The regulation of intrinsic signaling in *Brassica napus* defending against *Leptosphaeria maculans*

by

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A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Plant Science

University of Manitoba

Winnipeg

ABSTRACT

The regulation of intrinsic signaling in *Brassica napus* defending against *Leptosphaeria maculans*

Cunchun Yang, Ph.D., University of Manitoba, August 2021

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Plants are able to trigger multiple signaling pathways to cope with pathogenic invasion. Hypersensitive response (HR), one of the effective mechanisms, is triggered by the interaction between pathogenic Avr effectors from the pathogen and plant R proteins (also known as gene-for-gene interaction). Plant tissues induce distinct activities when they have, or have no HR, and those differences may help scientists to find out the crucial factors in efficient defense against plant pathogens. The general objective of this Ph.D. thesis is based on that background and applied on the studies of *Brassica napus – Leptosphaeria maculans* pathosystem. Three cultivars of *B. napus* (Westar, Surpass400 and 01-23-2-1) were inoculated by two *L. maculans* isolates (HCRT75 8-1 and HCRT77 7-2) to cause three distinct levels of severity: susceptible, intermediate and resistant. Expression studies (by RT-qPCR), histochemical assays (such as trypan blue staining) were applied on the cotyledons of those cultivars to search the differences in defense response from those cultivars (with distinct severities). Histochemical assays transcriptional analysis suggested that the intermediate and resistant genotypes (i.e. Surpass400 and 01-23-2-1) displayed earlier H_2O_2 accumulation and cell death on the cotyledons. and activation of the genes related to salicylic acid (SA) and ROS (as early as 3 and 5 dpi). The results indicated that the early activation of SA/ROS signaling is one of the crucial components for *B. napus* to defend against *L. maculans*. Environmental factors are essential components for plant growth/development; therefore, it is reasonable that the environmental changes are able to alter the actions in plant defense. The third part of this study was to explore the association between temperature and HR resistance. Lesion measurements suggested that high incubation temperature resulted in larger lesion size. RT – qPCR results reflected the distinct expression levels of putative temperature – sensitive genes among three incubating temperature conditions (28 ^{o}C/ 22 ^{o}C). The results indicated that 22 ^{o}C/16 ^{o}C condition is the peak point for *PR1/2*

expression. This study suggested that the defense in *B. napus* was affected by the temperature and might have an optimal temperature to elicit robust defense signals.

ACKNOWLEDGEMENT

I want to thank my advisor Dr. Dilantha Fernando for giving me the opportunity to do this research. I learned new knowledge in plant pathology, plant breeding, plant physiology and new lab techniques.

I also want to thank the other three-committee members: Dr. Mark Belmonte, Dr. Claudio Stasolla and Dr. Genyi Li for giving me useful advice during my study.

I particularly appreciated the help from Ms. Paula Parks for providing me a large amount of assistance. I also need to mention the support from currently and previous lab members including Dr. Rhodesia Celoy, Dr. Fei Liu, Dr. Carrie Selin, Dr. Aria Dolatabadian etc., without your help, I could not finish my Ph.D. study successfully.

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CONTRIBUTIONS OF AUTHORS

Literature Review

Part of this Literature Review has now been published: Cunchun Yang, Aria Dolatabadian, W. G. Dilantha Fernando. 2021. The Wonderful World of Intrinsic and Intricate Networks to Defend Plants against Pathogens. *Canadian Journal of Plant Pathology* DOI: 10.1080/07060661.2021.1960610 (IF=2.42)

The conceptualization and writing of the review was done by C.Y.; the revisions were done by W.G.D.F.; the figures and legends in the review (**Figure 2.3, 2.4, 2.5 and 2.6**) were drawn and prepared with the assistance by A.D..

Chapter 3 Hormonal Responses to Susceptible, Intermediate, and Resistant Interactions in the *Brassica napus–Leptosphaeria maculans* Pathosystem

Published in International Journal of Molecular Sciences: Cunchun Yang and W. G. D. Fernando 2021. Hormonal Responses to Susceptible, Intermediate, and Resistant Interactions in the *Brassica napus–Leptosphaeria maculans* Pathosystem. Intl. J. Mol. Sci. 22: 4714. DOI: 10.3390/ijms22094714 (IF=5.923)

Author Contributions: Conceptualization, C.Y. and W.G.D.F.; methodology, C.Y.; validation, C.Y. and W.G.D.F.; formal analysis, C.Y.; investigation, C.Y.; resources, data curation, C.Y.; writing—original draft preparation, C.Y.; writing—review and editing, C.Y. and W.G.D.F.; supervision, W.G.D.F.; project administration, W.G.D.F.; funding acquisition, W.G.D.F. All authors have read and agreed to the published version of the manuscript.

Chapter 4 Analysis of the Oxidative Burst and Its Relevant Signaling Pathways in *Leptosphaeria maculans—Brassica napus* Pathosystem

Published in International Journal of Molecular Sciences: Cunchun Yang and W. G. D. Fernando 2021. Analysis of the Oxidative Burst and Its Relevant Signaling Pathways in *Leptosphaeria maculans—Brassica napus* Pathosystem. Intl. J. Mol. Sci. 22: 4812. doi: /10.3390/ijms22094812 (IF=5.923)

Author Contributions: Conceptualization, C.Y. and W.G.D.F.; methodology, C.Y.; validation, C.Y. and W.G.D.F.; formal analysis, C.Y.; investigation, C.Y.; resources, W.G.D.F.; data curation, C.Y.; writing—original draft preparation, C.Y.; writing—review and editing, C.Y. and W.G.D.F.; supervision, W.G.D.F.; project administration, W.G.D.F.; funding acquisition, W.G.D.F. All authors have read and agreed to the published version of the manuscript.

Chapter 5 The Effect of Temperature on the Hypersensitive Response (HR) in the *Brassica napus–Leptosphaeria maculans* Pathosystem

Published in: Plants: Cunchun Yang, Zhongwei Zou and W. G. D. Fernando 2021. The Effect of Temperature on the Hypersensitive Response (HR) in the *Brassica napus–Leptosphaeria maculans* Pathosystem. Plants 10: 843. doi: 10.3390/plants10050843 (IF=3.935)

Author Contributions: Conceptualization, C.Y. and W.G.D.F.; methodology, C.Y.; validation, C.Y. and W.G.D.F.; formal analysis, C.Y.; investigation, C.Y.; resources, Z.Z.; data curation, C.Y.; writing—original draft preparation, C.Y.; writing—review and editing, C.Y. and W.G.D.F.; supervision, W.G.D.F.; project administration, W.G.D.F.; funding acquisition, W.G.D.F. All authors have read and agreed to the published version of the manuscript. Z.Z. did data curation for the manuscript.

1 GENERAL INTRODUCTION

Canola, as a brand of cultivars from rapeseeds and field mustards, has become very important as a crop to Canada, also to the world, since they have desired low amount of erucic acid and glucosinolate which have bad tastes and harmful effects on human and animal consumption (Canola Council of Canada, 2020). Blackleg (*Leptosphaeria maculans*) has become a great threat since 1975 in Canada (Bailey et al., 2003), it could wipe out 90% of the canola production. Blackleg can cause stem canker, root rot, leaf lesion (with black dots) and black dots on stubble (Canola Council of Canada, 2020; Zhang and Fernando, 2017).

Blackleg can be managed by many methods including crop rotation, fungicides, however, development of genetically resistant rapeseed (*Brassica napus*) is the most cost-effective and environmentally friendly strategy (Raman et al., 2011). There are two general strategies the host has to combat disease. Single or oligo-gene resistance (also called qualitative resistance, which is the strong and short-lived resistant mechanism to stop pathogenic growth by inducing rapid and localized cell death around the site of infection Multi-gene resistance (also called quantitative resistance, attained by the cooperation of many genes, which is a slow but long-lasting resistance) is the second method (Agrios, 2004).

The breeding for single/oligo-gene resistance is based on the principal of gene-for-gene interaction between AvrLm proteins from *L. maculans* and Rlm's from *B. napus*. The interactions between matched AvrLm and Rlm proteins triggered a mechanism called hypersensitive response (HR), which induces series of cell signaling cascades to interrupt the fungal development (Agrios, 2004; Van de Wouw et al., 2011). Localized signaling pathways triggered by HR include reactive oxygen species (ROS) production, hormonal biosynthesis/signaling, systemic acquired resistance (SAR) and so on (Knepper and Day, 2010).

To date, sixteen *AvrLm* genes (*AvrLm1* to *4-7*, *AvrLmJ1*, *AvrLm6* to *11*, *AvrLepR1 to 4*) (Rouxel and Balesdent, 2005; Gout et al., 2006; Fudal et al., 2007; Parlange et al., 2009; Kutcher et al., 2011; Balesdent et al., 2013; Van de Wouw et al., 2014) and sixteen *Rlm* genes (*Rlm1* to *11*; *LepR1* to *4*; *RlmS*) have been identified (Balesdent et al., 2002; Yu et al., 2005; Yu et al., 2012; Kutcher et al., 2011; Balesdent et al., 2013; Ghanbarnia et al., 2015). With the progresses of research, more *AvrLm* and *Rlm* genes will be identified in future.

According to Delourme et al., (2004), qualitative resistance is the major resistance during seedling stage, while quantitative resistance emerges during adult plant stage. Quantitative resistance in *L. maculans – B. napus* pathosystem is race non-specific defense attributed by polygenic network of minor genes. Studying quantitative resistance are based on identifying the QTLs from the host of which the existences may enhance the resistance to many pathogens. For example, *WRKY45* in rice was considered as an important QTL for resistance to bacterial and fungal diseases such as rice blast (Yang et al., 2009; Shimono et al., 2012). Delourme et al., (2014) confirmed that the breeding of *B. napus* varieties combining qualitative and quantitative resistance is an approach with stronger efficacy, the quantitative resistance delayed the *L. maculans* races from overcoming major genes (*Rlm* genes).

Recent studies are showing that both qualitative and quantitative resistances have complex downstream genetic networks to achieve the effects and there are potential connections between these two types of resistance (Tao et al., 2003; Poland et al., 2009; Stotz et al., 2014; Becker et al., 2017).

In general, plant defense involves a large and complicated signaling network and those signaling pathways alter the behavior of plant tissues including callose deposition, lignin biosynthesis, ROS production (Bari and Jones, 2009; Vlot et al., 2009; Knepper and Day, 2010; Baxter et al., 2013; Becker et al., 2017). It is necessary to investigate the crucial genes and activities that confer the effective resistance from both qualitative and quantitative background resistances. On the other hand, the timing and pattern of expression of certain types of genes may also affect the expression of plant defense (Tao, et al., 2003; Becker et al., 2017). Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) have been found as the three major hormones in plant defense, but other hormones may also participate (Kunkel and Brooks, 2002; Bari and Jones, 2009). The hormonal signaling is able to activate the production of anti-microbial proteins such as pathogenesis–related proteins (PRs), to stop the proliferation of the phytopathogens (Kunkel and Brooks, 2002; Bari and Jones, 2009; Berens et al., 2017).

Moreover, ROS signaling is also a crucial part of plant defense, which has been found to play roles in activities inhibiting pathogenic growth including cell death/lesion formation, electrolyte leakage, MAPK signaling, those types of signaling transduction are able to trigger the expression of defense related genes to stop pathogenic attacks (Overmyer et al., 2000; Rao

et al., 2002; Torres and Dangl, 2005; Zurbriggen et al., 2010; Baxter et al., 2013; Qi et al., 2017).

The signal programming between compatible and incompatible interactions are distinct, the compatible interaction exhibited more defense signals related to innate/quantitative resistance (Tao et al., 2003; Poland et al., 2009; Lowe et al., 2014; Becker et al., 2017). Localized PCD is considered as a remarkable feature of qualitative resistance achieving HR. However, Yu et al., (1998) revealed that PCD was not the sole component in HR. In Arabidopsis, mutants of the gene named "Defense, no death" 1 (*DND1*) conferred the HR without PCD, but still restricted the growth of the pathogen (Yu et al., 1998), similar situations also occurred to a *DND1* analog (*DND2*) demonstrated by Jurkowski et al., (2004). Both Yu et al., (1998) and Jurkowski, et al., (2004) found the reduction of cell death, SA accumulation and enhanced *PR* genes induction in their *dnd* mutants. In *dnd1* mutants, enhanced SAR was induced to compensate the lost expression of defense mediated by cell death, while *dnd2* mutants induced the resistance mediated by other *R* genes such as *RPS2* and *RPM1*. By considering the previous studies above, it is easy to consider that there are overlapping signaling pathways between qualitative resistance and quantitative or basal resistance, and even possibly two types of resistance have cooperation and their signaling pathways have effects on each other in plant defense. It is suggested that the general expression profiles between compatible and incompatible interactions are similar, however some defense genes are expressed in high amounts at certain time points from the incompatible interaction, which could be the key factor for effective hindrance of the pathogenic invasion (Tao et al., 2003; Becker et al., 2017).

Since, plant defense involves a complicated network of cellular signaling, it is important to explore the pivotal components within this network when studying plant–microbe interactions in the *B. napus–L. maculans* pathosystem, and it is necessary to study the onset patterns of the defense signals to see whether the timing of the defense activity is crucial for effective resistance. It is also necessary to study the switching of *B. napus* defense due to external factors such as temperature**.** The research towards essential factors related to plant defense is broken into three objectives.

Objective 1

Hormonal Responses to Susceptible, Intermediate, and Resistant Interactions in the *Brassica napus–Leptosphaeria maculans* Pathosystem

Objective 2

Analysis of the Oxidative Burst and Its Relevant Signaling Pathways in *Leptosphaeria maculans - Brassica napus* Pathosystem

Objective 3

The Effect of Temperature on the Hypersensitive Response (HR) in the *Brassica napus–Leptosphaeria maculans* Pathosystem

2 LITERATURE REVIEW

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2.1 Introduction

Like animal species, plants are often exposed to various types of invaders including viruses, bacteria, fungi, nematodes and insects. Through evolution, plants have developed a set of sophisticated strategies to cope with the potential stresses.

Generally, plant defense against biotic stresses is divided into basal and race-specific defense. The basal resistance confers a set of physiological activities such as structural change (e.g. lignification), generation of antimicrobial metabolites (e.g. phytoalexins) and programmed cell death (PCD) (Agrios, 2004). Plants usually only encounter high amounts of infection during specific time periods; moreover, because of the developmental stages and other factors such as environment, one plant may be affected by only certain type(s) of stresses. Therefore, the host plant may recognize certain races of the pathogen to elicit specific activities to combat it, which is considered as race-specific resistance (Agrios, 2004).

One major mechanism is the recognition of pathogen-associated molecular patterns (PAMPs), which triggers basal disease resistance and is referred to as PAMP-triggered immunity (PTI). PAMPs are the slow-evolving pathogenic molecules which are recognized by the host. Some important pathogenic components such as flagellin, elongation factor Tu, and chitin can act as PAMPs (Jones and Dangl, 2006; Anderson et al., 2010). The host recognition of PAMPs is triggered by direct and indirect interactions, from which the molecules activate a group of transmembrane proteins such as pattern recognition receptors (PRRs). The interactions are able to trigger signaling pathways including MAPK cascades, gene expression, and phytohormone secretion (Kushalappa et al., 2016). One well-studied PAMP is a conserved 22-amino acid domain from bacterial flagellin (named flg22) (Jones and Dangl, 2006). The flg22 domain binds to a host leucine (Leu)-rich transmembrane receptor FLAGELLIN SENSITIVE2 (FLS2),

from which the host cells are stimulated to initiate the subsequent defense signaling (Chinchilla et al., 2006; Jones and Dangl, 2006; Zipfel et al., 2004).

Race-specific defense is the type of plant defense in which plants elicit defense signaling against specific pathogens. Effectors secreted by pathogens during infection (also known as Avr proteins) are recognized by host receptors (R proteins) which is also well known as gene-for-gene interaction (**Figure 2.1**). Therefore, the race-specific defense caused by effector-host recognition is referred to as effector-triggered immunity (ETI). The defense following the recognition is known as hypersensitive response (HR). The largest class of R proteins is nucleotide binding site-leucine-rich repeat (NB-LRR) receptors with N-terminus of either Toll-Interleukin-1 Receptor (TIR) or Coiled-Coil (CC). NB-LRR protein has two motifs, a conserved nucleotide-binding (NB) site at the amino-terminus for ATP or GTP binding, and the LRR domain at the C-terminus which has variable spatial organization and length, possible for protein-protein interactions and peptide/ligand binding (Knepper and Day, 2010).

Though there is no fundamental difference in signaling between ETI and PTI, ETI is considered a more rapid and vigorous version of PTI, ETI reinstates and amplifies PTI in cellular signaling (Cui et al., 2015). The strategies of plant defense can be divided to incompatible and compatible interactions; the incompatible interaction is caused by ETI while the compatible interaction is the defense without it. The general profile of defense signaling from compatible and incompatible interactions could be very similar; however, genes expression abundance at specific time points were found to differ between these two interactions, ETI activates some expression from PTI with stronger and longer in time (Cui et al., 2015; Tao et al., 2003).

Physiologically, the HR-based defense is featured by a series of locally acquired resistant mechanisms. One of them is the localized tissue death by exploiting the nutrients from the living cells, which hinders the paths for further proliferation of pathogens (Van Loon, 1997; Thakur and Sohal, 2013). Microscopic observations suggested that HR caused DNA fragmentation, nuclear lobing, plasma membrane shrinkage and condensation of cytoplasm, which are the symptoms of localized cell death (Li et al., 2008b). However, HR-elicited PCD is not the only mechanism that slows down pathogenic proliferation on host tissues. The Arabidopsis mutant lines of *dnd1* and *dnd2* are defective in HR cell death, and the studies with

those lines revealed that HR was not fully abolished. Other defensive mechanisms such as SA accumulation and the induction of pathogenesis-related genes (*PR*s) compensated for the penalty of defective HR cell death towards plant defense (Yu et al., 1998, Jurkowski et al., 2004).

Obviously, there are many other mechanisms participating in the HR defense along with the PCD. Even the local cell death is initiated by oxidative burst as the earliest defense response against biotic/abiotic stresses (Lamb and Dixon, 1997; Jabs, 1999; Baxter et al., 2013).

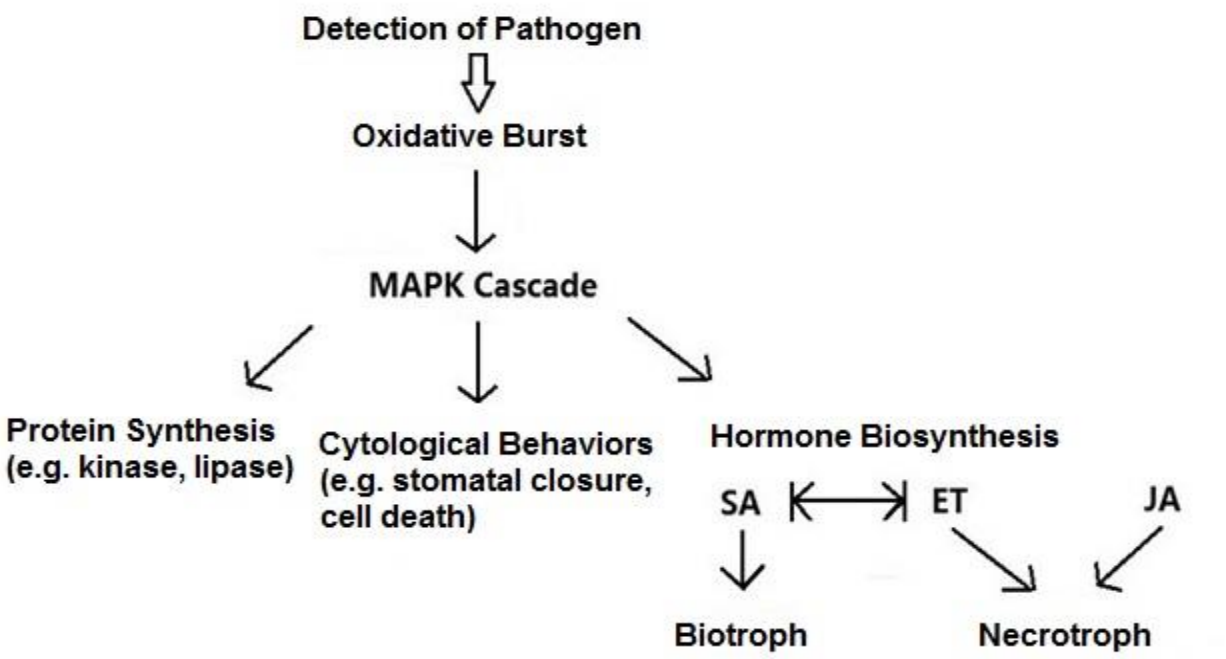

Figure 2.1. Brief scheme of signaling pathways following the detection of pathogen by the host. The early response after sensing of the pathogen is oxidative burst, which subsequently activates MAPK cascades. Activation of the MAPK factors further triggers a series of actions including protein secretion, cellular modifications and biomolecule (hormones, metabolite) synthesis, which propagate the signaling pathways for plant defense.

Superoxides (O_2^-) are the earliest ROS molecules produced from oxidative burst, which are generally formed by putting electrons on O_2 molecules (Wojtaszek, 1997). The molecules are produced wherever electron transport chains are present (Elstner, 1991). The superoxides can be further converted to a relatively stable ROS molecule, hydrogen peroxide (H_2O_2), which is able to move in apoplastic spaces and achieves long-distance signaling (Wojtaszek, 1997; Sharma et al., 2012). H_2O_2 as a major ROS molecule in oxidative burst is able to induce some cytological changes and gene activation which plays various roles in resistance to multiple stresses *in planta*. The over-accumulation of H_2O_2 is able to induce localized basic *PR* expression such as *PR1*, *PR2* to defend against infection (Chamnongpol et al., 1998). Aminotriazole is a catalase inhibitor which mitigates H_2O_2 metabolism, excessive H_2O_2 accumulation on the plant by aminotriazole treatment was found to activate the genes related to PCD such as heat shock proteins, WRKY factors and GSTs (Glutathione S-Transferases) (Gechev et al., 2005). ROS accumulation such as hydrogen peroxide (H_2O_2) initiates PCD by causing membrane damage when the pathogen establishes in the host cells (Bestwick et al., 1997).

Another mechanism connecting to both ROS and PCD is the hormonal signaling, from which salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the three major hormones in plant defense (Bari and Jones, 2009). In general, phytopathogens can be classified as biotrophic and necrotrophic in lifestyle, the biotrophic pathogens colonize the living tissues to exploit nutrients, while the necrotrophic ones expolit nutrients from the dead tissues (Perfect and Green, 2001; Agrios, 2004). The allocation and interaction among threads of signaling pathways depend on the lifestyles of various pathogens. Previous studies revealed that SA is more effective in defending against biotrophic pathogens while ET/JA signaling is more capable of resisting necrotrophic pathogens and herbivorous insects (Bari and Jones, 2009). Moreover, the signal transduction of SA has an antagonistic relationship with that of ET/JA (Kunkel and Brooks, 2002; Li et al., 2004; Bari and Jones, 2009). There are specific signaling pathways and factors attributed to each phytohormone, while those sets of pathways also modulate each other to build a large genetic network to adapt to various situations during pathogenic infection such as the lifestyles of pathogens, environmental factors, and location/onset patterns of infection.

Bordersen et al. (2005) revealed that the genes evoking accelerated PCD (*ACD11* and *SID2*) modulate SA biosynthesis. The generation of SA and ET also enhances lesion development and tissue death following oxidative burst in plants (Chamnongpol et al., 1998; Overmyer et al., 2000; Rao et al., 2002). The potential cooperation between SA and ET in plant defense was shown by the coexpression between SA and ET-responsive genes following the inoculation between *Leptosphaeria maculans* and *Brassica napus* in the incompatible interaction (Sašek et al., 2012).

JA is known to play a negative role in PCD and lesion development (Overmyer et al., 2000; Rao et al., 2002). The induction of *MPK4* is able to activate JA-responsive defensive protein PDF1.2, which suggested the positive regulatory roles for *MPK4* in ET/JA-dependent pathway (Wang et al. 2009). Both Overmyer et al., (2000) and Rao et al., (2002) indicated the reduced lesion development by JA signaling. However, other evidence suggested that incompatible interactions may also be involved in the cooperation between SA and JA signaling (Spoel et al., 2007; Becker et al., 2017). This makes the signaling network after gene-for-gene interaction more complicated. Spoel et al., (2007) showed that SA and JA-responsive genes triggered by unclear factor(s) are co-expressed for incompatible interaction to achieve both localized and systemic resistance, which confers a new model of plant defense.

Since plant defense against biotic stresses includes multiple elements/sections which trigger many threads of signaling pathways, it is necessary to describe how those elements activate their pathways and whether those elements are able to interact with each other. In this review, I firstly introduced the molecular mechanisms in plant detection of phytopathogen by plants, we also chose two major elements to discuss the signal transduction after the perception: reactive oxygen species (ROS) and hormones to depict how plant body manages intrinsic signaling transduction to exert proper cellular activities against pathogenic attack. The article focuses on major ROS molecules such as hydrogen peroxide (H_2O_2), major hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) to sketch a general picture of signaling networks of plant defense.

Brassica napus is in the same family as *Arabidopsis thaliana*. Although there are plenty of studies in plant defense based on *A. thaliana*, the physiological and genetic response of *B. napus* to pathogens may still have some differences. Most of the current canola breeding

projects against *L. maculans* aim to exploit the gene-for-gene interaction by identifying and introducing more *Rlm* genes. Understanding of incompatible interactions between *B. napus* and *L. maculans* still has many gaps. One key objective of this study is to explain why and how the HR following race-specific recognition is initiated and regulated. Despite the great efforts made from previous studies, there are still many challenges to elucidate the list of main components in *B. napus* when defending against *L. maculans*. The general goal of this Ph.D. is to study the important elements of intrinsic defense in *B. napus* against the fungal pathogen *L. maculans*, crucial defensive elements including gene expression, cellular modifications. These will be considered and analyzed to explain the expression of host defense during an incompatible interaction. In this Ph.D. study, two elements of intrinsic defense on *B. napus* will be considered: hormone signaling and oxidative burst. Finally, this study will hopefully give a profound understanding of the cellular and molecular arm races between *B. napus* and *L. maculans*.

2.2 Essential Aspects in Plant Disease Epidemics

Epidemics are defined as a process of the population change of a disease in time and space. Plant disease epidemiology is a study aiming to understand the cause and effect of the spreading of plant disease (Agrios, 2004). Understanding of the dynamics of plant epidemics is essential to the survival of humanity because some plant diseases can spiral out of control, and cause serious food shortages. One typical example is the Irish potato famine from 1845 to 1847, causing the mass emigration from Ireland and millions of deaths. The disease, known as potato late blight and caused by the fungal pathogen *Phytophthora infestans*, was first recorded in Philadelphia, United States, 1843 (Ristaino, 2002). Besides the event occurred in Ireland, there are several other cases of potato late blight epidemics recorded in history as well (Ristaino, 2002). The diversity of the *Phytophthora* species and flexibility of its genome make this fungus a dangerous pathogen even today (Fry, 2008).

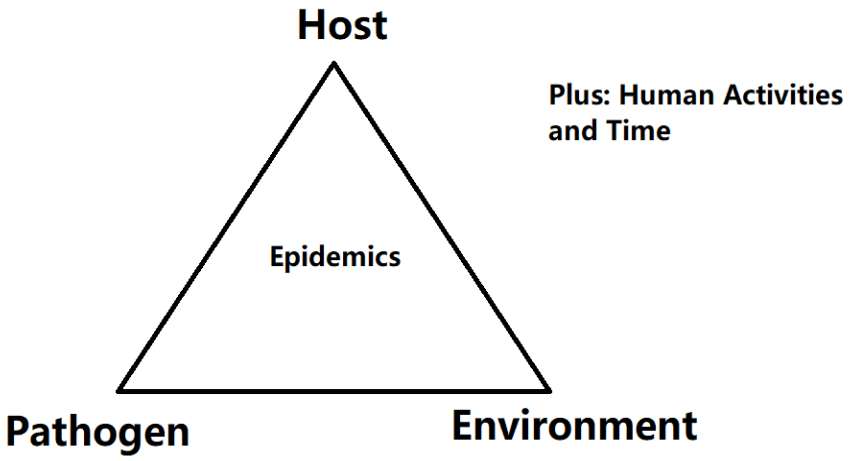

Figure 2.2. Scheme of the elements of plant disease epidemics and their connections during the spreading of the disease.

Plant disease epidemics depend on many factors; however, there are five general elements that affect plant disease development including host, pathogen, human activities, environment and time (**Figure 2.2**).

The physiological background of host plant may determine its level of resistance against pathogen, this includes both its genomic background, life stage and lifecycle. Host plants with susceptible genetic/physiological backgrounds would be easier for the pathogens to infect. For example, the fungal pathogen *Leptosphaeria maculans* is able to infect some susceptible varieties of *Brassica napus*, while some resistant varieties of *B. napus* are able to inhibit the colonization of the pathogen (Delourme et al., 2004; Delourme et al., 2006; Kutcher et al., 2011). However, *Arabidopsis thaliana*, another plant species within the same family as *B. napus*, can totally hinder the infection of *L. maculans*. Moreover, one host plant does not have the same level of resistance against a pathogen throughout its lifecycle. Some host-pathogen interaction changes during the aging of the host plants (Delourme et al., 2006). During its seedling stage, it is easier for *Brassica napus* to be severely infected by the fungal pathogen *L. maculans*. In the field, the type of host also affects the spread of disease; annual crops have

faster epidemics than perennial plants. Diseases like Dutch Elm Disease (DED) has long term (15 to 20 years) infection cycles because elm is a perennial host (Hartwood et al., 2011) while rice blast can spread very fast and become extremely destructive since rice, as the host, is an annual crop (Bonman et al., 1989; Roumen, 1992).

Virulence is another key factor in epidemics. Phytopathogens evolve various biological traits to overcome host resistance and infect successfully. Virulence factors, including enzymes, secreted proteins and toxins are effective weapons to assist pathogenic infection. For example, *Nectria haematococca,* which is the anamorph of *Fusarium solani,* a soil fungus, secretes an enzyme PDA1 (pisatin demethylase) demethylating the phytoalexin pisatin (Van Der Does and Rep, 2007). The plant cell wall acts as a natural structure against potential pathogenic attacks, therefore, penetration of the cell wall becomes a useful strategy for pathogens to attack the plant successfully. Fungal pathogens often secrete plant cell wall-degrading enzymes (CWDEs) which are able to break the plant cell wall by degrading cell wall components such as cellulose, pectin and xylan (Kubicek et al., 2014). Small secreted proteins (SSPs) are another type of biomolecule which pathogens eject into the cytoplasm of plant cells by Type III Secretion System (T3SS). Those proteins contribute to the virulence of pathogens while they may also act as the effectors which trigger the host defense reactions (Jones and Dangl, 2006; Knepper and Day, 2010). Toxins are another type of substance, which have various functions to colonize plant tissues efficiently. For instance, tabtoxin in the bacterial pathogen *Pseudomonas syringae* pv. *tabaci* causes chlorosis on tobacco leaves, and trichothecenes in *Fusarium* species inhibit protein synthesis - both of these toxins enhance the virulence of the pathogens during infection (Kimura et al., 2001).

Another important aspect of pathogenic infection is the type of lifestyle, including sexual/asexual reproduction (the type of reproduction) and mono/polycyclic (the type of lifecycle). Compared with asexual reproduction, sexual reproduction results in more population genetic diversity. Daverdin et al. (2012) suggested that the sexual reproduction of *L. maculans* promotes the mutation of effector genes by Repeat Induced Point mutation (RIP). Sexual reproduction is able to cause mutations and recombination among the different genomes of isolates to survive better during the natural selection (Schoustra et al., 2010).

Besides lifecycle, the spread mode determines the speed of disease reproduction and transmission. Phytopathogens can transmit by air, vectors (such as insects), soil and other factors. Those modes of disease transmission lead to distinct situations of epidemics in certain areas. For instance, bacterial pathogen *Xylella fastidiosa* which causes Pierce's disease on grapevines disperses itself via the foregut of sharpshooter *Homalodisca vitripennis* (Backus and Morgan, 2011). The ascospores of fungal pathogen *Leptosphaeria maculans* is transmitted from the stubbles of canola (*Brassica napus*) by wind (airborne) (West et al., 1999). The manner of dispersal is able to shape the cyclical nature of pathogens, which is divided into monocyclic and polycyclic cycles. A monocyclic pathogen finishes only one lifecycle within one lifecycle of the crop while a polycyclic pathogen is able to complete more than one lifecycle during one lifecycle of the crop. Usually, polycyclic epidemics can increase the rate of infection throughout time (usually being observed after several years) compared with monocyclic epidemics which may have more stable annual rate (APS website, Plant Epidemiology Topics, Temporal Aspects, Disease Progress).

Environmental factors and human activities are the two external factors related to host-pathogen interaction. Environmental factors (e.g. moisture, temperature, airflow) are essential for pathogenic growth and transmission, and those factors may also affect the expression of host when defending against pathogens (Agrios, 2004). Temperature is the factor affecting the biological processes of both host and pathogen. From the host side, temperature is able to affect the regulation of defense signaling. For example, in tobacco, the induction of salicylic acid and pathogenesis-related protein 1 (PR1) were found to decrease at higher temperature (Malamy et al., 1992). Iglesias et al. (2010) found the maximal spore concentration of *Phytophthora infestans* from 16 ^{o}C to 23 ^{o}C in A Limia, Spain. Usually, for pathogens, moisture and temperature are considered to influence epidemics. For fungal pathogen *L. maculan*s on *B. napus*, the maximal lesion development on leaves occurred at 18/15 ^{o}C (day/night temperature) with 96 hours of wetness while the maximal emergence of stem infection happened at 23/20 ^{o}C with 48 – 72 hours of wetness (Sosnowski et al., 2005).

Moreover, intrinsic signaling of plant defense is also impacted by temperature. The reduction of nuclear accumulation of R proteins was found when the temperature was higher (Zhu et al., 2010). Studies from *Arabidopsis thaliana* and *Nicotiana benthamiana* also revealed that the

raising temperature negatively effected both basal and *R* gene-mediated defense (against *Pseudomonas syringae*), also SA and two of its related defense genes, *PAD4* and *EDS1* were also inhibited in higher temperature (Wang et al., 2009b). With global warming, the plant-pathogen interaction will be shaped with the increasing temperature. Higher temperature may give some pathogens more favorable environment to infect hosts (Velásquez et al., 2018).

Besides all those environmental factors, human intervention is another factor to take into consideration, which is also crucial for the development of the epidemic of phytopathogens. Clean planting materials could reduce the spread of the disease cassava brown streak disease (CBSD) (McQuaid et al., 2017). The efforts of plant breeding towards more resistant host cultivars may change the population diversity or structure of pathogen. The microevolution triggered by natural selection encourages the new features and changes of genomic structure, from which the pathogens are able to overcome the challenges from the new conditions created by humans. The mutations and deletions of *Avr* genes from *L. maculans* are the typical examples for the microevolution followed by the application of newly bred resistant cultivars (Sprague et al., 2006; Fudal et al., 2009; Van de Wouw et al., 2010; Zhang et al., 2016a). For example, the *Brassica rapa* cultivar named "sylvestris", with major gene resistance (*LepR3*), was commercially grown in Australia starting in 2000, to defend against the epidemics of *L. maculans.* After 3 years, this cultivar was totally broken down because of the switch of races in the field population of *L. maculans* (Sprague et al., 2006). Moreover, Zhang et al. (2016)a also demonstrated that the cultivated *B. napus* with *Rlm3* predominantly in Canada had caused the selection pressure of *L. maculans* population to reduce its proportion of *AvrLm3*. This led to the breakdown of *Rlm3*.

In conclusion, the spread of phytopathogens depends on multiple factors, which either act independently or cooperatively. Each element may profoundly influence the initiation and development of epidemics. Adequate studies depicting the epidemic factors are necessary to develop new strategies to manage various plant diseases and increase the yield of crops.

2.3 Pathogen Recognition

2.3.1 Pathogen-Associated Molecular Pattern (PAMP)-Triggered Immunity (PTI)

Pathogen recognition consists of two levels. The first level is triggered in plants by the perception of microbial or pathogen-associated molecular patterns (MAMPs or PAMPs), which is one of the crucial mechanisms boosted by the detection of extrinsic molecules (Henry et al., 2012; Kushalappa et al., 2016). PAMPs are slow-evolving compounds, ranging from carbohydrates to proteins, recognized by host plants. Compounds such as flagellin, elongation factor Tu, and chitin act as PAMPs (Jones and Dangl 2006; Anderson et al. 2010). PAMPs trigger basal disease resistance and result in PAMP-triggered immunity (PTI). At the molecular level, PAMPs or MAMPs are recognized by transmembrane pattern recognition receptors (PRRs) with an extracellular ligand-binding domain and an intracellular leucine-rich repeat domain (LRR) (Henry et al., 2012). PAMPs or MAMPs are generally triggered by the detection of molecules released by pathogens known as elicitors, for instance, chitin or lipopolysaccharides, which consist of the significant components of pathogen structures (i.e., cell walls) and infectious factors (i.e., enzymes). The perception of the presence of a pathogen depends on the specific interactions between plants' cellular receptors and the pathogen elicitors.

One typical example of PAMP receptor recognition is the interaction between bacterial flagellin 22 (flg22) and *Arabidopsis* LRR receptor-kinase FLS2 (Flagellin Sensitive 2) (Jones and Dangl, 2006). The binding of flg22 and FLS2 triggers the intracellular interaction between the C-terminus of FLS2 and BRASSINOSTEROID INSENSITIVE-associated kinase 1 (BAK1), which further helps to activate plant immunity (Belkhadir et al. 2012; Sun et al. 2013). Evidence has suggested that the activation of FLS2 protein triggers a series of cellular responses related to plant defense (Navarro et al., 2004; Anderson et al., 2010; Sun et al., 2013).

Other molecular patterns similar to PAMPs are damage-associated molecular patterns (DAMPs), which refer to the recognizable molecules related to plant damage, such as cell wall fragments, protein fragments, peptides, nucleotides, amino acids, and lytic enzymes (Albert, 2013; Doughari, 2015; Kushalappa et al., 2016). DAMPs are perceived by plasma membrane-

localized receptors of surrounding cells to regulate immune responses against the invading organisms and promote damage repair. DAMPs overlap with PTI signalling components (Boller and Felix, 2009). In general, PAMPs, MAMPs and DAMPs elicit a series of defense signalling to respond to potential threats from invaders.

2.3.2 Effector-Triggered Immunity (ETI)

The second level of pathogen recognition encircles plant resistance (R) proteins, which identify specific receptors from a pathogen (Avr proteins) (Dangl and McDowell, 2006; Gouveia et al., 2017; Abdul Malik et al., 2020) and results in effector-triggered immunity (ETI). Avr proteins, also known as effectors, are secreted by pathogens and recognized by host receptors (R proteins) during infection. R proteins are produced by *R* genes that convey plant disease resistance against pathogens. This interaction is known as a gene-for-gene interaction. Therefore, the race-specific defense caused by effector-host recognition induces a stronger defense response known as effector-triggered immunity (ETI) (Henry et al., 2012; Kushalappa et al., 2016). Although there is no fundamental difference in signalling between ETI and PTI, ETI is considered a more rapid and vigorous version of PTI. ETI reinstates and amplifies PTI in cellular signalling (Cui et al., 2015). The defense following the recognition is known as the hypersensitive response (HR). HR is a mechanism to prevent the spread of infection. The rapid death of cells characterizes HR in the local region surrounding infection, and it serves to restrict the growth and spread of pathogens to other parts of the plant. Physiologically, the HR-based defense features a series of locally expressed resistance reactions at attempted penetration points. For example, localized tissue death occurs followed by nutrient exploitation from the living cells, which hinders the further proliferation of pathogens (Van Loon, 1997; Thakur and Sohal, 2013). For example, in some bacteria, when the pathogen penetrates the intercellular spaces and then the cell membrane, Type III Secretion System (T3SS) effectors are injected (Knepper and Day, 2010). R proteins from the host plant then recognize and bind the effectors to induce HR, which subsequently halts the further invasion of the pathogen (Dangl and Jones, 2006). Findings suggest that HR causes DNA fragmentation, nuclear lobing, plasma membrane shrinkage, and condensation of the cytoplasm, which are the symptoms of localized cell death (LCD) (Li et al., 2008). However, HR-elicited PCD is not the only

mechanism that slows down pathogen proliferation in host tissue. A study on *Arabidopsis* mutant lines of *dnd1* and *dnd2* (defective in HR cell death) revealed that HR was not entirely abolished. Other defensive mechanisms such as SA accumulation and the induction of pathogenesis-related genes (*PR*s) compensated for defective HR cell death (Yu et al., 1998, Jurkowski et al., 2004).

Plant defense strategies can be divided into incompatible and compatible interactions; ETI causes the incompatible interaction, while the compatible interaction is the defense without ETI. The general profile of defense signalling from compatible and incompatible interactions could be very similar; however, gene expression at specific time points differed between these two interactions. ETI activates some expression from PTI that is stronger and longer in duration (Cui et al., 2015; Tao et al., 2003).

Besides PTI and ETI, another general mechanism named effector-triggered defense (ETD) is postulated, which lies between PTI and ETI (Stotz et al., 2014). ETD is characteristic of microbes that move into the intercellular matrix or apoplastic spaces of the host. ETD is initiated by the interaction of the apoplastic effectors with the cell surface-localized receptors, from which this type of defense has both ETI and PTI characteristics (Stotz et al., 2014). The receptor-like proteins (RLPs) interact with a receptor-like kinase SOBIR1 (Suppressor of Bir-1), and this interaction is associated with another factor, BAK1 (BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1), to promote cell death and defense responses (Liu et al., 2016). Moreover, Ma and Borhan (2015) found a *Brassica napus* homolog of AtSOBIR1, which interacts with *B. napus* RLP LepR3. This BnSOBIR1 was found to elicit HR in the case of the *AvrLm1-LepR3* interaction.

2.3.3 R and Avr Proteins

In *Arabidopsis*, about 200 *R* genes are found with conserved domains (Meyer et al., 2003). Conserved domains can be used for *R* gene identification and classification. Nucleotide-binding site-leucine-rich repeat (NLR), receptor like kinase (RLK), and receptor like protein (RLP) genes are the main types of *R* genes. *R* genes duplicate in the genome so that more R proteins will be encoded. This could be advantageous for plants since the regulation of more R

proteins is beneficial, because it leads to a broader spectrum of disease resistance (Yi and Richards, 2007).

The largest class of R proteins are nucleotide-binding site-leucine-rich repeat (NB-LRR) receptors. LRRs consist of 2-45 motifs of 20-30 amino acids in length. Each motif is considered one repeat, and LRR proteins can have many repeats, forming the LRR domain. LRR is involved in specific ligand-receptor interactions (Chisholm et al., 2006). In contrast, the NBS domain at the N-terminus binds ATP or GTP molecules. This interaction causes conformational changes to trigger downstream signalling (DeYoung and Innes, 2006; McHale et al., 2006). NB-LRR domains can be subdivided further based on the presence or absence of an N-terminal Toll/interleukin1-like receptor (TIR) homology region or a CC motif in the N-terminal region (McHale et al., 2006; Knepper and Day, 2010) into the functionally distinct TIR-domain-containing (TNL), CC-domain-containing (CNL), and RPW8 domain-containing (RNL) subfamilies. The N-terminal motifs inhibit the ligand-binding of the LRR domain to keep the R protein persistently inactive.

The structure and function of effector proteins vary among different pathogens. Effector proteins are generally short peptides, and many are cysteine-rich and harbour N-terminal signal peptides. Secretory proteins carry a short signal peptide at their N-termini that assists with their secretion into the cytosol or host apoplastic spaces (Owji et al., 2018).

Cysteine residues form a disulphide bridge, stabilizing the protein structure (Chisholm et al. 2006). Effectors can have multiple bridges for folding, conformational and functional stability; this seems more important in the hostile apoplastic space. Avr proteins are a subset of effector proteins and are genetically identified as triggering HR, due to recognition by R genes.

The stability of the Avr protein is essential for host recognition. For example, the mutated proteins of Avr4 in *Cladosporium fulvum* can avoid the interaction with the R protein Cf-4 in tomatoes (Joosten et al., 1997). The mutated derivatives of Avr4 have a substitution of Cys to Tyr, which causes unstable cysteine–cysteine disulphide bonds to affect the protein structure. The failed interaction produced more severe disease symptoms in tomato leaves (Joosten et al. 1997).

Studies have suggested that Avr proteins may have specific biological roles in infection and colonization. For example, in *C. fulvum*, Avr2 inhibits tomato cysteine protease Rcr3 activity (Rep, 2005; Chisholm et al., 2006) and changes the structure of Rcr3, which triggers the HR initiated by R protein Cf-2 (Rep 2005). Furthermore, some Avr proteins have been implicated in activating plant transcription (Chisholm et al., 2006). For instance, the AvrBs3 protein family found in *Xanthomonas* has a nuclear localization domain (Zhu et al., 1998), and AvrXa7 in *Xanthomonas oryzae* binds DNA with a preference for dA- and dT-rich fragments (Yang et al., 2000). This interaction between Avr proteins and host nuclear content probably intervenes in the host transcriptional activation of defense genes.

R proteins can interact with Avr proteins directly and indirectly. For example, AvrPita from *Magnaporthe grisea* and Pita from *Arabidopsis thaliana* physically interact with each other in the LRR domain (DeYoung and Innes, 2006). Indirect interactions between R and Avr proteins may be described by the guard hypothesis. This hypothesis postulates that a host R protein (the product of an R gene) guards a protein, the guardee, that is the target of the Avr effector protein. Binding, deactivation, or cleavage of the target protein by the Avr effector protein is sensed by the R protein, sometimes through a conformational change, which in turn triggers the resistance function of the R protein (DeYoung and Innes, 2006; Knepper and Day, 2010). The guard model was first suggested to explain the mechanism of *Pseudomonas syringae* AvrPto perception by the tomato proteins Pto and Prf (Van der Biezen and Jones, 1998). One well-known example is the interaction between the effector AvrRpt2 (*Pseudomonas syringae*) and R protein RPS2 (*Arabidopsis thaliana*) mediated by RPM1-interacting protein 4 (RIN4). The interaction between AvrRpt2 and RIN4 disrupts the RIN4/RPS2 complex to switch RPS2 into the active formation (Mackey et al. 2003; DeYoung and Innes 2006). R protein-mediated defense also occurs by detecting the biological activity of Avr protein. For example, AvrPphB acts as a protease, which cleaves a protein kinase, and the R protein RPS5 in *Arabidopsis* can detect the cleavage from the Avr protein and elicits subsequent defense (Shao et al., 2003; Bent and Mackey, 2007).

Moreover, results about additional targets of AvrPto and AvrBs3 provoked suggestions of the concept that some host targets of effectors act as decoys (antagonistic interactions between hosts and pathogens). Decoys mimic effector targets to trap the pathogen into a recognition

event to detect pathogen effectors via R proteins (Zhou and Chai, 2008; Zipfel and Rathjen, 2008).

2.4 Plant Defense Molecular Signalling

2.4.1 Oxidative Burst

Reactive oxygen species are highly reactive chemical molecules formed via oxygen consumption in a so-called oxidative burst. ROS act as signal molecules in various biological processes in plants, including photorespiration, stomatal movement, and photosynthesis (Baxter et al., 2013; Das and Roychoudhury, 2014). ROS signalling acts as an early response in plants when combating biotic or abiotic stresses. The signalling itself controls a broad spectrum of biological processes. Common ROS molecules such as superoxide ($^{\bullet}O^{-}_{2}$), hydroxyl radical (·OH), singlet oxygen ($^{1}O_{2}$), and nitric oxide (NO) are generated in plant cells by the electron transport chain (ETC), NADPH oxidase, and peroxisomes (Baxter et al., 2013; Liu and He, 2016). Generally, ROS exist in ionic (hydroxyl radicals and superoxide anions) and molecular states (hydrogen peroxide and singlet oxygen) and can be produced by extracellular (environmental pollutants, radiation exposure, microbial infection, and exposure to engineered nanoparticles) and intracellular (mitochondria, the endoplasmic reticulum (ER), peroxisomes, microsomes, and NOX complexes) sources (Abdal Dayem et al., 2017).

Local and systemic ROS generation and signalling appear in biotic and abiotic stresses such as high light, drought, pathogenic attack, and plant-arbuscular mycorrhizal interactions (Wojtaszek, 1997; O'Brien et al., 2012; Baxter et al., 2013). Cellular activities such as stomatal closure, programmed cell death, and Ca^{2+} leakage during stress tolerance involve the modulation of ROS-derived signalling (Baxter et al., 2013). ROS generation also elicits extremely diverse signalling events from which an extensive and complicated genetic network is involved.

2.4.2 ROS Molecules

Superoxide radicals ($O^{-}_{.2}$) are the earliest ROS molecules produced from the oxidative burst, and are generally formed by adding electrons to an oxygen molecule (O_2) (Wojtaszek, 1997; Sharma et al. 2012). They form in various locations inside the plant cells, where electrons are available from the ETC in the chloroplast and mitochondria (Elstner, 1991; Das and Roychoudhury, 2014). They are short-lived and not frequently involved in biochemical reactions. They can be further converted to hydrogen peroxide (H_2O_2), a relatively stable molecule, by superoxide dismutase (SOD). Hydrogen peroxide is not a reactive molecule and can travel across a long distance through the apoplastic spaces and achieve long-distance signalling (Wojtaszek, 1997; Sharma et al., 2012). Hydrogen peroxide production mainly occurs during ETC in the chloroplast, mitochondria, endoplasmic reticulum, and cell membrane and during the β-oxidation of fatty acid and photorespiration (Das and Roychoudhury, 2014). Hydrogen peroxide can induce cytological changes and gene activation, playing various roles in resistance to multiple stresses. For example, the over-accumulation of hydrogen peroxide can induce localized basic *PR* expression such as that of *PR1* and *PR2* to defend against infection (Chamnongpol et al., 1998). The accumulation of reactive oxygen species or hydrogen peroxide initiates PCD by causing membrane damage when the pathogen is established in the host cells (Bestwick et al., 1997). Hydrogen peroxide accumulates during papillae formation. Peroxidases can use it to promote cross-linking of proteins and phenolics to reinforce cell wall appositions (Brown et al., 1998). The apoplastic generation of superoxide or hydrogen peroxide has been documented following the recognition of various pathogens (Grant et al., 2000).

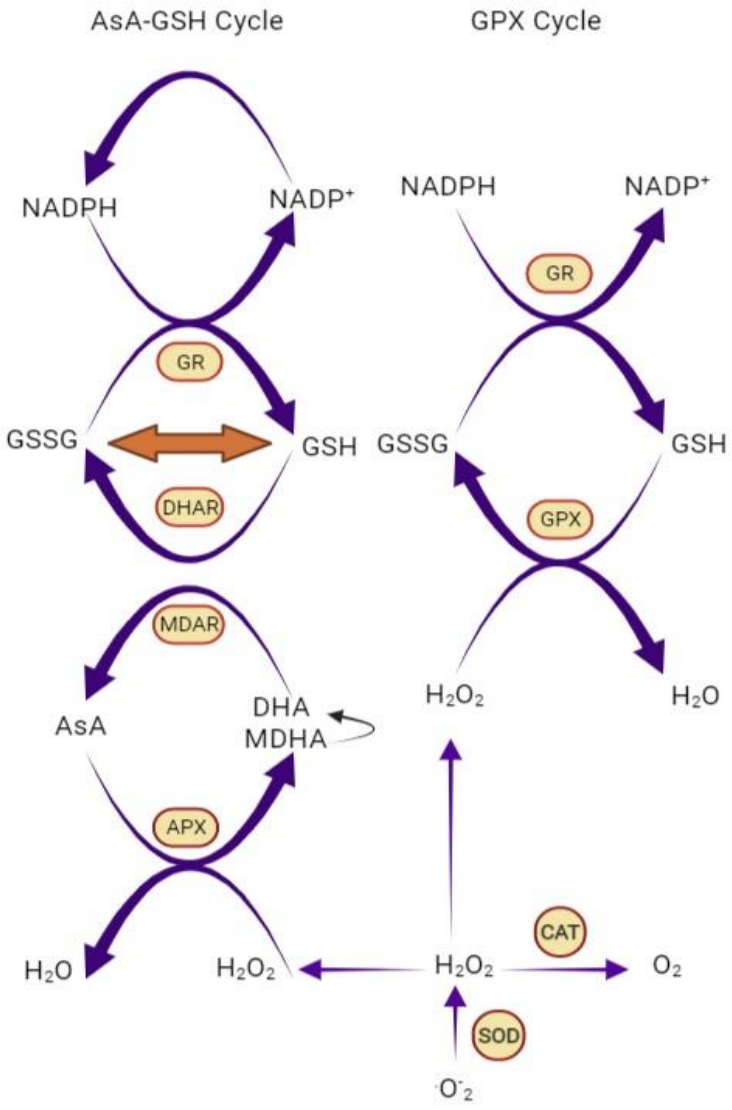

Figure. 2.3 The ascorbate glutathione (AsA-GSH) and glutathione peroxidase (GPX) cycles. GSSG: oxidized glutathione (glutathione disulphite); GSH: glutathione; GR: glutathione reductase; DHAR: dehydroascorbate reductase; MDAR: monodehydroascorbate reductase; AsA: ascorbate; DHA: dehydroascorbate; MDHA: monodehydroascorbate; APX: ascorbate peroxidase; GPX: glutathione peroxidase. **The image and legend were prepared with the assistance from Dr. Aria Dolatabadian (Fernando Lab).**

Hydrogen peroxide is the pivotal molecule for eliciting diverse downstream responses in various events, including cell cycle, senescence, lignification, electrolyte leakage, the MAPK cascade, SA, JA, ABA, or ET signalling, and stomatal closure (Quan et al., 2008). The formation of superoxide and hydrogen peroxide is catalysed by membrane-bound NADPH-oxidases (RBOHs) and cell wall-bound peroxidases (Lamb and Dixon, 1997). The reduced ROS production has been reported to block the expression or function of those two types of genes in transgenic plants (Lamb and Dixon, 1997; Torres et al., 2002; Daudi et al., 2012; Morales et al., 2016).

In *Arabidopsis*, *RBOH-D* and *-F* are the two most well studied NADPH-oxidase genes, which play crucial roles in cell death and basal defense by modulating hydrogen peroxide accumulation (Torres et al., 2002; Torres and Dangl, 2005; Morales et al., 2016). Disruption of the enzymes by mutation, knockdown, or chemical treatment can reduce or diminish plant resistance by attenuating the hydrogen peroxide-related defense such as cell death (Torres et al., 2002; Morales et al., 2016). For example, a dipenylene iodonium elicitor from *Phytophthora* spp. (or *C. lindemuthianum*) significantly inhibited hydrogen peroxide production in rose cells by targeting RBOH proteins (Bolwell et al., 1998). Reduced cell death, peroxide production, and electrolyte leakage were observed in *Arabidopsis* lines with *RBOH-D* and *-F* mutations when the plants were inoculated with *Pseudomonas syringae* DC3000 (Torres and Jones, 2002). The transgenic lines of antisense *NtRBOH-D* in tobacco showed reduced ROS and hydrogen peroxide production when the leaves were elicited by cryptogein (Simon-Plas et al., 2002).

The hydroxyl radical (·OH) is the neutral form of the hydroxide ion (OH^-). The molecule is formed by the cleavage of the O-O double bond in hydrogen peroxide. It is active and usually acts very near its production site. The hydroxyl radical (·OH) is the most reactive of the ROS and can react with all biological molecules. It is able to loose the cell wall by the oxidation of polysaccharides (Karkonen and Kuchitsu, 2015), and it can also induce DNA single-strand breakage (Hiramoto et al., 1996).

Singlet oxygen ($^1O_2$) is another ROS generated via energy transfer from excited chlorophyll to molecular oxygen during photosynthesis, mainly at the photosystem II (Wojtaszek, 1997; Das and Roychoudhury, 2014). Singlet oxygen has a short half-life but is highly reactive and

destructive, damaging photosystems I and II, along with other essential plant components such as proteins and nucleic acids (Das and Roychoudhury, 2014). Singlet oxygen is moderately reactive and can be protonated at lower pH to form a highly reactive hydroperoxyl radical (HO_2.). The hydroperoxyl radical is more hydrophobic and able to move through the cell membranes (Wojtaszek, 1997).

Nitric oxide (NO) is also known as an important signalling and regulatory molecule in plants that regulates multiple processes during growth, development, reproduction, responses to the external environment and biotic interactions. It has been noted that cellular levels of NO facilitate early establishment of the pathogen and restrict further pathogenic infections (Martínez-Medina et al., 2019). The interaction between NO and ROS is essential to initiation of cell death mechanisms in response to certain types of pathogens (Sadhu et al., 2019). Furthermore, the crosstalk of NO with other defense components such as hormones (Mur et al., 2013) has been documented by Sami et al. (2018).

2.4.3 ROS Scavenging

In plants, ROS molecules can either promote early signals to trigger various molecular events to respond to different conditions or impair tissues and cells when the amounts are excessive. Therefore, besides the signal network attributed to ROS molecules, plants have a series of enzymatic (SOD, catalase (CAT), glutathione peroxidase (GPX), and glutathione-S-transferase (GST)) and non-enzymatic (tocopherols, carotenoids, and flavonoids as a lipid phase; ascorbate, urate, glutathione, and other thiols as a liquid phase) protective antioxidant mechanisms; those enzymes and metabolites work altogether to protect the cells from oxidative damage and prevent the formation of radicals (Young and Woodside, 2001). These mechanisms constitute the ROS scavenging system. The first group of ROS scavengers belongs to the catalase family (CATs), which are the haem proteins catalysing the decomposition of hydrogen peroxide to water and dioxygen gas. The antioxidative activity of CATs mainly occurs in the peroxisomes, which are the hotspots for superoxide radical and hydrogen peroxide production (Das and Roychoudhury, 2014). Catalase enzymes show a high

affinity towards hydrogen peroxide and have a high turnover rate. Catalases can be highly expressed in the plant tissues where antioxidative activities are needed (Mhamdi et al., 2010).

Superoxide dismutase is an enzyme that catalyses the conversion of superoxide radicals to hydrogen peroxide in cells (Wojtaszek, 1997; Das and Roychoudhury, 2014). Superoxide dismutase enzymes are also regarded as metalloenzymes because they have metal ions, co-factors for achieving specific reactions. Superoxide dismutase enzymes can be classified into SODs associated with manganese (Mn), iron (Fe), or copper/zinc (Cu/Zn) by the ions with which they interact (Das and Roychoudhury, 2014). These enzymes can induce multiple stress tolerances and defense against pathogenic attacks. For example, Cu/Zn-SOD, Fe-SOD, and Mn-SOD activities were increased under mild and high drought stresses conditions (Sharma and Dubey, 2005). The overexpression of Cu/Zn-SOD can relieve oxidative stress by metabolizing superoxide radicals, reducing potential damage to the chloroplasts (Gupta et al., 1993). Superoxide dismutase activity is induced in defense against fungal pathogens in the genus *Cercospora*, since the pathogen-derived toxin cercosporin can produce singlet oxygen and superoxide molecules. Superoxide dismutase in mitochondria also plays a role in incompatible interactions between *N. plumbaginifolia* and *P. syringae* (Bowler et al., 1992).

Ascorbate peroxidases (APXs) are another critical group of enzymes adjusting ROS levels in the cells. Ascorbate (AsA), with a low molecular weight, is a highly abundant antioxidant, which donates electrons to various enzymes for their reactions, including APXs (Sharma et al., 2012). Ascorbate molecules are mainly located in the cytosol and mediated by APXs. The ROS scavenging reaction involving AsA and APXs can relieve oxidative damage and protect crucial macromolecules and organelles (Caverzan et al., 2012; Pandey et al., 2017).

In ROS scavenging pathways, ascorbate (AsA) associates with glutathione (GSH) to form an ascorbate-glutathione (AsA-GSH) cycle (**Figure 2.3**). The AsA-GSH cycle operates in the cytosol, mitochondria, plastids and peroxisomes. The first step of AsA-GSH cycle is the conversion of hydrogen peroxide to water, which is mediated by ascorbate peroxide (APX) and ascorbate (AsA) acts as the electron donor. The oxidized ascorbate (monodehydroascorbate, MDHA) is regenerated by monodehydroascorbate reductase (Wells and Xu, 1994). Monodehydroascorbate is a radical, which can disintegrate into ascorbate and dehydroascorbate (DHA). Dehydroascorbate is reduced to ascorbate by dehydroascorbate

reductase (DHAR) at the expense of GSH, yielding oxidized glutathione (GSSG). Finally, GSSG is reduced by glutathione reductase (GR) using NADPH as an electron donor (Noctor and Foyer, 1998; Pandey et al., 2017). Therefore, the AsA-GSH cycle plays a crucial role in hydrogen peroxide detoxification.

2.4.4 Mitogen-Activated Protein Kinase (MAPK) Cascades

Mitogen-activated protein kinase (MAPK) cascades represent a group of signalling pathways that are highly conserved among eukaryotes. This signalling module plays diverse roles, including the regulation of growth and development, programmed cell death, and responses to biotic and abiotic stresses (Pitzscheke et al., 2009; Bigeard and Hirt, 2018). Reactive oxygen species signalling induces MAPK cascades, electrolyte leakage, hormone secretion, programmed cell death and transcriptional reprogramming. Reactive oxygen species induce Ca^{2+} leakage and cause LCD by MAPK cascades starting with mitogen-activated protein (MAP)/extracellular signal-related kinases (ERK) (MEKs) (Zhang and Klessig, 2001). SA, JA, and ET may create a homeostatic network under oxidative stress; SA and ET promote cell death and lesion development, and JA attenuates those processes (Overmyer et al., 2000; Rao et al., 2002). Because of the importance of this biological section, the activation and timing of ROS signalling play important roles in effective plant defense.

The cascade consists of MAPKK kinase (MEKK), MAPK kinase (MEK), and MAPK (MPK). There are specific combinations among different MEKKs, MEK, and MPK factors. For example, MEKK1-MEK1/2-MPK4 in *Arabidopsis* negatively regulates signal transduction against biotrophic pathogens, but positively regulates it against necrotrophic pathogens (Ichimura et al., 2006: Petersen et al., 2010). Hydrogen peroxide regulates MEKK1 to attain ROS homeostasis (Nakagami et al. 2006), and it is found to negatively regulate the ROS signalling factors, including MPK3/6 (Ichimura et al., 2006). In tobacco (*Nicotiana tabacum*), the module of NPK1 (a MEKK1)-MEK1-NTF6 (an MPK) is crucial for *N*-gene (an *R* gene in tobacco) expression to defend against tobacco mosaic virus (TMV). The activation of defencss also involves the expression of *WRKY* and *MYB* genes (transcription factors) and JA responsive factor *COI1* (Liu et al., 2004).

The ROS production activates MAPK cascades such as *MPK3/6*, which regulate hormonal signals such as *ERF1* (as an ET transcription factor) (Moon et al., 2003). Wang et al. (2009) suggested that *MPK4* suppresses ROS production and activates ET-JA-responsive factor *PDF1.2* in canola (*Brassica napus*). The overexpression of *MPK4* in transgenic canola plants makes them more resistant to necrotrophic pathogens such as *Sclerotinia*. MPK4 in ET-JA binds a nuclear substrate called MAPK kinase substrate 1 (MKS1) to regulate *WRKY33*, which is a WRKY factor promoting ET-JA signalling (Petersen et al., 2010). In summary, MAPK cascades induce diverse mechanisms combatting various biotic and abiotic stresses. Since there are numerous plant MAPK factors, the genetic network of MAPK cascades can be diverse and fine-tuned to respond to various stresses.

2.5 Phytohormones

2.5.1 Introduction to Phytohormones

Plants activate another defense response to cope with pathogens, which is modulated by the induced production of a wide variety of hormones (Vos et al., 2015), including auxins, gibberellins (GA), abscisic acid (ABA), cytokinins (CK), salicylic acid (SA), ethylene (ET), jasmonates (JA), brassinosteroids (BR) and peptide hormones (Bari and Jones, 2009). The classical defense phytohormones SA, JA and ET are known to play major roles in regulating plant defense responses (Kunkel and Brooks, 2002).

Salicylic acid is a group of phenolic compounds containing an aromatic ring and a hydroxyl group. Previous studies have shown that SA plays various roles in plant defense and growth (Vlot et al., 2009; Rivas-San Vincente and Plasencia, 2011). In plants, the biosynthesis of SA starts with shikimic acid and chorismic acid, and the pathway divides into two routes: the route of isochorismic acid (catalysed by isochorismate synthase (ICS)) and the route of cinnamic acid (catalysed by phenylalanine ammonia-lyase (PAL)) (Dempsey et al., 2011) (**Figure 2.4**).

Since SA represents similar molecules, it has many derivatives that play different functions under various conditions. Salicyloyl glucose ester (SGE), SA *O-β*-glucoside (SAG), methyl salicylate (MeSA), and methyl salicylate *O-β*-glucoside (MeSAG) are four of them. MeSA is

the methylated type of SA and a phloem-mobile signalling molecule. MeSA is involved in systemic acquired resistance (SAR), and crosstalk occurs between SA and JA signalling (Dempsey et al., 2011). SAG is the glucosylated form of SA and is produced in the cytosol and actively transported from the cytosol to the vacuole as the storage for inactive SA (Dempsey et al., 2011; Rivas-San Vincente and Plasencia, 2011). Both MeSA and SAG are inactive; they are converted into free SA when the SA signalling is needed (Vlot et al., 2009).

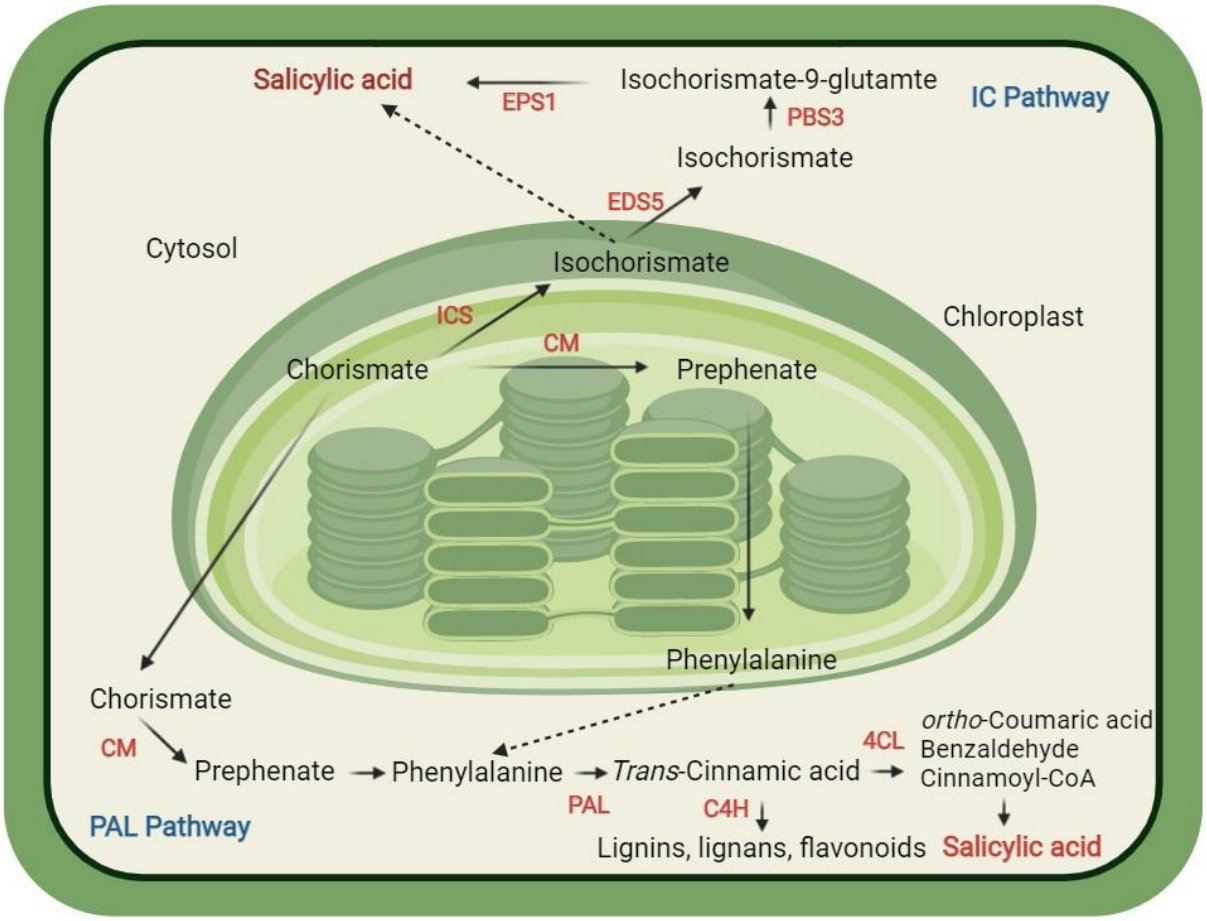

Figure. 2.4 Salicylic acid biosynthesis pathways. The pathway is divided into phenylalanine ammonia-lyase (PAL) and isochorismate (IC) routes, mediated by isochorismate synthase (ICS). Chorismate is converted to isochorismate. IC is moved from plastid to cytosol, and then IC is converted into isochorismate-9-glutamate (IC-9-Glu) mediated by avrPphB SUSCEPTIBLE3 (PBS3), and then finally, IC-9-Glu is spontaneously converted to salicylic acid. The PAL route involves the *trans*-cinnamic acid (made from phenylalanine by PAL) to

lignins, lignans, and flavonoids (mediated by cinnamate 4-hydroxylase, C4H), and precursors of salicylic acid (involving 4-coumarate: CoA ligase, 4CL). **The image and legend were prepared with the assistance from Dr. Aria Dolatabadian (Fernando Lab).**

SA plays a wide range of biological roles in plants. For example, the role of SA in seed germination has been controversial as contradictory reports suggest that it can either inhibit or increase seed germination. It has been reported that SA inhibits seed germination in *Arabidopsis* (Rajjou et al., 2006), maize (Guan and Scandalios, 1995), and barley (Xie et al., 2007), which might be due to SA-induced oxidative stress (Rivas-San Vincente and Plasencia, 2011). In contrast, during seed maturation, SA promotes the synthesis of proteins for germination (Rivas-San Vincente and Plasencia, 2011).

Previous studies revealed that SA effectively defends against biotrophic pathogens (Bari and Jones, 2009). SA-mediated immune responses are essential components of both PTI and ETI (Tsuda et al., 2009) and important for SAR activation (Durrant and Dong, 2004). Furthermore, one of the well-known roles of SA in plants is to provoke oxidative bursts. The role of SA related to ROS signalling is complicated. Salicylic acid regulates both provocative and inhibitive roles upon oxidative burst. As the critical mechanism in treating biotic and abiotic stresses, the modulation of ROS by SA can adjust multiple defensive activities, including stomatal closure, gene expression, PCD, and SAR. The initial defense and the subsequent SAR occur in different parts of the tissues with different SA levels (Vlot et al., 2009). Salicylic acid promotes ROS signalling via the ETC in early responses and growth-promoting defense priming in late response (Dong et al., 2016). Salicylic acid also has its feedback loop in the signalling pathway (Brodersen et al., 2005; Vlot et al., 2009). For example, SA triggers hydrogen peroxide signalling and causes cell death.

On the other hand, the ectopic expression of *nahG* (salicylate hydroxylase, a SA metabolizing enzyme) is found to block spontaneous lesion formation, which balances the effects from SA signalling (Vlot et al., 2009). Rao et al. (2002) also depicted a model that increases oxidative status (increased amounts of superoxide and hydrogen peroxide molecules) and activates SA secretion, which further upregulates ET secretion and signalling and induces cell death and leaf

lesion. RBOH-D suppresses the induction of the oxidative burst and cell death by SA and ET in *Arabidopsis*. RBOH-D plays a dual role in ROS signalling; it promotes hydrogen peroxide accumulation and attenuates SA and ET secretion. The *AtrbohD* mutant showed an excessive accumulation of free SA and ET and macroscopic cell death, while wild-type *Arabidopsis* induced cell death in distinct and single cells (Pogány et al., 2009). These findings suggest that the SA-induced oxidative burst and cell death have a homeostatic feedback loop to focus these processes more on pathogen-damaged tissues. Xu and Brosché (2014) found that the accumulation of SA in *Arabidopsis* attenuated the apoplastic ROS burst, while other defense mechanisms were induced. AtRBOH-D, as the factor producing apoplastic hydrogen peroxide, plays a role in suppressing SA accumulation and macroscopic cell death (Pogány et al., 2009). Collectively, this suggests that there is a homeostatic network between SA and ROS in plant defense. First, the SA accumulation supports ROS signalling for specific cell defense against pathogenic infection. Secondly, ROS signalling also restricts the extent of SA signalling, preventing unnecessary damage to the plant.

NON-EXPRESSOR of PR1 genes (*NPR1*) and *WRKY70* are the two pivotal regulators in SA signalling (Li et al., 2004; Vlot et al., 2009). The *NPR1* gene encodes a nuclear localization factor (a novel protein containing an ankyrin repeat domain involved in protein-protein interactions) induced by SA, which correlates with the expression of the genes in SAR such as *PR1*. *PR1* is also considered as one of the components in *R*-gene mediated defense. *WRKY70* is another regulator lying on the node between SA and JA signalling. The overexpression of *WRKY70* supports SA signalling and suppresses JA signalling. Moreover, microarray data reveals that WRKY70 upregulates genes in oxidative stress responses, cell death, and cell wall modification. When regulating JA, on the other hand, *WRKY70* downregulates the JA-related signals such as vegetative storage protein 1 (*VSP1*) and -*2* (Li et al., 2004). The accumulation and signalling of SA are also dependent on the activity of *PHYTOALEXIN DEFICIENT 4* (*PAD4*) and *ENHANCED DISEASE SUSCEPTIBILITY* (*EDS1*) (Kunkel and Brooks, 2002; Vlot et al., 2009). *PAD4* and *EDS1* regulate glycerol metabolism and play roles in basal resistance and *R*-gene mediated ETI (triggered by infection of biotrophic pathogens). The SIZ1-mediated sumoylation of EDS1 and PAD4 inhibits glycerol metabolism as one way of attenuating SA accumulation (Vlot et al., 2009). The signalling activated by those factors leads to the activation of the genes encoding antimicrobial proteins, including *PATHOGENESIS*-

*RELATED PROTEIN*s (*PR*'s). *PR1* and *PR2* are the two crucial *PR* genes regulated by SA. PR1 includes a group of proteins found in plants with antifungal activity at the micromolar level (Stintzi, et al. 1993; Borad and Sriram, 2008). The expression of PR1 proteins is positively regulated by *WRKY70* (Kunkel and Brooks, 2002; Li et al., 2004). The *PR1* gene is also found as one of the basal resistance QTLs in *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Ahmad et al., 2011). The *PR1* gene is also involved in PAMP-induced callose formation (Ahmad et al. 2011). *PR2* (also known as *BGL2*) encodes beta 1, 3-glucanase 2 that degrades the fungal cell wall. It cleaves the 1, 3-D-glucosidic linkage in a 1, 3-glucan, an essential part of the fungal cell wall (Stintzi et al., 1993; Borad and Sriram, 2008). *PR2* is activated by SA accumulation and is essential for plant defense against fungi (Thibaud et al., 2004). Another important SA-induced *PR* is *PR3*, a chitinase cleaving chitin polymers of the fungal cell wall (Stintzi et al., 1993; Borad and Sriram, 2008).

Jasmonic acid includes a group of fatty acid-derived compounds (linolenic acid, which is oxygenated by lipoxygenase (13-LOX), forming a peroxide) playing roles in various plant development processes, including seed and pollen development, root growth, flower development, tuber formation, and senescence (Bari and Jones, 2002; Kazan and Manners, 2008; Wasternack and Hause, 2013). An octadecanoid pathway follows the biosynthesis of JA. The pathway starts with the oxygenation of α-linolenic acid (α-LeA, 18:3) by 13-lipoxygenases (13-LOXs) in the chloroplast. Mediated by allene oxide cyclase (AOC) and allene oxide synthase (AOS), 13-HPOT is converted to *cis*-(+)-oxo-phytodienoic acid (OPDA), and OPDA is transported to the peroxisome. The final step of JA biosynthesis is β-oxidation, mediated by acyl-CoA-oxidase 1 (ACX1), converting OPC8 to (+)-7-iso-JA, which is further added to with one isoleucine residue by JA-amino acid synthetase 1 (JAR1) to become (+)-7-iso-JA-Ile (**Figure 2.5**). Attachment of this amino acid to JA results in nuclear localization where it can induce the expression of multiple JA-related genes such as *JAZ* and *MYC2* (Wasternack and Hause, 2013).

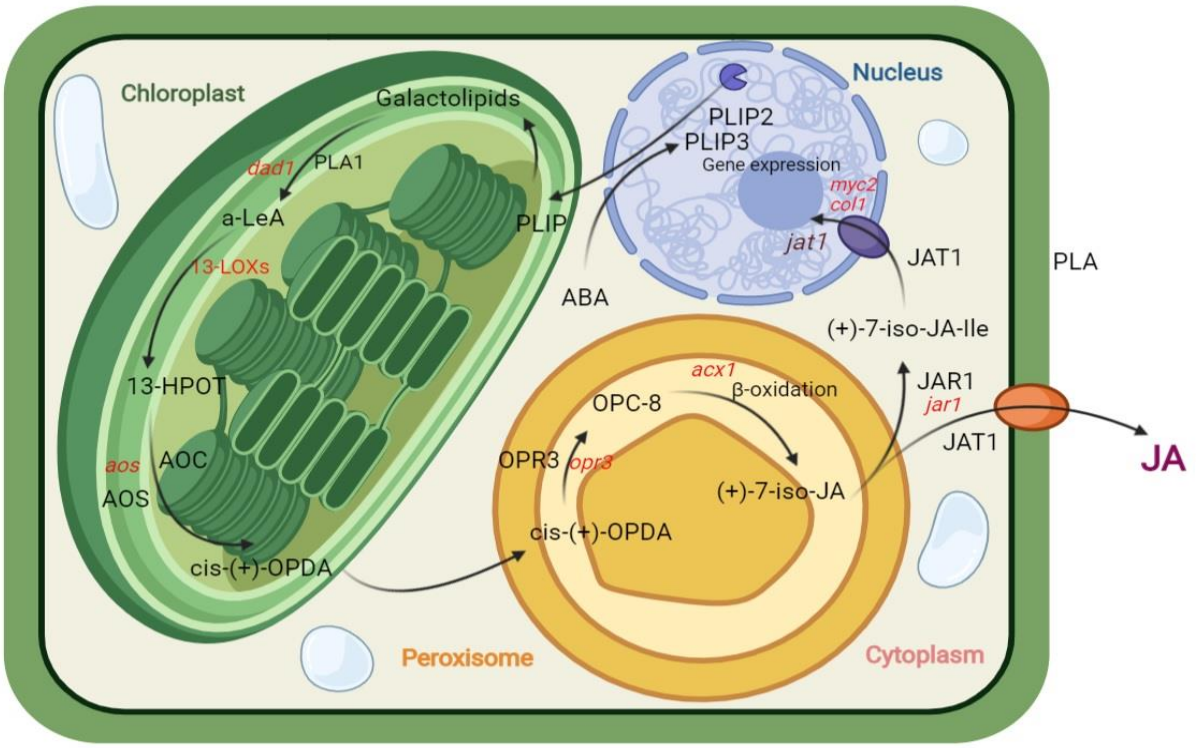

Figure. 2.5 Jasmonic acid biosynthesis pathway. The pathway starts with α-LeA in the chloroplast and moves to the peroxisome at the point of *cis*-(+)-oxo-phytodienoic acid (OPDA). The final product is (+)-7- iso-jasmonate conjugated with isoleucine, which is involved in JA-responsive signalling. A-LeA: α-linolenic acid; AOS: allen oxide synthase; AOC: allene oxide cyclase; 13-LOX: 13-lipoxygenase; 13-HPOT: (13-*S*)-hydroperoxy-octadecatrienoic; OPC-8: 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid; cis-(+)-OPDA: cis-(+)-12-oxophytodienoic acid; (+)-7-iso-JA: (+)-7-iso-jasmonic acid; (+)-7-iso-JA-Ile: (+)-7-iso-jasmonoyl-L-isoleucine. **The image and legend were prepared with the assistance from Dr. Aria Dolatabadian (Fernando Lab).**

Jasmonic acid is associated with ET and light response to inhibit root growth mediated by JA responsive factor COI1. Jasmonic acid also achieves this goal by regulating the synthesis and transport of auxin (Wasternack and Hause, 2013). The inhibitive role of JA towards auxins also causes reduced lateral and adventitious root formation since auxins play essential roles in root growth and development (Wasternack and Hause, 2013). One of the JA-responsive factors,

MYC2, promotes root elongation by inhibiting auxin transport (Dombrecht et al., 2007). Jasmonic acid is also involved in leaf senescence in conjunction with ET, *EIN3*, *ETR1*, *EIN2*, *EIN1,* and *CTR1*, which are essential components of senescence regulation. ET-related factors *EIN3* and *EIL1* positively regulate JA-mediated gene *HDA6* during leaf senescence. If considering JA-responsive signalling solely, factors such as *COI1* and *AOS* are found to regulate the timing of leaf senescence, while JA regulates ET-factor *EIN2* in leaf senescence (Kim et al., 2015).

Jasmonic acid is involved in wounding, insect herbivory responses, and pathogen defense (Kunkel and Brooks, 2002; Kazan and Manners, 2008; Wasternack and Hause, 2013). Among phytopathogens, JA is more specific for defense against necrotrophic pathogens so that JA signalling can enhance resistance to necrotrophic pathogens and herbivorous insects (Kazan and Manners, 2008; Bari and Jones, 2009). JA is also known to play a negative role in PCD and lesion development and reduced lesion development is seen following JA signalling (Overmyer et al., 2000; Rao et al., 2002). However, other evidence suggests that incompatible interactions may also involve the cooperation between SA and JA signalling (Spoel et al., 2007; Becker et al., 2017), making the signalling network more complicated. Spoel et al., (2007) showed that SA- and JA-responsive genes are co-expressed to achieve localized and systemic resistance, which confers a new model of plant defense.

The JA signalling in plant defense against biotic stresses starts early through MAPK cascades. There is an MSK1-MPK4-WRKY33 module when *A. thaliana* is inoculated with *P. syringae*. This pathway can induce antimicrobial camalexin (Qiu et al., 2008). Overexpression of *MPK4* in *B. napus* is also found to induce higher JA-responsive defensin *PDF1.2*, enhancing the resistance against the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Wang et al., 2009). The JA-related *WRKY* factor *WRKY33* enhances the resistance against two fungal pathogens, *B. cinerea* and *Alternaria brassicicola* in *A. thaliana*. They also upregulate the JA-responsive defense genes *PDF1.2* and *PR3* while downregulating the SA-related defense genes *PR1*, *-2*, and *-5* (Zheng et al., 2006). The overexpression of two other JA-related *WRKY* genes, *WRKY28* and *WRKY 75,* enhanced resistance to *S. sclerotiorum* and delayed disease symptom in *A. thaliana*, where the ET-JA-related defense genes *PDF1.2*, *VSP2*, and *LOX2* are upregulated (Chen et al., 2013). For more downstream transcription

factors (TFs) related to JA, a basic helix-loop-helix zip TF (bHLHzip), *MYC2* activates JA-related defense genes *VSP2* and MYC2 to elicit defense against herbivores by inducing *VSP2* (Dombrecht et al., 2007; Wasternack and Hause, 2013). Another important JA-related TF COI1, an F-box protein with Leu repeats, also assists MYC2 signalling. MYC2 protein is physically associated with and repressed by the JAZ proteins. COI1 binds both JAZs and SCFCOI, which activates the ubiquitination and 26S proteasome degradation of JAZs to release the function of MYC2 (Kazan and Manners, 2008; Wasternack and Hause, 2013). This function makes COI1 an essential factor activating JA signalling.

Ethylene (C_2H_4) is a gaseous hormone and signal molecule. ET plays diverse roles in plants, including plant defense, leaf development and senescence, flowering, and fruit ripening. This hormone is also considered an efficient molecular signal in cell-to-cell communication because it is the smallest plant hormone, which is also capable of plant–plant communication. The biosynthesis of ET starts with methionine, which is converted to *S*-adenosyl methionine (SAM) by ACCPage synthase (ACS). SAM is then changed to ACC (1-aminocyclopropane-1-carboxyl acid), which is in turn converted to C_2H_4 by ACC oxidase (ACO) (Dubois et al., 2018).

Exposure to ET can cause leaf yellowing, abscission, desiccation, and necrosis, which suggests its positive roles in leaf senescence. Exposure of rocket salad (*Eruca sativa*) to ET results in chlorophyll reduction and a shorter shelf life (Iqbal et al., 2017). The activation of ET signalling is associated with the expression of a group of genes called *Ethylene Responsive Factor*s (*ERF*s). Like SA- and JA-responsive TFs, *ERF*s interact with MAPK factors and activate ET signalling. The first cloned ethylene signalling component, CTR1, encodes a kinase with homology to mammalian Raf MAPK kinase kinase (MAPKKK) (Kieber et al., 1993). Based on genetic data, CTR1 is a negative regulator and is inactivated after ethylene sensing, which then leads to the activation of downstream components including ethylene-insensitive 2 and 3 (*EIN2*, *EIN3*), ethylene-insensitive like (*EIL*), and *ERF* transcription factors (Zhao and Guo, 2011). The MAPK factor MPK6 physically interacts with an ERF (ERF104) to regulate downstream genes in basal resistance (Bethke et al., 2009). ERF6 can be phosphorylated by the MPK3/6 cascade to induce *PDF1.1* and *PDF1.2* in *Arabidopsis*, enhancing its defense against *B. cinerea* (Meng et al., 2013). The *ERF*s activate multiple

processes, including defense against phytopathogens. The ectopic expression of *ERF1* enhances the resistance by upregulating defense genes such as *PR3* and *PDF1.2* in *Arabidopsis* (Adie et al., 2007). ET signalling is reported to enhance resistance to necrotrophic pathogens or to herbivorous insects (Bari and Jones, 2009).

The signal transduction of SA has an antagonistic relationship with ET and JA (Kunkel and Brooks, 2002; Li et al., 2004; Bari and Jones, 2009). There are specific signalling pathways and factors attributed to each phytohormone. Simultaneously, those sets of pathways also modulate each other to build an extensive genetic network to adapt to various situations during a pathogenic infection, such as the lifestyle of the pathogen, environmental factors, and the location or onset patterns of infection. Bordersen et al., (2005) found that the genes evoking accelerated PCD (*ACD11* and *SID2*) modulate SA biosynthesis. The secretion of SA and ET also enhances lesion development and tissue death following an oxidative burst in plants (Chamnongpol et al., 1998; Overmyer et al., 2000; Rao et al., 2002). The potential cooperation between SA and ET in plant defense was shown by the co-expression of SA- and ET-responsive genes following the infection of *B. napus* by *Leptosphaeria maculans* during an incompatible interaction (Sašek et al., 2012).

Generally, ET is associated with JA in plant defense, and ET-JA signalling shows an antagonistic relationship with SA responsive signalling (Kunkel and Brooks, 2002; Berens et al., 2017; Li et al., 2019). For instance, the JA factor ORA59 and the ET factor ERF1 induce the expression of plant defensin *PDF1.2* (Dubois et al., 2018). The cooperation between *EIN2* and JA signalling exerts defense gene expression such as *PDF1.2*, *THI2.1*, *PR4* (*HEL*), and *CHIB* (Kunkel and Brooks, 2002; Li et al., 2019). However, some studies also indicated that ET and JA also have an antagonistic relationship. For example, ERF1 induces the defense genes against plant pathogens while suppressing genes involved in wound responses (Adie et al., 2007). Moreover, the overexpression of SA-responsive *WRKY70* suppresses JA-signal vegetative storage proteins (*VSPs*), and the suppression of *WRKY70* activates JA/COI1 responsive genes (Li et al., 2004), suggesting an antagonistic relationship between SA and ET-JA. COI1 is the transcription factor for JA signalling, and is also involved in the antagonistic interactions between SA and JA. The overexpression of the master SA regulator *WRKY70* can suppress the expression of *COI1* (Li et al., 2004). Although there is a general theory

implicating the antagonistic relationship between SA and ET-JA signalling, SA-ET cooperation suggests that the interactions among hormones are not simple and straightforward. ET potentiates the SA-induced expression of *PR1*, and the co-expression of SA- and ET-induced defense genes is also found in the *B. napus–L. maculans* interaction (Adie et al., 2007; Sašek et al., 2012). ET has also been found to modulate SA and JA signalling by regulating *NPR1*, suggesting that ET (Leon-Reyes et al., 2009) adjusts the antagonism between SA and JA. Additionally, other hormones may also have an interplay in plant defense, including SA, ET, and JA. For example, SA and ABA work together in stomata-related plant defense; as a negative factor in plant defense, auxin signalling is repressed by SA; cytokinins are known to support the defense against biotrophs and hemi-biotrophs, and there is a synergetic relationship between CKs and SA (Bari and Jones, 2009; Berens et al., 2017).

Brassinosteroids (BRs) are well known for their influence on seed germination, cell division and elongation, and flowering (Bari and Jones, 2009). The function of BRs in plant defense is well known for its responsive factor BRI1-associated kinase 1 (BAK1), which is an essential protein in PAMP-triggered signalling. BAK1 is also involved in ETI (Bari and Jones, 2009). BR also has an antagonistic relationship with GA, stabilizes DELLA proteins (a key negative regulator of gibberellin (GA) signalling), inhibits GA biosynthesis, and activates GA inhibitors (De Bruyne et al., 2014).

Auxins comprise a group of signal molecules that plays numerous pivotal roles in plant development, including lateral root growth and photo- and gravitropism. Auxins also control cell division, elongation, and differentiation (Teale et al., 2006). Auxin signalling has two crucial factors: auxin responsive factors (*ARF*s) and their downstream *GH3* genes. Auxins affect basal defense positively and negatively (Bari and Jones, 2009; Fu and Wang, 2011). Auxins cause cell wall loosening by inducing expansions that can lead to susceptibility to some pathogens. They are also involved in stomatal opening and inhibition of SA signalling, which weaken the plant defense against many phytopathogens (Fu and Wang, 2011). Conversely, the inhibition of auxin signalling can benefit plant defense. For example, the overexpression of micro-RNA miR393, which silences the gene for auxin receptors, can enhance the resistance against *Pst* DC3000 in *Arabidopsis* (Bari and Jones, 2009). However, auxin signalling also contributes to plant resistance. For example, *GH3.5*, a member of the *GH3* family of early

auxin-responsive genes in *Arabidopsis*, encodes a protein possessing *in vitro* adenylation activity on both indole-3-acetic acid (IAA) and SA during pathogen infection, and *GH3-8* in rice enhances host resistance against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Bari and Jones, 2009). Moreover, there are potential pathways for biosynthesis of IAA, indole glucosinolate (IG) and camalexin. Camalexin (3-thiazol-2′-yl-indole), which is toxic to necrotrophic fungi, and IGs, which are broad-spectrum defense compounds, are indole derivatives that share overlapping biosynthesis steps with tryptophan (Trp) dependent IAA synthesis pathways in plant.

The auxin precursor Trp is applied to produce IGs by *CYP79B2* and *CYP79B. ARF1* and *AFR9* inhibit IG production, and *AFR9* supports camalexin accumulation (Fu and Wang, 2011).

Cytokinins (CKs) are crucial for shoot and root growth, inflorescence branching, seed development, and stress tolerance (Bari and Jones 2009). Cytokinin plays positive roles in plant defense, inducing SA-dependent genes such as *NPR1* and *WRKY45* in rice (Akagi et al., 2014). However, CK can also cause more severe pathogenic infections because low to moderate amounts of CK promote the growth of some pathogens, such as powdery mildew on wheat leaves (Albrecht and Argueso, 2017).

Gibberellins (GAs) belong to a large family of tetracyclic diterpenoids. Gibberellin was first identified in an extract from a culture of the fungus *Gibberella fujikuroi,* which causes the ‘foolish seedling’ Bakanae disease in rice (Navarro et al., 2008). Gibberellins regulate a wide range of plant physiological processes, including seed germination, root, leaf, stem, fruit growth, and flower and seed development (Hartweck, 2008; Hauvermale et al., 2012). Gibberellins stimulate plant growth by promoting the degradation of DELLA proteins, which act as the negative regulators of growth. It has been reported that DELLA proteins promote resistance to necrotrophic pathogens by activating JA-ET-dependent defense responses, but also susceptibility to biotrophic pathogens by repressing SA-dependent defense responses in *Arabidopsis* (Bari and Jones, 2009).

By contrast, GA likely promotes resistance to biotrophy and susceptibility to necrotrophy, as GA stimulates the degradation of DELLA proteins. This might be because DELLA proteins regulate immunity by modulating the balance between SA and JA in favour of JA. At the same

time, GA antagonizes JA action and promotes SA signalling perception. Another scenario is where GA antagonizes JA and SA signalling pathways and where the rice DELLA protein SLR1 integrates and strengthens both signalling pathways (De Vleesschauwer et al., 2016). Although the role of DELLA proteins in controlling plant immune responses by modulating SA- and JA-dependent defense responses is well acknowledged (Navarro et al. 2008), its role in hosts undergoing pathogen attack remains subject to debate.

In one study, GA application enhanced *Arabidopsis* resistance to the (hemi) biotrophic bacterial pathogen *Pst* DC3000 and compromised resistance to *A. brassicola,* a necrotrophic fungus (Navarro et al., 2008). Similarly, in rice, the *gid1* mutant (defective in the GA receptor) had higher GA levels. It also showed enhanced resistance to the blast fungus *Magnaporthe grisea* compared with wild-type plants, suggesting that GA signalling components play roles in defense signalling in rice (Tanaka et al., 2006). In contrast, GA treatment enhanced susceptibility to (hemi) biotrophic pathogens *M. oryzae* and *Xanthomonas oryzae* pv. *oryza* (*Xoo*) in rice (De Vleesschauwer et al., 2016). These findings suggest that the role of GA in plant immunity depends on the host and the pathogen (De Bruyne et al., 2014).

Abscisic acid (ABA) is a plant hormone involved in various development and stress responses, including seed germination and embryo maturation (Bari and Jones, 2009). ABA plays an essential role in stress tolerance to heavy metals, heat, drought, radiation, and salinity (Vishwakarma et al. 2017). Generally, ABA plays opposing roles in plant defense. Several mutants conferring ABA deficiency gain more resistance against various diseases (Bari and Jones 2009). For example, *aba2-1* from *Arabidopsis* showed stronger resistance against *Fusarium oxysporum* (Anderson et al. 2004). However, ABA could play positive roles in plant defense, such as defense against tobacco mosaic virus (TMV) in tobacco (plants); TMV infection increases the amount of ABA production of the host plant (Bari and Jones, 2009). Abscisic acid and SA induce resistance against the fungus in the *B. napus* and *S. sclerotiorum* pathosystem (Nováková et al. 2014). Transcripts of the two ABA factors NCED3 and RD26 are induced during fungal infection. Previous studies suggest that the defensive roles from ABA come from its ability in cell wall modification (such as callose deposition), and its involvement in ROS signalling could contribute to plant defense (Bari and Jones, 2009).

2.5.2 Interaction Among the Various Signalling Compounds

Plants have evolved sophisticated and efficient defense mechanisms to cope with biotic stresses and to mount effective defense mechanisms. Although these defense mechanisms are separately well understood, the interaction between these defense components is poorly evaluated. Here, we outline mechanisms underlying plant immunity and emerging roles for immune regulators in biotic stress tolerance. The plant immune system is regarded as consisting of two levels of defense, PTI and ETI, defined by the recognition mechanisms detecting invading pathogens. However, this distinction appears less pronounced than originally believed. Indeed, both PTI and ETI are associated with the activation of defense in the infected tissue, including the generation of ROS, increases in intracellular Ca^{2+} concentrations, the activation of MAPKs, the increased expression of various defense-associated genes, the synthesis of antimicrobial compounds, and the accumulation of SA. This recognition also results in the downregulation of growth, mediated by phytohormones. The components and events occurring following the detection of the pathogen by the host are depicted in **Figure 2.6**.

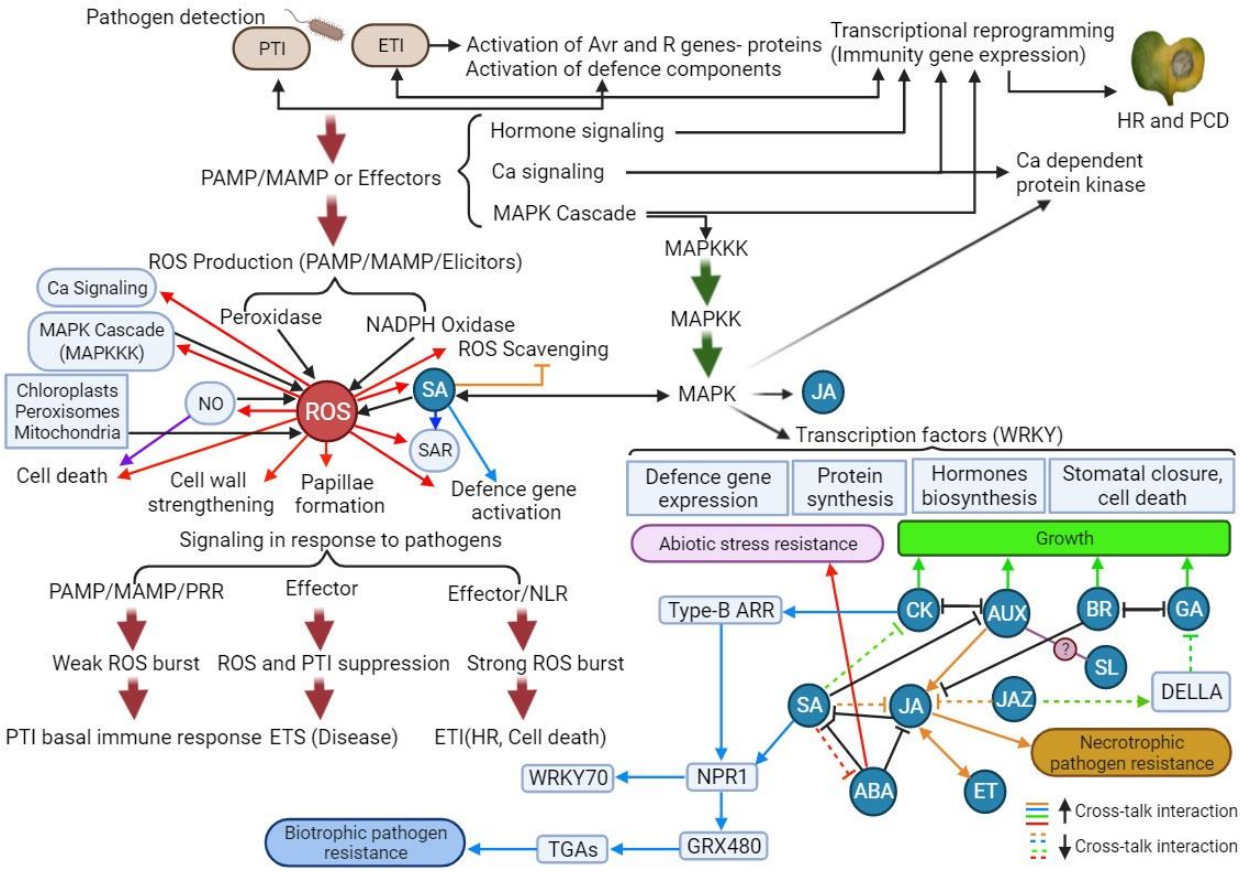

Figure. 2.6 Proposed model depicting the components and events occurring following a host's detection of a pathogen. The network represents relationships between PTI, ETI, ROS, MAPK cascade, and phytohormones. **The image and legend were prepared with the assistance from Dr. Aria Dolatabadian (Fernando Lab).**

ROS are involved in intra-organellar communication to trigger the immune response and are induced in both PTI and ETI. ROS play a central role in PTI responses towards attacking pathogens by a homologous NADPH oxidase. ROS accumulation is detected via different redox-based mechanisms. The perception of PAMPs or MAMPs induces weak ROS bursts and leads to PTI-dependent basal defense responses. In response, pathogens exude effector proteins to suppress the ROS burst and PTI, resulting in effector-triggered susceptibility (ETS). Effectors interact with intracellular nucleotide-binding domain leucine-rich repeat containing receptor (NLR) proteins, leading to a strong ROS burst and HR cell death response. PTI

induces a fast and transient ROS burst, while ETI is associated with a biphasic ROS burst with the second peak usually much stronger and more sustained than during PTI (Chandra et al., 1996).

In addition, the accumulation of ROS activates MAPK signalling cascades, which trigger various downstream pathways, including redox-modulated SA signalling, with NPR1 being the master redox sensor for SA-mediated gene expression in the defense response (**Figure 2.6**). PTI is dependent on the activation of MAPK cascades, while a ROS burst is independent of these factors. The rapid influx of Ca^{2+} into the cytosol, immediately after the activation of PRR signalling and PAMP recognition, is known as the major hallmark of early PTI responses. The subsequent change in cytosolic Ca^{2+} level is then thought to play a role in triggering downstream responses. Changes in cytosolic Ca^{2+} are known as ubiquitous events of signalling networks and are believed to be linked to many cellular functions and immune responses, including ROS production, stomatal closure, the expression of immunity genes and the synthesis of Ca-dependent protein kinases (Thor et al., 2020) (**Figure 2.6**). Nonetheless, how Ca^{2+} influx is regulated during ETI remains unclear.

Upon pathogen detection, ETI is also activated following recognition of effector proteins or their actions by resistance (R) protein receptors and results in HR, a specialized form of PCD, which usually leads to or is linked to resistance associated with NBS-LRR domains R-proteins. The activation of PTI and ETI enhances plant disease resistance and restricts pathogen growth. The detection of effectors or their actions reflects an evolutionary arms race between the host and pathogen to recognize pathogens and initiation of the defense mechanisms and to defeat host defenses and establish compatibility by the pathogen, respectively. This recognition triggers transcriptional reprogramming in plant and pathogens cells.

Rapid activation of the MAPK cascade is a well-known characteristic of PRR signalling. MAPK cascades are involved in both PTI and ETI. The activation of NLR signalling triggers a slower but longer-lasting MAPK activation (Su et al., 2018). MAPK cascades are also involved in ROS generation, HR, and signalling of plant defense hormones (**Figure 2.6**). MAPKs are involved in SA and JA signalling pathways as both positive and negative regulators. MAPKs regulate JA biosynthesis and the expression of JA-dependent genes (**Figure 2.6**). However,

downstream MAPK targets involved in SA- and JA-dependent processes and crosstalk between JA and MAPK signalling are not clearly understood.

SA, JA, and ET play roles in plant defense mechanisms. SA triggers the expression of antimicrobial proteins *PR1* and *PR2* and the activation of SAR. The non-expressor of PR genes 1 (NPR1) is the master regulator in the SA pathway and is required for the full activation of both PTI and ETI, and especially cell death as triggered by ETI (Zhang et al., 2018). SA is generally involved in the activation of defense responses against biotrophic and hemibiotrophic pathogens, while JA and ET are usually associated with defense against necrotrophic pathogens. In most cases, JA and SA defense signalling pathways are mutually antagonistic. Furthermore, JA and ET are associated by activating their responsive signalling factors and mainly play antagonistic roles against SA. Significant JA accumulation occurs when ETI is evoked. According to Rao et al., (2002), SA and ET have cooperative roles during oxidative bursts, causing cell death signals, and JA usually suppresses the related signals.

2.4 *Brassica napus – Leptosphaeria maculans* Pathosystem

2.4.1 Introduction to *Brassica napus*

The origin of *Brassica napus*, according to the model of Triangle U, *Brassica napus* (2n=4×=38, AACC) is formed by the interspecific cross between *B. rapa* (2n=2×=20, AA) and *B. oleracea* (2n=2×=18, CC) (U, 1935; Allender and King, 2010). The *B. napus* was cultivated in 2000 B.C. and introduced in China and Japan in 35 B.C. Rapeseeds were cultivated in Canada since 1942. In 1974, a University of Manitoba plant breeder, Dr. Baldur Stefansson, developed the first “double low” *B. napus* rapeseed variety, which means the variety was low in both erucic acid in the oil and glucosinolates in the meal (Canola Council of Canada, 2020).

Canola is in the genus *Brassica* (*Brassica napus*, *B. rapa* and *B. juncea*) which are bred for altered levels of erucic acid and glucosinolates. Studies suggested that high consumption of erucic acid is correlated with large accumulation of fat in heart muscle of rats when they were fed with it (Vaisey-Genser and Eskin, 1987), Charlton et al., 1975 also suggested that in rats, the dose of erucic acid is positively related to myocardiac necrosis and fibrosis. Therefore,

breeding of canola cultivars aims to reduce the level of erucic acid to lower than 2% (CODEX, 1999; Casséus, 2009). However, canola are also bred with high erucic acid (named HEARs) which are also useful in industry for use as lubricants. Furthermore, by chemical alteration such as nitrogen derivatives or hydrogenation, high erucic acid oils can be used for components in perfumes and water repellents (Nieschlag and Wolff, 1971).

Glucosinolates (GLSs) are a class of secondary metabolites with sulphur and nitrogen. They are products of the reactions between glucose and amino acids, and are considered as toxic compounds, which cause health problems. GLSs are sulphur-rich secondary metabolites in the order of Brassicales, generally classified to/as aliphatic, benzenic and indolic glucosinolates. The biosynthesis of GLSs starts with the elongation of amino acids continues with the formation of the core structures and ends with the secondary modification of the amino acid side chain to form various types of GLSs (Sønderby et al., 2010). Various studies have found a positive relationship between health problems caused by animal feed and the level of GLSs present. For example, the consumption with high GLSs shows the inhibition of thyroid hormones in pigs (McKinnon and Bowland, 1979) and liver hemorrhage in hens (Campbell and Slominski, 1991).

As one of the most valuable crops in Canada, the cultivation of canola generates 29.9 billion CAD annually for Canada and 207,000 jobs (Canola Council of Canada, 2020). In 2017, there were 22.9 million acres of canola cultivation in Canada which produced 21.3 million tonnes annually. Canola oil, meal and seeds give Canada tremendous profits from export. The United States is the biggest importer of canola oil and meal, while China and the European Union are the most important buyers of canola seed (Canola Council of Canada, 2020).

In Canada, canola varieties are mainly cultivated in the western Canadian provinces - Alberta, Manitoba and Saskatchewan. The canola can be sowed in either spring or fall, which results in different types of spring and winter types of canola respectively (Canola Council of Canada, 2020).

Nowadays, new technologies in genomic sequencing and transcriptional studies have been applied to understand the physiological/genetic potential of *B. napus*, which in future, will help

the plant breeders to improve the cultivation and production of canola varieties (Becker et al., 2017; Fu et al., 2019; Lu et al., 2019).

2.4.2 Major Diseases of *Brassica napus*

Plant diseases affect the canola growth and harvest. The pathogen-related diseases towards canola plants include clubroot, verticillium (*Verticillium longisporum*), blackleg, fusarium (*Fusarium oxysporum*), sclerotinia (*Sclerotinia sclerotiorum*) and downy mildew (Canola Council of Canada, 2020).

The first pathogen of interest is clubroot, caused by the soil-borne obligate parasite *Plasmodiophora brassicae*. The pathogen starts to infect host root hairs with the zoospore to form plasmodia on the root, and then, the zoospores from the plasmodia undergo secondary infection to induce visible symptoms (i.e. club-like galls) and damage the crop. The spores from the galls are able to spread by wind to induce further infestation and infection (Hwang et al., 2012). This disease is able to cause large-scale yield losses and reduction of seed quality. Adequate surveillance activities are needed, for example, by sampling of water, dust, soil; one can estimate the general situation of *P. brassicae* infestation in the field (Chai et al., 2014). Another essential action is the sanitation of farm equipment because clubroot is a soil-borne disease, and the infested soil with the resting spores is able to travel from field to field by the transportation of the machinery. Other strategies of management against clubroot include crop rotation, fungicides and bait crops (Chai et al., 2014). The breeding of resistant canola cultivars is also a good tool to hinder the disease. In China, breeding for clubroot resistant cultivars is focused on Chinese cabbage, European cabbage and canola, and in Canada, a 3-year period is needed for resistant cultivars of canola to grow on the clubroot – infested fields (Chai et al., 2014). However, the cultivars should be managed properly since the pathotypes of the pathogen can change due to the selection pressure (Chai et al., 2014; Strelkov and Hwang, 2014).

Sclerotinia stem rot (SSR) is another important disease of canola, which is caused by the fungus *Sclerotinia sclerotiorum* (Canola Council of Canada, 2020). The infection begins with the resting structure named sclerotia in the soil. The structure germinates apothecia, which

disseminate ascospores to infest on petals. The sclerotia are also able to produce hyphae. Apothecia are very essential for the initial infection. Both apothecia and hyphae, infect the pod, flower, stem and leaf and cause water-soaked lesions. The fungal hyphae also form white mold on the stems (McLaren et al., 2004). There are several ways to reduce the infection of *S. sclerotiorum*, such as fungicide, tilling, or crop rotation (McLaren et al., 2004). Fungicides like anilinopyrimidines, benzimidazoles and dicarboxamides have been used to control the disease (Derbyshire and Denton-Giles, 2016). The best time for the fungicide application is the period right before the outbreak of the infection, and the outbreak can be predicted by forecasting system (McLaren et al., 2004). The practice of tilling also helps to reduce the chance of apothecia formation from sclerotia since the sclerotia need enough depth in soil to develop apothecia (Derbyshire and Denton-Giles, 2016). Development of a forecasting system is also a useful way to predict the epidemic of the disease, since the spread of SSR is heavily reliant on environmental factors. For example, 15 to 20 ^{o}C is an optimal range of temperatures for apothecia germination. Soil moisture is also a positive factor for fungal development, which may increase the disease incidence (McLaren et al., 2004). Usually, the flowering stage of the crop is when it is most susceptible to infection by the fungus; therefore for fungicide application, the timing of flowering should also be considered (McLaren et al., 2004). Forecasting sclerotinia stem rot by petal testing system makes good prediction of disease risk and incidence (McLaren et al., 2004). In recent years, there have been efforts to develop biocontrols against SSR by inputting some capable microbes to directly or indirectly inhibit the pathogenic growth. One of the most notable biocontrol strategies against SSR is the bacteria *Pseudomonas chlororaphis* PA23 (Manuel et al., 2012). PA23 is able to stop the fungus through multiple mechanisms s such as inhibition of spore germination, hyphal lysis and vacuolation. PA23 produces the antibiotics phenazines and pyrrolnitrins to achieve those anti-fungal activities (Selin et al., 2012; Kamal et al., 2016).

The third major disease discussed herein is the verticillium stripe. In canola, this disease is caused by the fungus *Verticillium longisporum* (Heale and Karapapa, 1999; Zhou et al., 2006; Zou et al., 2020). Another close relative of *V. longisporum*, *V. dahliae* can also cause similar disease on *B. napus* (Zhou et al., 2006; Hwang et al., 2017). The major symptoms of verticillium stripe include blackening of the stem, leaf defoliation, deformation of pods and formation of microsclerotia (Hwang et al., 2017). However, the infection process from *V.*

longisporum and *dahliae* is different: in oilseeds, *V. longisporum* colonization starts at the root and spreads throughout the host, while *V. dahliae* generally stays at the basal regions (i.e. root, basal stem). Furthermore, *V. dahliae* tends to grow mycelia better from the low-glucosinolate cultivars compared with the high-glucosinolate cultivars, which did not happen in *V. longisporum* infection (Zhou et al., 2006). Generally, Verticillium species damage their host by blocking the water uptake and transport which eventually brings about/causes the wilting symptoms. Finally, after successful infection throughout the vascular tissues, the Verticillium species survive as microsclerotia in the soil which are able to infect in the following growing seasons (Carroll et al., 2018), and the microsclerotia can survive in the soil for several years (Canola Council of Canada, 2020). Verticillium stripe firstly occurred on the lettuce in California, 1995 (Carroll et al., 2018), and in Canada, it was first found in 2014 in Manitoba (Canola Council of Canada, 2020). The management of this disease includes crop rotation, soil fumigation, and adaption of resistant cultivars. Other management practices are to clean and monitoragricultural tools/equipment, since *V. longisporum* and *V. dahliae* are soil-borne fungi, and therefore it is easy for them to contaminate used tools to travel long-distance from one farm to another (Carroll et al., 2018).

Alternaria blackspot is caused by the fungus *Alternaria brassicae*, which is able to infect the *Brassicaceae* plants including *Brassica napus* (Nowicki et al., 2012). The pathogen usually causes the damping-off of seedlings, and black spots on the leaves. The infection upon the seeds results in reduction in seed germination and seedling vigor (Nowicki et al., 2012). *A. brassicae* is able to reside inside of the host seeds and debris. It penetrates the plant through stomata. Following the penetration, the hyphal development of the fungus creates brownish lesions on the leaves. Under favourable conditions, the hyphae sporulate and further damage the plant until it finally perishes (Nowicki et al., 2012). Cool temperature and high moisture are essential environmental factors for the fungus to prevail, e. g., the temperature range of 18 to 24 ^{o}C is optimal for hyphal growth, and high air humidity (95 to 100%) is favorable for plant infection (reference). The most feasible way to manage this disease is to breed resistant *B. napus* genotypes. Some *Brassica* crops have genetic resistance against the fungus (*A. brassicae*). The resistant genes are found to relate to the high activities of phenolases, high levels of leaf sugar and epicuticular wax layer (Nowicki et al., 2012).

2.4.3 Introduction to *Leptosphaeria maculans*

Blackleg, mainly caused by the fungus *Leptosphaeria maculans* (anamorph: *Phoma lignam*) is one of the most devastating diseases for *Brassica napus*. Blackleg is able to cause up to 50% yield loss in western Canada (Canola Council of Canada, 2020; Zhang and Fernando, 2017). Besides rapeseed, the fungus also infects other cruciferous taxa such as *Raphanus savitus* (radish) and *Sinapis alba* (white mustard) (Rouxel and Balesdent, 2005). *L. maculans* has been a worldwide problem, since it has spread globally, *L. maculans* and *biglobosa* travel throughout the world possibly because of the seed transmission (Fitt et al., 2006). The blackleg disease has caused massive problems in many countries including UK, Australia, France and Canada (Fitt et al., 2006).

Blackleg disease also can be caused by *Leptosphaeria biglobosa*, which does not cause stem canker but causes leaf and upper stem lesions (Thomas et al., 2009). *L. biglobosa* did not cause severe damage on canola in western Canada. The emergence of the new fungus *L. maculans* in 1975 caused severe damage to the canola crop (Bailey et al., 2003). *L. biglobosa* induces resistance against *L. maculans* from *Brassica napus* and *juncea* (Thomas et al., 2009). In China, only *L. biglobosa* was found in blackleg affected canola plants, and many Chinese cultivars are very susceptible to *L. maculans* (Fitt et al., 2006; Zhang and Fernando, 2017).

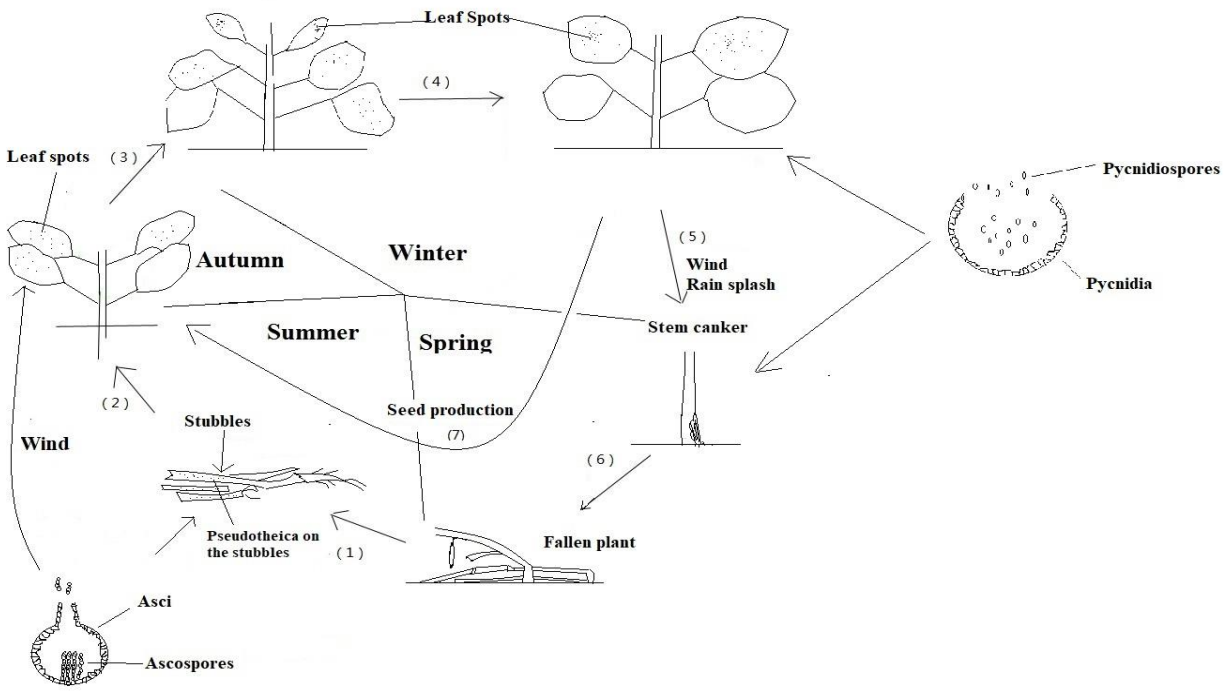

Figure 2.7. The life cycle of blackleg: (1) saprophytic stage/long-term survival pseudothecia, ascospores (sexual reproduction) from asci may be transmitted by wind (2) cotyledon primary infection which causes leaf spots/lesions (3) asexual reproduction (pycnidia) after primary infection, which causes leaf spots and lesions; ascospores may also infest on leaves by wind blow (4) pycnidia releases pycnidiospores (5) pycnidiospores cause stem canker and root rot, at the same time, water splash/wind may transmit pycnidiospores to infect other parts of the plant, enter the soil to be absorbed by the root or inoculate a new host (6) stem canker and root rot perish the host (7) pycnidiospores enter seeds.

The life cycle of *L. maculans* (**Figure 2.7**) starts with the black fruiting bodies pseudothecia which are located on canola stubble. Pseudothecia contain large numbers of yellow-brown ascospores from sexual reproduction. The ascospores are transmitted to leaves kilometers away by rainfall or wind, which cause white lesion on leaves (Zhang and Fernando, 2017). The infesting ascospores on the leaves proliferate by asexual reproduction to form a new type of fruiting body called pycnidia (usually black dots on tissue lesions). By rainfall or wind, the pycnidia ooze asexual pycnidiospores, which are able to infect the crop as the secondary infection. The fungal entrance from the leaf to the petiole and the stem, which results in stem

canker, triggers severe infection. Eventually, it causes the lodging of the whole plant; as well, the fallen plant pieces become stubble, which are contaminated with the fungal fruiting bodies. The fungus on the stubble can be viable for years to infect new hosts in the following growing seasons (Canola Council of Canada, 2020).

In Canada, the major source of inocula are pycnidiospores, while for other countries are ascospores, the difference is due to the heavy rain during the growing season of canola in Canada, which is the favorable condition for pycnidiospores to germinate and be rain-splashed (Guo et al., 2005; Fitt et al., 2006; Ghanbarnia et al., 2009; Canola Council of Canada, 2020).

2.4.4 Physiology of *L. maculans*

Plant pathogens are able to produce secondary metabolites called phytotoxins which assist in their infection of the hosts and exploitation of host nutrients. For *L. maculans*, there are several phytotoxins. One family of toxins is sirodesmin PL, and they belong to the epipolythiodioxopiperazine group. One of its members, sirodesmin PL, is a non-selective phytotoxin causing chlorotic leaf lesion (Rouxel et al., 1988; Rouxel and Balesdent, 2005; Mitrovic et al., 2012). In *L. maculans*, there are 18 (in 4 clusters) genes related to sirodesmin PL biosynthesis, which co-regulate each other in sirodesmin production (Gardiner et al., 2004).

Another remarkable host-selective toxin is phomalide, which is produced from first 30 to 60 hours of germinated conidia. Phomalide causes leaf lesion on *B. napus*, but does not inflict strong sensitivity upon *B. junce*a (Pedras et al., 1993; Rouxel and Balesdent, 2005).

Besides phytotoxins, *L maculans* possesses various enzymes to reinforce its infection upon hosts. Three cell-wall-degrading enzymes (CWDEs): endopolygalacturonase (*pg1*) and two cellulase (*cel1* and *2*), and *cel2* transcripts are found in the cotyledons and leaves from *B. napus* and *B. juncea* (Sexton et al., 2000). Another isocitrate lyase gene involved in glyoxylate pathway is essential for *L. maculans* pathogenicity, and the deletion of this gene reduced the fungal pathogenicity upon *B. napus* (Idnurm and Howlett, 2002).

2.4.5 Blackleg Management

There are three common strategies to manage blackleg: crop rotation, fungicide and resistant cultivars.

The crop rotation with cereals and pulses is an effective management strategy. Usually, a crop rotation in a period up to 3 years is recommended, this slows down the emergence of blackleg races overcoming the current resistant canola cultivars, also, the canola crop fields have to be separated to prevent the transmission of ascospores because there might be residues with fungal pseudothecia in the soil throughout 2 to 3 years (Canola Council of Canada, 2020).

Another common management strategy is fungicide application. Fungicides MBC (Methyl Benzimidazole Carbamate), flusilazole and tebuconazole inhibit mycelia growth of both *L. maculans* and *biglobosa* (Eckert et al., 2010). Other fungicides, such as fluopyram and flutriafol are also effective in reducing blackleg severity (Peng et al., 2020). Fungicides treating stem canker are sprayed during leaf spot phase, which is the period before the fungal cells enter the stem. However, fungicide treatment is uneconomic in the countries outside Western Europe, including Canada and Australia (West et al., 2001).

Among all the methods to stop the disease, developing resistant canola cultivars is both cost-effective and most promising to reduce yield loss (Raman et al., 2011). The resistant cultivars are able to reduce the severity of blackleg inside the stem where fungicides may not reach. Brassica species with various genetic backgrounds related to blackleg resistance have been studied and several varieties have been used for commercial cultivation, especially in Australia, France and Canada (Raman et al., 2011). For example, cultivars termed “sylvestris”, with major gene resistance, were commercially grown in Australia (starting in 2000) andfor several years those cultivars erased blackleg (Sprague et al., 2006). A study in AAFC (Agriculture and Agri-Food Canada) from 2000 to 2006, in Melfort and Scott, Saskatchewan, suggested that the rotation of two to four years with “R” hybrid cultivars reduced the blackleg severity (Canola Council of Canada, 2020). Rotation among canola cultivars with different *Rlm* genes is able to reduce blackleg severity, this management is able to relieve the disease pressure by manipulating the blackleg population (Marcroft et al. 2012; Zhang and Fernando, 2017).

The resistance of *B. napus* against *L. maculans* has two types: qualitative and quantitative resistance. Qualitative resistance is triggered by the interaction between the Avr proteins from the pathogens (AvrLm for *L. maculans*) and R proteins from the hosts (Rlm for *B. napus*). This type of interaction is called gene-for-gene interaction (also known as incompatible interaction). The plant-microbe interaction is called compatible interaction when the pathogenic Avr protein and the R protein of the host are lost or modified, whereby host is susceptible towards plant pathogen, and the pathogen usually causes more damage upon the host. R resistance triggers a series of rapid and localized host signaling cascades known as hypersensitive response (HR) which results in localized cell death to halt the further spread of the pathogen (Agrios, 2004; Chisholm et al., 2006; Knepper and Day 2010).

Quantitative resistance (QR), on the other hand, consists of multiple genes to confer the resistance, the studies towards QR currently are based on quantitative trait loci (QTL) (Agrios, 2004; Poland et al., 2009; Huang et al., 2009; Brun et al., 2010; Raman et al., 2018). In *Brassica napus*, unlike *Rlm* gene resistance, QR does not eliminate all *L. maculans* fungal cells, only relieves the severity of the disease (Huang et al., 2009b; Brun et al., 2010; Raman et al., 2018). Brun et al., (2010) has also shown that QR is able to increase the durability of *Rlm* resistance in the fields.

2.4.6 *AvrLm*s and *Rlm*s

The aforementioned interaction between matched *AvrLm* and *Rlm* proteins causes hypersensitive responses, therefore, the breeding of *B. napus* aims at creating the varieties with the *Rlm*s matching with the dominant *AvrLm*s in the *L. maculans* population from one region.

Currently, sixteen *AvrLm* genes (*AvrLm1* to *4-7*, *AvrLmJ1*, *AvrLm6* to *11*, *AvrLepR1 to 4*) (Rouxel and Balesdent, 2005; Gout et al., 2006; Fudal et al., 2007; Parlange et al., 2009; Kutcher et al., 2011; Balesdent et al., 2013; Van de Wouw et al., 2014) and sixteen *Rlm* genes (*Rlm1* to *11*; *LepR1* to *4*; *RlmS*) have been identified (Balesdent et al., 2002; Yu et al., 2005; Yu et al., 2012; Kutcher et al., 2011; Balesdent et al., 2013; Ghanbarnia et al., 2015).

For *R* genes, only *LepR3* and *Rlm2* have been cloned so far in *B. napus* (Larkan et al., 2013, 2015). For *AvrLm* genes, *AvrLm1*, *2*, *3*, *4-7*, *J1-9*, *6*, *10* and *11* have been cloned (Gout et al., 2006; Fudal et al., 2007; Parlange et al., 2009; Balesdent et al., 2013; Van de Wouw et al., 2014; Ghanbarnia et al., 2015; Plissonneau et al., 2016; Ghanbarnia et al., 2018; Petit-Houdenot et al., 2019). The *Avr* genes encode mostly but not exclusively cysteine-rich proteins (Rep, 2005). *AvrLm4-7* and *6* are the two Avr proteins with many cysteine residues (Fudal et al., 2007; Parlange et al., 2009), while *AvrLm1* has only one cysteine residue (Sašek et al., 2012). The plant *R* genes usually encode a nucleotide binding site-leucine-rich repeats (NB-LRR) class of proteins with N-terminus of either Toll-Interleukin-1 Receptor (TIR) or Coiled-Coil (CC). NB-LRR protein has conserved nucleotide-binding (NB) site at amino-terminus which is for ATP or GTP, and LRR domain at the C-terminus has variable spatial organization and length, possible for protein-protein interactions and peptide/ligand binding (Chisholm et al., 2006; Knepper and Day, 2010). The identified LepR3 may resemble the Receptor-Like Kinase (RLK) type of *R* gene.

In *L. maculans*, many SSPs, including *AvrLm* genes, are located in the AT-rich heterochromatin regions, which have intensive transposon invasions and RIPs (Parlange et al., 2009), and which are enforced by sexual reproduction and large population. As a result of this, the microevolution of the species is very powerful, with the *AvrLm* profiles of the fungal population being able to switch in a short time due to natural selection (Rouxel and Balesdent, 2005; Daverdin et al., 2012). Zhang et al. (2016a) reported the breakdown of *Rlm3* canola varieties in western Canada, *Rlm3* is the most prevalent *Rlm* gene from the canola accessions in this study, and Zhang et al. (2016), also reported the deletion of *AvrLm3* from the fungal population while the proportions of *AvrLm4*, *6* and *7* were still high. Besides, Canada, other countries also have their histories of *Rlm* gene breakdown. For example, In France, *Rlm6* was broken down within 1 year because of the RIP and retrotransposon mutations of the pathogen (Fudal et al., 2009), *AvrLm1* was 83% in the population in 1997-1998, and it was reduced to less than 13% in 1999-2000 (Rouxel et al., 2003). In Australia, the *AvrLm1* occupied 80% the tested *L. maculans* isolates in 2003, whereas, in 2006, this percentage had reduced to less than 40%, and *AvrLm6* was over 77% until 2003, and dropped to the range of 23% to 47% since then, except for 2009 (58%) and 2012 (80%) (Van de Wouw et al., 2017).

The fitness cost in losing *AvrLm4* is heavier than *AvrLm1*, since *AvrLm4* is reported to cause more severe symptoms, such as leaf lesions, stem cankers, hyphal development (Huang et al., 2009). Therefore, because of fitness and other reasons, the frequency of individual *AvrLm* genes can be different in nature. For example, Avr4 in *Cladosporium fulvum* inhibits chitinase activity in tomato and Avr2 is found to target to a cysteine protease, and both Avr4 and Avr2 are cysteine-rich small proteins (de Wit et al., 2009). The presence of *AvrLm4-7* is also found to mask the recognition between *AvrLm3* and *Rlm3*, however, *AvrLm4-7* and *AvrLm3* had no physical interaction (Plissonneau et al., 2016).

2.4.7 Molecular Interaction between *Brassica napus* and *Leptosphaeria maculans*

The downstream signaling after hypersensitive response involves a complicated signaling network in which some of the sections are also similar with other pathosystems.

When *Brassica napus* with specific *Rlm* recognizes its matching *AvrLm*, this will trigger various types of responses from plant cells to attenuate the spread of disease. For example, the recognition of *AvrLm1* by *Brassica napus* plants containing *Rlm1* gene triggers the biosynthesis of salicyclic acid (SA) and ethylene (ET), and these two molecules mediate resistance against blackleg disease (Sašek et al., 2012).

The global expression in the condition of HR (*AvrLepR1 – LepR1* interaction), and found that the genes related to WRKY factors, callose deposition, lignin deposition, hormone secretion, and glucosinolates were up-regulated (Becker et al. 2017). By inoculating *L. maculans* isolates on Arabidopsis mutants a group of genes had been identified such as *PEN1* (basal resistance) and *APK2* (glucosinolate biosynthesis) in plant defense to blackleg infection. Moreover, localized callose deposition and cellular collapse were observed around the site of inoculation.

Besides qualitative resistance, quantitative resistance exhibits slower and more prolonged defensive responses which involve more complicated intrinsic signaling networks (Agrios, 2004; Knepper and Day 2010). Qualitative resistance is the major resistance for a young canola plant (around seedling stage), while the adult plant relies on quantitative resistance (which is also named adult plant resistance, APR) (Dion et al., 1995; Delourme et al., 2006;

Rimmer, 2006; Zhang et al., 2016b). However, young *Brassica napus* plants (when each *B. napus* plant has three expanded leaves) are also able to induce quantitative resistance by leaf lamina or leaf petiole inoculation, and the variety with quantitative resistance hinders the fungal growth from both petiole and stem tissues (Huang et al., 2014).

Since quantitative resistance involves multiple factors and more complicated genetic networks, there are currently multiple theories to explain how this type of resistance works. Genes involved in quantitative resistance are possibly genes encoding phytoalexins, signaling factors in defensive pathways; there are even suggestions that quantitative resistant genes are the minor *R* genes *in planta* (Poland et al., 2009). Scientists characterize this type of defense by identifying the quantitative traits loci (QTLs) from which, it is feasible to construct the linkage maps locating potential genetic markers for resistance against *L. maculans* (Pilet et al., 2001; Rimmer, 2006; Kaur et al., 2009).

Furthermore, recent studies suggest that the combination of genetic backgrounds between qualitative and quantitative resistance helps the duration of resistance through *Rlm* genes, and the canola varieties with combined resistance are considered a useful tool to lengthen the effectiveness of *Rlm* genes. The combination of qualitative and quantitative resistance was able to retain the diversity of virulence alleles in *L. maculans* populations throughout/for 8 years, and because the quantitative resistance, the severity of stem canker was controlled even when the *Rlm6* was eroded (Delourme et al., 2014). The quantitative background increases the durability of *Rlm6* and controls the stem canker (Brun et al., 2009).

Plants possess a complicated network with multiple independent signaling pathways elicited from many cytological components (for example, hormones, effectors, ROS molecules). The regulation and interaction within pathways depend on the physiology of host and the lifestyle of pathogen, slight changes in defensive cellular components/signals could shape the plant defense remarkably.

2.5 Objectives of The Thesis

Since there have been plenty of studies in plant – microbe interactions, those studies start to sketch a vast map of signaling network in planta when being attacked by pathogens. However, many details are yet to be explored, for example, the onset pattern of certain genes could be a key element for effective resistance, and the effect of environmental factors (such as temperature) upon plant defense could predict the expression of defense in the fields. Moreover, the studies in plant-microbe interaction in molecular level are mostly based on *Arabidopsis thaliana*, pathosystems involving other plant species are limited in research. The recent studies in *Brassica napus – Leptosphaeria maculans* utilize the theories concluded in *Arabidopsis* to understand molecular signaling in *B. napus* defense against *L. maculans*. This thesis is the continuation of this field. The recent studies in molecular signaling in *B. napus* against *L. maculans* have been mostly based on the comparisons in cellular observation/transcriptional profiling between resistant (incompatible interaction) and susceptible (compatible interaction) cases. Since there are also *B. napus* cultivars which shows intermediate resistance against certain *L. maculans* isolates. They displayed the phenotypes that resemble both resistant and susceptible genotypes, studying the signal transduction in intermediate genotypes is another intriguing field to explore and compare/contrast with resistant and susceptible genotypes.

By considering all those blindspots of the current research, I decided to investigate three objectives in my PhD thesis. The first objective is to study the onset patterns of hormone-responsive genes in susceptible, intermediate and resistant genotypes. Hormones have been suggested to assist plant defense in various diseases, as a crucial element of plant defense (Kazan and Manners, 2008; Bari and Jones, 2009; Berens et al., 2017). However, studies are limited in timing of the hormone-related genes, since *L. maculans* is a hemi-biotrophic fungus, it undergoes biotrophic first and necrotrophic stage later (Perfect and Green, 2001). SA is responsible for the defense against biotrophs and JA is necrotrophs (Kazan and Manners, 2008), therefore, the SA signaling towards biotrophic stage of *L. maculans* could be beneficial for *B. napus* resistance. The chapter hypothesizes that the early expression of certain hormonal genes (such as SA responsive genes) is crucial for effective HR. The second objective explores the onset patterns of ROS related cellular behaviors and signaling in *B. napus* defense against *L. maculans* fungus. ROS signaling considered as an early response following pathogenic attack,

ROS signaling invokes various subsequent signal transduction (Lamb and Dixon, 1996; Torres and Dangl, 2005; Zurbriggen et al., 2010; Baxter et al., 2013; Qi et al., 2017). Therefore, it is necessary to look at the ROS related cellular activities and factors in a time course with susceptible, intermediate and resistant severities. This objective aimed at observing the timing patterns of those cellular activities among those three severities. I intended to see whether those timing patterns reflect that it is important to trigger certain ROS activities for strong and durable resistance. This objective hypothesizes that the secretion of ROS molecules and the activation of ROS – related genes appears to be earlier in more resistant *B. napus* genotype. The third objective of this thesis is to see the effects of distinct temperature conditions upon the intrinsic signaling in HR defense in *B. napus*. Temperature has been considered as an intriguing external factor in shaping instrinsic signaling pathways. Previous studies have shown that the switching of enviormental conditions altered the expression of some defense – related genes such as *PR1*, and *BON1* (Malamy et al., 1992; Jambunathan et al., 2001; Jambunathan and McNellis, 2003; Yang et al., 2006). Testing the effects of HR in different temperature condition will enable to predict the performance of *B. napus* defense against *L. maculans* in various incubating conditions or fields. The hypothesis of this chapter is that the expression of certain defense genes is influenced by temperature, and these alterations in expression correlate to the altered phenotypes of the HR.

Hormones have been suggested to assist plant defense in various diseases, as a crucial element of plant defense (Kazan and Manners, 2008; Bari and Jones, 2009; Berens et al., 2017). However, studies are limited in timing of the hormone-related genes, since *L. maculans* is a hemi-biotrophic fungus, it undergoes biotrophic first and necrotrophic stage later (Perfect and Green, 2001). This is necessary to study the relationship between onset patterns of hormone-responsive factors and *B. napus* defense against *L. maculans*.

CHAPTER 3

Hormonal Responses to Susceptible, Intermediate, and Resistant Interactions in the *Brassica napus–Leptosphaeria maculans* Pathosystem

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3.1 Abstract

Hormone signaling plays a pivotal role in plant–microbe interactions. There are three major phytohormones in plant defense: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). The activation and trade-off of signaling between these three hormones likely determines the strength of plant defense in response to pathogens. Here, we describe the allocation of hormonal signaling in *Brassica napus* against the fungal pathogen *Leptosphaeria maculans*. Three *B. napus* genotypes (Westar, Surpass400, and 01-23-2-1) were inoculated with two *L. maculans* isolates (H75 8-1 and H77 7-2), subsequently exhibiting three levels of resistance: susceptible, intermediate, and resistant. Quantitative analyses suggest that the early activation of some SA-responsive genes, including *WRKY70* and *NPR1*, contribute to an effective defense against *L. maculans*. The co-expression among factors responding to SA/ET/JA was also

observed in the late stage of infection. The results of conjugated SA measurement also support that early SA activation plays a crucial role in durable resistance. Our results demonstrate the relationship between the onset patterns of certain hormone regulators and the effectiveness of the defense of *B. napus* against *L. maculans*.

Keywords: *Leptosphaeria maculans*; *Brassica napus*; hormone signaling; gene expression; salicylic acid (SA); jasmonic acid (JA); ethylene (ET); defense

3.2 Introduction

Plant hormones (or phytohormones) refer to a group of small biomolecules that flow throughout the plant body and play various roles in the physiological processes and signal transduction. Plant defense, as one of these biological processes, involves the co-operation of multiple hormones (Kunkel and Brooks, 2002; Bari and Jones, 2009; Berens et al., 2017). Among these hormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are considered to play major roles (Bari and Jones, 2009; Berens et al., 2017). Conventional theory from previous studies (in Arabidopsis) suggested that the signals from SA and ET/JA have antagonistic relationships to each other. SA is more effective in defending against biotrophic and hemi-biotrophic pathogens, while ET/JA signaling is more capable of resisting necrotrophic pathogens and herbivorous insects (Bari and Jones, 2009; Berens et al., 2017).

Each hormone has certain responsive factors and signaling pathways, where the responsive pathways of different hormones also have different potential connections, building up an integrated and systemic signaling network in order to cope with various challenges (Kunkel and Brooks, 2002; Bari and Jones, 2009; Berens et al., 2017). *WRKY70* encodes a transcription factor that lies on the node between SA and JA signaling; the up-regulation of *WRKY70* activates SA signaling and suppresses JA signaling (Li et al., 2004). In *Arabidopsis thaliana*, *Coronatine-Insensitive 1* (*COI1*) has been found to regulate JA signaling in root growth, plant defense, and senescence (Vlot et al., 2009). *Ethylene Insensitive 3* (*EIN3*) encodes a nucleus-localized transcription factor that positively regulates ET signaling (Benavente et al., 2006). Hormonal pathways eventually induce responsive downstream proteins that have anti-microbial activities. For instance, Pathogenesis-Related Protein 1 (PR1) proteins (encoded by

PR1 genes) are the responsive factors of SA signaling (Kunkel and Brooks, 2002), while another *PR* gene—*Pathogenesis-Related Protein 4* (*PR4*)—is activated by ET/JA signaling (Norman-Setterblad et al., 2000).

B. napus has two types of in vivo resistance to *L. maculans*: qualitative and quantitative (Agrios et al., 2004). Qualitative resistance is triggered by the interaction between the Avr proteins of the pathogen (AvrLm for *L. maculans*) and R proteins from the host (Rlm for *B. napus*). This type of interaction is also called an incompatible interaction (resistance), while an interaction without the Avr–R protein interaction is called a compatible interaction (susceptible). Incompatible interactions trigger a series of rapid and localized host signaling cascades named hypersensitive response (HR), which includes reactive oxygen species (ROS) production, programmed cell death (PCD), and systemic acquired resistance (SAR) (Agrios, 2004; Knepper and Day, 2010).

Previous studies have suggested that compatible and incompatible interactions may have similar molecular signaling network profiles, including hormonal secretion and signaling. In the *Arabidopsis thaliana–Pseudomonas syringae* pathosystem, the expression profiles between compatible and incompatible interactions are similar; however, some genes in the incompatible interaction are activated earlier than in the compatible interaction, which makes the incompatible interaction more robust (Tao et al., 2003; Spoel et al., 2007). By studying the pathosystem between *Arabidopsis thaliana* and *Alternaria brassicicola/Pseudomonas syringae* pv. tomato DC3000, it has been shown that the R-protein resistance activates the hormone-regulated factors that are able to defend against both biotrophs and necrotrophs (Spoel et al., 2007). Moreover, studies of the gene-for-gene interaction in the *B. napus–L. maculans* pathosystem have also observed the early induction of SA/JA responsive factors, indicating the importance of those genes in the incompatible interaction (Sašek et al., 2012; Becker et al., 2017). Plants have developed a huge network of hormonal signaling pathways to cope with pathogenic invasion. Among plant hormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the three major phytohormones released in response to plant pathogens (Kunkel and Brooks, 2002; Borad and Sriram, 2008; Berens et al., 2017). Therefore, investigating the signaling and interactions among these three hormones becomes necessary to understand the three selected *B. napus* genotypes responded differently to *L. maculans*.

There have been studies in hormone signaling between *B. napus* and *L. maculans.* However, the comparisons (in hormones) among susceptible, intermediate and resistant interactions have not been well studied in *B. napus*. In this study, we selected three *B. napus* genotypes to be inoculated with two *L. maculans* isolates, such that the host cotyledons were able to produce three typical disease severities: susceptible, intermediate, and resistant phenotypes. The goal of this research is to find the connection between the *B. napus* defense and the hormonal signaling, we aimed to find which types of the hormonal regulation (in quantity and onset patterns) are optimal for *B. napus* to understand distinct ways of regulation of the hormonal signaling among the three levels of interaction (i.e., susceptible, intermediate, and resistant). The expression levels of several genes, which are crucial for hormonal–responsive defense, were analyzed in the aspects of both quantity and onset pattern; those analyses help to explain the relationship between hormone signaling and disease severity/host resistance.

3.3 Materials and Methods

3.3.1 Plant Growth and *Leptosphaeria maculans* Isolates

Three *Brassica napus* genotypes Westar (no *Rlm* genes), Surpass400 (*BLMR1/LepR3* and *BLMR2/RlmS*), and 01-23-2-1 (*Rlm7*)) were grown in Sunshine Professional Growing Mix (SumGro Horticulture), with a cycle of 16 h of light (light intensity: 323 μmol/S·m^2, 22 °C) and 8 h of night (16 °C) at 50–60% relative humidity. *L. maculans* isolates H75 8-1 (genotype: *avrLm1*, *AvrLm2*, *avrLm3*, *avrLm4*, *AvrLmJ1-5*, *AvrLm7*, *AvrLm6*, *avrLm9*, *AvrLm11*, *avrLepR1* and *AvrLepR2*) and H77 7-2 (genotype: *AvrLm1*, *avrLm2*, *avrLm3*, *AvrLm4*, *AvrLmJ1-5*, *AvrLm7*, *AvrLm6*, *avrLm9*, *AvrLm11*, *avrLepR1*, and *avrLepR2*) were cultured on V8 juice agar medium (Campbell's, Camden, NJ, USA) at room temperature in the light. The culturing of isolates lasted for 10–14 days to produce pycnidiospores. Each culture was scraped and washed by 2 mL of distilled water to collect pycnidiospores.

3.3.2. Cotyledon Inoculation

The harvested pycnidiospores were adjusted to a concentration of 2×10^7 spores/mL for cotyledon inoculation tests.

The cotyledons of seven-day-old seedlings were punctured by a modified tweezer and inoculated by 10 µL diluted inoculum. Each cotyledon lobe was punctured by a modified tweezer; thus, there were four points of inoculation on each seedling canola cotyledon.

3.3.3 Lesion Size Quantification

The cotyledons from 3 to 14 days post-inoculation (dpi) were scanned, and the lesion size was measured using the APS Assess 2.0 software (American Phytopathological Society, Saint Paul, MN, USA, 2008).

3.3.4 Trypan Blue Staining

Cotyledons (5, 7, and 11 dpi) were cut into 1×1 cm^2 segments, immersed with 4 mL of clearing solution A (acetic acid/ethanol = 1:3, *v*/*v*), and shaken at a low speed overnight. Solution A was discarded, changed to clearing solution B (acetic acid/ethanol/glycerol = 1:5:1, *v*/*v*/*v*), and shaken at a low speed for at least 3 h. After the removal of clearing solution B, the cotyledons were stained with 2 mL of staining solution (0.01% trypan blue in lactoglycerol; lactic acid/glycerol/dH_2O = 1:1:1, *v*/*v*/*v*) and shaken at a low speed overnight. The staining solution was changed to 60% glycerol as washing solution with low-speed shaking for at least 2 h. Finally, the washed cotyledon segments were ready to observe on clean slides. The staining experiment followed the protocol of Chung et al. (2006).

3.3.5 Analysis of Bound Salicylic Acid (Bound SA)

The cotyledons at 3, 7, and 11 dpi were collected, lyophilized, and stored at −80 °C. The SA content from bound SA was released by acidic (HCl) hydrolysis. The levels of freed SA were

measured by HPLC (Ratzinger et al., 2009). Both the control (water-inoculated) and inoculated cotyledons were measured in 3 biological replicates (0.1 g dry mass for each).

3.3.6 Gene Expression Analysis

Frozen cotyledons (1, 3, 5, 7, and 11 dpi) were ground in liquid nitrogen with a pestle and mortar. Total RNA was extracted with TRI reagent (Sigma-Aldrich, St. Louis, MO 63103, USA). Total RNA was purified by DNaseI treatment with a recombinant DNaseI RNase-free kit (Millipore Sigma, Oakville, ON, Canada). Purified RNA (1 µg) was used to synthesize cDNA using the GOScript Reverse Transcription System (Promega, Madison, WI, USA). The cDNA stock solution was diluted to 100 ng/µl. Quantitative-PCR was performed by loading 1 µL of cDNA (100 ng) into the 10 µL reaction system of the IQ™ SYBR® Green Supermix (BioRad, Hercules, CA, USA). Experiments were based on three biological replicates (4 cotyledons, 2 seedlings, per replicates).

The qPCR program used for all of the analyzed genes (except for *ACO1*) was 95 °C for 3 min; followed by 39 cycles of 95 °C for 15 s and 60 °C for 20 s; followed by a melting curve analysis.

As the qPCR for *ACO1* using the program mentioned above showed low quality, the qPCR program used for *ACO1* was 95 °C for 3 min; followed by 39 cycles of 95 °C for 15 s, 55 °C for 1 min, and 72 °C for 1 min; followed by a melting curve analysis.

All qPCR primers are listed in **Appendix I**. The relative level of gene expression was analyzed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001). *Actin* was used as a reference gene to normalize the expression of the target genes.

3.3.7 Statistical Analysis

Unless specified, the analyses of samples used at least three biological replicates. The statistical analyses were performed using the Fisher's least significant difference (LSD) method with the SAS 9.4 software. The Fisher's LSD was applied to lesion test, gene

expression, and bound SA measurement, in order to observe effectiveness of resistance in three genotypes when inoculated with two isolates.

3.4 Results

3.4.1 Distinct Levels of Disease Severities from the *B. napus* Cotyledons with Different Inoculation Pairs

To obtain *B. napus* cotyledons with various levels of disease severity, three *B. napus* genotypes (Westar, Surpass400, and 01-23-2-1) were selected for inoculation with two *L. maculans* isolates (HCRT75 8-1 and HCRT77 7-2; the term "HCRT" will be shortened to "H" for the rest of the article). For the selected *B. napus* cultivars, Westar, which has no *Rlm* genes, was regarded as a typical example for susceptible phenotypes, Surpass400 (*BLMR1/LepR3* and *BLMR2/LepR2*) as intermediate and resistant phenotypes, and 01-23-2-1 (*Rlm7*) as resistant phenotypes. Isolate H75 8-1 exhibited a compatible interaction (susceptible) with Westar, an intermediate incompatible interaction (intermediate resistant) with Surpass400, and an incompatible interaction (resistant) with 01-23-2-1 (**Figure 3.1**), while isolate H77 7-2 exhibited a resistant phenotype with Surpass400 (**Figure 3.1**) and the same phenotypes as H75 8-1 with Westar and 01-23-2-1. Westar had a compatible interaction, as it had no *Rlm* genes, while the intermediate resistance for Surpass400 inoculated with H75 8-1 was due to the interaction between AvrLepR2 and BLMR2/LepR2 (Surpass400: *BLMR1/LepR3* and *BLMR2/LepR2*) (Long et al., 2011). The incompatible interaction between Surpass400 and H77 7-2 was triggered by the interaction between LepR3/BLMR1 and AvrLm1, as both Rlm1 and LepR3/BLMR1 recognize AvrLm1 (Larkan et al., 2013; Dandena et al., 2019), while the resistance from 01-23-2-1 against HCRT75 8-1/HCRT77 7-2 was caused by the recognition of AvrLm7 by Rlm7 in 01-23-2-1.

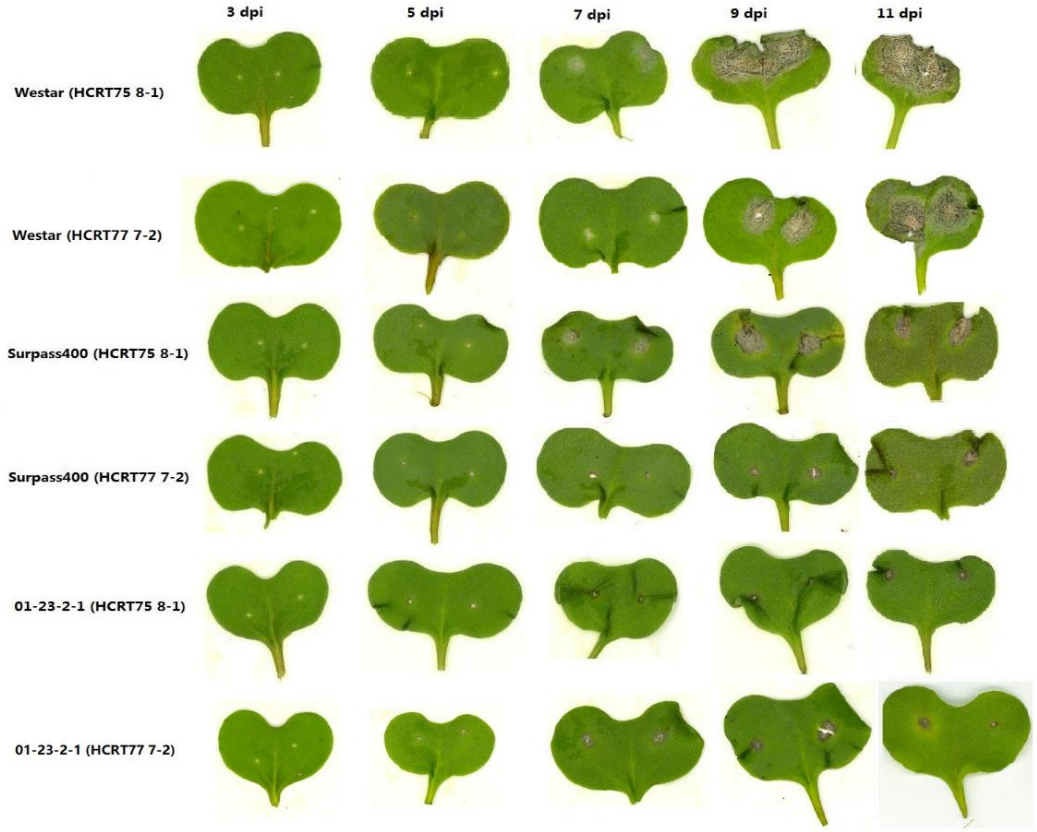


Figure 3.1. Lesion development on six pairs of *B. napus* cotyledons inoculated *with L. maculans* isolate: Westar—HCRT75 8-1/HCRT 77 7-2, Surpass400—HCRT75 8-1/HCRT77 7-2, and 01-23-2-1—HCRT75 8-1/HCRT 77 7-2 at 3, 5, 7, 9, and 11 days post-inoculation (dpi).

As shown in **Figure 3.1**, the emergence of distinct phenotypes among six sets of host–pathogen combinations did not appear until 5 dpi. From 5 dpi, Westar started to develop lesions at the inoculation sites, while brownish lesions emerged on Surpass400 and 01-23-2-1 cotyledons; in the case of both H75 8-1 and H77 7-2, the HR phenotype (brownish lesions) appeared at 5 dpi. To show the development of lesions in a numerical way, the lesion area was measured for each genotype–isolate pair from 3 to 14 dpi. As the phenotypes from all inoculation pairs emerged at 5 dpi, the lesion size at 3 dpi was set as zero. As shown in **Figure 3.2**, Westar–H75 8-1 displayed a rapid development of lesions from 7 dpi and the cotyledons collapsed at 11 dpi, due to the massive fungal colonization. Both Surpass400–H77 7-2 and 01-

23-2-1–H75 8-1/–H77 7-2 had slowly increasing lesion areas (**Figures 3.1 and 2**). Surpass400–H75 8-1 displayed the gradual development of HR necrotic lesions (brownish lesions) and reached a large size at 11 dpi; however, the plant was still viable and exhibited HR phenotype, this is called the intermediate resistance (Long et al., 2011; Dandena et al., 2019).

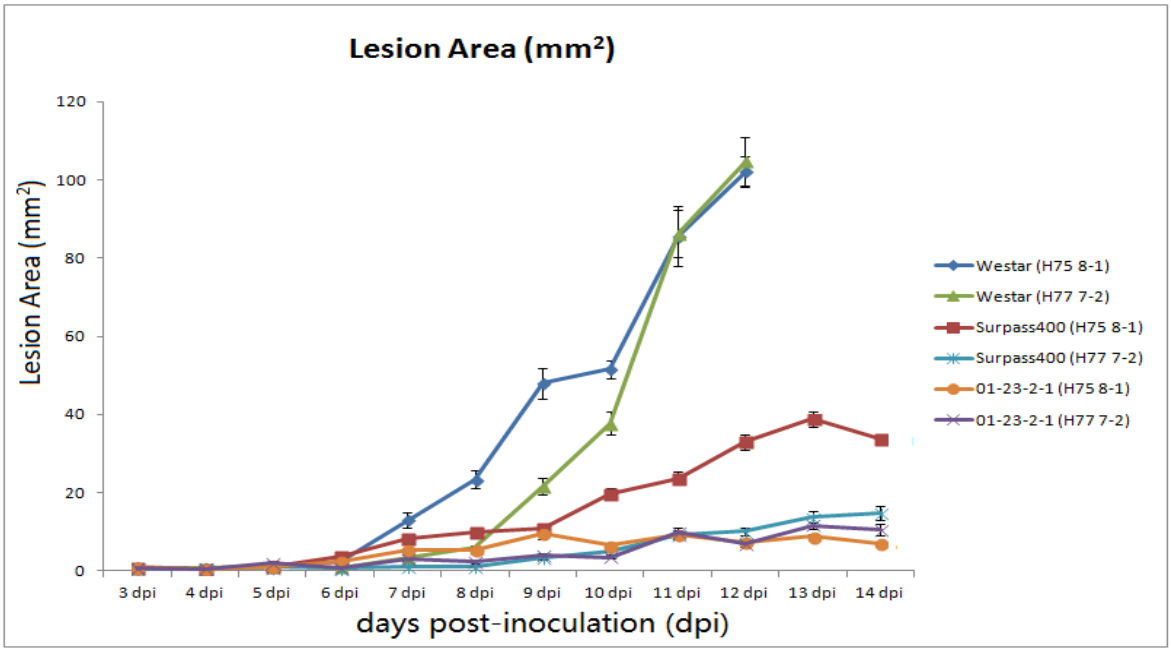


Figure 3.2. Changes in lesion size (mm^2) from 3 to 14 dpi in Westar–H75 8-1 (blue curve), Surpass400–H75 8-1, (red curve), Surpass400–H77 7-2 (light green curve), and 01-23-2-1–H75 8-1 (purple curve). The lesion sizes were calculated as the average from the cotyledons of 20 plants (each genotype at each time point).

3.4.2 Fungal Development of *L. maculans* Isolates from Compatible, Intermediate, and Incompatible Interactions

The presentation of microscopic views of infected cotyledons started at 5 dpi, when the hyphal development (compatible) and necrotic lesions (incompatible) were visible in the microscope (**Figure 3.3**). Li et al. (2008) demonstrated hyphal development in intercellular spaces at 5 dpi. At 7 dpi, the three types of severity exhibited distinct patterns of fungal development on the host tissues. On the leaves of oilseeds, *L. maculans* is a fungus that starts its growth as a

biotrophic pathogen; after several days of infection, the fungus reaches its necrotrophic stage and fruiting bodies (pycnidia) are formed (Hammond and Lewis, 1987). As shown in **Figure 3.3**, isolates H75 8-1 and H77 7-2 initiated the necrotrophic stage at 7 dpi on susceptible Westar and intermediate Surpass400 (infected by H75 8-1 only), growing pycnidia on the cotyledon tissues on the same day; meanwhile, Surpass400–H77 7-2 and 01-23-2-1–H75 8-1/–H77 7-2 showed few to no pycnidia at the same time point, as the fungal cells on those cotyledons were still in the biotrophic stage (i.e., hyphae only).

For susceptible cotyledons (Westar–H75 8-1/–H77 7-2), the formation of pycnidia occurred at 7 dpi and became dominant at 11 dpi, while 01-23-2-1 (resistant) did not have any pycnidia, and few hyphae emerged at 7 dpi and 11 dpi after inoculation with H75 8-1 or H77 7-2. For the intermediate Surpass400–H75 8-1 cotyledons, the symptoms lay between susceptible and incompatible interaction phenotypes, the production of pycnidia was, somehow, restricted within the region of necrotic lesions.

To sum up, the different *B. napus* genotypes exhibited distinct responses towards *L. maculans* isolates. For susceptible responses (Westar–H75 8-1/–H77 7-2), apparent hyphal development started at 5 dpi, and the fungus transited to necrotrophic stage at 7 dpi, with the formation of pycnidia; subsequent development was the enhancement of what happened at 7 dpi. Nevertheless, Surpass400 and 01-23-2-1, as more resistant genotypes, displayed delayed fungal development compared with Westar, with the intermediate response from Surpass400–H75 8-1 exhibiting limited pycnidia formation, and 01-23-2-1 had little hyphal development throughout the timeline of observation.

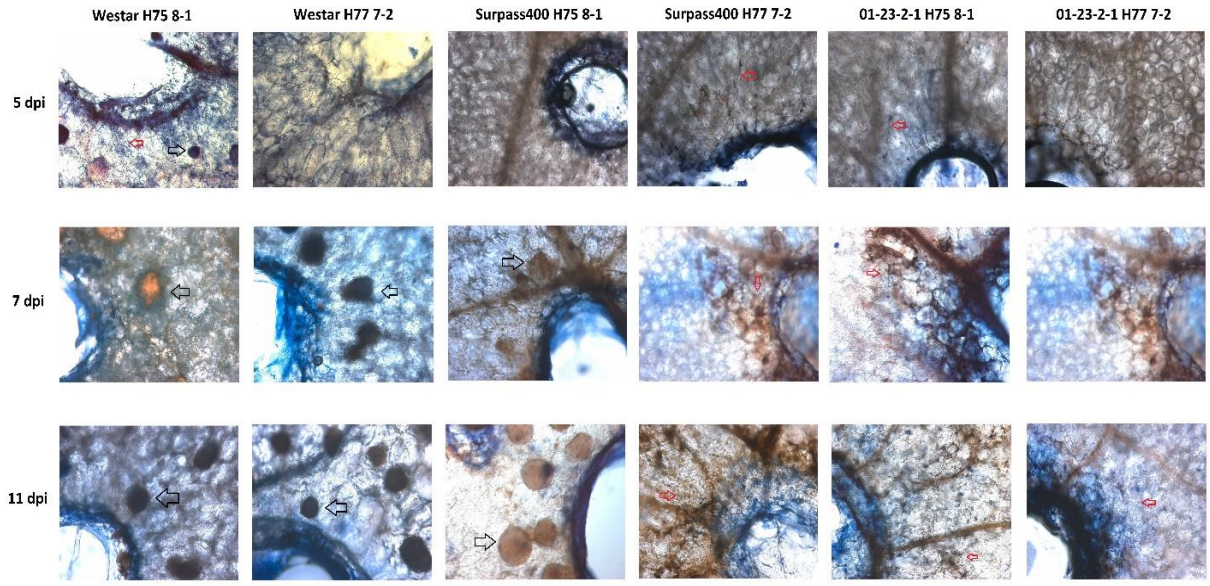


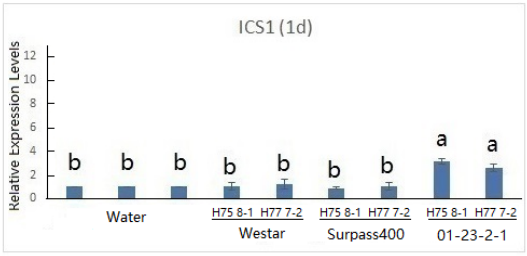
Figure 3.3. Fungal growth and development on the cotyledons of *B. napus* cv. Westar, Surpass400, and 01-23-2-1 inoculated with *L. maculans* isolates H75 8-1/H77 7-2, as shown by trypan blue staining. Hyphae (red arrows) started to grow on the cotyledon tissue at 5 dpi. The compatible (Westar–H75 8-1/–H77 7-2) and intermediate incompatible (Surpass400–H75 8-1) interactions allowed for the formation of pycnidia (black hollow arrows) at 7 dpi and the fungal tissues formed pycnidia at only 11 dpi. The incompatible interactions (Surpass400–H77 7-2 and 01-23-2-1–H75 8-1/–H77 7-2) did not have pycnidia and only few hyphae grew up to 7 dpi and 11 dpi. The images were taken at 100× magnification.

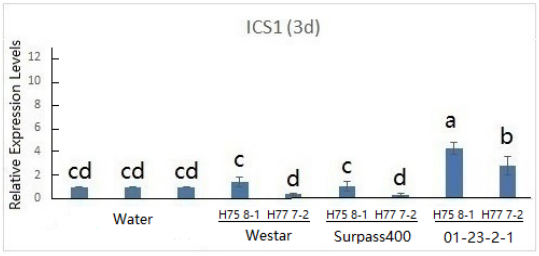
3.4.3 Gene Expression Analysis in Hormone Signaling

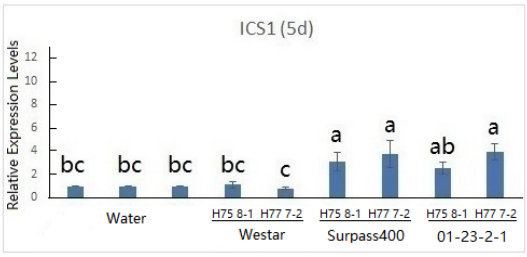
The characterization of hormonal signals pathways started with quantitative analyses of the genes responsible for the biosynthesis of the three phytohormones (SA, JA, and ET). To achieve this goal, three hormonal biosynthetic genes were chosen: *ICS1*, *AOS*, and *ACO1*. *ICS1* encodes an enzyme called isochorismate synthase 1, which is involved in salicylic acid biosynthesis (Wildermuth et al., 2001). *AOS* encodes an enzyme called allene oxide synthase, which is an enzyme involved in the JA biosynthetic pathway and the octadecanoid pathway (Laudert and Weiler, 1998). *ACO1* encodes an enzyme called 1-aminocyclopropane-1-

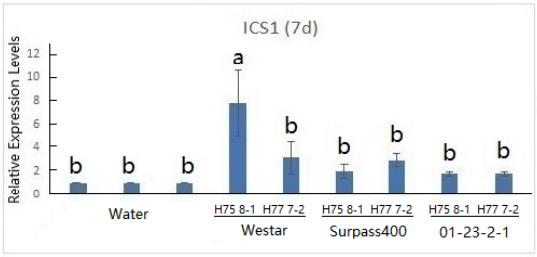
carboxylate oxidase 1, which is involved in ethylene biosynthesis in different situations (Garcia et al., 2010). We analyzed the expression of these three biosynthetic genes in both *L. maculans*-inoculated (H75 8-1 and H77 7-2) and water-inoculated cotyledons at multiple time points after inoculation. The expression levels of the genes from water-inoculated cotyledons were normalized to the level of "1", in order to find the differential expression levels between control and inoculated cotyledons at each time point, which indicated how the hormonal signals were modulated, when encountering fungal infection. By analyzing the three genes (*ICS1*, *AOS*, and *ACO1*) with regard to the production of SA, JA, and ET, the temporal pattern of biosynthesis-related genes was found to be distinct to the cotyledons among three genotypes.

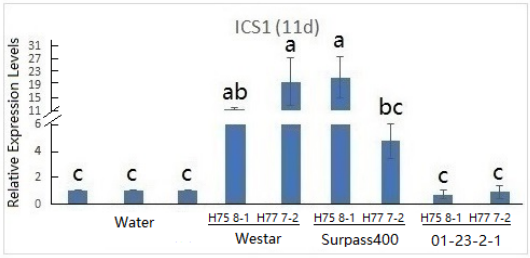
As shown in **Figure 3.4**, the activation of *ICS1* and *AOS* from Surpass400 and 01-23-2-1 were earlier than in Westar; for Surpass400, the earliest timepoint of significantly higher expression of *ICS1* and *AOS* started at 5 and 3 dpi, respectively; however, Westar had lower expression of these two genes at the same timepoints. For 01-23-2-1 cotyledons (resistant), all three biosynthetic genes showed earlier expression, as early as 1 dpi. *ICS1* in 01-23-2-1 (both inoculated by H75 8-1 and H77 7-2) showed higher levels of expression at all early time points (i.e., 1, 3, and 5 dpi). Surpass400 – H75 8-1 and 01-23-2-1 displayed late up-regulation of *ACO1* (i.e., 7 and 11 dpi) whilst Westar did not have apparent activation throughout infection. Altogether, the intermediate and resistant genotypes have distinct transcriptional programming compared with the susceptible one, featured by the early activation of SA/JA biosynthetic marker genes *ICS1* and *AOS1*.

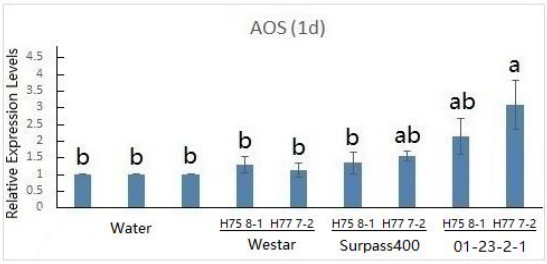

ICS1 (1d)
Relative Expression Levels
b b b b b b b a a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

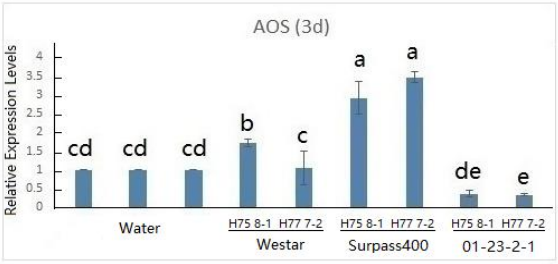

ICS1 (3d)
Relative Expression Levels
cd cd cd c d c d a b
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

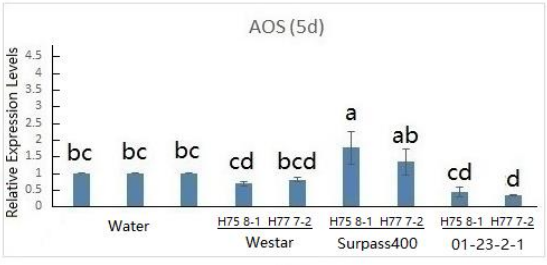

ICS1 (5d)
Relative Expression Levels
bc bc bc bc c a a ab a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

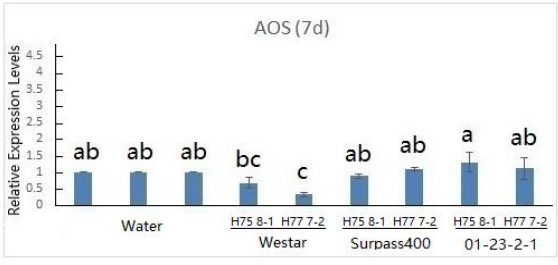

ICS1 (7d)
Relative Expression Levels
b b b a b b b b b
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

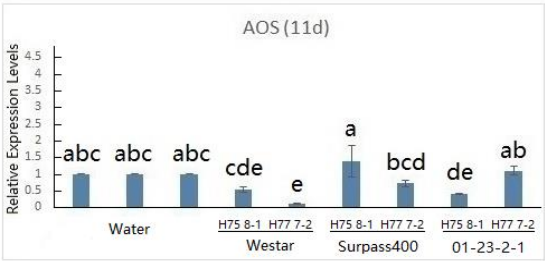

ICS1 (11d)
Relative Expression Levels
c c c ab a a bc c c
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


AOS (1d)
Relative Expression Levels
b b b b b b ab a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


AOS (3d)
Relative Expression Levels
cd cd cd b c a a de e
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


AOS (5d)
Relative Expression Levels
bc bc bc cd bcd a ab cd d
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


AOS (7d)
Relative Expression Levels
ab ab ab bc c ab ab a ab
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


AOS (11d)
Relative Expression Levels
abc abc abc cde e a bcd de ab
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

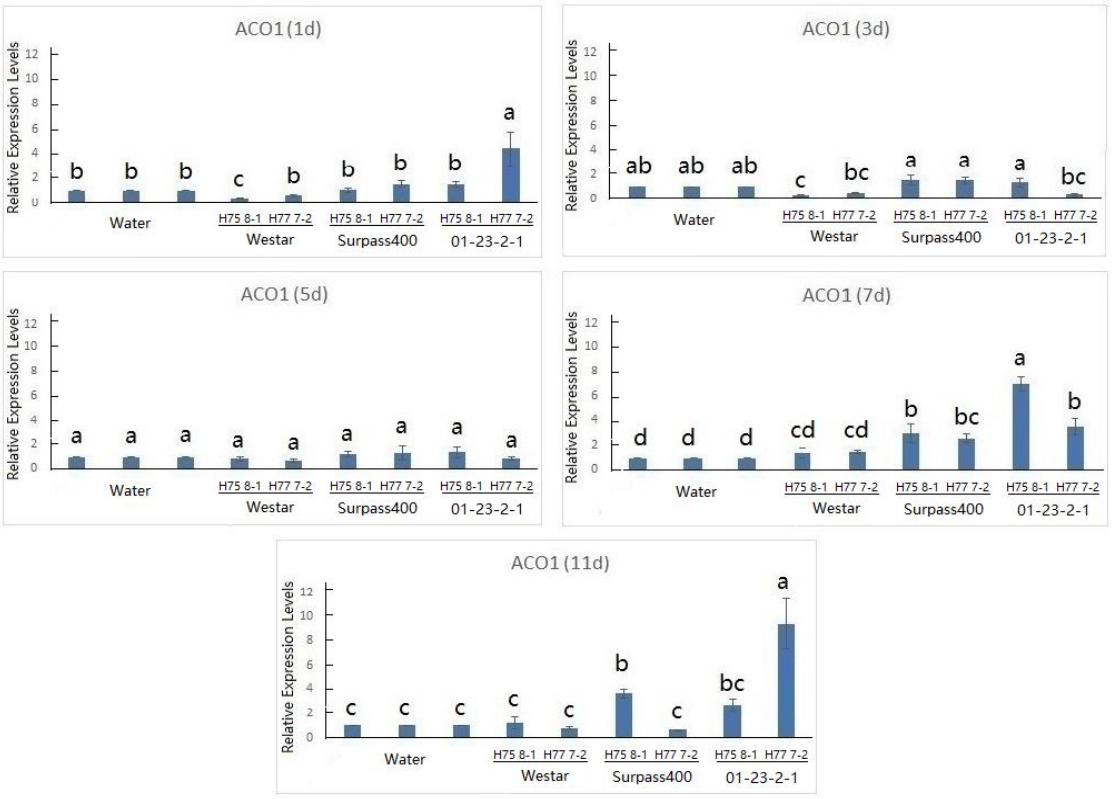
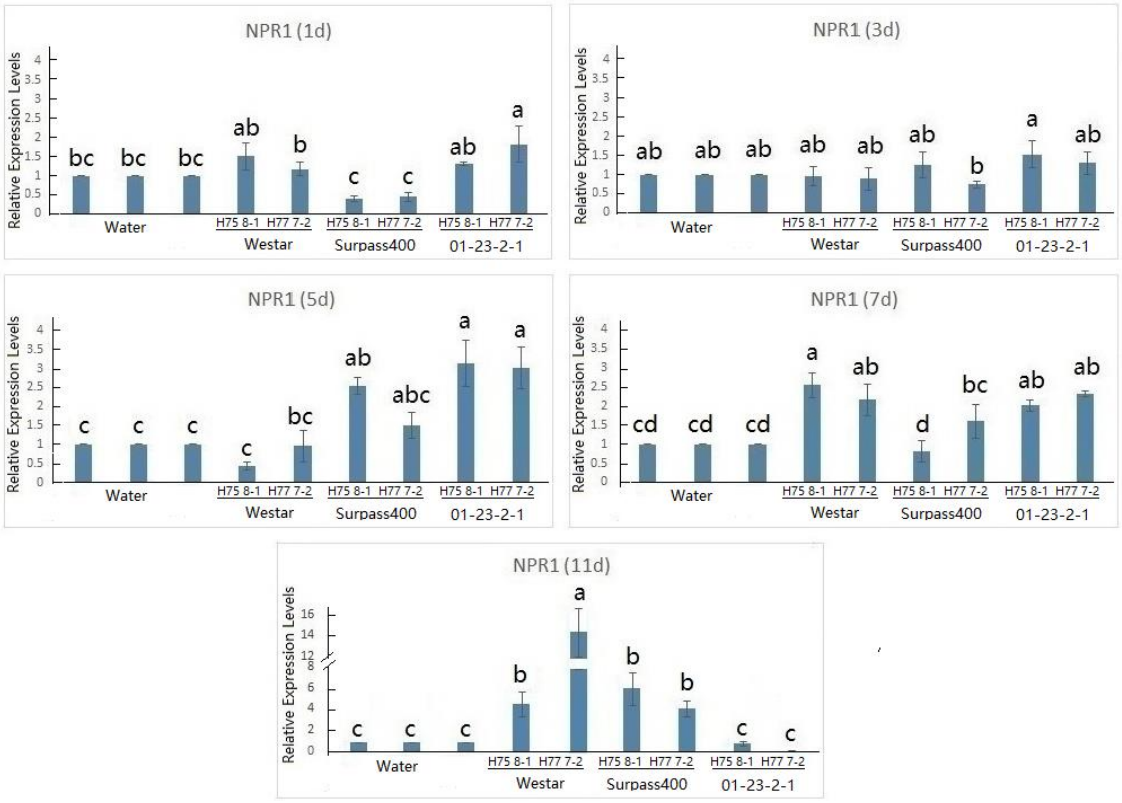


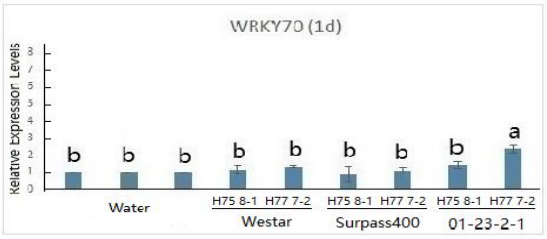
Figure 3.4. Gene expression in hormonal biosynthesis (*ICS1*, *AOS*, and *ACO1*): the levels of the bars are the expression levels from the inoculated cotyledons (inoculated by H75 8-1 and H77 7-2), compared to the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). Error bars represent standard error of the mean. For time point, different lowercase letters suggest the significant differences among mean values (Fisher's least significant difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

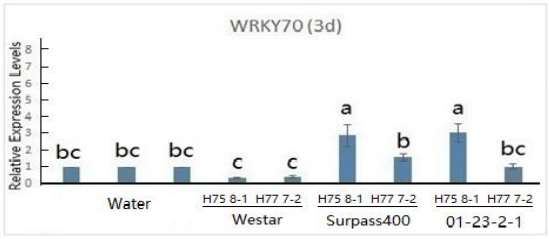
3.4.4 The Potential Relationship between Hormonal Biosynthesis and the Regulatory Patterns of Hormonal Signals throughout the *B. napus* and *L. maculans* Interaction

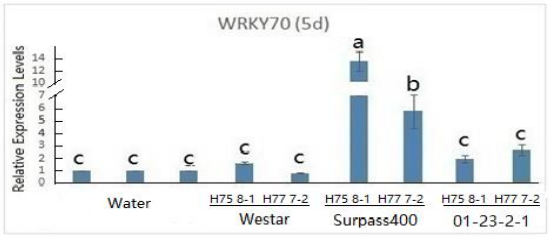
Surpass400–H75 8-1 (intermediate) and 01-23-2-1 (resistant) had earlier induction of *NPR1* and *WRKY70*, compared with Westar. For *NPR1*, Surpass400 (H75 8-1) and 01-23-2-1 had pronounced expression from 5 dpi; while, for Westar (susceptible), this pattern did not occur until 7 dpi. For the downstream factor *WRKY70*, Surpass400 and 01-23-2-1 genotypes

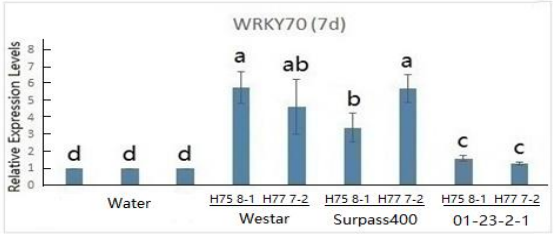
displayed similar trends, suggesting that the intermediate and resistant cotyledons had earlier SA-related responses (**Figure 3.5**). Surprisingly, Surpass400 and 01-23-2-1 also had early activation of ET/JA responsive factor *WRKY33*: both of them induced this gene at 1 dpi, and 01-23-2-1 also had high expression at 3 dpi. Moreover, Surpass400 and 01-23-2-1 also tended to have stronger expressions of *EIN3* than Westar at 3 and 5 dpi; 01-23-2-1 showed high expression of this gene at 1 dpi. In Westar cotyledons (inoculated by both H75 8-1 and H77 7-2), the defense genes started to induce at 7 dpi and reached high levels at 11 dpi. For Westar cotyledons, 7 and 11 dpi are the timepoints when the fungus formed pycnidia and transited into the necrotrophic stage, respectively. The lesions on the infected tissues quickly developed (as shown in **Figure 3.1**); therefore, the high levels of defense genes reflected the non-HR-related responses against this deteriorating situation.

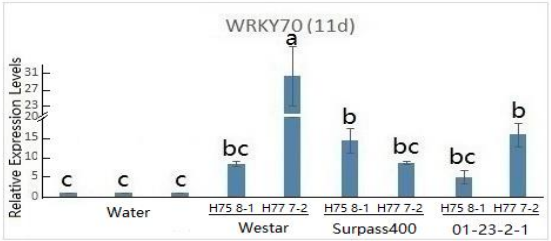


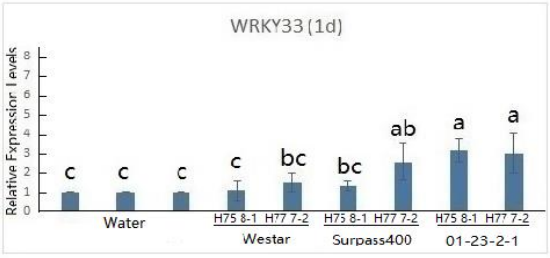

WRKY70 (1d)
Relative Expression Levels
b b b b b b b b a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

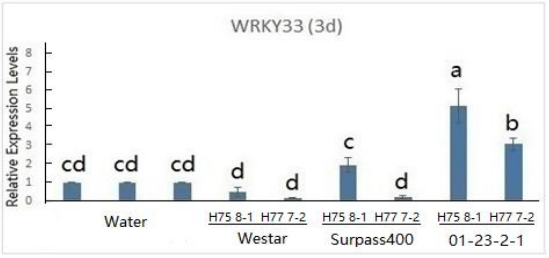

WRKY70 (3d)
Relative Expression Levels
bc bc bc c c a b a bc
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

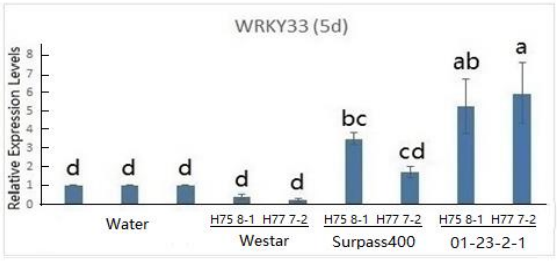

WRKY70 (5d)
Relative Expression Levels
c c c c c a b c c
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

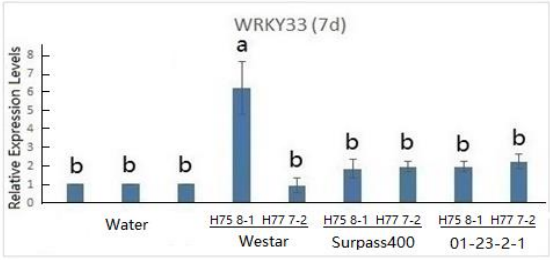

WRKY70 (7d)
Relative Expression Levels
d d d a ab b a c c
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

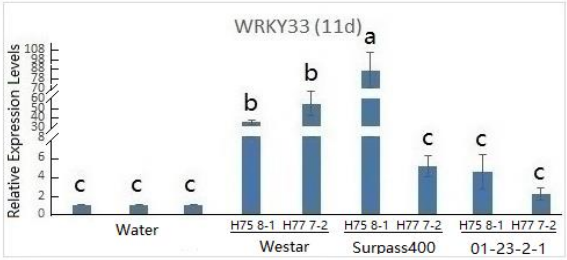

WRKY70 (11d)
Relative Expression Levels
c c c bc a b bc bc b
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


WRKY33 (1d)
Relative Expression Levels
c c c c bc bc ab a a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


WRKY33 (3d)
Relative Expression Levels
cd cd cd d d c d a b
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


WRKY33 (5d)
Relative Expression Levels
d d d d d bc cd ab a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


WRKY33 (7d)
Relative Expression Levels
b b b a b b b b b
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


WRKY33 (11d)
Relative Expression Levels
c c c b b a c c c
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

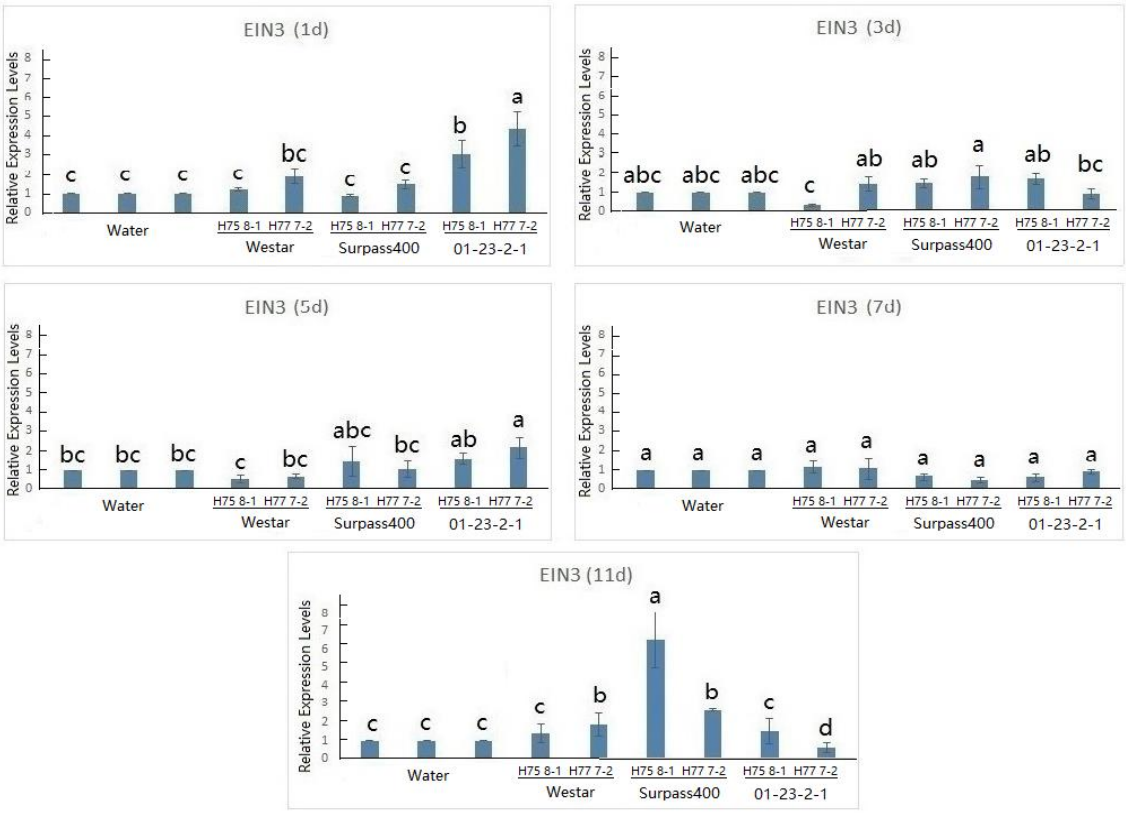
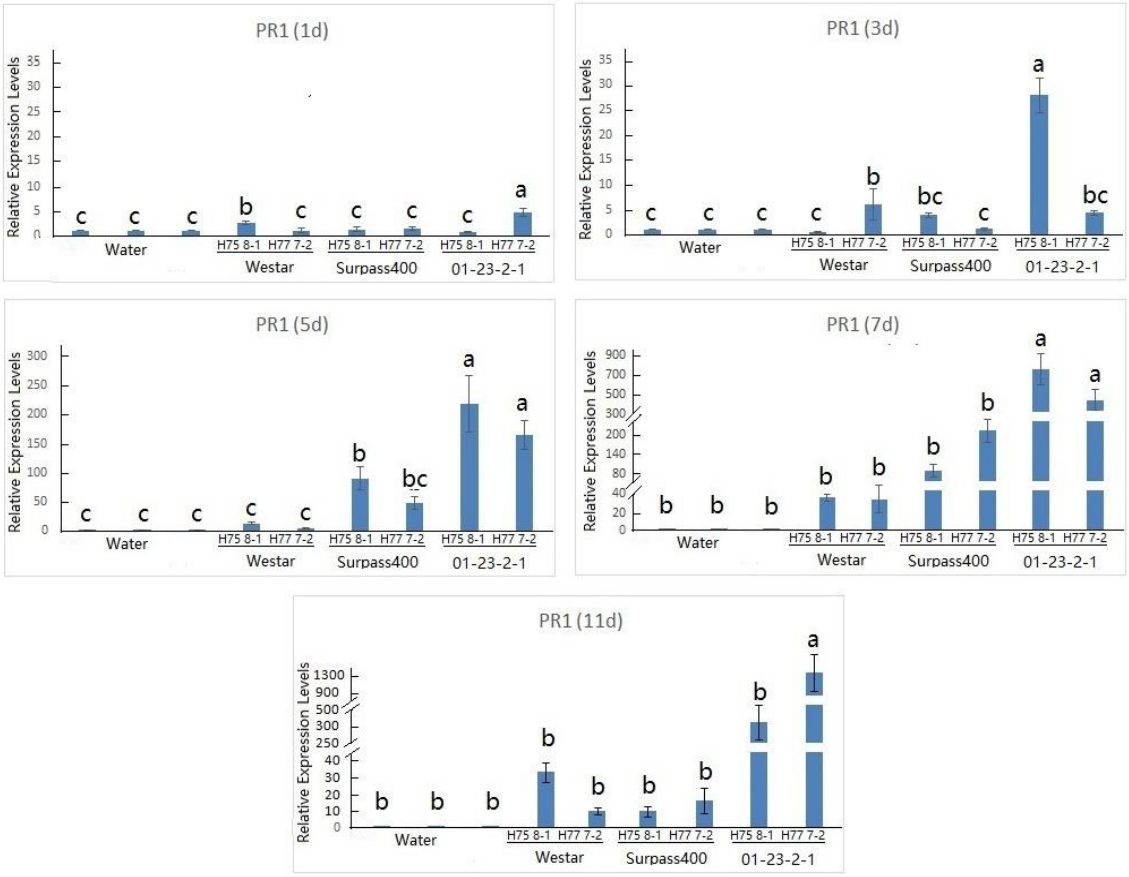
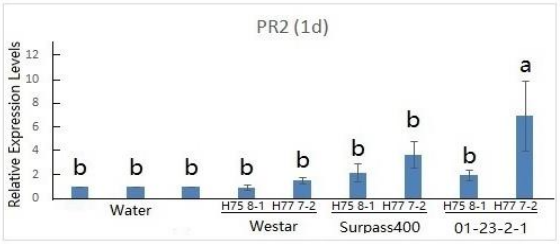


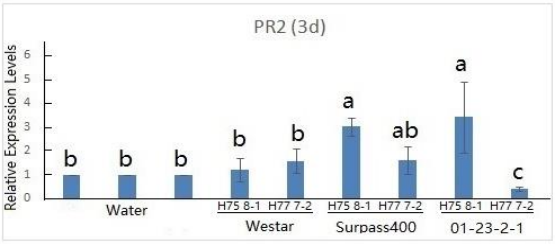
Figure 3.5. Gene expression in regulation of hormonal signals (*NPR1*, *WRKY70*, *WRKY33*, and *EIN3*): the levels of the bars are the expression levels in the inoculated cotyledons (inoculated by H75 8-1 and H77 7-2), compared to the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). Error bars represent standard error of the mean. For time point, different lowercase letters suggest the significant differences among mean values (Fisher's least significant difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

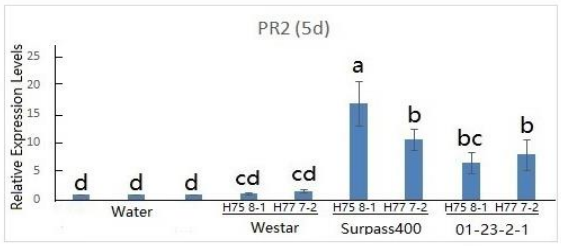
Surpass400 (intermediate/resistant) and 01-23-2-1 (resistant) started to induce high expression of *PR1*, *PR2*, and *PR4* at 5 dpi, while, for susceptible Westar (H75 8-1), the massive induction of *PR4* started at 11 dpi (**Figure 3.6**). WRKY70, as an SA regulator, positively regulates the expression of pathogenesis-related 1 (PR1) proteins (Kunkel and Brooks, 2002; Li et al., 2004). It seems that the studied transcription factors had somewhat synchronization with the studied *PR* genes; as such, both Surpass400 and 01-23-2-1 showed the activation of regulators (*WRKY70* and *WRKY33*) and *PR* genes (*PR1*, *2*, and *4*) at 5 dpi. On the other hand, there were

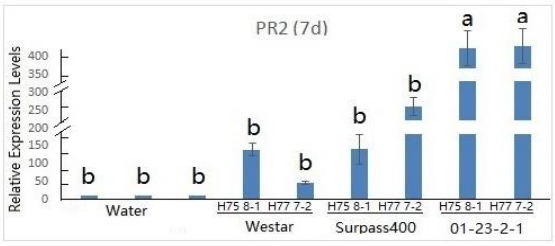
also discrepancies between transcription factors and downstream *PR* genes. For example, 01-23-2-1 did not show the high induction of *WRKY33* at 7 and 11 dpi, but *PR4* was still very high at the same time points. These results reflect the potential influences of upstream signaling upon the downstream proteins in plant defense; besides, there may have been other factors affecting the expression of downstream proteins.

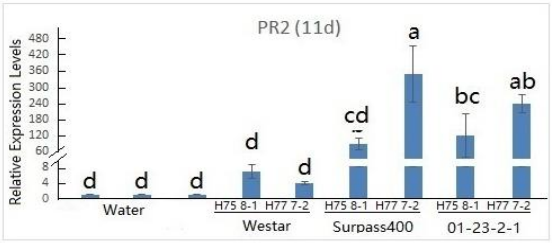


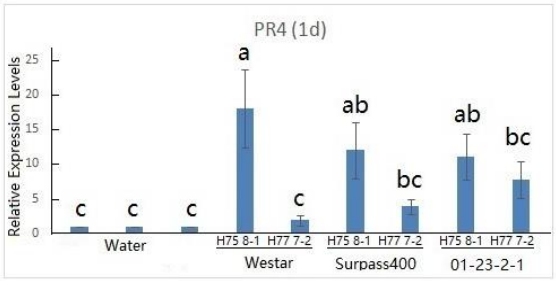

PR2 (1d)
Relative Expression Levels
b b b b b b b b a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

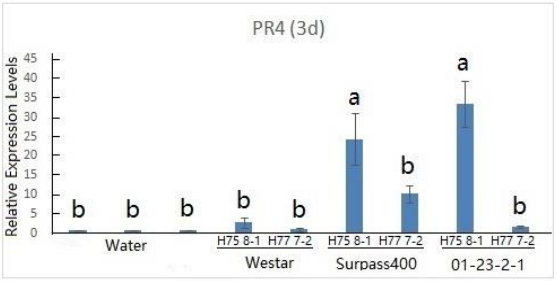

PR2 (3d)
Relative Expression Levels
b b b b b a ab a c
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

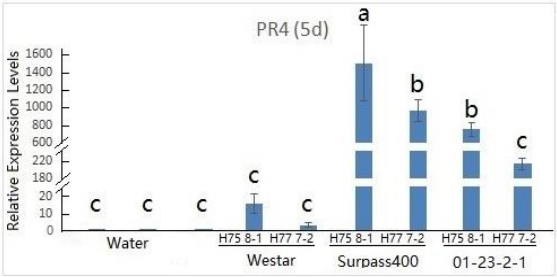

PR2 (5d)
Relative Expression Levels
d d d cd cd a b bc b
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

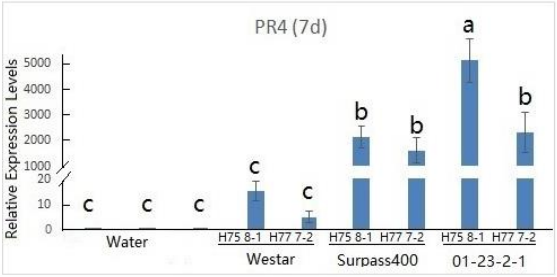

PR2 (7d)
Relative Expression Levels
b b b b b b a a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


PR2 (11d)
Relative Expression Levels
d d d d d cd a bc ab
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


PR4 (1d)
Relative Expression Levels
c c c a c ab bc ab bc
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


PR4 (3d)
Relative Expression Levels
b b b b b a b a b
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


PR4 (5d)
Relative Expression Levels
c c c c c a b b c
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1


PR4 (7d)
Relative Expression Levels
c c c c c b b a b
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

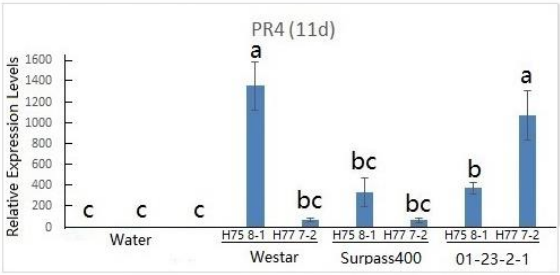

PR4 (11d)
Relative Expression Levels
c c c a bc bc bc b a
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

Figure 3.6. Gene expression of downstream proteins from hormonal signaling (*PR1*, *PR2*, and *PR4*). The levels of the bars are the expression levels from the inoculated cotyledons (inoculated by H75 8-1 and H77 7-2), compared to the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). Error bars represent standard error of the mean. For time point, different lowercase letters suggest the significant differences among mean values (Fisher's least significant difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

As free SA can be toxic for the living plant, SA signaling induces electrolyte leakage, oxidative burst, and cell death (Kawano et al., 2004; Brodersen et al., 2006; Vlot et al., 2009). As shown in **Figure 3.7**, in the intermediate and resistant cotyledons from Surpass400 and 01-23-2-1 genotypes, the presence of bound SA was detected as early as 3 dpi; while it was not detected in the two Westar sample pairs (H75 8-1 and H77 7-2). However, Westar showed later accumulation of SA (at 11 dpi), at which point its levels exceeded those in Surpass400 and 01-23-2-1 cotyledons at the same time point.

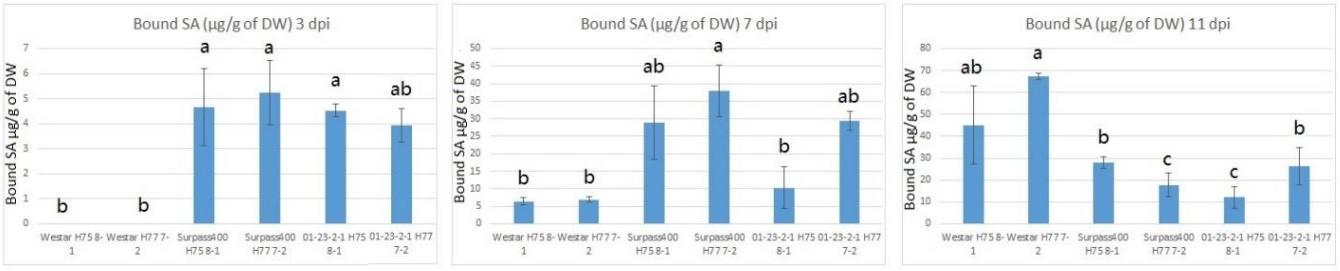


Figure 3.7. Amount of bound salicylic acid (µg/g of dry weight (DW)) in Westar/Surpass400/01-23-2-1 inoculated with isolates H75 8-1/H77 7-2 at 3, 7, and 11 dpi. Conjugated salicylic acids were hydrolyzed by HCl first, in order to free SA for measurement by HPLC–fluorescence. Error bars represent standard error of the mean. For time point, different lowercase letters suggest the significant differences among mean values (Fisher's least significant difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

3.5 Discussion

Certain hormonal-related factors displayed earlier activation from in intermediate and resistant cases, while susceptible *B. napus* possessed distinct onset patterns of hormonal signal responses. The study suggested that the timing of gene activation might be important to trigger the effective hindrance of fungal growth and development; this type of signal transduction seems to be correlated with the manipulation of fungal development by the host.

3.5.1 The Fungal Development of *L. maculans* Was Hindered due to the Host Resistance

According to a study of the susceptible adult leaves of *B. napus* (cv. Westar), the intercellular development of fungal hyphae was observed from the microscope as early as 5 dpi, massive hyphal development throughout the mesophyll was initiated at 7 dpi, and finally, pycnidia were produced on the dead tissues after 11 or 12 dpi (Li et al., 2008a).

In the case of the susceptible Westar–H75 8-1/–H77 7-2 cotyledons, fungal development followed the regular lifecycle of hemi-biotrophic fungus, in which the fungus starts its biotrophic stage from 7 dpi by spreading hyphae, in order to absorb nutrients from the living tissues. Then, it shifted into the necrotrophic stage by producing pycnidia. On the other hand, the incompatible interactions in 01-23-2-1 and Surpass400 restricted the growth and slowed the development of the fungus. One of the associated mechanisms is to induce regional cell death, which creates necrotic lesions on the tissues; this mechanism causes inhibitive growth conditions for biotrophic pathogens, which need living tissues to exploit nutrients (Coll, 2011; Stotz et al., 2014). On the other hand, Surpass400 and 01-23-2-1 displayed some inconsistency in the further development of necrotic lesions, suggesting unequal intrinsic signaling among the different forms of incompatible interactions. The intermediate Surpass400–H75 8-1 combination triggered the gene-for-gene interaction between AvrLmS/AvrLepR2 and RlmS/BLMR2, while that in resistant Surpass400–H77 7-2 was between AvrLm1 and LepR3/BLMR1, and those in 01-23-2-1–H75 8-1/–H77 7-2 were between AvrLm 4-7 and Rlm7 (Parlange et al., 2009; Larkan et al., 2013). These different types of incompatible interactions may have caused distinct defense signaling network patterns. Therefore, some different onset patterns among the three interactions (i.e., Surpass400–H77 7-2 and 01-23-2-1–

H75 8-1/–H77 7-2) may have been due to the different mechanisms of AvrLm–Rlm interactions and/or the subsequent signaling cascades. Surpass400 was remarkable, due to the presence of identified *R* genes *LepR3/BLMR1* (ressistant) and *BLMR2/LepR2* (intermediate), associated with these two genes (Long et al., 2011; Dandena et al., 2019; Neik et al., 2020). *AvrLmS/AvrLepR2* was considered as an independent *AvrLm* gene, conferring HR by interacting with *RlmS* (Van de Wouw et al., 2019; Neik et al., 2020), and the intermediate *R* genes in Surpass400 (*LepR3*/*BLMR1* and *RlmS/BLMR2/LepR2*) worked co-operatively with major *Rlm* genes, but also functioned independently of those major genes (Dandena et al., 2019; Neik et al., 2020).

3.5.2 Fine-Tuning of Hormonal Signals in *B. napus* Is Able to Resist to *L. maculans* by Controlling Its Developmental Stages

As there was no apparent hyphal development in all six inoculation pairs before 5 dpi, the differential expression of some of the genes at 1, 3, and 5 dpi implicated that the three genotypes possessed unequal priming response strengths, which were linked to the ability of early sensing of fungal invasion and the anticipated release of defense signals. Studies have revealed that the intrinsic signaling before the emergence of symptoms determines further trends of the host–microbe interaction (Stotz et al., 2014; Becker et al., 2017). During the biotrophic stage of *L. maculans*, small secreted proteins (SSPs), including AvrLm proteins, are released into the intercellular space and cytoplasm. The recognition of *L. maculans* AvrLm proteins by *B. napus* Rlm proteins is able to trigger early defense responses (Stotz et al., 2014; Becker et al., 2017).

During plant defense, the biosynthesis of each of the hormones triggers their responsive transcription factors to activate the downstream genes responsible for curtailing the spread of the disease. Hormonal transcription factors are more downstream proteins, following the activation of MAPKs and biosynthetic enzymes. These factors are triggered by the hormone molecules and impact the expression of some anti-microbial elements, in order to effectively stop further invasion of the pathogens.

3.5.3 The Early Activation of SA-Related Factors (from 1 to 7 Dpi) Was One of the Common Features of the Intermediate and Resistant Cotyledons

Generally, Surpass400 and 01-23-2-1 had SA-responsive factors (*ICS1*, *NPR1, WRKY70*, *PR1*, and *PR2*) expression higher than that in mock inoculations before 7 dpi, while Westar activated the same set of genes at 11 dpi. SA- and JA-related factors play pivotal roles in plant defense, including HR. *NON-EXPRESSOR OF PR1* (*NPR1*) lies on the node between SA- and JA-dependent defensive signaling, ET modulated the role of *NPR1* to buffer SA and JA signaling, NPR1 positively regulated SA-related defense and negatively regulated JA-related defense, and ET controlled *NPR1* by its responsive factor *ETHYLENE-INSENSITIVE PROTEIN 2* (*EIN2*). NPR1 may also be involved in the full-scale expression of one *WRKY* gene, *WRKY70*, the over-expression of constitutive resistance to some disease by constitutive SA defensive signals, while suppression of *WRKY70* showed increased JA-dependent signals. *WRKY70* encodes a transcription factor that positively regulates SA-related signaling; the over-expression of *WRKY70* also triggers the constitutive expression of *PR1* (Garcia et al., 2010). *Ethylene-Insensitive 3 (EIN3*) encodes an ethylene-responsive transcription factor; constant ET signaling has been observed as a result of the over-expression of *EIN3* (Adie et al., 2007).

Becker et al. (2017) also observed the early induction of *ICS1* and *PR1* at 3 dpi in the case of incompatible interaction (AvrLepR1–LepR1), indicating that the early activation of those genes correlates with effective resistance. *PR1* was found to be one of the components and activators of SAR (Vlot et al., 2009). SAR has defense activity in planta, which is triggered by the primary infection; plant cells secrete mobile substances throughout the plant body in order to prevent secondary infection from the pathogens. Those molecules include many defense-related molecules/proteins, such as PR1 proteins and beta-glucanase (PR2) (Conrath, 2006). *PR2* (also known as *BGL2*) encodes an enzyme called beta-1,3-glucanase, which is also up-regulated following SA accumulation (Thibaud et al., 2004). The PR4, also known as Helvin-Like Protein, HEL protein is regulated by ET-/JA-responsive transcription factors (Kunkel and Brooks, 2002); the PR4 protein is a chitinase that is able to degrade fungal cell walls (Borad and Sriram, 2008). The activation of *PR4* indicated the induction of the ET/JA signaling pathways, which are usually responsible for the defense against necrotrophic pathogens (Bari and Jones, 2009).

As the fungal development of *L. maculans* (as a hemi-biotroph) initially starts with the biotrophic stage, in this study, around 7 dpi was the transitive time point between biotrophic and necrotrophic phases when colonizing the susceptible *B. napus* genotype (**Figure 3.1**). The early activation of SA-responsive factors in Surpass400 and 01-23-2-1 suggested that resistant *B. napus* genotypes are able to effectively slow down the lifecycle of the pathogen and the associated SAR reinforced the defense throughout the plant body; therefore, these two genotypes were able to hinder the fungal development during biotrophic stage. However, the susceptible genotype Westar, after 7 dpi, started to induce hormonal-related defense genes, as the other two genotypes did from 1 to 5 dpi. The late activation of defense genes might be due to the massive colonization during necrotrophic stage, at this stage, the host was barely able to stop the infection, since the amount of fungal load (mycelia) was too large.

Moreover, bound SA measurement also reflected the priming of SA activation, in agreement with the qPCR results (**Figures 3.4 and 5**). Usually, SA is synthesized in the chloroplast and transported to the cytosol, where some SA molecules are transformed into bound versions and the inactive bound SA molecules are subsequently displaced in the vacuole for inactive storage (Vlot et al., 2009). Salicylic acid glucoside (SAG) is one of the derivatives of conjugated salicylic acid (glucolysated form). SAG becomes a slow inducer of SAR and a storage molecule to form free SA. Both SA and SAG play roles in abiotic/biotic stresses, but SAG is a safer and slower agent for oxidative burst and Ca^{2+} leakage (Kawano et al., 2004). In *Brassica napus*, infection by *Verticillium longisporum* caused the accumulation of SA and SAG from the xylem sap (Ratzinger et al., 2009). Stored SAG is able to release SA by hydrolysis to induce oxidative burst and Ca^{2+} leakage for disease resistance (Kawano et al., 2004; Ratzinger et al., 2009). SA/SAG also activates SAR and SA-responsive signaling factors, such as *PR1*, which play roles in plant defense (Hennig et al., 1993; Kawano et al., 2004; Sašek et al., 2012). A previous study suggested the connection between SA level and programmed cell death/ROS production (Pogány et al., 2009). The early induction of conjugated SA in Surpass400 and 01-23-2-1 suggested that early SA storage might slowly induce free SA to trigger defense responses, such as oxidative burst and cell death, by which the plant cells are able to stop the fungal cells during their initial development. On the other hand, Westar showed the accumulation of bound SA at 11 dpi. Similar to the late activation of SA/JA factors, the susceptible Westar genotype could not recognize the presence of fungus at the early stage and

trigger SA-responsive signals in a timely manner. It was not until the fungus had reached its necrotrophic stage that the increase in SA storage/accumulation was initiated. The accumulation of stored SA seemed to synchronize with SA-related transcription factors in all three genotypes, suggesting that a large section of SA and its responsive signals came from the hydrolysis of stored (i.e., inactive) SA.

3.5.4 Unconventional Signaling Transductions Were Observed from qPCR Results

There was also an unusual co-operation between SA and ET/JA signaling observed in Surpass400–H75 8-1 and Westar–H75 8-1/H77 7-2 during the late stage of infection. According to the conventional hormonal signaling theory, SA and ET/JA have an antagonistic relationship (Bari and Jones, 2009); however, Sašek et al., (2012) also observed the co-expression of both SA- and ET-related genes in the case of incompatible interaction in the *B. napus–L. maculans* pathosystem. The SA-/ET-/JA-related genes showed a gradual increase in expression after the transitive time point (Nováková et al., 2016), and the defense genes downstream of these three major hormones coincided in induction from the transitive time point (7 dpi) to the necrotrophic stage (11 dpi). Similar patterns have also been observed by Sašek et al. (2012) and Becker et al. (2017). It seems that the early recognition of AvrLm proteins by the resistant genotypes caused the distinct onset patterns of certain genes between susceptible and resistant backgrounds, such as the genes in hormone signaling (i.e., *ICS1* and *PR1*) and sulfur metabolism (i.e., *APR* genes).

This type of co-operation has been observed in other studies. Sašek et al. (2012) also found the earlier activation of SA and ET/JA factors after the inoculation of avirulent *L. maculans* isolate. Genes such as *WRKY70* (SA-responsive), *ACS2* (ET-responsive), and *CHI* (ET-/JA-responsive) were expressed in the case of resistant genotype (by incompatible interaction) before or at 7 dpi. Becker et al. (2017) also observed the early activation (3 dpi) of SA-/ET-/JA-responsive genes from the resistant *B. napus* genotype. The co-expression of the factors from both SA/ET and JA aspects can be explained as a balanced general defense signaling strategy. SA and ET have been found to promote oxidative burst and lesion formation, while JA is able to reduce the effects of ROS-induced cell senescence (Overmyer et al., 2000; Rao et

al., 2002). As both susceptible (Westar–H75 8-1/H77 7-2) and intermediate (Surpass400–H75 8-1) cotyledons showed high induction of defense signaling around 11 dpi, the plant body may trigger innate balancing mechanisms to prevent the detrimental effects of excessive defense activities. Spoel et al. (2007) postulated that the PCD induced by avirulent pathogen may attract the necrotrophs, and the activation of JA signaling is able to hinder the spread of necrotroph. From the situation of *L. maculans* infection, it is possible that the fungus would transit to necrotrophic stage when dead cells are formed by HR, the activation of JA may prevent the necrotrophic *L. maculans* among the dead tissues.

3.5.5 Two Isolates (HCRT75 8-1 and HCRT77 7-2) Induced Differential Patterns of Hormonal Gene Expression in Incompatible Interactions

Both resistant Surpass400–H77 7-2 (AvrLm1—BLMR1/LepR3) and 01-23-2-1–H75 8-1/–H77 7-2 (AvrLm4-7/Rlm7) displayed total resistant phenotypes. The RT-qPCR results suggested that distinct expression profiles were observed among those genotypes.

It is possible that different versions of gene-for-gene interactions (i.e., Avr–R pairs) have distinct subsequent patterns of defense signaling cascades. In the gene-for-gene interaction between *Arabidopsis thaliana* and *Pseudomonas syringae*, Century et al. (1995) found that RPS2-mediated resistance is largely repressed in *ndr1* mutant Arabidopsis lines, while RPM1-mediated resistance was partially suppressed under the same mutant background. Two Avr proteins reacting to one R protein also exert distinct defense signaling responses: both AvrRpt2 and AvrRpm1 caused a defense response towards RPM1; however, AvrRpt2 only resulted in a weak defense response, while AvrRpm1 was able to trigger the typical HR phenotype. Even the different versions of gene-for-gene interactions may exert different ways of signal transductions for some genes.

3.6 Conclusions

In conclusion, this study showed that the regulation of hormonal signaling is crucial for plant defense in *B. napus* under the pressure of *L. maculans*. Different trade-off patterns for some

hormonal responsive factors led to distinct levels of severity. Among them, SA-responsive factors were found to play pivotal roles in stronger resistance in *B. napus*, in which early SA signaling and subsequent SAR, such as *WRKY70* and *PR1*, possibly play a central role in the defense against *Brassica napus*. Compared with the incompatible interaction, the compatible interaction showed later activation of the SA-/JA-/ET-related genes studied in this research, suggesting that the late activation of massive defense signals may not rescue *B. napus* from *L. maculans* invasion. Again, it implicates the advantages of priming of defense activities in *B. napus* from more resistant genotypes (i.e., Surpass400 and 01-23-2-1). The distinct onset patterns of the hormone-responsive genes between these two types of interactions reflect the importance of early activation of essential defense genes to stem the early hyphal development of *L. maculans*.

Author Contributions: Conceptualization, C.Y. and W.G.D.F.; methodology, C.Y.; validation, C.Y. and W.G.D.F.; formal analysis, C.Y.; investigation, C.Y.; resources, data curation, C.Y.; writing—original draft preparation, C.Y.; writing—review and editing, C.Y. and W.G.D.F.; supervision, W.G.D.F.; project administration, W.G.D.F.; funding acquisition, W.G.D.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by NSERC (Discovery) and NSERC-CRD.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

Acknowledgments: The authors wish to thank a NSERC Discovery grant and a NSERC-CRD grant awarded to W.G.D. Fernando to carry out this work. We acknowledge the assistance of the Plant Science greenhouse staff in assisting with watering and maintaining our experiments.

Conflicts of Interest: The authors declare no conflict of interest.

Published in International Journal of Molecular Sciences: Cunchun Yang and W. G. D. Fernando 2021. Hormonal Responses to Susceptible, Intermediate, and Resistant Interactions in the *Brassica napus–Leptosphaeria maculans* Pathosystem. Intl. J. Mol. Sci. 22: 4714. DOI: 10.3390/ijms22094714 (IF=5.923)

ROS signaling considered as an early response following pathogenic attack, ROS signaling invokes various subsequent signal transduction including hormone signaling (Lamb and Dixon, 1996; Torres and Dangl, 2005; Zurbriggen et al., 2010; Baxter et al., 2013; Qi et al., 2017). Therefore, it is necessary to look at the ROS related cellular activities and factors in a time course with susceptible, intermediate and resistant severities. This objective aimed at observing the timing patterns of those cellular activities among those three severities. I intended to see whether those timing patterns reflect that it is important to trigger certain ROS activities for strong and durable resistance.

CHAPTER 4

Analysis of the Oxidative Burst and Its Relevant Signaling Pathways in *Leptosphaeria maculans—Brassica napus* Pathosystem

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4.1 Abstract:

An oxidative burst is an early response of plants to various biotic/abiotic stresses. In plant-microbe interactions, the plant body can induce oxidative burst to activate various defense mechanisms to combat phytopathogens. A localized oxidative burst is also one of the typical behaviors during hypersensitive response (HR) caused by gene-for-gene interaction. In this study, the occurrence of oxidative burst and its signaling pathways was studied from different levels of disease severity (i.e., susceptible, intermediate, and resistant) in the *B. napus–L. maculans* pathosystem. Canola cotyledons with distinct levels of resistance exhibited differential regulation of the genes involved in reactive oxygen species (ROS) accumulation and responses. Histochemical assays were carried out to understand the patterns of H_2O_2 accumulation and cell death. Intermediate and resistant genotypes exhibited earlier

accumulation of H_2O_2 and emergence of cell death around the inoculation origins. The observations also suggested that the cotyledons with stronger resistance were able to form a protective region of intensive oxidative bursts between the areas with and without hyphal intrusions to block further fungal advancement to the uninfected regions. The qPCR analysis suggested that different onset patterns of some marker genes in ROS accumulation/programmed cell death (PCD) such as *RBOHD*, *MPK3* were associated with distinct levels of resistance from *B. napus* cultivars against *L. maculans*. The observations and datasets from this article indicated the distinct differences in ROS-related cellular behaviors and signaling between compatible and incompatible interactions.

Keywords: *Leptosphaeria maculans*; *Brassica napus*; reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), programmed cell death (PCD), respiratory burst oxidase (RBOH)

4.2 Introduction

Canola, as a brand of cultivars from rapeseed and field mustard, has become a very important crop to Canada as well as to the world, since they have low amounts of erucic acid and glucosinolate, which have bad tastes and harmful effects on human and animal consumption (Dupont et al., 1989; Lin et al., 2013). Blackleg (caused by fungus *Leptosphaeria maculans*) has become such a great threat since 1975 in Canada (Bailey et al., 2003) that it may cause a 50% reduction in the production of canola. The major symptoms caused by the pathogen include stem canker, root rot, leaf lesion (with pycnidia), and pycnidia and pseudothecia on stubble (Canola Council of Canada/Blackleg, 2020).

Traditionally, the blackleg disease is managed by crop rotation, fungicide, etc., however, the development of genetically resistant rapeseed (*Brassica napus*) is the most cost-effective and environmentally friendly strategy for dealing with the disease (Raman et al., 2011). *L. maculans* possesses various enzymes to reinforce its infection upon hosts. Three cell-wall-degrading enzymes (CWDEs): endopolygalacturonase (*pg1*) and two cellulases (*cel1* and *2*), and *cel2* transcripts are found in the cotyledons and leaves from *B. napus* and *B. juncea* (Sašek et al., 2012). Moreover, *L. mauclans* also secretes phytotoxins such as sirodesmin PL, which causes leaf lesion (Gardiner et al., 2004; Mitrović et al., 2012).

Generally, there are two types of disease resistance in plants (triggered by phytopathogenic infection): qualitative and quantitative resistance. Qualitative resistance is triggered by gene-for-gene interactions, and it represents one type of interaction between the Avr proteins from the pathogens (AvrLm for *L. maculans*) and R proteins from the hosts (Rlm for *B. napus*). The interaction induces a hypersensitive response (HR) which triggers a series of rapid localized signaling cascades including ROS production, programmed cell death (PCD), and systemic acquired resistance (SAR). On the other hand, quantitative resistance exhibits a slower but more persistent defense in which more complicated internal signaling networks are involved (Lamb and Dixon, 1997; Agrios, 2004; Poland et al., 2009).

Oxidative burst including reactive oxygen species (ROS) production is an early response to various biotic/abiotic stresses in plants, which is considered a crucial part of the defense against biotic/abiotic stresses (Jabs, 1999; Poland et al., 2009; Baxter et al., 2013). During the plant defense, ROS accumulation, and the following signaling cascades exert various defense mechanisms that halt the pathogen invasion (Poland et al., 2009; Balint-Kurti, 2019). Those mechanisms include electrolyte leakage, modification of plant cells, programmed cell death (PCD), hormonal signaling, and protein production (Quan et al., 2008; Poland et al., 2009; Zubriggen et al., 2010; Baxter et al., 2013; Yoshioka et al., 2016).

Various studies correlated the electrolyte leakage and ROS-responsive defense activities. Electrolyte leakage has been found potentially connected with PCD and ROS generation/signaling (Overmyer et al., 2000; Ranf et al., 2012; Demidchik et al., 2014; Imanifard et al., 2018). Localized H_2O_2 secretion is the early response of HR from the origins of infection, including cell wall cross-linking and membrane damage (Tenhaken et al., 1995; Bestwick et al., 1997). Apoplastic peroxidases (such as peroxidases 33 and 34) are also involved in the PAMP Triggered Immunity (PTI) against plant pathogens (Bindschedler et al., 2006; Daudi et al., 2012), the binding between PAMP molecules and receptor-like R protein results in the activation of ROS-related factors including RBOHs (Respiratory Burst Oxidase Proteins), MAPK (Mitogen-Activated Protein Kinase) signaling and Ca^{2+} transportation (Yoshioka et al., 2016; Qi et al., 2017). Moon et al., (2003), suggested that the two MAPK cascade factors, *MPK3,* and *6* are activated by ectopic H_2O_2 accumulation. Furthermore, a gene named *ETHYLENE RESPONSE FACTOR6* (*ERF6*) was activated by MPK3/6 cascade to

induce *PDF1.1* and *PDF1.2*, which enhance plant defense in Arabidopsis (Meng et al., 2013). Wang et al., (2009) suggested that *MPK4* suppresses the ROS production in *Brassica napus*, and *MPK4* was found to induce jasmonic acid (JA) induced *PDF1.2*; the overexpression of *MPK4* connected with enhanced resistance against a necrotrophic pathogen, *Sclerotinia sclerotiorum*.

Evidence also shows that hormones such as salicylic acid also respond to oxidative burst. Salicylic acid (SA) and ethylene (ET) secretion respond to oxidative burst to elicit cellular signals towards lesion extension (i.e., programmed cell death), while JA responsive factors played the opposite roles (Overmyer et al., 2000; Rao et al., 2002). According to Overmyer et al. (2000), ethylene (ET) has its dependent pathway to induce cell death ahead of lesion formation before the symptoms emerge, and this process was activated by superoxide, JA response factors such as *JAR1* played a negative role in superoxide/ET-induced cell death.

The interaction between R and Avr proteins leads to hypersensitive response (HR), which involves various defense mechanisms including Ca^{2+} signaling and MAPK signaling, localized cell death (LCD) to hinder further pathogenic progression (Torres et al., 2005; Zubriggen et al., 2010; Baxter et al., 2013; Qi et al., 2017). Studies have shown that HR triggers a set of defense mechanisms that are similar to those from basal resistance, while the signal transduction is activated earlier and more localized (Tenhaken et al., 1995; Zubriggen et al., 2010; Becker et al., 2017), and the subsequent cell-cell communication sends the defense signals towards adjacent plant cells using ROS molecules as the messenger (Tenhaken et al., 1995; Bestwick et al., 1997; Zubriggen et al., 2010; Yoshioka et al., 2016; Becker et al., 2017).

Both basal resistance and gene-for-gene interaction utilize ROS accumulation to combat pathogenic invasion, these two types of resistance also represent two types of the genetic background of *B. napus*, which the plant breeders have been attempting to breed for efficient blackleg resistance. Therefore, it is necessary to have a deeper understanding of ROS responsive signaling pathways.

Although the relationship between plant defense and ROS signaling has been well studied, the onset patterns of ROS is not intensely explored in *B. napus* when defending against *L. maculans*. The goal of the study is to describe how ROS production and signaling function in

the *Brassica napus–Leptosphaeria maculans* interaction, we intended to elucidate the role of ROS signaling with different severities of *B. napus* defense. By analyzing and comparing the pivotal genes within ROS signaling pathways, the onset patterns and expression levels of those studied genes can explain the various disease severities among different canola cultivars. The observations of cytological behaviors were also able to visualize the effects of ROS signaling on disease resistance.

4.3 Materials and Methods

4.3.1 Plant Materials

Brassica napus plants were grown in Sunshine Professional Growing Mix (SumGro Horticulture, Agawam, MA, USA), in 16 h of light (22 °C) (Photosynthetically Active Radiation (PAR) 300 μmole(m^{-2}·s^{-1})) and 8 h dark (16 °C) at 50 to 60% relative humidity.

4.3.2. Pathogen Cultivation

Leptosphaeria maculans isolates were cultured on V8 juice (Campbell's, Camden, NJ, USA) at room temperature under the fluorent tube light. The isolates were cultured for 10 to 14 days to obtain a sufficient amount of pycnidiospores. Each plate was scraped off and washed with 2 mL of distilled water to collect pycnidiospores and make inoculum stock solutions. The stock solutions were stored at −20 °C.

4.3.3 Pathogen Inoculation

Two *L. maculans* isolates were selected for inoculation: HCRT75 8-1 (Genotype: *avrLm1, AvrLm2, avrLm3, avrLm4, AvrLmJ1-5, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1* and *AvrLepR2*) and HCRT77 7-2 (Genotype: *AvrLm1, avrLm2, avrLm3, AvrLm4, AvrLmJ1-5, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1* and *avrLepR2*).

Three *B. napus* genotypes were selected to be inoculated: Westar (no *Rlm* gene), Surpass400 (*BLMR1/LepR3* and *BLMR2/RlmS*), and 01-23-2-1 (*Rlm7*).

The cotyledons of *B. napus* cultivars were inoculated 7 days after sowing (seedling stage) by puncture inoculation. Each lobe of cotyledons was punctured by a sterile needle twice from each side, to have 4 inoculation points on each seedling of the canola plant.

Two selected *L. maculans* isolates (HCRT75 8-1 and HCRT77 7-2) produced three distinct levels of severity on three *B. napus* cultivars (Westar, Surpass400, and 01-23-2-1). The genotype Westar without any *Rlm* genes produced susceptible phenotypes with both isolates, while Surpass400 (*Rlm* genes: *BLMR1/LepR3* and *BLMR2/RlmS*) exhibited some level of resistance on both isolates, as such, intermediate towards HCRT75 8-1 and resistant (hypersensitive response, HR) towards HCRT77 7-2. Finally, the cultivar 01-23-2-1 (*Rlm* genes: *Rlm7*) showed typical HR resistance on both isolates (**Figure 4.1**).

4.3.4 Electrolyte Leakage Measurement

The cotyledons (6 cotyledons from 3 biological replicates) were cut into small leaf disks (round, 5 mm in diameter) with the cork borer. The leaf disks were washed for 30 min in 10 mL ultrapure water and transferred into another round of fresh ultrapure water (25 mL). After 5 h, the electrolyte leakage was measured in voltage from the soaked ultrapure water by the VWR sympHony conductivity meter (Radnor, PA, United States).

4.3.5 3,3′-Diaminobenzidine (DAB) Staining

The DAB staining solution was prepared by dissolving 40 mg of DAB (Sigma-Aldrich, St. Louis, MO, USA) in 200 μL of dimethylformamide in 40 mL of water. Cotyledons were soaked in the staining solution in the dark and shaken overnight. The stained cotyledons were discolored by 95% ethanol. The experiment is followed by the protocol of Sašek et al. (2012).

4.3.6 Trypan Blue Staining (TBS)

The trypan blue stock solution was prepared by mixing 10 mL of phenol, 10 mL of glycerol, 10 mL of lactic acid, 10 mL of water, and 0.02 g of trypan blue powder (Sigma-Aldrich, St. Louis, MO, USA). The working solution was prepared by dissolving the stock solution with ethanol (96%; 1:2, *v*/*v*). *B. napus* cotyledons were soaked in the working solution and boiled in a water bath for 1 min, incubated in the solution overnight and washed in chloral hydrate solution (2.5 g of choral hydrate in 1 mL of distilled water).

4.3.7 Gene Expression Analysis

Frozen cotyledons (12 cotyledons, 6 seedlings, 3 biological replicates) were ground in liquid nitrogen using pestles and mortars. Total RNA was extracted with TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). Total RNA was purified by DNase I treatment with DNase I recombinant, RNase-free kit (Roche). Purified RNA was used to synthesize cDNA with the GOScript Reverse Transcription System (Promega). The cDNA stock solution was diluted into a concentration of 100 ng/µL. The quantitative-PCR was performed by mixing 1 µL of cDNA (100 ng) into the 10 µL reaction system of IQ^{TM} SYBR$^{®}$ Green Supermix (BioRad, Hercules, CA, USA).

The qPCR program used for all analyzed genes was: 95 °C for 3 min; followed by 39 cycles of 95 °C for 15 sec, and 60 °C for 20 sec; followed by a melting curve analysis.

All qPCR primers are listed in **Appendix I**. The relative level of gene expression was analyzed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen, (2001). The expression of the studied genes was normalized by the house-keeping gene *ACTIN* (NM_001316010.1).

4.3.8 Statistical Analysis

Unless specified, the analyses of samples used at least three biological replicates. The statistical analyses were performed using the Fisher's Least Significant Difference (LSD) method with the SAS 9.4 software (SAS Institute, Cary, NC, USA). The Fisher's LSD was

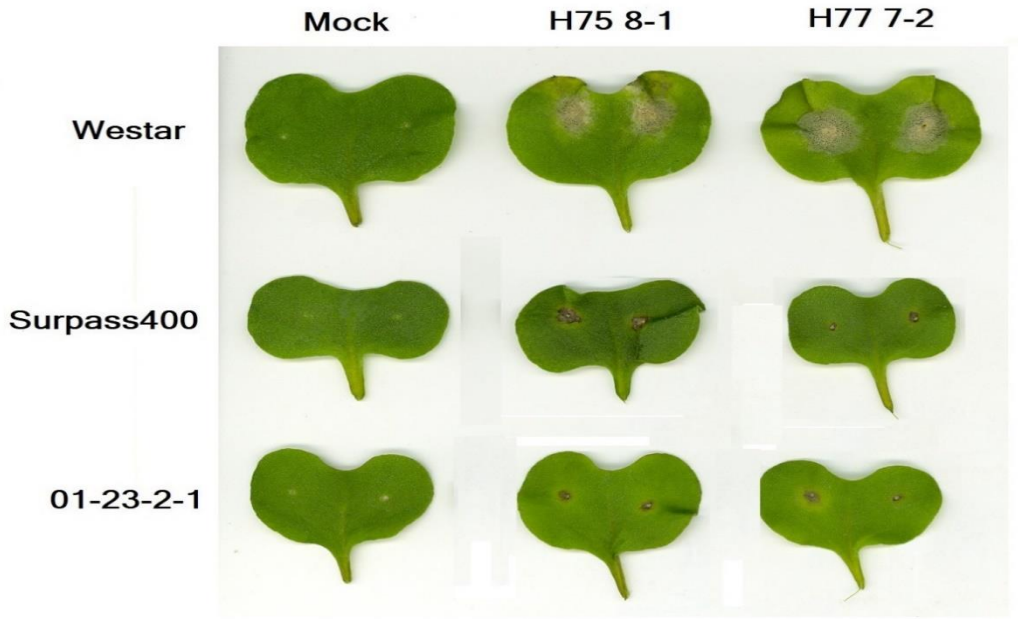
applied to gene expression and electrolyte leakage measurement, in order to observe the effectiveness of resistance in the three genotypes when inoculated with two isolates.

4.4 Results

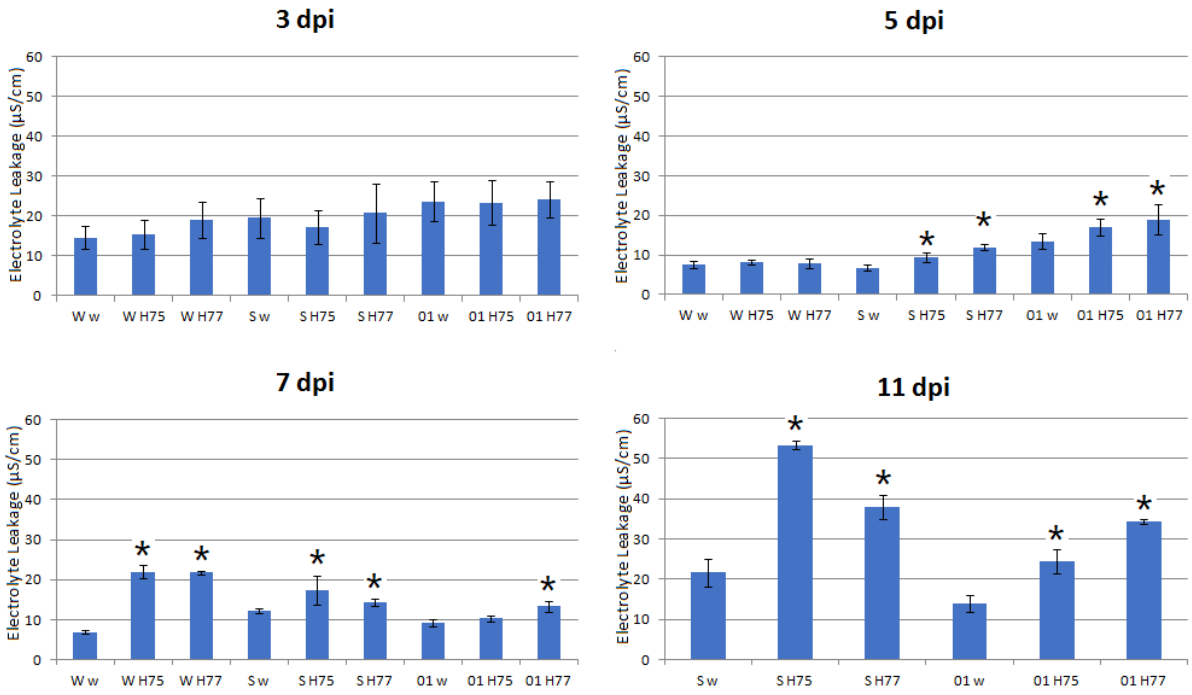
4.4.1 Early Induction of Electrolyte leakage Occurring from Intermediate and Resistant Phenotypes

As shown in **Figure 4.1**, two selected *L. maculans* isolates (HCRT75 8-1 and HCRT77 7-2) produced three distinct levels of severity on three *B. napus* cultivars (Westar, Surpass400 (*BLMR1/LepR3* and *BLMR2/RlmS*), and 01-23-2-1 (*Rlm7*)). The inoculation caused susceptible phenotypes on Westar cotyledons; Surpass400 and H75 8-1 had intermediate incompatible interaction (AvrLepR2–BLMR2) while Surpass400–H77 7-2 (AvrLm1–BLMR1), 01-23-2-1–H75 8-1/H77 7-2 (AvrLm 4-7–Rlm7), these three cases showed strong incompatible (resistant) interaction (Dandena et al., 2019; Neik et al., 2020). The differences in severity reflected the distinct modulation of defense signaling in those cultivars, and the study of these differences helps explain how susceptibility and resistance occur in canola when combating the blackleg pathogen.

(**a**)



(**b**)


3 dpi
5 dpi
7 dpi
11 dpi
Electrolyte Leakage (µS/cm)
0
10
20
30
40
50
60
W w
W H75
W H77
S w
S H75
S H77
01 w
01 H75
01 H77
*

(**c**)

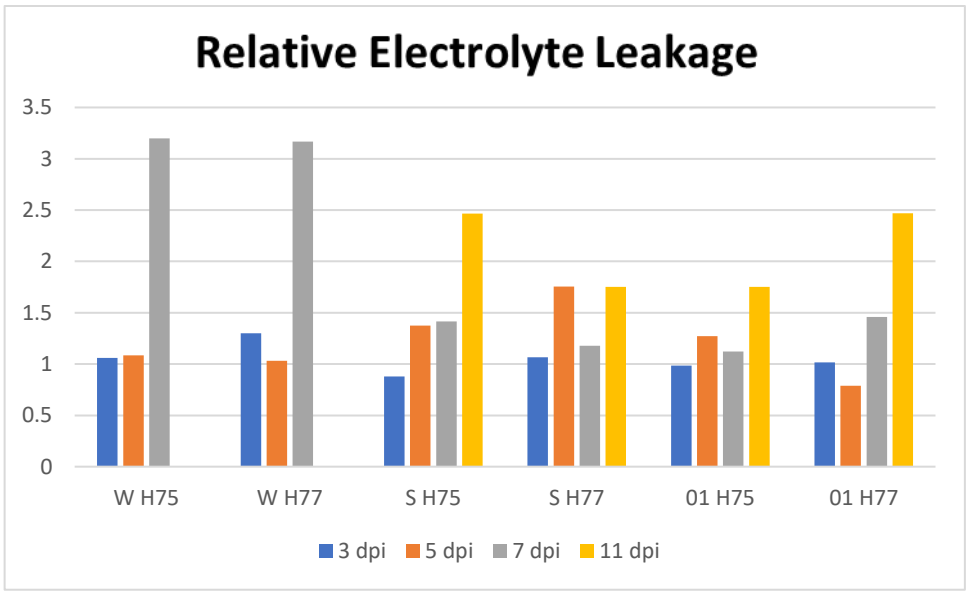

Relative Electrolyte Leakage
3.5
3
2.5
2
1.5
1
0.5
0
W H75
W H77
S H75
S H77
01 H75
01 H77
3 dpi
5 dpi
7 dpi
11 dpi

Figure 4.1. Lesion development and electrolyte leakage of the inoculated cotyledons (**a**) The lesion development and the appearance of phenotypes were observed from the three genotypes (Westar, Surpass400 and 01-23-2-1) and two isolates (H75 8-1 and H77 7-2) at 11 days post-inoculation (dpi). (**b**) The measurement of electrolyte leakage at 3, 5, 7, and 11 dpi. At 11 dpi, because Westar cotyledons were generally degraded and heavily infected, the measurement of electrolyte leakage at this stage became incapable and inaccurate, the measurement of Westar genotype at this time point was not included. The x-axis indicates the inoculation pair between genotypes (W: Westar; S: Surpass400; 01: 01-23-2-1) and isolates (w; water; H75: H75 8-1; H77: H77 7-2), the y-axis indicates the voltage detected from the cotyledon-soaked solution suggesting the leaking of ions (unit: μS/cm, S; Siemens). The asterisks indicate the significant differences of the electrolyte leakage measurement among mean values when compared with mock inoculation (Fisher's Least Significant Difference; $p < 0.05$). (**c**) The relative electrolyte leakage at 3, 5, 7, and 11 dpi. The relative leakage is calculated by dividing the average measurements of inoculated cotyledons by mock inoculated ones. For time point, different lowercase letters suggest the significant differences among mean values (Fisher's Least Significant Difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

To understand how oxidative burst works at a physiological level, the measurement of electrolyte leakage is a useful tool. As one of the earliest responses to various stresses, electrolyte leakage is found to trigger multiple defensive mechanisms in planta, which includes hormonal secretion, programmed cell death, oxidative burst, etc. (Overmyer et al., 2000; Ranf et al., 2012; Demidchik et al., 2014). For this study, the electrolyte leakage was measured from the excised cotyledons, the voltage caused by the leaked electrolytes from both mock and inoculated samples were measured with the VWR sympHony conductivity meter.

As shown in **Figure 4.1**, the two resistant genotypes Surpass400 and 01-23-2-1 exhibited a significantly higher level of electrolyte leakage (compared with mock-inoculated cotyledons) as early as 5 dpi, when the susceptible Westar cotyledons did not have the induction of significant electrolyte leakage. Westar started to induce higher leakage at 7 dpi, and at 11 dpi,

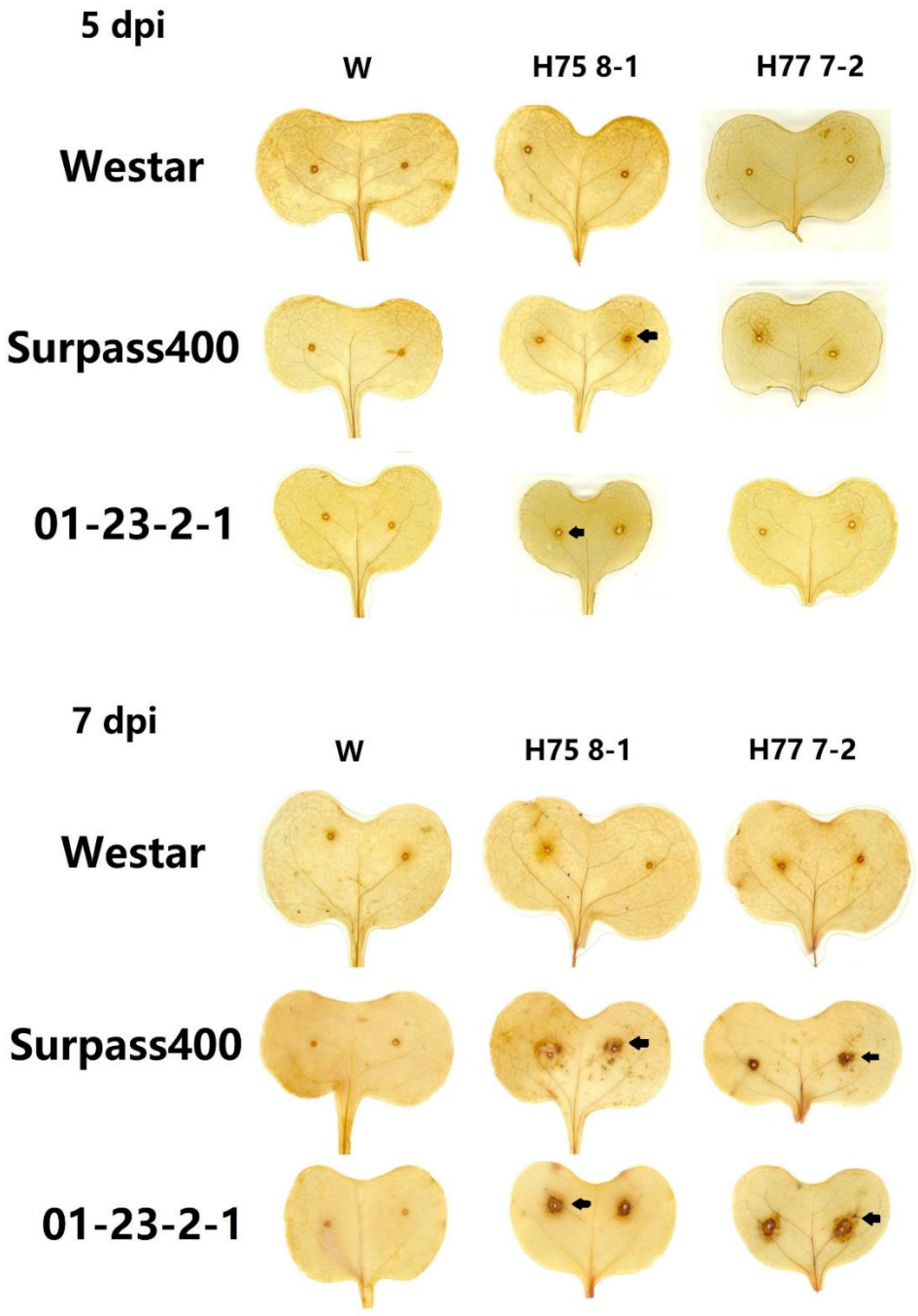
the cotyledons were collapsed and severely damaged to perform a further measurement, therefore, there was no data about Westar leakage at 11 dpi. Surprisingly, Surpass400–H75 8-1 seemed to have retained the secretion of electrolyte at 11 dpi according to the conductivity measurement. The results suggested that resistant genotypes had earlier activation of electrolyte secretion (at 5 dpi) while the compatible interaction (i.e., Westar) had a later triggering process (at 7 dpi).

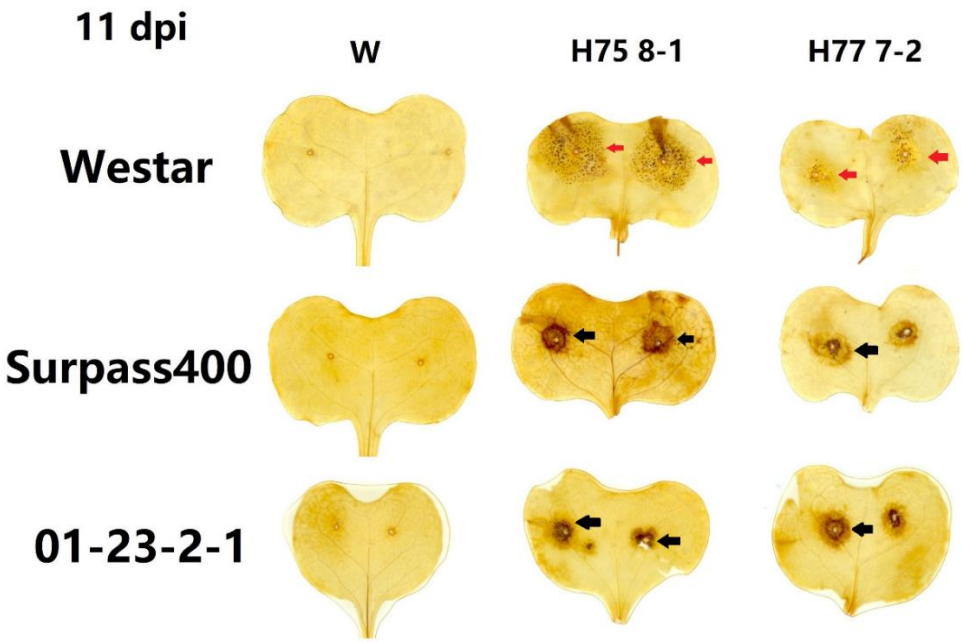
4.4.2. Distinct Detection of Hydrogen Peroxide in Susceptible, Intermediate and Resistant *B. napus* Plants

As a stable and reactive ROS molecule, H_2O_2 plays multiple roles in plants during normal physiological functioning and stress resistance, its membrane-permeable property makes it a useful messenger in cell-cell communication, thus coordinates cellular signaling mechanisms which are time/space-dependent (Wojtaszek et al., 1997; Quan et al., 2008; Baxter et al., 2013). By 3,3′–Diaminobenzidine (DAB) staining, the diffusion of hydrogen peroxide (H_2O_2) was visualized as brown-colored stains.

As shown in **Figure 4.2**, at 5 dpi, it is difficult to compare/contrast the patterns of H_2O_2 among the six inoculation treatments. At 7 and 11 dpi, both the intermediate and resistant genotypes Surpass400 and 01-23-2-1 exhibited more captured brownish color, formed a ring-like pattern surrounding of the origins of inoculation (**Figure 4.2a, red arrows**). On the other hand, Westar cotyledons had no intense brownish color around inoculation sites and the pycnidia were visible at 7 dpi.

(a)


5 dpi
W
H75 8-1
H77 7-2
Westar
Surpass400
01-23-2-1
7 dpi
W
H75 8-1
H77 7-2
Westar
Surpass400
01-23-2-1



(**b**)

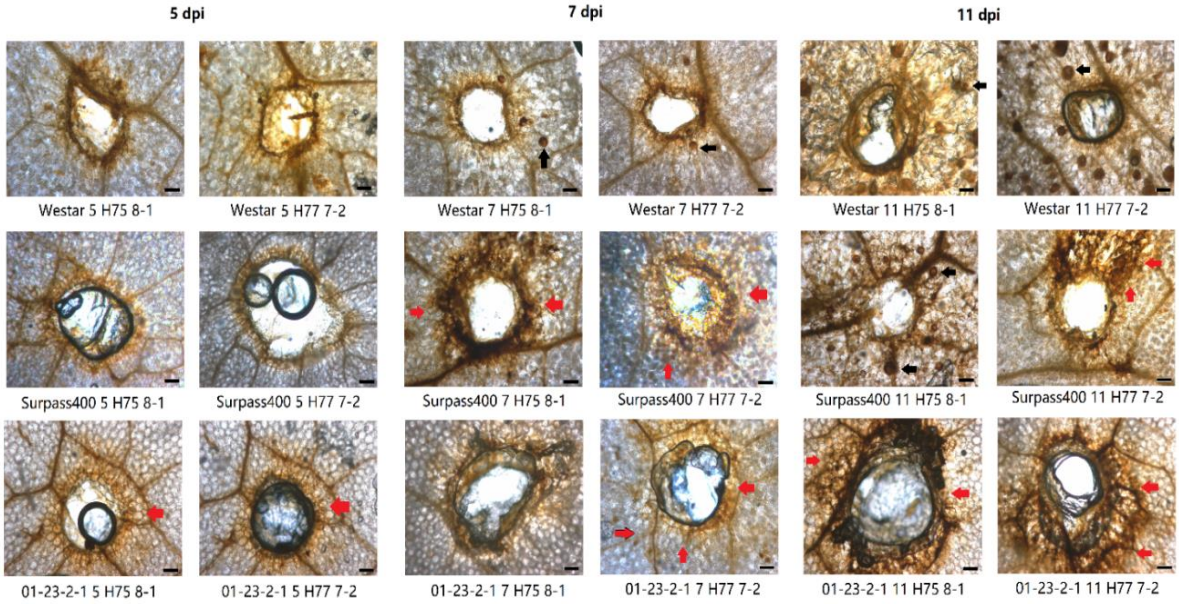


Figure 4.2. *In situ* detection of hydrogen peroxide by DAB staining on cotyledons of Westar/Surpass400/01-23-2-1 inoculated with H75 8-1/H77 7-2 (genotype/isolate). (**a**) The scanned cotyledon images (representative images) of stained cotyledons displayed the

diffusion of ROS molecule H_2O_2 after inoculation (with mock, H75 8-1 and H77 7-2). The brownish stains (black arrows) suggested the diffusion regions of H_2O_2. Westar at 11 dpi also showed some pycnidia production (red arrows). (**b**). The representative microscopic images taken from the origins of inoculation (magnitude: 50×), the images showed the details about H_2O_2 accumulation when the fungus progressed from the puncture holes for inoculation. The brownish color shown from Surpas400 and 01-23-2-1 had more captured H_2O_2 around the origins of inoculation (red arrows) and some pycnidia from Westar and Surpass400 were also captured (black arrows). The microscopic images were taken at 5, 7, and 11 days post-inoculation (dpi). Bars = 100 μm.

The microscopic observation revealed a similar pattern of H_2O_2 (**Figure 4.2b red arrows**). The localized secretion of H_2O_2 was visible as early as 5 dpi under the microscope from the cotyledons,01-23-2-1, which displayed some detected brownish (i.e., H_2O_2) distribution around the punctured holes (**Figure 4.2**). At 7 dpi and 11 dpi, Westar samples (both H75 8-1 and H77 7-2) had a large amount of pycnidia (**Figure 4.2b, black arrows**), while Surpass400–H75 8-1/H77 7-2 (intermediate/resistant) and 01-23-2-1- H75 8-1/H77 7-2 (resistant) cotyledons showed an apparent trace of H_2O_2 accumulation on the cotyledonary tissues (**Figure 4.2b, red arrows**). Adequate H_2O_2 accumulation induces considerable signaling, which triggers defense responses at the cellular level, such as MAPK cascades and Ca^{2+} signaling (Gechev et al., 2005; Quan et al., 2008; Zurbriggen et al., 2010). Since the accumulation of H_2O_2 plays central roles in the activation of plant defense signaling, the intense accumulation of H_2O_2 on Surpass400 (7 dpi) and 01-23-2-1 (5 and 7 dpi) cotyledons indicated that the gene-for-gene interaction (for both intermediate and resistant cases) can induce early H_2O_2 outburst to trigger anticipated and localized defense activities to inhibit fungal development.

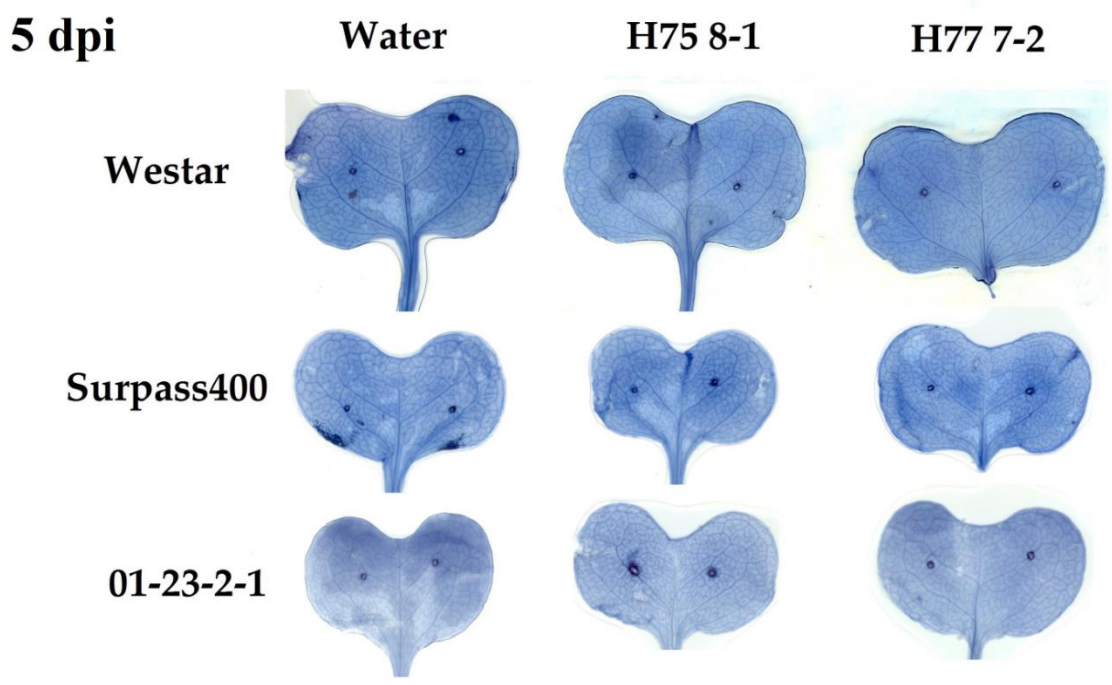
4.4.3 The Impacts of ROS Upon Cell Death

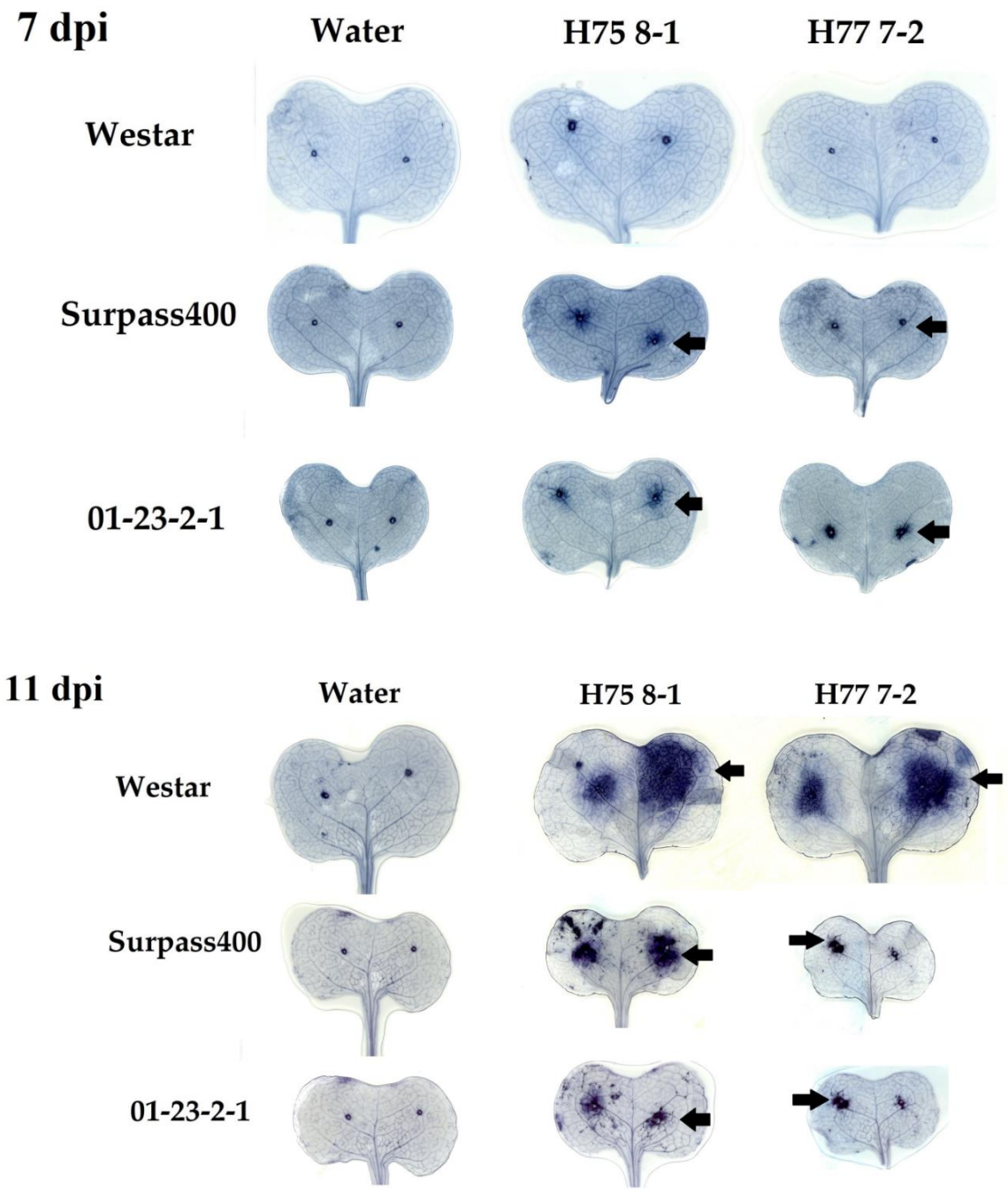
Followed by H_2O_2, various physiological activities can be triggered to stop further pathogenic progression, those activities include callose deposition, and cell wall cross-linking (Lamb and Dixon, 1997; Quan et al., 2008; Knepper and Day, 2010; Daudi et al., 2012; Baxter et al.,

2013). Another biological process highly regulated by H_2O_2 is the programmed cell death (PCD) (Lamb and Dixon, 1997; Zurbriggen et al., 2010; Baxter et al., 2013).

By observing the cotyledons treated with trypan blue staining (TBS), the bulk of stained senescent cells were visible from both Surpass400 and 01-23-2-1 cotyledons around the origins of inoculation at 7 dpi (**Figure 4.3a, b**). At 11 dpi, Surpass400 and 01-23-2-1 had a further enlargement of death regions, which was an enhanced situation to what happened at 7 dpi. From the microscopic images, the incompatible interaction did not hinder the hyphal formation of *L. maculans* fungus but formed a buffering zone with dead cells (**Figure 4.3a, black arrows, Figure 4.3b, yellow arrows**) to inhibit the chance for hyphae to invade more living tissues for nutrition. On the other hand, Westar only had hyphae (5 dpi) and pycnidia (7 and 11 dpi) formed around the punctured holes for inoculation (**Figure 4.3b, red arrows**), suggesting that the LCD was not observed in compatible interaction and this defense mechanism must be the feature for incompatible interaction (HR cell death) (**Figure 4.3b**).

(**a**)




7 dpi
Water
H75 8-1
H77 7-2
Westar
Surpass400
01-23-2-1
11 dpi
Water
H75 8-1
H77 7-2
Westar
Surpass400
01-23-2-1

(**b**)

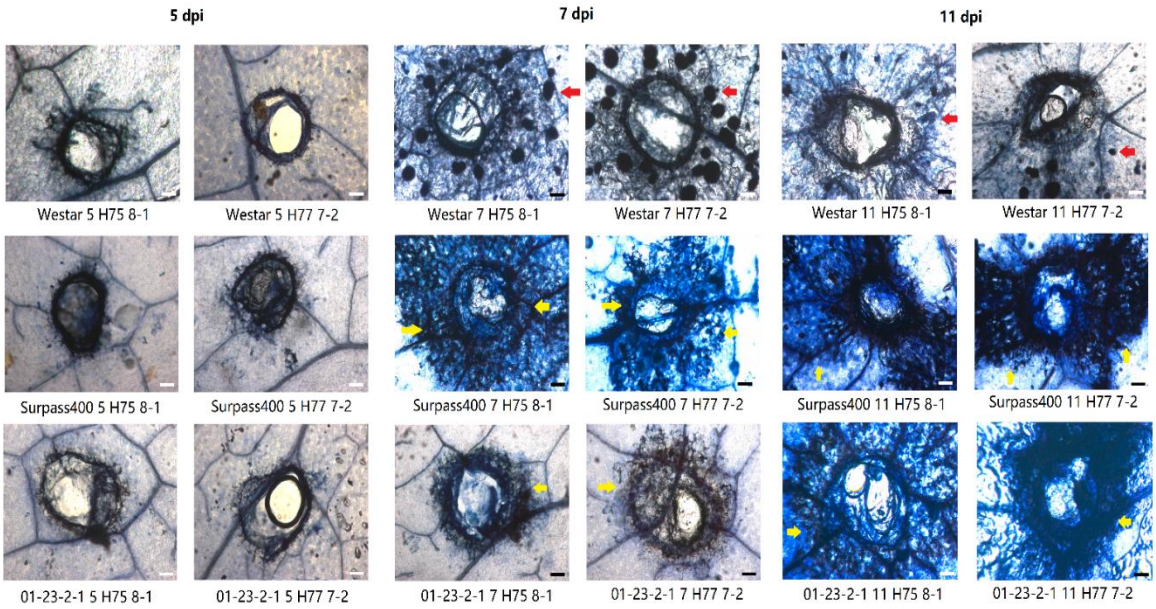
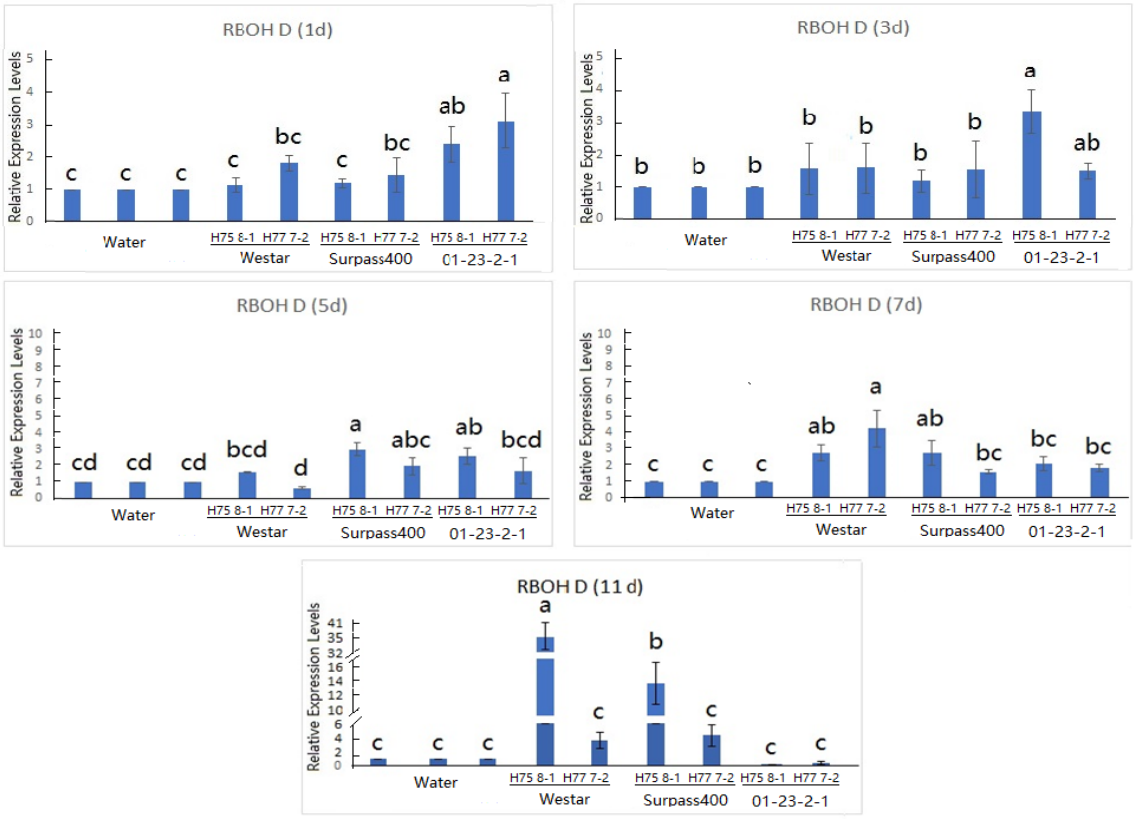


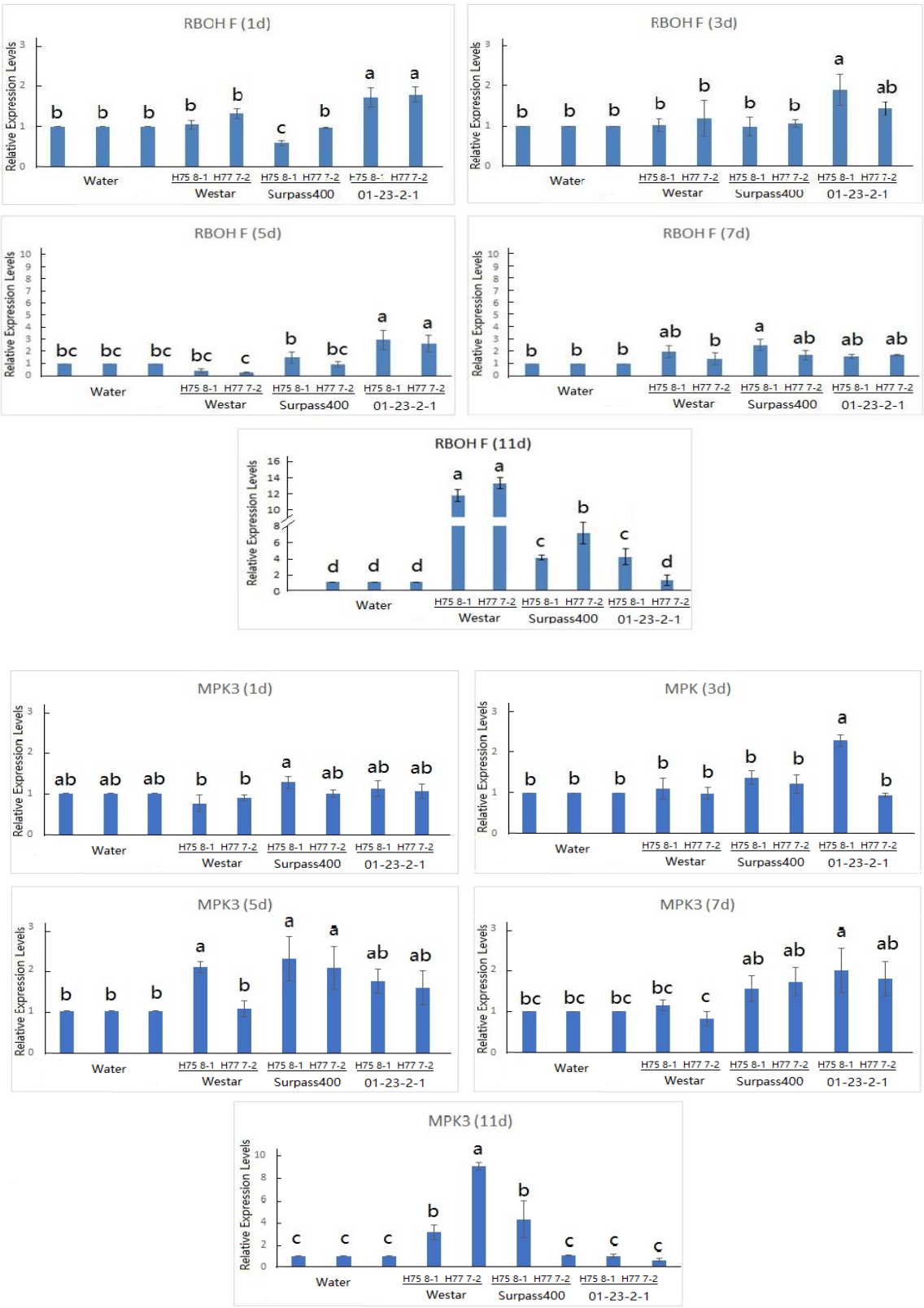
Figure 4.3. Regions of cell death (stained with trypan blue) at 5, 7, and 11 dpi adjacent to the origin of fungal development from 6 inoculation pairs (genotype/isolate: Westar-H75 8-1/H77 7-2, Surpass400 – H75 8-1/H77 7-2 and 01-23-2-1 – H75 8-1/H77 7-2). (**a**) The scanned cotyledons stained with trypan blue (representative images) showed the spread of cell death (dark blue) throughout the cotyledons initiated from the sites of inoculation (center of each lobe). The potential regions of cell death on the cotyledons were highlighted by black arrows. (**b**) The representative microscopic images (magnitude: 50×) taken around the sites of inoculation, the formations of senescent cells (yellow arrows), hyphal development (red arrows), and pycnidia (red arrows) formations were visualized under the microscope. Bars (black and white) = 100 µm.

4.4.4. Signal Allocation Patterns in ROS Production and Subsequent Responsive Factors among Susceptible, Intermediate and Resistant B. napus Plants

Triggered by oxidative burst, the plant body can trigger a series of defensive mechanisms including expression of responsive genes in hindering further pathogenic progression. These defensive mechanisms include the early apoplastic accumulation of ROS by membrane-bound NADPH oxidases (Lamb and Dixon, 1997).

As shown in **Figure 4.4**, Surpass400 and 01-23-2-1 exhibited an earlier induction of *RBOHD* and *F* compared with Westar. 01-23-2-1 showed the relatively high expression of *RBOHD/F* as early as 1 dpi. Surpass400 (both H75 8-1 and H77 7-2) did not show straightforward early induction of both *RBOHD* and *F* (1, 3 and 5 dpi). Remarkably, both Surpass400 H75 8-1 and H77 7-2 showed higher expression *RBOHD* at 5 dpi compared with Westar (Surpass400–H77 7-2 is not significant enough). For Westar, both genes were not expressed until 7 dpi and displayed a high expression level at 11 dpi. As early as 3 dpi, the blackleg fungus started to secrete cell wall degrading proteins (CWDBs) in *B. napus* (Sexton et al., 2000). Becker et al., (2017) also found the early cell collapse in resistant *B. napus* (incompatible interaction against *L. maculans*) at 3 dpi, and at the same time point, genes related to SA and JA signals are also induced. The results indicated that RBOH enzyme may be an important factor to initiate ROS production during plant defense in *B. napus* since early defense against *L. maculans* seems to be one of the features for effective defense.




RBOH F (1d)
Relative Expression Levels
b b b b b c b a a
RBOH F (3d)
b b b b b b b a ab
RBOH F (5d)
bc bc bc bc c b bc a a
RBOH F (7d)
b b b ab b a ab ab ab
RBOH F (11d)
d d d a a c b c d
MPK3 (1d)
ab ab ab b b a ab ab ab
MPK (3d)
b b b b b b b a b
MPK3 (5d)
b b b a b a a ab ab
MPK3 (7d)
bc bc bc bc c ab ab a ab
MPK3 (11d)
c c c b a b c c c
Water
H75 8-1 H77 7-2
Westar
Surpass400
01-23-2-1

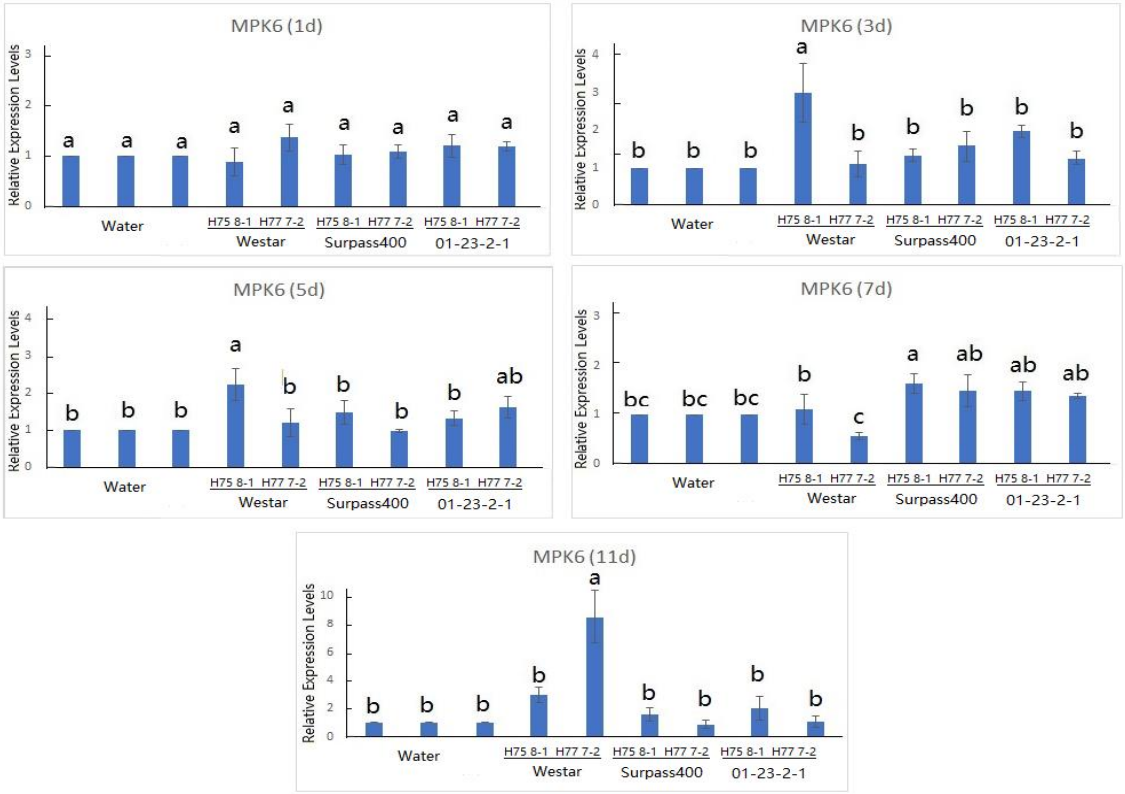
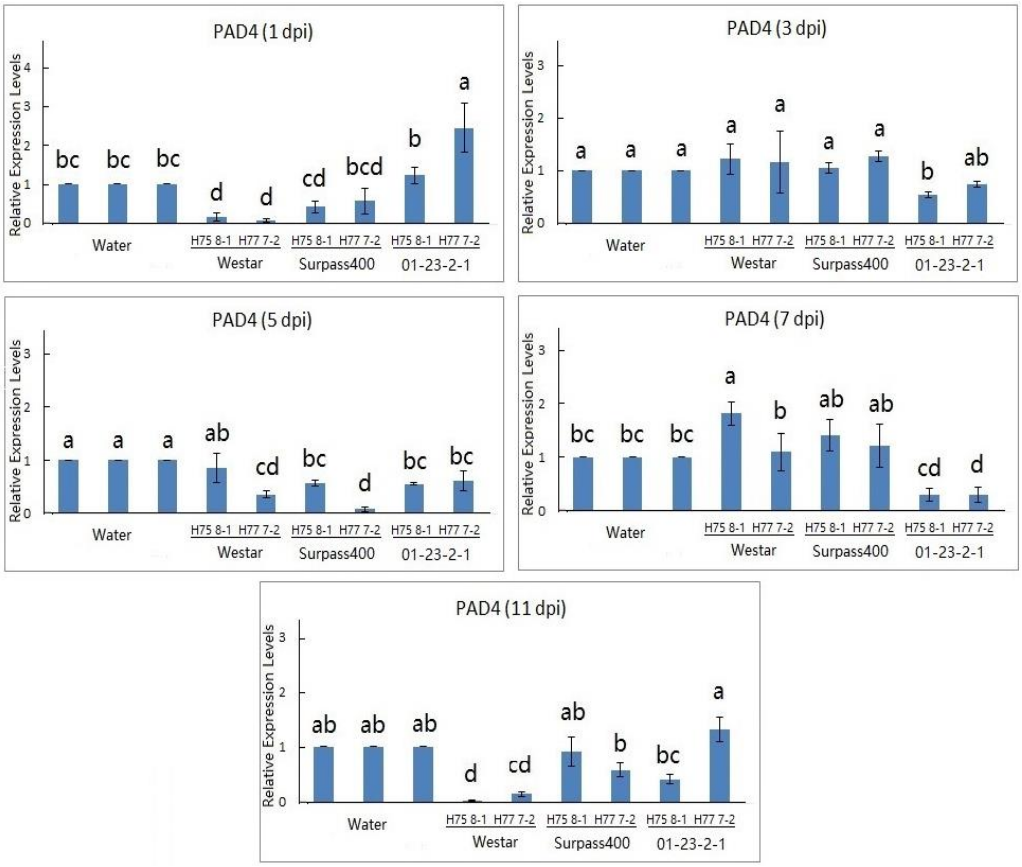


Figure 4.4. Gene expression of genes related to ROS production (*RBOHD* and *F*) and ROS signaling (*MPK3* and *6*). The level of the bars are the expression levels from the inoculated cotyledons (three genotypes: Westar, Surpass400 and 01-23-2-1, two isolates: H75 8-1 and H77 7-2) normalized with the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). For time point, different lowercase letters suggest the significant differences among mean values (Fisher's Least Significant Difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

Since PCD is one of the mechanisms of HR defense, the pathogen is not able to get enough nutrients to replicate when it is surrounded by dead cells (Sexton et al., 2000; Knepper and Day, 2010; Zubriggen et al., 2010). *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) and *PHYTOALEXIN DEFICIENT 4* (*PAD4*) are found to play pivotal roles in *R* gene-mediated signaling of resistance (Knepper and Day, 2010; Lin et al., 2013). By analyzing the expression of *EDS1* (**Figure 4.5**), 01-23-2-1 (inoculated with H75 8-1 and H77 7-2) displayed higher

expression at 3 and 5 dpi while Westar (with H75 8-1 and H77 7-2) had the peak expression at 11 dpi. Intermediate interaction for cotyledons of Surpass400 with H75 8-1 had early induction of the same gene at 3 dpi, and also exhibited up-regulation at 11 dpi like Westar. The onset patterns of *EDS1* expression suggested that resistant interaction had earlier activation of *EDS1*, possibly due to the earlier recognition of the pathogen by gene-for-gene interaction.



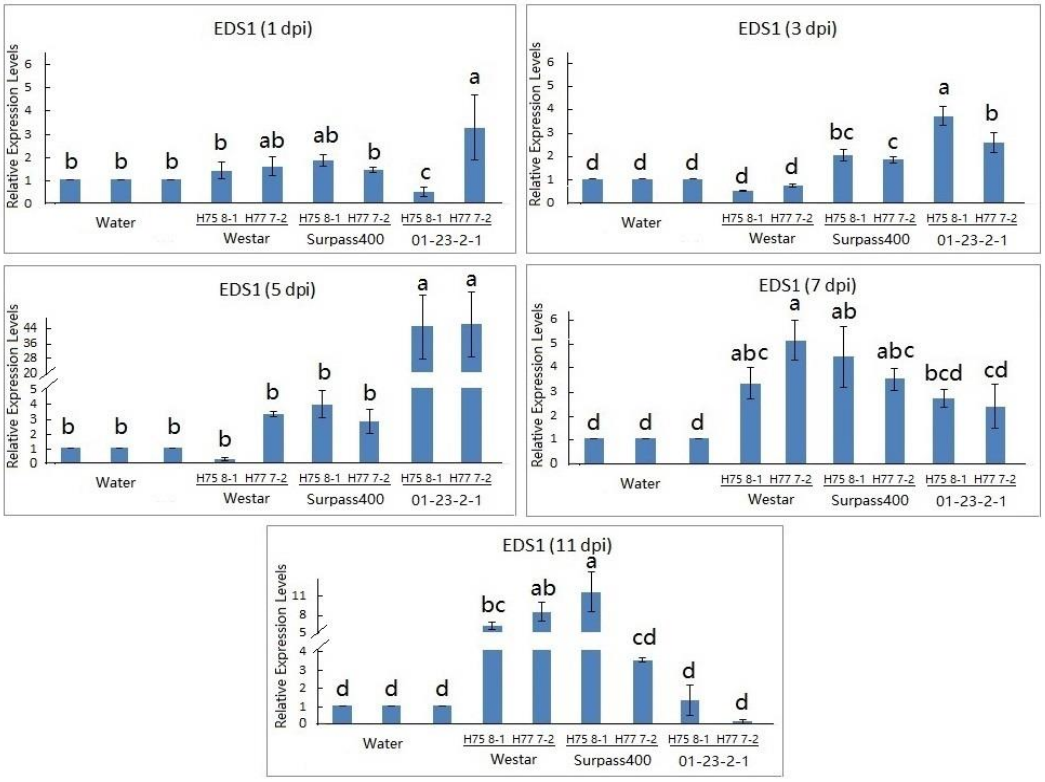


Figure 4.5. Expression analysis of genes related to cell death (*PAD4* and *EDS1*). The level of the bars are the expression levels from the inoculated cotyledons (three genotypes: Westar, Surpass400 and 01-23-2-1, two isolates: H75 8-1 and H77 7-2) normalized with the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). For the time point, different lowercase letters suggest the significant differences among mean values (Fisher's Least Significant Difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

Surprisingly, *PAD4* did not show co-expression with *EDS1*. According to other studies, PAD4 and EDS1 interact with each other to trigger basal resistance and HR (Feys et al., 2001; Rietz et al., 2011). *PAD4* did not have a high expression at 5 dpi in Surpass400 and 01-23-2-1 when expression of *EDS1* peaked at this time point in these two genotypes. EDS1 can induce other resistant activities without PAD4 (Feys et al., 2001; Rietz et al., 2011) and EDS1 is found to bind multiple factors in plant defense (Bhattacharjee et al., 2011).

MPK3 and MPK6 are also found to support ROS signaling, these two factors also assist the production of camalexin and ethylene (Bethke et al., 2009; Meng et al., 2013). An ethylene-responsive factor, *ERF6*, was phosphorylated and activated by MPK3/MPK6 cascade to induce *WRKY33*. *PDF1.1* and *PDF1.2*, two defensins to enhance plant defense, were also activated (Pitzschke et al., 2009; Su et al., 2017). *MPK3* and *MPK6* displayed induction from Westar at 11 dpi (with both HCRT75 8-1 and HCRT77 7-2), while Surpass400 and 01-23-2-1 did not show very high expression (**Figure 4.5**). Westar depends more on the expression of *MPK3* and *MPK6* expression levels at a later period (necrotrophic) of the infection, when the fungal progression was too severe that the host needed massive ROS signaling and other defense activities to stop further infection. The high expression of *MPK3* and *MPK6* on Westar cotyledons at 11 dpi also linked to the expression of *RBOHD* and *-F*, suggesting that susceptible Westar cotyledons lately activated massive ROS signaling to stop the necrotrophic phase of *L. maculans*, the ROS molecules are able to exert multiple factors and signaling pathways to activate plant defense activities (Ranf et al., 2012; Yoshioka et al., 2016).

4.5 Discussion

In this article, the genotypes with stronger resistance Surpass400 (intermediate/resistant) and 01-23-2-1 (resistant) exhibited earlier emergence of electrolyte leakage, H_2O_2 diffusion and cell death, compared with susceptible control Westar. Moreover, ROS-responsive genes such as *RBOHD/F* tended to be activated from 01-23-2-1.

Electrolyte leakage has been found in many studies as the early physiological signal for stress response. It is also observed from plant tissues during hypersensitive response and cell death (Overmyer et al., 2000; Imanifard et al., 2018). Ions such as K^+ and Ca^{2+} are transported via ion channels to induce signals related to stress tolerance. The efflux of K^+ is found in various biological processes including PCD, ROS, stomata closure, and hormonal secretion (Garcia-Brugger et al., 2006; Demidchik et al., 2014). Besides, another remarkable electrolyte Ca^{2+} is originated from the vacuole and induced as the second signal when the MAMP/DAMP (Microbe/Damage-Associated Molecular Pattern) factors are precepted, and the defense signals also lead to PCD (Ranf et al., 2012; Demidchik et al., 2014). Thus, electrolyte leakage

becomes a reliable measure of cell death and stress response. The early observation of electrolyte voltage from the inoculated cotyledons of Surpass400 and 01-23-2-1 implicated the early activation of defense response since the HR is found to trigger defense mechanisms including ion leakage, ROS signaling, hormonal signaling, etc. (Sexton et al., 2000; Demidchik et al., 2014; Becker et al., 2017). On the other hand, Westar samples started to have higher conductivity at 7 dpi, as suggested in Becker et al. (2017), the RNA sequencing data revealed that the susceptible *B. napus* genotype triggered the same defense-related genes as the resistant genotype, however, the incompatible interaction activates the earlier expression of those genes compared with the compatible interaction, causing the different disease severity between susceptible and resistant genotypes.

Since *L. maculans* is hemibiotrophic, it undergoes the biotrophic stage first and then reaches the necrotrophic stage. Biotrophs usually exploit the nutrient from the living cells, it penetrates the plant cell wall and membrane with fungal structures such as haustoria and hyphae (Perfect and Green, 2001; Coll et al., 2011; Stotz et al., 2014). Evidence also showed that around the early stage of *L. maculans* infection upon *B. napus*, the fungus secretes cell wall degrading enzymes (CWDEs), and this physiological process is considered as one aspect of its pathogenicity (Annis and Goodwin, 1996; Sexton et al., 2000). Sexton et al. (2000) reported that the highly virulent *L. maculans* races secrete the CWDEs at an early stage.

When blackleg fungus infects successfully, fungal hyphae develop in intercellular space during the biotrophic stage, and no obvious damage was made upon host cells (Li et al., 2008a). Thus, early cell senescence becomes an effective strategy against biotrophic pathogens, to prevent further colonization and exploitation of host nutrients (Li et al., 2008a; Zubriggen et al., 2010; Stotz et al., 2014).

The early intensive diffusion of H_2O_2 from Surpass400 (intermediate/resistant) and 01-23-2-1 (resistant) connect their resistant responses against the fungus, the accumulation of brownish discoloration (i.e., H_2O_2) around the origins of inoculation indicated a series of defensive responses including cell senescence from the host, which will hinder the further fungal growth. This may explain the similar pattern of cell death that occurred around the sites of inoculation, which was validated by trypan blue staining (TBS). The intermediate and resistant cotyledons tended to induce a protective region together with early hyphal development, so that the further

intercellular penetration by the hyphae could be suppressed. The regional secretion of H_2O_2 and cell death were also found from other HR cases, this also accompanies other defense responses such as papillae development and cell wall alteration (Bestwick et al., 1997; Thordal-Christensen et al., 1997). Moreover, the co-existence of regional cell death and H_2O_2 accumulation was also found from other examples of HR (Xiao et al., 2003; Balint-Kurti, 2019); Nováková et al. (2015) also suggested the potential function of H_2O_2 in restricting *L. maculans* development in *B. napus*. The findings from DAB and cell death assays revealed that the two types of incompatible interaction, intermediate (Surpass400–H75 8-1) and resistant (Surpass400–H77 7-2 and 01-23-2-1–H75 8-1/H77 7-2) were able to induce intense early (5 dpi) H_2O_2 accumulation and cell death as the priming defense to achieve effective defense against fungal proliferation on the plant tissues.

ROS generation and signaling play versatile roles in stress tolerance in the plant body. The superoxide (O_2^-) molecules are initially produced by NADPH oxidases or respiratory burst oxidase homologues (RBOH's) and converted into hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD's) (Wojtaszek, 1997; Quan et al., 2008). The stable and membrane-permeable properties make H_2O_2 molecules able to induce systemic responses against various biotic and abiotic stresses (Baxter et al., 2013). RBOHD and F are two the NADPH oxidases inducing ROS accumulation during plant defense response (Torres et al., 2002; Morales et al., 2016). RBOHD and F are the two NADPH oxidases that have been well studied in *Arabidopsis thaliana* defense (Torres et al., 2002; Torres and Dangl, 2005; Pogány et al., 2009; Morales et al., 2016). Calcium leakage, reactive oxygen intermediates (ROI) and peroxide were reduced in *rbohD*, *rbohF*, and *rbohD/rbohF* double mutants (Torres et al., 2002). RBOHs are regarded as the central factors to trigger ROS signaling in plant cells (Lamb and Dixon, 1997; Pogány et al., 2009; Yoshioka et al., 2016). RBOHD initiates the cell-to-cell ROS signaling which is called "ROS wave", by transmitting H_2O_2 extracellularly. Evidence suggested that RBOHD was involved in early acute ROS signaling in defense and tolerance against various challenges (Baxter et al., 2013; Yoshioka et al., 2016), and RBOHD plays important roles in ROS production when the host recognizes the pathogen successfully on the site of infection (Morales et al., 2016). *RBOHD* and *F* working together can fully activate basal resistance, the mutation of both genes abolishes ROS production and makes it easier for pathogens to infect (Torres et al., 2002; Morales et al., 2016). As shown in **Figure 4.4**, the early expression of

RBOHD from Surpass400 and 01-23-2-1 implied that this gene might also play crucial roles in *B. napus* such as in Arabidopsis during pathogenic infection. There are also some differences between the onset pattern between *RBOHD* and *F*, as such, *RBOHF* did not show high expression at 5 dpi in Surpass400, and 01-23-2-1 cotyledons were found to have the most pronounced up-regulation at 5 dpi while for *RBOHD*, the gene was up-regulated earlier at 1 dpi. These two genes were found to be regulated differently in *Arabidopsis thaliana*, and *RBOHD* plays more dominant roles in activation against pathogenic invasion (Morales et al., 2016). It is also noted that the Westar genotype also displayed high expressions of both *RBOH* genes during necrotrophic stage (11 dpi). When infected, both compatible and incompatible interactions can trigger an oxidative burst. Therefore, it is normal to see massive a regulation of ROS-related genes when the plant tissue is heavily infected, however, the timing of the coordination of various regulators seems to be more important. By analyzing host-cell-wall-degrading enzymes (CWDEs) from the pathogen, Sexton et al. (2000) implied that early restriction of fungal development is a crucial factor for *B. napus* cotyledon to achieve resistance towards *L. maculans*.

ROS signaling plays important roles in lesion development and cell senescence on plants, on the other hand, other factors such as JA signaling at the same time, can also attenuate to prevent the excessive damage by ROS (Overmyer et al., 2000; Rao et al., 2002). Moreover, Becker et al. (2017) also listed various types of genes which were activated from resistant *B. napus* genotype at 3 dpi. They include the factors in pathogen perception, callose deposition, sulfur metabolism, and lignification, whereas at the same time point, genes related to the negative regulation of plant defense and senescence were also activated. It seems that the resistant genotypes trigger massive signals from both up-regulation and down-regulation sides of defense at an early stage of infection, which hinders the fungal development during hyphal stage. On the other hand, during late stage (necrotrophic), the pathogen colonizes too widely. Thus, it is impossible for the host to achieve effective resistance, and the defense signals including ROS tend to express in a large amount to halt the further development, which produced such high levels of *RBOH* genes at 11 dpi in Westar.

Therefore, the high amount of the fungal cells pushed the host to evoke more defense signals to cope with the pressure of self-defense, similar to the cases in susceptible Westar and

intermediate Surpass400–H75 8-1 after 7 dpi. Surpass400–H75 8-1, as the intermediate interaction, displayed both resistant and susceptible traits, as the samples taken from the inoculation pair both had the anticipated activation of *RBOHD* expression and H_2O_2 (same as resistant interaction), and the late induction of electrolyte leakage and *EDS1* expression (same as susceptible interaction).

On the other hand, the absence of co-expression between *PAD4* and *EDS1* was not expected. According to previous studies, the expression of *PAD4* is dependent upon *EDS1*, the interaction between EDS1 and PAD4 seems to enhance the HR by further SA accumulation (Feys et al., 2001; Cui et al., 2016). In protein level, EDS1 and PAD4 interact each other, and trigger *R* gene-related resistance (Feys et al., 2001; Rietz et al., 2011; Cui et al., 2016; Bhandari et al., 2019). However, the function of *EDS1* is not totally dependent on *PAD4*, EDS1 is also able to dimerize itself (i.e., EDS1–EDS1 interaction) or bind with another PCD factor SENESCENCE ASSOCIATED GENE 101 (SAG101), moreover, those types of interaction contribute to innate immunity (Feys et al., 2001; Feys et al., 2005; Rietz et al., 2011). EDS1 itself also triggers partial *R* gene-related defense and SA accumulation (Feys et al., 2001; Cui et al., 2016). Therefore, in this study, *EDS1* is highly expressed in defense response alone, without the cooperation with *PAD4*.

Finally, yet importantly, there was no strong trend of the early activation of *MPK3/6* from Surpass400 and 01-23-2-1. Besides gene expression, the function of MAPK factors is also related to phosphorylation, which activates downstream defense factors (Pitzschke et al., 2009; Bigeard and Hirst, 2018). It is necessary to postulate that early ROS activation in *B. napus* might promote more on phosphorylation than expression.

4.6 Conclusions

The data from this chapter revealed that ROS metabolism and signaling played pivitol roles in the host-microbe interaction in the *B. napus–L. maculans* pathosystem. Intermediate and resistant genotypes displayed intense hydrogen peroxide (H_2O_2) diffusion and cell death around the site of inoculation. Moreover, ROS/PCD-responsive genes tended to express earlier in the intermediate and incompatible interactions. Those findings suggested that earlier

activation of ROS-related defense mechanisms is an essential component of effective resistance in *B. napus* against *L. maculans*.

Author Contributions: Conceptualization, C.Y. and W.G.D.F.; methodology, C.Y.; validation, C.Y. and W.G.D.F.; formal analysis, C.Y.; investigation, C.Y.; resources, W.G.D.F.; data curation, C.Y.; writing—original draft preparation, C.Y.; writing—review and editing, C.Y. and W.G.D.F.; supervision, W.G.D.F.; project administration, W.G.D.F.; funding acquisition, W.G.D.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by NSERC (Discovery) and NSERC-CRD.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

Acknowledgments: The authors wish to thank a NSERC Discovery grant and a NSERC-CRD grant awarded to W.G.D. Fernando to carry out this work. We acknowledge the assistance of the Plant Science greenhouse staff in assisting with watering and maintaining our experiments.

Conflicts of Interest: The authors declare no conflict of interest.

Published in International Journal of Molecular Sciences: Cunchun Yang and W. G. D. Fernando 2021. Analysis of the Oxidative Burst and Its Relevant Signaling Pathways in *Leptosphaeria maculans—Brassica napus* Pathosystem. Intl. J. Mol. Sci. 22: 4812. doi: /10.3390/ijms22094812 (IF=5.923)

Previous studies have shown that the switching of enviormental conditions altered the expression of some defense – related genes such as *PR1*, and *BON1* (Malamy et al., 1992; Jambunathan et al., 2001; Jambunathan and McNellis, 2003; Yang et al., 2006). Testing the effects of HR in different temperature condition will enable to predict the performance of *B. napus* defense against *L. maculans* in various incubating conditions or fields.

CHAPTER 5

The Effect of Temperature on the Hypersensitive Response (HR) in the *Brassica napus*–*Leptosphaeria maculans* Pathosystem

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5.1 Abstract

Temperature is considered one of the crucial environmental elements in plant pathological interactions, and previous studies have indicated that there is a relationship between temperature change and host–pathogen interactions. The objective of this research is to investigate the link between temperature and the incompatible interactions of the host and pathogen. In this study, two *Leptosphaeria maculans* isolates (HCRT75 8-1 and HCRT77 7-2) and two *Brassica napus* genotypes (Surpass400 and 01-23-2-1) were selected. The selected *B. napus* genotypes displayed intermediate and resistant phenotypes. The inoculated seedlings were tested under three temperature conditions: 16 °C/10 °C, 22 °C/16 °C and 28 °C/22 °C (day/night: 16 h/8 h). Lesion measurements demonstrated that the necrotic lesions from the 28 °C/22 °C treatment were enlarged compared with the other two temperature treatments (i.e., 16

°C/10 °C and 22 °C/16 °C). The results of expression analysis indicated that the three temperature treatments displayed distinct differences in two marker genes (*PATHOGENESIS–RELATED (PR) 1* and *2*) for plant defense and one temperature-sensitive gene *BONZAI 1* (*BON1*). Additionally, seven dpi at 22 °C/16 °C appeared to be the optimal pre-condition for the induction of *PR1* and *2*. These findings suggest that *B. napus* responds to temperature changes when infected with *L. maculans*.

Keywords: *Brassica napus*; *Leptosphaeria maculans*; gene-for-gene interaction; temperature; *BONZAI1 (BON1)*; pathogenesis-related protein (PR)

5.2 Introduction

Plants develop sets of mechanisms to combat the threat from phytopathogens. Plants secrete a set of metabolites, proteins and gene factors after the triggering of defense responses. According to two studies on the *Arabidopsis thaliana–Pseudomonas syringae* pv. tomato DC3000, the elicitation of innate immunity induces salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) responsive genes (Martin-Rivilla et al., 2020a; Martin-Rivilla et al., 2020b) *Brassica napus* was also found to express hormone/ROS-related signals when coping with the fungal pathogen *Leptosphaeria maculans* (Sašek et al., 2012; Lowe et al., 2014; Becker et al., 2017).

Plant disease epidemics are affected by various environmental factors, including the temperature, humidity and wind, and these factors can be crucial elements influencing the development of disease in nature. Among these factors, temperature is an important element which influences both hosts and pathogens. Moreover, there is molecular evidence suggesting that these organisms (i.e., hosts and pathogens) have developed various adaptive genetic backgrounds to interact with changes in temperature by evolution; as such, certain genes related to infection/defense can be regulated to change the general physiology of an organism when temperatures reach specific levels.

In nature, there is plenty of evidence indicating that a change in temperature alters disease epidemics by changing the infectivity (from pathogens) and defense (from hosts). For example, the rust pathogen *Puccinia striiformis* is less effective when the temperature increases (starting

at 15.4 °C), and the pathogen is unable to infect seedlings of wheat when the temperature is over 21 °C. Shayka et al. (2015), by studying potato late blight, suggested that an oscillation of temperature as low as 5 °C was able to increase the infection efficiency, lesion growth development and sporulation. Iglesias et al. (2010), found the maximal spore concentration of *Phytophthora infestans* within a temperature range of from 16 to 23 °C in Spain, and the optimal temperature for oomycete formation was 21 °C. In the *Brassica napus–Leptosphaeria maculans* pathosystem, an alteration in the temperature could also change the interaction between the host and fungus. One study based on the Effector–Triggered Immunity (ETI) between *Arabidopsis thaliana* and *Pseudomonas syringae* revealed that the hypersensitive response (HR) was suppressed at an elevated temperature of 28–30 °C compared with the ambient temperature (21–24 °C) (Menna et al., 2015). Based on the *B. napus–L. maculans* pathosystem, Huang et al. (2006), suggested that the fungus could grow faster and become more aggressive with an increased incubation temperature, and this happened in the case of both compatible and incompatible interactions. Studies have shown the connection between temperature and the expression of plant defense signaling pathways. A study regarding tobacco resistance against Tobacco Mosaic Virus (TMV) in 1992 (Malamy et al., 1992) suggested that, at an elevated temperature (32 °C), tobacco compromised its salicylic acid (SA)-related resistance with a reduction in the *PR1* gene expression. Compared with a lower temperature (22 °C), both free SA and conjugated SA were increased and *PR1* expression was induced, which was able to cause necrotic lesions (Malamy et al., 1992). In *Arabidopsis*, the gene *BONZAI1* (*BON1*) has been found to modulate the plant defense in a temperature-sensitive manner. The *BON1* gene is part of the *COPINE* gene family, which supports plant growth and development, and negatively regulates plant defense and programmed cell death (PCD) (Hua et al., 2001; Jambunathan et al., 2001; Jambunathan and McNellis, 2003; Liu et al., 2005). The family of *BON* genes consists of three homologs: *BON1*, *2* and *3*. *BON1* plays a major role while the other two *BON* genes are more redundant. Moreover, the triple mutant of all three *BON* genes (*bon1bon2bon3*) of *Arabidopsis* also displays difficulty in germination (Hua et al., 2001). Studies have suggested that *BON1* positively regulates plant growth at lower temperatures, and the mutation of *BON* genes induces excessive cell death at 22 °C (Hua et al., 2001; Yang et al., 2006; Li et al., 2009). *BON1* also represses an *R* gene named *SNC1* (*suppressor of NPR1, constitutive 1*), and the *BON1* mutant *bon1–1* induces constitutive

resistant responses (Yang and Hua, 2004; Li et al., 2007). In the field, the temperature may change in each growing season and the strength of host resistance may be affected because of changes in the climate (Chellappan et al., 2005). By considering all of the information mentioned above, it is possible to conduct a set of experiments to detect the effects of temperature on the defense of *B. napus* against the blackleg pathogen.

As mentioned above, the effects of temperature upon plant defense have been studied, however, their effect on resistance in *B. napus* and defense under different temperature conditions have not been reported. In this study, a set of experiments were conducted to explore the effects of temperature upon hypersensitive resistance against *L. maculans* races on *B. napus* genotypes at the seedling stage. By analyzing the inoculated *B. napus* seedlings at three different growing temperatures, distinct patterns of fungal development were observed and molecular evidence suggested that the intrinsic signaling also responded differently at those temperatures.

5.3 Materials and Methods

5.3.1 Plant Cultivation and Temperature Treatments

Two *Brassica napus* genotypes (Surpass400 (*BLMR1/LepR3* and *BLMR2/RlmS*) and 01-23-2-1 (*Rlm7*)) were grown under three temperature conditions: 16 °C/10 °C (day/night: 16 h/8 h); 22 °C/16 °C (day/night: 16 h/8 h); 28 °C/22 °C (day/night: 16 h/8 h). All flats with seedlings were grown under 22 °C/16 °C (day/night: 16 h/8 h) first, and the seedlings to be tested under the other two conditions were moved into the growth cabinets 24 h before inoculation.

5.3.2 Pathogen Inoculation

Two *L. maculans* isolates were selected for inoculation: HCRT75 8-1 (Genotype: *avrLm1, AvrLm2, avrLm3, avrLm4, AvrLmJ1, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1* and *AvrLepR2*) and HCRT77 7-2 (Genotype: *AvrLm1, avrLm2, avrLm3, AvrLm4, AvrLmJ1, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1* and *avrLepR2*).

The cotyledons of *B. napus* cultivars were inoculated seven days after sowing (cotyledon stage) by puncture inoculation. Each lobe of cotyledons was punctured by a sterile needle twice from each side, to have four inoculation points on each seedling of the canola plant.

5.3.3 Lesion Measurement

The cotyledons at 11 days post-inoculation (dpi) were scanned and the lesion size was measured by ImageJ (National Institutes of Health, Bethesda, MD, USA).

5.3.4 Gene Expression Analysis

Frozen cotyledons (7 and 11 dpi) were ground in liquid nitrogen with pestles and mortars. The total RNA was extracted with TRI reagent (Sigma-Aldrich) (St. Louis, MO 63,103 USA). According to the manual, the total RNA was purified by DNaseI treatment with a recombinant DNaseI, RNase-free kit (Roche). Purified RNA was used to synthesize cDNA by employing the GOScript Reverse Transcription System (Promega). The cDNA stock solution was diluted to 100 ng/µL. Quantitative-PCR was performed by loading 1 µL of cDNA (100 ng) into the 10 µL reaction system of the IQTM SYBR® Green Supermix (BioRad, Hercules, CA, USA). The experiments were based on three biological replicates (4 cotyledons, two seedlings, as one biological replicate). The RT-qPCR experiments were run by Touch Real-Time PCR System (BioRad).

The qPCR program used for all of the analysed genes (except for *COI1* and *ACO1*) was 95 °C for 3 min, followed by 39 cycles of 95 °C for 15 s and 60 °C for 20 s. This was followed by a melting curve analysis.

All qPCR primers are compiled in **Appendix I**. The relative level of gene expression was analysed with the 2-ΔΔCT method described by Livak and Schmittgen, (2001). Actin was used as a reference gene to normalize the expression of the target genes.

5.3.5 Statistical Analysis

Unless specified, the analyses of the samples used at least three biological replicates. The statistical analyses were performed using the Tukey ANOVA method with SAS 9.4 software.

5.4 Results

By measuring the lesions on the cotyledons after three temperature treatments, both Surpass400–H75 8-1/H77 7-2 and 01-23-2-1–H75 8-1/H77 7-2 exhibited phenotypes of incompatible interactions; as such, brownish necrotic lesions formed around the sites of inoculation (**Figure 5.1**). The *L. maculans* isolate HCRT75 8-1 (Genotype: *avrLm1, AvrLm2, avrLm3, avrLm4, AvrLmJ1-5, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1* and *AvrLepR2*) induced incompatible interaction upon Surpass400 (*BLMR1/LepR3 and BLMR2/LepR2*) by AvrLepR2-BLMR2/LepR2 interaction, and upon 01-23-2-1 (*Rlm7*) by AvrLm4-7–Rlm7 interaction, respectively. The *L. maculans* isolate HCRT77 7-2 (Genotype: *AvrLm1*, *avrLm2, avrLm3, AvrLm4, AvrLmJ1-5, AvrLm7, AvrLm6, avrLm9, AvrLm11, avrLepR1* and *avrLepR2*) induced incompatible interaction upon Surpass400 (*BLMR1/LepR3 and BLMR2/LepR2*) by AvrLm1–BLMR1/LepR3 and upon 01-23-2-1 (*Rlm7*) by AvrLm4-7–Rlm7 interaction, respectively (Larkan et al., 2013; Dandena et al., 2019; Neik et al., 2020). Generally, the results indicated that a higher temperature caused larger lesion sizes and, at 28 °C/22 °C, the lesions were the largest compared with the other two temperature treatments (**Figure 5.2**). Surpass400–H75 8-1, as the inoculation combination showing an intermediate phenotype (22 °C/16 °C), displayed an apparent increase in lesion development when responding to an increasing temperature. The other three cases, which were usually identified as resistant interactions (i.e., Surpass400–H77 7-2 and 01-23-2-1–H75 8-1/H77 7-2), displayed relatively mild increases in lesion development as the temperature became higher.

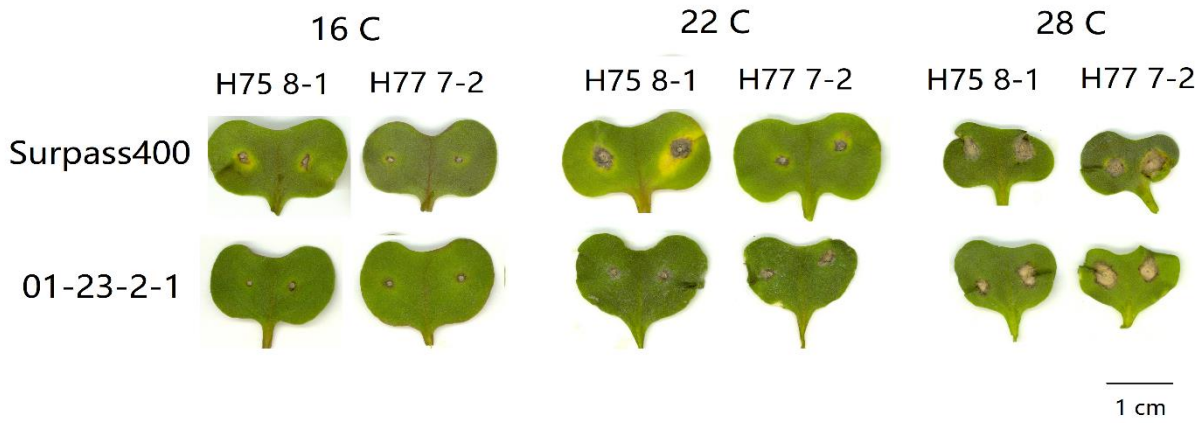

Figure 5.1. Lesion development from six pairs of *Brassica napus* cotyledon–*Leptosphaeria maculans* isolate inoculation: Surpass400–HCRT75 8-1/HCRT77 7-2 and 01-23-2-1–HCRT75 8-1/HCRT 77 7-2 at 11 days post-inoculation (dpi) with three temperature treatments: 16 °C/10 °C, 22 °C/16 °C, and 28 °C/22 °C (day/night: 16 h/8 h). Bar = 1cm.

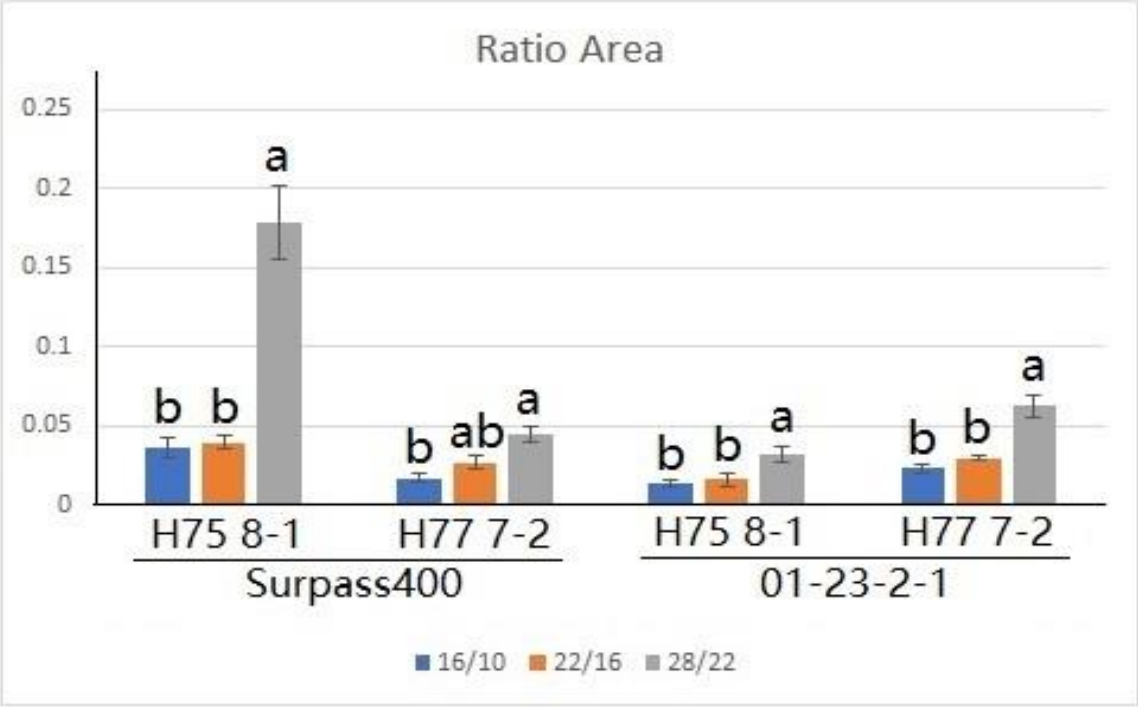

Figure 5.2. The extent of blackleg lesion development at 11 dpi from Surpass400 and 01-23-2-1 inoculated by the isolates HCRT75 8-1 and HCRT77 7-2. The inoculated plants were exposed to three temperature treatments: 16 °C/10 °C, 22 °C/16 °C and 28 °C/22 °C

(day/night: 16 h/8 h). The lesion development was calculated by the ratio between the area of the lesion and the area of the cotyledon. Error bars represent standard error of the mean. Different lowercase letters suggest the significant differences among mean values (Fisher's Least Significant Difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

Studies have suggested that *BON* genes are related to the growth/defense balance at a low temperature (22 °C), and BON1 has been found to play a dominant role in repressing the plant defense (Jambunathan et al., 2001; Yang et al., 2006). As can be seen by the expression of *BON1* in **Figure 5.3**, *BON1* was more pronounced at the 22 °C/16 °C condition compared with the other two conditions at 7 dpi. Similar to at 7 dpi, *BON1* was still induced at 11 dpi when the plant was treated with the 28 °C/22 °C condition (**Figure 5.4**). Moreover, Surpass400–H75 8-1/H77 7-2 maintained a pronounced up-regulation in all three conditions at 11 dpi, while 01-23-2-1–H75 8-1 appeared to have higher levels of *BON1* at 16 °C/10 °C than at 22 °C/16 °C. Additionally, the gene also exhibited a high level of induction at 28 °C/22 °C.

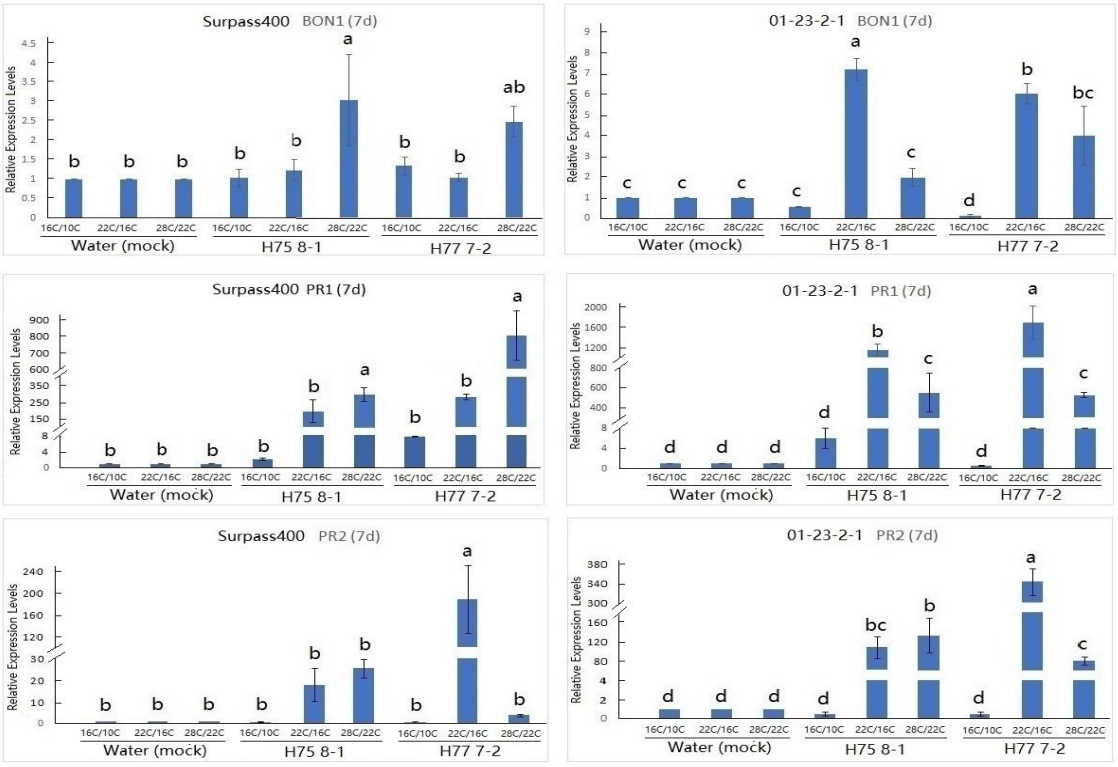

Figure 5.3. Gene expression of temperature-dependent regulator *BONZAI1 (BON1)*, *PATHOGENESIS-RELATED* (*PR)1* and *PATHOGENESIS-RELATED* (*PR)2* (in *B. napus*) in the regulation of hormonal signals at 7 dpi from Surpass400 and 01-23-2-1 inoculated by the blackleg isolates HCRT75 8-1 and HCRT77 7-2. The inoculated plants were exposed to three temperature treatments: 16 °C/10 °C, 22 °C/16 °C and 28 °C/22 °C (day/night: 16 h/8 h). The levels of the bars are the expression levels obtained from the inoculated cotyledons (inoculated by H75 8-1 and H77 7-2) compared to the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). Error bars represent standard error of the mean. Different lowercase letters suggest the significant differences among mean values (Fisher's Least Significant Difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

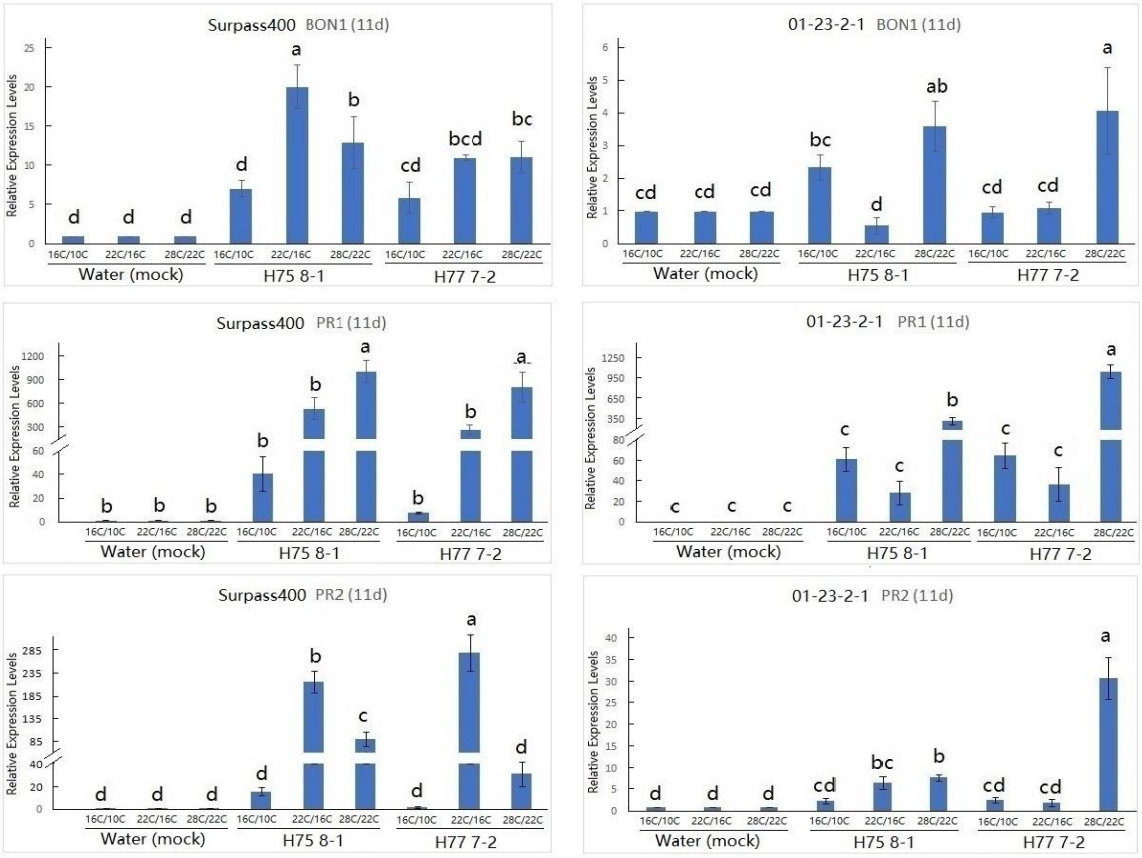

Figure 5.4. Gene expression of *BONZAI1 (BON1)*, *PATHOGENESIS-RELATED* (*PR)1* and *PATHOGENESIS-RELATED* (*PR)2* (in *B. napus*) in the regulation of hormonal

signals at 11 dpi from Surpass400 and 01-23-2-1 inoculated by the blackleg isolates HCRT75 8-1 and HCRT77 7-2. The inoculated plants were exposed to three temperature treatments: 16 °C/10 °C, 22 °C/16 °C and 28 °C/22 °C (day/night: 16 h/8 h). The levels of the bars are the expression levels obtained from the inoculated cotyledons (inoculated by H75 8-1 and H77 7-2) compared to the cotyledons inoculated with water (assuming that the expression of each studied gene in the cotyledons inoculated with water is 1). Error bars represent standard error of the mean. Different lowercase letters suggest the significant differences among mean values (Fisher's Least Significant Difference; $p < 0.05$). The results are based on three replicates in three independent experiments.

According to previous studies on *Arabidopsis*, the up-regulation of the *PR1* gene was observed in the *bon1* mutants at the lower temperature (22 °C), suggesting that this gene might have the function of defense suppression in some conditions, such as temperature (Stintzi et al., 1993). Looking at the *PR1* expression in **Figures 5.3** and **4**, the genotype 01-23-2-1 exhibited the strongest induction at 7 dpi when the temperature was 22 °C/16 °C. On the other hand, for Surpass400, the optimal circumstance for the highest induction of *PR1* appeared to be at 11 dpi, at 28 °C/22 °C. It seemed that *PR1* expression in 01-23-2-1 synchronized with the expression of *BON1*, in which the trends of up- and down-regulation between these two genes were similar (both 7 and 11 dpi). On the other hand, *PR1* and *BON1* generally exhibited an antagonistic relationship in Surpass400, which matched with previous studies in *Arabidopsis*.

Another *PR* gene tested was *PR2*. *PR2* encodes an enzyme called beta-1, 3 glucanase 2. It is SA-responsive and functions as a regulator of sugar metabolism and fungal cell wall degradation (Stintzi et al., 1993; Thibaud et al., 2004; Borad and Sriram, 2008). According to **Figures 5.3 and 4**, thermal fluctuation did not seem to have apparent effects on *PR2* expression. Except for Surpass400–H77 7-2 (at 7 dpi) and 01-23-2-1 – H77 7-2 (11 dpi), *PR2* was highly induced at 22 °C/16 °C, suggesting that, unlike *PR1*, *PR2* expression was only induced at 22 °C/16 °C. It seemed that *PR2* had no apparent connection with *BON1*, but it had generally lower expression in the 28 °C/22 °C condition, which indicated that *PR2* expression might be affected by other factors besides temperature and/or *BON1* regulation.

5.5 Discussion

Our experiments suggested that the HR lesions were increased at the higher temperature treatment (28 °C/22 °C). The expression profiles of *BON1*, *PR1* and *PR2* were distinct among three temperature conditions. The temperature 22 °C/16 °C is generally optimal for *PR1/2* to express. The *PR1/2* was also induced (higher than water inoculation) when the temperature treatment reached 28 °C/22 °C.

Previous evidence has suggested that a change in temperature is able to alter the general defense in plants. Malamy et al. (1992), observed a reduction in the SA level and *PR1* expression in tobacco leaves (inoculated with tobacco mosaic virus (TMV)) when the temperature was increased to 32 °C from 22 °C; on the other hand, a higher temperature (i.e., 32 °C) enabled the virus to replicate and infect the host. Other signaling regulators, such as *BON* family genes, are involved in temperature-dependent regulation of plant defense.

According to previous studies on *Arabidopsis*, *BONZAI1* (*BON1*) is expressed at a lower temperature (22 °C) to regulate the general plant defense (Hua et al., 2001; Yang et al., 2006). *BON* genes appear to regulate SA-related signals, such as *PR1*, and the expression of *BON1* represses SA-related pathogenesis-related proteins (*PR1*, *PR2*, and *PR5*) (Jambunathan and McNellis, 2003; Yang et al., 2006). The results from lesion measurement matched those presented in previous studies of pathogenic development, with an increasing temperature-promoting pathogenic development and suppressing host defense (Malamy et al., 1992; Chellappan et al., 2005; Wang et al., 2009; Velásquez et al., 2018). The lesion size, especially that obtained from the Surpas400–H75 8-1 case, increased significantly at 28 °C/22 °C compared with 16 °C/10 °C and 22 °C/16 °C, whilst the other three cases (Surpass400–H77 7-2 and 01-23-2-1–H75 8-1/H77 7-2) also exhibited moderate increases in the lesion size. The enlargement of the lesion size on those genotypes suggested that the effect of the hypersensitive response (HR) on the suppression of fungal growth was mitigated when the temperature was raised. Previous studies have indicated that the temperature has effects on the strength of Effector–Triggered Immunity (ETI). *SNC1* (*suppressor of NPR1, constitutive 1*), as an *R* gene in *Arabidopsis*, is repressed by *BON1* on its promoter region (Li et al., 2007). *SNC1* has been found to play a role in defense against *Nicotiana benthamiana*, together with the *N* gene (another *R* gene specific for *N. benthamiana*). Moreover, the nuclear accumulation of

SNC1 and *N* genes is reduced when the temperature is elevated (Zhu et al., 2010). *SCN1* is also suppressed by factors other than *BON1* at a higher temperature. In *Arabidopsis*, HOPZ-ETI-DEFICIENT 1 (ZED1) and ZED1-related kinases (ZRKs) suppress *SNC1* expression at a temperature of 25 °C, and the mutation of *ZED1* was shown to activate defense genes *PR1* and *PR2* at 25 °C (Wang et al., 2017).

According to the qPCR results, the genes *BON1*, *PR1* and *PR2* were found to react to the thermal changes by observing the transcriptional analyses; however, some changes did not follow the indicated rules set out in previous studies. One remarkable discrepancy is that both *BON1* and *PR1* displayed a high level of induction at 28 °C/22 °C. Conversely, in previous research, the expression of these genes was shown to be lower than in the other low-temperature conditions.

In the 22 °C/16 °C condition, an antagonistic relationship between *BON1* and *PR1* appeared to be shown. *PR1* displayed a very high expression when the expression of *BON1* was not very high, it was more obvious from Surpass400 (except for 11 dpi, Surpass400 H75 8-1), and this finding matched that of the repression of *BON1* upon defense genes and cell death (Jambunathan et al., 2001; Jambunathan and McNellis, 2003; Yang et al., 2006). In the same condition (22 °C/16 °C, Surpass400), *PR1* and *2* were induced at 7 dpi and repressed in 11 dpi, which synchronized with the lower expression of *BON1* at 7 dpi and its high expression at 11 dpi.

Surprisingly, in the 28 °C/22 °C condition, both *BON1* and *PR1* were induced at 11 dpi. By considering the larger lesion size in this condition for both genotypes, the high expression of *PR1* can be explained as the physiological response towards more severe infectious situations, similar trends also happened to 01-23-2-1 at 22 °C/16 °C. The infected hosts with compatible interactions were found to have a high level of induction of defense-related genes at a later stage of infection compared with cases of incompatible interactions (Tao et al., 2003; Becker et al., 2017). On the other hand, the induction of *BON1* was also observed, which did not display its negative regulation upon *PR1*, as mentioned in previous studies. It is possible that, in a high-temperature growth condition, the resistant *B. napus* genotypes obtain a homeostatic status, where both the activation and repression of defense mechanisms occur. Yang et al. (2006) suggested the balance between defense-related cell death and cell growth. This balance

is mediated by BON proteins, which negatively regulate defense triggering factor, SNC1. SA and ET are found to have a homeostatic interplay when regulating the defense at lower temperature (Li et al., 2020).

There are still many questions that need to be answered in order to explain the relationship between the regulation of plant defense and changing of temperature. This signaling system may be more complicated and depends on different species. In this article, *PR2* did not follow the presumed pattern of expression when changing the temperature, and other factors may affect the expression of downstream proteins like PR proteins. One possible explanation for this unusual situation is the homeostatic regulation between plant growth and defense. A plant body may suppress excessive defensive activities when there are few pathogens present inside. Plants may develop certain mechanisms, such as a guard model, to activate their resistance when a large amount of pathogen inoculum are recognized, since *R* gene-related defense is destructive to plant bodies (McDowell et al., 2006). In addition, ZED1 and ZRKs repress SNC1-triggered defense when there is no pathogen present. Moreover, these genes negatively regulate the SNC1-activated autoimmunity at an elevated temperature (relative to ambient temperature) (Wang et al., 2017). Plant body has developed a complicated network when responding to temperature changes (Menna et al., 2015; Desaint et al., 2020). The alteration of defense signalling according to temperature is not a black-and-white situation. Cheng et al., (2013) suggested that temperature may affect PTI and ETI differently, whereas the initiation of R-protein detection of the pathogen is not affected. All those studies suggested that the switching of intrinsic signalling is able to alter in an unpredictable way due to the multi-faceted signaling pathways.

5.6 Conclusions

Taken together, the evidence displayed in this article suggests that infected genotypes growing at a higher temperature cause **larger lesion sizes on the resistant genotype by shaping the effects of the hypersensitive response.** Expression analysis revealed that, in a higher temperature condition (28 °C/22 °C), both ***BON1*** **and** ***PR1*** were **triggered at 7 and 11 dpi**, and were presumed to have an antagonistic relationship with each other based on previous

studies on *Arabidopsis*. The results indicated that, at a higher temperature, *B. napus* seems to display a balance between the plant defense and growth mechanism, at the same time as exhibiting incompatible interactions, from which the expression of defense-repressing factor *BON1* and defense gene *PR1* coincides during a pathogen attack.

Author Contributions: Conceptualization, C.Y. and W.G.D.F.; methodology, C.Y.; validation, C.Y. and W.G.D.F.; formal analysis, C.Y.; investigation, C.Y.; resources, Z.Z.; data curation, C.Y.; writing—original draft preparation, C.Y.; writing—review and editing, C.Y. and W.G.D.F.; supervision, W.G.D.F.; project administration, W.G.D.F.; funding acquisition, W.G.D.F. All authors have read and agreed to the published version of the manuscript. Z.Z. did data curation for the manuscript.

Funding: This research was funded by NSERC (Discovery) and NSERC-CRD.

Institutional Review Board Statement: Not applicable,

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

Acknowledgments: The authors wish to thank a NSERC Discovery grant and a NSERC-CRD grant awarded to W.G.D. Fernando to carry out this work. We acknowledge the assistance of the Plant Science greenhouse staff in assisting with watering and maintaining our experiments.

Conflicts of Interest: The authors declare no conflict of interest.

Published in: Plants: Cunchun Yang, Zhongwei Zou, Z. and W. G. D. Fernando 2021. The Effect of Temperature on the Hypersensitive Response (HR) in the *Brassica napus*–*Leptosphaeria maculans* Pathosystem. Plants 10: 843.
doi: 10.3390/plants10050843 (IF=3.935)

6 GENERAL DISCUSSION

This thesis provided a deep understanding of molecular aspects under gene-for-gene interaction. The research projects were based on the pathosystem of *Brassica napus* – *Leptosphaeria maculans*. As the Canada's most valuable crop, *B. napus* (canola) has been under great threat from the severe damage caused by fungal pathogen *L. maculans* (blackleg disease). Understanding the molecular basis becomes a useful strategy for future canola breeding against blackleg disease. In this thesis, the first two chapters have explained the connection between hormone/ROS signaling and effective resistance in *B. napus*, and the third chapter discussed the potential link between incubating temperature and expression of host defense.

Plant defense consists of various components which involves multiple signaling pathways and constructs a signaling network against pathogen attack. Previous studies have demonstrated the crucial roles of hormone/ROS responsive factors in plant defense (Bari and Jones, 2009; Zurbriggen et al., 2010; Baxter et al., 2013; Berens et al., 2017). Salicylic acid (SA) is found to play important roles in defense against biotrophic pathogens while Jasmonic Acid (JA) supports the defense against necrotrophic pathogens (Kazan and Manners, 2008; Bari and Jones, 2009; Berens et al., 2017); ROS molecules also activate a set of defense activities such as MAPK cascades, stomatal closure (Quan et al., 2008; Baxter et al., 2013). From this thesis, the induction of hormone/ROS signaling during early stage of infection was observed from more resistant genotypes (intermediate/resistant genotypes, i.e. Surpass400 and 01-23-2-1). From the resistant genotypes, our results demonstrated the emergence of H_2O_2 and cell death as early as 3 and 5 dpi and electrolyte leakage was also detected from the same time points. Moreover, hormone analysis also found the induction of SA storage (i.e. bound SA) at 3 dpi, which implicated intrinsic SA – responsive signaling (Vlot et al., 2009; Berens et al., 2017). Those observations suggested that the priming defense as early as 3 dpi has initiated in the intermediate/resistant genotypes, previous studies have suggested that the priming defense activities *in planta* is one crucial element to induce effective resistance (Sašek et al., 2012; Becker et al., 2017). Transcriptional analysis also revealed the early induction of several defense – related genes from both hormone and ROS – responsive pathways. At 3 and 5 dpi, SA factors such as *ICS1*, *WRKY70* and *NPR1* showed higher expression than mock

controls/Westar samples from Surpass400 and 01-23-2-1 (Surpass400 – H75 8-1 as intermediate; and Surpass400 – H77 7-2 and 01-23-2-1 – H75 8-1/H77 7-2 as resistant). The microscopic morphology of the inoculated Surpass400 and 01-23-2-1 revealed that there was a weak hyphal development at these two time points, suggesting that the resistant genotypes tried to halt the infection during biotrophic stage. Moreover, ROS – related factors for examples, *RBOH-D/F*, which encode two enzymes involved in ROS production (Lamb and Dixon, 1997; Torres et al., 2002; Torres and Dangl, 2005), ROS signaling is found to regulate cell death/lesion formation and supported by SA (Overmyer et al., 2000; Rao et al., 2002). The study upon adult *B. napus* leaves revealed that for susceptible genotypes, *L. maculans* started to spread hyphae at 3 and 5 dpi (Li et al., 2008a), and *L. maculans* starts to secrete enzymes degrading cell wall as early as 3 dpi (Sexton et al., 2000). Therefore, intense hormone/ROS signaling during early biotrophic stage of the fungus could stop the hyphal development from exploiting enough nutrients from the host (Perfect and Green, 2001), thus this strategy slows down the fungal growth and possibly gives longer time period for the host to react to the pathogen attack (Sašek et al., 2012). This theory was also indirectly suggested by the data from the susceptible Westar genotype which activated all the hormone/ROS responsive genes at 11 dpi. This time point already reached the necrotrophic stage on Westar cotyledons. The hyphal development had been immense and the fungus killed the host cells to take in nutrients, this is probably too late for the host to react, since the fungus does not only infect the cotyledons, but also other parts of the plant body, there must be systemic infection during the late stage of the infection.

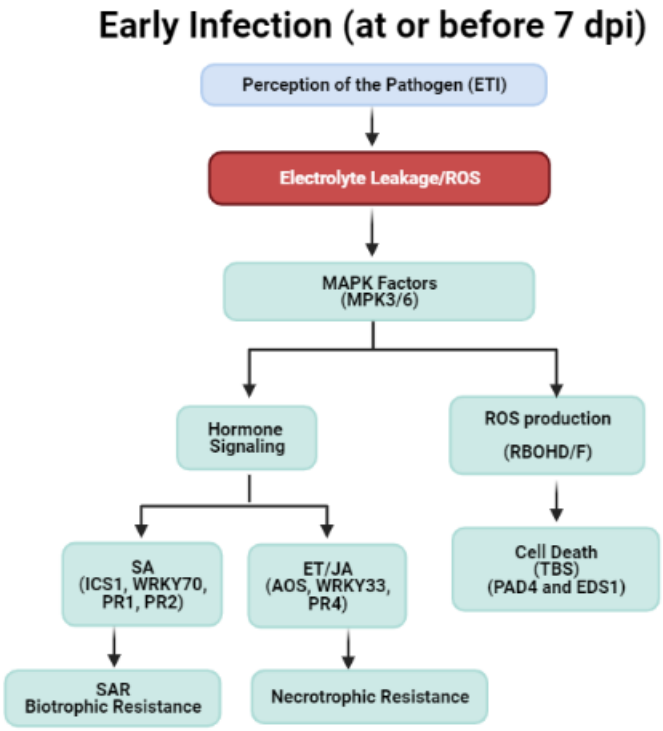

Figure 6.1. General conclusion of my major findings from Chapter 3 and 4. The scheme exhibited the signaling reprogramming of *B napus* with intermediate/resistant responses. The genes indicated in the boxes showed early induction (except MPK3/6) when inoculated by *L. maculans isolates*.

The third research chapter discussed about the potential influence of temperature and the regulation of the resistant signals. In Arabidopsis, *BONZAI1* (*BON1*) regulates the plant defense in a temperature – sensitive manner, the incubated *bon1* mutants in lower temperature (22 ^{o}C) showed excessive defense and cell death (Hua et al., 2001; Yang et al., 2006). A high temperature condition (32 ^{o}C) in tobacco also reduced the SA – responsive defense against tobacco mosaic virus (TMV) (Malamy et al., 1992). Lesion measurement showed that higher temperature caused larger lesion size where the 28 ^{o}C/22 ^{o}C (day/night) condition exhibited more extreme increase. However, the qPCR analysis was different compared with the previous theory of *BON1* regulation and temperature – sensitive defense. In 01-23-2-1 genotype, the *BON1* and *PR1* coincided each other in the aspects of time point and temperature while

Surpass400 seemed to show the antagonistic relationship between these two genes, however, *BON1* and *PR1* in Surpass400 also had higher expression in the high temperature condition (28 ^{o}C/22 ^{o}C, day/night). These results suggested that even influenced by the temperature, *B. napus* may have its own system to adjust the expression of defense. This is different from *A. thaliana*, in the high temperature incubation (Jambunathan et al., 2001; Jambunathan and McNellis, 2003; Wang et al., 2017), the *B. napus* genotypes with a functional gene – for – gene interaction might have a homeostatic and pleotropic signaling network which both defense – supportive and repressive pathways are active at the same time.

This Ph.D. study discussed some of the important internal and external components of plant defense. The research is based on *Brassica napus – Leptosphaeria maculans* pathosystem. The study upon role of hormone/ROS signaling is supported by the previous studies and the findings from this study may also help further research elucidating which genes would be important in regulating *B. napus* defense and whether other environmental factors such as temperature are able to influence the defense activities. In summary, this thesis will help further understanding of host – microbe interactions between *B. napus* and *L. maculans* at the molecular level.

7 FUTURE DIRECTIONS

Plant defense consists of multiple defense pathways, which may also modulate each other. The analysis of the defense genes in this study only scratched the surface, in future, more genes need to be analyzed, possibly we can also clone some of the defense genes and perform genetic transformation to test whether the alteration of *B. napus* genetic background could improve/weaken its combat against *L. maculans*. Moreover, the studies upon microarray and RNA sequencing also revealed that the fundamental difference between compatible and incompatible interaction, is the dynamic expression of certain defense genes at certain time points of infection. Further, the host performing incompatible interaction is able to induce some essential defense genes during early periods following pathogen attack, due to the detection of the pathogen (Tao et al., 2003; Becker et al., 2017). Therefore, transcriptional analysis upon *B. napus* defense can focus more on early time points following *L. maculans* inoculation, the selected time points can be within hours after inoculation.

Finally yet importantly, environmental factors will be another element to consider when testing the expression of plant defense. This will give more input for breeding of resistant canola cultivars in fields since the climate and/or human activities may have positive/negative effects upon the potential of *B. napus* resistance against the blackleg fungus. The third chapter of this thesis has indicated the potential effects of temperature upon the expression of incompatible interactions. Other factors like humidity, light can also be taken into account in future studies on this important host-pathogen interaction.

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APPENDICES

Appendix I qPCR primers used in this study

Name	Sequence 5' to 3'	
Actin-F	CCTCAGCACTTTCCAACAGA	NM_001316010.1
Actin-R	CCAGAAGGCAGAAACACTTAGA	
WRKY70-F	AGCAAGTGCAGAAGCTAGAG	FJ384113.1
WRKY70-R	GCTTGATCTTGGGTGCTACT	
WRKY33-F	GTGAAACAAACGGTGGGAATG	KF712488.1
WRKY33-R	CATCTGTAACCGTCGTCAAGAA	
NPR1-F	GCAGAGGTTCATGGAGATACAG	EF613226.1
NPR1-R	TTGGATGTGGAAGAAGAGGAAG	
EIN3-F	GAGTTCCTGAAGATGGACAGAAG	XM_013839374.2
EIN3-R	TGACCATAGAAGTTTGGC	
ICS1-F	CTTCCAGCTACAATCCCTGTC	XM_013827053.2
ICS1-R	TGGCGAGGAGAGTGAATTTG	
AOS-F	TGCAACAACCTCTCTCCTTC	XM_013888091.2
AOS-R	GATATCAACCGCTTGCGACTA	
ACO1-F	TGAGAGGGCTGAGAGAACATA	XM_013804747.2
ACO1-R	TCAAGAACTCAAGACCAGGAAC	
PR1-F	AAGCTCAAGACAGTCCACAAG	U70666.1
PR1-R	TAGTCGGTCGGCGTAGTT	
PR2-F	CGTCTCTCTACAATTCGCTCTG	XM_013886724.2
PR2-R	GAGATTGGCGTCGAAGAAGT	
PR4-F	TACGGTTGGACAGCTTTCTG	XM_013880328.2
PR4-R	GCGCTTGAGTCCCAGTATTT	
PAD4-F	GCATTAGCCGTGGAATCTCT	XM_013881638.2
PAD4-R	TTGGAGTTCCTGTTGAGTTGAG	
EDS1-F	GGAGGTGCTACTGCAATCTTAG	XM_013849617.2
EDS1-R	GGAGCTCCAAACGTCATACAA	
RbohD-F	GCCTATGACGTGATGGGTTAT	XM_013788801.2
RbohD-R	CGACAAACAGGCAGCAATATG	
RbohF-F	AGCCGATGAAACAACAAAGAAA	MF039316.1
RbohF-R	CAATGCCAAGACCAACCAATAA	
MPK3-F	TGGTTCCTCCACCACTTAGA	KU363194.1

MPK3-R	**CCCTGGTTGGATCTGATGATTT**	
MPK6-F	**CATAATACCACCACCGCTAAGA**	**XM_022699164.1**
MPK6-R	**GCCTTGGTTAGAGCGTATGA**	
BON1-F	**CAATGGCAAACACAGTCTCATC**	**XM_013787352.2**
BON1-R	**CTTTGTCCAGCACCAGTAGTAG**	

Appendix II List of abbreviations

ABA, Abscisic Acid

AOS, Allene Oxide Synthase

ACC, 1-Amino-Cyclopropane-1-Carboxylate

ACO1, ACC OXIDASE 1

BON1, BONZAI 1

BR, Brassinosteroid

DAB, 3, 3’ - Diaminobenzidine

DAMP, Damage-Associated Molecular Pattern

DPI, Days Post-Inoculation

EIN3, ETHYLENE INSENSITIVE 3

EDS, ENHANCED DISEASE SUSCEPTIBILITY 1

ET, Ethylene

ETD, Effector-Triggered Defense

ETI, Effector-Triggered Immunity

FLS2, FLAGELLIN SENSITIVE2 (FLS2)

HR, Hypersensitive Response

ICS1, Isochorismate Synthase

JA, Jasmonic Acid

MAMP, Microbe-Associated Molecular Pattern

MAPK, Mitogen-Activated Protein Kinase

NPR1, NON-EXPRESSOR of PR1 Genes

PAD4, PHYTOALEXIN DEFICIENT 4

PAMP, Pathogen-Associated Molecular Pattern

PCD, Programmed Cell Death

PCR, Polymerase chain reaction

PR, Pathogenesis-Related Protein

PTI, PAMP-triggered immunity

QTLs, Quantitative Traits Loci

RBOH, Respiratory Burst Oxidase Protein

ROS, Reactive Oxygen Species

SA, Salicylic Acid

SAG, Salicylic Acid Glucoside

SAR, Systemic Acquired Resistance

TBS, Trypan blue staining

TIR, Toll-Interleukin-1 Receptor