dehydrin gene transcription (Chandler and Robertson, 1994). It has also been suggested that dehydrins and small heat shock proteins may protect cells from the dehydration effects (Pnueli et al. 2002).

Heat stress proteins (HSPs) are molecular chaperones that are required for maintenance and restoration of protein homeostasis during stress conditions (Baniwal et al. 2004). In our microarray experiments, some of the heat stress related transcripts such as heat shock protein hsp70 (At3g12580, 2-fold) and heat shock protein hsp70-1 (At5g02500, 2.4-fold) were up-regulated at 48 h (Table A-2). A role of heat shock proteins as molecular chaperones was explained (van Montfort et al. 2001), who indicated thay they help in folding of nascent polypeptide chains, refolding of denatured proteins and prevention of irreversible protein aggregation and insolubilization. Chaperones increase the rate of folding and thus increase the resistance of cells under stress conditions (Golkari et al. 2007).

Our microarray results, therefore, suggest that upon challenge with *S. sclerotiorum*, there is a burst of ROS, which may lead to programmed cell death (PCD) as well as increased Ca^{2+} influx into the stomatal guard cells. Furthermore, our detection of a large number of dehydration and heat stress responsive genes in these microarrays indicates significant cross-talk between pathogen-responsive signal transduction pathways and those that are known to be responsive to abiotic stresses. These responses also appear to be mediated by ROS through Ca^{2+} signal transduction. Such cross-talk has been previously described in other pathosystems (Bowler and Fluhr, 2000; Jones et al. 2006; Keates et al. 2003; Kurkcuoglu et al. 2007). However, to the best of our knowledge, this is the first report of such a possibility the *S. sclerotiorum–B. napus* interaction.

Plant defensins.

Defensins are small, basic peptides that have a characteristic three-dimensional structure stabilized by eight disulfide bonds (Thomma et al. 2002). At least 13 putative plant defensin genes (PDF) have been detected in *A. thaliana*, encoding 11 different plant defensins and members of this family display differential activity against different types of microorganisms (Thomma et al. 2002). Based on phylogenetic analysis of putative protein sequences, members of defensins were classified into two families, seven peptides PDF1.1–1.5 in the first family and PDF 2.1–2.6 in the second family (Thomma et al. 2002). However, the molecular components involved in signaling, and putative intracellular targets, are still unknown for most of the plant defensins (Thomma et al. 2002). The transcripts of six plant defensin genes, PDF1.1 (At1g75830, 5.3-fold); PDF1.5 (At1g55010, 4.8-fold);PDF1.2c (At5g44430, 4.3-fold);PDF1.2b (At2g26020, 3.8-fold);PDF1.3 (At2g26010, 3-fold) and PDF1.2a (At5g44420, 4-fold) exhibited an increase in abundance at 48 h (Table A-2). Our results are consistent with the previously reported induction of the leaf-specific PDF1.2 expression by pathogen infection and that mutants impaired in either jasmonate (JA) or ethylene signaling do not express this defensin gene and are hyper-susceptible to infection (Penninckx et al. 1996; Thomma et al. 1998). The role of plant defensins in disease tolerance has been demonstrated through studies where transgenic expression of defensin genes has led to increased tolerance to pathogen attack (Gao et al. 2000).

Leucine-rich repeat proteins (LRR).

Leucine-rich repeats (LRRs) are 20–29-residue sequence motifs present in a number of proteins with diverse functions, such as hormone–receptor interactions, enzyme inhibition, cell adhesion and cellular trafficking (Kobe and Kajava, 2001). Plant responses to pathogens involve complex signal transduction pathways and many defense genes are activated by processes that rely on LRRs present in those genes (Shanmugam 2005). We observed that the transcript abundance for three genes containing LRR motifs increased at different time-

points following pathogen challenge. These included a member of the extensin family (At1g21310, 2.1-fold) at 12 h; putative leucine-rich repeat transmembrane protein kinase (At1g09970; 2.1-fold) at 24 h and disease resistance protein-related (LRR; At1g33590, 3-fold) at 48 h post-pathogen challenge (Table A-2). There are several reports in the literature that describe role of LRRs for various actions following ligand recognition (Shanmugam 2005). Extracellular plant proteins such as polygalacturonase inhibiting proteins (PGIPs), which are capable of inhibiting fungal endopolygalacturonases (PGs) have specific recognition abilities against many PGs produced by fungi (De Lorenzo et al. 2001; Di et al. 2006). The beta-turn motifs of the LRRs are responsible for the specific interaction of PGIPs with PGs (De Lorenzo et al. 2001). The importance of LRRs in plant disease responses was further illustrated by a study in flax (*Linum usitatissimum* L.) where differences in the specificities of resistance were determined by six amino acid changes, that were confined to beta-turn motif of LRR repeats (Dodds et al. 2001). Overexpression of PGIPs and/or extensins has been suggested as a possible strategies for engineering disease resistance (Shanmugam, 2005) and the results from our microarray analysis also suggest an important role for these genes in the response of *B. napus* to *S. sclerotiorum*.

Steroid sulfotransferase.

Sulfotransferases (ST) catalyze the transfer of a sulfate group from a donor to various acceptors including steroid molecules (Negishi et al. 2001). Another gene identified in our microarray experiment with increased transcript abundance following *S. sclerotiorum* challenge was a putative steroid sulfotransferase (At2g03760; eight-fold) at 48 h (Table A-2). The sulfate-conjugation reaction plays an important role in growth, development and adaptation to stress in plants (Klein and Papenbrock, 2004). Moreover, the sulfate-conjugation reaction also facilitates transport and excretion of hydrophobic molecules and abolishes the biological activity of steroid molecules such as estrogens (Gidda et al. 2003). Specifically, it has been reported by others (Lacomme and Roby, 1996) that transcripts of At2g03760 (the putative steroid ST identified in our studies as well)

accumulated after seedlings mature plants were treated with either salicylic acid (SA) or methyl jasmonate (mJA) and after challenge with *Xanthomonas campestris* pv. *campestri.* The three dimensional structure of At2g03760 from *A. thaliana*, which has high structural similarity to two human steroid STs was characterized (Staswick et al. 1998). In addition to deactivation of steroids, plant brassinosteroid ST also uses the sulfonation reaction to control the biological activity of hormones (Rouleau et al. 1999). Therefore, an increase in abundance of ST transcript in our microarray experiment may be indicative of a potential role for sulfonation in plant defense responses following pathogen challenge. Additional studies are currently underway in our laboratory aimed at investigating the role of this ST gene product in regulating the biological activities of hormones involved in mediating plant disease responses.

Other defense-related transcripts.

Many transcripts that play a crucial role in cross-talk between abiotic and biotic stress response pathways increased in abundance at 48 h post-*S. sclerotiorum* challenge and included genes encoding a low temperature and salt responsive protein LTI6A (At3g05880, 3.7-fold); cold-regulated protein COR6.6 or stress-induced protein KIN2 (At5g15970, 2.4-fold); formate dehydrogenase (FDH; At5g14780, 3-fold); stress related protein (At3g05500, 2.7-fold); symbiosis-related protein (At4g21980, 2-fold, At4g04620, 3-fold); glycine-rich protein (At3g06780, 2.9- fold) and putative myrosinase-associated protein (At1g54020, 3-fold and At1g52030, 2.2-fold; Table A-2). The roles of these genes in mediating plant responses to various stresses have been previously reported. However, this is the first report in which their involvement in *B. napus–S. sclerotiorum* interaction has been demonstrated and must be investigated further.

Transcription factors

Transcription factors (TFs) transmit pathogen-derived defense signals to either activate or suppress downstream defense gene expression, and regulate cross-talk between different signaling pathways (Anderson et al. 2004; Lorenzo et al. 2003; Singh et al. 2002). Virtually every major TF gene family harbors at least some

TF genes that have been implicated in some aspect of plant defense. We observed that, among the genes with increased transcript abundance, 15% at 12 h, 10% at 24 h and 6% at 48 h were composed of those encoding TFs and constituted 6.2% of all genes with increased transcript abundance (Figure 2-4A). However, in the case of the genes whose transcripts were observed to be lesser in abundance following pathogen challenge, only 1.2% was composed of TFs (Figure 2-4B). Among the TFs with increased transcript abundance, members of several families were detected including heat stress transcription factors (HSFs) and ethylene responsive factors (ERFs) and are discussed below.

Heat stress transcription factor (HSFs).

HSFs mediate the activation of genes responsive to heat and a large number of chemical stressors, which is followed by the rapid accumulation of heat shock proteins (Morimoto et al. 1992). HSFs also play a central role not only in protection against stress damage but also in folding, intracellular distribution and degradation of proteins and the functioning of signal transduction cascades (Baniwal et al. 2004; Nover et al. 2001). In our microarray results, we identified heat shock transcription factor 4 (HSF4; At4g36990) which demonstrated 3.1-, 5.5- and 3.4-fold increases in abundance at 12, 24 and 48 h post-pathogen challenge, respectively (Table A-2). Other members of heat shock proteins such as hsp70-1 (At5g02500, 3.2-fold) at 24 h; hsp70 (At3g12580, 2.7-fold) at 24 h; hsp70-2 (At5g02490, 2.7- fold) at 24 h (Table A-2); and hsc70-7 (At5g49910, 0.5-fold) at 48 h were also detected (Table A-3). HSFs have been classified into three classes: A (HSF1 and HSF3), B (HSF4 and HSF7), and C (Lohmann et al. 2004; Nover et al. 2001). HSF1 and HSF3 are fast response regulators that may be important for the coordination of stress gene expression and generation of stress tolerance under rapidly changing environmental conditions in natural habitats (Lohmann et al. 2004). In our microarray experiments we observed an increase in abundance of the transcript for HSF4. The role of HSF4 is still unclear. However, HSF4 and HSF7, which are members of class B, have been suggested as transcriptional repressors or attenuators of the heat shock response

(Czarnecka-Verner et al. 2000; Lohmann et al. 2004). The isolation and analysis of HSF4 knock-out mutants will be required to investigate its functional roles in plants particularly in response to pathogens.

Ethylene responsive factors (ERFs).

Genes in the ERF family encode transcriptional regulators with a variety of functions that are involved in various developmental and physiological processes in plants. Our microarray results demonstrated an increase in transcript abundance of various ERFs such as ethylene responsive element binding factor 5 (AtERF5; At5g47230, 3.8-fold) at 12 h; ethylene responsive element binding factor 4 (ERF4; At3g15210, 4.4-fold) at 24 h; ERF5 (At5g47230, 3.4-fold) at 24 h; AP2 domain transcription factor family (At5g13330, 2.6-fold) at 24 h; ERF4 (At3g15210, 2.7-fold) at 48 h; ERF5 (At5g47230, 2-fold) at 48 h and AP2 (At3g50260, 2.4-fold) at 48 h (Table A-2). The action of ethylene upon gene expression involves ethylene responsive element binding factors (ERFs) containing a highly conserved DNA binding domain (the ERF domain containing 58–59 amino acids; (Ohmetakagi and Shinshi, 1995)), which binds with high affinity to the GCC box (Hao et al. 1998). A model has been established in which ERFs, bound to the GCC box contained in ethylene and/ or stress responsive genes, recruit the SAP (Sin3)-HDA19 co-repressor complex to the genes, which then mediates transcriptional repression through histone deacetylase activity (Viiri et al. 2006). Our findings suggest a relationship between plant defensin gene expression and ERFs. For example, the defensin gene PDF1.2, which is regulated by the JA pathway (Anderson et al. 2004), contains a GCC-box motif in its promoter (McGrath et al. 2005) and it is known that AtERF4 negatively regulates the MeJA-responsive expression of PDF1.2 (McGrath et al. 2005). Therefore, it appears that during the *S. sclerotiorum–B. napus* interaction, the regulation of MeJAresponsive genes might be important for mediating plant responses to this pathogen and is discussed later within the context of genes involved in JA biosynthesis and signal transduction.

Calcium signal transduction and G-protein coupled receptors.

Calcium is an important messenger in signal transduction pathways and functions as a central node to coordinate and synchronize diverse stimuli in various stress conditions (Mahajan and Tuteja, 2005). Reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), nitric oxide and Ca^{2+} participate in apoptosis or PCD (Wang et al. 2006). Downstream signaling by ROS might involve Ca^{2+} and Ca^{2+} binding proteins (Mittler et al. 2004) which may be also important for cross-talk between abiotic and biotic stress responses in plants (Sanders et al. 2002). Transcripts of several genes involved in calcium signal transduction were observed to be increased in abundance at more than one time-point following pathogen challenge. These included C2 domain-containing protein (At4g34150, 3.4-fold) at 24 h; calmodulin-related protein (At3g50770, 3-fold) at 24 h; calcium-transporting ATPase plasma membrane-type (Ca^{2+}-ATPase, isoform 2; At4g37640, 4.6-fold) at 24 h (Table A-2). Influx of Ca2+ into the cytosol of guard cells control stomatal opening and it is known that ABA regulates the Ca^{2+} concentration in guard cells (Allen et al. 1999; McAinsh et al. 1990). It has also been demonstrated that hydrogen peroxide plays a crucial role in the ABA-mediated influx of Ca^{2+} into the guard cells (Pei et al. 2000).

At present, the receptors for ROS are unknown. It has been proposed that plant cells sense ROS via at least three different mechanisms (i) unidentified receptor proteins; (ii) redox sensitive transcription factors (e.g. NPR1 or HSFs); and (iii) direct inhibition of phosphatases (Mittler et al. 2004 and references therein). We observed that the abundance of the transcript for heat shock transcription factor 4 (HSF4; At4g36990, 3.4-fold) was increased in response to *S. sclerotiorum* at 48 h after challenge (Table A-2). G-protein coupled receptors (GPCR) are direct targets of ROS (Kokkola et al. 2005). GPCR have seven transmembrane spanning domains and in plants there are more than 20 proteins which have been demonstrated to possess similar structure (Fujisawa et al. 2001 and references therein). The heteromeric G-proteins comprised of a-/b-subunits, which have been demonstrated to be localized in plasma membrane of Arabidopsis (Weiss et al. 1997). The function of these proteins are still unknown,

however, an in vitro experiment in tomato demonstrated activation of plasma membrance Ca^{2+} channel by a subunit of G proteins (Aharon et al. 1998). It has also been suggested that plant G proteins may be involved in regulation of Ca^{2+} movement and elicitor signaling (Fujisawa et al. 2001). The transcripts for two genes involved in G-protein coupled receptor protein signaling pathway (At2g16630, 0.4-fold and At1g21500, 0.5-fold) were detected as those with decreased transcript abundance in our microarray experiments (Table A-3). Our results are consistent with previous reports describing a decrease in abundance of these transcripts in plants following both abiotic and biotic stresses (Golkari et al. 2007; Lu, 2003; Wang et al. 2004).

Other transcription factors.

Members of b-ZIP TF family, which regulate diverse biological processes such as pathogen defense, light and stress signaling, seed maturation and flower development (Jakoby et al. 2002), also exhibited modulation in transcript levels post *S. sclerotiorum*-challenge. Members exhibiting transcript abundance changes in this family included, homeobox-leucine zipper protein HAT22 (At4g37790, 2.1-fold) at 24 h; bZIP family transcription factor (At1g42990, 2.3-fold) at 48 h; MADS-box protein (At1g31630, 2.2-fold) at 48 h; transducin/WD-40 repeat protein family (At1g18830, 2-fold) and putative ovule development protein (At2g41710, 3.8-fold) at 48 h (Table A-2). In addition, other important TFs that demonstrated increases in transcript abundance included: salt-tolerance zinc finger protein (At1g27730, 6-fold), 12 h; zinc-finger protein-related (At2g28200, 3.3-fold) at 24 h; no apical meristem (NAM) protein family (At3g15500, 2.5-fold) at 24 h and zinc-finger protein-related (At2g28200, 2.2-fold) at 48 h (Table A-2). Only C2H2-type zinc finger protein-related transcript exhibited a decrease in abundance (At2g41940, 0.4-fold, Table A-3). Further research on these candidate TF genes will most likely reveal other new aspects of regulation of plant defense and related signaling pathways in this pathosystem.

JA biosynthesis and signaling

Jasmonate (JA) plays an important role in modulating various physiological events such as resistance to pathogens and insects, fruit ripening, maturation of pollen, root growth and senescence (Creelman and Mullet, 1997). JA synthesis involves oxygenation of linolenic acid by lipoxygenase (LOX), which is then converted to 12-oxo-phytodienoic acid by allene oxide synthase (AOS) and allene oxide cyclase (AOC). An additional reduction step by oxophytodienoic acid reductase (OPR) and three steps of boxidation lead to the synthesis of JA (Sasaki et al. 2001). In our microarray experiments, JA biosynthesis-related transcripts that exhibited increases in abundance included allene oxide cyclase family members (At3g25780, 4.7-fold) at 12 h and 9.2-fold at 24 h; cytochrome P450 83B1 (At4g31500, 2.2-fold) at 12 h; 12-oxophytodienoate reductase (OPR3; At2g06050, 2.7-fold) at 24 h; allene oxide synthase/ cytochrome P450 74A (At5g42650, 12-fold at 24 h and 20-fold at 48 h) and 12- oxophytodienoate reductase (OPR1; At1g76680, 2.6-fold) at 48 h (Table A-2). Our results suggest that one of the responses to *S. sclerotiorum* infection might be increased JA biosynthesis, as evidenced by the increased abundance of transcripts for enzymes involved in the JA biosynthetic pathway. A proteomics-based investigation currently underway in our laboratory revealed an increase in the level of JA-responsive protein JR1, at 12 and at 48 h post-challenge with *S. sclerotiorum* (Liang et al, unpublished observations) providing additional evidence for JA-mediated processes in responses of *B. napus* to this pathogen. Furthermore, as mentioned earlier, JA and ethylene signaling pathways are essential for the expression of the plant defensin 1.2 (PDF1.2) gene, which encodes a JA-responsive, antimicrobial plant protein (Penninckx et al. 1998). The importance of JA-dependent defense gene expression is further illustrated by the fact that Arabidopsis mutants defective in this process exhibited compromised resistance to necrotrophic fungal pathogens (Staswick et al. 1998; Thomma et al. 1998). In addition, transgenic plants overexpressing transcription factors (TFs) which are involved in the ethylene- and JA-responsive pathways demonstrated an increased tolerance to several necrotrophic pathogens (Berrocal-Lobo and Molina, 2004;

Berrocal-Lobo et al. 2002). The extent of crosstalk between JA- and other signaling pathways during the interaction of *B. napus* with *S. sclerotiorum* is unclear. However, results from our microarray analysis suggest an important role for JA biosynthesis and signaling during the early events of this host-pathogen interaction.

Transcripts related to cell wall structure and function

Plant cell walls play an active role in growth, development, signaling, intercellular communication and plant defense (Showalter, 1993). A complex network of cellulose, hemicelluloses, pectins and structural proteins in plant cell walls determines their dynamic nature and function (Campbell and Braam, 1999). Transcripts of many genes encoding proteins putatively related to cell wall structure and function exhibited increased abundance and included those for arabinogalactan- protein AGP2 (At2g22470, 4.1-fold) and AGP16 (At2g46330, 2.3-fold) at 24 h; AGP12 (At3g13520, 2.6-fold) and AGP2 (At2g22470, 4.5-fold) at 48 h; extensin family protein (At1g21310, 2-fold) at 12 h; glycine-rich protein (GRP; At3g06780, 2.9-fold) at 48 h and proline-rich protein (PRPs; At4g13390, 2.1-fold; At4g08370, 2.2-fold and At5g06630, 2.3- fold) at 48 h (Table A-2). Among the cell wall-related transcripts that exhibited modulation in our microarray experiments, many genes encoded for proline/hydroxyproline rich proteins (PHPs). The PHP family is classified into three classes: PRPs, extensins and AGPs (Schultz et al. 2002). AGP proteins are proteoglycans that are secreted by plant cells and suggested to be involved in many aspects of plant growth and development, including cell wall deposition, signaling and differentiation (Showalter, 2001).

In Arabidopsis, there are at least 35 genes that encode AGP proteins. The backbones of most of the AGPs are anchored to the plasma membrane by glycosylphosphatidylinositol (GPI) anchors (Schultz et al. 2002; Youl et al. 1998) , which transiently attaches AGPs to the plasma membrane before they are released into the cell wall following hydrolysis of the GPI anchor (Gilson et al. 2001). The structural proteins extensins are present in the cell walls of higher plants, and their levels are regulated by wounding and pathogen infection

(Shanmugam, 2005). As discussed previously, extensins in association with LRR motifs are important for a plant's defense response against a pathogen. GRPs have important functions with respect to the plant vascular systems and their synthesis is part of the plant's defense mechanism (Mousavi and Hotta, 2005). Our results suggest that PRPs, and synergistic activities with GRP, may play crucial roles in the defense of *B. napus* from *S. sclerotiorum* invasion. Members of the glycosyl hydrolase family exhibited an increase in transcript abundance (At2g27500, 2.8-fold and At3g13790, 2.6-fold) at 24 h and (At5g49360, 4.3-fold; At5g28510, 2-fold; At2g14690, 2-fold) 48 h post-*S. sclerotiorum* challenge (Table A-2). A member of the glycosyl hydrolase family 17 (At2g27500) hydrolyzes 1,3-b-glucan polysaccharides found in cell walls and may play an important role in the defense response by attacking fungal cell walls (Davies and Henrissat, 1995; Hrmova et al. 1997; Masoud et al. 1996). Although a number of genes presumably involved in increasing cell wall integrity were observed to be induced in response to *S. sclerotiorum*, a decrease in the abundance of transcript involved in modification of cell wall such as xyloglucan endotransglycosylase (XEGs; At4g03210, 0.3-fold) at 24 h was observed (Table A-3). We also observed a decrease in the abundance of many transcripts that are related to carbohydrate metabolism such as starch synthase (At5g24300, 0.5-fold) at 48 h; phosphoglucomutase (At5g51820, 0.5-fold) at 48 h; aldose-1-epimerase (At5g66530, 0.5-fold) at 48 h and isoamylase (At2g39930, 0.5- fold, and At1g31190, 0.4-fold) at 48 h (Table A-3). Modulation in transcript abundance for many potentially cell wall-related lipid transfer proteins (LTPs) and enzymes involved in lignification were also observed in our microarray experiments. In addition, a few transcripts involved in phenylpropanoid metabolism including an O-methyltransferase (At1g21130, 4.3-fold and At1g21120, 4.3-fold) at 12 h and (At1g21130, 4.3-fold; At1g21120, 3.9-fold and At4g35150, 3.8-fold) at 24 h exhibited increased transcript abundance (Table A-2). Members of the protease inhibitor/seed storage/lipid transfer protein (LTP) family also exhibited increased transcript abundance (At3g22600, 22-fold) at 24 h (At3g22620, 2.3-fold and At3g22600, 8.4-fold) and at 48 h (Table A-2) and decreased transcript abundance

(At2g45180, 0.3-fold; At2g38530, 0.4- fold; At1g12090, 0.4-fold) at 48 h (Table A-3). Involvement of a putative LTP in systemic resistance signaling was demonstrated in *A. thaliana* (Maldonado et al. 2002) and the transcript abundance of many LTPs in our experiments also suggest an important role of LTPs in the *B. napus–S. sclerotiorum* interaction.

Other transcripts

Roles for specific proteins in transporting molecules across plasma and vacuolar membranes during abiotic and biotic stresses have been previously reported (Jiang and Deyholos, 2006; Liu et al. 2005; Wan et al. 2002). Pathogen associated increases in abundance of transcripts for many putative transporters involved in the transport of water, sugar, lipid and other molecules were also observed in our microarray experiments. These included mitochondrial phosphate transporters (At3g48850, 5-fold) at 24 h; MATE efflux family proteins (At1g61890, 4-fold) at 24 h; putative hexose transporter (At5g26340, 3.8-fold) at 24 h; ABC transporter family protein (At2g29940, 13-fold) at 48 h and a putative sugar transporter (At2g48020, 2.6-fold) at 48 h (Table A-2). Increases in abundance of transcripts for these transporters have been reported in other pathosystems, for example, amino acid and sugar transporters in the *Triticum aestivum–Mycosphaeiella graminicola* interaction (Keon et al. 2007); a sucrose transporter in the *A. thaliana–Meloidogyne spp*. (Hammes et al. 2005) and an ABC transporter in the *A. thaliana–Agrobacterium tumefaceins* (Ditt et al. 2006). Many transcripts for components of the glycolytic pathway exhibited a decrease in abundance, whereas those involved in tricarboxylic (TCA) cycle, and the pentose phosphate pathway (PPP) exhibited an increase in abundance post-*S. sclerotiorum* challenge (Tables A-2 and A-3). We observed an increase in transcript abundance of monodehydroascorbate reductase (At5g03630, 10-fold) at 48 h; quinine reductase (At5g54500, 2.6-fold) at 48 h; glucose-6-phosphate dehydrogenase (G6PDH; At5g40760, 2.5-fold) and malate dehydrogenase (At5g25880, 2.6-fold) at 48 h in our microarray experiment, which are reported to be up-regulated under oxidative stresses (Chen et al. 2004; Jiang and Deyholos, 2006; Rizhsky et al. 2002; Saher et al. 2005).

Validation of microarray results by qRT-PCR

Quantitative real-time PCR (qRT-PCR) experiments were performed to validate selected genes, which exhibited at least 2-fold increase or 0.5-fold decrease in transcript abundance in our microarray experiments (Figure 2-5). These genes included glutaredoxin, 2-cys peroxiredoxin, RD20, defensin PDF1.1, defensin PDF1.5 and steroid sulfotransferase; salt-tolerance zinc finger TF; allene oxide synthase, allene oxide cyclase; and glyoxalase, xyloglucan endotransglycosylase, starch synthase, jacalin, glutamate ammonia ligase and glutamate dehydrogenase. As evident from Figure 2-5, our qRT-PCR results are consistent with the microarray data and validate not only the results of our microarray experiments but also suggests a crucial role for these genes in cell rescue and defense, JA biosynthesis and signaling, cell wall integrity as well as other metabolic pathways during *B. napus–S. sclerotiorum* interaction.

Concluding remarks

We have profiled the transcriptional changes that accompany *S. sclerotiorum* infection of *B. napus* using microarrays containing 23,686 unique *A. thaliana* genes at three different time points following pathogen infection. A similar study performed by Liu et al. (Liu et al. 2005) used microarrays containing 6,120 cDNA probes and identified 61 and 25 genes that exhibited a *S. sclerotiorum*-induced two-fold increase or decrease in transcript abundance, respectively, at one time-point (32 h). Our study is a substantially more detailed investigation where oligonucleotide arrays representing 23,686 unique genes were used. Our results indicated that 258 and 84 genes exhibited a two-fold increase or decrease in transcript abundance, respectively at the three (12, 24 and 48 h) time-points investigated in this study. Genes encoding proteins involved in the scavenging of ROS, plant defensins, those involved in JA biosynthesis and transcriptional factors exhibited more than a two-fold increase in transcript abundance and were not previously reported as being responsive to *S. sclerotiorum* (Liu et al. 2005). Similarly, many genes which exhibited a two -fold decrease in transcript abundance were also identified only in this study.

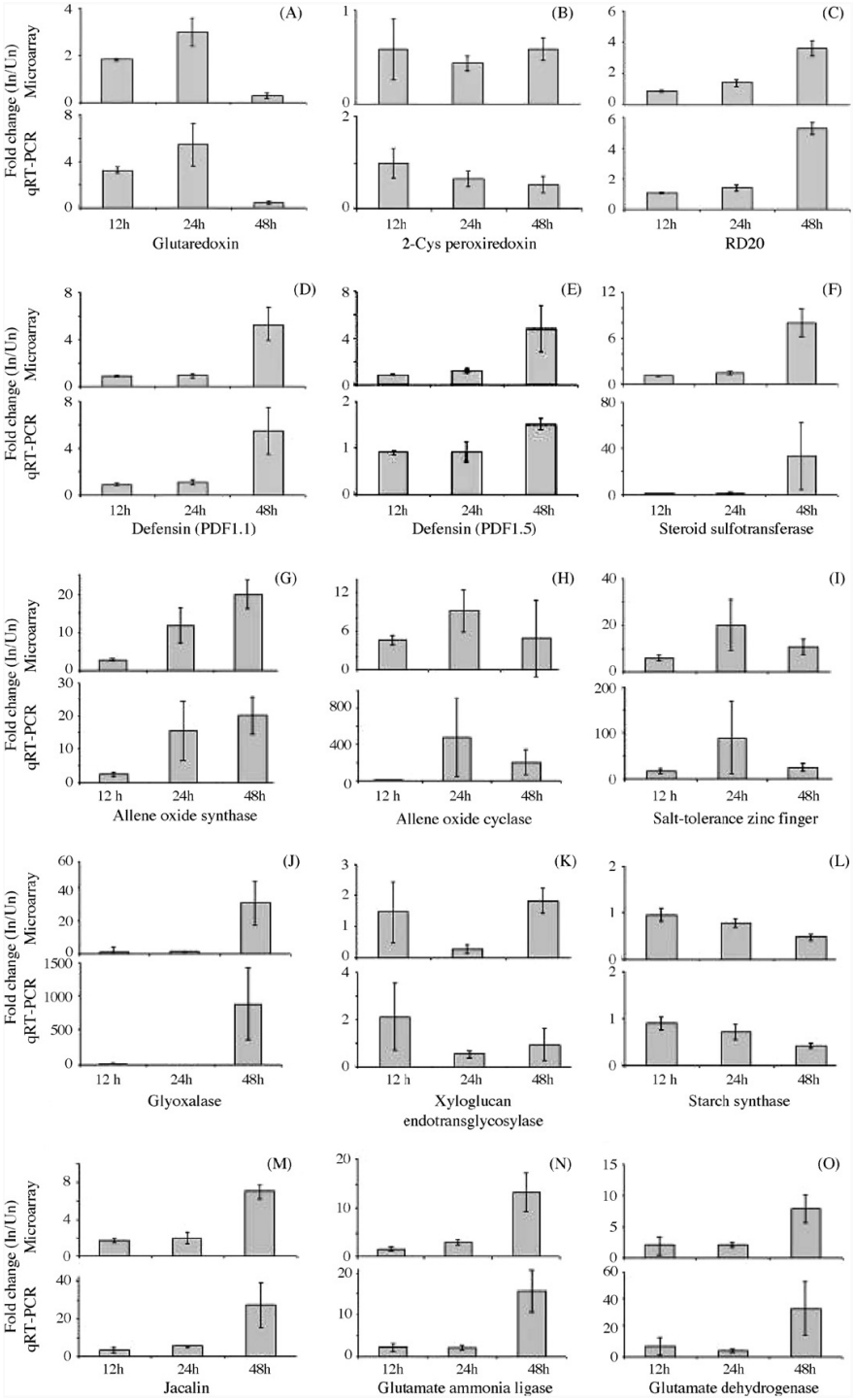

Figure 2-5 Comparison of microarray and qRT-PCR results for the relative expression of selected genes at 12, 24 and 48 h post-*S. sclerotiorum*-challenge. The fold changes in transcript abundance were expressed relative to control (uninoculated), and for qRT-PCR all the data were normalized against the expression of the actin gene.

This is the first detailed report describing the use of spotted oligonucleotide arrays containing this large number of genes to investigate changes in gene expression during this host pathogen interaction, which were subsequently validated using qRT-PCR. Future studies will be directed towards characterizing the role of JA and JA-mediated gene expression, the expression of those genes involved in ROS metabolism, cell wall integrity and defense, within the context of the compatible/incompatible interaction of *S. sclerotiorum* with plant hosts.

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Chapter 3 Characterization of defense signaling pathways of *Brassica napus* and *Brassica carinata* in response to *Sclerotinia sclerotiorum* challenge

Introduction

When plants are challenged by pathogens, a complex and integrated set of defense responses are triggered (Dong 1998; Kunkel and Brooks 2002). These include wax layers, pre-formed antimicrobial enzymes, secondary metabolites and toxic compounds under the constitutive responses and systemic acquired resistance (SAR) as well as induced systemic resistance (ISR) under the induced responses (Kunkel and Brooks 2002). A series of events are initiated in plants during interactions between plants and pathogens including the production of signaling compounds such as jasmonic acid (JA), ethylene (ET), salicylic acid (SA) or reactive oxygen species (ROS) (Kunkel and Brooks 2002). Signaling pathways, mediated by JA/ET and SA, are important components of plant defense systems (Dong 1998; Feys and Parker 2000; Martinez et al. 2001; McDowell and Dangl 2000). They are regulated and can act independently, synergistically or antagonistically, which depends on the system of host and pathogens and are discussed below (Glazebrook 2005; Kachroo 2007).

JA and its derivate methyl jasmonate (MeJA) are signaling molecules important for initiating and/or maintaining developmental processes and defense responses in various plants (Clarke et al. 2001; van der Fits et al. 2000). In Arabidopsis, as revealed by microarray analysis, five out of 41 genes responding to JA are those involved in its biosynthesis, indicating the existence of a positive feedback regulatory system for JA biosynthesis (Sasaki et al. 2001). JA induces the transcription of genes coding for lipoxygenase (*LOX2*), defective in anther

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dehiscence1 (*DAD1*), allene oxide synthase (*AOS*), allene oxide cyclase (*AOC*), 12-oxophytodienoate reductase (*OPR3*), and jasmonic acid carboxyl methyltransferase (*JMT*) (Heitz et al. 1997; Ishiguro et al. 2001; Laudert and Weiler 1998; Mussig et al. 2000; Seo et al. 2001; Stenzel et al. 2003). Among the aforementioned, AOC is a key enzyme in the biosynthesis of JA and functions to release the first cyclic and biologically active metabolite - 12-oxo-phytodienoic acid (OPDA) (Schaller, et al. 2008).

ET signaling has been reported to play an important role in mediating a broad-spectrum resistance against pathogens in Arabidopsis (Bent, et al. 1992; Thomma, et al. 1999) and rice (Singh, et al. 2004). Some of the genes involved in ET signaling have been identified and characterized in Arabidopsis (Wang, et al. 2002) and tomato (Wilkinson et al. 1995). In Arabidopsis, cDNAs encoding five different ERF proteins (AtERF1 to AtERF5), which show GCC box–specific binding activity, are differentially regulated by ET, wounding, cold, high salinity or drought, via ethylene-insensitive2 (*EIN2*)-dependent or -independent pathways (Fujimoto et al. 2000). The transcript abundance of *AtERF1*, *AtERF2* and *AtERF5* increased two- to three- fold 12 h after ET treatment, whereas *AtERF3* and *AtERF4* transcripts did not increase (Fujimoto et al. 2000). Hence, it was proposed that AtERF1, AtERF2 and AtERF5 act as transcriptional activators whereas AtERF3 and AtERF4 act as transcriptional repressors (Fujimoto et al. 2000). Moreover, senescence has been reported to be induced by ET (Bleecker et al. 1988; Grbic and Bleecker 1995), and necrotrophic pathogens benefit from host cell death (Glazebrook 2005) by producing an environment conducive to the growth and colonization of such fungi. However, it has also been reported that ET plays an important role in mediating resistance to pathogens (Ohtsubo et al. 1999; van Loon et al. 2006). In addition to the aforementioned studies on the ET signaling pathways, the role of ET in plant development and its responses to environmental stimuli has been investigated using several strategies aimed at modulating the levels of endogenous ET (Stearns and Glick 2003). Among these, the application of a gene encoding 1-aminocyclopropane- 1-carboxylate (ACC)

deaminase isolated from *Pseudomonas putida* strain UW4 (Shah et al. 1998) has been demonstrated to lower the level of ET in plants because ACC deaminase can metabolize the ET precursor ACC to α-ketobutyrate and ammonia (Glick 1995; Glick et al. 1999; Jacobson et al. 1994). This gene has been reported to alleviate ET exposure and thereby down-regulate ET responsive genes (Glick et al. 1998), increase root elongation (Penrose et al. 2001) and protect plants from some phytopathogens (Lund et al. 1998; Robison et al. 2001).

SA signaling pathways in defense responses have been well characterized including SA accumulation, pathogenesis related (PR) gene expression, and the induction of local and systemic acquired resistance (Delaney et al. 1994; Friedrich et al. 1995; Meuwly et al. 1995; Shah and Klessig 1996). Nonexpresser of PR gene 1 (*NPR1*, also known as *NIM1* and *SAI1*), a key regulator of the SA signaling pathway, controls SA-mediated SAR signaling pathway in Arabidopsis (Cao et al. 1994; Cao et al. 1997; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). It has been demonstrated that the expression of *NPR1* is increased as a result of treatment with SA, 2,6-dichloroisonicotinic acid (INA), benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), or pathogen infection (Cao et al. 1997; Ryals et al. 1997). Moreover, the ability to induce PR gene expression and generate an SAR response is impaired in Arabidopsis *npr1/nim1* mutants after treatment with SA or INA (Chern et al. 2005). *AtNPR1* can interact with the TGA subfamily of the basic leucine zipper protein (bZIP) family of transcription factors (Kesarwani et al. 2007); TGA2, TGA5 and TGA6 are positive regulators of SAR (Zhang et al. 2003). All these provide evidence that SA plays a crucial role in mediating plant defense responses.

Canola (*Brassica napus* L.) is a major edible oil crop worldwide and 12.6 million tons were harvested in Canada alone in 2008 (http://www.canola-council.org/acreageyields.aspx). Canola yield is significantly affected by plant diseases and Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (another necrotrophic phytopathogen) is an important disease. Considerable efforts are underway to understand the comprehensive molecular responses of plants to this pathogen (Liang et al. 2008; Yang et al. 2007; Zhao et al. 2007). Among the

Brassica species, *B. carinata* is reported to be tolerant to *S. sclerotiorum* (Bansal et al. 1990). Therefore, in order to investigate possible differences in the JA, ET and SA-mediated signaling pathways between *B. napus* and *B. carinata,* the expression of five genes (*AOC3, ERF2*, *NPR1*, *TGA5* and *TGA6*) was characterized. The availability of an ACC-deaminase transgenic canola which was reported to produce low levels of ET permitted us to investigate whether ET plays any role in the susceptibility/tolerance to *S. sclerotiorum* (Shah et al. 1998). Our results are discussed within the context of the *Brassica*-Sclerotinia interaction and possible strategies to engineer durable tolerance to this pathogen.

Materials and methods

Identification of signaling genes in canola

Orthologs of five Arabidopsis genes implicated in SA or JA/ET signaling were identified in *B. napus* by Blastn analysis using the cDNA sequences of the Arabidopsis genes from both the non-redundant (nr) and expressed sequence tag (EST) databases. These presumptive orthologs were validated by performing a reciprocal Blastn search of the identified canola genes in the Arabidopsis database. Except in the case of *BnNPR1,* whose cDNA was available in the nr database, only EST sequences were available. These ESTs were assembled using the Seqman function of DNASTAR (DNASTAR Inc., USA).

Plant growth and treatments

A strain of *S. sclerotiorum* was kindly provided by Dr. Stephen Strelkov (University of Alberta). *S. sclerotiorum* was subcultured on potato dextrose agar (PDA) media (Becton Dickinson, Sparks, MD, USA) under continuous light. Preparation of agar plugs of *S. sclerotiorum* for inoculation was performed as previously described (Yang et al. 2007).

Wild type *B. napus* (Westar) and *B. carinata* plants were grown in Sunshine Soil Mix 4 (Sungro Horticulture, Vancouver, Canada) in the greenhouse (22°C day/18°C night; 16 h photoperiod, a combination of natural light and T5 fluorescent tubes with a light intensity of 300 μE (μmol) m^{-2} s^{-1}) for 18 days. *B. napus* was sprayed with 50 mM SA (Sigma, MO, USA (Hegedus et al. 2008)), 20

µM JA (Sigma (Profotova, et al. 2006)) or 20 µM ACC (Sigma (Chen and Chang 2003)). Ethanol 0.1% (v/v) in water was used as a control for ACC treatment and water was used as a control for both SA and JA treatments. Leaves were harvested at 2, 4, 8, 24 h post-treatment (Hegedus et al. 2008; Profotova et al. 2006; Chen and Chang 2003), flash-frozen in liquid nitrogen and stored at −80 °C.

PDA plugs with mycelia prepared as described above were placed on the first and second true leaves which were wounded with a needle slightly before inoculation. Leaves of uninoculated plants were treated similarly with PDA plugs from control plates without any mycelial growth. Plants, both uninoculated and inoculated, were placed in a humidity chamber for 24 h after which they were returned to the greenhouse. Leaves from inoculated and uninoculated plants were harvested at 12, 24 and 48 h (Liang et al. 2008; Yang et al. 2007) after inoculation, flash frozen in liquid nitrogen and stored at −80 °C. The stems of five-week old *B. napus* and *B. carinata* plants were inoculated with a plug of mycelia that was placed between the 3rd and 4th internodes. The plugs were wrapped with parafilm and disease symptoms were evaluated at 24, 48 and 72 h post-inoculation. Three independent biological replicates of the entire experiment were performed.

Reverse Transcription-PCR (RT-PCR) and isolation of cDNA

Total RNA was isolated from leaf tissue using the RNeasy Plant Mini kit (Qiagen, ON, Canada) with on-column DNA digestion according to manufacturer's instructions. RNA integrity was checked on an agarose gel and quantified by with a NanoDrop 1000 (NanoDrop Technologies, Inc., DE, USA). First-strand cDNA was synthesized from 2.5 µg of total RNA using Superscript II (Invitrogen, CA, USA) and primed with Oligo$(dT)_{18}$ (Fermentas, ON, Canada). PCR was performed in a 50-µL final volume including 0.5 µL of cDNA template, 1× amplification buffer, 200 µM deoxynucleotide triphosphates (dNTPs) (Fermentas), 400 nM of each primer, and 2 units of Platinum *Taq* high-fidelity polymerase (Invitrogen). PCR conditions included an initial denaturing step at 94

°C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 68 °C for 1 min per kb, with a final extension at 68 °C for 5 min. PCR products were gel purified using the QIAquick gel extraction kit (Qiagen) and cloned into a pJET1.2 vector supplied with the CloneJET PCR cloning kit (Fermentas) and sequenced from the two ends using the BigDye reagent on an ABI3700 sequencer (Applied Biosystems, Foster City, CA, USA).

Quantitative qRT-PCR

Primers were designed using PrimerExpress3.0 (Applied Biosystems) targeting an amplicon size of 100 bp. The list of genes as well as the primers used is shown in Table 3-1. All primers used were submitted to the NCBI database for a Blastn search and all primers were confirmed to specifically anneal only with their corresponding genes. Hence, the results from qRT-PCR analysis represents the expression pattern of specific genes. The qRT-PCR assay was performed as described previously (Yang et al. 2007) and each gene was assayed in duplicate for each of the three biological replicates. Data were analyzed by Students' *t*-test and ANOVA ($p<0.05$) using PAST (http://folk.uio.no/ohammer/past/) and SAS 9.2 (SAS Institute Inc., NC, USA).

Characterization of ACC-deaminase transgenic canola

A triple response assay was performed as described by others (Jing et al. 2005; Qin et al. 2006) with minor modifications. A stock solution of 100 mM ACC dissolved in water was filtered through a 0.22 µm filter. Transgenic ACC-deaminase (T4-177, T5-130B, T5-163) and wild-type ('Westar') seeds were sown on ½ × LS media (Caisson Laboratories Inc., UT, USA) supplemented with or without 10 µM ACC (Sigma). The seeds were allowed to grow for 4 days in the dark before being scored and photographed. The stems of 7-week old transgenic ACC-deaminase (T4-177, T5-130B, T5-163) and wild- type canola plants were inoculated with a plug of mycelium being placed between the 3rd and 4th internodes of the stem and wrapped with parafilm. Disease symptoms were evaluated at 48, 72, 96 and 336 h post inoculation. Three independent biological

Table 3-1 Primers used in this study (F1 and R1 primers are for real-time while F2 and R2 primers are for gene cloning to get a full length sequence)

Gene	At AGI#	Accession No.	Forward and reverse primers
BnAOC3-F1	At3g25780	CD817484	5’-CAAGCAAAAACCCGAGGAGTT-3’
BnAOC3-R1			5’-CTGGTGGCATATTGACTCGAAA-3’
BnAOC3-F2			5'-CAAACCAAGTTCCAAGTCTTCC-3'
BnAOC3-R2			5'-GTATTCCACCAACACAGCGTTA-3'
BnERF2-F1	At5g47220	CN737061.1	5'-GGGTTTGGTTAGGGACGTTTG-3'
BnERF2-R1			5'-GGAACCACGCATCCTAAAAGC-3'
BnERF2-F			5'-ATGTACGGACAGAGCGAGGT-3'
BnERF2-R			5'-AAGCTTCGAAACCAACAAGTAACTG-3'
BnNPR1-F1	At1G64280	AF527176	5'-TGCTCTGTTGATCGCGAAAC-3'
BnNPR1-R1			5'-CGCCTTTGGCAGCTAACTTC-3'
BnTGA5-F1	At5g06960	EE463024	5'-CAGACGCTGGCAGGAAGATAA-3'
BnTGA5-R1			5'-GCTCAGGTTCACTCGCATGA-3'
BnTGA6-F1	At3g12250	CN732338	5'-CAGCCAAGAATGATGTCTTCCA-3'
BnTGA6-R1			5'-CCCACCAAGCCACAAGAAAC-3'
BnActin-F1	At3g18780	AF111812	5’-ACGAGCTACCTGACGGACAAG-3’
BnActin-R1			5’-GAGCGACGGCTGGAAGAGTA-3’

replicates (5 plants each) were performed.

Results and discussion

Identification of presumptive representative genes and analysis of their response to hormone treatments

We hypothesized that orthologs between *Brassica* species and Arabidopsis may play similar roles in mediating plant responses to hormone treatments and pathogen infection. We first utilized cDNA sequences of the Arabidopsis SA and JA/ET signaling genes to search for orthologs of those genes in canola using public non-redundant and EST databases. A reciprocal Blastn search of the identified genes or assembled contigs also indicated that they are the most likely orthologs of corresponding Arabidopsis genes (data not shown). We chose five genes as representatives of JA/ET or SA signaling pathways, which were *BnAOC3* for JA (Stenzel, et al. 2003), *BnERF2* for ET (Fujimoto et al. 2000) and *BnNPR1* as well as the two bZIP transcription factors (*BnTGA5, BnTGA6*) for SA (Cao et al. 1994; Kesarwani et al. 2007; Zhang et al. 2003).

The cDNA sequences of these genes (*BnAOC3.1*, *BnAOC3.2*, *BnAOC3.3*, *BnERF2.2*, *BnERF2.3*, *BcERF2.2*, *BcERF2.3*, *BnTGA5.1*, *BnTGA5.2*, *BcTGA5.1*, *BcTGA5.2*, *BnERF2.1*, *BcERF2.1*) were cloned from *B. napus* and *B. carinata*. However, *BcAOC3*, *BnTGA6* and *BcTGA6* could not be amplified. After alignment, *BnACO3*, *BnERF2* and *BcERF2* had three different alleles whereas the sequences of *BnERF2.1* and *BcERF2.1* are the same (Figure A-1). Both *BnTGA5* and *BcTGA5* have two different alleles (Figure A-1). Due to the high homology between the *B. napus* and *B. carinata* sequences, the primers of these five genes were designed based on the *B. napus* sequence and used to investigate the transcript level changes in both *B. napus* and *B. carinata*.

To test the response of these presumptive representative genes to JA, SA and ET, we employed qRT-PCR to investigate the transcript levels of these genes in hormone-treated *B. napus* plants compared to their untreated controls. We observed that the transcript levels of these genes were significantly ($p<0.05$) up-regulated by the corresponding hormones as shown in Figure 3-1. The relative

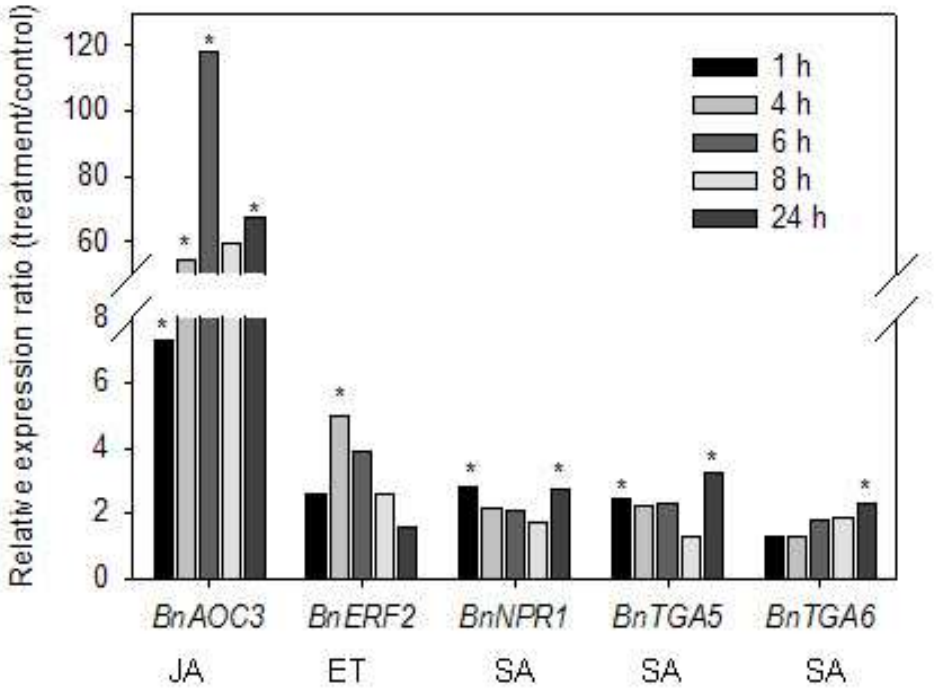

Figure 3-1 Changes in relative transcript abundance for the five selected genes in leaves of *B. napus* in response to hormone treatments. Changes in the relative abundance of *BnAOC3* to 20 µM JA, *BnERF2* to 20 µM ACC and *BnNPR1*, *BnTGA5* as well as *BnTGA6* to 50 mM SA treatment were investigated using qRT-PCR. Results are presented as a ratio of relative transcript abundance in treatment/control on a linear scale. Data are mean of three biological replicates. The asterisk indicates that the treatment-induced changes in relative transcript abundance were significant ($p<0.05$) according to Student's *t*-test analysis.

abundance of transcript for *BnAOC3* increased significantly after treatment with JA; that of *BnERF2* after ACC treatment and *BnNPR1*, *BnTGA5* and *BnTGA6* after SA application. Based on these results, the aforementioned genes were subsequently used as representatives of the hormone signaling pathways investigated in this study.

Symptoms on both *B. napus* and *B. carinata* upon *S. sclerotiorum* infection

It has been reported in the literature that *B. carinata* is tolerant to *S. sclerotiorum* (Bansal et al. 1990). In order to verify this, stem inoculation experiments were performed in both *B. napus* and *B. carinata*. The *S. sclerotiorum*-induced stem lesions were considerably smaller in *B. carinata* than in *B. napus* (Figure 3-2). However, when we inoculated leaves of *B. napus* and *B. carinata* and compared the disease symptoms on inoculated leaves, the results were quite different from stem inoculation. In both *Brassica* species, necrotic lesions of leaves appeared after 12 h post inoculation and expanded rapidly with the lesions reaching leaf margins by 24 h and spread further by 48 h and 72 h post-inoculation (Figure 3-3A and C) compared to control plants (Figure 3-3B and D). The extent of tissue degeneration in both genotypes was quite similar leading us to conclude that when subjected to leaf inoculations, both *Brassica* species tested were identical with respect to the degree of tolerance exhibited. Remarkably, the infection of the leaves can contribute to the disease cycle of Sclerotinia stem rot and may often precede (although is not absolutely required) stem infection (Agrios 1997; Bolton, et al. 2006; http://www.agriculture.gov.sk.ca/Sclerotinia_Infection_Field_Crops). The differences between *B. carinata* and *B. napus* with respect to the size of stem lesions, despite similar reactions on leaves, led us to hypothesize that phytohormone-mediated signaling pathways during pathogen infection may be different in the two species. We therefore investigated the expression of the five genes representing the JA/ET and SA-signaling pathways in both *B. napus* and *B. carinata* at 12 h, 24 h, 48 h and 72 h post-inoculation and the results obtained are described below.

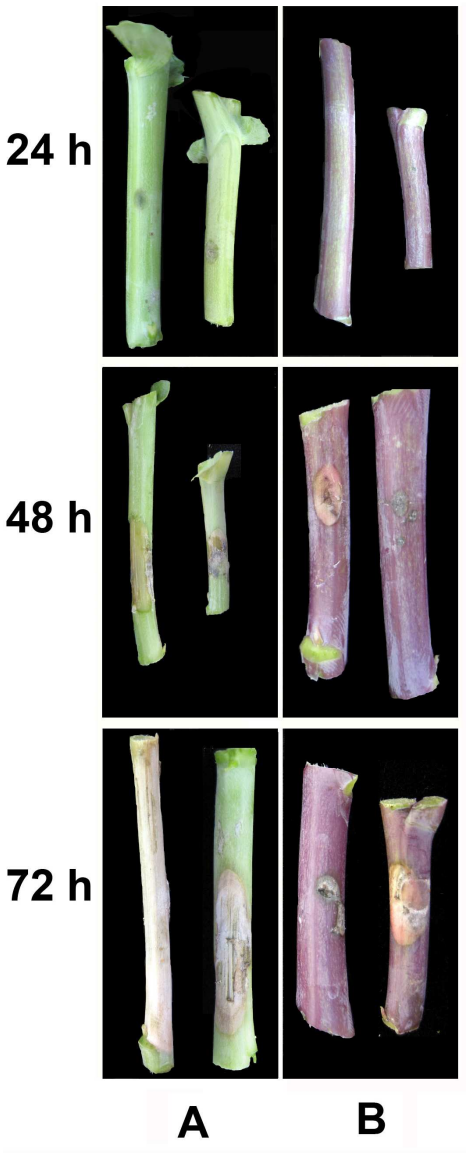

Figure 3-2 Responses of *B. napus* (A) and *B. carinata* (B) to stem inoculation with *S. sclerotiorum*. Stem lesions induced by the pathogen challenge 24, 48 and 72 h are shown.

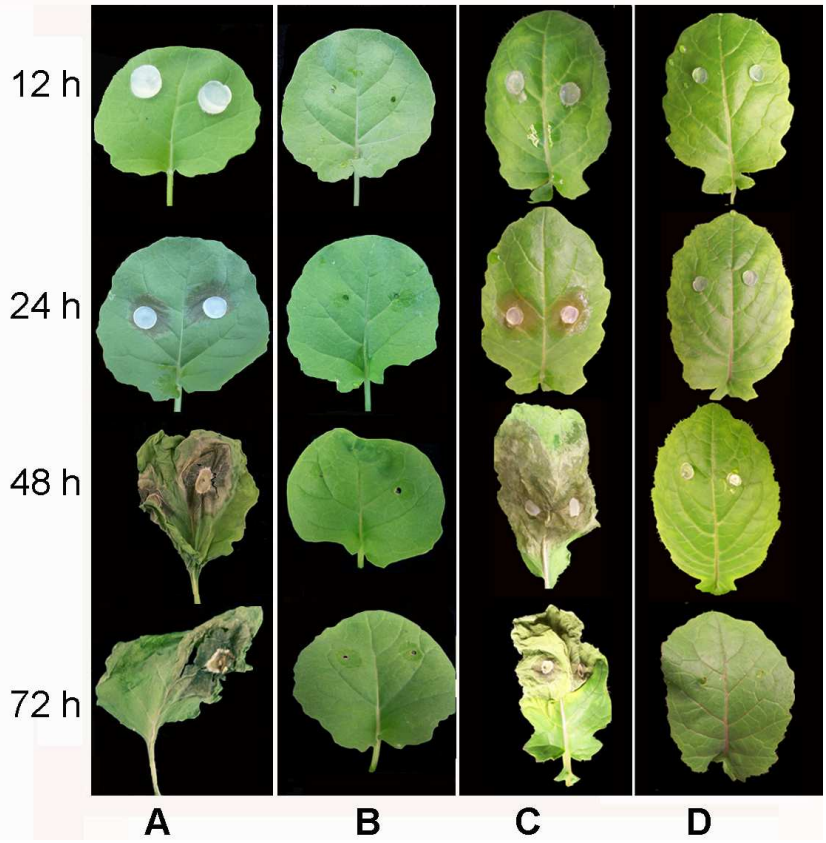

Figure 3-3 Responses of *B. napus* and *B. carinata* leaves in responses to leaf inoculation with *S. sclerotiorum*. Appearance of *B. napus* leaves challenged with, A, *S. sclerotiorum*; B, agar control and appearance of *B. carinata* leaves challenged by C, *S. sclerotiorum*; D, agar control at 12, 24, 48 and 72 h.

Differential responses of JA/ET- and SA-related genes to *S. sclerotiorum* infection in two *Brassica* species

The expression of *AOC3*, an important gene involved in JA biosynthesis, was observed to be significantly different at 24 h in the *B. carinata-S. sclerotiorum* pathosystems (Figure 3-4A, $p<0.05$). However, the relative abundance of the transcript for this gene was not observed to be significantly affected *S. sclerotiorum- B. napus* interaction over the time-course of the experiment. Similarly, in the case of the ET-responsive *ERF2*, the relative abundance of its transcripts was observed to be significantly increased at 48 h (~73-fold) and 72 h (~86-fold) in *B. carinata* by the *S. sclerotiorum* challenge (Figure 3-4B, $p<0.05$), while remaining relatively unchanged at the earlier time-points. However, in the case of *B. napus*, no significant temporal changes in the relative abundance of the transcript for this gene were observed in response to pathogen challenge. In summary, it appears that significant temporal differences in the relative abundance of transcripts for the genes responsive to both JA and ET can be observed in the tolerant *B. carinata* but not in the susceptible *B. napus*.

We also investigated the response of three genes that are known to be involved in SA signaling. Our results demonstrate that, once again, significant ($p<0.05$) temporal differences in the relative abundance of transcript for *NPR1*, *TGA5* and *TGA6* exist in *B. carinata* but could not be observed in *B. napus* in response to the pathogen (Figure 3-4). The relative transcript abundance for all three genes increased significantly at a late disease stage (72 h) in *B. carinata*, after remaining unchanged at of the earlier time-points investigated.

In conclusion, significant temporal changes in the expression of all five genes were observed in *B. carinata* but not in *B. napus*. We observed that at time points where the levels of transcripts for JA-responsive genes were higher, the levels of SA-responsive gene transcripts were unchanged and only after the levels of the transcripts for JA-responsive genes declined did the levels of the three SA-responsive genes increase. This is consistent with the reports in the literature that

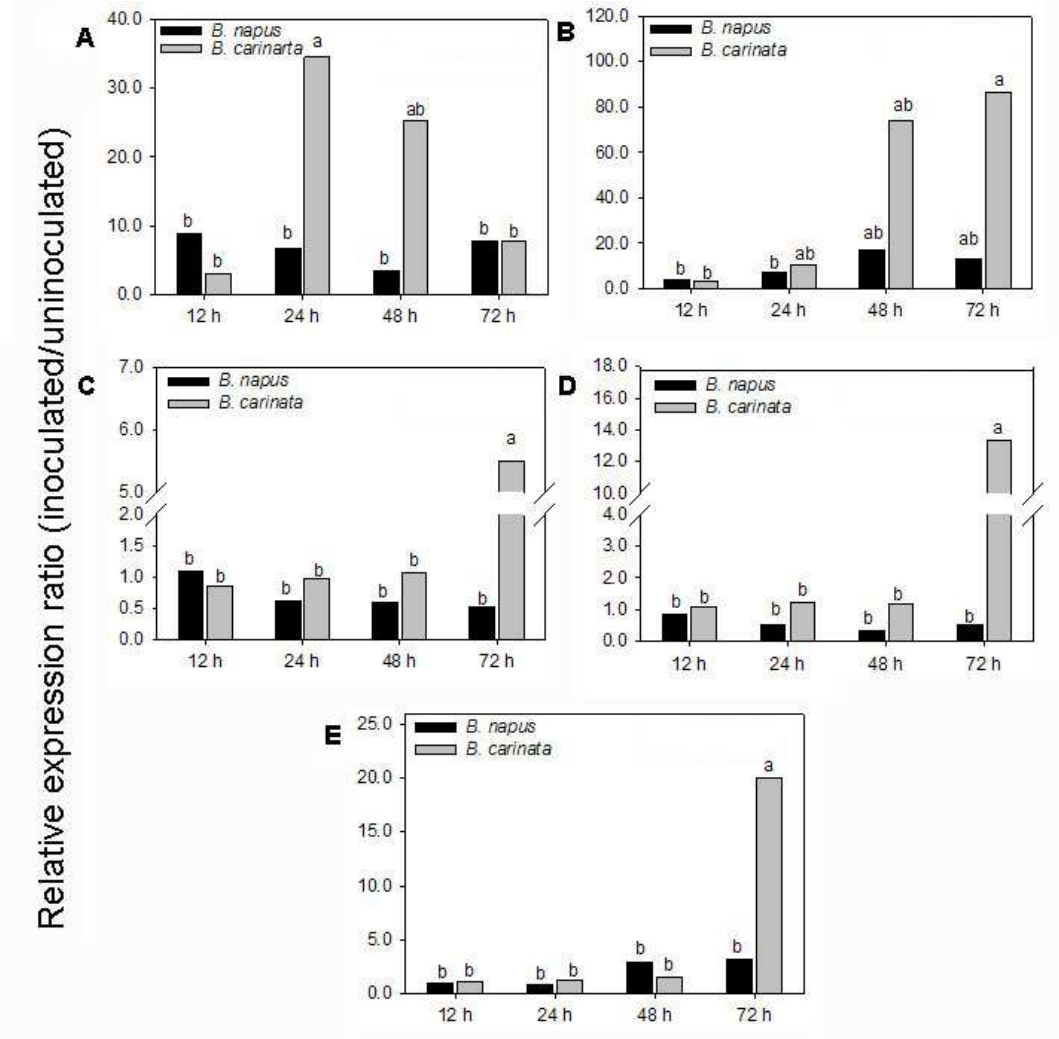

Figure 3-4 Changes in relative abundance of transcripts for the five selected genes in the two *Brassica* species in response to *S. sclerotiorum* challenge. Changes in relative transcript abundance were investigated using real-time PCR at 12, 24, 48 and 72 h. The relative expression ratios were analyzed between the two *Brassica* species at four time-points by SAS (ANOVA, $p<0.05$). Different letters above the columns indicate significant differences between the two *Brassica* species with respect to the relative abundance of transcripts for A, *BnAOC3*; B, *BnERF2*, C, *BnNPR1*; D, *BnTGA5*; E, *BnTGA6*.

JA-triggered signaling defense can interfere with the SA-responsive genes in the Arabidopsis and *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae* interactions (Ellis et al. 2002). It is tempting to speculate that the suppression of SA-responsive pathways as a result of pathogen-induced triggering of JA-responsive signaling response contributes to lesion development. This delay in SA-dependent signaling response may account for its lack of complete tolerance in *B. carinata*. Furthermore, the ET-responsive signaling appeared to occur at a late disease stage (72 h) in the moderately tolerant *B. carinata*, whereas no such increase was observed in the susceptible *B. napus* at any of the time points investigated. Since ET has been reported to play an important role in mediating the resistance of plants to necrotrophic pathogens (Berrocal-Lobo et al. 2002; Thomma et al. 1999; Wang et al. 2002), we further investigated the relative susceptibility/tolerance of transgenic canola plants with less endogenous levels of ET and described below.

Characterization of ACC-deaminase transgenic canola and phenotypic assay

A simple genetic screen using ET effects on dark-grown seedlings known as the 'triple response' has been used to identify many key components of the ET signal transduction pathway and the triple response includes inhibition of elongation and stem thickening, enhanced apical hook curvature and horizontal growth (Chen et al. 2005). ACC-deaminase can compete with ACC-oxidase to metabolize ACC into 2-oxobutanoate, not ET, leading to a reduced ET level (Honma and Shimomura 1978; Ververidis and John 1991). We utilized a previously characterized canola line transformed with an ACC-deaminase gene from *Pseudomonas putida* strain UW4 (Shah et al. 1998) to examine the effect of decreased endogenous ET levels during disease development upon *S. sclerotiorum* challenge. We expected that transgenic canola plants producing less ET may show a weak triple response. We therefore subjected transgenic canola plants ectopically expressing ACC-deaminase to ACC treatment. We observed that wild type canola seedings showed significant inhibition of hypocotyl length and enhanced apical hook curvature and two lines of transgenic ACC-deaminase

canola lines (T5-130B and T4-177) showed a weak triple response: significant reduction of length of hypocotoyl upon ACC treatment compared to wild type seedlings and decreased apical hook curvature (Figure 3-5A and B). Our results indicate that the transgenic ACC-deaminase canola plants used in this study produce less ET (Shah et al. 1998), which may lead to the alleviation of the triple response.

ET has been observed to play a role in the regulation of programmed cell death (PCD) in Arabidopsis (Asai et al. 2000; Greenberg et al. 2000) and PCD might help the necrotrophic pathogen obtain nutrients for growth and development (Glazebrook 2005) and, therefore, we proposed that blocking ET biosynthesis may alleviate stem rot in canola. However, stem inoculation of ACC-d canola plants showed that lesion size caused by the infection of *S. sclerotiorum* in lines of T5-130B, T4-177 and T5-163 were significantly larger than that in wild-type plants at 336 h (14-days) (Figure 3-5C, $p<0.05$). Among them, the size of lesions on T5-130B and T4-177 was bigger than those observed on T5-163 at 336 h (Figure 3-5C), while at other time points (72, 96 and 120 h), the lesion sizes of all the transgenic lines were larger than that of wild-type plants, although the differences were not statistically significant (Figure 3-5C). Our results therefore suggest that decreased levels of ET in transgenic canola plants can accelerate the symptoms of Sclerotinia stem rot. Previous studies have also reported that ET seems to inhibit symptom development during necrotrophic pathogen infection (Berrocal-Lobo et al. 2002; Thomma et al. 1999; Wang et al. 2002). For example, the Arabidopsis *ein2* mutant displays enhanced susceptibility to the necrotrophic fungus *Botrytis cinerea* (Thomma et al. 1999) and overexpression of *ERF1* in Arabidopsis, which activates ET responses, is sufficient to confer resistance to necrotrophic fungi such as *B. cinerea* and *Plectosphaerella cucumerina* (Berrocal-Lobo et al. 2002). However, transgenic tomato with heterologous expression of ACC-deaminase showed a significant decrease in the symptoms of Verticillium wilt caused by *Verticillium sp* (Robison et al. 2001). This may be due to the diverse response of various pathogens to

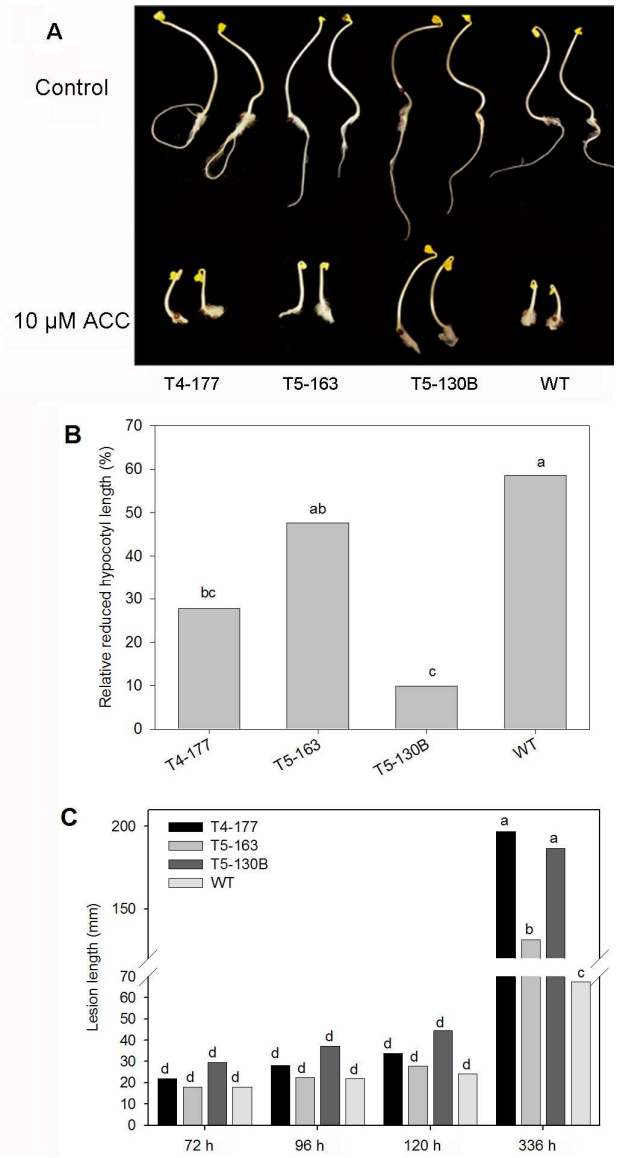

Figure 3-5 Characterization of transgenic ACC-deaminase canola plants. A, The triple response of transgenic ACC-deaminase canola was evaluated and the appearance of four-day-old WT and transgenic seedlings on ½ × LS medium containing ACC (10 µM) are shown; B, The hypocotyl lengths were measured and the relative reduced hypocotyl length of transgenic ACC-deaminase canola as a result of ACC treatment is shown (n=45), letters indicate statistically significant differences (ANOVA, $p<0.05$); C, Size of lesions induced on the stems of WT (Westar) and ACC-deaminase transgenic canola plants by *S. sclerotiorum* at 72, 96, 120 and 336 h. Different letters above the columns indicate significant differences (ANOVA, $p<0.05$).

different levels of ethylene insensitivity, either negatively or positively (Hoffman et al. 1999; Knoester et al. 1998; Thomma et al. 1999; Robison et al. 2001).

Based on our current results, it seems reasonable to conclude that ET may have a significant role in determining the eventual outcome of infection (i.e., susceptibility or tolerance). This suggestion is supported by the fact that ET-responsive genes are triggered only in the relatively tolerant species, *B. carinata* and the observation that transgenic canola with less endogenous ET is relatively more susceptible than the wild-type. Taken together with reports from the literature (Ellis et al. 2002), the abundant JA-responsive genes are induced earlier in the infection process and it suggests that JA may be repressing the SA-induced defense responses. The SA-induced responses taking place in *B. carinata*, along with the ET responses previously discussed may be responsible for the relatively higher tolerance. Upon verification of this suggestion, it may be possible in the future, to engineer canola varieties that are capable of generating earlier defense responses mediated by SA to enhance tolerance to the ubiquitous plant pathogen.

GenBank accession

The sequences reported in this study were deposited in NCBI with the Acc. No. of *BnAOC3.1*, FJ788937; *BnAOC3.2*, FJ788938; *BnAOC3.3*, FJ788939; *BnERF2.2*, FJ788940; *BnERF2.3*, FJ788941; *BcERF2.2*, FJ788942; *BcERF2.3*, FJ788943; *BnTGA5.1*, FJ788944; *BnTGA5.2*, FJ788945; *BcTGA5.1*, FJ788946; *BcTGA5.2*, FJ788947; *BnERF2.1*, FJ788948; *BcERF2.1*, FJ788949.

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Chapter 4 Identification and expression analysis of *WRKY* transcription factor genes in canola *(Brassica napus* L.*)* in response to fungal pathogens and hormone treatments

Introduction

Canola is an agriculturally and economically important crop in Canada, and its growth and yield are frequently affected adversely by fungal pathogens. Among these, *Sclerotinia sclerotiorum* causing stem rot (Agrios 1997), and *Alternaria brassicae*, causing Alternaria black spot (Agrios 1997), have the potential to cause significant crop losses (Bolton et al. 2006). Considerable efforts are underway to develop canola varieties that are better able to tolerate these pathogens. Our own previous research using proteomics and genomics has identified many of the global changes in gene expression that occur as a result of pathogen challenge in canola (Liang et al. 2008; Sharma et al. 2008; Sharma et al. 2007; Yang et al. 2007).

Plant defense responses include the transcriptional control of expression of stress-responsive genes (Chen et al. 2002; Durrant et al. 2000; Maleck et al. 2000; Mysore et al. 2002), including a number of transcription factors (TFs) whose abundance is altered as a result of pathogen challenge. These TFs are presumably involved in regulating the expression of defense-related genes, and specifically include those containing Ethylene Response Factor (ERF)/Apetala2 (AP2)-domain, homeodomain, basic Leucine Zipper (bZIP), MYB, WRKY families and other zinc-finger factors, all of which have been observed to increase in response to pathogen challenge (Singh et al. 2002). These defense-associated TFs can regulate downstream defense-related genes and may themselves be regulated by phosphorylation (Eulgem et al. 2000; Korfhage et al. 1994; Rushton et al. 1996; Zhou et al. 1997).

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The name of the WRKY family itself is derived from the most prominent feature of these proteins, the WRKY domain, which constitutes 60 amino acids (Eulgem et al. 2000). In this WRKY domain, a conserved WRKYGQK heptapeptide is followed by a C_2H_2- or C_2HC-type of zinc finger motif (Eulgem et al. 2000). They contain one or two WRKY zinc-finger motifs, which can bind to the W-box motif (C/T)TGAC(C/T) on DNA (dePater et al. 1996; Eulgem et al. 1999; Rushton et al. 1995; Wang et al. 1998; Yang et al. 1999). Previous studies demonstrated that for the Group I WRKY TFs containing two WRKY domains, the C-terminal domain plays a major role in binding to the W-box, while the N-terminal WRKY domain affects the binding strength (dePater et al. 1996; Eulgem et al. 1999). The deletion of the N-terminal WRKY domain resulted in reduced binding affinity (dePater et al. 1996; Eulgem et al. 1999). Furthermore, a *cis*-element besides TTGACY has also been identified as being recognized by the WRKY domain of a barley *WRKY* TF (Ciolkowski et al. 2008; Sun et al. 2003).

WRKY proteins belong to a superfamily of zinc finger proteins [WRKY-Glial Cell Missing (GCM1)] containing six members (Babu et al. 2006). For example, genes coding WRKY proteins were found not only in plants but also in the slime mold *Dictyostelium discoideum* and the protist *Giardia lamblia*, which indicates that WRKYs in these two lineages may be of ancient origin and they may have evolved prior to the evolution of plant phyla (Ulker and Somssich, 2004; Zhang and Wang, 2005; Zheng et al. 2007). Furthermore, the *G. lamblia* WRKY protein containing two WRKY-domains (formerlly group I) is closely related to the lineage fungal/animal and not that of plants (Zhang and Wang, 2005). WRKY protein function might in part be evolutionarily conserved over large phylogenetic distances including Arabidopsis and barley (Mangelsen et al. 2008).

WRKY TF genes form large families in plants, with 72 members in Arabidopsis and close to 100 in *Oryza sativa* (rice) (Ross et al. 2007). Previous studies have demonstrated that *WRKY* TFs are implicated in plant defense responses (Zhou et al. 1997), sugar signaling (Sun et al. 2003) and chromatin remodeling (Kim et al. 1998). Furthermore, *WRKY*s have been found to play

essential roles in various normal physiological processes, including embryogenesis, seed coat and trichome development, senescence, regulation of biosynthetic pathways, and hormonal signaling (Johnson et al. 2002; Lagace and Matton, 2004; Xie et al. 2005; Xu et al. 2004; Zhang et al. 2004; Zou et al. 2004). As alluded to earlier, abiotic and biotic stresses are among the major external factors influencing the expression of *WRKY* genes in plants (Eulgem et al. 2000; Jiang and Deyholos, 2008; Jiang and Deyholos, 2006; Ramamoorthy et al. 2008; Ryu et al. 2006; Ulker and Somssich, 2004) and have been demonstrated to be involved in the defense against phytopathogens such as bacteria (Asai et al. 2002; Chen and Chen, 2002; Dellagi et al. 2000; Dong et al. 2003; Zheng et al. 2007); fungi (Marchive et al. 2007; Shimono et al. 2007; Zheng et al. 2006); and viruses (Oh et al. 2008; Yoda et al. 2002).

The responses of Arabidopsis to pathogens have been observed to be mediated by signaling pathways (Liu et al. 2005; McDowell and Dangl, 2000; Thomma et al. 1998). For example, salicylic acid (SA) plays a positive role in plants against biotrophic pathogens, whereas jasmonic acid/ethylene (JA/ET) appears to be important in the case of necrotrophic pathogens (Berrocal-Lobo and Molina, 2004; Berrocal-Lobo et al. 2002; Staswick et al. 1998; Thomma et al. 1998). It is also known that these (SA and JA/ET) signaling pathways are mutually antagonistic (Kunkel and Brooks, 2002). In Arabidopsis, it was observed that 49 out of 72 *AtWRKY* genes are regulated by *Pseudomonas syringae* or SA treatment (Dong et al. 2003). On the other hand, of JA-responsive TF in Arabidopsis, AtWRKY TFs are in some of the greatest number (Zheng et al. 2006). Moreover, it is observed that cross-talk of SA- and JA-dependent defense response could be mediated by AtWRKY70, which is downstream of nonexpressor of pathogenesis-related gene 1 (*NPR1*) (Li et al. 2004).

Previous studies showed that abscisic acid (ABA), as a negative factor in the SA and JA/ET signaling defense response, did not increase disease resistance (Audenaert et al. 2002; Henfling et al. 1980; Koga et al. 2004; Mohr and Cahill, 2003; Thaler and Bostock, 2004). However, recent research demonstrated that ABA has a positive effect on callose deposition, which could lead to resistance of

plants towards pathogens (Mauch-Mani and Mauch, 2005; Ton et al. 2005; Ton and Mauch-Mani, 2004). Although WRKY TFs have been demonstrated to be involved in abiotic stress and ABA signaling (Jiang and Deyholos, 2006; Pnueli et al. 2002; Xie et al. 2005; Xie et al. 2006; Zhou et al. 2008), there are no reports available on the role of WRKYs in ABA-mediated biotic stress responses. The role of other hormones, such as cytokinins, has been investigated by many groups and it was observed that cytokinins serving as endogenous inducers for distinct classes of pathogenesis-related (PR) proteins are necessary for the biosynthesis of SA and JA (Sano and Ohashi, 1995; Sano et al. 1996; Sano et al. 1994). Others have observed that the effect of cytokinins is mediated through the stimulation of ethylene production (Cary et al. 1995). However, whether cytokinins induce the expression of PR genes through WRKYs is not presently clear.

Despite the obvious importance of WRKYs in responses to pathogens and hormone signaling, there are no reports yet describing *WRKY* TFs in canola and their role(s) in mediating responses to pathogens. In our previous microarray analysis of canola response to *S. sclerotiorum*, we identified three WRKY genes whose transcript abundance was significantly affected by this fungus (Yang et al. 2007). These results prompted us to systematically identify and examine *WRKY* TF genes in canola using the large set of available expressed sequence tags (ESTs). In this study, we analyzed ESTs from publicly available sequence information of canola and identified 46 sequences with similarities to Arabidopsis *WRKY* TFs. We investigated their evolutionary relationships with counterparts from Arabidopsis and rice. We examined the subcellular localization of four BnWRKY proteins using green fluorescent protein (GFP). Subsequently, we studied the responses of representative members of monophyletically distinct WRKY clades to two fungal pathogens as well as five plant hormones molecules in order to gain further insights into their roles in canola defense responses.

Materials and Methods

BnWRKY gene identification

Thirty-six WRKY domain sequences (WRKY-seed) downloaded from Pfam http://pfam.sanger.ac.uk/family?acc=PF03106 were used to search the dbEST (http://www.ncbi.nlm.nih.gov/dbEST/index.html) datasets (release 053008) for WRKY genes in *B. napus* (oilseed rape and canola) using the tBlastn program. The significant hits (E < 1E-4) were retrieved and Microsoft Excel 2003 was then used to obtain unique sequences based on the GenBank Accession numbers. 177 unique ESTs were retrieved and organized into a FASTA format file before input into ESTpass program (Lee et al. 2007) for cleansing, clustering, and assembling of the unique ESTs. To confirm that the obtained contigs and singlets encode WRKY proteins, the nucleotide sequences were translated in six possible reading frames using OrfPredictor (Iseli et al. 1999; Min et al. 2005) into amino acid sequences, which were then examined for the existence of the heptapeptide WRKYGQK and its variants. The resulting 36 contigs and 38 singlets were used as query sequences in a BLASTn search against *B. napus* EST dataset in NCBI dbEST and Shanghai RAPESEED database (http://rapeseed.plantsignal.cn/, (Wu et al. 2008)) in order to obtain maximum sequence length for each *BnWRKY*, and 339 unique ESTs were retrieved. We also used a key word search of *WRKY* genes in *B. napus* in the non-redundant (nr) database of NCBI and obtained two cDNA sequences (GenBank Acc. DQ539648 and DQ209287), which were annotated to be *BnWRKY40*. Altogether we obtained 341 unique sequences based on the accession numbers. We then used the ESTpass program for cleansing, clustering, and assembling of the unique ESTs. The resultant contigs and singletons were then used as query sequences in a Blastn search against Arabidopsis to find the best hit (putative orthologs) among the 72 *AtWRKY* genes. Afterwards, the putative transcripts were analyzed using OrfPredictor (Iseli et al. 1999; Min et al. 2005) to predict open reading frames (ORFs) and obtain the translated amino acid sequences. The amino acid sequence of the largest ORF for each putative transcript was filtered out and entered into the SMART program (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) to predict the

WRKY domain. In case of the absence of the characteristic features of the WRKY domain for a particular transcript, it was translated in six possible reading frames in DNAMAN (V4.0, Lynnon BioSoft) and manually checked to output the amino acid sequences. At this step, we obtained 46 unique *BnWRKY* genes and identified those *BnWRKY* genes that contain incomplete or no WRKY domain and therefore we used RT-PCR together with 3'RACE to extend the WRKY domain sequences.

Plant growth and gene cloning

Wild-type canola ('Westar') plants were grown in Sunshine Soil Mix 4 (Sungro, Vancouver, BC, Canada) in the greenhouse with a photoperiod of 16 h light (combination of natural light and T5 fluorescent tubes with a light intensity of approximately 200 µE (µmol) m^{-2} s^{-1})/ 8 h dark, and a temperature of 21 °C day/18 °C night for 18 days. Young leaves were harvested for RNA isolation using the RNeasy Plant Mini kit (Qiagen, Mississauga, ON, Canada). RNA integrity was checked by electrophoresis on a formaldehyde agarose gel and quantified using the NanoDrop 1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). First-strand cDNA was synthesized from 2 µg of total RNA using Superscript II (Invitrogen, Burlington, ON, Canada) and $Oligo(dT)_{18}$ primers (Fermentas, Burlington, ON, Canada). PCR primers were designed using PrimerSelect (DNAStar Inc.) or Primer 3 (v0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are listed in Table 4-1. PCR was conducted in a 50-µL final volume including 0.5 µL of cDNA template, 1x *Pfx* buffer, 200 µM deoxynucleotide triphosphates (dNTPs) (Fermentas), 400 nM of each primer, and 2 units of Platinum *Pfx* polymerase (Invitrogen). The PCR conditions included an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 68 °C for 1 min per kb, with a final extension at 68 °C for 5 min. PCR products were gel purified using the QIAquick gel extraction kit (Qiagen) and cloned into the pJET1.2 vector supplied with the CloneJET PCR cloning kit (Fermentas) and sequenced from the two ends using the BigDye reagent on an ABI3700 sequencer (Applied Biosystems, Foster City, CA, USA).

Table 4-1 Primers used in this study.

gene	primer sequence (5'→3')
BnWRKY1	F: ATCTTGGTCGGATGGTGATCTT
	R: TCCCGCCTGAAACACCAAACT
	QF:AGGGAAACCACGACCACGAT
	QR:GGGTCTTTACCACTCGGTTCTTT
BnWRKY2	F:CACAGTTTAAGCAAAGACTCAGTC
	R:CCGGTAATTTCCCTTGACCA
BnWRKY3	F: TTTTTGGTTTGAGCTTGGTTTGTTA
	R: TCCCCATGTACAAAAGAATCCAC
BnWRKY4	F: CGATGTCGGAGAAAGAAGAACGTC
	R: AACTGGCTGCTGCTGCTGATTT
BnWRKY6	QF: GCG GCC GCA ACA ACA AAT GAC
	QR: CGA CGG AGA GCC ACT GGA AAA CTG
	GFP-F:TTAGGTCATGATGGACATAGGATGGTCT GGTCT
	GFP-R:TTAGGTCATGACAGCTCCACCTCCACCTCC
	TTGATTTTTGTTGTTTCCTTCGCC
BnWRKY7	F: TTTGGTCTCTCGGTGCTCTT
	R: CAGTTTAGGGCCCACTCTGT
BnWRKY8	F: GACCGAAGCGTAGACCGACTAA
	R: TGCACCCAAAACAATTTTTCATCAAG
BnWRKY11	F:CATCATGGCCGTCGATCTAATG
	R:ACAAGTCAAAGTCATGCCGAAGC
	QF:CATCGAGCATGAGCACTCTCA
	QR:GGTTCTCTTCGGCCGATTTT
BnWRKY15	F:TAGATCGTACTCCAACCGGACA
	R:CTTCCGCAGAAGGTTTTTGATTAC
BnWRKY17	F:TTTTCCCAGATCTTCAAACTTTTC
	R:CTAATTCTCAACCATTCAAGCAGAGC
BnWRKY18	F:AATGGACTGTTCATCTTTTCTTGAC
	R:CACTAACGTTTCCATCTTTTTCTCC
	QF:GTGCAGAGGATTCGTCCATACTG
	QR:GATCCACCTTGGCTTGTAGCA
BnWRKY20	F:AGATAATGAACCCCCAAGTTGATA
	R:TTCTAAGGACCCGATTGTATTCTC
	QF:CCATGGATCCTCACACTCACCTT
	QR:GGTTGGTGGCGTCTGAAGAG
BnWRKY21	F:GTTTTGCTTTCGCTTCCTCATCAT
	R:AATCCTACGTTTACCCCTTAAACC
BnWRKY22	F:CAAATGGCCGACGATTGGGATCTC
	R:CTAGTCCCCCGCGAATCATA
BnWRKY24	F:TTGATGAAACCCTAATGATGATGC
	R:AGATGTTGGGTAGCGGGTTTGACT
BnWRKY25	F:CCATGTCGTCCACTTCTTTCACCG

	R:TAACTTCAGCCCCAAGGATG
	QF:AGACGAGAGAGCGGTTCTCACT
	QR:TTCAGCCCCAAGGATGTTCT
	GFP-F:CATGCCATGGTGAGTAGGAACTCTAAC
	GFP-R:CATGCCATGGCAGCTCCACCTCCACCTCC
	TGAGCGACGTGGCGCGGTTGG
BnWRKY26	RACE-F:ATGGCCTCTTTCAACCAGCAAAG
BnWRKY27	F:CGTCCTTTACGCCATAGTTTGTC
	R:CCACCGTCCCCGGCGGACCC
BnWRKY28	F:CGCAATCCCCAAACCTAAAAATCT
	R:TGTACATGCAAACAAGAGAGACAA
	QF:GCCGTCAAAAACAGCCCTTA
	QR:CCGTTGGATCTTGGAACGAT
BnWRKY29	RACE-F:GAGAAAGAGAGATATGGGTGAG
BnWRKY32	F:GCCTGGAGGAGACAGACAGAA
	R:CAAGAACAAGGAGACTGAAAATGGA
	QF:ACCAGAGCCAAAACGGAGGTT
	QR:ACCAGCTGCGTGAACTACGA
BnWRKY33	QF:AGAGGACGGTTACAACTGGAGAAA
	QR:TGTCGGACAGCTTGGGAAAG
	GFP-F:CATGCCATGGCTGCTTCTTCCCTTCTTC
	GFP-R:TTAGGTCATGACAGCTCCACCTCCACCTCC
	AGACAAAAACGAATCAAAGAAAGA
BnWRKY35	F:TAGACATGGACAACTTCCAAGGAG
	R:CGAATTTTTATAACGACCTTGAGCA
BnWRKY36	RACE-F:CAGTGAGGGGTCCGATAAGA
	F:AAGAGCGGATCCAACTGTTCTC
	R:ATGGGTTGCTGATGTTTATCACTTT
BnWRKY39	F:CTAGTTTGGGTGGAAGGAACTAAA
	R:AAATCCAAGCGTAGACGCAAA
BnWRKY40	F:CCATGGACCAGTACTCATCGT
	R:TCTCTCACATATGCCAAGTTCAAT
	QF:TGCTCCAAGCTGTTCTGTCAA
	QR:TGGCATTGGATGGTTATGTTCA
BnWRKY42	F:ATATGGCACACACAGAGAGTGGA
	R:AAAAACTCAAAACCGGCCAAT
BnWRKY44	RACE-F: TGTCCGGTCAAGAAGAAAGTAGAG
	F:GGTACGACCGATACATCAACGA
BnWRKY45	F:GGGGAGCAAGAAATGGAGGATAG
	R:GTACAATTCACGCATTGCTCAG
	QF:TGCACATCTTCCCTCCCTTT
	QR:TGCGCCAGTTTCATTCATCTAA
BnWRKY50	F:TTTGAGTTATGAATGATGAAGAGACA
	R:CATTGTTAAGTCTCAAGAACAGATTCA
BnWRKY53	F:CTAAATGGAAGGTCAAAAAGCTATGT
	R:CAAGAAGAGTGCTGCGGCTACGAC

	QF:CAGAACTGTTGGGCAACGAA
	QR:GGGTCCCCTGCTGACAAGTG
BnWRKY65	F:CCTCGTCTCCCTTCAGCAACTCAG
	R:CACCACGTGTCAAAAGATCTCCAC
	QF:CATGGGCCTGGCGTAAGTAC
	QR:TTCCTTGCCGGACAACCTTT
BnWRKY66	F:AGACTCTCATCTACAACTCTAAACG
	R:CCATTCTCTGTGTACATCAACCA
BnWRKY69	RACE-F: GATGCACCGTAGAGGAATTCAAGA
	QF:GGTTCTACGACGCGTCCATCT
	QR:TCTCCGCCTACTGAAAAACCA
BnWRKY70	F: CATGCCATGGATGTTGCTAATAATAACA
	R:GCCTGATAATTATTTCTCTAGACAAGATGA
	QF:CTCTTGTCACCGCCGTTGA
	QR:TTAACGGGTCCAAGTCTTTTCC
BnWRKY72	F:AATGGAGGTTCTTCTGAAATTACCC
	R:CTAGCTTTTCTCTTCCTTGTTCACGA
BnWRKY74	F:ATTTGGGGGTTTCAATGTTTTGGC
	R:TGCAGATCGCAGCTACTAGACC
BnWRKY75	QF:TCGAGCATATCCTCACCCAAA
	QR:GCAGCACGAGCTCTCAGAATT
	GFP-F:CATGCCATGGAGGGATATCAAAATGGA
	GFP-R:TTAGGTCATGACAGCTCCACCTCCACCTCC
	ATTAAAAGAAGAGTAGATTTG
BnACT2	QF:ACGAGCTACCTGACGGACAAG
	QR:GAGCGACGGCTGGAAGAGTA
BnGAPDH	QF:CCGGTATGTCCTTCCGTGTT
	QR:TGCCCTCAGATTCCTCCTTGA

F, forward primer for RT-PCR; R, reverse primer for RT-PCR; QF, qRT-PCR forward primer; QR,qRT-PCR forward primer; RACE-F, 3'RACE forward primer; GFP-F, forward primer for N-terminal GFP fusion; GFP-R, reverse primer for N-terminal GFP fusion.

For rapid amplification of cDNA ends (3'RACE), first-strand cDNA was made from 2 µg of total RNA extracted from wild-type canola (cv. Westar) using Superscript II and an oligo$(dT)_{17}$ adaptor sequence (Frohman et al. 1988), and 0.5 µL of cDNA template was used for 3' RACE. Reactions were conducted in a 50-µL final volume including 1x Taq buffer, 0.2 mM dNTPs, 0.4 µM of each primer, and 0.2 µL (1 unit) of Platinum *Taq* polymerase (Invitrogen). The primers designed with PrimerSelect (DNAstar) are outlined in the Table 4-1 and the adaptor sequence was 5'-GACTCGAGCGACATCGAT-3' (Frohman et al. 1988). The PCR conditions included an initial denaturation of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, with a final extension at 72°C for 5 min. PCR products were purified and cloned into the pGEM-T vector (Promega, Madison, WI, USA) or the pJET1.2 vector (Fermentas) and sequenced. Sequences were analyzed and translated using DNAStar. Based on the sequenced cDNA sequences from 3'RACE, new primers were designed, which were then used to clone the full-length cDNAs of some BnWRKY genes. At least two independent clones were sequenced from both ends.

Phylogenetic tree construction and bioinformatics

The WRKY domain boundary was defined as previously described (Eulgem et al. 2000). The peptide sequences of the domains were aligned using ClustalX (v1.83) with a gap opening penalty of 35 and gap extension penalty of 0.75 in pairwise alignment, and a gap opening penalty of 15 and gap extension penalty of 0.30 in multiple alignment parameters settings. The multiple alignments were adjusted with gaps manually inserted for optimal alignment based on the conserved features of the WRKY domains. The maximum parsimony algorithm implemented in MEGA4 (Tamura et al. 2007) for amino acid sequences were used for phylogenetic tree reconstruction according to Baldauf, (2003) and Hall, (2007). One hundred bootstrapped data sets were used to estimate the confidence of each tree clade. The protein sequences of Arabidopsis WRKY TFs were retrieved from TAIR (www.arabidopsis.org) and rice WRKY TFs from the

Database of Rice Transcription Factors (DRTF, http://drtf.cbi.pku.edu.cn/). The nomenclature of rice (*Oryza sativa*. cv japonica) WRKY TFs was as previously proposed (Ross et al. 2007). Putative orthologs of *BnWRKY* genes were identified in both Arabidopsis and rice using the translated amino acid sequences in InParanoid (Remm et al. 2001).

Subcellular localization and confocal microscopy

The coding regions (CDS) of *BnWRKY6, BnWRKY25, BnWRKY33,* and *BnWRKY75* were amplified by RT-PCR from canola (Westar) cDNAs using the primers listed in Table 4-1. PCR products were purified using a QIAquick PCR purification kit (Qiagen), restricted by *Nco* I (New England Biolabs, Ipswich, MA, USA) and/or *Bsp* HI (Fermentas), purified again and cloned into a *Nco* I digested pCsGFPBT (GenBank: DQ370426) vector with a Gly-Ala- rich peptide linker between CDSs and sGFP. All constructs were sequenced and mobilized into *Agrobacterium tumefaciens* GV3101 through the freeze-thaw method and transformed into wild-type *Arabidopsis thaliana* (Col-0) employing the floral dip method (Clough and Bent, 1998). Resistant lines were selected on ½ × MS containing 1% (w/v) sucrose and 50 mg/L hygromycin B (Sigma-Aldrich) for 7 d before being transferred into soil to grow the plants to maturity and to harvest T_2 seeds, which were further sown on the same type of hygromycin-containing medium. Preliminary experiment was performed to identify the position of nuclei using nuclei staining chemical-Hoechst (Sigma-Aldrich). Five-day-old seedlings from ten independent T_2 lines were mounted on slides for GFP observation under a confocal microscope (Carl Zeiss). At least five cells were screened for each line.

Fungal pathogen inoculation and hormone treatments

Wild-type canola (cv. Westar) plants were grown as described previously in a greenhouse for 18 days. Potato dextrose agar (PDA) agar plugs of *S. sclerotiorum* were prepared as described earlier (Yang et al. 2007) and placed on the first and second true leaves, which were wounded slightly. The preparation of spores of *A.*

brassicae and inoculation of canola leaves were performed as described previously (Sharma et al. 2007). Leaves of uninoculated/mock plants were treated similarly with PDA agar plugs without the mycelia or with water in the case of *A. brassicae*. Plants were placed in a humidity chamber for 24 h before being placed in the greenhouse. Tissues were harvested 12, 24, 48 and 72 h post inoculation and kept at -80 ˚C after being flash-frozen in liquid nitrogen. JA, SA, BAP and ABA were applied by spraying 50 µM JA, 1 mM SA, 20 µM BAP or 50 µM (±)-ABA (Sigma-Aldrich, St. Louis, MO, USA). A stock solution (500 µM) of JA in water first prepared and then diluted with 0.1% (v/v) ethanol to 50 µM. ABA was dissolved in absolute ethanol to prepare a 20 mM stock solution and then diluted with 0.1% (v/v) ethanol to the final 50 µM solution. SA was dissolved in water to prepare a 100 mM stock solution and the pH adjusted to 6.5 using 1 M KOH, before dilution in water to make the 1 mM working solution, BAP was dissolved in 1 M NaOH to prepare a 1 mM stock solution after which it was diluted with water to the 20 µM working solution. The mock were 0.1% (v/v) ethanol for JA and ABA, water adjusted to pH 6.5 with 1 M KOH or 1 M NaOH for SA or BAP treatments, respectively. Ethylene treatment was carried out in an airtight clear acrylic chamber (1.5 m × 0.6 m × 0.6 m) placed in the same greenhouse, into which 100 ppm ethylene gas in air (Praxair, Mississauga, ON, Canada) was passed at a rate of 2 L/min. Mock plants were placed in a separate chamber into which air (Praxair) was passed at the same rate. Leaves from mock and hormone-treated plants were harvested at 6 and 24 h time-points, flash frozen in liquid nitrogen and stored at −80 °C. The entire sample preparation was repeated three times at separate times.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from mock, inoculated or hormone-treated leaf tissue using the RNeasy Plant Mini kit (Qiagen) with on-column DNA digestion. RNA was quantified with a NanoDrop ND-1000 (NanoDrop Technologies, Inc.) and the integrity of the RNA was assessed on a 1% (w/v) agarose gel. Primers were designed using PrimerExpress3.0 (Applied Biosystems), targeting an amplicon size of 80-150 bp. The primers used are listed in the Table 4-1. The specificity of

all primers designed was submitted to a BLASTn search against the NCBI *B. napus* nr and EST databases and any nonspecific primers were eliminated or redesigned. Hence, the results from qRT-PCR analysis might represent the response of specific *BnWRKY* genes. The assay was performed as described previously (Yang et al. 2007). qRT-PCR for each gene was performed in duplicate for each of the three independent biological replicates. Significance was determined with SAS software version 9.1 (SAS Institute Inc.) (p value <0.05).

Results

Identification of 46 WRKY transcription factor genes in canola

Although the complete sequence of the canola genome has not yet been determined, the number of publicly available ESTs was 593,895 as of May 30, 2008. It is well known that gene discovery and genome characterization through the generation of ESTs is one of the most widely used methods (Rudd 2003). We searched the dbEST database and identified 343 unique GenBank EST accessions from *B. napus* that showed significant similarity to the 36 *WRKY* seed sequences and 72 *AtWRKY* genes. A keyword search in NCBI "nr" dataset returned two previously annotated *BnWRKY* sequences. We then used ESTpass to remove four chimerical ESTs and clustered the remaining 339 ESTs into 69 contigs and 66 singlets. For subsequent analyses, we also identified the largest open reading frame of each of the 135 contigs or singlets using OrfPredictor (Iseli et al. 1999; Min et al. 2005). We next performed a tBlastn search using the 36 *WRKY* seed sequences in the Dana-Farber Cancer Institute (DFCI) oilseed rape gene index (BnGI, release 3.1) and identified 70 tentative consensuses (TC) and 79 singlets, which consisted of 314 ESTs. We further compared our identified 339 EST sequences to those 314 ESTs from BnGI and found that those 314 ESTs were included in our dataset. The difference in the numbers of *WRKY* ESTs between the two datasets could be explained by the fact that we utilized a more recent release of the dbEST and also mined the Shanghai RAPESEED database (http://rapeseed.plantsignal.cn/, (Wu et al. 2008)) with the ESTs not available in NCBI dbEST database until the end of 2008. Also, the currently available

information may be skewed because of the tissue bias of the database (e. g most of the ESTs are from seed coat and embryo, see http://www.brassicagenomics.ca/ests/), which may result in the over-representation of some genes. As the contigs/singlets output from ESTpass were annotated based on their similarity to Arabidopsis *WRKY* genes, we were able to identify the presumptive orthlogs of the respective canola *WRKY* genes. Therefore, the generic names were given according to the Arabidopsis *WRKY*s to provide a unique identification for each *BnWRKY* (Table 4-2).

We noted that among all the *BnWRKY* genes we annotated, *BnWRKY11* has the largest number (40) of ESTs, followed by *BnWRKY32* with a total of 26 ESTs, while *BnWRKY*26, *30*, *36*, and *66* have only one EST each (Table 4-2 and Table A-4). To facilitate the following phylogenetic, GFP fusion, and qRT-PCR analyses, we designed primers based on the identified ESTs for each of the 46 *BnWRKY* genes to obtain full length cDNA sequences, at least for each of the coding regions, employing RT-PCR together with 3'RACE. As a result, we succeeded in cloning the cDNA sequences of 38 of these 46 *BnWRKY* genes, among which we identified two different alleles for 13 *BnWRKY* genes (Table 4-2). At this step, we were able to identify putative orthologs of these *BnWRKY* genes in both Arabidopsis and rice using the program InParanoid (Remm et al. 2001) (Table 4-2).

Although WRKY proteins have a conserved heptapeptide WRKYGOK (Eulgem et al. 2000), many studies observed slight variations of the sequence for a number of WRKY proteins in Arabidopsis, rice, tobacco and barley (Mangelsen et al. 2008; van Verk et al. 2008; Xie et al. 2005; Zhang and Wang, 2005). Similarly, a number of BnWRKYs have amino acid sequence substitutions in the conserved WRKY signature. For example, the following variations were noted; WRKYGKK in BnWRKY50, and WRKYGRK in BnWRKY51 (Figure A-2). We also observed a 25 amino acid insertion in the C-terminal WRKY domain of BnWRKY26, compared to AtWRKY26 (Figure A-2), suggesting a divergence between them during the evolutionary process.

Table 4-2 *B. napus* (canola) WRKY transcription factors identified in this study. AGI, Arabidopsis genome initiative.

gene	GenBank Acc No.	EST count	Arabidopsis homolog/AGI No.	Rice homolog/locus[a]	cloning strategy
BnWRKY1	EU912389	22	*AtWRKY1/At2g04880*	*OsWRKY78/Os07g39480*[b]	RT-PCR
BnWRKY2	FJ012166, FJ384104	13	*AtWRKY2/At5g56270*	*OsWRKY30/Os08g38990*	RT-PCR
BnWRKY3	EU912401, FJ384106	5	*AtWRKY3/At2g03340*	*OsWRKY85/Os12g32250*	RT-PCR
BnWRKY4	EU912405	3	*AtWRKY4/At1g13960*	*OsWRKY85/Os12g32250*[b]	RT-PCR
BnWRKY6	EU912410	2	*AtWRKY6/At1g62300*	*OsWRKY2/Os01g14440*	RT-PCR
BnWRKY7	EU912414, FJ384112	18	*AtWRKY7/At4g24240*	*OsWRKY51/Os04g21950*	RT-PCR
BnWRKY8	EU912418	3	*AtWRKY8/At5g46350*	*OsWRKY49/Os05g49100*	RT-PCR
BnWRKY10	N/A	5	*AtWRKY10/At1g55600*	*N/A*	
BnWRKY11	EU912390, FJ384101	40	*AtWRKY11/At4g31550*	*OsWRKY51/Os04g21950*	RT-PCR
BnWRKY15	EU912391	4	*AtWRKY15/At2g23320*	*OsWRKY83/Os12g40570*	RT-PCR
BnWRKY17	EU912392, FJ384102	13	*AtWRKY17/At2g24570*	*OsWRKY51/Os04g21950*	RT-PCR
BnWRKY18	FJ210288, FJ384103	6	*AtWRKY18/At4g31800*	*OsWRKY76/Os09g25060*[b]	RT-PCR
BnWRKY19	N/A	5	*AtWRKY19/At4g12020*	*N/A*	
BnWRKY20	EU912393	14	*AtWRKY20/At4g26640*	*OsWRKY78/Os07g39480*[b]	RT-PCR
BnWRKY21	EU912394	25	*AtWRKY21/At2g30590*	*OsWRKY83/Os12g40570*	RT-PCR
BnWRKY22	EU912395, FJ384105	3	*AtWRKY22/At4g01250*	*OsWRKY39/Os02g16540*	RT-PCR
BnWRKY24	FJ210289	3	*AtWRKY24/At5g41570*	*OsWRKY23/Os01g53260*	RT-PCR
BnWRKY25	EU912396	10	*AtWRKY25/At2g30250*	*OsWRKY24/Os01g61080*[b]	RT-PCR
BnWRKY26	EU912397	1	*AtWRKY26/At5g07100*	*OsWRKY53/Os05g27730*[b]	3'RACE
BnWRKY27	EU912398	3	*AtWRKY27/At5g52830*	*OsWRKY39/Os02g16540*	RT-PCR
BnWRKY28	EU912399	3	*AtWRKY28/At4g18170*	*OsWRKY11/Os01g43650* *or* *OsWRKY49/Os05g49100*	RT-PCR
BnWRKY29	EU912400	5	*AtWRKY29/At4g23550*	*OsWRKY39/Os02g16540*	3'RACE
BnWRKY30	N/A	1	*AtWRKY30/At5g24110*	*N/A*	
BnWRKY31	N/A	6	*AtWRKY31/At4g22070*	*N/A*	
BnWRKY32	EU912402	26	*AtWRKY32/At4g30935*	*OsWRKY82/Os08g17400*[b]	RT-PCR

BnWRKY33	EU912403	12	*AtWRKY33/At2g38470*	*OsWRKY53/Os05g27730*	RT-PCR
BnWRKY34	N/A	2	*AtWRKY34/At4g26440*	*N/A*	*in silico*
BnWRKY35	FJ012167	2	*AtWRKY35/At2g34830*	*OsWRKY66/Os02g47060 OR OsWRKY37/Os04g50920*	RT-PCR
BnWRKY36	EU912404	1	*AtWRKY36/At1g69810*	*OsWRKY97/Os01g09080*[b]	3'RACE
BnWRKY39	FJ210290, FJ384108	4	*AtWRKY39/At3g04670*	*OsWRKY83/Os12g40570*	RT-PCR
BnWRKY40	EU912406, FJ384109	13	*AtWRKY40/At1g80840*	*OsWRKY71/Os02g08440*	RT-PCR
BnWRKY42	EU912407, FJ384110	4	*AtWRKY42/At4g04450*	*OsWRKY43/Os05g49210*[b]	RT-PCR
BnWRKY44	EU912408	3	*AtWRKY44/At2g37260*	*OsWRKY85/Os12g32250*[b]	3'RACE
BnWRKY45	FJ012169	2	*AtWRKY45/At3g01970*	*OsWRKY72/Os11g29870*	RT-PCR
BnWRKY46	N/A	3	*AtWRKY46/At2g46400*	*OsWRKY74/Os05g27730*	*in silico*
BnWRKY50	FJ012170	2	*AtWRKY50/At5g26170*	*OsWRKY67/Os05g09020 or OsWRKY7/Os05g46020*	RT-PCR
BnWRKY51	N/A	2	*AtWRKY51/At5g64810*	*OsWRKY7/Os05g46020*	*in silico*
BnWRKY53	EU912409, FJ384111	11	*AtWRKY53/At4g23810*	*OsWRKY93/Os06g06360*	RT-PCR
BnWRKY56	N/A	2	*AtWRKY56/At1g64000*	*N/A*	*in silico*
BnWRKY65	EU912411	9	*AtWRKY65/At1g29280*	*OsWRKY13/Os01g54600*	RT-PCR
BnWRKY66	EU912412	1	*AtWRKY66/At1g80590*	*N/A*	RT-PCR
BnWRKY69	EU912413	2	*AtWRKY69/At3g58710*	*OsWRKY13/Os01g54600*	3'RACE
BnWRKY70	EU912415, FJ384113	6	*AtWRKY70/At3g56400*	*OsWRKY45/Os05g25770*	RT-PCR
BnWRKY72	FJ012171, FJ384114	13	*AtWRKY72/At5g15130*	*OsWRKY73/Os06g05380*	RT-PCR
BnWRKY74	EU912416	6	*AtWRKY74/At5g28650*	*OsWRKY83/Os12g40570*	RT-PCR
BnWRKY75	EU912417	2	*AtWRKY75/At5g13080*	*OsWRKY72/Os11g29870*	RT-PCR

[a] Putative orthologs were identified by InParanoid (http://inparanoid.sbc.su.se/cgi-bin/index.cgi) with a score of 1 (the maximum score).

[b] No ortholog was identified (score <1), instead homology was identified by InParanoid. NA, the full-length cDNA sequences were not cloned and/or complete protein sequences are not available for using InParanoid.

Phylogenetic analysis of BnWRKY proteins

From the 46 canola WRKY genes identified, we were able to extract 52 WRKY domains which are approximately 60 amino acids in length. In 11 BnWRKY TF proteins, we identified two separate WRKY domains (Figure A-2), and both N- and C-terminal WRKY domains of these proteins were included in the phylogenetic analysis. The amino acid sequences were aligned with each other (Figure A-2) and a consensus maximum parsimony (MP) tree was inferred (Figure 4-1). To gain insights into the WRKYs from canola and the models, Arabidopsis and rice, we selected putative orthlogs of the identified BnWRKY TFs from these two model plants. Subsequently, we reconstructed a rooted MP tree using a WRKY protein from the world's smallest unicellular green algae *Ostreococcus tauri* WRKY as the outgroup (Figure A-3). The generated MP tree of BnWRKY TFs demonstrated a polyphyletic nature, which is consistent with previous studies (Babu et al. 2006; Mangelsen et al. 2008; Ulker and Somssich, 2004).

We further classified these identified *BnWRKY* TFs into three major groups as previously described (Eulgem et al. 2000). Accordingly, the Group II proteins are divided into five subgroups. From our study, at least two representatives for all subgroups of WRKY proteins were identified in the canola genome (Figure 4-1). For example, twelve *BnWRKYs* (*BnWRKY1*, *2*, *3*, *4*, *19*, *20*, *25*, *26*, *32*, *33*, *34* and *44*) code for proteins with two WRKY domains and clearly cluster with Group I of the AtWRKYs. The N- and C-terminal domains of these twelve BnWRKY form two different clusters named Group IN and Group IC (Figure 4-1). Interestingly, the 28 identified Group II WRKY members of canola were distributed unevenly among the five subgroups (subgroups IIa-e, Figure 4-1). Two BnWRKYs (BnWRKY18, 40*)* formed a distinct subclade with the characteristic members of subgroup IIa. Five canola WRKYs (BnWRKY6, 31, 36, 42, 72) belong to Group IIb; eight (BnWRKY8, 24, 28, 45, 50, 51, 56, 75) belong to Group IIc; seven (BnWRKY7, 11, 15, 17, 21, 39, 74) belong to Group IId, and six canola WRKY (BnWRKY22, 27, 29, 35, 65, 69) belong to Group IIe. Group III is represented by four single WRKY domain canola proteins

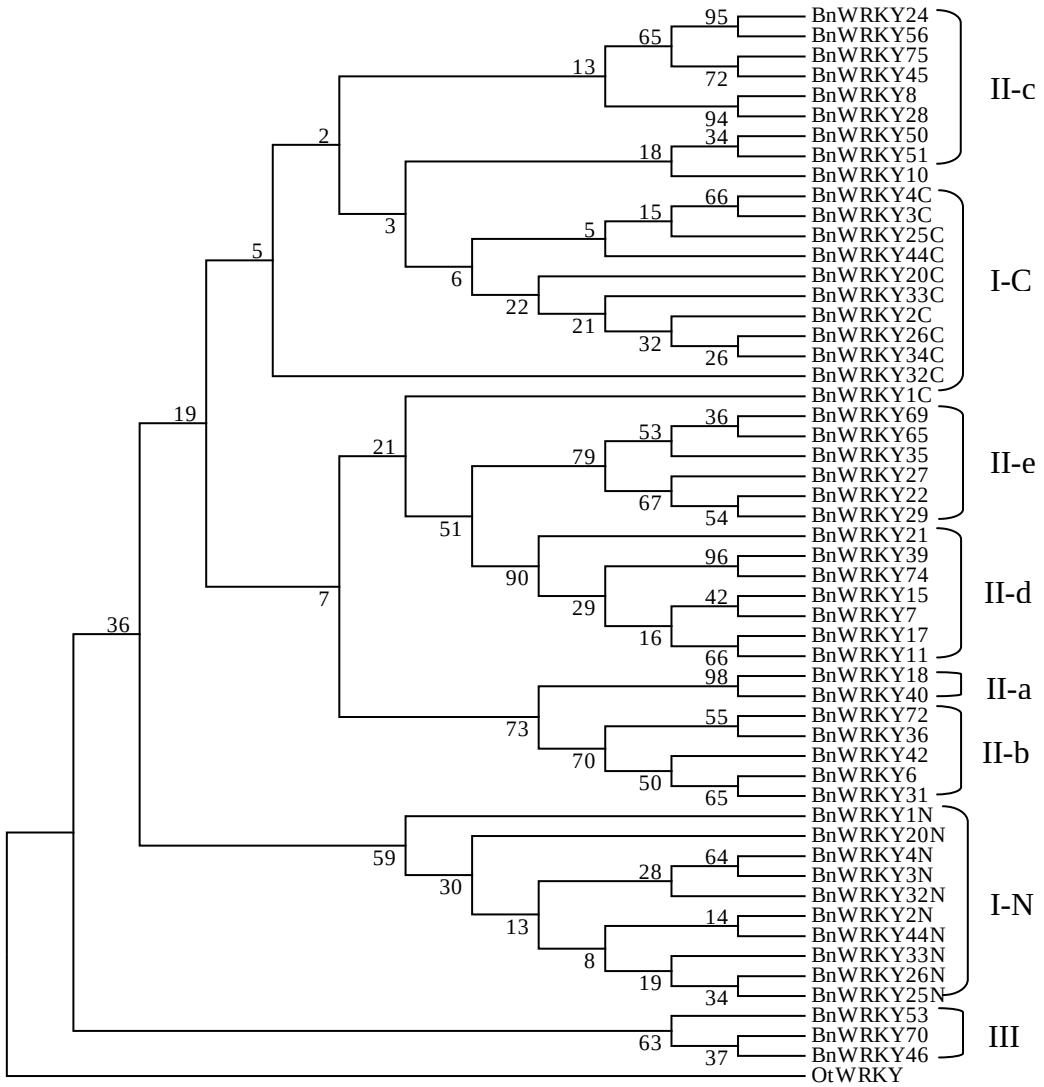

Figure 4-1 A bootstrap consensus maximum parsimony tree of WRKY TFs in canola. The phylogenetic tree was based on the amino acid sequences from WRKY domains only. Only the ~ 60 amino acid residues in the WRKY domain were aligned using ClustalX (v1.83) and were further examined manually for optimal alignment. The parsimony tree was drawn using MEAG4.0. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The two letters N and C after group I represent the N-terminal and the C-terminal WRKY domains of group I proteins, respectively.

(BnWRKY46, 53, 66 and 70). The total number of canola WRKY genes will remain unknown until the whole canola genome and its analysis is completed.

Nuclear localization of four BnWRKY proteins

The function of a TF requires that it can be localized in the nuclei, although TFs targeting chloroplasts, mitochondria, or endoplasmic reticulum (ER) have also been identified (Schwacke et al. 2007). To investigate whether the identified BnWRKY TFs can be targeted to the nucleus, we selected four *BnWRKY* genes, based on their known functions in mediating defense responses in Arabidopsis (Genevestigator, (Zimmermann et al. 2004)) for our studies. We fused the coding regions of *BnWRKY6*, *25*, *33*, and *75* to the N-terminus of synthetic green fluorescent protein (sGFP) (Chiu et al. 1996) and expressed them in Arabidopsis under the control of the constitutive cauliflower mosaic virus (CaMV) *35S* promoter. Analysis of conceptually translated *BnWRKY6*, *25*, and *33* coding sequences revealed the presence of a monopartite nuclear localization signal (NLS), when submitted to PSORT (prediction program of protein localization sites, http://psort.nibb.ac.jp). However, no NLS was detected in the translated BnWRKY75 sequence.

In order to determine whether the aforementioned WRKYs are targeted to the nucleus, we analyzed transgenic Arabidopsis seedlings harboring the respective four constructs. Firstly, we stain the seedling with Hoechst, only nucli can be stained by this chemical and later on the same nuclei was observed to harbor green signal under fluroresence microscopy (data not shown). In all four cases, green fluorescent signals were observed only in the nucleus (Figure 4-2A-D). With the control vector alone, GFP signals were distributed in both the cytoplasm and nucleus (Figure 4-2E). Our results indicate that *BnWRKY6*, *25*, *33*, and *75* are indeed nuclear-localized proteins, which is consistent with their predicted function as transcription factors.

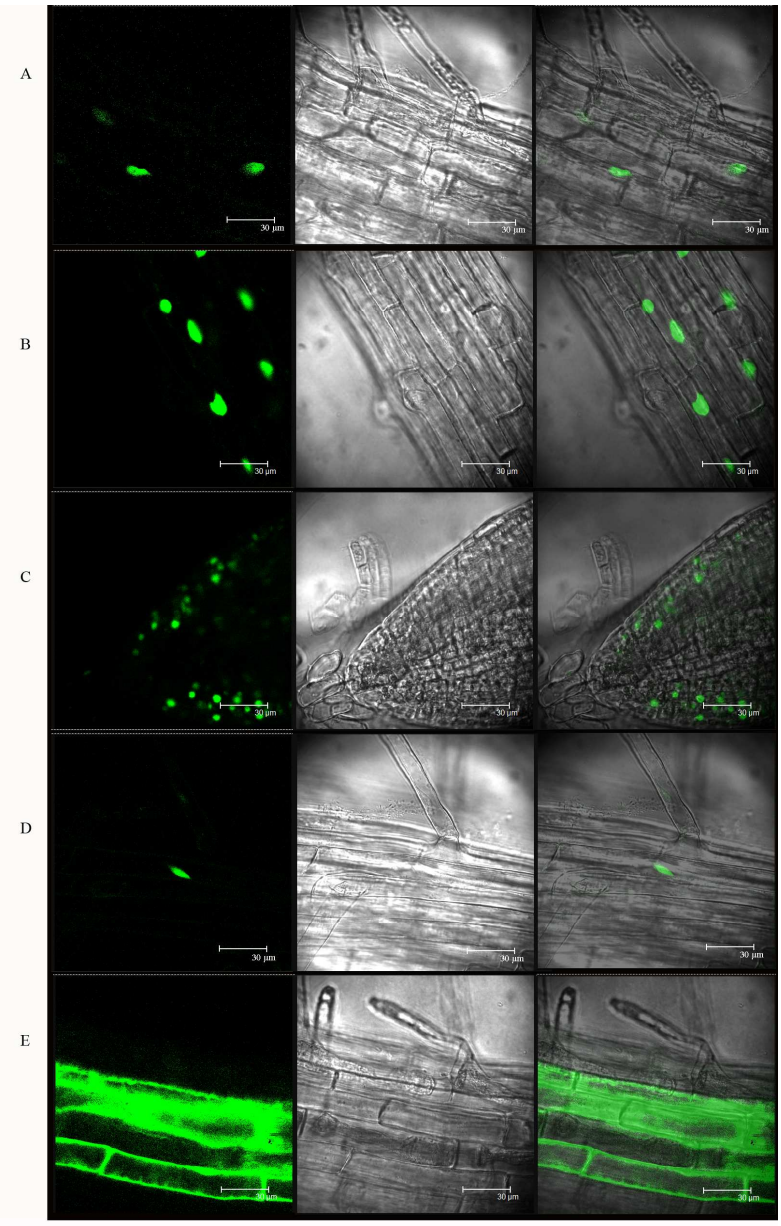

Figure 4-2 Nuclear localization of four BnWRKY proteins. Transgenic (T_2) Arabidopsis roots of five-day old seedlings were observed under confocal microscopy. Panels A-E represent the subcellular localization of BnWRKY6-sGFP, BnWRKY25-sGFP, BnWRKY33-sGFP, BnWRKY75-sGFP and pCsGFPBT vector control, respectively. In each case, the extreme left panel is GFP fluorescence, the middle bright field and the right represents an overlay of the two images.

Expression analysis of *BnWRKY* genes in response to fungal pathogens- *S. sclerotiorum* and *A. brassicae*

Because sub-functionalization often follows the divergence of paralogous genes (Duarte et al. 2006), we investigated the responses of representatives of each of the three major *WRKY* clades through quantitative real time-PCR (qRT-PCR). We selected 16 *BnWRKY* genes, *WRKY1*, *6*, *11*, *18*, *20*, *25*, *28*, *32*, *33*, *40*, *45*, *53*, *65*, *69*, *70* and *75*, as representatives of each clade (Table 4-2, Figure 4-1). After challenge with the fungal pathogen *S. sclerotiorum*, transcript abundance of 13 *BnWRKY* genes was observed to be significantly (t-test, $P<0.05$) changed with10 being increased, two being decreased and one being decreased at 12 h but subsequently increased at 72 h (Figure 4-3A). *BnWRKY6*, *25*, *28*, *33*, *40*, *45*, 53, *65*, *69* and *75* were highly induced at 48 h after inoculation. However, *BnWRKY20* and 32 were repressed by *S. sclerotiorum* infection. *BnWRKY1* was observed to be repressed at an earlier time-point (12 h) but induced later (72 h, Figure 4-3A). We then examined the changes in transcript abundance of these 16 *BnWRKY* genes in response to a second fungal pathogen, *A. brassicae*, which is also a necrotrophic pathogen. The symptom development in these two pathosystems (*S. sclerotiorum* and *A. brassicae*) is different with respect to time required, with *A. brassicae* requiring a much longer period before visible disease symptoms could be observed (Agrios 1997; Liang et al. 2008; Yang et al. 2007; Sharma et al. 2007). Accordingly, the transcript abundance of only four *BnWRKY* genes were significantly affected by *A. brassicae* with two (*BnWRKY33* and *75*) being significantly increased at 48 h post-pathogen challenge and two (*BnWRKY70* at both 48 and 72 h and *BnWRKY69* only at 72 h) with decreased transcript abundance (Figure 4-3B). In summary, our results indicate that *BnWRKY33* and *75* are induced by both *S. sclerotiorum* and *A. brassicae*, with *BnWRKY75* exhibiting a similar temporal pattern of changes in transcript abundance in respond to the two fungi. However, *BnWRKY69* and *70* had different responses to *S. sclerotiorum* and *A. brassicae*. Our results suggest that even though both pathogens investigated in this study are necrotrophic, they elicit

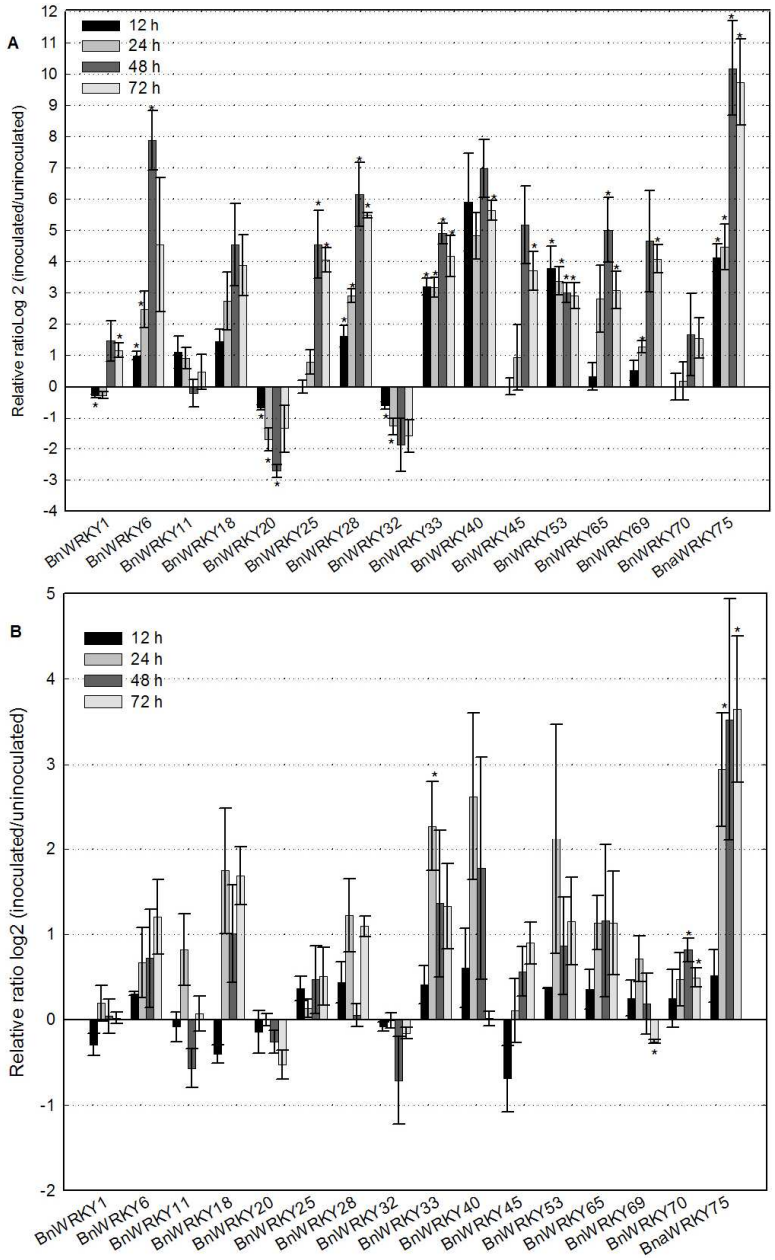

Figure 4-3 Expression analyses of *BnWRKY* genes in response to fungal challenge. Changes in *BnWRKY* transcript abundance in response to (A) *S. sclerotiorum* and (B) *A. brassicae* infection. Data is the mean of three biological replicates ± S.E. Asterisks denote significantly differences by Student t-test analysis.

slightly different responses with respect to changes in transcript abundance of *BnWRKY* genes.

Response of selected *BnWRKY* genes to hormone treatments

To investigate the hormonal control mechanisms underlying *BnWRKY* gene expression, we treated canola plants with five phytohormones, JA, SA, ABA, BAP and ET and analyzed the changes in transcript abundance of these 16 *BnWRKY* genes using qRT-PCR. In order to ensure that the hormone applications were eliciting appropriate responses in plants, we first examined the responses of a few additional canola genes that are proposed to be orthologs of Arabidopsis genes previously reported to respond to these hormones. These Arabidopsis genes were two *bZIP* transcription factors, *TGA2, TGA5* for SA (Cao et al. 1994; Kesarwani et al. 2007; Zhang et al. 2003); allene oxide cyclase (*AOC*) (Stenzel et al. 2003) and plant defensin 1.2 (*PDF1.2*) for JA (Thomma et al. 1998); ethylene insensitive 2 (*EIN2*) (Alonso et al. 1999) and ethylene responsive factor (*ERF2* and *ERF4*) (Fujimoto et al. 2000) for ET; ABA insensitive 5 (*ABI5*) (Finkelstein et al. 2002; Finkelstein and Lynch, 2000; Li et al. 2005) for ABA, and Arabidopsis response regulator 6 (*ARR6*) (Imamura et al. 1998) and cytokinin response 1 (*CRE*) (Mahonen et al. 2006) for BAP. We observed that the abundance of transcripts for all of these genes was significantly increased in response to the hormone treatments (data not shown), confirming that our hormone treatments elicited appropriate responses.

Our results demonstrated that, among the 16 *BnWRKY* genes studied, *BnWRKY40, 69* and *75* were induced by ET and *BnWRKY53* was repressed by ABA at 6 h (Table 4-3, Figure 4-4A). In contrast, *BnWRKY25, 32, 45, 69* and *70* were repressed by BAP at 6 h (Table 4-3, Figure 4-4A). At 24 h, *BnWRKY1, 28, 32, 33, 45,* and *75* were specifically induced by ET and *BnWRKY70* was repressed by ET (Table 4-3, Figure 4-4A). Three *BnWRKY* genes exhibited modulation of expression in response to two hormones (Table 4-3). At 6 h, both JA and ET repressed *BnWRKY11* and both ET and BAP repressed *BnWRKY1, 20* and *32* (Figure 4-4C, Table 4-3). However, none of the genes were observed to be

Table 4-3 Expression analyses of *BnWRKY* genes to five plant defense-related hormone treatments assayed by qRT-PCR. Results are presented as a ratio of transcript abundance in treatment/mock on a linear scale. Data were mean of three biological replicates ± S.E. The asterisk indicates that the corresponding gene was significantly up- or down-regulated under a stress treatment by *t*-test (* for $p<0.05$ and ** for $p<0.01$).

gene	JA		ET		SA		ABA		BAP	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
BnWRKY1	2.47 (±1.40)	1.11 (±0.06)	0.83 (±0.03)*	1.13 (±0.03)*	1.53 (±0.26)	1.10 (±0.2)	2.54 (±1.31)	1.03 (±0.08)	0.59 (±0.02)**	0.58 (±0.08)*
BnWRKY6	0.64 (±0.14)	0.88 (±0.29)	1.07 (±0.59)	3.98 (±0.81)	1.99 (±0.39)	1.29 (±0.37)	2.17(±0.17)	1.23 (±0.13)	0.72 (±0.13)	0.64 (±0.22)
BnWRKY11	0.63 (±0.01)**	0.85 (±0.12)	0.70 (±0.00)**	1.02 (±0.10)	1.63 (±0.21)	1.22 (±0.29)	1.16 (±0.03)	1.06 (±0.37)	0.64 (±0.11)	0.97 (±0.05)
BnWRKY18	1.99 (±0.64)	1.31 (±0.33)	1.74 (±0.20)	1.60 (±0.33)	11.65 (±4.02)	6.48 (±0.59)	3.01 (±0.62)	1.07 (±0.39)	0.71 (±0.27)	0.38 (±0.11)*
BnWRKY20	0.85 (±0.24)	0.95 (±0.12)	0.68 (±0.04)*	1.38 (±0.13)	1.38 (±0.35)	1.19 (±0.15)	0.77 (±0.15)	0.87 (±0.03)	0.61 (±0.07)*	0.59 (±0.14)
BnWRKY25	1.71 (±0.54)	1.53 (±0.16)	1.82 (±0.16)	2.34 (±0.50)	1.69 (±0.39)	0.92 (±0.09)	2.51 (±1.45)	1.83 (±0.19)*	0.55 (±0.07)*	0.42 (±0.13)*
BnWRKY28	0.75 (±0.11)	1.55 (± 0.25)	0.86 (±0.26)	1.49 (±0.05)**	1.37 (±0.45)	3.64 (±3.00)	1.00 (±0.09)	1.75 (±1.02)	1.00 (±0.38)	1.32 (±0.73)
BnWRKY32	1.11(±0.15)	1.14 (±0.15)	0.81 (±0.04)*	1.33 (±0.06)*	1.44 (±0.34)	0.88 (±0.08)	1.27 (±0.17)	0.97 (±0.12)	0.73 (±0.04)*	0.82 (±0.14)
BnWRKY33	0.68 (±0.30)	0.76 (±0.09)	3.89 (±0.09)	2.38 (±0.31)*	4.80 (±1.23)	1.40 (±0.36)	0.50 (±0.15)	1.00 (±0.21)	1.09 (±0.17)	0.87 (±0.18)
BnWRKY40	0.84 (±0.2)	1.17 (±0.44)	4.74 (±0.05)*	6.49 (±1.63)	2.17 (±0.47)	0.99 (±0.24)	1.28 (±0.47)	1.69 (±0.74)	0.61 (±0.05)*	0.56 (±0.16)
BnWRKY45	1.5 (±0.51)	0.91 (±0.21)	1.29 (±0.17)	3.41 (±0.39)*	1.39 (±0.17)	1.11 (±0.20)	2.35 (±1.07)	1.71 (±0.21)	0.63 (±0.01)**	0.94 (±0.21)
BnWRKY53	0.39 (±0.14)	0.89 (±0.50)	2.14 (±0.07)	0.75 (±0.10)	8.14 (±1.69)	2.33 (±1.05)	0.45 (±0.00)**	0.81 (±0.37)	1.43 (±0.19)	2.08 (±0.69)
BnWRKY65	1.41(±0.36)	1.58 (±0.51)	1.82 (±0.70)	1.46 (±0.33)	1.64 (±0.40)	1.84 (±0.71)	0.77 (±0.19)	1.16 (±0.42)	0.78 (±0.17)	0.50 (±0.05)**
BnWRKY69	0.71(±0.10)	1.04 (±0.27)	1.29 (±0.01)**	1.76 (±0.29)	1.29 (±0.16)	1.15 (±0.19)	0.42 (±0.08)*	1.17 (±0.41)	0.65 (±0.08)*	0.56 (±0.10)*
BnWRKY70	0.84 (±0.16)	1.12 (±0.21)	1.43 (±0.24)	0.52 (±0.00)**	13.98 (±6.01)	3.66 (±2.00)	0.85 (±0.12)	1.01 (±0.36)	0.46 (±0.04)**	0.83 (±0.26)
BnWRKY75	1.55 (±0.36)	2.30 (±0.93)	2.39 (±0.03)*	9.21 (±0.63)*	17.58 (±12.54)	8.19 (±4.61)	4.53 (±2.14)	2.03 (±0.43)	0.50 (±0.24)	0.60 (±0.30)

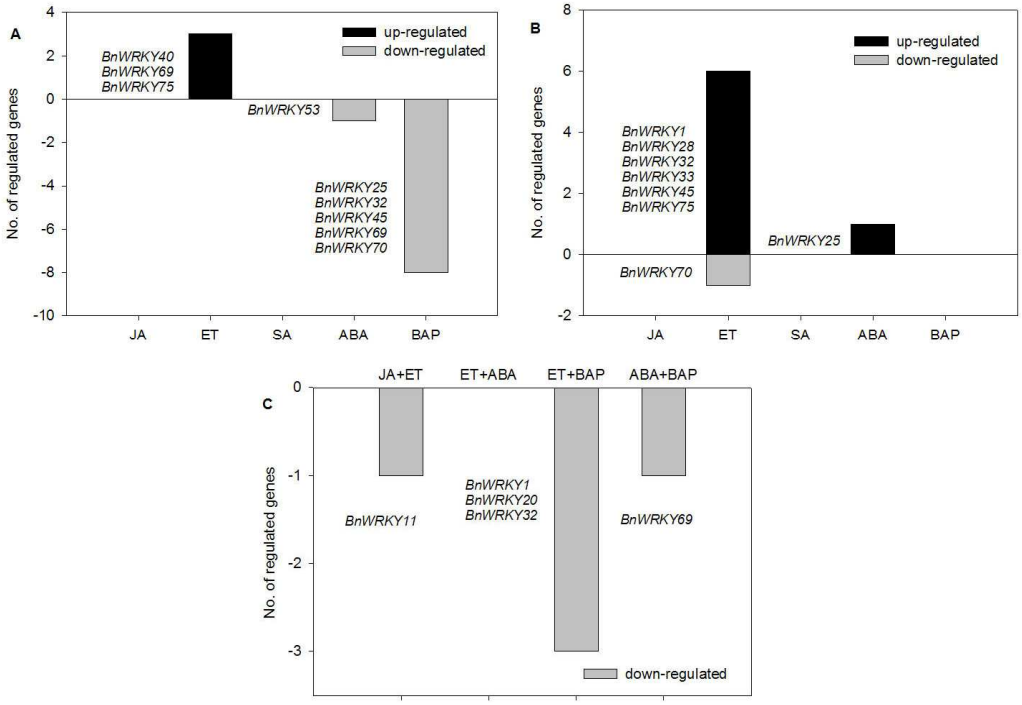

Figure 4-4 Expression analyses of *BnWRKY* genes in response to different hormone treatments. Changes in *BnWRKY* transcript abundance as a result of hormone application at (A) 6 h, (B) 24 h and (C) those that respond to more than one hormone at 6 h.

affected by the two hormones at 24 h. In addition, both ABA and BAP repressed *BnWRKY69* (Table 4-3, Figure 4-4A). None of these *BnWRKY* genes were affected by three or more hormones (Table 4-3).

As indicated earlier, JA and SA are important signaling molecules which are implicated in plant defense responses (Dong, 1998; Durrant and Dong, 2004); and other phytohormones, through their effect on SA or JA signaling, may influence disease outcomes (Robert-Seilaniantz et al. 2007). *BnWRKY11* was observed to be repressed by JA at 6 h although no significant change was observed at 24 h (Table 4-3). In response to SA treatment, we observed that that the transcript abundance for seven genes (*BnWRKY6*, *18*, *33*, *40*, *53*, *70* and *75*) exhibited modulation at 6 h and three (*BnWRKY53*, *70* and *75*) at 24 h (Table 4-3). However, these observed changes were not statistically significant.

In summary, SA did not significantly affect the transcript abundance of any of the *BnWRKYs* tested whereas ET, ABA, JA and the cytokinin BAP did affect the transcript abundance of various *BnWRKY* genes investigated in this study (Table 4-3). Athough the 16 genes tested did not show significant change in expression levels after exogenous treatments with SA, there is the possibility that other *BnWRKY* genes may be responsive to SA.

Discussion

In this study, we describe the identification and annotation of cDNA sequences of 46 members of the *WRKY* gene family in canola and their classification into groups I to III (Figure 4-1, Table 4-2). Among the 46 *BnWRKY* genes identified, both the hallmark WRKYGQK motif and its variants were identified in the translated amino acid sequences. A recent study demonstrated that AtWRKY TFs bearing the WRKYGQK motif exhibit binding site preferences, which are partly dependent on the adjacent DNA sequences outside of the TTGACY-core motif (Ciolkowski et al. 2008). For those WRKY TFs that do not contain the canonical WRKYGQK motif, a binding sequence other than the W-box element ((C/T)TGAC(C/T)) may exist. For instance, the binding sequence of tobacco (*Nicotiana tabacum*) NtWRKY12 with a WRKYGKK motif is TTTTCCAC, which deviates significantly from a W-box (van Verk et al. 2008). Moreover,

soybean (*Glycine max*) GmWRKY6 and GmWRKY21 lose the ability to bind to a W-box containing the variant WRKYGKK motif (Zhou et al. 2008). Finally, mutation of amino acid Q to K of AtWRKY 1 was observed to finally lead to the affected binding activity to the consensus W-box (Duan et al. 2007). We propose that the BnWRKY TFs that lack the canonical WRKYGQK motif might not be able to interact with the W-box and, therefore, may have different target genes and divergent roles, a proposal that must be verified in future studies.

Complete or partial WRKY domains are found in ESTs from many species of land plants (Zhang and Wang, 2005). Recently, 37 *WRKY* genes were identified in the moss, *Physcomitrella patens* (Guo et al. 2008). So far, no *WRKY* genes have been identified in the archaea, eubacteria, the fungi, or animal lineages (Zhang and Wang, 2005). However, in the genomes of the protist, *Giardia lamblia* and *Dictyostelium discoideum*, a single *WRKY* gene with two *WRKY* domains was recently identified (Ulker and Somssich, 2004; Zhang and Wang, 2005). Further examination of the two WRKY domains existing in the two organisms indicates that the *G. lamblia* WRKY TF has a WRKYGSK heptapeptide at its N-terminal and a WKKYGHK at its C-terminal, whereas in *D. discoideum*, both WRKY domains have a classical WRKYGQK heptapeptide. This suggests an ancient origin of the canonical WRKYGQK heptapeptide and its variants. In the green algae, *Chlamydomonas reinhardtii*, a WRKY TF containing two WRKY domains (Acc. XM_001692290) was also identified (Guo et al. 2008; Zhang and Wang, 2005). In the genome of the recently sequenced of world's smallest free-living eukaryote- the unicellular chlorophytic alga, *Ostreococcus tauri*, a WRKY gene containing a single WRKY domain and a WRKYGCK heptapeptide is also present (Acc. CAL54953).

The identification of WRKY genes in primitive eukaryotes suggests an ancient origin of the WRKY family, and that this family emerged before the evolution and diversification of the plant phyla (Zhang and Wang 2005). During this long evolutionary history, the WRKY gene family greatly expanded, as demonstrated by the increased numbers of *WRKY* genes in higher plants (Guo et al. 2008), and this expansion may be due primarily to segmental duplications of

genomic fragments as a result of independent polyploidy events (Bowers et al. 2003; Cannon et al. 2004; Thomas et al. 2006; Zhang and Wang, 2005). Comparison of a genomic region harboring five genes, one of which is *WRKY10*, between tomato, Arabidopsis and *Capsella rubella*, has revealed a great degree of microsynteny between closely and distantly related dicotyledonous species (Rossberg et al. 2001). An overall genomic duplication event has been identified to exist in the tribe *Brassiceae* after a comparative genomic analysis, and many genomic units that are conserved between canola and Arabidopsis have also been identified (Lysak et al. 2005; Parkin et al. 2005; Town et al. 2006). A further comparative genomic analysis of the *WRKY*-containing regions between canola and Arabidopsis should enable us to reveal the extent of microcolinearity between these closely related species and a better understanding of the expansion of the *WRKY* gene family in canola.

WRKY TFs are involved in the regulation of various biological processes, including pathogen responses and hormone signaling (Eulgem and Somssich, 2007). A previous expression analysis of AtWRKY genes demonstrated that nearly 70% are differentially expressed in response to pathogen infection or SA treatment, suggesting important roles for *WRKY* in defense responses (Dong et al. 2003). Recently, two studies on the rice *WRKY* genes also demonstrated that many are responsive to JA, SA and ABA treatments (Ramamoorthy et al. 2008; Ryu et al. 2006). Transcriptional activation of SA- and JA-responsive genes is essential for the induced resistance conferred by the two signaling pathways (Durrant and Dong, 2004; Penninckx et al. 1996; Penninckx et al. 1998). *WRKY* TFs are also reported to participate in disease resistance in Arabidopsis and tobacco, through modulation of SA- or JA-responsive gene expression similar to that induced by the TGA class of basic leucine-zipper transcription factors (Asai et al. 2002; Chen and Chen, 2002; Robatzek and Somssich, 2002; Xu et al. 2006; Zheng et al. 2006).

Previous studies from our laboratory, as well as those of others, revealed that few genes related to SA-signaling were modulated by infection of canola with *S. sclerotiorum* (Liang et al. 2008; Yang et al. 2007), suggesting that SA does not

play a crucial role in mediating responses of canola to this pathogen. The responses of Arabidopsis to *A. brassicicola*, which causes black spot in canola as *A. brassicae* does, also appear to be mediated through JA instead of SA (Vijayan et al. 1998), which is similar to responses to other necrotrophic fungi including *Pythium* species (Lippok et al. 2007; Robatzek and Somssich, 2001). Hence, it is possible that WRKY TFs may play an important role in suppressing the involvement of SA in response to those pathogens. This suggestion is consistent with the conclusion that *AtWRKY33*, which is induced by many pathogens, acts as a positive regulator of JA- and ET-mediated defense signaling but as a negative regulator of SA-mediated responses (Zheng et al. 2006). As mentioned earlier, both *A. brassicae* as well as *S. sclerotiorum* are able to induce *BnWRKY33*, one of the genes belonging to group I. Moreover, it has been demonstrated that pathogen-induced *AtWRKY33* expression does not require SA signaling (Lippok et al. 2007). Similar to *AtWRKY33*, *AtWRKY25* acts as a negative regulator of the SA-mediated signaling pathway (Zheng et al. 2007). The increased abundance of *BnWRKY25* due to infection by *S. sclerotiorum*, but not in the case of *A. brassicae* challenge, also suggests that it might work as a negative regulator of SA-related signaling pathways in the canola-*S. sclerotiorum* pathosystem but not in the canola-*A. brassicae* pathosystem. Of the other group I members investigated in our study (*BnWRKY1, 20 and 32*), BnWRKY1 was observed to be significantly induced by *S. sclerotiorum* only at 72 h (Figure 4-3) and *BnWRKY20* and -*32* were repressed by *S. sclerotiorum* (Figure 4-3), indicating the differences in behavior of group I BnWRKYs in response to fungal pathogens (*S. sclerotiorum* and *A. brassicae*).

It is possible that several BnWRKY TFs may also be involved in signaling the responses of canola to the pathogens *S. sclerotiorum* and *A. brassicae*. For instance, the transcript levels of two genes of the group IIa: *BnWRKY18* and *40*, orthlogs of which are known to act as negative regulators of plant defense in Arabidopsis (Mahonen et al. 2006), were observed to increase in response to *S. sclerotiorum* and *A. brassicae* challenge. For *A. brassicae*, the differences in transcript abundance between controls and inoculated plants were not statistically

significant (Figure 4-3). In addition, it has been reported that *AtWRKY6*, acts as a positive regulator of the senescence- and pathogen defense-associated *PR1* promoter activity, and is also induced by SA and bacterial infection (Robatzek and Somssich, 2001). Since leaf senescence is often linked to plant defense (Quirino et al. 1999), the induction of *BnWRKY6* by *S. sclerotiorum*, ABA and SA at an early time-point (6 h), but not *A. brassicae* may play a role in leaf senescence, which is observed very early in the *S. sclerotiorum*-canola pathosystem. We also observed that the group IIc (*BnWRKY2*8, *45*) and III (*BnWRKY75*) BnWRKYs in our study were all induced by *S. sclerotiorum* infection and ET, whereas *BnWRKY75* was induced only by *A. brassicae* (Figure 4-3, Table 4-3). Changes in expression of *BnWRKY75* induced by both pathogens suggest that an ET-mediated signaling pathway may be involved in mediating the responses of canola to necrotrophic pathogens. In Arabidopsis, mining of a public microarray database revealed that *AtWRKY45* and *AtWRKY75* were also induced by ET, while *AtWRKY28* is repressed by ET (Genevestigator, (Zimmermann et al. 2004), data not shown), suggesting that orthologs between canola and Arabidopsis may not necessarily have the same role in ET-mediated signaling. Further studies about the role of these three WRKY TFs in mediating defense responses are ongoing in our laboratory.

Although *BnWRKY11* (Group IId) was not affected by either *S. sclerotiorum* or *A. brassicae* in this study, our previous microarray profiling of transcriptome changes in canola as a result of *S. sclerotiorum* infection revealed that transcript levels of *BnWRKY11* and *15* increased while that of *BnWRKY17* decreased at specific time points, although the magnitude of response was less than two-fold (Yang et al. 2007). Arabidopsis *AtWRKY11* and *AtWRKY17* are both known to act as negative defense regulators, and *WRKY11* appears to act upstream of JA (Journot-Catalino et al. 2006), because of the fact that it does not respond to JA (Zimmermann et al. 2004) (Genevestigator). However, the expression of *AtWRKY11* has been reported to correlate with the induction of the JA biosynthesis enzymes AOS and LOX 2 h after challenge with *P. syringae* (Journot-Catalino et al. 2006). Incidentally, the accumulation of JA also occurs

within the first hour of the interaction with *P. syringae* (De Vos et al. 2005). Taken together with our observations that *BnWRKY11* was repressed by JA and ET treatments at 6 h, and was not induced by the pathogens, it is possible that the pathogen-induced accumulation of JA might repress the expression of *BnWRKY11,* a suggestion that will form the basis for future studies in our laboratory.

With the recently emerging role for ABA in defense responses (Mauch-Mani and Mauch, 2005; Ton et al. 2005; Ton and Mauch-Mani, 2004), it is possible that ABA exerts some of these effects through the modulation of *BnWRKY* genes, specifically *BnWRKY53* and *BnWRKY69*. Similarly, the cytokinin BAP has been implicated in both alleviating and exacerbating the hypersensitive response (HR), which is characterized by tissue necrosis and is frequently accompanied by the subsequent induction of systemic acquired resistance (SAR) throughout the plant (Hare et al. 1997). Furthermore, cytokinins can promote the susceptibility of biotrophs by inducing the necrotroph resistance pathway, which is responsive to JA/ET (Robert-Seilaniantz et al. 2007). As suggested for ABA-mediated plant defense responses, it is possible that the BnWRKYs, which were observed to be modulated by exogenous BAP application, may be responsible, at least in part, for mediating the observed effects with the necrotrophic pathogens.

Conclusions

In summary, we identified 46 BnWRKY TFs based on the publicly available EST resources of canola and cloned the cDNA sequences for 38 of them. We characterized the responses of 16 selected genes, based on their phylogenetic relationship in response to two fungal pathogens and five hormone treatments. Based on our data, we propose that BnWRKY TFs might play an important role in the plant defense response, possibly by acting as positive or negative regulators of plant defense, and canola may respond differently to *S. sclerotiorum* and *A. brassicae* from the standard point of a BnWRKY-mediated plant defense system. Our results also confirm that there is cross-talk between biotic stress and hormone signaling. Functional redundancy in defense programs is an inherent feature of

WRKY genes (Eulgem and Somssich, 2007) and future studies will be directed towards delineating the specific roles of individual WRKY TFs in those and related pathosystems, in order to explore the possibility that manipulation of abundance of one or several of these proteins may lead to durable and robust resistance to the pathogen, apart from contributing to our understanding of the molecular processes that occur during host-pathogen interactions.

Accession numbers

The cDNA sequences of 38 BnWRKY TF genes cloned in this study were deposited in GenBank under the accession No. EU912389- EU912407, EU912409-EU912418, FJ012166- FJ012171, FJ210288- FJ210290 and FJ384101- FJ384114.

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Chapter 5 Isolation, expression and characterization of two single- chain variable fragment antibodies against an endo-polygalacturonase secreted by *Sclerotinia sclerotiorum*

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic, highly destructive and non-host–specific fungus which affects more than 400 plant species (Boland and Hall, 1994; Bolton et al. 2006; Bom and Boland, 2000) and is prevalent in temperate regions of North America, Europe and New Zealand (Hambleton et al. 2002). Sclerotinia stem rot is a very serious disease in canola, which is an important North American crop with 8.7 million tonnes harvested in Canada in 2007 (Anonymous, 2007). It is very difficult to control this fungus due to long survival of sclerotia (an overwintering structure), ascospore dispersal potential, limited sources of resistance in canola and related species as well as the fact that its host range is so broad, which can limit the effectiveness of crop rotations (Bolton et al. 2006). There are several fungicides effective in controlling *S. sclerotiorum*, but it is important to determine the need for fungicide application early using risk assessment tools (Thomas, 1984; Turkington and Morrall, 1993; Turkington et al. 1991a; Turkington et al. 1991b).

Secreted virulence factors are essential for *S. sclerotiorum* pathogenesis and include oxalic acid (Bateman and Beer, 1965; Cessna et al. 2000; Godoy et al. 1990; Guimaraes and Stotz, 2004; Kim et al. 2008; Kolkman and Kelly, 2000; Noyes and Hancock, 1981), hydrolytic enzymes (Kasza et al. 2004; Li et al. 2004a; Li et al. 2004b; Yajima and Kav, 2006) and several other factors such as oleic acid and sclerin (Erental et al. 2007; Harel et al. 2006; Rollins, 2003; Vega et al. 1970). The full virulence of *S. sclerotiorum* is thought to be due to the synergistic activities of oxalic acid and endo-polygalacturonases (Rollins, 2003).

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Polygalacturonases (PGs), encoded by multigene families, are produced in several molecular forms with different specificities and are subject to differential regulation. This multiplicity can grant adaptive flexibility to pathogens when infecting different hosts or plant organs (De Lorenzo et al. 2001). Internal α(1-4) glycosidic bonds in de-esterified regions of the middle lamella and primary cell wall homogalacturonans are hydrolyzed by PGs, and when produced in sufficient amounts, determine the degree of maceration of infected tissues (Zuppini et al. 2005). One of the endo-polygalacturonases, SSPG1d, is detected in the early stages of *S. sclerotiorum* pathogenesis and its expression is optimal in low pH environments (Li et al. 2004b; Rollins and Dickman, 2001). SSPG1d is highly homologous at the nucleotide level to other *S. sclerotiorum* endo-polygalacturonases such as SSPG1a-c (Fraissinet-Tachet et al. 1995).

By expressing recombinant antibodies against virulence factors or coat proteins, resistance or tolerance to the detrimental effects of phytopathogenic organisms can be introduced into plants (Peschen et al. 2004; Tavladoraki et al. 1993). The detection of these polygalacturonases using antibody-based tests may have utility in confirming the presence of this fungus, especially when used in a panel format assay with other Sclerotinia-specific antibodies, for example a single chain variable fragment (ScFv) that we have previously reported (Yajima et al. 2008). Towards achieving this objective, we have isolated, expressed, purified and characterized two recombinant ScFvs against SSPG1d using phage display technology. In addition, we have expressed the cDNAs encoding the ScFvs in *Arabidopsis thaliana* in order to characterize the transgenic plants for enhanced tolerance to *S. sclerotiorum*. The properties of these two antibodies with respect to specificity, sensitivity, affinity, their ability to detect SSPG1d in plant material as well as the tolerance of ScFv-expressing transgenic *A. thaliana* to *S. sclerotiorum*, were characterized and our results are discussed.

Materials and Methods

Culture of *S. sclerotiorum*

A strain of *S. sclerotiorum* (isolated from canola) was kindly provided by Dr. Stephen Strelkov, University of Alberta. Sclerotia were subcultured on solid potato dextrose agar (PDA) medium (Becton Dickinson, MD, USA) under continuous light at room temperature (RT). After 3 days, agar plugs (0.2 cm diameter) were removed with a sterile cork borer from the leading edge of the mycelia and were subcultured on fresh PDA plates for an 2 additional days. Agar plugs (0.5 cm) were removed from the leading edge of mycelia and were used for inoculation of liquid pectin media (50 mM NH_4Cl, 7.3 mM KH_2PO_4, 4.2 mM $MgSO_4$, 6.7 mM KCl, 0.07 mM $FeSO_4$, and 1% (w/v) pectin; (Yajima and Kav, 2006)). The cultures were incubated at RT with agitation (180 rpm) on a platform shaker for 5 days. Mycelia were harvested from these cultures by centrifugation at 13,800x*g* for 20 min.

Source of antigens

A cDNA encoding SSPG1d (GenBank accession no. AF501307) was kindly provided by Dr. Dwayne Hegedus (Agriculture and Agri-Food Canada, SK, Canada). This cDNA was subcloned into the expression vector pET30a (+) (Novagen, WI, USA) at *Nde* I/*Xho* I (New England Biolabs, MD, USA) sites for the expression of an N-terminal polyhistidine tagged protein. This expression construct was used to transform *E. coli* strain BL21 (DE3) (Stratagene, CA, USA) and gene expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG,1 mM, Fisher, PA, USA) and growth at 25 °C for 6 h. The expressed protein was purified using Ni-NTA (Qiagen, ON, Canada) as per the manufacturer's recommendations. The amino acid sequence NGSPTGKPTSGVPI from SSPG1d, corresponding to amino acid sequence 294-307 of the mature SSPG1d primary structure, predicted to be hydrophilic using the Protein Hydrophobicity Plots (http://arbl.cvmbs.coloctate.edu/molkit/hydropathy/index.html) and, therefore, likely to be antigenic was synthesised at the Alberta Peptide Institute (API) at the

University of Alberta. This peptide, henceforth referred to as peptide 3796, was conjugated with both KLH for immunization and BSA for screening by ELISA.

Antigen preparation and mouse immunizations

Both antigens were used to immunize three-week old BALB/c mice as described below. Tail bleeds were obtained from mice prior to each immunization. The mice were primed with a maximum of 200 µl (100 µl x 2 subcutaneous sites) of Freund's Complete Adjuvant (FCA, Difco, MI, USA) containing 20 µg of antigen (either peptide 3796 or recombinant SSPG1d (rSSPG1d)). Three boosts of 20 µg antigen using Freund's Incomplete Adjuvant (FIA, Difco) were given with a two-week interval between boosts. One week after each boost, blood samples were obtained by tail bleeds and the serum was used to determine antibody titers as described below. When titers were sufficiently high, and four days prior to euthanasia, 20 µg of the appropriate antigen suspended in sterile 1xPBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) was injected into the tail vein. The mice were euthanized by cervical fracture or with an overdose of carbon dioxide and the spleens of the euthanized mice were collected aseptically and used in the construction of heavy and light chain antibody libraries.

Antibody titres were determined by coating microtiter plate wells (Costar, NY, USA) with 100 µl of 10 µg/ml solution of the appropriate antigen, overnight at 4 °C. Wells were washed thrice with 1xPBS and all subsequent incubation steps were performed at RT unless otherwise stated. Wells were blocked with 200 µl of 2% (w/v) BSA for 2 h, followed by incubation with 100 µl of diluted mouse serum (1:10, 1:100, 1:1000, 1:10,000 dilutions) for 1 h following three rinses with 1xPBS. Horse Radish Peroxidase (HRP) conjugated goat anti-mouse IgG+IgM (Cell Sciences, CA, USA) was diluted 1:5,000 in 1% (w/v) BSA in 1xPBS, added to wells (100 µl) which were incubated for another 2 h. Following three rinses with 1xPBS, Sure Blue Reserve TMB Microwell Peroxidase Substrate (100 µl /well, Kirkegaard & Perry Labs, MD, USA) was added to each well and incubated for 1-5 min. Once a clear difference in colour development between sample and background (controls) was observed, the reaction was

stopped by adding 100 µl of 1 N HCl and absorbance values at 450 nm were determined using a plate reader (Spectra Max 190, Molecular Devices, CA, USA).

Isolation of ScFv using phage display technology

Total RNA was extracted from spleens with the RNeasy Mini kit (Qiagen, MD, USA) followed by purification of mRNA using the mRNA Purification Kit (GE Healthcare, QC, Canada**).** cDNA was synthesized from the purified mRNA using the First strand cDNA synthesis kit (GE Healthcare). Heavy and light chains were amplified from the newly synthesized cDNA using an oligonucleotide primer mix Burmester and Plcükthun (2001) and Krebber et al. (1997) according to the PCR conditions described in (Burmester and Plcükthun, 2001; Krebber et al. 1997). Heavy and light chains were then spliced using overlapping PCR, cloned into the pJB12 vector (the pJB12 vector was kindly provided by Dr. Andreas Pluckthun, University of Zurich), which was digested with *Sfi* I (New England Biolabs, MD, USA), and used to transform *E. coli* TG1 strain. Transformation reactions were plated and cultured on 2xYT agar containing 1% (w/v) glucose and 30 µg/ml chloramphenicol (Burmester and Plcükthun, 2001; Krebber et al. 1997).

The rescue of ScFv-displaying phages and selection of antigen binders by panning was performed according to Krebber et al. (1997) and Tout et al. (2001) with minor modifications. After transformation, colonies were resuspended and used to inoculate fresh 5 ml 2xYT liquid medium containing 1% (w/v) glucose and 30 µg/ml chloramphenicol, which were incubated at 37°C with agitation (200 rpm) until the optical density (OD) at 600 nm reached 0.5. At this point, another 5 ml of 2xYT medium containing 1% (w/v) glucose, 30 µg/ml of chloramphenicol, 1 mM IPTG and VCSM13 helper phage ($5x10^9$ CFU; Stratagene, WI, USA) was added and the cultures incubated overnight at RT with agitation (200 rpm) for the rescue of phage particles expressing ScFv. The overnight culture was centrifuged at 1,500x*g* for 20 min, 2 ml of PEG/NaCl solution (20 % (w/v) PEG-8000; 14.6 % (w/v) NaCl) was added and the supernatant was placed on ice for 1 h for the precipitation of phage particles. The

precipitated phage particles were collected by centrifugation at 4 °C for 20 min at 20,000x*g* and the phage pellet was resuspended in 1 ml of 1xPBS.

In order to obtain phage-presenting antibodies with affinity towards the antigen, microtiter plates were coated overnight at 4 °C with 100 µl of 10 µg/ml of the appropriate antigen (rSSPG1d or peptide 3796). Coated wells were blocked with 200 µl of 3% (w/v) BSA in 1xPBS for 2 h, and phage suspension (10^9 phages/100 µl) was added to each well and incubated for another 2 h. Wells were then washed 10 times with 1xPBS containing 0.05% (v/v) Tween-20 (PBST) followed by another 10 washes with 1xPBS. Phage particles that remained bound to the antigen were eluted with 100 µl sodium acetate buffer (0.1 M acetic acid, pH 2.8 containing 0.15 M NaCl) and incubated for 8 min, followed by neutralization with 12 µl of 2 M Tris buffer (pH 9.5). The eluted phage particles were used to infect a fresh culture of *E. coli* TG1 cells (OD_{600} of 0.5) at 37 °C for 30 min followed by another 30 min of incubation at RT. Cells were plated on 2xYT agar plates containing 1% (w/v) glucose and 30 µg/ml of chloramphenicol and incubated at 30 °C for 16 h. This panning procedure was repeated 4 times as described above, except that the PBST and 1xPBS washes were increased to 20 in subsequent rounds of panning.

Screening of phage display library by phage ELISA

After each round of panning, 8-16 colonies were picked randomly and cultured in 2xYT medium (1 ml) containing 1% (w/v) glucose and 30 µg/ml of chloramphenicol at 37 ºC, for the rescue and isolation of phage particles as described above. Phage ELISAs were performed on microtiter wells coated with 10 µg/ml (100 µl/well) antigen and blocked with 3% (w/v) BSA in 1xPBS (200 µl/well) for 2 h, washed three times with 1xPBS and incubated with the phage solution for 2 h. The wells were washed three times with PBST followed by five washes with 1xPBS and then HRP-conjugated anti-M13 secondary antibody (1:5,000 dilution; GE Healthcare), was added to each well (100 µl/well) and incubated for 1 h. Wells were washed three times with PBST followed by five washes with 1xPBS and Sure Blue Reserve TMB Microwell Peroxidase Substrate was added to each well (100 µl/well) and incubated for 1-5 min. The reaction

was stopped by adding 100 µl of 1 N HCl and absorbance values at 450 nm were determined using a plate reader (Spectra Max 190). Generally, a background subtracted absorbance value >0.2 was used as the threshold value to select clones that were considered to be "good binders". The nucleotide sequences for these clones were sequenced using a *BigDye* Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

Production and purification of ScFvs

The sequence encoding both ScFvs (ScFv-rSSPG1d and ScFv-3796) from phage clones identified as being the binders that produced the highest background subtracted signals were amplified by PCR using primers containing *Nde* I/*Xho* I restriction endonuclease sites and sub-cloned into pET28a(+) expression vector (Novagen, WI, USA). The sequence of the ScFvs introduced into the expression vector in this manner was verified and then the ScFv construct was transformed into *E. coli* BL21 strain. The expression of ScFvs was induced with 1 mM of IPTG and growth at RT for 6 h. Cells were harvested by centrifugation (5,000x*g* for 5 min at 4 °C) and subsequently resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Cells were lysed by the addition of lysozyme (5 µg/ml; Sigma, MO, USA) to the cell suspension and incubated on ice for 30 min. Inclusion bodies containing the expressed ScFv were collected by centrifugation at 13,800x*g* for 10 min. ScFvs were extracted from the inclusion bodies with a second buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, 5 mM imidazole, pH 8.0) and were purified using Ni-NTA resin as per the manufacturer's instructions. The purified ScFv antibodies were refolded according to Das et al. (2004).

Biotinylation of ScFvs

Since both rSSPG1d and ScFv-rSSPG1d contain a polyhistidine tag, to characterize the interaction between them, ScFv-rSSPG1d was biotinylated for subsequent detection with streptavidin-HRP. ScFv-3796 or ScFv-rSSPG1d (100-1,000ng) was mixed with biotinamidohexanoic acid-3-sulfo-N-hydroxysuccinimide ester (10 µl of 30 µg/ml in 1xPBS; Long arm biotin, Sigma,

ON, Canada) and incubated at RT for 1 h with intermittent mixing. Ten µl of glycine (10 mg/ml in water) was added to stop the reaction and the mixture was dialyzed against 1xPBS overnight at 4°C, followed by an additional 4 h of dialysis in fresh 1xPBS (Delos et al. 2000). In order to confirm biotinylation, 10 µl of diluted (10-, 100-, and 1,000-fold) biotinylated and non-biotinylated ScFvs were applied to hybond-N^+ membrane (GE Healthcare) and dried at RT for 10 min. The membrane was then blocked with 2% (w/v) BSA in 1xPBS and washed thrice with 1xTBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). Following washes, streptavidin-HRP (1:1,000, Invitrogen, CA, USA) was added to the membrane and incubated at RT for 1 h. The membrane was then washed once with 1xTBS containing 0.05% (v/v) Tween-20 (TTBS), twice with 1xTBS, and then stained with TMB peroxidase substrate (Vector Laboratories, CA, USA). The reaction was stopped by immersion of the membrane in water.

ELISAs with ScFvs

In order to characterize the ScFvs, the respective antigens [100 µl of 10 µg/ml of peptide 3796 or rSSPG1d and control peptides/proteins (BSA conjugated CipC peptide from *S. sclerotiorum* corresponding to amino acids 38-49 of the CipC protein; GenBank accession no. CD645941: KMFEDRQRREGK; BSA conjugated peptide 3795 corresponding to amino acids 114-127 of the mature SSPG1d sequence: WDGKGTNGGKTKPK and BSA alone)] were used to coat microtiter plates at 4 ºC overnight. Peptide 3795, CipC peptide and BSA were used as controls to test the specificity of ScFv-3796. BSA and mycelium from *S. sclerotiorum* were used as controls to test the specificity of ScFv-rSSPG1d. Wells were washed three times with 1xPBS and then blocked with 3% (w/v) BSA in 1xPBS (200 µl/well) for 2 h at RT. After washing with 1xPBS three times, refolded, biotinylated ScFv-rSSPG1d or non-biotinylated ScFv-3796 were added to the wells (7 µg/ml, 100 µl/well) and the plates were incubated at RT for 1 h, after which the secondary antibody (1:2,000 dilution, 100 µl of streptavidin-HRP for ScFv-rSSPG1d; 1 µg/ml; or 1:1,000 dilution, 100 µl HRP-anti-His antibody for ScFv-3796, Qiagen, ON, Canada) was added and the plates incubated for another hour at RT. Subsequent steps in the ELISA were similar to those used for

phage ELISAs described earlier. HRP-conjugated anti-His antibody could not be employed as the secondary antibody for detection of rSSPG1d because it would bind to the polyhistidine tag of rSSPG1d in addition to the ScFv.

Secreted proteins from culture filtrates of 5-day old *S. sclerotiorum* cultures were prepared as previously described (Yajima and Kav, 2006). Wells were coated with 20 µg/ml (100 µl/well) of culture filtrate at 4 ºC overnight. Subsequent ELISA steps were similar to those described above except that a series of dilutions (0, 0.50, 1.00, 1.50, 2.00 and 5.00 µg/ml, 100 µl) of ScFv-rSSPG1d were added to wells. In this case, due to the absence of a polyhistidine tag on the secreted SSPG1d, we were able to use HRP-conjugated anti-His antibody as the secondary antibody.

Western blotting

The secreted fungal proteins and purified rSSPG1d were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane using a trans-blot SD semi-dry transfer cell (Bio-Rad, CA, USA). Following transfer, the membrane was blocked with 3% (w/v) BSA in 1xPBS at RT for 1 h. The membrane was incubated with biotinylated ScFv (ScFv-3796 or ScFv-rSSPG1d; 7 µg/ml, 5 ml) for 1 h at RT, followed by streptavidin-HRP (1 µg/ml, 5 ml) for an additional hour. The membrane was then washed 5 times with TTBS and three times with 1xTBS. Immunoreactive bands were visualized with TMB peroxidase substrate.

Determination of affinity constants

Surface plasmon resonance (SPR) using a Biacore 3000 system (BIAcore, Uppsala, Sweden) was employed to analyze the binding of ScFv-rSSPG1d and ScFv-3796 to rSSPG1d and/or peptide 3796. For the characterization of rSSPG1d and ScFv-rSSPG1d interaction, CM5 (BIAcore) sensor chips were coupled with 1,500 Resonance Units (RU) of rSSPG1d dissolved in 10 µM sodium acetate using the amine coupling method (Wizard procedure, (Corr et al. 1994)). Ethanolamine-HCl was used to block the remaining, uncoupled sites on the chip. A range of concentrations of ScFv-rSSPG1d or ScFv-3796, which was dissolved in running buffer (20 mM Tris, pH 7.0, 135 mM KCl, 2 mM $CaCl_2$, 0.05% (v/v)

Tween-20, 200 µM PMSF, 100 µM Benzamidine, SL inhibitors (1:1,000 dilution)) was then passed over the immobilized antigens at a flow rate of 30 µl/min at 21 °C. BIAevaluation (BIAcore) version 3.1 was used to determine the *ka*, *kd* and K_D of each ScFv using a 1:1 binding with drifting baseline model. High salt solution (20 mM Tris, pH 7.0, 800 mM KCl, 2 mM $CaCl_2$, 0.05% (v/v) Tween-20) was used as the regeneration buffer.

A streptavidin-coated sensor chip was used to analyze the binding of ScFv-3796 and peptide 3796. To accomplish this, 50 nM to 1 mM of streptavidin (Sigma, MO, USA) was coupled to a CM5 sensor chip (790 RU) following the amine coupling method described above. After blocking of uncoupled sites with ethanolamine-HCl, the chip was washed by the injection of 5 µl of 0.1% (w/v) SDS thrice for 1 s each time after which biotinylated peptide 3796 (50 nM) in running buffer was passed through the chip. In total, 25-50 RU of peptide was bound to the chip and used for kinetic analysis as described previously for rSSPG1d and the ScFv-rSSPG1d interaction.

Diagnostic assays with ScFv

Extracts of canola leaves or flowers from 6-week old plants grown in a greenhouse with a photoperiod of 16 h light (combination of natural light and T5 fluorescent tubes with a light intensity of 300 µE (µmol) m^{-2} s^{-1} or µV)/8 h dark were obtained by grinding the tissue in 1x PBS (2.5g leaf/3.5ml 1xPBS; 1g flower/1.4ml 1xPBS) followed by centrifugation at 10,000xg for 10 min and collection of the supernatants. The leaf and flower extracts were diluted 100-fold in 1xPBS to which a series of rSSPG1d dilutions (0.1, 0.5, 1.0, 5.0 and 10.0 ng/ml) were added. Plates were coated overnight at 4°C using 100 µl/well of the mixture and then washed thrice by 1xPBS. Coated wells were blocked with 3% (w/v) BSA in 1xPBS (200 µl/well) for 2 h and biotinylated ScFv-rSSPG1d (7 µg/ml, 100 µl/well) was added to the wells and incubated for another 1 h. The plates were washed thrice with PBST and 5 times with 1xPBS. Subsequent ELISA steps were as described in previous sections.

Plasmid construction and transformation of Arabidopsis

The open reading frames of ScFv-3796, ScFv-rSSPG1d were re-amplified using primers ScFv-3796-F1, and ScFv-3796-R1 (Table 5-1) for ScFv-3796 and ScFv-rSSPG1d-F1 and ScFv-3796-R1 (Table 5-1) for ScFv-rSSPG1d, and cloned into pKYLX 71 vector. The two constructs together with the blank vector pKYLX71 were individually transformed into Agrobacteria GV3101 using freeze-thaw and introduced into Arabidopsis (WS) by floral dipping. Seeds (T1) were harvested and selected on half strength LS media (Caisson Laboratories Inc., UT, USA) supplemented with kanamycin (50 mg/l) for 10 d before being transplanted into soil to grow to maturity (T2), with further selecting the same type of medium. Homozygous lines were used in the q-RT-PCR and phenotypic assay.

Quantitative RT-PCR (qRT-PCR)

Plants were grown in Sunshine Soil Mix 4 (Sungro Horticulture, Vancouver, Canada) in the greenhouse for three weeks and leaves were harvested for RNA isolation using RNeasy Plant Mini kit (Qiagen, Mississauga, ON, Canada). RNA was quantified using NanoDrop 1000 (NanoDrop Technologies, Inc., Wilmington, USA). First-strand cDNA was synthesized from 2.5 µg of total RNA using Superscript II (Invitrogen, Carlsbad, CA, USA) and Oligo$(dT)_{18}$ primers (Fermentas, Vilnius, Lithuania). PCR primers were designed using PrimerExpress3.0 (Applied Biosystems, Foster city, CA, USA) targeting an amplicon size of approximately 80-100 bp. The list of genes as well as the primers used are shown in Table 5-1. The qRT-PCR assay was performed as described previously (Yang et al. 2007) and each gene was assayed in duplicate using RNA from each of the three biological replicates. A two-tailed students' *t*-test and one-way ANOVA were performed using PAST (http://folk.uio.no/ohammer/past/) and SAS (SAS Institute Inc., NC, USA).

Table 5-1 Primers used in this study

Primer name	sequence of primers
ScFv-3796-F1	CGCACGAAGCTTATGGACTACAAAGACATTGTG
ScFv-3796-R1	GCGCATTCTAGATTACGCAGAGACAGTGAC C
ScFv-SSPG1d-F1	CGCACGAAGCTTATGGACTACAAAGATATTGTG
ScFv-3796-F2	AGCATGGAGGCTGAAGATGTTG
ScFv-3796-R2	CACCACGTTTGATTTCCAGCTT
ScFv-rSSPG1d-F2	CAGCTCAGCAGCCTGACATC
ScFv-rSSPG1d-R2	GAGTCCCTTGGCCCCAGTAA
At-UBQ-F	GGCCTTGTATAATCCCTGATGAA
At-UBQ-B	AGAAGTTCGACTTGTCATTAGAAAGAAA

Characterization of transgenic plant responses to *S. sclerotiorum*

Agar plugs of *S. sclerotiorum* for inoculation were obtained as previously described (Yang et al. 2007). Wild-type *Arabidopsis thaliana* (WS) and transgenic Arabidopsis plants were grown in Sunshine Soil Mix 4 in the green house (22°C day/18°C night; 16 h photoperiod combination of natural light and T5 fluorescent tubes with a light intensity of 300 µE (µmol) m^{-2} s^{-1}) for three weeks. PDA plugs with mycelia were placed on two of the rosette leaves. After inoculation, the plants were placed in a humidity chamber for 24 h before being returned them to the greenhouse. Photographs of the inoculated leaves were taken at 36 and 60 h post-inoculation and lesion size was measured by Image Analysis Software for Plant Disease Quantification (APS Press, St. Paul, MN, USA). The whole treatments were repeated three times at separate times and data were analyzed using SAS statistical analysis.

Results and Discussion

Immunization of mice, construction of a ScFv library and isolation of specific ScFvs

Both peptide 3796 and rSSPG1d were used to immunize mice to obtain specific ScFvs against these antigens. The rSSPG1d (~37 kDa, pI 7.21) used for immunization was purified as an N-terminal polyhistidine-tagged protein. Typically, the yield of rSSPG1d ranged from 120-500 µg per litre of cell culture. Its identity was verified by tandem mass spectrometry (data not shown). After immunization of mice, it was observed that the antibody titres were >10,000 for all mice after the third boost injection, at which time, the mice were euthanized and their spleens collected. The variable regions of heavy (V_H, 386-440 bp) and light (V_L, 375-402 bp) chains were amplified using specific primer sets (Krebber et al. 1997). Full-length ScFvs (~800 bp) were assembled in the 5'-V_L-linker-V_H-3' configuration where the linker sequence is $(GlyGlyGlyGlySer)_4$ (Das et al. 2004; Schouten et al. 1997). Two phage display libraries were made using the phagemid vector pJB12. The first library was for ScFv-3796 ($3.3x10^9$ cfu/ml after phage rescue) and the second was for ScFv-rSSPG1d ($1.9x10^9$ cfu/ml after

phage rescue). After panning, 13 “binders” specific to peptide 3796 and 8 against rSSPG1d were identified.

Sequence analysis and identification of complementarity determining regions (CDRs)

Nucleotide sequences of cDNAs encoding all 13 peptide 3796-specific ScFvs and 8 rSSPG1d-specific ScFvs were determined. The nucleotide-derived amino acid sequence revealed that all 13 peptide-specific binders were identical and all 8 rSSPG1D-specific binders were identical. The nucleotide sequence for ScFv-3796 and ScFv-rSSPG1d were submitted to GenBank (Accession nos. FJ526992 and FJ526993, respectively). The CDRs were predicted, based on the Kabat database (Krebber et al. 1997), from these amino acid sequences. Interestingly, when the amino acid sequences of ScFv-rSSPG1d (~27 kDa, pI 5.65) and ScFv-3796 (~27 kDa, pI, 9.13) were aligned with other Sclerotinia-specific ScFvs isolated in our laboratory, it was observed that the amino acid sequence of CDR2 (V_L) of ScFv 3796 was identical to CDR2 (V_L) of two such previously described ScFvs (Yajima et al. 2008). When the sequence of ScFv-3796 and ScFv-rSSPG1d were compared, differences in the amino acid sequences of CDR1, 2 and 3 in both V_H and V_L sequences were observed. Among the differences, CDR3 of V_H was observed to be the most significant in terms of sequence as well as length; ScFv-3796 had 8 amino acids in this CDR as compared to 11 in ScFv-rSSPG1d.

Purification of ScFvs and characterization of ScFvs

The yield of recombinant antibodies ranged from 120-500 µg per litre of cell culture. The purity as well as the molecular weight of these recombinant antibodies were confirmed by SDS-PAGE (Figure 5-1). When the purified, refolded ScFvs were used in ELISAs against their respective antigens, it was observed that ScFv-3796 could specifically recognize and bind peptide 3796 (Figure 5-2A). However, ScFv-3796 could not detect rSSPG1d based on ELISA results (data not shown). The absorbance values obtained from the ELISAs were significantly higher for peptide 3796 (around 0.2 and 1.3 at 10 and 20 µg/ml peptide respectively, $p<0.05$) compared to the other peptides tested. It is evident

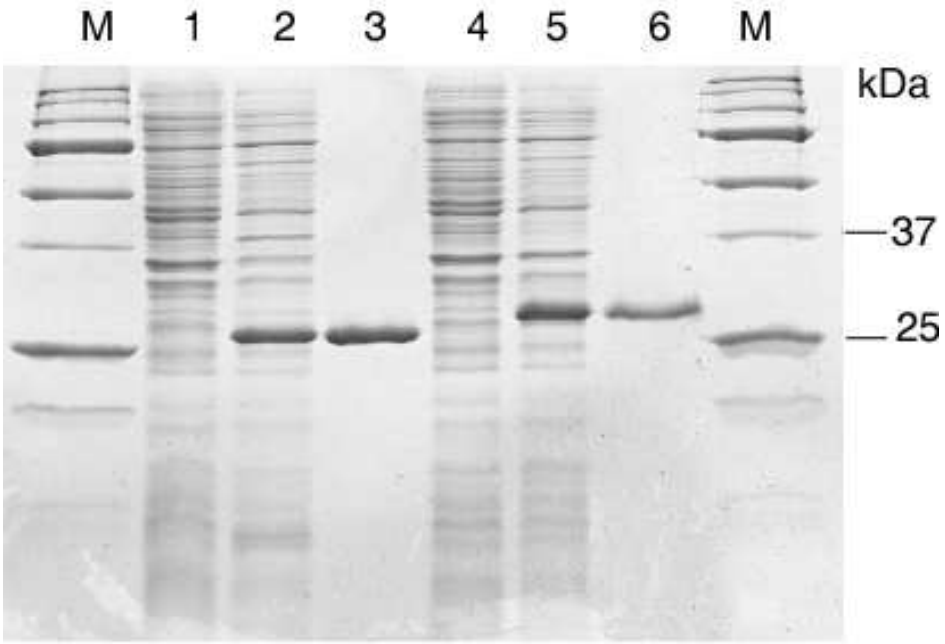

Figure 5-1 Overexpression and purification of ScFv-3796 and ScFv-rSSPG1d. Lanes from left to right were protein marker, total protein of uninduced ScFv-3796, total protein of induced ScFv-3796, purified ScFv-3796, total protein of uninduced ScFv-rSSPG1d, total protein of induced ScFv-rSSPG1d, purified ScFv-rSSPG1d and protein molecular mass marker.

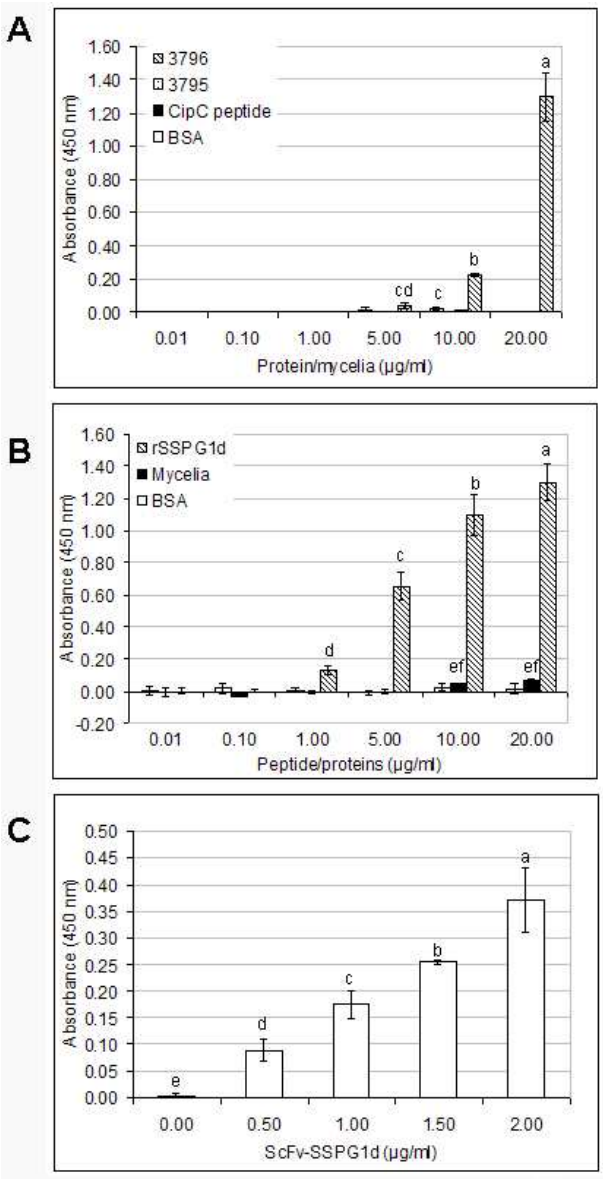

Figure 5-2 Characterization of ScFvs. ELISA was used to demonstrate (A) the specificity of ScFv-3796 towards peptide 3796; (B) the specificity of ScFv-rSSPG1d towards rSSPG1d; and (C) the detection of SSPG1d in 5-day old culture filtrate by ScFv-rSSPG1d. Data are presented as mean ± SE of three biological replicates and SAS statistical analysis ($p<0.05$) was used to identify significant differences among the samples as indicated by the different letters above the columns.

from the results presented in Figure 5-2A that ScFv-3796 did not recognize even peptide 3795, which is also derived from SSPG1d, attesting to the specificity of this ScFv. ScFv-rSSPG1d binds rSSPG1d (Figure 5-2B) and the absorbance values for the rSSPG1d and ScFv-rSSPG1d interaction were 1.1 and 1.3 at 10 and 20 µg/ml of rSSPG1d, respectively, while the other controls resulted in absorbance values close to baseline. In addition, when ScFv-rSSPG1d was tested against crude protein extracts prepared from *S. sclerotiorum* culture supernatants (i.e. the secretome), our results indicated that ScFv-rSSPG1d was able to recognize the target in a concentration-dependent manner (Figure 5-2C). We were unable to detect SSPG1d in culture filtrates using ScFv-3796.

Detection of SSPG1d

To further verify the identity of the protein recognized by the recombinant antibodies, we performed Western blotting experiments with 5-day-old culture filtrates of the fungus. When the blots were probed with ScFv-3796 or ScFv-rSSPG1d, a single immunoreactive band was observed in the case of ScFv-rSSPG1d but not with ScFv-3796, which produced additional faint bands, possibly indicating that the observed bands in the Western blot for ScFv-3796 were the result of non-specific binding (Figure 5-3A and B). The fact that only one band is observed in the culture filtrate lane for ScFv-rSSPG1d served to confirm the specificity of the ScFv-rSSPG1d for SSPG1d, as additional bands would have been seen if ScFv-rSSPG1d was non-specific or if the HRP-streptavidin could bind to other secreted proteins. The identity of the immunoreactive band detected by ScFv-rSSPG1d was confirmed to be SSPG1d by Mass Spectrometry (data not shown) further demonstrating that ScFv-rSSPG1d was able to recognize the correct protein from a crude sample of secreted proteins. The observed size difference between native SSPG1d and rSSPG1d is the result of the presence of a signal peptide in rSSPG1d that is cleaved from the native SSPG1d during secretion by the fungus.

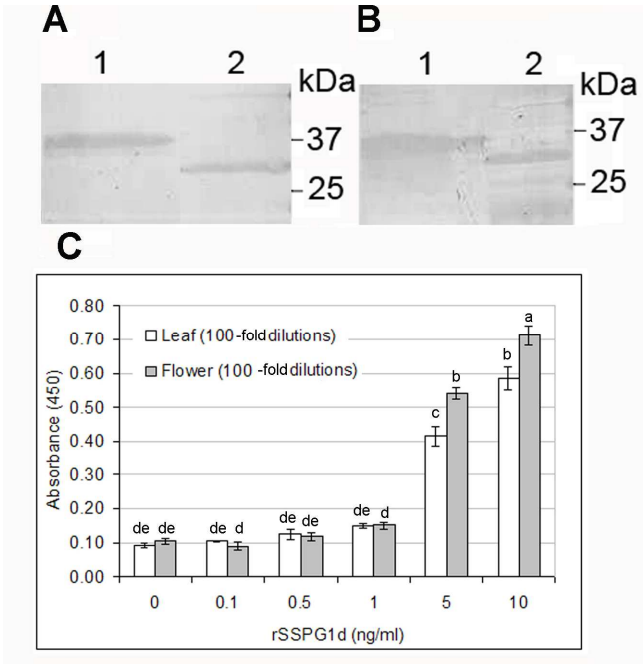

Figure 5-3 Utility of ScFvs in Western blots and ELISA. Western blot detection of rSSPG1d using (A) ScFv-rSSPG1d and (B) ScFv-3796. In both panels, lanes 1 and 2 refer to rSSPG1d and protein from *S. sclerotiorum* culture supernatants, respectively. (C) Detection of rSSPG1d in plant extracts by ELISA using ScFv-rSSPG1d. Results indicate a concentration-dependent increase in absorbance when rSSPG1d was used to spike both leaf and flower extracts. Data are presented as mean ± SE of three biological replicates and SAS statistical analysis ($p<0.05$) was used to identify significant differences among the samples as indicated by different letters above columns.

In order to evaluate the ability of ScFv-rSSPG1d and ScFv-3796 to detect the presence of SSPG1d, we added different amounts of rSSPG1d to plant extracts to determine their detection limits. Based on our results, the presence of 5 ng/ml rSSPG1d could be clearly detected by ScFv-rSSPG1d in both leaf and flower extracts after these extracts were diluted (Figure 5-3C, $p<0.05$), however, ScFv-3796 was unable to detect rSSPG1d at all concentrations tested. We acknowledge that the observed detection limit for ScFv-rSSPG1d might not be physiologically relevant and that a significant amount of work is still required to determine the utility of ScFv-rSSPG1d in an effective detection assay. However, the fact that this ScFv was able to bind rSSPG1d in plant material demonstrates the potential that this ScFv may find use as part of diagnostic assays to detect the presence of fungal SSPG1d in infected plants. We also acknowledge that there is a possibility of cross reactivity with polygalacturonases secreted by other fungi that may be found in the field, and this will need to be addressed during the field validation of such antibody-based diagnostic tests.

Characterization of ScFvs using surface plasmon resonance

The kinetics of association and dissociation between the ScFvs and their respective antigens were determined by employing SPR. When ScFv-rSSPG1d was passed over immobilized rSSPG1d and ScFv-3796 was passed over immobilized peptide 3796, we observed a very specific interaction between the antigen and the antibody (Table 5-2). Negative controls, which involved passing similar dilutions of BSA over the immobilized antigens, did not reveal any specific interaction. When ScFv-3796 was passed over immobilized rSSPG1d, no observable interaction was detected (data not shown). Taking this result into consideration with the observation described earlier indicating that ScFv-3796 was unable to detect rSSPG1d in ELISA-based assays, we arrive at the conclusion that ScFv-3796 is not suitable for potential use in tests for this enzyme. These results are also consistent with the fact that the use of ScFv-3796 in Western blots results in the detection of multiple bands, unlike ScFv-rSSPG1d which is able to

Table 5-2 Affinity and rate constants for the interaction between ScFv-rSSPG1d and ScFv-3796 with rSSPG1d and peptide 3796, respectively. The apparent association (*ka*) and dissociation rate constants (*kd*) are averaged over three independent experiments. Data are presented as mean ± SE of three biological replicates.

	$ka \times 10^{3}$ Ms^{-1}	$kd \times 10^{-3}$ S^{-1}	$K_D \times 10^{-7}$ M
ScFv-rSSPG1d	3.58(±0.26)	2.95(±0.50)	8.43 (±1.28)
ScFv-3796	5.18 (±3.04)	2.58 (±0.66)	8.89 (±3.34)

detect a specific band identified by tandem mass spectrometry as SSPG1d. Although it appears that ScFv-3796 is unable to detect rSSPG1d, it does show high affinity for peptide 3796, which was a region predicted to be antigenic. Although it must be verified by experimentation, it is tempting to speculate that the lack of reactivity of ScFv-3796 with SSPG1d may be due to lack of exposure of this region, illustrating the limitations of predictive approaches in generating useful antibodies.

The calculated K_D value for both ScFv-rSSPG1d and ScFv-3796 for their respective targets (Table 5-2) is within the normal range for ScFv antibodies (Altschuh et al. 1992 ; Choulier et al. 2001; Huang et al. 2006; Leonard et al. 2007; Reiter et al. 1999). However, the affinities are lower than those of monoclonal antibodies, which is not unexpected since monoclonal antibodies possess two antigen binding sites as opposed to the single antigen binding sites of ScFvs. Therefore, higher avidity for ScFvs could possibly be achieved by the covalent linking of the single chain antibodies or by multimerization (Krebber et al. 1997).

Characterization of transgenic Arabidopsis expressing ScFvs

In order to evaluate the utility of the cDNAs encoding the ScFvs to induce durable tolerance to *S. sclerotiorum*, we generated constructs for the in planta expression of these cDNAs in the binary vector pKYLX71, which directs the expression of the transgene under a control of double *35S* promoter (Figure 5-4). These constructs were used to transform *A. thaliana*.

Six and five homozygous lines of transgenic Arabidopsis expressing ScFv-3796 and ScFv-rSSPG1d, respectively, were characterized for the presence of the ScFv transcripts using q-RT-PCR. Four out of the six lines of Arabidopsis expressing ScFv-3796 (ScFv-3796-123H, ScFv-3796-101E, ScFv-3796-111C and ScFv-3796-62H) showed significantly elevated transcript levels (10-10^5 folds, Student's t-test; Figure 5-5A). Similarly, four (ScFv-rSSPG1d-1B, ScFv-rSSPG1d-5A, ScFv-rSSPG1d-11A and ScFv-rSSPG1d-22A) independently derived homozygous lines of Arabidopsis expressing ScFv-rSSPG1d showed high transcript levels (10^2-10^5 folds, Figure 5-5B). However, the expression of these

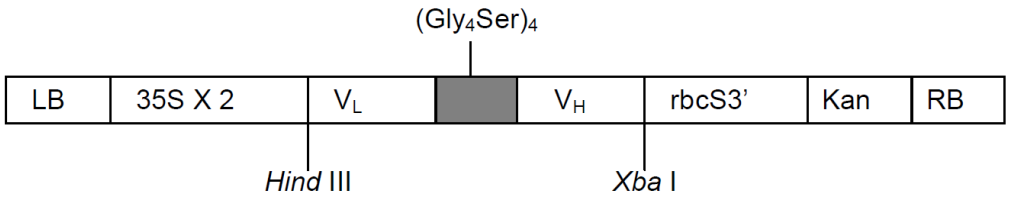

Figure 5-4 Structure of ScFv constructs expressed in plants. The V_L and V_H regions were connected by $(Gly_4Ser)_4$. The ScFv genes were inserted into pKYLX 71 using *Hind*III and *Xba* I restriction sites.

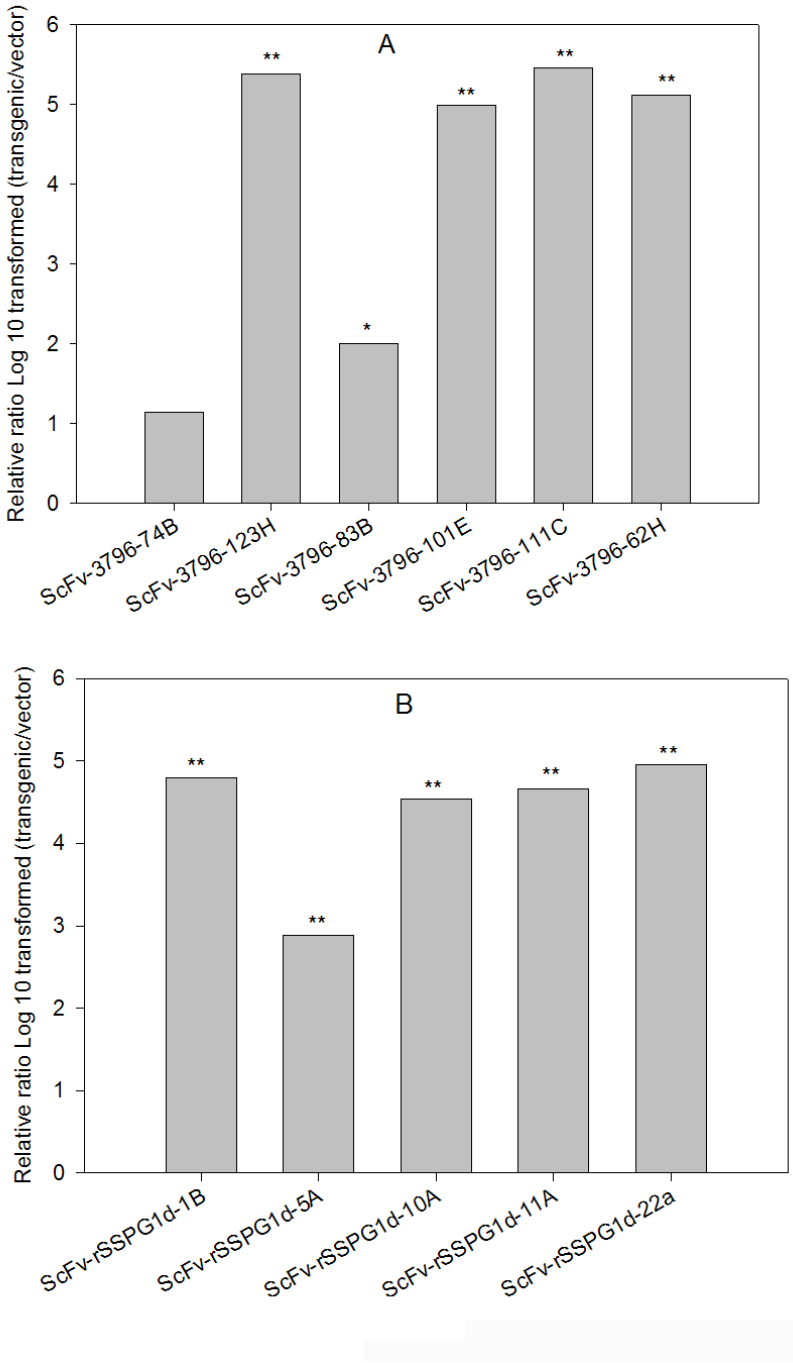

Figure 5-5 Transcript level of transgenic Arabidopsis expressing A: ScFv-3796; B: ScFv-rSSPG1d. The asteriskes indicate the significant change by Students' t-test ($p<0.05$).

ScFvs can not be detected by Western blotting with anti-ScFv antibody (data not shown).

To investigate the response of these homozygous transgenic Arabidopsis linee to challenge with *S. sclerotiorum*, leaf inoculation was performed and lesion size was measured and quantified at 36 and 60 h post-inoculation. The lesion sizes of these transgenic plant leaves compared to wild-type and vector control did not show any significant difference for either of the ScFvs tested (Figure 5-6 and 5-7). One reason for the lack of observed tolerance may be that the levels of ScFv were not sufficiently high, as evidenced by our inability to detect them using Western blots, to be of utility in this regard. Indeed, it has been reported that the overall levels and activities of ScFv antibodies accumulating in the plant cytosol may be determined by the intrinsic properties of the expressed ScFv fragments (De Jaeger et al. 1999). Due to the reducing conditions and the absence of protein disulfide isomerases and chaperones, ScFv antibodies generally accumulate to low or undetectable levels in the cytosol. However, there are exceptional cases in which the high-level expression of a functional ScFv has been achieved (up to 4% total soluble protein) (De Jaeger et al. 1999; Eeckhout et al. 2000; Nolke et al. 2005; Santos et al. 2004). Strategies developed to stabilize expressed antibodies have been reported including addition of C-terminal KDEL peptide before ScFv (Schouten et al. 1997; Schouten et al. 1996). Compared to the ScFv fragment without KDEL, which is undetectable, inclusion of KDEL result in maximum expression levels of up to 0.2% of the total soluble protein (Schouten et al. 1997; Schouten et al. 1996). However, in our case, the retention of the ScFv in the ER would not be expected to afford tolerance to the pathogen, and hence was not attempted.

Conclusion

In this communication, we report the isolation of two recombinant ScFv antibodies that were targeted to a cell wall degrading enzyme secreted by the phytopathogen *S. sclerotiorum*. Among these ScFvs, ScFv-rSSPG1d was observed to be capable of detecting both rSSPG1d as well as native SSPG1d

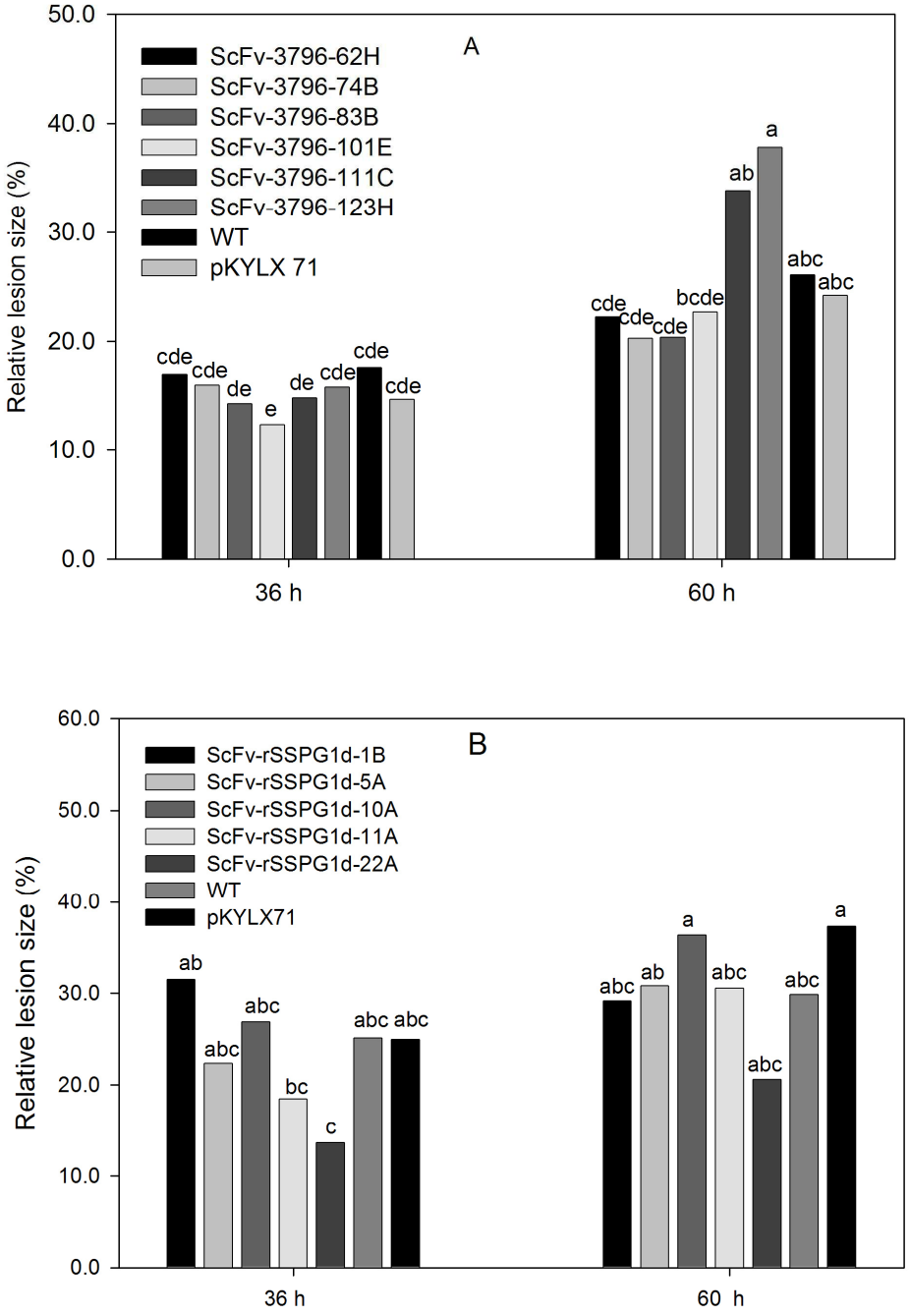

Figure 5-6 Phenotypic assay of transgenic Arabidopsis expressing A: ScFv-3796; B: ScFv-rSSPG1d

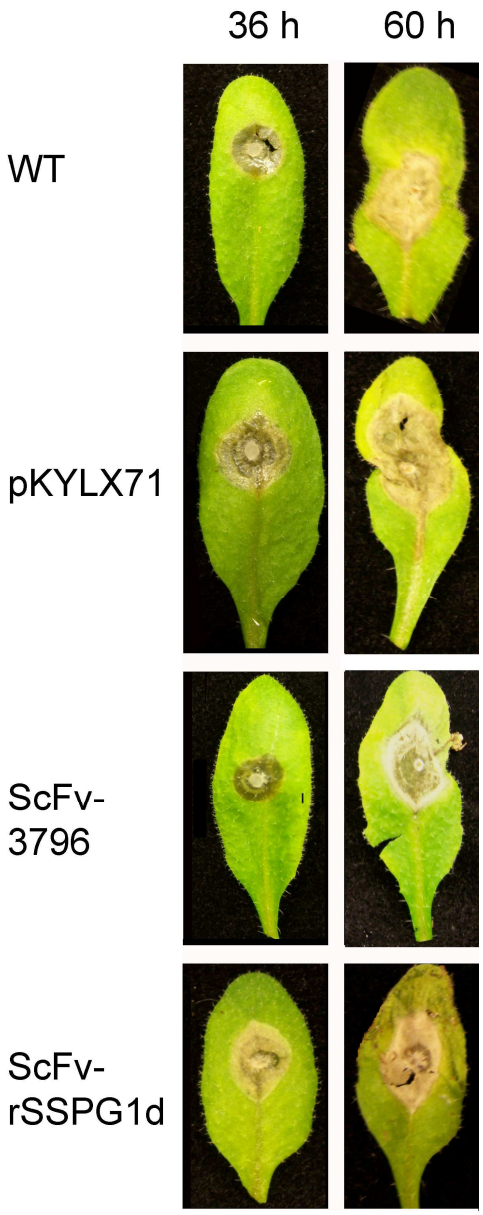

Figure 5-7 Disease symptoms on Arabidopsis inoculated with hyphal masses (agar plugs) of *S. sclerotiorim*. Transgenic Arabidopsis expressing ScFv-3796/ScFv-rSSPG1d also was infected as was the pKYLX71 vector control and the wild type plants at 36 h and 60 h post inoculation.

secreted by this fungus. This ScFv-rSSPG1d may eventually have utility in a diagnostic test capable of better assessing the risks posed by this pathogen. In addition, future studies will be directed towards determining whether ScFv-rSSPG1d can inhibit the target cell wall degrading enzyme.

As a trial assay to hinder the infection of *S. sclerotiorum* at an early stage, we transformed three ScFv genes, which can recognize not only the recombinant but also the native SSPG1d, into Arabidopsis. High transcript levels of those genes in transgenic Arabidopsis were analyzed by qRT-PCR. However, at the protein level, expression of the trangenes could not be detected by Western blotting. It has been shown that multivalent recombinant antibody fragments provide high binding avidity and unique specificity to a wide range of target antigens (Hendy et al. 1999; Hudson and Kortt, 1999) because the instability of ScFv antibodies, especially in the cytosol, and monovalency, resulting in slower on-rates and faster off-rates when compared to the corresponding full-size antibody, may be overcome by the development of diabodies or dimerized antibodies (Schillberg et al. 2001).

In summary, we have successfully isolated and characterized ScFvs against one of the virulence factors secreted by *S. sclerotiorum*. ScFv-rSSPG1d has potential applications in disease diagnosis, immunopurification of SSPG1d and inhibition of infection on host by *S. sclerotiorum*. We demonstrated the application of phage display in isolating ScFvs against virulence factors secreted by this fungus and that ScFv-rSSPG1d is promising as both a diagnostic tool and also for its apparent inhibitory activities against fungal pathogens. However, transgenic approachs to engineer Arabidopsis resistance against *S. sclerotiorum* have not been successful most probably due to the low level expression and/or the stability of ScFv products in plants. The problem may be solved by improving the stability of these antibodies in plants, along with the possible targeting of the ScFvs to the apoplast, where they can effectively neutralize the cell wall degrading enzymes. Finally, a complete characterization of the ability of these two ScFvs to inhibit the activity of SSPG1d is warranted.

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Chapter 6 General discussion and conclusions

Canola is an important crop for Canada and is also one of the top three oil crops in the world (Snowden et al. 2007). *S. sclerotiorum* and *A. brassicae* are two significant pathogens that decrease the harvestable yield of canola in Canada (http://www.canola-council.org/contents10c.aspx). With a goal to better understand canola responses to *S. sclerotiorum* infection, microarray analysis was performed and genes responsive to pathogen challenge were identified. Similar studies with Arabidopsis-*L. maculans* and Arabidopsis-*A. brassicicola* have recently been reported (Schaller et al. 2008; Staal et al. 2008). The results presented in this dissertation demonstrate that, in canola after infection with *S. sclerotiorum*, the expression ratio of 1674 genes differed significantly (P<0.05, FDR <5%) from 0 (log-scale) at various time-points (12, 24 and 48 h). This included 1134 genes that decreased in abundance and 541 that increased in abundance in the infected leaves when compared with uninoculated controls. Several genes involved in JA biosynthesis, ROS-mediated defense responses and cell wall structure and function were identified and validated using qRT-PCR. Although JA/ET-dependent pathways have been reported by others to be involved in plant responses to necrotrophic pathogens (Egusa et al. 2009; Spoel et al. 2007; Trusov et al. 2008), gene expression changes in genes of the JA/ET mediated signal transduction pathways that are associated with *S. sclerotiorum* infection had not been previously reported. Results presented in this dissertation, as well as those from other groups published almost simultaneously (Zhao et al. 2007), revealed that few genes known to be regulated by SA were induced by the pathogen, whereas several that are regulated by JA/ET were modulated by the pathogen. These results suggest that plant responses to *S. sclerotiorum* are SA-independent, and mainly JA/ET-dependent. In the contrast, another study found that the response of Arabidopsis to *S. sclerotiorum* is dependent on both SA and JA/ET signaling (Guo and Stotz 2007). The observed discrepancy between different plant genera belonging to the same family in response to *S. sclerotiorum* requires closer examination in the future.

B. napus is a species susceptible to *S. sclerotiorum*, which suggests that the observed changes of the abundance of transcripts for genes responsive to the pathogen are the result of the compatible interactions between the host and the pathogen. In addition, it has been observed that the partially resistant line RV289 of *B. napus* tended to induce the expression of genes associated with defense mechanisms earlier (24 h) than the susceptible genotype 'Stellar', in which many of these same genes are expressed later (48 h) (Zhao et al. 2007). Based on the aforementioned results, Zhao et al. (2007) also concluded that the temporal and quantitative differences in gene expression may determine the outcome of these two genotypes to *S. sclerotiorum* infection. In the future, studies towards characterizing the role of JA/ET and JA/ET-mediated gene expression within the context of compatible/incompatible interactions between *S. sclerotiorum* and plant hosts will be crucial in order to explore the mechanisms underlying host susceptibility or tolerance to this pathogen.

To limit the invasion of pathogens by plants, it trigger defense responses through modulation of other hormones and balance the energy and nutrient needs between defense and development (Thomma et al. 2001). Correspondingly, pathogens can counterbalance the responses from plants as an approach to enhancing their virulence by regulating phytohormones and its related signal transduction events (Lopez et al. 2008). All these observations indicate that hormones play an important role in mediating plant-pathogen interactions (Thomma et al. 2001). In order to test our hypothesis that canola plants respond to necrotrophic fungi by triggering JA/ET signaling pathways, *S. sclerotiorum*-induced changes in the transcript abundance of canola genes that were presumed to respond to SA, JA/ET signaling were investigated. We observed that *S. sclerotiorum*, like other necrotrophic pathogens, can trigger the JA/ET signaling pathway in plants (Figure 6-1; modified from Staal), which was consistent

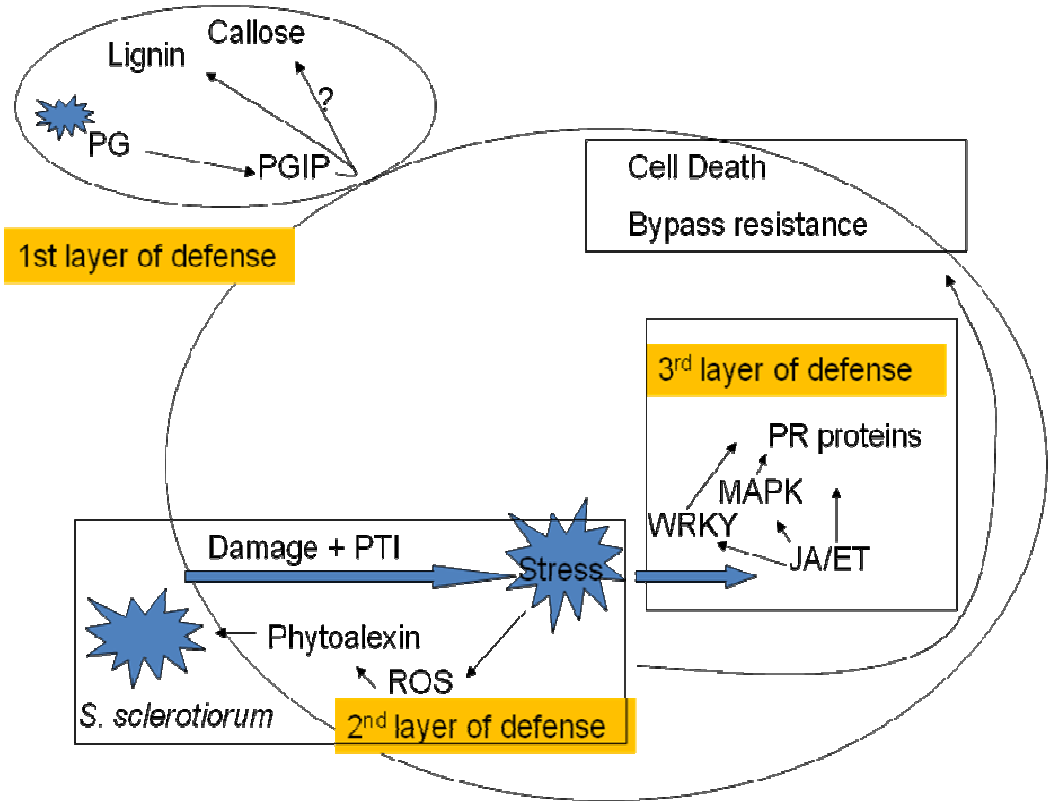

Figure 6-1 Overview of the different layers of defense in plants response to the pathogen (*S. sclerotiorum*, modified from Staal, 2006). The 1st layer of defense response is to inactivate virulence factors including PGs through PGIP and this may also lead to the cell wall strengthening through deposition of callose and lignin. The 2nd layer of defense is that triggered ROS as well as activated phytoalexins by *S. sclerotiorum* protect plants from pathogens. The 3rd layer of defense of plants is that the defense hormones JA and ET quantitatively and qualitatively influence disease development but do not determine resistance. If the pathogen can overcome these three layers of defense, infection and finally leads to cell death.

with our microarray analysis (Chapter 2). Studies reported in the literature have also revealed that symptom development following necrotrophic pathogen infection was negatively affected by ET (Berrocal-Lobo et al. 2002; Wang et al. 2002). Our own observations reported in this dissertation also revealed that canola plants with decreased ethylene levels, as a result of the expression of *ACC*-deaminase, were more susceptible to *S. sclerotiorum* infection. When plants respond to *S. sclerotiorum*, current evidence suggests the presence of three layers of defense responses (Guyon et al. 2004; Hegedus et al. 2008; Kim et al. 2008; Liang et al. 2008; Sexton et al. 2009; Soledade et al. 2007; Yang et al. 2007; Zhao et al. 2007). The first layer of defense response is to inactivate virulence factors including endopolygalacturonase (PG) through polygalacturonase-inhibiting protein (PGIP) and the strengthening of cell walls, through the deposition of callose and lignin (Guyon et al. 2004; Hegedus et al. 2008; Kim et al. 2008; Liang et al. 2008; Sexton et al. 2009; Soledade et al. 2007; Yang et al. 2007; Zhao et al. 2007). The second layer of defense triggers ROS and phytoalexin production (Guyon et al. 2004; Hegedus et al. 2008; Kim et al. 2008; Liang et al. 2008; Sexton et al. 2009; Soledade et al. 2007; Yang et al. 2007; Zhao et al. 2007). Finally, the third layer of defense involves the plant hormones JA and ET which influence, quantitatively and qualitatively, disease development but do not determine resistance (Guyon et al. 2004; Hegedus et al. 2008; Kim et al. 2008; Liang et al. 2008; Sexton et al. 2009; Soledade et al. 2007; Yang et al. 2007; Zhao et al. 2007). Therefore, it is possible that the blocking of ET signaling in canola impairs the third layer of the defense system, leading to increased susceptibility to *S. sclerotiorum* compared to the wild-type. Finally, the third layer as proposed by Staal (2006), also for the Arabidopsis-*L. maculans* interaction, suggests that the hormones SA, JA and ET quantitatively and qualitatively influence disease outcome but do not determine resistance. Overcoming of these three layers of defense by the pathogen *S. sclerotiorum* result in the infection and finally lead to plant death. Our results are consistent with the observations made by others that ET plays a very important role in determining the disease outcome (Bohman et al. 2004; Staal, 2006; Staal et al. 2006). Hence, it is proposed that ethylene is

indispensable for the defense response of Brassica plants to *S. sclerotiorum* infection. Additional research using plants resistant/susceptible to this pathogen is required in order to fully delineate the roles of JA/ET in determining the eventual outcome of infection.

It is well known that downstream or cross-talk of hormone signaling pathways (JA/ET and SA), and transcription factors (TFs) such as TGA, WRKY, ERF and JIN1/MYC2 play important roles in binding and activating defense-related genes (Dong, 2004; Eulgem and Somssich, 2007; Laurie-Berry et al. 2006; McGrath et al. 2005; Wang et al. 2006). That TFs can mediate the defense reactions of plants to biotic stresses and the can transcriptionally control the expression of stress-responsive genes have been documented by several large-scale transcription-profiling experiments (Chen and Chen, 2002; Durrant et al. 2000; Maleck et al. 2000; Mysore et al. 2002). For example, many TFs including ERF/AP2-domain, homeodomain, bZIP, Myb, WRKY families and other zinc-finger factors are observed to be up-regulated during the multiple compatible and incompatible interactions (Riechmann and Ratcliffe, 2000; Rushton and Somssich, 1998; Singh et al. 2002). Furthermore, in our microarray analysis of canola response to *S. sclerotiorum*, we identified three *WRKY* genes whose transcript abundance was significantly affected by this fungus (Yang et al. 2007). In order to understand their role(s) in mediating canola responses to two necrotrophic pathogens (*S. sclerotiorum* and *A. brassicae*) and exogenous hormone treatments, we identified and characterized members of the *WRKY* gene family in *B. napus* (canola). The transcript abundance of 13 *BnWRKY* genes was observed to be significantly (t-test, $P<0.05$) affected in canola upon the infection of *S. sclerotiorum*, and the transcript abundance of only four *BnWRKY* genes were significantly affected by *A. brassicae*. It is possible that, in canola, the signaling pathway response to *S. sclerotiorum* and *A. brassicae* may involve several BnWRKY TFs (Figure 6-1).

While SA did not significantly affect the transcript abundance of any of the *BnWRKYs* tested, ET, ABA, JA and CK did affect the transcript abundance of various *BnWRKY* genes investigated in this study. The WRKY domain contains a

conserved WRKYGQK heptapeptide and also a C_2H_2- or C_2HC-type of zinc finger motif (Eulgem et al. 2000). As in Arabidopsis, tobacco and soybean, an atypical WRKY domain, WRKYGKK was also identified in the current study (van Verk et al. 2008; Zhou et al. 2008; Duan et al. 2007). We propose that the BnWRKY TFs that lacks the canonical WRKYGQK motif might not be able to interact with the W-box and, therefore, may have different target genes and divergent roles. However, further work to identify the direct target can be elucidated through Tilling microarray and/or Chromatin immunoprecipitation (CHIP)-Chip (Gong et al. 2008; O'geen et al. 2000; Lander 1999) in future studies. Moreover, investigation of the molecular events occurring in the stem of *B. carinata* inoculated with *S. sclerotiorum* may help to solve the puzzle of defense response in plants since stem inoculation results indicated that *B. carinata* is more resistant to *S. sclerotiorum* than *B. napus* (Liang, Strelkov and Kav, unpublished observations). Further work needs to be performed to elucidate the response of other stress-related TFs such as the ERF/AP2-domain, homeodomain, bZIP, and Myb to infection by *S. sclerotiorum* and hormonal stimuli, in order to gain more insight in compatible/incompatible interaction.

ScFv antibodies targeted to virulence factors of fungal pathogens may be a practical way to engineer disease resistance in plants (Schillberg et al. 2001). ScFvs are very attractive for antibody-based genetic engineering due to its small and almost equal affinity (Nolke et al. 2006). Recombinant antibodies of the ScFv type directed against fungal and viral pathogens have been successfully used to engineer disease resistance (Peschen et al. 2004; Tavladoraki et al. 1993). As an alternate approach to preventing infection by *S. sclerotiorum* at an early stage, we isolated two recombinant ScFv antibodies that were targeted to a cell wall-degrading enzyme, SSPG1d. *In vitro* experiments demonstrated that ScFv-rSSPG1d is capable of detecting both rSSPG1d as well as native SSPG1d secreted by this fungus. This ScFv-rSSPG1d may eventually have utility in a diagnostic test capable of better assessing the risks posed by this pathogen. This diagnosis of the inoculum load in nature could help producers in determining the timing and extent of prophylactic measures to be undertaken to prevent the occurrence and

the spread of the disease. However, our strategy to engineer canola and Arabidopsis producing these ScFvs specific to SSPG1d needs to be improved further to increase the functional ScFvs antibodies, since ScFvs could not be detected by Western blotting, although several homozygous lines of transgenic Arabidopsis showed significantly increased transcript levels. Furthermore, none of the transgenic Arabidopsis lines expressing ScFvs were observed to be tolerant to the fungal pathogen (Chapter 5).

It is worthy undertaking to express two to five ScFvs targeting different virulence/pathogenecity factors in proper compartments for the purpose of development of durable and broad spectrum resistance (Schillberg et al. 2001) because the fact of the high binding avidity binding and specificity to many target antigens (Hendy et al. 1999; Hudson and Kortt 1999). In addition, the isolation and identification of cytosol-stable ScFv scaffolds could allow grafting of the antigen-binding loops of low-stability cytosolic ScFvs to further improve their accumulation (Worn et al. 2000). Cytosolic antibody expression may be further enhanced by the fusion of ScFvs to fusion protein partners, such as maltose-binding protein (MBP), glutathione-S-transferase (GST) and other chaperones that stabilize the antibodies (Shaki-Loewenstein et al. 2005). Therefore, alternate avenues for engineering durable resistance to *S. sclerotiorum* using the antibodies (ScFvs) described in this dissertation must be explored in the future. In addition, a lack of resistance in Arabidopsis does not mean that these antibodies will not be able to afford tolerance against *S. sclerotiorum* in canola. Transgenic canola plants expressing the cDNAs encoding these ScFvs have been generated and will be characterized in the future once we obtain homozygous plants. Nevertheless, the enhancement and stabilization of ScFvs production in plants is an issue that needs to be solved in the future. In addition, future studies can be directed towards determining whether ScFv-rSSPG1d can inhibit target cell wall degrading enzymes, or if it can be engineered to possess such inhibitory activity, which may ultimately have applications in crop protection.

Conclusion

Taken together, in the compatible interaction between *B. napus* and *S. sclerotiorum*, JA/ET-dependent pathways were shown to be involved in the plant response, in addition to the ROS-mediated defense responses, we observed from the transcript profiling study (Yang et al. 2007). To further confirm this hypothesis, presumptive genes related to JA/ET and SA were investigated in the compatible and incompatible pathosystems, and ET was observed to be indispensable for the defense response of *Brassica* to *S. sclerotiorum* infection (Yang et al. 3 chapter). Moreover, some BnWRKYs were observed to be induced by both necrotrophic fungi and hormone stimuli, which may indicate that in canola, defense-signaling response towards *S. sclerotiorum* and *A. brassicae* may also involve several BnWRKY TFs (Yang et al. 4 chapter). As an alternate approach towards hindering the infection of *S. sclerotiorum* at an early stage, ScFvs specific to rSSPG1d were isolated through phage display. Out of the two ScFvs isolated, ScFv-rSSPG1d was observed to be capable of detecting both rSSPG1d and native SSPG1d secreted by this fungus. Further optimization and utilization of the generated ScFvs may be interesting and should be performed in the future.

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Appendix

Table A-1 Temporal changes in transcript abundance of the major clusters of genes shown in Figure 2-3 (A-F)

Group A Symbol	Spot	Operon annotation	FC[a] 12h	24h	48h
AT1G23800	11799	mitochondrial aldehyde dehydrogenase (ALDH3)	1.1	1.36	1.69
AT4G39950	22542	cytochrome P450 79B2	1.02	1.2	2.14
	24947;42				
AT3G53990	11	expressed protein	0.95	1.04	1.83
AT3G61220	6772	short-chain dehydrogenase/reductase family protein	0.86	1.11	1.61
AT5G06630	27608	proline-rich protein family	1.03	1.19	2.32
AT5G48950	25041	thioesterase-related	1.49	1.34	2.18
AT5G06760	19887	late embryogenesis abundant protein LEA like	0.95	0.99	10.99
AT5G24610	15924	expressed protein	0.94	0.67	1.92
AT4G08370	27482	proline-rich protein family	1.3	1.14	2.15
AT1G18270	18577	Genomic sequence for Arabidopsis thaliana BAC F15H18 from chromosome I	0.91	1.17	1.85
AT5G13170	17796	nodulin MtN3 family protein	0.98	0.6	2.48
AT3G56310	10130	glycosyl hydrolase family 27 (alpha-galactosidase/melibiase)	0.85	1.35	1.87
AT1G55010	18012	plant defensin protein, putative (PDF1.5)	0.8	1.24	4.79
AT5G10980	24871	histone H3	1.02	1.08	1.69
AT5G46180	17753	ornithine--oxo-acid aminotransferase (ornithine aminotransferase/ornithine ketoacid aminotransferase), putative	0.97	1.13	2.87
AT5G49360	10620	glycosyl hydrolase family 3	0.98	1.1	4.27
AT5G59290	6819	NAD-dependent epimerase/dehydratase family	0.87	0.93	2.21
AT2G26020	13424	plant defensin protein, putative (PDF1.2b)	0.91	1.29	3.8
AT2G36680	7480	expressed protein	1.02	1.41	2.03
AT1G02300	6893	Arabidopsis thaliana BAC T7I23, complete sequence	0.97	1.11	2.66
AT5G28510	20083	glycosyl hydrolase family 1	2.14	1	2.13
AT3G22200	12236	4-aminobutyrate aminotransferase (gamma-amino-N-butyrate transaminase/GABA transaminase/beta-alanine--oxoglutarate aminotransferase)	0.92	1.27	2.01

AT4G36040	24825	DnaJ protein family	0.95	0.96	2.96
AT3G15530	5657	expressed protein	0.8	1.64	1.88
AT5G57655	15606	expressed protein	0.97	0.82	2.22
AT4G27450	26667	expressed protein	1.2	1.01	3.23
AT3G47340	8702	glutamine-dependent asparagine synthetase	1.1	1.65	4.46
AT5G44420	15678	plant defensin protein, putative (PDF1.2a)	0.95	1.16	4.06
AT1G07040	2458	expressed protein	1	1	6.77
AT3G07870	12523	F-box protein family	0.85	0.92	1.75
AT5G64370	26635	beta-ureidopropionase	0.98	1.23	2.08
AT3G03080	14837	NADP-dependent oxidoreductase (P2), putative	0.66	0.9	2.71
AT5G59530	6753	2-oxoglutarate-dependent dioxygenase, putative	0.88	1.29	4.33
AT4G27350	19739	membrane lipo protein lipid attachment site-containing expressed protein	0.96	1.03	2.97
AT1G42990	20863	bZIP family transcription factor	2	1.46	2.28
AT1G64190	11429	6-phosphogluconate dehydrogenase -related	1.2	1.13	1.98
AT1G21410	20828	F-box protein family	0.67	1.26	1.84
AT2G48020	23701	sugar transporter, putative	0.87	1.37	2.62
AT2G03760	723	steroid sulfotransferase, putative	1.15	1.49	8
AT2G26010	18032	plant defensin protein, putative (PDF1.3)	0.9	1.06	2.99
AT3G12490	21651	cysteine proteinase inhibitor -related	0.95	0.99	1.9
AT5G42040	6462	hypothetical protein	0.75	1.04	2.06
AT5G05340	17529	peroxidase, putative	0.76	0.45	2.42
AT5G44430	11070	plant defensin protein, putative (PDF1.2c)	0.86	1.22	4.26
AT2G38400	5318	alanine--glyoxylate aminotransferase (beta-alanine-pyruvate aminotransferase/AGT), putative	0.84	0.89	2.26
AT4G35770	12794	senescence-associated protein sen1	0.87	1.13	4.63
AT5G51570	17554	expressed protein	1.09	0.85	1.84
AT3G06500	984	neutral invertase -related	1.04	2.03	8.01
AT1G80920	11073;21027	DnaJ protein family	0.83	1.1	3.51
AT1G67360	11033	stress related protein -related	0.77	1.16	1.5
AT1G07890	15623	ascorbate peroxidase, putative (APX)	0.89	1.19	2.01
AT1G15380	9000	glyoxalase family protein (lactoylglutathione lyase family protein)	1.25	1.49	30.68
AT1G05100	16562	protein kinase family	1.14	1.39	5.43
AT5G40670	12917	expressed protein	0.74	1.27	2.32

		protease inhibitor/seed storage/lipid transfer protein			
AT3G22620	10047	(LTP) family	0.92	1.12	2.31
AT1G70780	24810	expressed protein	1.18	0.85	4.65
AT1G20450	11667	dehydrin (ERD10)	0.89	1.14	1.86
AT1G31630	2101	MADS-box protein	0.98	1.4	2.18
AT5G61820	6221	expressed protein	0.86	1.26	2.73
		succinate dehydrogenase, iron-sulphur subunit,			
AT3G27380	20341	mitochondrial (sdh2-1)	0.93	1.2	2.34
AT1G17170	15921	glutathione transferase, putative	1.69	1.29	6.59
AT5G61510	24634	NADP-dependent oxidoreductase, putative	0.85	0.79	1.57
AT1G52030	13671	myrosinase binding protein, putative	1.37	1.2	2.2
AT1G52720	20965	expressed protein	1	1.17	5.31
AT5G14780	24471	formate dehydrogenase (FDH)	1.06	1.11	2.95
AT2G16830	13357	hypothetical protein	1.08	1.14	2.22
AT3G05880	1888	low temperature and salt responsive protein LTI6A	0.99	1.53	3.69
AT1G21000	16592	expressed protein	0.9	1.28	4.18
AT1G03910	6970	hypothetical protein	0.94	1.01	2.22
AT5G38540	11391	jacalin lectin family	1.48	1.21	1.89
AT4G37010	15620	caltractin (centrin), putative	0.91	1.25	4.52
AT3G11900	14585	amino acid transporter family	0.74	1.13	1.65
AT5G65860	22272	hypothetical protein	1	0.97	2.72
AT5G53560	27132	cytochrome b5	1.27	1.22	2.14
AT1G75830	17900	plant defensin protein, putative (PDF1.1)	0.89	0.92	5.3
AT5G40760	15576	glucose-6-phosphate dehydrogenase	1.07	1.28	2.49
AT4G04620	27462	symbiosis-related protein -related	1.02	1.03	3.01
		ESTs, Weakly similar to T06126 calcium-dependent			
AT3G10300	6712	protein kinase [A.thaliana]	1.02	1.83	2.33
AT3G17860	21520	expressed protein	0.86	1.77	2.6
		abscisic acid responsive elements-binding			
AT4G34000	8212	factor(ABF3)	0.77	0.98	1.86
		glyoxalase II, putative (hydroxyacylglutathione			
AT1G53580	8659	hydrolase)	0.93	0.95	5.82
AT3G02230	19236	reversibly glycosylated polypeptide-1	1.06	1.15	2.28
AT4G19860	4229	hypothetical protein	0.8	1.21	1.85
AT5G10040	13007	expressed protein	0.85	1	3.29
AT4G21980	22581	symbiosis-related like protein	1.02	1.11	1.99

	24649;19				
AT5G65110	10	EST	1.12	1	2.18
AT5G11520	24558	aspartate aminotransferase, chloroplast (transaminase A/Asp3)	0.98	1.33	3.54
A020750_01	6422	EST	0.99	1.21	5.52
AT5G47720	17712	acetyl-CoA C-acetyltransferase (acetoacetyl-coenzyme A thiolase), putative	1.04	0.93	4.17
AT5G03630	10934	monodehydroascorbate reductase, putative	1.02	1.72	10.04
AT4G29270	8137	acid phosphatase-related protein	0.69	0.64	2.65
AT4G35830	27408	aconitate hydratase (citrate hydro-lyase/aconitase/ACO) [cytoplasmic]	1.14	1.02	2
AT1G13080	15718	cytochrome P450 family	1.05	2.32	6.39
AT2G41710	26046	ovule development protein, putative	1.24	1.61	3.75
AT3G50260	14669	AP2 domain transcription factor, putative	0.91	1.07	2.42
AT1G08630	13380	expressed protein	0.96	1.15	4.78
AT3G17810	13625	dehydrogenase -related	0.96	1.66	4.25
AT3G02480	24087	expressed protein	1.17	0.82	4.92
AT2G33150	688	acetyl-CoA C-acyltransferase (3-ketoacyl-CoA thiolase), putative	0.78	1.31	4.58
AT3G05500	9979	stress related protein -related	1.24	1.81	2.69
AT2G33380	16821	RD20 protein	0.89	1.4	3.63
AT1G76180	1822	dehydrin -related	0.94	1.11	4.42
AT3G29575	12410	expressed protein	0.82	1.42	4.08
AT4G39730	5885	expressed protein	0.96	0.87	2.49
AT1G54020	6284	myrosinase-associated protein, putative	1.07	1.68	2.99
AT2G29940	18093	ABC transporter family protein	1	1	12.55
AT1G61120	2459	terpene synthase/cyclase family	0.95	1.41	6.23
AT3G06780	19426	glycine-rich protein	1	1.25	2.9
AT2G39330	8948	jacalin lectin family	1.69	1.94	7
AT3G11410	13257	protein phosphatase 2C (PP2C), putative	0.98	1.55	3.23
AT2G47770	22481	expressed protein	0.94	1.15	8.18
AT5G15970	22646	cold-regulated protein COR6.6 (stress-induced protein KIN2)	1.14	0.56	2.41
AT5G13180	8203	No apical meristem (NAM) protein family	0.92	1.24	1.88
AT1G10070	27154	tat-binding protein -related	1.33	1.72	2.16
AT1G47128	23156	cysteine proteinase RD21A	0.75	1.38	2.62

AT2G28400	9849	expressed protein	0.83	1.5	2.82
AT5G47860	6131	expressed protein	0.87	1.34	2.29
AT4G16190	21950	cysteine proteinase	1	1.17	3.04
AT4G38060	5869	expressed protein	0.74	1.32	2.54
AT3G14310	23969	pectin methylesterase -related	0.84	0.79	1.43

Group B

				FC[a]	
Symbol	**Spot**	**Operon annotation**	**12h**	**24h**	**48h**
	1885;207				
AT5G20620	19	polyubiquitin (UBQ4)	1.09	1.39	1.66
AT4G34150	1760	C2 domain-containing protein	1.84	3.37	3.12
AT1G33590	25193	disease resistance protein-related (LRR)	1.6	1.67	3.01
AT1G06030	27227	fructokinase, putative	1.37	1.91	2.47
AT1G28480	21101	glutaredoxin protein family	1.3	2.31	2.2
AT3G26200	9034	cytochrome P450 71B22	1.72	2.71	2.63
AT1G76680	20628	12-oxophytodienoate reductase (OPR1)	0.96	1.73	2.62
AT3G57280	7715	expressed protein	1.11	1.54	1.64
AT5G47240	3681	MutT/nudix family protein	0.89	1.21	2.14
AT2G29460	9874	glutathione transferase, putative	1.31	1.58	2.56
AT2G28200	9873	zinc-finger protein -related	1.48	3.32	2.19
AT1G23440	20486	expressed protein	1.06	1.52	2.19
AT5G42050	26930	expressed protein	2.44	3.17	3.25
	16105;22				
AT5G07440	947	glutamate dehydrogenase 2	1.83	2.3	6
AT3G19970	5713	expressed protein	1.68	2.66	2.04
AT2G24550	3055	expressed protein	1.33	1.61	2.61
AT3G02360	21642	6-phosphogluconate dehydrogenase -related	1.26	1.6	2.33
AT2G43510	21295	trypsin inhibitor -related	1.98	1.96	6.4
AT4G37410	1304	cytochrome P450, putative	3.31	6.62	6.78
AT5G59820	20489	zinc finger protein Zat12	3.63	2.86	4.55
AT5G39050	27428	transferase family	1.91	1.85	3.05
AT5G54500	4083	quinone reductase, putative	1.66	2.55	2.59
AT5G25880	13602	malate dehydrogenase - like protein	1.77	2.3	2.64
AT4G23100	8187	gamma-glutamylcysteine synthetase	1.24	1.54	1.83
AT3G45640	17104	mitogen-activated protein kinase (MAPK), putative (MPK3)	1.31	2.55	3.78

AT4G02380	17450	late embryogenesis abundant protein family	5.96	6.05	12.44
AT5G58310	17654	hydrolase, alpha/beta fold family	1.02	1.5	2.11
AT5G22270	17754	expressed protein	1.49	2.78	10.88
AT4G30530	5906	glutamine amidotransferase class-I domain-containing protein	2.14	4.25	3.67
AT1G07610	4181	metallothionein-related protein	1.44	2.71	10.83
AT5G13420	15640	transaldolase - like protein	1.14	1.37	2.5
AT1G27980	13931	sphingosine-1-phosphate lyase -related	1.76	1.9	3.3
A021142_01	4077	Genomic sequence for Arabidopsis thaliana BAC F15O4 from chromosome I	1.02	1.67	2.09
AT2G39980	21487	transferase family	1.33	2.03	3.33
AT5G05600	25211	oxidoreductase, 2OG-Fe(II) oxygenase family	1.93	2.58	8.28
AT2G32210	4189	expressed protein	2.56	8.07	5.81
AT2G22470	2939	arabinogalactan-protein (AGP2)	1.94	4.15	4.52
AT4G37370	9068	cytochrome P450, putative	1.08	2.15	8.35
AT2G43020	22836	amine oxidase family	0.96	1.62	2.76
AT3G53180	3980	nodulin / glutamate-ammonia ligase - like protein	1.5	2.92	13.32
AT5G15080	18117	serine/threonine specific protein kinase -related	1.05	1.84	3.48
AT4G23920	10885	UDP-glucose 4-epimerase (UDP-galactose 4-epimerase) (Galactowaldenase), putative	0.93	4.46	3.51
AT4G26080	20194	protein phosphatase ABI1	0.93	1.77	3.06
AT2G29420	15629	glutathione transferase, putative	1.49	2.11	5.91
AT5G51070	8366	ATP-dependent Clp protease ATP-binding subunit (ClpD), ERD1 protein precursor	0.84	2.24	6.06
AT1G54650	4785	expressed protein	2.56	1.06	2.93
AT4G23600	15515	aminotransferase family	0.99	1.66	2.83
AT5G53050	11170	hydrolase, alpha/beta fold family	1.57	3.76	3.32
AT3G10260	5647	expressed protein	0.97	1.68	1.94
AT5G42650	26886	allene oxide synthase / cytochrome P450 74A	2.78	11.8	19.98
AT1G19670	20738	coronatine-induced protein 1 (CORI1)	1.74	8.81	8.88
AT2G43910	2865	thiol methyltransferase	0.92	1.85	3.71
AT4G16500	12789	cysteine proteinase inhibitor like protein	1.25	1.97	3.64
AT5G13330	10737	AP2 domain transcription factor family	1.29	2.55	9.01
AT1G09970	9606	leucine-rich repeat transmembrane protein kinase, putative	1.23	2.14	2.16
AT1G70700	23372	expressed protein	1.4	2.36	2.41

AT2G06050	16793	12-oxophytodienoate reductase (OPR3)(DDE1)	1.38	2.71	1.98
AT5G26340	24836	hexose transporter, putative	2.95	3.81	13.35
AT4G37990	10471	mannitol dehydrogenase (ELI3-2), putative	1.18	3.07	6.83
AT3G07030	14834	hypothetical protein	1.13	3.68	1.97
AT1G72330	25587	alanine aminotransferase, putative	1.65	2.46	2.05
AT4G11800	10528	calcineurin-like phosphoesterase family	0.91	1.61	2.47
AT3G14050	716	Arabidopsis thaliana RSH2 mRNA, complete cds	1.31	2.92	5.91
AT2G23810	13275	senescence-associated protein family	2.13	4.94	7.72
AT5G01750	24854	expressed protein	1.46	2.79	3.74
AT3G26190	13642	cytochrome P450 71B21	1.58	2.47	2.26
AT2G36950	14530	heavy-metal-associated domain-containing protein	1.21	2.2	3.44

Group C

			FC[a]		
Symbol	**Spot**	**Operon annotation**	**12h**	**24h**	**48h**
AT5G03120	3692	expressed protein	1.2	0.64	0.45
AT2G40100	21413	light-harvesting chlorophyll a/b binding protein	0.92	0.4	0.48
AT5G08050	26943	expressed protein	0.43	0.63	0.43
AT1G42970	21012	glyceraldehyde-3-phosphate dehydrogenase	0.56	0.46	0.52
AT4G34090	9113	expressed protein	0.76	0.66	0.52
AT1G31190	9118	expressed protein	0.57	0.58	0.42
AT1G32220	7119	expressed protein	0.94	0.87	0.49
AT1G69120	21115	floral homeotic gene APETALA1	0.93	0.84	0.48
AT2G21530	14522	expressed protein	0.82	0.64	0.53
AT5G39530	13343	expressed protein	1.09	0.87	0.49
AT4G15560	8662	DEF (CLA1) protein	1.07	0.72	0.5
AT4G15630	5835	expressed integral membrane protein common family	1.04	0.84	0.61
		ESTs, Weakly similar to RS8_HUMAN 40S			
AT5G20290	2751	RIBOSOMAL PROTEIN S [*H.sapiens*]	0.79	0.76	0.45
AT1G29670	18236	GDSL-motif lipase/hydrolase protein	1.3	0.51	0.37
AT3G63410	3138	chloroplast inner envelope membrane protein, putative	0.61	0.58	0.48
AT5G66530	17762	aldose 1-epimerase family	0.96	0.75	0.47
AT3G11950	3242	UbiA prenyltransferase family	0.7	0.6	0.49
AT4G27520	26581	expressed protein	1.11	0.63	0.47
AT1G08380	9644	expressed protein	0.89	0.63	0.31
AT5G24300	22324	starch synthase, putative	0.94	0.78	0.47
AT5G51820	22266	phosphoglucomutase (emb|CAB64725.1)	0.78	0.78	0.47

AT1G66200	18889	glutamine synthetase -related	0.66	0.37	0.64
AT3G26000	3222	F-box protein family	0.73	0.65	0.51
AT3G61080	7959	expressed protein	0.95	0.57	0.51
AT1G09340	16297	RNA-binding protein -related	0.78	0.59	0.58
AT4G37230	12827	photosystem II oxygen-evolving complex like protein	0.86	0.58	0.37
AT5G52100	17710	dihydrodipicolinate reductase-related protein	0.76	0.67	0.56
AT4G16980	22409	arabinogalactan-protein family	0.92	0.81	0.54
AT2G38530	25852	nonspecific lipid transfer protein 2 (LTP 2)	0.77	0.68	0.35
AT4G38860	4182	auxin-induced (indole-3-acetic acid induced) protein, putative	0.73	0.84	0.53
AT4G29140	26641	MATE efflux protein - related	0.91	0.33	0.55
AT3G59840	3410	expressed protein	0.89	0.68	0.57
AT5G66670	15830	At14a, putative	1.18	0.77	0.55
AT1G79850	21015	chloroplast 30S ribosomal protein S17 (CS17)	0.85	0.55	0.59
AT3G17930	14876	expressed protein	1.15	0.67	0.52
AT3G51820	15491; 5027	chlorophyll synthetase, putative	0.99	0.62	0.67
AT4G15510	19659	oxygen-evolving complex 25.6 kD protein, chloroplast precursor, putative	0.85	0.52	0.53
AT3G52380	5691	33 kDa ribonucleoprotein, chloroplast (RNA-binding protein cp33), putative	1.14	0.66	0.66
AT2G38140	21484	chloroplast 30S ribosomal protein S31	0.88	0.64	0.59
AT5G51010	6362	expressed protein	1.22	0.47	0.55
AT5G13630	10595	cobalamin biosynthesis protein	1.08	0.68	0.62
AT4G15930	21901	dynein light chain like protein	0.91	0.79	0.62
AT5G47550	10746	expressed protein	1.04	0.9	0.45
AT1G44446	23044	chlorophyll a oxygenase (chlorophyll b synthase)	1.12	0.81	0.54
AT3G47520	6366	malate dehydrogenase [NAD], chloroplast, putative	0.85	0.76	0.78
AT4G17050	22547	expressed protein	0.62	0.82	0.59
AT2G35260	12225	expressed protein	1.17	0.81	0.56
AT5G09650	1748;9 26832	inorganic pyrophosphatase - like protein	0.55	0.61	0.66
AT5G01530	634	light-harvesting chlorophyll a/b binding protein	0.92	0.57	0.5
AT5G55730	22316	fasciclin-like arabinogalactan-protein (FLA1)	0.82	0.6	0.63
AT1G29070	27106	plastid ribosomal protein L34 precursor -related	0.78	0.68	0.65
AT5G23060	15412	expressed protein	1.28	0.4	0.57

AT3G54210	14858	ribosomal protein L17 -related protein	0.94	0.66	0.64
AT5G58250	12928	unknown protein (sp|P72777) -related	1.17	0.48	0.46
AT5G44600	18130	hypothetical protein	0.74	0.59	0.56
AT5G06290	24660	2-cys peroxiredoxin-related protein	0.58	0.43	0.58
AT5G55220	24541	trigger factor-related protein	0.7	0.69	0.61
AT3G49220	10141	pectinesterase family	0.94	1.13	0.65
AT3G54600	8587	expressed protein	0.87	0.75	0.52
AT3G53900	3981	uracil phosphoribosyltransferase-related protein	1.03	0.6	0.71
AT5G04970	10751	pectinesterase, putative	0.83	0.6	0.5
AT1G08520	13666	magnesium-chelatase, subunit chlD, chloroplast (Mg-protoporphyrin IX chelatase) (CHLD), putative	1.03	0.63	0.63
AT3G18560	12320	expressed protein	0.92	0.67	0.65
AT3G57610	24145	EST	0.62	0.78	0.64
AT5G55280	17658	plastid division protein FtsZ, putative	0.7	0.75	0.62
AT2G03350	1769	expressed protein	0.83	0.62	0.53
AT2G40490	26049	uroporphyrinogen decarboxylase (UPD), putative	0.97	0.81	0.58
AT5G61980	13125	ARF GTPase-activating domain-containing protein	1.09	0.34	0.52
AT4G33680	24331	aminotransferase family	0.54	0.74	0.73
AT2G05990	5340	enoyl-[acyl-carrier protein] reductase [NADH] (enr-A), putative	1.07	0.65	0.82
AT5G20630	10796	germin-like protein (AtGER3)	1.53	0.55	0.93
AT3G08920	5524	rhodanese-like domain protein	0.98	0.64	1.61
AT4G35250	21952	vestitone reductase-related	0.9	0.71	0.51
AT3G63190	24958	expressed protein	0.75	0.48	0.46
AT1G73060	4006	expressed protein	0.83	0.67	0.49
AT3G62030	16614	peptidylprolyl isomerase ROC4	0.72	0.5	0.65
AT4G01460	8107	bHLH protein family	1.53	0.65	0.85
AT3G53460	15624	29 kDa ribonucleoprotein, chloroplast (RNA-binding protein cp29)	1.04	0.6	0.84
AT3G14930	21762	uroporphyrinogen decarboxylase (UPD), putative	1.03	0.66	0.6

Group D			**FC[a]**		
Symbol	**Spot**	**Operon Annotation**	**12h**	**24h**	**48h**
AT3G11930	7812	ethylene-responsive protein -related	0.83	2.14	0.41
AT5G22620	26859	expressed protein	0.75	1.02	0.44
AT1G03340	2383	hypothetical protein	0.91	0.95	0.49

AT3G47860	26131	expressed protein	0.8	1.35	0.59
AT3G01120	8689	cystathionine gamma-synthase -related	1.05	0.96	0.46
AT5G14060	6423	aspartate kinase, lysine-sensitive	1.16	0.52	0.47
AT5G15230	13040	GASA4	1.04	0.8	0.58
AT2G21280	2964	expressed protein	0.69	0.98	0.51
AT5G12860	8509	oxoglutarate/malate translocator, putative	0.7	0.76	0.5
AT3G52360	4389	expressed protein	1.02	0.87	0.56
AT1G78140	23377	expressed protein	0.99	0.94	0.49
AT2G30540	21300	glutaredoxin protein family	1.01	1.03	0.54
AT2G34430	10963	photosystem II type I chlorophyll a /b binding protein	0.86	0.77	0.55
AT5G02160	17492	expressed protein	0.71	0.74	0.5
AT2G05100	16768	light-harvesting chlorophyll a/b binding protein	1.01	0.73	0.39
AT2G34420	2838	photosystem II type I chlorophyll a /b binding protein	0.89	0.89	0.53
AT2G25830	23672	expressed protein	0.78	1.02	0.57
AT5G52640	26947	heat shock protein 81-1 (HSP81-1/heat shock protein 83/HSP83)	0.55	0.88	0.47
AT2G26500	21328	expressed protein	0.91	0.7	0.6
AT3G48740	21517	nodulin MtN3 family protein	0.87	1.11	0.46
AT3G08940	15635	chlorophyll a/b-binding protein -related	0.96	0.89	0.55
AT5G54270	9030	light-harvesting chlorophyll a/b binding protein, putative	1.12	0.47	0.45
AT4G27700	15646	rhodanese-like domain protein	0.89	0.66	0.44
AT1G48600	18340	Genomic sequence for Arabidopsis thaliana BAC T1N15 from chromosome I	0.97	1.01	0.41
AT3G51895	27506	sulfate transporter ATST1	0.66	1.19	0.6
AT2G15620	9838	ferredoxin--nitrite reductase	1.11	0.68	0.51
AT3G19380	6275	expressed protein	0.95	1.89	0.68
AT2G16630	16687	proline-rich protein family	1.21	1.14	0.37
AT5G45950	27083	GDSL-motif lipase/hydrolase protein	0.29	0.76	0.31
AT1G51400	15881	photosystem II 5 KD protein	0.9	0.73	0.55
AT2G29630	21493	thiamin biosynthesis protein -related	0.7	0.74	0.35
AT4G02770	24276	photosystem I reaction center subunit II precursor -related	0.66	0.67	0.57
AT1G21500	9443	expressed protein	1.12	0.63	0.5
AT3G43720	1110	protease inhibitor/seed storage/lipid transfer protein (LTP) family	0.79	0.99	0.54

	14916;14913;14891;17193;14914;14892;14915;17220;17194;17195;14890;14889;				
AT5G49910	17196;18856	heat shock protein cpHsc70-2 (hsc70-7)	0.8	0.85	0.65
AT2G18230	14553	inorganic pyrophosphatase -related	0.86	0.76	0.48
AT5G10180	17543	sulfate transporter	1	1.01	0.38
AT1G06830	7061	glutaredoxin protein family	1.84	2.99	0.3
AT2G02500	11104	sugar nucleotide phosphorylase -related	0.85	0.86	0.58
AT3G54890	7912	light-harvesting chlorophyll a/b binding protein	0.96	0.87	0.65
AT2G45180	16887	protease inhibitor/seed storage/lipid transfer protein (LTP) family	1.15	0.93	0.33
AT1G12090	2456	protease inhibitor/seed storage/lipid transfer protein (LTP) family	0.37	1.14	0.43
AT3G16140	5720	photosystem I subunit VI precursor	0.73	0.76	0.64
AT2G29980	21313	omega-3 fatty acid desaturase, endoplasmic reticulum (FAD3)	0.82	0.95	0.52
AT4G26520	15986	fructose-bisphosphate aldolase, cytoplasmic	0.71	0.44	0.45
AT5G59080	4089	expressed protein	1.2	1.17	0.62
AT2G24020	23632	expressed protein	1.04	0.58	0.51
AT1G58080	16586	ATP phosphoribosyl transferase	1.09	1.06	0.66
AT1G15140	18847	oxidoreductase NAD-binding domain-containing protein	0.94	1.23	0.66
AT2G25890	12245	oleosin	1.01	1	0.56
AT4G26530	13680	fructose-bisphosphate aldolase, putative	0.71	0.44	0.49
AT2G04570	2910	GDSL-motif lipase/hydrolase protein	1.24	1.14	0.38
AT4G17230	12843	scarecrow-like transcription factor 13 (SCL13)	1.37	1.69	0.4
AT2G47810	19153	CCAAT-box binding trancription factor -related	1.21	1.24	0.77

Group E			**FC[a]**		
Symbol	**Spot**	**Operon annotation**	**12h**	**24h**	**48h**
AT3G16150	10179	L-asparaginase -related	3.14	4.65	4.14
AT4G28460	8777	hypothetical protein	1.7	2.85	1.75
AT5G17380	3672	2-hydroxyphytanoyl-CoA lyase-related protein	1.28	2.28	1.79
AT3G15210	9770	ethylene responsive element binding factor 4 (ERF4)	1.74	4.44	2.74
AT5G40370	17876	glutaredoxin, putative	1.28	3.07	2.03

AT2G32190	8797	expressed protein	4.77	16.76	2.76
AT4G24570	22457	mitochondrial carrier protein family	2.88	4.22	1.91
AT3G12580	13210	heat shock protein hsp70	1.96	2.73	2.08
AT5G35475	13122	hypothetical protein	1.84	4.71	2.1
AT4G36990	9682	heat shock transcription factor 4 (HSF4)	3.11	5.47	3.37
AT4G34120	5863	CBS domain containing protein	1.03	2.5	1.67
AT4G31500	22842	cytochrome P450 83B1	2.21	3.72	2.06
AT1G61890	2557	MATE efflux protein family	2.27	3.95	1.6
AT5G12170	4027	expressed protein	1.72	2.45	1.57
AT5G67600	24760	expressed protein	1.43	4.65	1.6
AT3G03480	26193	transferase family	0.51	3.88	2.36
AT4G07840	11209	transposon protein -related	3.26	10.96	4.04
AT5G54510	27063	auxin-responsive-related protein	2.38	3.3	1.73
AT3G50770	14763	calmodulin-related protein, putative	2.21	2.97	3.44
AT5G66650	4017	expressed protein	1.41	2.26	1
AT2G29330	25055	short-chain dehydrogenase/reductase family protein (tropinone reductase, putative)	2.02	4.87	1.39
AT1G11310	20539	seven transmembrane MLO protein family (MLO2)	1.11	2.17	1.08
AT3G12600	20218	MutT/nudix family protein	1.52	2.46	2.23
AT1G19180	13993	expressed protein	1.42	2.87	1.22
AT5G42010	6093	expressed protein	2.15	3.63	1.34
AT3G13790	25146	glycosyl hydrolase family 32	1.59	2.6	1.21
AT2G32200	22805	hypothetical protein	1.6	3.25	2.31
AT3G22890	17001	ATP sulfurylase -related	1.26	2.46	1.22
AT4G35150	5939	O-methyltransferase family 2	1.23	3.76	1
AT3G05290	7921	mitochondrial carrier protein family	1.09	2.25	1
AT5G02230	1718	haloacid dehalogenase-like hydrolase family	2.1	3.18	1.15
AT4G37640	6426	calcium-transporting ATPase 2, plasma membrane-type (Ca2+-ATPase, isoform 2)	2.05	4.59	1.85
AT1G12010	25564	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), putative	1.45	2.54	1.74
AT4G34710	15467	arginine decarboxylase SPE2	1.98	2.96	1.28
AT3G09020	10136	glycosyltransferase-related	2.31	3.89	1.81
AT2G31945	21491	expressed protein	1.37	3.98	1.06
A000098_01	9221	Genomic sequence for Arabidopsis thaliana BAC F5O11 from chromosome I	9.78	15.01	6.71

		short-chain dehydrogenase/reductase family protein			
AT2G30670	3014	(tropinone reductase, putative)	1.45	3.34	2.24
AT1G63000	6715	expressed protein	1.28	2.5	0.88
AT5G43030	22716	CHP-rich zinc finger protein, putative	3.27	7.9	3.64
AT1G19770	2737	purine permease-related	1.72	2.63	1.1
AT1G67920	7302	expressed protein	2.04	6.58	3.76
AT1G27730	2108	salt-tolerance zinc finger protein	5.97	20.09	10.75
		EST, Moderately similar to T08924 hypothetical protein T15N24.30 - *Arabidopsis thaliana*			
AT5G55970	22243	[*A.thaliana*]	1.11	1.95	1.13
AT4G34138	6869	glycosyltransferase family	2.23	2.95	2.12
AT3G15500	15625	No apical meristem (NAM) protein family	1.16	2.49	2.73
AT2G46330	9023	arabinogalactan-protein (AGP16)	1.3	2.32	1.34
AT1G27100	23283	expressed protein	1.46	2.02	1.04
AT5G27520	25785	ESTs	1.28	2.69	1.3
AT3G25780	10062	allene oxide cyclase family	4.66	9.17	4.94
		Homeobox-leucine zipper protein HAT22 (HD-ZIP			
AT4G37790	6360	protein 22)	1.01	2.1	1.12
AT2G27500	11274	glycosyl hydrolase family 17	1.24	2.83	1.57
AT2G40000	11025	nematode-resistance protein -related	1.18	2.83	1.34
AT1G16030	11931	heat shock protein hsp70b	1.85	2.38	1.47
AT4G24380	19766	expressed protein	1.63	3.8	1.32

Group F			**FC[a]**		
Symbol	**Spot**	**Operon annotation**	**12h**	**24h**	**48h**
AT5G02490	18254	heat shock protein hsc70-2 (hsc70.2) (hsp70-2)	1.88	2.66	1.6
AT4G08780	22923	peroxidase, putative	3.61	3.79	1.73
AT3G22160	17240	expressed protein	1.48	1.71	0.66
AT1G14870	8822	expressed protein	5.13	7.18	4.13
AT2G35930	4096	expressed protein	2.01	2.81	1.1
		Sequence of BAC F7G19 from Arabidopsis thaliana			
AT1G08930	6802	chromosome 1	2.25	2.61	1.64
AT2G18200	13401	hypothetical protein	4.05	9.37	2
AT1G69930	15632	glutathione transferase, putative	6.59	10.15	2.77
AT5G05320	10656	monooxygenase 2 (MO2), putative	3.16	5.07	1.55
AT1G21120	20669	O-methyltransferase 1, putative	4.26	3.89	1.91
AT1G21130	11428	O-methyltransferase 1, putative	4.34	4.34	2.16

AT1G74000	1725	strictosidine synthase family	1.71	1.6	1.15
AT4G33050	24320	expressed protein	2.42	2.6	0.99
AT4G11280	12717	1-aminocyclopropane-1-carboxylate synthase 6 (ACC synthase 6) (ACS6)	4.15	7.51	1.72
AT1G13210	22390	haloacid dehalogenase-like hydrolase family	1.85	3.29	0.9
AT1G78850	18331	EST	3	2.6	1.23
AT1G78860	2195	curculin-like (mannose-binding) lectin family	3.54	3.31	1.59
AT4G37290	3611	expressed protein	21.46	53.83	1.23
AT3G14225	13683	GDSL-motif lipase/hydrolase protein	4.67	7.15	1
AT5G54490	15413	calcium-binding protein, putative	2.37	3.08	0.96
AT4G04610	20468	5'-adenylylsulfate reductase	2.57	5.05	1.5
AT1G53060	18761	protein kinase -related	4.75	7.27	2.55

Footnote:

FC[a] Fold Change in transcript abundance

Table A-2 Transcriptional profiling: Genes whose transcript abundance exhibited more than 2-fold increase

12 h	Operon Annotation	FC [a]	SE [b]
Cellular communication/Signal transduction			
At5g54490	calcium-binding protein, putative	2.37	0.28
At4g37640	calcium-transporting ATPase 2, plasma membrane-type (Ca^{2+}-ATPase, isoform 2)	2.05	0.23
Cell rescue & defense			
At2g43510	trypsin inhibitor -related	1.98	0.24
At4g08780	peroxidase, putative	3.61	0.84
At3g49120	peroxidase, putative	2.49	0.37
At4g02380	late embryogenesis abundant protein family	5.96	1.19
At1g21310	extensin family protein	2.09	0.37
Cellular structure & organization			
At1g21130	O-methyltransferase 1, putative	4.34	0.67
At1g21120	O-methyltransferase 1, putative	4.26	0.28
Cellular transport			
At1g08930	early-responsive to dehydration stress protein (ERD6)	2.25	0.26
Horomone biosynthesis			
At3g25780	allene oxide cyclase family	4.66	0.74
At4g31500	cytochrome P450 83B1	2.21	0.32
Nucelotide metabolism			
At4g30530	glutamine amidotransferase class-I domain-containing protein	2.14	0.38
Protein fate			
At5g02500	heat shock protein hsc70-1 (hsp70-1) (hsc70.1)	2.59	0.37
At3g12580	heat shock protein hsp70	1.96	0.18
At3g16150	L-asparaginase -related	3.14	0.66
Transcriptionn factors (TFs)			
At1g27730	salt-tolerance zinc finger protein	5.97	1.29
At5g47230	ethylene responsive element binding factor 5 (AtERF5)	3.76	0.30
At4g36990	heat shock transcription factor 4 (HSF4)	3.11	0.71
At5g43030	CHP-rich zinc finger protein, putative	3.27	0.58
Unclassified			
At1g14870	expressed protein	5.13	1.12

At2g32190	expressed protein	4.77	0.40
At1g53060	protein kinase -related	4.75	1.06
At1g78850	curculin-like (mannose-binding) lectin family	3.00	0.38
At2g32210	expressed protein	2.56	0.87
At4g33050	expressed protein	2.42	0.15
At4g04610	5'-adenylylsulfate reductase	2.57	0.37

24 h	**Operon Annotation**	**FC** [a]	**SE** [b]
Amino acid metabolism			
At5g07440	Arabidopsis thaliana glutamate dehydrogenase 2 (T2I1_150) mRNA, complete cds	2.30	0.64
At5g07440	glutamate dehydrogenase 2	2.05	0.50
At1g72330	alanine aminotransferase, putative	2.46	0.33
At4g34200	D-3-phosphoglycerate dehydrogenase (3-PGDH), putative	2.04	0.30
At1g13210	haloacid dehalogenase-like hydrolase family	3.29	0.50
Carbohydrate metabolism			
At2g31390	fructokinase, putative	1.98	0.14
At4g23920	UDP-glucose 4-epimerase (UDP-galactose 4-epimerase) (Galactowaldenase), putative	4.46	0.65
At2g27500	glycosyl hydrolase family 17	2.83	0.33
At3g13790	glycosyl hydrolase family 32	2.60	0.39
Cellular communication/Signal transduction			
At5g54510	auxin-responsive-related protein	3.30	1.22
At3g45640	mitogen-activated protein kinase (MAPK), putative (MPK3)	2.55	0.29
At5g67600	expressed protein	4.65	0.58
At4g34150	C2 domain-containing protein	3.37	0.41
At5g42010	expressed protein	3.63	0.82
At3g50770	calmodulin-related protein, putative	2.97	2.97
At4g33050	expressed protein	2.60	0.37
At4g37640	calcium-transporting ATPase 2, plasma membrane-type (Ca^{2+}-ATPase, isoform 2)	4.59	1.41
At1g09970	leucine-rich repeat transmembrane protein kinase, putative	2.14	0.36
At1g11310	seven transmembrane MLO protein family (MLO2)	2.17	0.30
Cell death & aging			
At2g23810	senescence-associated protein family	4.94	1.22
Cell rescue, defense			

At4g08780	peroxidase, putative	3.79	1.36
At3g49120	peroxidase, putative (PER34)	2.52	1.53
At2g29420	glutathione transferase, putative	2.11	0.35
At1g69930	glutathione transferase, putative	10.15	11.39
At4g37990	mannitol dehydrogenase (ELI3-2), putative	3.07	0.86
At4g34710	arginine decarboxylase SPE2	2.96	0.80
At4g02380	late embryogenesis abundant protein family	6.05	5.49
At2g40000	nematode-resistance protein -related	2.83	0.35
At5g40370	glutaredoxin, putative	3.07	0.45
At1g06830	glutaredoxin protein family	2.99	0.61
Cellular structure & organization			
At1g63000	expressed protein	2.50	0.36
At2g46330	arabinogalactan-protein (AGP16)	2.32	0.23
At2g22470	arabinogalactan-protein (AGP2)	4.15	1.11
At1g21130	O-methyltransferase 1, putative	4.34	1.41
At1g21120	O-methyltransferase 1, putative	3.89	1.36
At4g35150	O-methyltransferase family 2	3.76	1.18
Cellular transport			
At3g22890	ATP sulfurylase -related	2.46	0.39
At3g48850	mitochondrial phosphate transporter, putative	4.99	2.23
At4g24570	mitochondrial carrier protein family	4.22	0.69
At5g27520	mitochondrial substrate carrier family protein	2.69	0.42
At3g05290	mitochondrial carrier protein family	2.25	0.40
At1g61890	MATE efflux protein family	3.95	0.68
At5g26340	hexose transporter, putative	3.81	1.89
At1g08930	Sequence of BAC F7G19 from Arabidopsis thaliana chromosome 1	2.61	0.65
At2g36950	heavy-metal-associated domain-containing protein	2.20	0.14
At1g19770	purine permease-related	2.63	0.27
At1g07610	metallothionein-related protein	2.71	0.99
Energy			
At5g54500	quinone reductase, putative (NAPDH)	2.55	0.62
Horomone biosynthesis			
At3g25780	allene oxide cyclase family	9.17	3.23
At4g11280	1-aminocyclopropane-1-carboxylate synthase 6 (ACC synthase 6) (ACS6)	7.51	1.61
At2g06050	12-oxophytodienoate reductase (OPR3)(DDE1)	2.71	0.80

At1g12010	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), putative	2.54	0.36
At1g05010	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) (ethylene-forming enzyme) (EFE)	2.03	0.26
At5g42650	allene oxide synthase / cytochrome P450 74A	11.80	4.70
Lipid metabolism			
At2g29330	short-chain dehydrogenase/reductase family protein (tropinone reductase, putative)	4.87	1.50
At2g30670	short-chain dehydrogenase/reductase family protein (tropinone reductase, putative)	3.34	0.46
Nucleotide metabolism			
At4g30530	glutamine amidotransferase class-I domain-containing protein	4.25	1.03
At3g14050	Arabidopsis thaliana RSH2 mRNA, complete cds	2.92	0.41
Photosynthesis			
At1g19670	coronatine-induced protein 1 (CORI1)	8.81	3.22
Protein fate			
At5g02500	heat shock protein hsc70-1 (hsp70-1) (hsc70.1)	3.21	0.97
At3g12580	heat shock protein hsp70	2.73	0.37
At5g02490	heat shock protein hsc70-2 (hsc70.2) (hsp70-2)	2.66	0.38
At5g51070	ATP-dependent Clp protease ATP-binding subunit (ClpD), ERD1 protein precursor	2.24	0.52
At5g53050	hydrolase, alpha/beta fold family	3.76	2.03
At5g05320	monooxygenase 2 (MO2), putative	5.07	2.02
At5g02230	haloacid dehalogenase-like hydrolase family	3.18	1.18
At3g16150	L-asparaginase -related	4.65	1.89
At3g05640	protein phosphatase 2C (PP2C), putative	1.99	0.27
Secondary metabolism			
At4g34138	glycosyltransferase family	2.95	0.48
At5g05600	oxidoreductase, 2OG-Fe(II) oxygenase family	2.58	0.78
At3g19010	flavonol synthase family	2.04	0.35
Transcription factors (TFs)			
At3g15210	ethylene responsive element binding factor 4 (ERF4)	4.44	1.01
At5g47230	ethylene responsive element binding factor 5 (AtERF5)	3.39	2.02
At2g28200	zinc-finger protein -related	3.32	0.67
At5g13330	AP2 domain transcription factor family	2.55	0.87
At4g36990	heat shock transcription factor 4 (HSF4)	5.47	3.43

At3g15500	No apical meristem (NAM) protein family	2.49	0.33
At1g19180	expressed protein	2.87	0.61
At1g70700	expressed protein	2.36	0.43
At4g37790	Homeobox-leucine zipper protein HAT22 (HD-ZIP protein 22)	2.10	0.18
At1g27730	salt-tolerance zinc finger protein	20.09	11.05
At5g43030	CHP-rich zinc finger protein, putative	7.90	3.19
At5g59820	zinc finger protein Zat12	2.86	0.54
Unclassified			
At5g25880	malate dehydrogenase - like protein	2.30	0.22
At4g37290	expressed protein	53.83	27.78
At3g22600	protease inhibitor/seed storage/lipid transfer protein (LTP) family	21.72	27.20
At2g32190	expressed protein	16.76	5.13
A000098_01	Genomic sequence for Arabidopsis thaliana BAC F5O11 from chromosome I	15.01	9.07
At4g07840	transposon protein -related	10.96	3.83
At2g18200	hypothetical protein	9.37	5.34
At2g32210	expressed protein	8.07	2.97
At1g14870	expressed protein	7.18	4.26
At3g14225	GDSL-motif lipase/hydrolase protein	7.15	1.04
At1g67920	expressed protein	6.58	1.75
At5g35475	hypothetical protein	4.71	0.97
At2g31945	expressed protein	3.98	0.89
At3g09020	glycosyltransferase-related	3.89	0.73
At3g07030	hypothetical protein	3.68	0.83
At1g78860	curculin-like (mannose-binding) lectin family	3.31	0.67
At2g32200	hypothetical protein	3.25	0.70
At5g42050	expressed protein	3.17	1.42
At4g28460	hypothetical protein	2.85	1.11
At2g35930	expressed protein	2.81	0.76
At5g01750	expressed protein	2.79	0.42
At5g22270	expressed protein	2.78	0.74
At3g19970	expressed protein	2.66	0.49
At1g78850	curculin-like (mannose-binding) lectin family	2.60	0.40
At4g34120	CBS domain containing protein	2.50	0.33
At3g12600	MutT/nudix family protein	2.46	0.41

At5g66650	expressed protein	2.26	0.45
At2g39980	transferase family	2.03	0.16
At1g27100	expressed protein	2.02	0.18
At4g16500	cysteine proteinase inhibitor like protein	1.97	0.67
At4g37410	cytochrome P450, putative	6.62	2.05
At3g26200	cytochrome P450 71B22	2.71	0.34
At3g26190	cytochrome P450 71B21	2.47	0.41
At4g37370	cytochrome P450, putative	2.15	0.34

48 h	**Operon Annotation**	**FC** [a]	**SE** [b]
Amino acid metabolism			
At5g07440	glutamate dehydrogenase 2	7.86	2.25
At5g07440	Arabidopsis thaliana glutamate dehydrogenase 2 (T2I1_150) mRNA, complete cds	5.99	2.05
At1g08630	expressed protein	4.78	0.69
At3g47340	glutamine-dependent asparagine synthetase	4.46	1.13
At1g27980	sphingosine-1-phosphate lyase -related	3.30	0.56
At5g46180	ornithine--oxo-acid aminotransferase (ornithine aminotransferase/ornithine ketoacid aminotransferase), putative	2.87	0.58
At2g38400	alanine--glyoxylate aminotransferase (beta-alanine-pyruvate aminotransferase/AGT), putative	2.26	0.29
At4g35830	aconitate hydratase (citrate hydro-lyase/aconitase/ACO) [cytoplasmic]	2.00	0.17
At4g39950	cytochrome P450 79B2	2.14	0.37
Carbohydrate metabolism			
At1g15380	glyoxalase family protein (lactoylglutathione lyase family protein)	30.68	13.43
At5g49360	glycosyl hydrolase family 3	4.27	2.65
At5g57655	expressed protein	2.22	0.38
At5g28510	glycosyl hydrolase family 1	2.13	0.59
At2g14690	glycosyl hydrolase family 10	2.16	0.42
At4g23920	UDP-glucose 4-epimerase (UDP-galactose 4-epimerase) (Galactowaldenase), putative	3.51	0.40
At3g02360	6-phosphogluconate dehydrogenase -related	2.33	0.41
At1g64190	6-phosphogluconate dehydrogenase -related	1.98	0.31

At5g59290	NAD-dependent epimerase/dehydratase family	2.21	0.54
At1g60140	trehalose phosphatase family	2.14	0.43
At5g13420	transaldolase - like protein	2.50	0.32
At3g06500	neutral invertase -related	8.01	2.69
At3g27380	succinate dehydrogenase, iron-sulphur subunit, mitochondrial (sdh2-1)	2.34	0.38
At4g39800	myo-inositol-1-phosphate synthase	2.06	0.32
Cellular communication/Signal transduction			
At3g45640	mitogen-activated protein kinase (MAPK), putative (MPK3)	3.78	0.90
At4g34150	C2 domain-containing protein	3.12	0.89
At1g05100	protein kinase family	5.43	4.90
At5g15080	serine/threonine specific protein kinase -related	3.48	0.34
At4g26080	protein phosphatase ABI1	3.06	0.30
At3g11410	protein phosphatase 2C (PP2C), putative	3.23	0.22
At4g37010	caltractin (centrin), putative	4.52	0.93
At3g06780	glycine-rich protein	2.90	0.22
At3g10300	Arabidopsis thaliana chromosome III BAC F14P13 genomic sequence	2.33	0.37
Cell death and aging			
At4g35770	senescence-associated protein sen1	4.63	1.11
At5g11520	aspartate aminotransferase, chloroplast (transaminase A/Asp3)	3.54	0.55
Cell rescue & defense			
At2g03760	steroid sulfotransferase, putative	8.00	1.77
At2g43510	trypsin inhibitor -related	6.40	3.80
At1g75830	plant defensin protein, putative (PDF1.1)	5.30	1.40
At1g55010	plant defensin protein, putative (PDF1.5)	4.79	2.02
At5g44430	plant defensin protein, putative (PDF1.2c)	4.26	1.21
At2g26020	plant defensin protein, putative (PDF1.2b)	3.80	1.12
At2g26010	plant defensin protein, putative (PDF1.3)	2.99	0.70
At5g44420	plant defensin protein, putative (PDF1.2a)	4.06	1.36
At3g05880	low temperature and salt responsive protein LTI6A	3.69	0.83
At5g15970	cold-regulated protein COR6.6 (stress-induced protein KIN2)	2.41	0.05
At2g33380	RD20 protein	3.63	0.50
At1g33590	disease resistance protein-related (LRR)	3.01	0.68
At5g14780	formate dehydrogenase (FDH)	2.95	0.61

At5g05340	peroxidase, putative	2.42	0.68
At3g49120	peroxidase, putative	2.05	0.48
At3g03080	NADP-dependent oxidoreductase (P2), putative	2.71	0.40
At1g07890	ascorbate peroxidase, putative (APX)	2.01	0.38
At1g28480	glutaredoxin protein family	2.20	0.54
At5g40370	glutaredoxin, putative	2.03	0.35
At2g43020	amine oxidase family	2.76	0.28
At2g29460	glutathione transferase, putative	2.56	0.49
At2g29420	glutathione transferase, putative	5.91	0.93
At1g17170	glutathione transferase, putative	6.59	4.54
At3g03480	transferase family	2.36	0.19
At3g53990	expressed protein	2.01	0.62
At1g76180	dehydrin -related	4.42	0.59
At4g04620	symbiosis-related protein -related	3.01	0.46
At4g21980	symbiosis-related like protein	1.99	0.20
At4g02380	late embryogenesis abundant protein family	12.44	13.83
At5g06760	late embryogenesis abundant protein LEA like	10.99	3.74
At4g23600	aminotransferase family	2.83	0.20
At3g05500	stress related protein -related	2.69	0.29
At1g54020	myrosinase-associated protein, putative	2.99	0.25
At1g52030	myrosinase binding protein, putative	2.20	0.26
At5g06630	proline-rich protein family	2.32	0.45
Cellular structure & organization			
At2g45220	pectinesterase family	2.63	3.54
At2g22470	arabinogalactan-protein (AGP2)	4.52	0.78
At3g13520	arabinogalactan-protein (AGP12)	2.57	0.89
Cellular transport			
At2g29940	ABC transporter family protein	12.55	3.01
At2g48020	sugar transporter, putative	2.62	0.50
At5g13170	nodulin MtN3 family protein	2.48	0.63
At3g22620	protease inhibitor/seed storage/lipid transfer protein (LTP) family	2.31	0.25
At3g22600	protease inhibitor/seed storage/lipid transfer protein (LTP) family	8.38	2.78
At2g16830	hypothetical protein	2.22	0.28
At1g07610	metallothionein-related protein	10.83	5.67

Energy			
At5g03630	monodehydroascorbate reductase, putative	10.04	2.44
At5g54500	quinone reductase, putative	2.59	0.49
At5g40760	glucose-6-phosphate dehydrogenase	2.49	0.38
At1g06030	fructokinase, putative	2.47	0.56
At5g25880	malate dehydrogenase - like protein	2.64	0.54
Horomone biosynthesis			
At5g42650	allene oxide synthase / cytochrome P450 74A	19.98	3.69
At1g76680	12-oxophytodienoate reductase (OPR1)	2.62	0.60
At3g44310	nitrilase 1	2.08	0.26
Lipid metabolism			
At5g65110	acyl-CoA oxidase (ACX2)	2.22	0.17
At5g65110	acyl-CoA oxidase (gb|AAC13497.1)	2.18	0.23
At2g33150	acetyl-CoA C-acyltransferase (3-ketoacyl-CoA thiolase), putative	4.58	0.95
At5g47720	acetyl-CoA C-acetyltransferase (acetoacetyl-coenzyme A thiolase), putative	4.17	0.66
At5g48950	thioesterase-related	2.18	0.36
Nucleotide metabolism			
At4g30530	glutamine amidotransferase class-I domain-containing protein	3.67	0.58
Other metabolism			
At3g17810	dehydrogenase -related	4.25	0.58
At5g64370	beta-ureidopropionase	2.08	0.39
At3g22200	4-aminobutyrate aminotransferase (gamma-amino-N-butyrate transaminase/GABA transaminase/beta-alanine--oxoglutarate aminotransferase)	2.01	0.35
At5g59530	2-oxoglutarate-dependent dioxygenase, putative	4.33	2.14
At4g31500	cytochrome P450 83B1	2.06	0.21
Photosynthesis			
At1g19670	coronatine-induced protein 1 (CORI1)	8.88	0.90
At5g59820	zinc finger protein Zat12	4.55	1.55
Protein fate			
At3g53180	nodulin / glutamate-ammonia ligase - like protein	13.32	3.95
At1g53580	glyoxalase II, putative (hydroxyacylglutathione hydrolase)	5.82	1.16
At3g16150	L-asparaginase -related	4.14	1.10
At1g80920	DnaJ protein family	3.51	1.16

At4g36040	DnaJ protein family	2.96	0.99
At5g02500	heat shock protein hsc70-1 (hsp70-1) (hsc70.1)	2.38	0.47
At3g12580	heat shock protein hsp70	2.08	0.35
At1g23440	Arabidopsis thaliana unknown protein (F28C11.8) mRNA, complete cds	2.19	0.35
At4g10540	Arabidopsis thaliana BAC F3H7	2.11	0.29
At5g42040	hypothetical protein	2.06	0.47
At5g51070	ATP-dependent Clp protease ATP-binding subunit (ClpD), ERD1 protein precursor	6.06	1.01
At5g20620	polyubiquitin (UBQ4)	2.80	0.39
At1g02300	Arabidopsis thaliana BAC T7I23, complete sequence	2.66	0.75
At4g02890	polyubiquitin (UBQ14)	2.20	0.38
At5g53050	hydrolase, alpha/beta fold family	3.32	0.22
At5g58310	hydrolase, alpha/beta fold family	2.11	0.36
Protein synthesis			
At5g54940	translation initiation factor-related protein	1.98	0.41
Secondary metabolism			
At1g61120	terpene synthase/cyclase family	6.23	1.04
At5g05600	oxidoreductase, 2OG-Fe(II) oxygenase family	8.28	1.95
At2g30490	cytochrome P450 73 / trans-cinnamate 4-monooxygenase / cinnamate-4-hydroxylase (CYP73) (C4H)	2.30	0.51
Transcription factors (TFs)			
At3g15210	ethylene responsive element binding factor 4 (ERF4)	2.74	0.60
At5g47230	ethylene responsive element binding factor 5 (AtERF5)	2.03	0.57
At2g28200	zinc-finger protein -related	2.19	0.42
At3g50260	AP2 domain transcription factor, putative	2.42	0.23
At4g36990	heat shock transcription factor 4 (HSF4)	3.37	0.83
At2g41710	ovule development protein, putative	3.75	0.53
At1g42990	bZIP family transcription factor	2.28	0.47
At1g31630	MADS-box protein	2.18	0.30
At1g21000	expressed protein	4.18	1.16
At3g17860	expressed protein	2.60	0.31
At1g18830	transducin / WD-40 repeat protein family	2.01	0.40
Unclassified			
At5g22270	expressed protein	10.88	4.07
At2g47770	expressed protein	8.18	0.57
At2g39330	jacalin lectin family	7.00	0.72

At1g07040	expressed protein	6.77	2.73
At2g32210	expressed protein	5.81	1.45
A020750_01	EST	5.52	1.30
At1g52720	expressed protein	5.31	1.17
At3g02480	expressed protein	4.92	1.10
At1g70780	expressed protein	4.65	1.64
At5g55420	hypothetical protein	4.16	5.52
At3g29575	expressed protein	4.08	0.62
At4g07840	transposon protein -related	4.04	0.46
At2g43910	thiol methyltransferase	3.71	0.17
At2g39980	transferase family	3.33	0.69
At5g10040	expressed protein	3.29	0.47
At3g02910	expressed protein	3.25	0.57
At5g42050	expressed protein	3.25	1.10
At4g27450	expressed protein	3.23	0.75
At5g39050	transferase family	3.05	0.74
At4g34180	expressed protein	2.98	2.05
At4g27350	membrane lipo protein lipid attachment site-containing expressed protein	2.97	0.47
At1g54650	expressed protein	2.93	0.24
At5g61820	expressed protein	2.73	0.51
At5g65860	hypothetical protein	2.72	0.38
At4g29270	acid phosphatase-related protein	2.65	0.29
At3g44870	methyltransferase-related	2.63	0.50
At2g24550	expressed protein	2.61	0.63
At4g39730	expressed protein	2.49	0.22
At5g40670	expressed protein	2.32	0.38
At3g02230	reversibly glycosylated polypeptide-1	2.28	0.32
At4g08370	proline-rich protein family	2.15	0.47
At5g47240	MutT/nudix family protein	2.14	0.33
At4g13390	proline-rich protein family	2.13	0.59
At5g35475	hypothetical protein	2.10	0.28
At5g01730	expressed protein	2.09	0.22
A021142_01	Genomic sequence for Arabidopsis thaliana BAC F15O4 from chromosome I	2.09	0.18
At3g19970	expressed protein	2.04	0.27
At2g36680	expressed protein	2.03	0.38

At1g03910	hypothetical protein	2.22	0.32
At2g32190	expressed protein	2.76	0.76
At4g37370	cytochrome P450, putative	8.35	2.07
At4g37410	cytochrome P450, putative	6.78	1.54
At1g13080	cytochrome P450 family	6.39	1.46
At3g26200	cytochrome P450 71B22	2.63	1.03
At5g53560	cytochrome b5	2.14	0.24

Footnotes:

The expression ratios are significant (α=0.05) and in a linear scale where fold change = Inoculated/Uninoculated.

FC [a] – Fold Change

SE [b] – Standard Error

Table A-3 Transcriptional profiling: Genes whose transcript abundance exhibited more than 2-fold decrease*

12 h	Operon Annotation	FC [a]	SE [b]
Cellular structure & organization			
At3g53650	histone H2B, putative	0.81	0.03
Energy			
At5g20000	26S proteasome AAA-ATPase subunit RPT6a	0.86	0.02
Secondary metabolism			
At2g47730	glutathione transferase, putative (GST6)	0.79	0.02
Protein fate			
At4g30810	ESTs	0.72	0.04
Unclassified			
At4g33640	expressed protein	0.77	0.02

24 h	Operon Annotation	FC [a]	SE [b]
Carbohydrate metabolism			
At5g14740	CARBONIC ANHYDRASE 2	0.41	0.09
At3g63140	mRNA binding protein precursor - like	0.46	0.09
At4g03210	xyloglucan endotransglycosylase, putative	0.27	0.14
Cellular communication/Signal transduction			
At3g62030	peptidylprolyl isomerase ROC4	0.50	0.07
Cell rescue & defense			
At5g06290	2-cys peroxiredoxin-related protein	0.43	0.08
Cellular structure & organization			
At5g22740	glycosyltransferase family 2	0.45	0.08
Energy			
At1g20020	ferredoxin--NADP(+) reductase (adrenodoxin reductase), putative	0.42	0.09
At3g16250	ferredoxin - related	0.43	0.06
At5g51010	expressed protein	0.47	0.08
At1g42970	glyceraldehyde-3-phosphate dehydrogenase	0.46	0.07
Nucleotide metabolism			
At5g08610	DEAD box RNA helicase, putative	0.50	0.08

Photosynthesis			
At1g03630	protochlorophyllide reductase C (PCR C/POR C)	0.50	0.07
Protein fate			
At5g45680	immunophilin / FKBP-type peptidyl-prolyl cis-trans isomerase	0.44	0.10
Protein synthesis			
At3g63190	Arabidopsis thaliana DNA chromosome 3, BAC clone F16M2	0.48	0.07
Transcription factors (TFs)			
At2g41940	C2H2-type zinc finger protein -related	0.43	0.03
Unclassified			
At3g13470	chaperonin, putative	0.38	0.08
At2g42840	protodermal factor 1	0.41	0.15
At1g15930	40S ribosomal protein S12 (RPS12A)	0.47	0.10
A012624_01	Arabidopsis thaliana genomic DNA, chromosome 3, P1 clone:MKA23	0.49	0.10
At1g68590	expressed protein	0.50	0.06

48 h	**Operon Annotation**	**FC** [a]	**SE** [b]
Amino acid metabolism			
At2g29630	thiamin biosynthesis protein -related	0.35	0.10
At3g01120	cystathionine gamma-synthase -related	0.46	0.04
At5g14060	aspartate kinase, lysine-sensitive	0.47	0.05
Carbohydrate metabolism			
At1g31190	expressed protein	0.42	0.04
At5g51820	phosphoglucomutase (emb|CAB64725.1)	0.47	0.08
At5g66530	aldose 1-epimerase family	0.47	0.06
At2g39930	isoamylase, putative	0.49	0.07
Cellular communication/Signal transduction			
At2g16630	proline-rich protein family	0.37	0.07
At1g21500	expressed protein	0.50	0.10
Cell death and aging			
At4g27700	rhodanese-like domain protein	0.44	0.09
Cell rescue & defense			
At3g11930	ethylene-responsive protein -related	0.41	0.01
At1g06830	glutaredoxin protein family	0.30	0.12

Cellular structure & organization			
At5g04970	pectinesterase, putative	0.50	0.15
Cellular transport			
At2g45180	protease inhibitor/seed storage/lipid transfer protein (LTP) family	0.33	0.11
At2g38530	nonspecific lipid transfer protein 2 (LTP 2)	0.35	0.10
At1g12090	protease inhibitor/seed storage/lipid transfer protein (LTP) family	0.43	0.12
At4g24120	expressed protein	0.49	0.10
At5g10180	sulfate transporter	0.38	0.10
At3g48740	nodulin MtN3 family protein	0.46	0.08
Development			
At1g69120	floral homeotic gene APETALA1	0.48	0.05
Energy			
At4g26530	fructose-bisphosphate aldolase, putative	0.49	0.13
At4g26520	fructose-bisphosphate aldolase, cytoplasmic	0.45	0.11
At5g22620	expressed protein	0.44	0.02
Nucleotide metabolism			
At5g24300	starch synthase, putative	0.47	0.07
Other metabolism			
At4g13770	cytochrome P450 family	0.44	0.20
At2g18230	inorganic pyrophosphatase -related	0.48	0.10
At3g63410	chloroplast inner envelope membrane protein, putative	0.48	0.06
At4g15560	DEF (CLA1) protein	0.50	0.06
Photosynthesis			
At2g05100	light-harvesting chlorophyll a/b binding protein	0.39	0.07
At5g54270	light-harvesting chlorophyll a/b binding protein, putative	0.45	0.09
At2g40100	light-harvesting chlorophyll a/b binding protein	0.48	0.03
Protein fate			
At5g49910	heat shock protein cpHsc70-2 (hsc70-7)	0.47	0.12
At5g49910	heat shock protein cpHsc70-2 (hsc70-7)	0.50	0.12
At5g12860	oxoglutarate/malate translocator, putative	0.50	0.06
At3g11950	UbiA prenyltransferase family	0.49	0.06
At5g52640	heat shock protein 81-1 (HSP81-1/heat shock protein 83/HSP83)	0.47	0.09

Protein synthesis			
At5g20290	ESTs, Weakly similar to RS8_HUMAN 40S RIBOSOMAL PROTEIN S [H.sapiens]	0.45	0.06
Unclassified			
At1g08380	expressed protein	0.31	0.08
At5g45950	GDSL-motif lipase/hydrolase protein	0.31	0.10
At1g29670	GDSL-motif lipase/hydrolase protein	0.37	0.07
At4g37230	photosystem II oxygen-evolving complex like protein	0.37	0.08
At2g04570	GDSL-motif lipase/hydrolase protein	0.38	0.17
At1g48600	Genomic sequence for Arabidopsis thaliana BAC T1N15 from chromosome I	0.41	0.08
At5g08050	expressed protein	0.43	0.03
At2g46100	expressed protein	0.43	0.09
At5g03120	expressed protein	0.45	0.01
At5g47550	expressed protein	0.45	0.09
At5g58250	unknown protein (sp|P72777) -related	0.46	0.12
A004996_01	Arabidopsis thaliana BAC T15F16	0.47	0.06
At1g78140	expressed protein	0.49	0.07
At1g03340	hypothetical protein	0.49	0.03
At1g32220	expressed protein	0.49	0.05
At5g61050	hypothetical protein	0.49	0.04
At5g48930	anthranilate N-hydroxycinnamoyl/benzoyltransferase family	0.50	0.10
A004039_01	Genomic sequence for Arabidopsis thaliana BAC F1N21 from chromosome I	0.50	0.04
At5g39530	expressed protein	0.49	0.05
At5g02160	expressed protein	0.50	0.07
At4g27520	expressed protein	0.47	0.05

Footnotes:

* Less than 0.5 fold, except 12 h

The expression ratios are significant (α=0.05) and in a linear scale where fold change = Inoculated/Uninoculated.

FC [a] – Fold Change

SE [b] – Standard Error

Table A-4 Expression sequence tags (ESTs) identified for *BnWRKY* genes.

gene	No. of ESTs	EST components
BnWRKY1	22	DT469158,CD837211,CD837250,EV096280,EV047798,EE410429,EV036426,EV192534,EE417846,EV082903,ES978073,ES987430,EV066236,EV192462,EV191916,EV096200,FG555897,FG559810,EL629346,EL680858,EL680931,EL627918
BnWRKY2	13	EV158462,EV158373,EV194691,EE550855,EV020176,EV165306,EV136394,EV136185,EE553016,ES990089,EV194778,EL630589,FG560352
BnWRKY3	5	EE474685,EV103205,EE444852,EL626082,EL630352
BnWRKY4	3	CD837784,EE447978,EE565682
BnWRKY6	2	DY017333,FG559398
BnWRKY7	18	CD837871,CD837904,EE511922,ES990929,EV004846,EV007063,CD832622,EV009666,EV004551,ES995585,EV145568,EV145880,EV072011,EE440038,EE449594,ES958216,FG558193,FG565394
BnWRKY8	3	EV226150,EV225804,DY012193
BnWRKY10	5	EV225008,EV225336,EE568483,DY030626,ES994380
BnWRKY11	40	DY022859,EV199012,EV220154,EE421634,EV168434,ES902933,ES922025,DV643318,EV222591,EV222773,DY017702,CN730450,CN732732,EV057941,EV220578,EV027305,EV117836,EE471365,EV198928,EV220289,EV038247,EV181284,EV181367,EV168383,EE552575,EV071576,DW998334,DY022055,ES902980,H07803,EV100178,ES986857,EV100110,EE557476,EE434934,EV128579,EE560606,ES928140,EE476951,ES913094,FG555052
BnWRKY15	4	ES905501,EE409487,ES898331,BP999905
BnWRKY17	13	EV109970,EV185875,CN732624,CX278263,EV186271,ES912748,ES966918,EE422062,EE549995,ES961630,FG557424,FG573734
BnWRKY18	6	EV180393,EV180322,EV022164,DY020324,EV218409,EE565297
BnWRKY19	5	ES266880,EV117746,DY000072,EE429793,EL591134
BnWRKY20	14	CD836659,ES987965,ES905087,DY020787,EV191690,CD827221,EV191751,CN727401,ES978085,CN726232,ES907877,EV073321,EL626446,EL623865
BnaWRKY21	25	EV055736,EV031231,EV110595,CX194536,ES902472,ES989407,CX190008,EE549913,EE545018,ES960297,EV199108,EV198463,CD833115,CD836643,DY023699,CX189812,EE441904,EE426569,FG557000,FG557222,FG560824,FG562549,FG563803,FG571543,EL626852
BnWRKY22	3	EV068697,EE473343,EV193324
BnWRKY24	3	EV179750,ES914021,EV179662

BnWRKY25	10	EV139852,EV142157,EV173812,EV173832,EV226261,EV225959,EV141772,EV139663,EE423196,ES967361
BnWRKY26	1	ES900871
BnWRKY27	3	ES900582,EV083616,FG557795
BnWRKY28	3	EV137958,EV138384,EV138299
BnWRKY29	5	EV222778,EV222601,EV216404,EE447187,EV070763
BnWRKY30	1	EE541499
BnWRKY31	6	EV159546,EV149466,EV149791,EV149270,EV148981,EV159465
BnWRKY32	26	CX279701,EV174315,EV140393,EV139807,EV116543,ES905270,EV140601,EV140622,EV140633,EV140653,EV164258,EV174278,EV168577,EV140569,EV140553,EV140545,EV140525,EV186567,EV140028,EV068440,EV086727,EV089397,EE409285,EE411621,EE411622,EV164510,EV116630
BnWRKY33	12	EV116083,EV115998,EV115582,CX191595,DV643313,EV115494,DT469131,EE462750,EV137878,EV132514,EV131869,EV138268,EV105711
BnWRKY34	2	EV156771,EV156681
BnWRKY35	2	EV121815,EV121903
BnWRKY36	1	EV118852
BnWRKY39	4	ES951988,CX194758,ES926580,EV176491
BnWRKY40	11	CX192308,DY025052,EV028236,EV028308,EG020011,EV125065,EV144982,EV145203,EV124973,H74419,H74877
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BnWRKY46	3	DY011024,EG020824,EV037660
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BnWRKY51	2	EV177355,EV177290
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BnWRKY72	13	ES903951,EV114675,EV118438,EE454582,ES912067,EV220022,ES903554,EV118360,EV217436,EV217628,EE410362,ES903639,EV219849
BnWRKY74	6	CN737398,EE434455,EV025301,ES908505,EV113654,EV113567
BnWRKY75	2	EV107944,EV107580

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BnAOC3_2      1 ------------------------------------------------------------
consensus     1

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BnAOC3_2      1 --------------TCCNAGTTCCAAGTCTTCCCTTAACATACGAAATAAAATGGCTACT
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BnAOC3_2    287 GAACTCAACGTGTACGAACTCAACGAAGGAGATAGAAACAGCCCAGCTGTTCTAAAACTC
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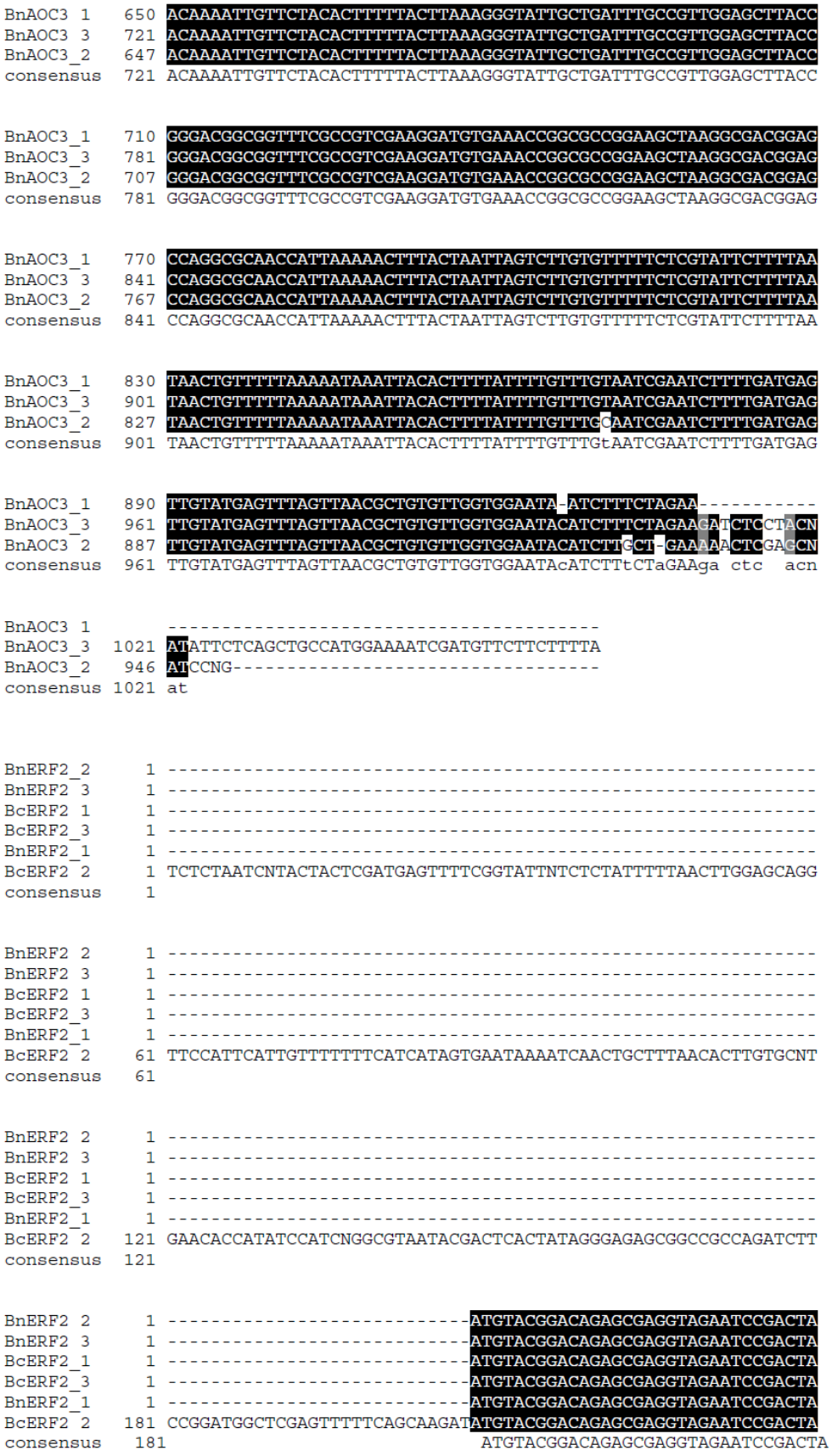
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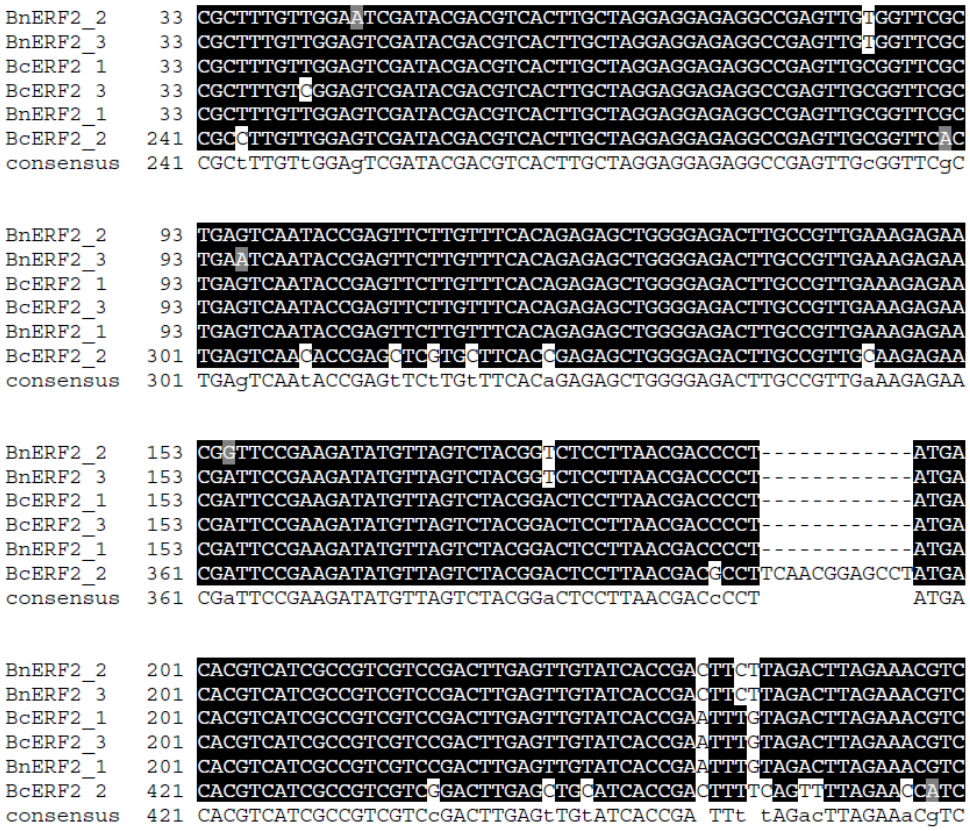
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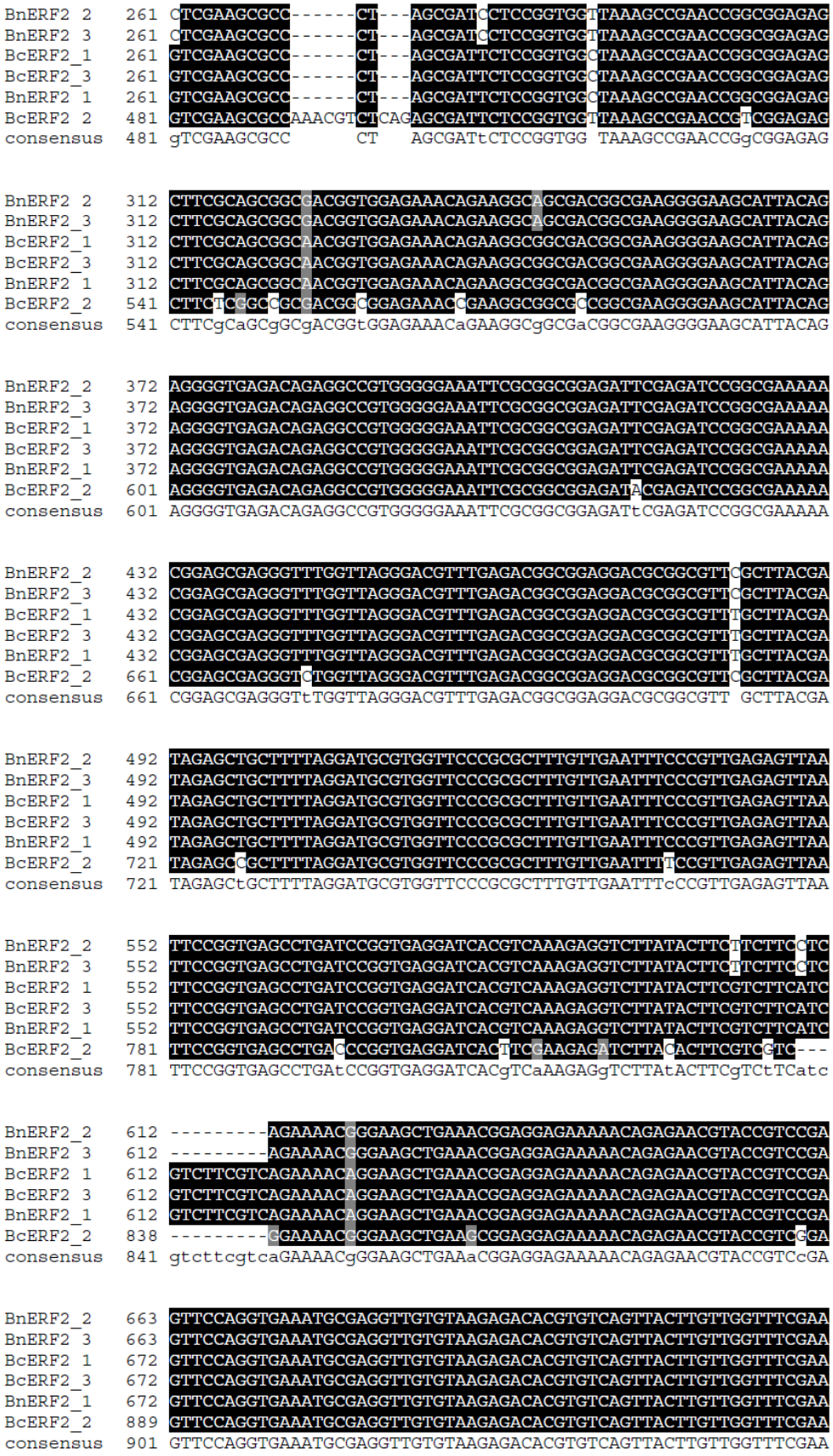
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BcTGA5_2    241 GAATCTAGCGACCGTTCAAAGGAAAAAATGGATCAAAAGACGGTTCGTAGGCTCGCTCAA
consensus   241 GAaTCTAGCGACCGTTCAAAGGAaAAAATGGATCAAAAGACGGTTCGTAGGCTCGCTCAA

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BcTGA5_1    300 AACCGTGAGGCTGCAAGGAAAAGCAGATTGAGGAAGAAAGCATATGTTCAGCAGCTAGAG
BcTGA5_2    301 AACCGTGAGGCTGCAAGGAAAAGCAGATTGAGGAAGAAAGCATATGTTCAGCAGCTAGAG
consensus   301 AACCGTGAGGCTGCAAGGAAAAGCAGATTGAGGAAgAAAGCATATGTTCAGCAGCTAGAG

BnTGA5_1    246 AACAGCCGTTTGAAGCTAACACAACTTGAACAGGAGCTGCAAAGAGCTAGGCAACAAGGT
BnTGA5_2    361 AACAGCCGTTTGAAGCTAACACAGCTTGAACAGGAGCTGCAAAGAGCAAGGCAACAAGGT
BcTGA5_1    360 AACAGCCGTTTGAAGCTAACACAGCTTGAACAGGAGCTGCAAAGAGCTAGGCAACAAGGT
BcTGA5_2    361 AACAGCCGTTTGAAGCTAACACAGCTTGAACAGGAGCTGCAAAGAGCTAGGCAACAAGGT
consensus   361 AACAGCCGTTTGAAGCTAACACAgCTTGAACAGGAGCTGCAAAGAGCtAGGCAACAAGGT

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BnTGA5_2    421 GTCTTTATCTCAAGCTCTGGAGACCAAGCCCATTCAACTACTGGAAATGGGGCAATGGCG
BcTGA5_1    420 GTCTTTATCTCAAGCTCTGGAGACCAAGCCCATTCTACTACTGGAAATGGGGCAATGGCG
BcTGA5_2    421 GTCTTTATCTCAAGCTCTGGAGACCAAGCCCATTCTACTACTGGAAATGGGGCAATGGCG
consensus   421 GTCTTTATCTCAAGCTCTGGAGACCAAGCCCATTCtACTACTGGAAATGGGGCAATGGCG

BnTGA5_1    366 TTTGATGCAGAGTACAGACGCTGGCAGGAAGATAAGAACAGAAAGATGAAGGAGCTGAGC
BnTGA5_2    481 TTTGATGCAGAGTACAGACGCTGGCAGGAAGATAAGAACAGAAAGATGAAGGAGCTTAGT
BcTGA5_1    480 TTTGATGCAGAGTACAGACGCTGGCAGGAAGACAAAAACAGAAAGATGAAGGAGCTGAGC
BcTGA5_2    481 TTTGATGCAGAGTACAGACGCTGGCAGGAAGACAAAAACAGAAAGATGAAGGAGCTGAGC
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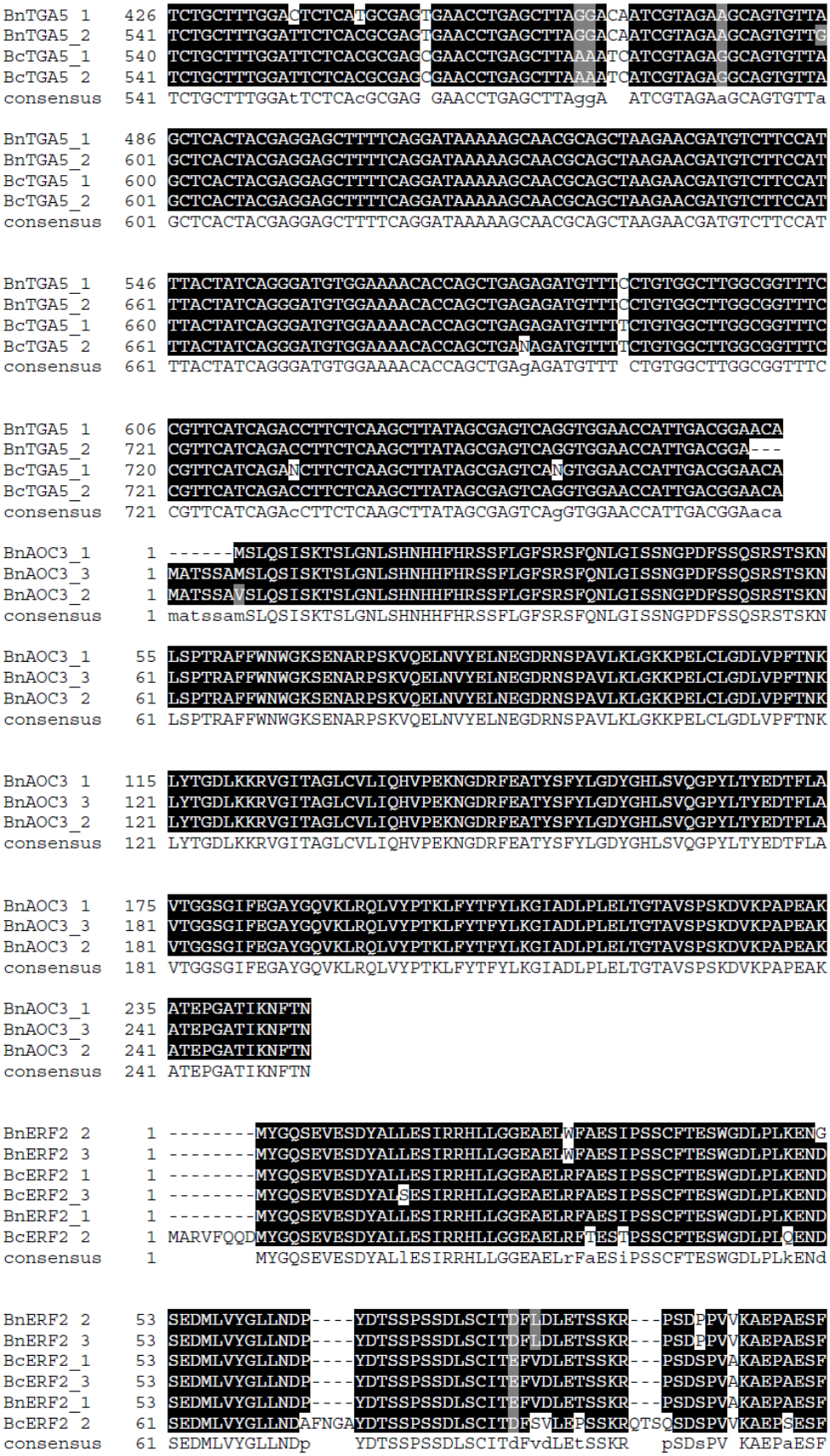
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BcTGA5_2 541 TCTGCTTTGGATTCTCACGCGAGCGAACCTGAGCTTAAAATCATCGTAGAGGCAGTGTTA
consensus 541 TCTGCTTTGGAtTCTCAcGCGAG GAACCTGAGCTTAggA ATCGTAGAaGCAGTGTTa
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BcTGA5_2 601 GCTCACTACGAGGAGCTTTTCAGGATAAAAAGCAACGCAGCTAAGAACGATGTCTTCCAT
consensus 601 GCTCACTACGAGGAGCTTTTCAGGATAAAAAGCAACGCAGCTAAGAACGATGTCTTCCAT
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BnTGA5_2 721 CGTTCATCAGACCTTCTCAAGCTTATAGCGAGTCAGGTGGAACCATTGACGGA---
BcTGA5_1 720 CGTTCATCAGANCTTCTCAAGCTTATAGCGAGTCANGTGGAACCATTGACGGAACA
BcTGA5_2 721 CGTTCATCAGACCTTCTCAAGCTTATAGCGAGTCAGGTGGAACCATTGACGGAACA
consensus 721 CGTTCATCAGAcCTTCTCAAGCTTATAGCGAGTCAgGTGGAACCATTGACGGAaca
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BnAOC3_3 1 MATSSAMSLQSISKTSLGNLSHNHHFHRSSFLGFSRSFQNLGISSNGPDFSSQSRSTSKN
BnAOC3_2 1 MATSSAVSLQSISKTSLGNLSHNHHFHRSSFLGFSRSFQNLGISSNGPDFSSQSRSTSKN
consensus 1 matssamSLQSISKTSLGNLSHNHHFHRSSFLGFSRSFQNLGISSNGPDFSSQSRSTSKN
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BnAOC3_2 61 LSPTRAFFWNWGKSENARPSKVQELNVYELNEGDRNSPAVLKLGKKPELCLGDLVPFTNK
consensus 61 LSPTRAFFWNWGKSENARPSKVQELNVYELNEGDRNSPAVLKLGKKPELCLGDLVPFTNK
BnAOC3_1 115 LYTGDLKKRVGITAGLCVLIQHVPEKNGDRFEATYSFYLGDYGHLSVQGPYLTYEDTFLA
BnAOC3_3 121 LYTGDLKKRVGITAGLCVLIQHVPEKNGDRFEATYSFYLGDYGHLSVQGPYLTYEDTFLA
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consensus 121 LYTGDLKKRVGITAGLCVLIQHVPEKNGDRFEATYSFYLGDYGHLSVQGPYLTYEDTFLA
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consensus 181 VTGGSGIFEGAYGQVKLRQLVYPTKLFYTFYLKGIADLPLELTGTAVSPSKDVKPAPEAK
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BnAOC3_3 241 ATEPGATIKNFTN
BnAOC3_2 241 ATEPGATIKNFTN
consensus 241 ATEPGATIKNFTN
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BnERF2_3 1 --------MYGQSEVESDYALLESIRRHLLGGEAELWFAESIPSSCFTESWGDLPLKEND
BcERF2_1 1 --------MYGQSEVESDYALLESIRRHLLGGEAELRFAESIPSSCFTESWGDLPLKEND
BcERF2_3 1 --------MYGQSEVESDYALSESIRRHLLGGEAELRFAESIPSSCFTESWGDLPLKEND
BnERF2_1 1 --------MYGQSEVESDYALLESIRRHLLGGEAELRFAESIPSSCFTESWGDLPLKEND
BcERF2_2 1 MARVFQQDMYGQSEVESDYALLESIRRHLLGGEAELRFTESTPSSCFTESWGDLPLQEND
consensus 1 MYGQSEVESDYALlESIRRHLLGGEAELrFaESiPSSCFTESWGDLPLkENd
BnERF2_2 53 SEDMLVYGLLNDP----YDTSSPSSDLSCITDFLDLETSSKR---PSDPPVVKAEPAESF
BnERF2_3 53 SEDMLVYGLLNDP----YDTSSPSSDLSCITDFLDLETSSKR---PSDPPVVKAEPAESF
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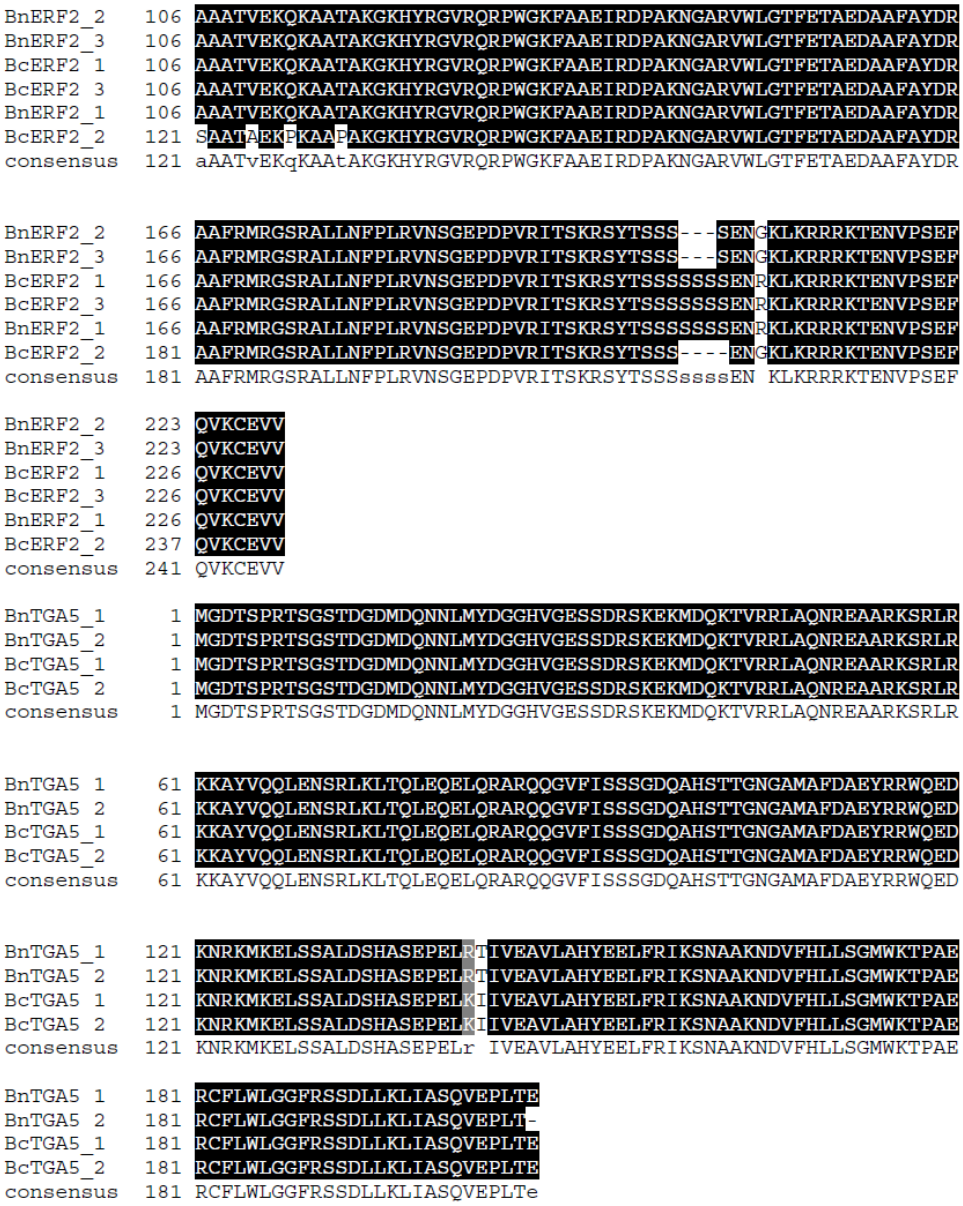


Figure A-1 Mutiple alignments of cDNA/amino acid sequence of different alleles of genes *AOC3*, *ERF2* and *TGA5* by BOXSHADE3.21 (http://www.ch.embnet.org/software/BOX_form.html).

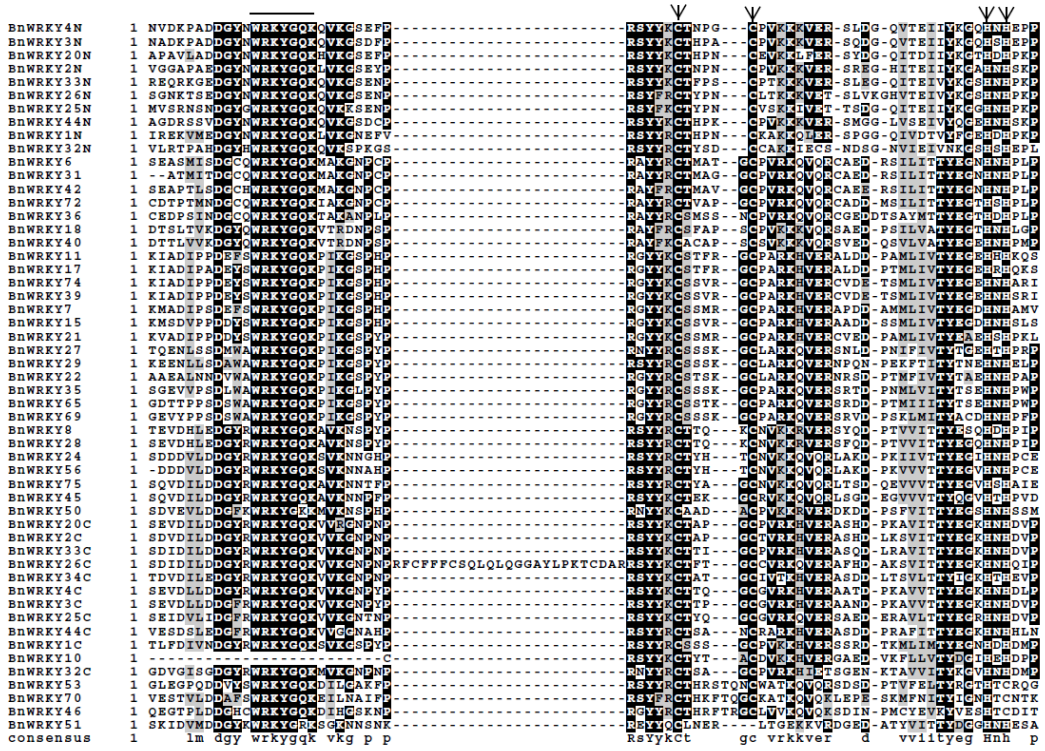
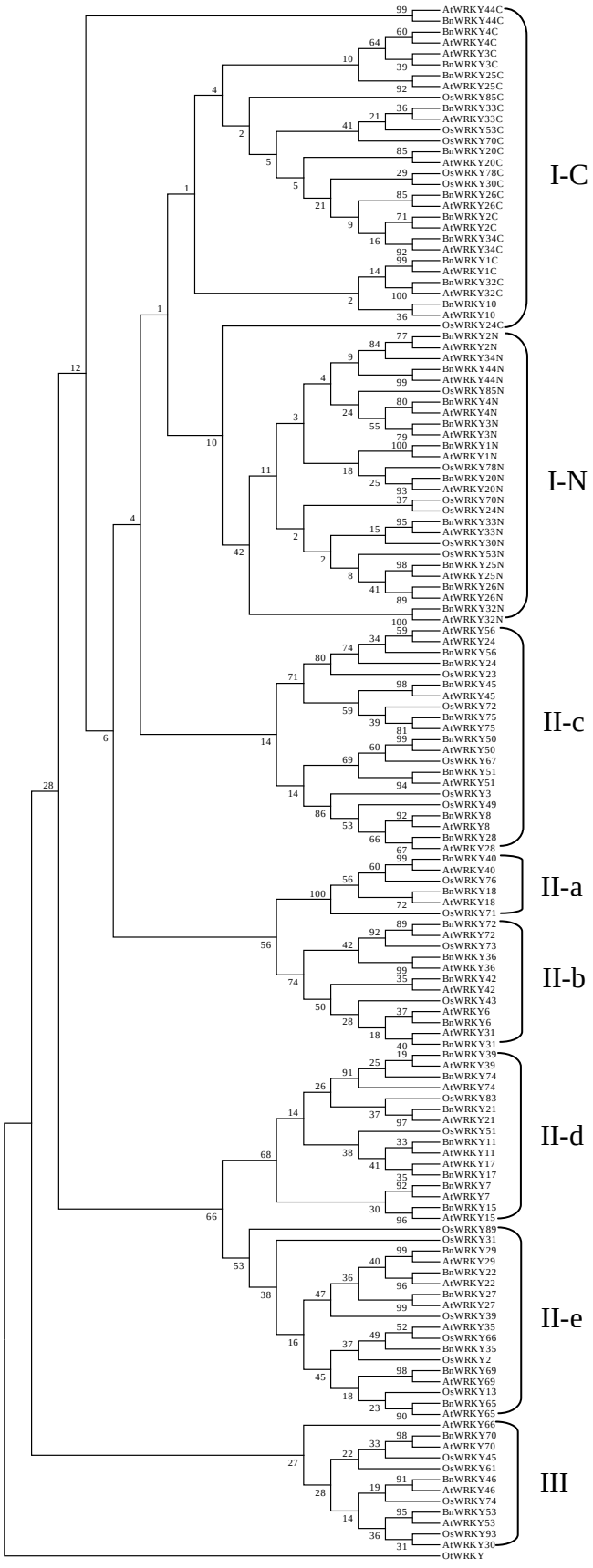


Figure A-2 Alignment of Sequences of 52 WRKY domains of BnWRKY transcription factors. Identical amino acids are shaded in black, and similar amino acids are shaded in gray. The conserved WRKYGQK heptapeptide or its variants are underlined at the top of the alignment and, the cysteines and histidines of the C2H2- or C2HC-type zinc finger motif are indicated by arrows. The consensus amino acids are shown at the bottom of the alignment. This alignment was produced by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).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BnWRKY72
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III
OtWRKY

Figure A-3 A bootstrap consensus maximum parsimony tree of WRKY TFs in canola, Arabidopsis and rice (*japonica*). Only the WRKY domain residues were aligned using ClustalX (v1.83) and the evolutionary history was inferred using the maximum parsimony method in MEAG4. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. All alignment gaps were treated as missing data. There were a total of 98 positions in the final dataset, out of which 55 were parsimony informative. The two letters N and C after group I represent the N-terminal and the C-terminal WRKY domains of group I proteins, respectively. A chlorophyte alga, *Ostreococcus tauri* (Ot) WRKY (Acc. CAL54953) is used as the outgroup.