Exploring Disease Resistance in the *Brassica napus* - *Leptosphaeria maculans* Pathosystem

by

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ABSTRACT

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Blackleg disease, caused by the ascomycete fungal pathogen *Leptosphaeria maculans*, is a devastating disease of canola (*Brassica napus*) in Australia, Canada and Europe. The pathogen is considered a global invasive species and poses a threat to canola production in China, where only the weakly aggressive strain *L. biglobosa* is present. In Canada, breakdown of blackleg resistance has been shown. In order to develop a more effective disease management strategy, there is a need to elucidate host resistance and defense mechanisms underlying the *B. napus-L. maculans* pathosystem. This is the very first study to investigate major resistance genes (*R* genes) and adult plant resistance (APR) in Canadian canola germplasm. This study also analyzed the avirulence allele frequency in *L. maculans* populations in western Canada. *R* genes were detected in the majority of these *B. napus* germplasm, with the *Rlm3* gene being predominant. The frequency of *AvrLm3* allele in field fungal populations was extremely low. APR was identified in more than 50% of the germplasm. This indicated the breakdown of *Rlm3* resistance, which could be due to the widespread use of this single resistance gene in Canadian *B. napus* germplasm and varieties. To address concerns of introducing *L. maculans* from Canada into China, this study further characterized *R* genes and APR to *L. maculans* in a collection of Chinese *B. napus* germplasm. *R* genes were detected in more than 40% of the germplasm tested, with *Rlm3* and *Rlm4* being predominant. A large portion of the Chinese germplasm showed high to moderate

APR in field trials at three locations in MB, SK and AB in western Canada. This study highlighted the availability of fair to good resistance in the Chinese *B. napus* germplasm against blackleg disease and was the first study to investigate a large number of Chinese germplasm against Canadian fungal populations in different environments. RNA sequencing of resistant and susceptible host tissues and a streamlined bioinformatics pipeline identified unique genes and plant defense pathways specific to plant resistance in the *B. napus-L. maculans LepR1-AvrLepR1* interaction. The sequencing data coupled with functional characterization of some unique genes, in depth histological analysis, and in situ gene activity analysis directly at the site of infection provide unprecedented spatial and temporal resolution of the plant defense response to *L. maculans*.

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FOREWORD

This thesis follows the manuscript style outlined by Department of Plant Science and Faculty of Graduate Studies at the University of Manitoba. This thesis started with a general introduction, followed by literature review, three manuscripts, a general discussion and future directions, and references. The manuscripts follow the style recommended by European Journal of Plant Pathology, and contains abstract, introduction, materials and methods, results and discussion. The first manuscript has been published in European Journal of Plant Pathology, the second manuscript has been accepted by Plant Pathology and the third manuscript has been submitted to The Plant Journal for publication.

1 GENERAL INTRODUCTION

Brassica napus (canola, oilseed rape) is an economically important oilseed crop cultivated worldwide (Snowdon et al. 2007; Hayward 2012). Canada is one of the world's leading producers and exporters of canola seeds. Canola is not only widely used as a major source of vegetable oil, but also applied for a broad range of industrial purposes (Hayward 2012). However, the production of canola is hindered by many diseases including blackleg, sclerotinia stem rot, and club root (Li and McVetty 2013).

Blackleg, caused by the ascomycete fungal species *Leptosphaeria maculans*, is one of the major diseases in many *B. napus* growing regions including Canada, Australia, and Europe, excluding China (Fitt et al. 2006). This disease can be controlled by crop rotation, utilization of resistant varieties, and fungicide application (Fernando et al. 2007; Kutcher et al. 2011). The use of resistant varieties has been proven to be the most effective and environmentally friendly strategy to control the disease (Raman et al. 2013; Li and McVetty 2013). However, field populations of *L. maculans* display a high evolutionary potential and are able to overcome major resistance genes within a few years. For example, *Rlm1*-carrying varieties became ineffective in France only a few years after commercial release (Rouxel et al. 2003a), and *LepR3* resistance was broken down in Australia within 3 years (Sprague et al. 2006a, b; Van de Wouw et al. 2010).

Brassica species fight against the infection of *L. maculans* by activating a set of defense pathways (Staal et al. 2006; Kaliff et al. 2007; Šašek, et al. 2012; Lowe et al. 2014). Genetic studies identified that there are two types of resistance against *L. maculans* infection:

qualitative resistance (seedling resistance, major gene resistance) mediated by a single major resistance (*R*) gene and quantitative resistance mediated by multiple minor genes (field resistance, adult plant resistance, APR) (Pongam et al. 1998; Balesdent et al. 2001; Jestin et al. 2011, 2015). The *R* gene mediated resistance follows the gene for gene concept proposed by Flor (1971). In this theory, proteins encoded by *R* genes are able to recognize small proteins encoded by *Avr* genes in the pathogen. A specific '*R-Avr*' interaction generally can result in hypersensitive response (HR) in the plant to restrict further invasion of the pathogen (Flor 1971; Jones and Dang 2006). To date, at least 16 *R* genes and many quantitative trait loci (QTLs) in *Brassica* species have been identified that confer resistance to *L. maculans* infection (Raman et al. 2013). Although *R* gene mediated resistance can be very effective in disease control, repeated use of a single *R* gene may result in resistance breakdown (Rouxel et al. 2003; Sprague, et al. 2006; Van de Wouw, et al. 2010; Marcroft et al. 2012a).

While the knowledge on genetics of resistance against the disease is accumulating, little is known about the *R* genes carried by Canadian canola varieties and advanced breeding lines (Rimmer 2006; Kutcher et al. 2010a). Although many Canadian canola varieties were labeled as resistant to blackleg, disease incidence of blackleg in canola fields have continued to increase within the last ten years (Canadian plant disease survey, http://phytopath.ca/publication/cpds; Fernando et al. unpublished). China, where only the less aggressive *L. biglobosa* is present, is one of the major buyers of Canadian canola seeds. However, due to the high risk of introducing *L. maculans* into China through blackleg infected canola seeds, a transitional period of restriction in canola seeds importation from countries where blackleg is present was announced by China in 2009 (Zhang et al. 2014). To

better evaluate the risk of *L. maculans* introduction into China, it is important to understand the presence of *R* genes in Chinese *B. napus* germplasm. Although earlier studies reported some Chinese *B. napus* varieties were very susceptible to blackleg in field trials done in England, however, these results were based on a small number of samples (Li et al. 2008; Zhang et al. 2014), and the resistance to the Canadian population of *L. maculans* was unknown. The high risk of introducing *L. maculans* into China requires a better understanding of resistance genes in Chinese *B. napus* germplasm to be better prepared for risk mitigation via the facilitatation of disease resistance breeding programs in China.

To achieve better and durable control of blackleg, it is important to understand resistance genes in the host and defense mechanisms underlying the *B. napus*-*L. maculans* pathosystem. In spite of some studies attempting to unravel defense mechanisms against *L. maculans* in *Arabidopsis* and *B. napus*, our understanding in defense mechanisms in this pathosystem is still at the early stages (Staal et al. 2006; Kaliff et al. 2007; Šašek, et al. 2012; Lowe et al. 2014). Rapid development of next generation sequencing (NGS) technologies and associated bioinformatics tools provide opportunities to use high-throughput sequencing technologies to unravel host defense mechanisms at the transcriptomic level.

Therefore, the overall objectives of this study can be summarized as follows:

1. Characterize *R* genes and APR in a collection of Canadian *B. napus* germplasm and seed samples collected from growers' fields; identify avirulence allele frequency of field *L. maculans* populations.
2. Characterize *R* genes and APR in a collection of Chinese *B. napus* germplasm.

3. Unravel key genes and regulatory pathways associated with disease resistance in the *B. napus-L. maculans* pathosystem, and compare dynamics of defense mechanisms in compatible and incompatible *B. napus-L. maculans* interactions.

2 LITERATURE REVIEW

2.1 Introduction

Brassica napus L. (oilseed rape, canola, rapeseed) is a major oilseed crop cultivated worldwide. In 2015, the production of canola seeds in Canada was approximately 13.3 million tonnes (Statistics Canada, 2015). Canola is not only used as a source of edible vegetable oil, but also applied for a broad range of industrial purposes (Hayward 2012). Blackleg (stem canker), caused by the fungal pathogen, *Leptosphaeria maculans*, is one of the most devastating diseases of canola (Fernando et al. 2007; Raman et al. 2013). This disease can cause significant yield loss up to 80% depending on disease severity in the field (Marcroft et al. 2004). In Canada, *L. maculans* was first identified in Saskatchewan in 1975 (McGee and Petrie 1978), and later in Manitoba, Alberta and British Columbia (Gugel and Petrie 1992). Although cultural strategies such as crop rotation, fungicide application, and tillage are adopted to control the disease, the most promising disease control strategy is the utilization of resistant canola varieties (Fitt et al. 2006; Fernando et al. 2007; Raman et al. 2013). To facilitate the use of genetic resistance in disease control, it is important to identify blackleg resistance genes in *B. napus* germplasm and unravel defense mechanisms underlying the *B. napus*-*L. maculans* pathosystem. This literature review provides insights into the research progress in understanding host resistance in *B. napus*, pathogen virulence in *L. maculans*, and their interactions.

2.2 The host

2.2.1 *Brassica* species

The genus *Brassica* in the Brassicaceae family, containing 37 different species, is of great economic importance in agriculture worldwide. *Brassica* species have undergone two duplication events and two triplication events and resulted in a large number of duplicated regions in the genome (Jenczewski et al. 2013; Fopa Fomeju et al.2015). Three basic *Brassica* genomes, A (n=10), B (n=8), and C (n=9) have been considered to be partially homologous based on genetic and gemonic studies (Chen et al. 2014). There are six cultivated *Brassica* species: *B. nigra* (L.) Koch (n=8, BB genome), *B. oleracea* L. (n=9, CC genome), *B. rapa* L. (n=10, AA genome), *B. carinata* (A.) Braun (n=17, BC genomes), *B. juncea* (L.) Czern (n=18, AB genomes), and *B. napus* L. (n=19, AC genomes). The genetic relationships of the six *Brassica* species were described by 'the triangle of U' (U 1935).

Brassicas are considered as ideal model species to elucidate the evolution of polyploid plants, therefore their genome sequencing projects were of major interest to scientists (Gaeta et al. 2007). The application of the high-throughput next-generation sequencing (NGS) technologies and advanced bioinformatics tools enabled the completion of genome sequences of many *Brassicas*, including *B. rapa* (Wang et al. 2011), *B. oleracea* (Liu et al. 2014), and *B. napus* (Chalhoub et al. 2014). The availability of these genome sequences provides new insights and opportunities for *Brassica* research. Moreover, rapid development of NGS also facilitated sequence-based transcriptome analysis in *B. napus* to understand mechanisms underlying developmental and defense processes (Lowe et al. 2014; Haddadi et

al. 2015; Sonash et al. 2016).

2.2.2 *Brassica napus* L./canola/rapeseed

During the past three decades, the cultivation and production of canola has grown rapidly and canola has become the second most important oilseed crop, after soybean, with an annual production of 53.3 million tonnes globally (FAO 2013, http://faostat.fao.org/). In Canada, acreage of canola is the second largest in recent years (Statistics Canada, 2014).

Evolutionally, *B. napus* (2n=38, AACC) is a relatively new species (5,000-10,000 million years ago) that most likely originated from inter-specific hybridizations between *B. rapa* (2n=20, AA) and *B. oleracea* (2n=18, CC) during medieval times (Gupta and Pratap 2007). *Brassica napus* is closely related to *Arabidopsis thaliana*, a well studied model species thus allowing comparative genetic and genomic studies to unravel homoeologous regions regulating important traits. Due to high concentration of two toxicants, erucic acid and glucosinolates in traditional *B. napus*, oil extracted from these cultivars was not suitable for human and animal consumption (Gupta and Pratap 2007). Therefore, intensive breeding programs aimed for breeding high quality *B. napus* varieties with significantly lower erucic acid and glucosinolates were initiated in the 1970s. This resulted in a milestone in *B. napus* industry leading to the appearance of CanOLA (Canadian Oil Low Acid) during the 1970s (Buzza 1995). Canola varieties must meet with the standard of low erucic acid (<2% in the oil), and low in glucosinolates (<30 mg/g in the meal). The first canola variety, Tower, developed by Canadian scientists from University of Manitoba was released in 1974. The fatty acids profile in canola oil, i.e., high monosaturated fatty acids, low saturated fatty acids,

and rich in omega-3 fatty acids, granted canola oil as a suitable vegetable oil for human consumption (Stringam et al. 2003).

2.2.3 Major diseases of *Brassica napus* L.

During the whole life cycle of *B. napus*, there are many pathogenic organisms that can cause diseases. According to the causal agents, diseases of *B. napus* can be categorized into four groups: fungal disease, bacterial disease, viral disease, and phytoplasma-initiated disease (Li and McVetty 2013). Most of the economically important diseases are caused by fungal pathogens, except for clubroot and aster yellow which are caused by protist and phytoplasma, respectively. Globally, major diseases of *B. napus* include sclerotinia stem rot (caused by fungal pathogen *Sclerotinia sclerotiorum*), blackleg (caused by *Leptosphaeria maculans* and *L. biglobosa*), clubroot (caused by the obligate protist *Plasmodiophora brassicae*), aster yellows (caused by a pytoplasma), Fusarium wilt (caused by *Fusarium avenaceum* and *F. oxysporum*), white rust (caused by the fungus *Albuga candida*), downey mildew (caused by the fungus *Peronspora parasitica*), light leaf spot (caused by *Pyrenopeziza brassica*), and Verticillium stripe (caused by *Verticillium longisporum*). Among these diseases, blackleg and sclerotinia stem rot are the most economically important diseases in Canada, and clubroot and Verticillium stripe are emerging diseases in Canada (Fernando et al. 2007; Gossen et al. 2015; Peng et al. 2015). Currently, blackleg is the most severe disease of canola, causing more than $ 900 million of economic losses per growing season worldwide (Fitt et al. 2008).

2.3 The pathogen

2.3.1 Taxonomy of *Leptosphaeria maculans*

The causal agent of blackleg was first discovered on dead cabbage stems and was classified as *Sphaeria lingam* (Tode 1791). Later, the same fungal pathogen was found in living *B. oleraces* and reclassified to the genus Phoma (*Phoma lingam*) (Desmaziere 1849). The sexual stage of *P. lingam* was first reported in New Zealand and the pathogen was confirmed as *L. maculans* (Desm.) Ces & De Not. (Punithalingam and Holliday 1972). Due to the morphological similarity of a few distinct blackleg-causing species, the taxonomy associated with *L. maculans* was confusing for a period of time (Howlett et al. 2001). In 1994, the International Blackleg of Crucifers Network (ICBN) was established to address the taxonomic problems. To date, *L. maculans* is considered as a hemibiotrophic fungal pathogen, belonging to the kingdom Fungi, phylum Ascomycota, class Dothideomyctes, order pleosporales, genus *Leptosphaeria* and species *maculans*. The sexual stage of the pathogen is *L. maculans* (Desm) Ces. & de Not., and the asexual stage of the pathogen is *P. lingam* (Tode) Desm (Kaczmarek and Jędryczka 2011).

Until 2001, strains of *L. maculans* were classified into two pathotypes: the highly virulent, aggressive ‘A’ group strains that cause stem cankers on canola, and the nonaggressive, weakly virulent, ‘B’ group strains that do not cause stem cankers on canola (Williams and Fitt 1999). Later, ‘A’ pathotype isolates were divided into different pathogenicity groups (PG) according to the differential *B. napus* varieties test, whereas ‘B’ pathotype isolates (PG1 group) were classified as another species, termed *L. biglobosa*

(Shoemaker and Brun 2001; Kuusk et al. 2002; Chen and Fernando 2006). 'A' pathotype isolates were classified into PG2, PG3, PG4, and PGT based on their interaction phenotypes on a few differential *B. napus* varieties, including Glacier (*Rlm2* and *Rlm3*), Quinta (*Rlm1* and *Rlm3*), and Westar (no resistance) (Koch et al. 1991; Balesdent et al. 2005). Interaction phenotypes of PG3 isolates are: Westar (S), Glacier (S), Quinta (IR). Interaction phenotypes of PG4 isolates are: Westar (S), Glacier (S), Quinta (S). Interaction phenotypes of PGT isolates are: Westar (S), Glacier (IR), Quinta (S) (Mengistu et al. 1991). *L. biglobosa* and *L. maculans* are closely related and may have evolved from a common ancestor (Mendes-Pereira et al. 2003).

2.3.2 Host range of *Leptosphaeria maculans*

L. maculans has a broad host range within the Brassicaceae family, including cultivated *Brassica* crops *B. napus*, *B. rapa*, *B. juncea* and *B. oleracea*, and many cruciferous species, such as radish (*Raphanus sativus*) and white mustard (*Sinapis alba*) (Johnson and Lewis 1994; Williams and Fitt 1999). Another member of the Brassicaceae family, *Arabidopsis thaliana*, is either considered as a host or non-host to *L. maculans* depending on the Arabidopsis genotypes used (Rouxel and Balesdent 2005; Jones and Dangl 2006).

2.3.3 Pathogenicity of *Leptosphaeria maculans*

Although a bank of characterized *L. maculans* mutants was developed by *Agrobacterium tumefaciens*-mediated mutagenesis to analyse the role of pathogenicity genes, pathogenicity mechanisms of *L. maculans* were largely unstudied (Howlett et al. 2004). Using a reverse genetics approach, Idnurm and Howlett (2002) found isocitrate lyase encoded by an isocitrate

lyase gene (*icl1*) is essential for pathogenicity of *L. maculans* to canola. To date, a few pathogenesis related genes have been functionally studied, mainly including the *THIOL* gene (Elliott and Howlett 2006), the *Ipa* gene (Elliott and Howlett 2008), the *Lmpma1* gene (Remy et al. 2008a), the *Lmgpi15* gene (Remy et al. 2008b), the *LmIFRD g*ene (van de Wouw et al. 2009b), *Lmepi* gene (Remy et al. 2009), and the *LmSNF1* gene (Feng et al. 2014). In addition, *L. maculans* is able to produce phytotoxins that are essential for virulence, with sirodesmin PL being the well studied major phytotoxin (Rouxel et al.1988; Elliott et al. 2011).

2.3.4 Epidemiology of blackleg caused by *Leptosphaeria maculans*

The severity of blackleg has increased in recent years mainly due to the intensive cultivation of canola, rapid evolution and adaptation of fungal populations, and improper use of management practices. *L. maculans* has been recorded on crucifers since 1791, but the severe damage to *Brassica* species was only recorded in the last four decades (Rouxel et al. 2005). *L. maculans* is able to attack nearly all parts of the whole plant, including cotyledons, leaves, stems, roots and pods (Gabrielson 1983). The pathogen causes both leaf lesions and stem canker (West et al. 2001). Leaf lesions are dirty-whitish spots with fruiting bodies (pycnidia). During infection, the pathogen generally first infects cotyledons or true leaves, and then grows down towards the stem and the root, causing severe symptoms in the form of stem canker at the adult plant stage (Huang et al. 2014; Appendix I, II, III). The fungus can survive on infected stems or other parts of crop residues for several years and can produce both sexual and asexual fruiting bodies (West et al. 2001).

Prior to the 1970s, only *L. biglobosa* was identified in Canada and blackleg was not a

big concern in canola production. However, *L. maculans* was detected in Saskatchewan in the 1970s and further spread to other canola-growing regions in Canada by the late 1980s (Gugel and Petrie 1992). Later, *L. maculans* spread throughout countries including USA, Mexico, Brazil and Argentina (Fitt et al. 2006; Chen and Fernando 2006; Moreno-Rico et al. 2001; Fernando and Parks 2003; Gaetán 2005). To date, these two species have been found coexist in North America, Australia and Europe, whereas only *L. biglobosa* has been identified in China (West and Fitt 2005; Fitt et al. 2006; Magyar et al. 2006; Karolewski et al. 2007; Brazauskiene et al. 2011; Zhang et al. 2014).

L. maculans has both sexual and asexual stages on host plants and can either be monocyclic or polycyclic according to the source of inoculum (Li et al. 2007a). The fungus can remain on crop residues in the form of mycelium, pycnidia and pseudothecia (Li et al. 2007b). Sexual mating occurs between isolates with different mating type alleles and results in the production of ascospores which can travel for long distances (West et al. 2001; Marcroft et al. 2012b). Both ascospores and pycnidiospores can adhere to cotyledons or young leaves, germinate and produce hyphae to penetrate through stomata and wounds (Li et al. 2004). The pathogen generally spreads from leaf lesions through the lamella and petiole, and further colonizes either the upper parts or the crown (Li et al. 2008). Stem cankers can be observed at the end of the growing season even if leaf lesions were not visible at earlier growth stages.

The epidemiology of blackleg differs between continents and regions because of differences in climate, growing season, cultivars and especially fungal populations (West et al.

2001; Fitt et al. 2006). Under humid or wet conditions, both ascospores and conidia (pycnidiospores) can infect leaves of new crops via stomatal spores and wounds (Li et al. 2004). Although the incidence of seed infection by *L. maculans* and *L. biglobosa* is relatively low, seed-borne inoculum is a major concern in transporting *L. maculans* into countries where *L. maculans* has not been identified, such as China (Fitt et al. 2006; Zhang et al. 2014; Van de wouw et al. 2015; Fernando et al. 2016). The most common primary inoculum of Phoma stem canker is ascospores released from pseudothecia formed on residues of infected plants (West et al. 2001). The period of ascospores release varies from region to region and generally coincides with the emergence of young plants (Savage et al. 2013). In the case of ascospores as the primary inoculum, the disease is considered as monocyclic. However, the disease may be considered as polycyclic when pycnidiospores is the primary inoculum or even the secondary inoculum (Li et al. 2007a).

Ascospores are released in June in western Canada (Kharbanda 1993; Guo and Fernando 2005a), May in Australia (Khangura et al. 2001) and late September/early October in western and central Europe (Huang et al. 2005). In Europe, ascospore showers are believed to be the major inoculum (Fitt et al. 2006). In Australia, the major inoculum of blackleg is ascospores, in combination with pycnidiospores (Barbetti 1975, 1976; Marcroft et al. 2004). In western Canada, pycnidiospores is the major inoculum in infection and disease development (Petri 1995; Guo and Fernando 2005; Ghanbarnia et al. 2011; Dilmaghani et al. 2011, 2013). Pycnidiospores are mainly dispersed by rain-splash and can only travel short distances. Conidial inoculum of *L. maculans* is able to colonize stubble. Therefore, pycnidiospores may have contributed greatly to increase the genetic diversity of the pathogen

on individual stubble (West and Fitt 2005).

The timing of onset of seasonal release of ascospores is affected by many environmental factors, and models to predict the date of ascospore release have been developed by some researchers (Salam et al. 2007). During initial infection stage, the fungus grows as a biotroph, and switches into a necrotrophic pathogen and produces pycnidia in dead plant tissues (Rouxel and Balesdent 2005). After infection, the incubation period until the formation of leaf lesions may differ under different environmental conditions (Biddulph et al. 1999; Huang et al. 2001; Hadrami et al. 2010; Lob et al. 2013). *L. maculans* then spread from infected leaves through petiole to produce lesions on stems. The fungus can invade and kill cells of the cortex and form a blackened canker that may girdle the base of the stem. There is a symptomless growth stage of the fungus between initial leaf infection and the formation of stem cankers (Pilet et al. 1998; Delourme et al. 2006; Huang et al. 2014). The symptomless stage can be divided into two parts; one is in leaf petioles before the pathogen reaches the stem, and the other one is in stem tissues before the appearance of stem canker symptoms (West et al. 1999; Fitt et al. 2006). The term phoma stem canker was used to describe all symptoms on stems, including phoma stem lesions, crown canker and blackleg. Phoma stem canker may lead to premature ripening of the pods, and even cause lodging and plant death (West et al. 2001). After harvest, the infected plant residues remain in the field and will be supplied as inoculum for the following season. The detailed life cycle of *L. maculans* in western Canada is described in Fig. 2.1.

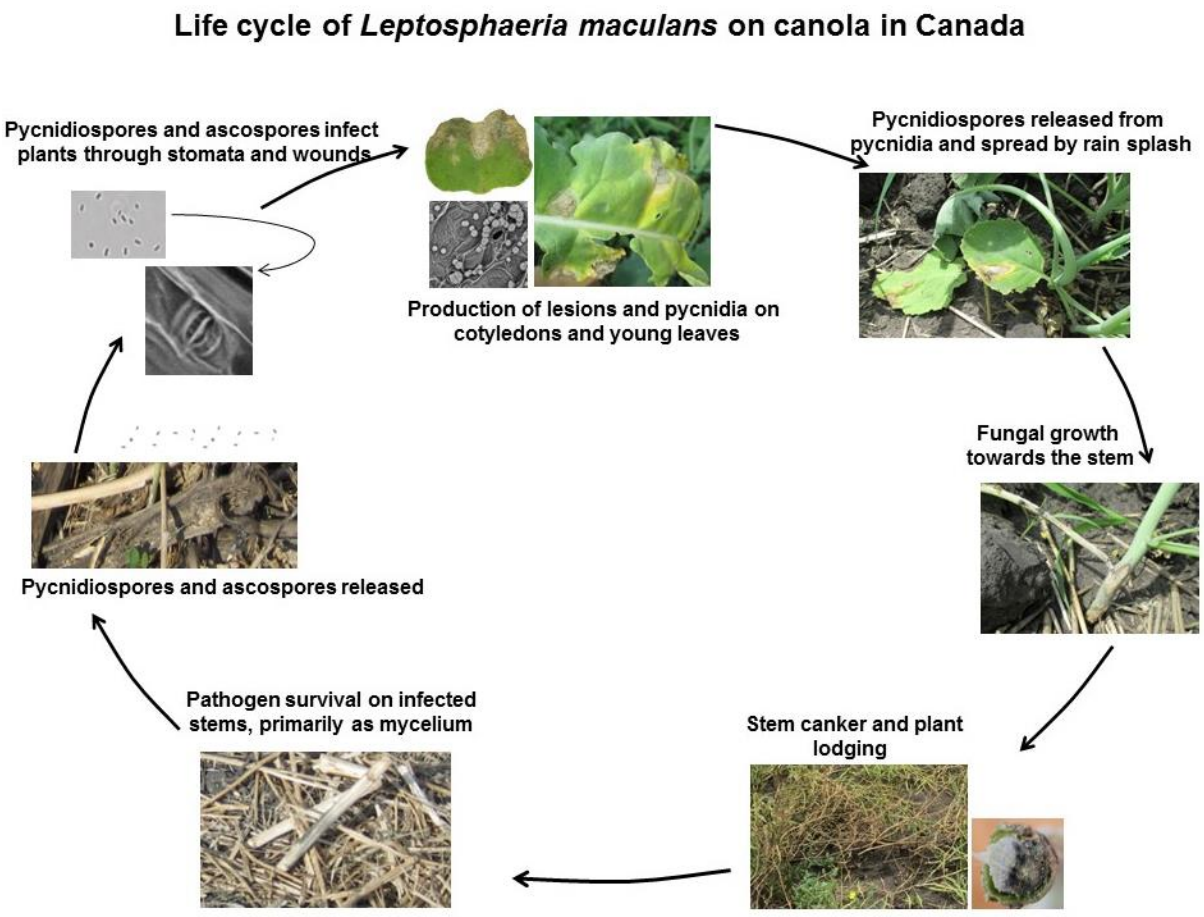

Fig. 2.1 Life cycle of *Leptosphaeria* maculans in western Canada.

2.3.5 Genomics of *Leptosphaeria maculans*

In 2004, the *Leptosphaeria* Genome Consortium was established and the 45-Mb genome of *L. maculans* 'brassicae' (Lmb) was published in 2011 (Rouxel et al. 2011; http://www.genoscope.cns.fr). The genome of *L. maculans* is organized with gene abundant GC-rich and gene poor AT-rich blocks. The AT-rich regions contain many class I long terminal repeat (LTR) retrotransposons and house 3.5% of the total genes found within the genome. About 20% of these genes encode small secreted proteins, including fungal effectors such as avirulence genes. The degenerated transposable elements (TEs) that surround the avirulence genes results in the loss or inactivation of avirulence genes caused by repeat-point

mutation during sexual reproduction.The genome of *L. maculans* is predicted to encode 10,000 to 13,000 genes within 16 chromosomes, and TEs account for 33% of the genome (Rouxel et al. 2011). The base composition (GC) of Lmb genome was relatively homogeneous locally, but variation was present over scales of hundreds of Kbs to Mbs. This variation in base composition, or the so called 'isochore' is a remarkable genomic structure of mammalian chromosomes that affects both coding and non-coding regions of the genome (Eyre-walker and Hurst, 2001). TEs associated with pathogenicity also likely contribute to the evolution of fungal virulence by promoting the translocations of effector genes to highly unstable regions. High percentage of TEs along with RIP mutation in Lmb genome generates large AT-rich regions, termed AT-isochores (Grandaubert et al. 2014). About 36% of Lmb genome is covered by AT-isochores, enriched in genes encoding effectors and gene clusters involved in the biosynthesis of secondary metabolites. The formation of large AT-isochores is in favour of the adaptation of the pathogenicity determinants to new plant resistance genes. The plasticity of *L. maculans* genome appears to be the basis of evolutionary potential of *L. maculans* that results in the rapid breakdown of resistance genes (Van de Wouw et al. 2010).

Two interesting observations within the *L. maculans* genome are chromosomal length polymorphisms and RIP mutations (Rouxel et al. 2011). Sexual crossing of *L. maculans* can be achieved *in vitro*, and genetic studies of *L. maculans* can be pursued. Since *L. maculans* isolates have different-sized chromosomes, progenies can have chromosomes in different length compared with the parental isolates. This was postulated to be due to different amounts of repetitive DNA homologs and unequal paring during meiosis (Plummer and Howlett 1993, 1995). Higher frequency of RIP mutations was shown to commonly occurr in the resulting

progenies of *L. maculans* isolates (Idnurm and Howlett 2003). Such active RIP is considered as a genome defense strategy that hypermutates repetitive DNA and therefore limits the accumulation and movement of these repetitive DNA sequences (Idnurm and Howlett 2003).

To better understand the genome structure of the *L. maculans* species complex, genomes of *L. biglobosa* "brassicae", *L. biglobosa* "thlaspi", *L. biglobosa* "Canadensis", *L. biglobosa* "lepidii", and *L. maculans* "brassicae" were sequenced (Grandaubert et al. 2014). Genomes of these species all encode similar numbers of small secreted proteins, which include putative effectors. Unlike *L. maculans* genome, the genomes of these *L. biglobosa* species are more compact (30-32 Mb), with lower proportion of TEs (less than 4% of the genome) mainly at the chromosome ends. These TEs contain both class I and class II type transposons (DNA transposons). High amounts of repetitive DNA (33%) and an isochore structure appeared to be unique to *L. maculans* "brassicae". These unique characteristics of *L. maculans* genome structure correlate with the rapid evolution of the *L. maculans* populations and its ability to cause severe blackleg epidemics in canola growing regions (Grandaubert et al. 2014).

2.3.6 Population variations of *Leptosphaeria maculans*

High levels of genetic variation have been found in *L. maculans* field populations in Australia (Barrins et al. 2002, 2004), Canada (Mahuku et al. 1997; Chen and Fernando 2006; Dilmaghani et al. 2009; Kutcher et al. 1993, 2007, 2010b; Liban et al. 2016) and Europe (Gout et al. 2006). All isolates collected from Manitoba and Saskatchewan in 1991 belonged to PG2 group (Kutcher et al. 1993). PG2 isolates remained the most common isolates found

in western Canada until the year 2000, but new PGT isolates were identified in isolates collected between 1998 and 2000, and a new PG3 isolate was detected in Manitoba in 1999 (Fernando and Chen 2003; Rimmer 2006; Chen and Fernando, 2006). Later, PG4 isolates were isolated in North Dakota, USA (Bradley et al. 2005). This phenomenon of PG groups change in western Canada was unusual. Theoretically, isolates of all four PG groups were expected to be present in western Canada if sexual recombination happened between PGT and PG3 isolates. This highlighted the fact that sexual recombination may not be common in Canadian *L. maculans* populations and ascospores may not be the major inoculum in blackleg epidemics each year (Guo and Fernando 2005a; Ghanbarnia et al. 2011).

To better address population variation of *L. maculans*, a new term, race structure was introduced by Balesdent et al. (2005) to describe population structures of *L. maculans* populations. Avirulence allele analysis in field *L. maculans* populations can provide information to guide proper use of resistance sources. For example, blackleg disease surveys in Europe (Balesdent et al. 2006; Stachowiak et al. 2006) and western Canada (Kutcher et al. 2010b; Liban et al 2016; Fernando et al. unpublished) demonstrated race structures of *L. maculans* and provided guidance in utilization of resistance genes. Population structure analysis of *L. maculans* in western Canada demonstrated high frequency of a few avirulence genes such as *AvrLm4*, *AvrLm6* and *AvrLm7* (Kutcher et al. 2010b). Therefore, continuous monitoring of local fungal populations is essential for the deployment of effective resistance genes (Liban et al. 2016).

2.3.7 Disease management

As large number of ascospores can be released from infected stems and contribute to an increase in subsequent blackleg disease severity (Wherrett et al. 2004), the integration of genetic resistance and cultural strategies such as tillage, fungicide application, and crop rotation are able to affect the concentration of ascospores (West and Fitt 2005; Aubertot et al. 2006; Fernando et al. 2007). In western Canada, the combination of appropriate rotation and tillage were proven to reduce the amount of airborne inoculum and the infection level (Guo et al. 2005, 2008). However, the most important approach to control blackleg is through genetic breeding and using resistant canola varieties (Rimmer 2006; Fernando et al. 2007; Kutcher et al. 2011, 2013). The durability and long-term effectiveness of resistance genes in resistant varieties are affected by the biology of the pathogen and its potential to undergo mutation and recombination of the avirulence genes (Kutcher et al. 2011; Howlett et al. 2015).

In spite of the effectiveness of resistance genes in disease control, rapid breakdown of blackleg resistance in commercial crops due to the increase in the frequency of the virulent isolates has been reported. In France, *Rlm1* resistance was overcome within 5 years of release of *Rlm1*-carrying varieties (1996-1999) (Rouxel et al. 2003). Similarly, in Australia, "sylvestris" resistance (*Rlm1* and *LepR3*) was broken down in three years after commercial release in the Eyre Peninsula area (Sprague et al. 2006a, b). This is not unusual as there is a typical boom and bust plant pathogen cycle in blackleg resistance under field conditions (Rouxel et al. 2003; Brun et al. 2010; Delourme et al. 2014). With new resistance introduced, the phenomenon that well-performing varieties are grown extensively in the following years

is described as the boom phase of the cycle. Changes in the pathogen population occurred under selection pressure and the frequency of the virulent isolates increased, resulting in the increase in disease severity, or even breakdown of the resistance. The bust cycle then comes when the variety was not grown in the field, and the frequency of the virulent isolates decrease over time (Delourme et al. 2014; Brun et al. 2010). The breakdown of "sylvestris" resistance in the Eyre Peninsula in 2003 resulted in the withdrawal of these cultivars from cultivation. By 2006, the frequency of virulent *L. maculans* isolates declined on trial sites in the Eyre Peninsula, where the variety was maintained (Sprague et al. 2006a, b; Van de wouw et al. 2014b).

Pyramiding of multiple resistance genes in a single cultivar is not a cost-effective strategy in the control of blackleg, as this strategy results in direct selection towards all corresponding avirulence genes (Fitt et al. 2006). Furthermore, when dealing with fungal populations with sexual recombination, multiple resistance genes pyramided in a single cultivar may lose their effectiveness rapidly (McDonald and Linde 2002; Fitt et al. 2006). Rotations of cultivars with different components of resistance genes have become evidently effective, but it requires the identification of resistance genes in commercial canola cultivars (Marcroft et al. 2012b). The combination of qualitative resistance and quantitative resistance to *L. maculans* in canola variety is able to improve the durability of blackleg resistance (Brun et al. 2010; Marcroft et al. 2012b; Delourme et al. 2014).

L. maculans can reside in infected stubbles in the field or infected seeds (Bailey et al. 2003; Van de Wouw et al. 2015; Fernando et al. 2016). Destruction of blackleg-infested

stubbles by tillage has been recommended. A paddy rice crop following winter canola in some canola growing regions of China has been proven to be able to minimize the impact of inoculum in stubble (Peluola et al. 2013). In Australia, new canola crops are recommended to be grown at least 500 meters from previous year's canola stubble (Marcroft et al. 2003). In western Canada, blackleg resistant canola varieties were first released in the 1990s and more resistant varieties were released in recent years (Kutcher at al. 2010a). Although resistance genes in these resistant canola varieties were not publicly known, blackleg was well controlled by using resistant varieties. Initially, a 4-year rotation of canola with other crops was the standard recommendation in western Canada (Rimmer et al. 2003; Kutcher et al. 2014). However, more frequent rotations were adopted by growers, and therefore, 3-year rotation was suggested to be sustainable (Cathcart et al. 2006). In recent years, due to the economic return of canola, many growers adopted two-year rotation or even grew canola in successive years (Backie et al. 2011; Marcroft et al. 2012b; Kutcher et al. 2014). To achieve a more effective blackleg management strategy, rotations of canola with other crops every four years seem to be a practical rotation strategy for western Canada canola growers (Kutcher et al. 2014).

Fungicide applications have been proven to be of limited value to maintain canola yield (Huang et al. 2011; Liu et al. 2014). A few studies have investigated the effect of fungicide on *L. maculans* and *L. biglobosa*, and most of these studies revealed that *L. maculans* was more sensitive to fungicides than *L. biglobosa* (Karolewski et al. 1998; Cavelier et al. 1999; Griffiths et al. 2003; Eckert et al. 2009; Huang et al. 2011). Among different *L. maculans* isolates, variations in sensitivity to QoI fungicides (fungicides with the

action mode of Quinone outside inhibitor) were observed (Liu 2014).The timing of fungicide application is crucial in blackleg control as the fungicides are not able to control the disease once the pathogen has reached the stem (Gladders et al. 1998; Steed et al. 2007; Peng et al. 2012; Liu 2014). Although foliar fungicides have been shown to reduce disease severity and increase yield in blackleg susceptible canola varieties (Kutcher et al. 2008), there is no economic benefit of using fungicide in resistant canola varieties (Bailey et al. 2000; Liu 2014).

Wind-dispersed ascospores that can travel for long distances are the major source of inoculum in Australia, whereas pycnidiospores (asexual) are the major source of inoculum in western Canada (Guo and Fernando 2005; Fernando et al. 2007; Ghanbarnia et al. 2011). Therefore, in western Canada, the distance between canola fields was recommended at least 50 to 100 meters to reduce the impact of inoculum movement (Guo and Fernando 2005). However, the recommended distance is more than 400 meters as canola plants grown within 400 m are in higher risk of infection than that of more than 400 m (Marcroft et al. 2004).

2.4 Host-pathogen interactions

2.4.1 Host resistance genes and disease resistance breeding

In *B. napus*, there are two types of resistance against blackleg, qualitative resistance (*R* gene, major gene) mediated by single major genes and quantitative resistance (adult plant resistance, APR) controlled by multiple genetic factors (QTLs) (Rimmer 2006; Raman et al. 2013). *R* gene mediated resistance is qualitative, race specific and generally expressed at seedling stage (Delourme et al. 2006; Elliott et al. 2016). *R* gene resistance is effective and can prevent

the spread of initial infection from spread to the whole plant (Johnson and Lewis 1994; Raman et al. 2013). Quantitative resistance is considered as non-race specific mediated by multiple QTLs (Delourme et al. 2006; Jestin et al. 2011, 2015). However, in some cases, adult plant resistance seems to be isolate-specific (Marcroft et al. 2012a). The effectiveness of quantitative resistance is affected by environmental conditions (Huang et al. 2014, 2016). Resistance of *B. napus* to *L. maculans* is evaluated based on disease severity under both controlled and field conditions (Marcroft et al. 2012a, b). The characterization of major gene resistance is performed under controlled condition using cotyledon inoculation test. Resistance to *L. maculans* has been identified in some wild *Brassica* species, such as *B. rapa* subsp. *sylvestris* (L.) Janchen, *B. oxyrrhina* Coss, and *B. insularis* Moris (Mithen et al. 1987, Salisbury 1989). Resistance from *B. rapa* subsp. sylvestris has been successfully introgressed into *B. napus* and was subsequently incorporated into an Australian cultivar 'Surpass 400' and other cultivars. *A. thaliana* is another source of resistance to blackleg. Adult-leaf resistance from *A. thaliana* has been transferred into *B. napus* by asymmetric somatic hybrids (Bohman et al. 2002). In Canada, blackleg resistant canola varieties were released in the 1990s and have been performing well for a period of time (Kutcher et al. 2010a).

Major gene resistance has been introgressed into *B. napus* from other *Brassica* species (Li and Cowling 2003). To date, there are at least 18 well studied major blackleg resistance (*R*) genes in *Brassica* species (Table 2.1): *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* from *B. napus*; *Rlm5* and *Rlm6* from *B. juncea*; *Rlm10* from *B. nigra*; *LepR1*, *LepR2*, *LepR3*, *LepR4* and *RlmS* from re-synthesized *B. rapa subsp. sylvestris*; *Rlm8* and *Rlm11* from *B. rapa*; and *BLMR1* and *BLMR2* from Surpass 400 (Raman et al. 2013). Most of the *R* genes are located

on chromosome A7 and A10 (Raman et al. 2013). Only two *R* genes, *LepR3* that interacts with *AvrLm1* and *Rlm2* that confers resistance to *AvrLm2*, have been cloned so far (Larkan et al. 2013, 2015). *LepR3* and *Rlm2* are two allelic variants located on the *LepR3/Rlm2* blackleg resistance locus encoding alternate forms of the same Leucine-rich repeat receptor-like protein (LRR-RLP) (Larkan et al. 2013, 2015).

It has been considered that complete resistance to *L. maculans* is present in all B genome *Brassica* species (Rimmer and van den Berg 1992). However, Keri et al. (1997) and Fernando et al. (unpublished) identified some *B. juncea* varieties that were susceptible to *L. maculans*, indicating that complete resistance in B genome is not completely correct. Most of the identified *R* genes are located on A genome, with a few on B genome and none on C genome. By using a set of *L. maculans* isolates with known *Avr* gene profile, *R* genes in *B. napus* can be identified (Williams and Delwiche 1979; Balesdent et al. 2002; Marcroft et al. 2012a). For example, *Rlm1* was identified in Quinta (Ansan-Melayah et al. 1998), *Rlm4* in Jet Neuf (Basesdent et al. 2001), and *Rlm9* in Darmor (Delourme et al. 2004).

Table 2.1 *R* genes conferring blackleg resistance in *Brassica* species.

R gene	Originated from	Chromosome	References	Note
Rlm1	*B. napus*	A7	Ferreria et al. 1995	*R* gene cluster
Rlm3	*B. napus*	A7	Ansan Melayah et al. 1998	
Rlm4	*B. napus*	A7	Zhu and Rimmer, 2003	
Rlm7	*B. napus*	A7	Rimmer 2006	
Rlm9	*B. napus*	A7	Delourme et al. 2006	
BLMR1	*B. napus*	A10	Long et al. 2011	
BLMR2/RlmS	*B. napus*	A10	Van de Wouw et al. 2009; Long et al. 2011; Larkan et al. unpublished	
LepR1	*B. rapa ssp.sylvestris*	A2	Yu et al. 2005	
LepR2	*B. rapa ssp.sylvestris*	A10	Yu et al. 2007	
LepR3	*B. rapa ssp.sylvestris*	A10	Larkan et al. 2013	Allelic variants
Rlm2	*B. napus*	A10	Mayerhofer et al. 1997, Larkan et al. 2015	
LepR4	*B. rapa ssp.sylvestris*	A6	Yu et al. 2008	
Rlm8	*B. rapa*	-	Balesdent et al. 2002	
Rlm11	*B. rapa*	-	Balesdent et al. 2013	
Rlm5	*B. juncea*	-	Chèvre et al. 1997	
Rlm6	*B. juncea*	B8	Balesdent et al. 2002	
Rlm10	*B. nigra*	B4	Chèvre et al. 1996; Eber et al. 2011	

Quantitative resistance evaluation is generally performed under field conditions (Li et al. 2008; Huang et al. 2016). Inoculum is provided in the forms of spraying fungal spore suspension or spreading infected stubbles. Both disease severity and disease incidence are used to evaluate disease resistance against *L. maculans* infection (Marcroft et al. 2012b). However, field evaluation is affected by many environmental factors and there are genotye by environment (G×E) interactions (Huang et al. 2016). Jestin et al. (2015) adopted a multi-cross connected design using a few populations derived from four resistant lines and one susceptible line. Using this strategy, they identified population-common and population-specific QTLs. Association mapping approach has been adopted to confirm the markers located with QTLs in Darmor (Jestin et al. 2011). The combination of quantitative resistance and qualitative resistance can maximize the durability of resistance (Brun et al. 2010; Marcroft et al. 2012b). Compared with single *Rlm6* resistance, the combination of *Rlm6* resistance with quantitative resistance was shown to provide 4-year-long effective control of blackleg (Brun et al. 2010). Moreover, the disease severity remained at lower level even after the major gene had been overcome by the fungal populations (Delourme et al. 2014).

Quantitative resistance is associated with reduced disease susceptibility controlled by a set of ‘minor’genes that produce resistance matabolites and proteins (Kushalappa et al. 2016). In spite of efforts in dissecting genetics and genomics of quantitative resistance, the mechanisms underlying quantitative resistance are still poorly understood. A few pathosystems such as wheat-*Puccinia triticina*, *Arabidopsis-Botrytis* have been used to explore quantitative resistance underlying the host immune system (Niks et al. 2015; Corwin

et al. 2016). Thousands of genes associated with a wide variety of cellular processes including hormone signaling and reactive oxygen signaling were activated in the *Arabidopsis-Botrytis* pathosystem (Corwin et al. 2016). Although quantitative resistance is generally considered as non-race-specific, race-specific quantitative resistance has been reported in some pathosystems. These include rice blast, leaft rust and leaf stripe in barley, black stem in sunflower, and rose blackrot (Poland et al. 2009). In addition, quantitative resistance may have a qualitative inheritance and vice versa (Niks et al. 2015). Molecular mechanisms of adult plant resistance in the *B. napus-L. maculans* pathosystem is generally unknown.

2.4.2 Pathogen avirulence genes

Avirulence genes as well as other effectors are usually located in AT-rich gene-poor regions of the genome (Rouxel et al. 2011). These regions comprise repetitive DNA derived from TEs and therefore provide an unstable genomic environment that promotes the gain and loss of avirulence and other effector genes (Soyer et al. 2014). The availability of reference genome facilitated the identification of candidate avirulence genes, and to validate the interaction between *Avr* genes and *R* genes (Selin et al. 2016). To date, at least seven avirulence (*Avr*) genes have been cloned: *AvrLm1* (Gout et al. 2006), *AvrLm2* (Ghanbarnia et al. 2014), *AvrLm3* (Plissonneau et al. 2016), *AvrLm5/AvrLmJ1* (Van de Wouw et al. 2014a; Balesdent and Howlett unpublished data), *AvrLm4-7* (Parlange et al. 2009), *AvrLm6* (Fudal et al. 2007), and *AvrLm11* (Balesdent et al. 2013). *AvrLm1* is located in a recombination-deficient, transposon-rich region, and linked with *AvrLm6* and five other avirulence genes (Gout et al.

2006; Parlange et al. 2009). Four cloned *Avr* genes, *AvrLm1*, *AvrLm6*, *AvrLm4-7* and *AvrLm11* encode small secreted proteins (Gout et al. 2006; Fudal et al. 2007; Parlange et al. 2009; Balesdent et al. 2013). The other three cloned *Avr* genes, *AvrLm2*, *AvrLmJ1*, and *AvrLm3* encode small cysteine-rich secreted protein (Ghanbarnia et al. 2014; Van de Wouw et al. 2014a; Plissonneau et al. 2016). It is quite interesting to notice that although *LepR3* and *Rlm2* are two allelic variants, their corresponding *Avr* genes *AvrLm1* and *AvrLm2* are not allelic variants and encode different proteins.

Polymorphisms of *AvrLm1* in *L. maculans* isolates collected before and after the breakdown of 'sylvestris' resistance in Australia demonstrated deletions, amino acid substitutions, and RIP mutations (Van de Wouw et al. 2010). The *AvrLm6* gene was adjacent to a single-copy non-coding sequence at the 3' end. This gene had six different RIP alleles conferring virulence. During the breakdown of 'sylvestris' resistance, there was an eightfold increase in isolates lacking *AvrLm1*, where no RIP was identified. Surprisingly, although varieties with *Rlm6* were not grown in that region, the frequency of isolates lacking *AvrLm6* increased six fold. These findings strongly suggest that selection of one avirulence gene affects other linked avirulence genes and could potentially lead to a selective sweep (Barton et al. 2013). Therefore, widespread use of one *R* gene could lead to the breakdown of other *R* genes (Rouxel et al. 2003; Sprague et al. 2006a, b; Zhang et al. 2016). Some known *L. maculans Avr* genes are organized in the form of a gene cluster: the *AvrLm1-AvrLm2-Avrlm6* cluster, and the *AvrLm3-AvrLm4-AvrLm7* cluster (Balesdent et al. 2002). This suggests the possible relationship between an *Avr* gene cluster in *L. maculans* and the counterpart *R* gene cluster in *B. napus* (Delourme et al. 2004).

2.4.3 Molecular interactions between *Arabidopsis thaliana* and *L. maculans*

Success in disease resistance breeding relies on a solid understanding of the genetic basis of resistance and defense mechanisms in the host plant. Using *A. thaliana* as a model system to study defense mechanisms against *L. maculans* infection has many advantages. For example, Arabidopsis lines can be used to screen for pathogenicity of *L. maculans* transformants (Elliott et al. 2008). Resistance in Arabidopsis against *L. maculans* isolates is believed to be controlled by a dominant *R* gene encoding a nucleotide binding site-leucine-rich repeat (NBS-LRR) (Staal et al. 2006, 2008). At least three genes in Arabidopsis have been identified so far: *AtRlm1*, *AtRlm2*, and *AtRlm3* (Staal et al, 2006, 2008). As a model plant, Arabidopsis, a non-host of *L. maculans*, has been used to unravel defense mechanisms against *L. maculans* infection (Bohman et al. 2004). Although defense mechanisms in Arabidopsis and *B. napus* may differ, studies in *A. thaliana-L. maculans* pathosystem provide insights in the *B. napus-L. maculans* pathosystem. The defense responses induced by RLM1 were proven to be associated with physical barriers and complex cross-talk among hormone signaling pathways including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Staal et al, 2006, 2008). Major gene-mediated resistance in *Arabidopsis* against *L. maculans* was shown to be dependent on callose deposition promoted by abscisic acid (ABA) through the repression of *PR2* (Oide et al. 2013). The fungus can infect the plants and form pycnidia when the single Toll interleukin-1 receptor-nucleotide binding-leucine-rich repeat *R* gene was lost (Staal et al. 2006). Oxidative burst and ABA contributed to resistance in Arabidopsis to *L. maculans* (Jindrichova et al. 2011; Oide et al. 2013). ABA has also proven to be involved in *L. maculans* resistance in Arabidopsis (Kaliff et al. 2007). Although multiple defense pathways

restricting the growth and infection of *L. maculans* on *A. thaliana* has been studied, the roles of these pathways in *B. napus* are still uncertain.

2.4.4 Molecular interactions between *B. napus* and *L. maculans*

Plants have evolved to defend themselves from attack by a wide range of pathogens. After pathogen attack, receptor proteins called pattern recognition receptors (PRRs) are stimulated upon recognition of conserved microbial elicitors, known as pathogen-associated molecular patterns (PAMPs) (Boller and Felix 2009; Dodds and Rathjen 2010; Denancé et al. 2013). The interaction of PRRs and PAMPs leads to the first class of plant immunity, PAMP-triggered immunity (PTI) (Jones and Dangl 2006; Bigeard et al. 2015). The second class of perception is the recognition of pathogen avirulence molecules called effectors by intracellular receptors; this recognition leads to effector-triggered immunity (ETI) (Dangl et al. 2013). Compared with the conservation of PAMPs, effectors are variable and dispensable (Dodds and Rathjen 2010). Generally, PTI is effective against non-adapted pathogens, and this type of resistance is called non-host resistance, whereas ETI is active against adapted pathogens leading to a rapid localized cell death called hypersensitive response (HR) (Tsuda and Katagiri 2010).

Compared to other intensive studies on host resistance and fungal avirulence genes, resistance mechanisms underlying the *B. napus-L. maculans* pathosystem is largely unresolved. For qualitative resistance, the interaction between *B. napus* and *L. maculans* follows the gene-for-gene concept (Flor 1971; Ansan-Melayah et al. 1998). This concept states that for each resistance gene in the host, there is a corresponding specific avirulence

gene in the pathogen. Gene for gene interaction involved the direct or indirect recognition of pathogen effectors by the proteins encoded by the corresponding *R* genes (Flor 1971). Generally, most *R* genes encode proteins with NBS-LRR proteins (Marone et al. 2013). The successful interaction between R protein and the corresponding Avr effectors will lead to the induction of signaling pathways and downstream defense responses (Dangl et al. 2013). The mechanism and genetic factors involved in quantitative resistance against blackleg appears to be more complicated and largely unknown. Some *R* gene-mediated host resistance in *B. napus* has been recently considered as an example of effector-triggered defense (ETD) as proposed by Stotz et al. (2014). Unlike ETI, effectors of the apoplastic pathogens are recognized by R proteins on the cell surface during ETD. *R* genes involved in ETD encode cell surface-localized receptor-like proteins (RLPs) that contain the receptor like kinase SOBIR1 (Stotz et al. 2014).

The gene for gene interaction involves in direct or indirect recognition of pathogen effectors (A = avirulence genes) by the R protein are encoded by the *R* gene (Fig. 2.2). Many of the cloned *R* genes of plant species encode NBS-LRR proteins (Bent 1996). The response of *B. napus* after *L. maculans* infection include HR, callose, lignin, and phytoalexin production (Howlett et al. 2001). Early molecular studies demonstrated the complexity of host responses against *L. maculans* infection (Fristensky et al. 1999). During *L. maculans* infection, several cell-wall-degrading enzymes are activated (Hassan et al. 1991). In *B. napus*, more callose deposition was observed in compatible interaction (*Rlm1-avrLm1*), whereas enhanced callose deposition was observed in incompatible interaction in Arabidopsis (Staal et al. 2006). In the *B. napus-L. maculans* pathosystem, JA, ET and SA signaling pathways were

activated during the host-incompatible interaction (Sašek et al. 2012). *Rlm1* mediated resistance in *B. napus* involved in the increase in biosynthesis of SA and induced the expression of SA-associated genes, such as *PR-1*, *ICS1*, and *WRKY70* (Šašek, et al. 2012). The induction of ET signaling related genes, *HEL*, *CHI*, and *ASC2a* were also observed (Šašek, et al. 2012).

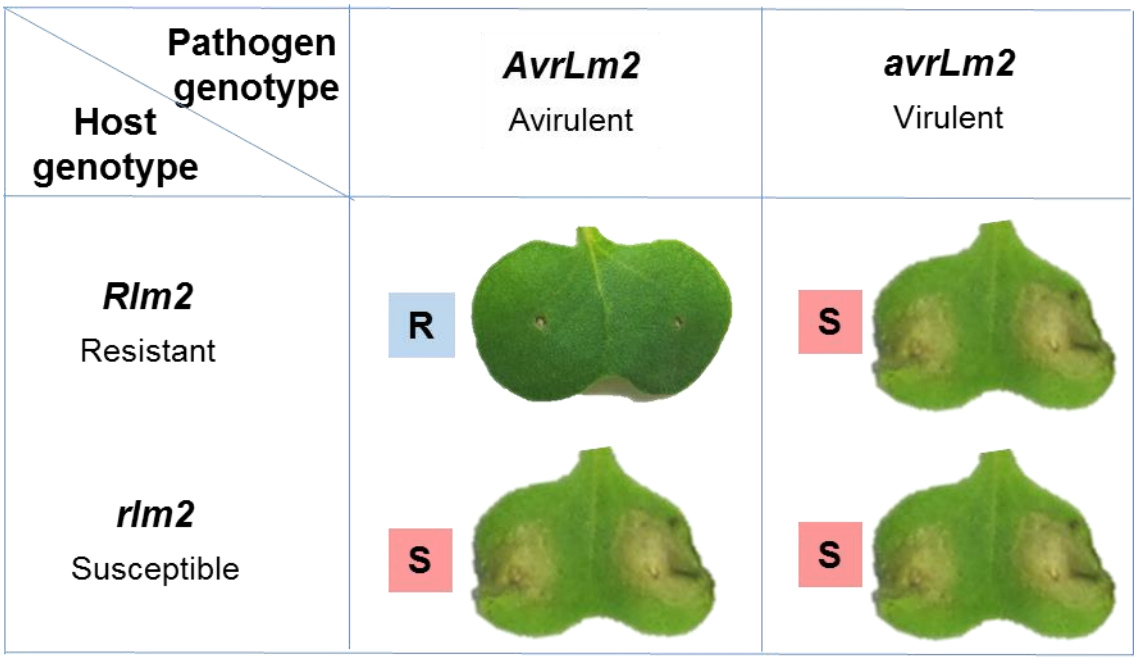

Fig. 2.2 Gene for gene interaction between *R* genes and *Avr* genes in the *B. napus-L. maculans* pathosystem. Resistant reaction only resulted from the recognition of *Avr* gene by the protein of the corresponding *R* gene.

With an increase in understanding both *R* genes and *Avr* genes, a set of differential interactions between Brassica species and *L. maculans* isolates were adopted to predict *R* genes in the host and *Avr* genes in the pathogen (Williams and Delwiche 1979, Balesdenr et al. 2002), such as the interaction between *Rlm1*/*LepR3* and *AvrLm1* (Larkan et al. 2013), and the interaction between *Rlm2* and *AvrLm2* (Larkan et al. 2014).

2.5 RNA sequencing

2.5.1 The development of sequencing technology

In 1977, DNA sequencing technologies based on chain-termination method (Sanger sequencing) and chemical modification method were developed by Frederick Sanger and Walter Gilbert, respectively (Bräutigam and Gowik 2010). Sanger sequencing was widely applied and later considered as the "first generation sequencing" because of its low radioactivity and high efficiency (Liu et al. 2012). Since DNA sequencing at that time required radioactive materials and was laborious, a more accurate and faster sequencing platform was in high demand. In 1987, the first automatic sequencing machine (AB370) adopting capillary electrophoresis and Sanger sequencing was introduced by Applied Biosystems. The completion of the human genome project in 2001 largely stimulated the development of more powerful sequencing technologies. In 2005, the 454 system was launched (http://my454.com/products/technology.asp). In 2006, the Genome Analyzer was released by Solexa (purchased by Illumina in 2007) and Sequencing by Oligo Ligation Detection (SOLiD) was launched by Agencourt (purchased by Applied Biosystems in 2006). These are the three typical massively parallel sequencing systems that were referred to as "second-generation sequencing" (Liu et al. 2012; Egan et al. 2012; Mardis 2013).

The Roche 454 system uses pyrosequencing technology that relies on the detection of pyrophosphate released during nucleotide incorporation (Liu et al. 2012; Frese et al. 2013). The AB SOLiD system uses the technology of two-base sequencing centered on ligation sequencing. The Illumina GA/HiSeq system adopts the technology of sequencing by

synthesis (SBS) (Mardis 2008). The Roche 454 system has advantages including long read length and rapid in terms of sequencing, with disadvantages such as error rate with poly base more than 6, low throughput and high cost. Compared with Roche 454 system, both Illumina and SOLiD generate shorter reads and much higher output data per run (Varshney et al. 2009; Liu et al. 2012; Egan et al. 2012). NGS technologies are able to produce huge amounts of DNA sequences at a much lower cost in a high-throughput manner (Wall et al. 2009). To date, NGS has been successfully applied in studying genomes and transcriptomes of a broad range of species (Bräutigam and Gowik 2010; Frese et al. 2013).

The technology evolution in NGS affords new opportunities to answer biological questions in a genome- or transcriptome- wide manner. How to manage the ever-growing amount of NGS data, and extract biological knowledge from these data, however, poses unprecedented challenges to research scientists. Processing NGS data requires intensive computation and the development of new software tools. In spite of efforts in developing new tools for NGS data analysis, the barrier of using these tools remains to be resolved since most tools are only compatible with Unix (or Linux) environment. Prior to NGS data analysis, biologists or students without bioinformatics background had to acquire a broad range of skills including familiarity with Unix/Linux, basic knowledge of programming languages in R and Perl, basic knowledge of key concepts in computational biology and biostatstics, and basic understanding of a relational database and computer software (Wang 2016).The development of some user-friendly systems or projects, such as Galaxy (Goecks et al. 2010) and Bioconductor (www.bioconductor.org) based on R (www.r-project.org) enables scientists to analyse NGS data with less training in bioinformatics.

2.5.2 The application of RNA sequencing in host-pathogen interactions

Transcriptomics mainly aimed to analyze all species of transcripts (mRNAs, small RNAs and non-coding RNAs); to quantify gene expression levels of transcripts (mainly mRNAs); and to reveal transcriptional structures (5' and 3' ends, splicing patterns, and post-transcriptional modifications) of genes (Wang et al. 2009). Technologies applied in transcriptome analysis mainly include hybridization-based and sequencing-based approaches. Microarray is a hybridization-based approach that has been the most popular approach for transcriptomic analysis particularly for model organisms with high-quality gene annotation data since the invention of this technology in the 1990s (Marguerat et al. 2008). However, there are several limitations when using microarray, mainly including high background levels caused by cross-hybridization; reliance upon existing knowledge about genome sequence; lack of reproducibility; and a limited dynamic range in detection due to background and saturation of signals (Guarnaccia et al. 2014). Tag-based sequencing approaches were developed to overcome these limitations in microarray. These approaches included massively parallel signature sequencing (MPSS) (Brenner et al. 2000; Reinartz et al. 2002), serial analysis of gene expression (SAGE) (Velculescu et al. 1995; Harbers and Carninci 2005), and cap analysis of gene expression (CAGE) (Kodzius et al. 2006; Shiraki et al. 2003). Although these approaches are of high throughput and can quantify gene expression levels, they have significant disadvantages, including reliability on expensive Sanger sequencing, limitation in the number of transcripts analyzed and mapping bias of these short tags. RNA sequencing (RNA-Seq) overcome limitations in microarray and has shown great potential to replace microarray in gemone-wide transcriptome analysis. RNA-Seq is a sequencing-based

technology that allows a user to survey the entire transcriptome of any eukaryote in a high-throughput manner. RNA-seq has a wider dynamic range, more accurate estimation of gene expression levels, and higher technical reproducibility (Marioni et al. 2008; Fu et al. 2009).

RNA-seq is a recently developed technology based on NGS technologies. In general, cDNA libraries constructed from RNA samples are sequenced in a high-efficient manner within high-throughput sequencing platforms (Holt and Jones 2008). This technology can not only perform whole transriptomic analysis, reveal RNA sequence variations, but also characterize alternative splicing patterns. Theoretically, any high-throughput sequencing technology can be applied in RNA-Seq, and some pioneering studies have been performed on Illumina (Marioni et al. 2008; Morin et al. 2008), SOLiD (Cloonan et al. 2008) and Roche 454 (Emrich et al. 2007; Vera et al. 2008) platforms. In recent years, with the rapid development and reduced cost of NGS, NGS platforms such as the Illumina system, their application has led towards unraveling many biological questions (Wolf 2013). In a RNA-Seq experiment, a population of RNA (total RNA or fractionated RNA) is converted into a barcoded and fragmented cDNA library. This cDNA library, with or without PCR amplification, is then sequenced using NGS platforms to obtain one end or two end short sequences (30-400 bp). The raw reads are processed and aligned to a reference genome (transcripts), or assembled *de novo* when reference genome sequences are not available (Wang et al. 2009). The mapped reads contain three types: exonic reads, junction reads and poly(A) end-reads. These mapped reads are further used to generate gene expression profile of each gene and further biological analysis (Wang et al. 2009).

The available reference genome sequences of both *B. napus* and *L. maculans* make sequencing-based transcriptome (RNA-Seq) studies more advanced. Although RNA-seq is a relatively new approach towards transcriptome studies, recent advances in NGS technologies make RNA-Seq based transcriptome studies more affordable and applicable in many host-pathogen interactions; such as *Xanthomonas axonopodis* pv. *glycines* in soybean (Kim et al. 2011), *Pseudoperonospora cubensis* in cucumber (Adhikari et al. 2012), *Magnaporthe oryzae* in rice (Kawahara et al. 2012), *X. arboricola* pv. *pruni* in peach (Socquet-Juglard et al. 2013). In the *B. napus*-*L. maculans* pathosystem, RNA-Seq has been utilized in unraveling host-pathogen interactions (Lowe et al. 2014; Haddadi et al. 2015; Sonash et al. 2016).

2.6 Major research objectives

To better understand disease defense in *B. napus* against *L. maculans* infection, the characterization of disease resistance genes (both known and potentially novel resistance genes) and dissection of defense mechanisms in *B. napus* are essential to provide essential knowledge for disease resistance breeding. Therefore, the first objective of this thesis was to characterize blackleg resistance in Canadian *B. napus* germplasm and seed samples, and identify avirulence profiles in *L. maculans* field populations. This involved identification of *R* genes and APR in *B. napus* germplasm under controlled environment. In addition, *R* genes in seed samples collected from growers' fields and *Avr* genes in *L. maculans* field populations were identified to predict the effectiveness of *R* genes in western Canada. Since 1994, China imports several million tons of canola seeds from Canada. However, in 2009, due to limited knowledge on blackleg resistance in Chinese *B. napus* germplasm and the high risk of introducing *L. maculans* from imported canola seeds into China, the Chinese government

restricted the importation of canola seeds from Canada (Fernando et al. 2016). Although some earlier studies evaluated blackleg resistance of Chinese *B. napus* cultivar/lines, only a limited number of plant materials were included in these studies, and the resistance of Chinese *B. napus* cultivars to the Canadian population of *L. maculans* was unknown.The second objective was to evaluate blackleg resistance in a collection of Chinese *B. napus* germplasm. This would allow the identification of *R* genes and APR (field conditions) in Chinese *B. napus* germplasm to facilitate blackleg resistance breeding to assist mitigating the risk of blackleg caused by introduced *L. maculans* when it happens. The third objective was to understand defense mechanisms in the *B. napus-L. maculans* pathosystem. This involved temporal and spatial comparison of differences in defense responses between compatible and incompatible *B. napus-L. maculans* interactions, using high-throughput RNA-Seq, rigorous bioinformatics, laser microdissection, and histological analysis. Thus, the advances in sequencing technology will enable the identification of a large number of candidate genes and regulatory pathways involved in disease resistance in the *B. napus-L. maculans* pathosystem at the transcriptome level.

BREAKDOWN OF *RLM3* RESISTANCE IN THE *BRASSICA NAPUS–LEPTOSPHAERIA MACULANS* PATHOSYSTEM IN WESTERN CANADA

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3 BREAKDOWN OF *RLM3* RESISTANCE IN THE *BRASSICA NAPUS-LEPTOSPHAERIA MACULANS* PATHOSYSTEM IN WESTERN CANADA

3.1 Abstract

Blackleg disease, caused by the fungal pathogen *Leptosphaeria maculans*, is a serious disease of *Brassica napus*. The disease is mainly controlled by genetic resistance and crop rotation. However, *L. maculans* has displayed a high evolutionary potential to overcome major resistance genes in *B. napus*. This study aimed to analyze the major-gene and adult-plant resistance (APR) of Canadian *B. napus* varieties/lines (accessions) and the avirulence allele frequency in *L. maculans* populations in western Canada. For resistance identification, a set of *L. maculans* isolates with known avirulence genes were used to characterize major resistance (*R*) genes in 104 Canadian *B. napus* accessions and 102 seed samples collected from growers' fields; with 104 *B. napus* accessions further evaluated for APR under controlled conditions. In addition, avirulence genes of 300 *L. maculans* isolates collected from infected canola stubbles in growers' fields were determined by cotyledon inoculation and gene-specific PCR assays. The results indicated that *R* genes were present in the majority of these *B. napus* accessions, with the *Rlm3* gene being predominant while other *R* genes rarely detected. APR was identified in more than 50% of the accessions. Predominance of *Rlm3* in 102 seed samples from growers' fields suggested *Rlm3*-carrying *B. napus* varieties were currently widely used in western Canada. Avirulence allele frequency identification of field *L. maculans* isolates revealed the scarcity of the avirulence allele towards *Rlm3, AvrLm3*.

This indicated the breakdown of *Rlm3* resistance, which could be due to the over use of this single resistance gene in Canadian *B. napus* germplasm.

3.2 Introduction

Canola (oilseed rape, *Brassica napus*) is one of the major oilseed crops of the world. Blackleg, caused by the fungal pathogen *Leptosphaeria maculans*, is a serious disease of canola in North America, Australia, Europe and many other regions around the world (Fitt et al. 2006). The disease can be controlled by crop rotation, fungicide application as well as the use of resistant varieties (West et al. 2001; Fitt et al. 2006). As an environmentally friendly strategy, genetic resistance is generally very effective in disease control. Both seedling resistance controlled by major or seedling *R* genes and adult plant resistance (APR) mediated by quantitative resistance (minor) genes to *L. maculans* have been identified in *B. napus* varieties (Pongam et al. 1998; Balesdent et al. 2001; Pilet et al. 1998, 2001; Jestin et al. 2011, 2015). *R* genes confer race-specific resistance and follow the gene-for-gene concept proposed by Flor (1971). To date, at least 18 major *R* genes against *L. maculans* have been identified in *Brassica* species: *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* from *B. napus*, which have been mapped to two *B. napus* linkage groups, N7 and N10 (Ferreria et al. 1995; Mayerhofer et al. 1997; Ansan Melayah et al. 1998; Zhu and Rimmer 2003; Rimmer 2006; Delourme et al. 2006); *Rlm8* and *Rlm11* from *B. rapa* (Balesdent et al. 2002, 2013); *Rlm5* and *Rlm6* from *B. juncea* (Chèvre et al. 1997; Balesdent et al. 2002); *Rlm10* from *B. nigra* (Chèvre et al. 1996; Eber et al. 2011); *LepR1*, *LepR2*, *LepR3*, *LepR4* and *RlmS* from re-synthesized *B. rapa subsp. sylvestris* (Yu et al. 2005, 2007, 2008; Van de Wouw et al. 2009); and *BLMR1* and *BLMR2* from Surpass 400 (Long et al. 2011). To date, two *R* genes, *LepR3* (that interacts with

AvrLm1) and *Rlm2*, have been cloned (Larkan et al. 2013, 2015). By contrast, at least seven of the corresponding avirulence (Avr) genes have been cloned: *AvrLm1* (Gout et al. 2006b), *AvrLm2* (Ghanbarnia et al. 2014), *AvrLm3* (Plissonneau et al. 2016), *AvrLm5/AvrLmJ1* (Van de Wouw et al. 2014a; Balesdent & Howlett unpublished data), *AvrLm4-7* (Parlange et al. 2009), *AvrLm6* (Fudal et al. 2007), and *AvrLm11* (Balesdent et al. 2013).

The cotyledon inoculation assay has been used to identify resistance to *L. maculans* (Williams and Delwiche 1979; Rimmer and van den Berg 1992; Rouxel et al. 2003b; Marcroft et al. 2012a). The characterization of *R* genes in a given canola variety can be achieved by analyzing its interactions with a set of *L. maculans* isolates carrying known avirulence genes. Based on reactions to isolates with known avirulence alleles, Rouxel et al. (2003b) deduced race-specific resistance genes to blackleg in accessions of *B. napus* mainly originating from Europe. Marcroft et al. (2012a) identified seedling resistance genes in Australian *B. napus* varieties using *L. maculans* isolates harbouring known avirulence genes. In Canada, blackleg resistance breeding programs have successfully developed resistant varieties for commercial release. However, *R* genes for blackleg resistance in Canadian *B. napus* varieties are unknown (Rimmer 2006).

Both seedling and adult plant resistance play important roles in blackleg control. It has been shown that a combination of major gene resistance and adult plant resistance can provide effective and durable resistance against blackleg (Kiyosawa 1982; Brun et al. 2010). Selection of blackleg resistant breeding materials is usually based on field evaluations without genetic characterization of *R* genes (Rouxel et al. 2003b). Moreover, the interaction

between specific *R* genes and their corresponding avirulence genes in the seedling stage normally results in low disease severity at the adult plant stage. Therefore, it is difficult to dissect blackleg resistance evaluated under field conditions into major gene resistance and/or adult plant resistance. To develop varieties with a combination of seedling resistance and adult plant resistance, it is necessary to characterize *R* genes in breeding lines and then evaluate APR by reducing the interference of *R* genes.

Large-scale utilization of single gene resistance sources in commercial fields will exert strong selection pressure on *L. maculans* populations through the co-evolution of host and pathogen. In France, the increased commercial use of *Rlm1* resistance resulted in a rapid decrease of the proportion of isolates carrying *AvrLm1* (Rouxel et al. 2003a). Similarly, 'sylvestris' resistance in Australia was overcome within three years after commercial release of the cultivar (Sprague, et al. 2006; Van de Wouw, et al. 2010). It has been reported that pathogenicity of *L. maculans* populations changed over time in western Canada. In early studies, *L. maculans* isolates were classified into pathogenicity groups (PGs) based on the interaction phenotypes (IP) of the isolate on a few *B. napus* varieties. The majority of *L. maculans* isolates collected during 1984-2000 in western Canada were classified as PG2 (Kutcher et al. 1993; Chen and Fernando 2006). Keri et al. (2001) and Kutcher et al. (2007) observed additional PGs (PG3 and PGT) from collections between 1998 and 2004. Chen and Fernando (2006) observed more aggressive isolates (PG4) in 2002-2004 collections. Kutcher et al. (2010b) also reported changes in the population structure of *L. maculans* in western Canada, which was believed to be the result of the use of specific *R* gene(s). Liban et al. (2016) provided further evidence of this when they reported a shift in avirulence allele

frequency in isolates collected in 2010 and 2011. The association between the specific *R* gene(s) in canola varieties as mentioned by Kutcher et al. (2010b) and the corresponding avirulence gene(s) in *L. maculans* populations can be revealed by investigating *R* genes in canola varieties and avirulence allele frequencies in field fungal populations.

The objectives of this study were to characterize *R* genes and evaluate adult plant resistance of Canadian *B. napus* varieties/lines. Furthermore, *R* genes in seed samples collected from growers' fields were characterized to investigate the proportion of *R* genes used in the fields. Additionally, avirulence alleles of *L. maculans* populations were assessed to understand the effectiveness of *R* genes identified in Canadian canola cultivars.

3.3 Materials and Methods

3.3.1 *Brassica napus* varieties/lines and seed sample collection

B. napus varieties/lines with known *R* genes were considered as differentials and used to characterize avirulence genes in *L. maculans* isolates. The *B. napus* differentials used in this study are listed in Table 3.1. A total of 104 *B. napus* varieties/lines, which will be referred to as *B. napus* accessions, included commercial varieties released since 1980's and advanced breeding lines, were kindly provided by commercial seed companies and research institutions. These 104 *B. napus* accessions were used to investigate *R* genes in Canadian *B. napus* germplasm. A collection of 102 *B. napus* seed samples were directly collected from different growers' fields across Manitoba in 2012. These 102 seed samples were used to determine the proportion of *R* genes used in the fields. Of 102 seed samples, 35 were from fields where canola stems were collected and *L. maculans* isolates were identified in this

study. These 35 samples were considered a subset of field seed samples.

Table 3.1 *Brassica napus* varieties/lines used as differentials to identify avirulence genotypes of *Leptosphaeria maculans* isolates.

Variety/line	Resistance genes	Reference
Darmor	*Rlm9*	Delourme et al. 2004
MT29	*Rlm1, Rlm9*	Delourme et al. 2008
Falcon	*Rlm4*	Rouxel et al. 2003b
Cooper	*Rlm1, Rlm4*	Dilmaghani et al. 2009
Samourai	*Rlm2, Rlm9*	Rouxel et al. 2003b
01-23-2-1	*Rlm7*	Dilmaghani et al. 2009
Quinta	*Rlm1, Rlm3*	Kutcher et al. 2010b
Surpass 400	*LepR3, RlmS*	Larkan et al. 2013
1065	*LepR1*	Kutcher et al. unpublished
Verona	*Rlm2, Rlm4*	Kutcher et al. 2010b
1135	*LepR2*	Kutcher et al. unpublished
Columbus	*Rlm1, Rlm3*	Balesdent et al. 2002
Jet Neuf	*Rlm4*	Gout et al. 2006a
Goéland	*Rlm9*	Balesdent et al. 2006
Bristol	*Rlm2, Rlm9*	Balesdent et al. 2005
02-22-2-1	*Rlm3*	Gout et al. 2006a
Westar	No resistance gene	Balesdent et al. 2002

3.3.2 *Leptosphaeria maculans* isolates and canola stem collection

Isolates of *L. maculans* previously characterized for avirulence genes were used to identify *R* genes in canola varieties/lines; these were referred to as differential isolates. A total of 12 isolates (D1-D10, D13, and D14) were provided and previously characterized by scientists at the University of Melbourne to identify 10 avirulence genes (*AvrLm1-AvrLm9* and *AvrLmS*). The avirulence genotypes of *AvrLm1-9* and *AvrLmS* in isolates D8, D9, D13 and D14 were described in Marcroft et al. (2012a). In addition, seven *L. maculans* isolates (ICBN14, PHW1223, JN2/v23.1.2, JN3/v23.1.3, S7, R2, and AD746) were characterized previously. The genotypes of *AvrLm1-AvrLm9* in 4 isolates (ICBN14, PHW1223, and JN2/ v23.1.2) were

described in Balesdent et al. (2005), in isolates R2 and S7 were described in Leflon et al. (2007), and in isolate JN3/v23.1.3 was described in Balesdent et al. (2013). Genotypes of *AvrLepR1* and *AvrLepR2* in these 19 differential isolates were characterized in this study. Genotypes of some *Avr* genes in these 19 differential isolates were further confirmed in this study, using differential varieties/lines listed in Table 3.1. Additional isolates were collected from western Canada and characterized in this study. The *L. maculans* differentials used in this study are described in Table 3.2. Canola stems collected after harvest were randomly sampled from 37 growers' canola fields across Manitoba in 2012. Seed samples of 35 of these fields were collected and included in 102 seed samples that were used to determine the proportion of *R* genes used in the fields, whereas seed samples were not available for two fields.

Table 3.2 Avirulence genotypes of *Leptosphaeria maculans* differential isolates.

Isolates	Avirulence genotypes											
	AvrLm1	*AvrLm2*	*AvrLm3*	*AvrLm4*	*AvrLm5*	*AvrLm6*	*AvrLm7*	*AvrLm8*	*AvrLm9*	*AvrLmS*	*AvrLepR1*	*AvrLepR2*
D1	-[a]	+[a]	-	-	+	+	-	-	+	+	+	+
D2	-	-	-	-	+	+	-	+	-	+	+	-
D3	-	-	-	-	+	-	-	-	-	-	+	-
D4	-	-	-	+	+	+	+	+	-	-	+	+
D5	+	+	-	+	-	-	+	-	-	+	+	+
D6	+	-	-	-	+	+	-	+	-	+	-	-
D7	+	-	+	-	+	+	-	+	-	nd	+	-
D8	-	-	-	-	+	-	+	nd	-	-	+	-
D9	-	-	-	-	+	+	+	nd	-	-	+	-
D10	-	-	-	-	+	+	-	+	+	+	-	-
D13	-	-	-	+	nd[b]	+	+	nd	-	-	-	-
D14	+	-	-	-	nd	-	+	nd	-	+	+	-
S7	+	-	-	-	+	+	+	nd	-	nd	+	-
ICBN14	-	-	-	-	+	+	-	-	-	nd	+	-
PHW1223	-	-	-	-	+	+	-	+	+	nd	-	-
R2	-	-	-	-	+	-	+	nd	-	nd	+	-
AD746	-	-	+	-	−	+	-	nd	-	nd	+	-
JN2	-	-	-	-	+	+	+	+	-	nd	+	-
JN3	+	-	-	+	+	+	+	+	-	nd	-	-
J3	-	+	+	-	+	+	-	nd	-	+	-	-
J20	-	+	+	-	−	+	-	nd	-	+	+	-
Q12	-	+	-	+	+	-	+	nd	-	-	+	-
L-MD7-14	-	-	-	+	+	+	+	nd	-	-	-	-
L-PC4-1	-	+	-	+	−	-	-	nd	-	-	-	-
L-MP1-8	-	+	-	+	+	+	+	nd	-	-	-	-
L-Sb1	-	+	+	-	+	+	+	nd	-	+	-	-
L-MP1-6	-	-	-	+	+	+	+	nd	-	-	-	-
L-Sb7-6	-	-	-	+	+	+	+	nd	-	-	+	-
L-Br17-1	-	-	-	nd	+	+	+	nd	-	-	+	-
L-Mo5-1	-	+	-	+	+	+	+	nd	-	-	-	+
L-Br1-16	+	-	-	+	+	+	+	nd	-	nd	-	-
L-RL25	-	-	-	-	+	+	+	nd	-	+	-	-
L-DS103	-	-	-	-	+	-	-	nd	+	-	-	-
L-CV8-7	-	+	-	+	+	+	+	nd	-	+	-	-

[a] +/- indicates the presence/absence of a specific avirulence gene.
[b] nd indicates the genotype was not determined.

3.3.3 *Leptosphaeria maculans* isolation, inoculum preparation and DNA extraction

A total of 300 *L. maculans* isolates from 37 growers' canola fields were collected from blackleg infected canola stems. *L. maculans* isolates isolation and characterization was performed as described by Chen and Fernando (2006) with some modification: the stems were surface disinfected with 5% bleach treatment for 1 min, and V8® agar medium was amended with 0.35% (w/v) streptomycin sulfate. All *L. maculans* isolates were stored as pycnidiospores at -20℃ on small sterile filter paper discs in 1.5 mL centrifuge tubes for further use.

Fungal inoculum was prepared according to Chen and Fernando (2006). The concentration of spores was diluted to a final spore concentration of 2×10^7 spores mL^{-1}. DNA was extracted from fungal mycelium according to Calderon et al. (2002) with some modification. Briefly, fungal mycelium was homogenised using 0.2 mm ceramic beads for 45 sec at 6500 rpm in a Precellys® 24 homogenizer (Bertin Technologies, France) before DNA extraction.

3.3.4 Characterization of avirulence genotypes of *Leptosphaeria maculans* isolates

Cotyledon inoculation and gene-specific PCR assays were used to identify avirulence genotypes of the *L. maculans* isolates. In the cotyledon inoculation assay, *B. napus* differentials used to confirm/characterize the *L. maculans* differential isolates were Westar (no known resistance gene, susceptible check), Darmor, MT29, Falcon, Cooper, Samourai, 01-23-2-1, Quinta, Surpass 400, 1065, Verona, 1135, Columbus, Jet Neuf, Goéland, Bristol, and 02-22-2-1 (Table 3.1). Avirulence genotyping of 300 *L. maculans* isolates collected from

the field was performed with 11 *B. napus* differential varieties/lines: Westar, Quinta, Bristol, Jet Neuf, 01-23-2-1, Goéland, 1065, 1135, 02-22-2-1, Surpass 400, and MT29 (Table 3.1).

In the cotyledon inoculation assay, plant material were seeded in a growth chamber at 16℃ (night) and 21℃ (day) with a 16-h photoperiod. Cotyledons of seven-day-old seedling were punctured with a modified tweezer and inoculated with a 10-µL droplet (2×10^7 spores mL^{-1}) of inoculum (four inoculation sites per plant). Inoculated cotyledons were air dried for at least 12 hours before watering. Each isolate was inoculated onto at least 8 different plants of each variety. Symptoms on the cotyledons were scored 14 days post inoculation (dpi) using the rating scale of 0-9 (Williams and Delwiche 1979; Appendix IV). The average rating score (ARS) was calculated from 32 inoculation sites: ARS 6.1-9.0 was considered susceptible (S), ARS 4.6-6.0 intermediate (I) and ARS ≤4.5 resistant (R). When intermediate reactions were observed the assay was repeated to confirm the scoring.

Polymerase chain reaction (PCR) characterization of six cloned avirulence (Avr) genes in *L. maculans* isolates collected from growers' field (2012) was performed: *AvrLm1* (Gout et al. 2006b), *AvrLm2* (Ghanbarnia et al. 2015), *AvrLmJ1/AvrLm5* (Van de Wouw et al. 2014a, Balesdent & Howlett unpublished data), *AvrLm4-7* (Parlange et al. 2009), and *AvrLm6* (Fudal et al. 2009) and *AvrLm11* (Balesdent et al. 2013). *HaeIII* enzyme (GG^CC) was used to digest the PCR product of *AvrLm4*-7 to detect the SNP mutation of C^{358} to G^{358} that leads to virulence against *Rlm4*. The avirulence/virulence of *AvrLm1*, *AvrLm2*, *AvrLmJ1, AvrLm6,* and *AvrLm11* were decided by presence/absence of the corresponding PCR products. The avirulence genotypes of *AvrLm1*, *AvrLm2,* and *AvrLm4* in 300 isolates were a combination of

PCR assay and differential test results. The genotypes of *AvrLmJ1/AvrLm5*, *AvrLm6*, and *AvrLm11* were only determined by gene-specific PCR assay as we do not have access to any differential varieties that can identify the presence/absence of these three genes.

3.3.5 Characterization of *R* genes in *Brassica napus* varieties/lines

A total of 206 *B. napus* accessions/seed samples were collected for *R* gene characterization, and two trials were performed. Trial I included 104 Canadian *B. napus* accessions. A set of 22 ((D1-D10, D13, D14, S7, ICBN14, PHW1223, R2, AD746, JN2, JN3, J3, J20 and Q12); Table 3.2) differential isolates, which were able to detect 12 major blackleg *R* genes (*Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, RlmS, LepR1, LepR2*), were used to characterize *R* genes in these accessions. Three canola accessions (DF78, DF79 and DF80) were resistant to 21 differential isolates and as a result the *R* genes they carried were difficult to postulate. Thus, twelve more *L. maculans* isolates from Canada (L-MD7-14, L-PC4-1, L-MP1-8, L-Sb1, L-MP1-6, L-Sb7-6, L-Br17-1, L-Mo5-1, L-Br1-16, L-RL25, L-DS103 and L-CV8-7) were used to further detect *R* genes in these three accessions (Table 3.2). Trial II included 102 canola seed samples collected from different growers' fields in Manitoba in 2012. This analysis identified the *R* genes present in the canola varieties grown by Manitoba growers in 2012. For this trial, a set of 11 *L. maculans* differentials (D3, D4, D5, D7, D10, AD746, JN3, J3, ICBN14, PHW1223 and R2) were used (Table 3.2). In trial II, 35 seed samples were collected from fields where blackleg infected stems were collected and analyzed in this study.

In both trials, methods for inoculum and plant preparation, inoculation and disease

evaluation followed the same methods as described in the avirulence gene characterization section; however, at least 12 different plants were used for each isolate-variety/line combination. Due to the genetic heterogeneity of seed samples collected from the field and some canola varieties/lines, the percentage of resistant reactions (rating scores 0, 1, 3) was calculated from inoculation sites. When the percentage of resistant reactions was over 50% but less than 100%, genetic heterogeneity was considered as the major cause of the variation and the variety was considered resistant. The *R* genes were postulated based on the gene-for-gene theory. For example, if a variety was resistant to all differential isolates that carried *AvrLm3*, but was susceptible to all isolates carrying *avrLm3*, the *R* gene deduced to be present in this variety was *Rlm3*.

3.3.6 Adult plant resistance evaluation

Adult plant resistance of 104 Canadian *B. napus* accessions provided by companies and research institutions were evaluated under controlled conditions, where cv. Westar was used as a susceptible check. Among differential isolates, D3 infected 101 accessions and caused lesions on the cotyledons as early as 12 dpi. Three accessions, DF78, DF79 and DF80 were resistant to isolate D3 but susceptible to isolate D13. To reduce the interference of seedling resistance during adult plant resistance evaluation, isolate D13 was used to inoculate accessions DF78, DF79 and DF80, and isolate D3 was used to inoculate the other 101 accessions. Seeds were directly seeded into plastic pots (18 cm in diameter), and inoculated with a single *L. maculans* isolate. The experiment was a completely randomized design of three replicates, each with 9 plants. The inoculation methods and spore concentration were

the same as for the *R* gene identification. Seedling infection was observed 14 days after inoculation and plants without visible symptoms were removed. Infected plants were grown to maturity and evaluated for their blackleg resistance by inspecting internal infection on the cross-section of the crown. Disease severity of basal stems was scored on a 0 - 5 rating scale (Western Canada Canola/Rapeseed Recommending Committee (WCC/RRC); Appendix V): 0 - no noticeable infection, 1 - diseased tissue occupies ≤ 25% of the cross-section, 2 - diseased tissue occupies 25-50% of cross-section, 3 - between 50–75% of the cross-section infected, 4 - more than 75% of the cross-section infected, 5 - 100% of cross-section were diseased, plant dead.

The blackleg resistance category system used was based on relative disease severity: the percentage of the mean disease severity of a canola line was assessed as a proportion of the susceptible cv. Westar. Relative disease severity scores of ≤35% were considered resistant (R), 35-50% as moderately resistant (MR), 51-65% as moderately susceptible (MS), and 66-100% as susceptible (S).

3.3.7 Data analysis

Excel 2010 was employed for data recording and preliminary analysis. APR data analysis was carried out using analysis of variance (ANOVA) in SAS 9.1. Relative disease severity was root square transformed before ANOVA for normal distribution of data. Diversity of *L. maculans* populations were analysed with two indices: the Margalef index (which measures the richness in species/races of a population) and the Simpson index of diversity (Balesdent et al. 2006).

3.4 Results

3.4.1 Prevalence of *Rlm3* in Canadian canola varieties/lines

In Experiment I, 85% of the accessions showed seedling resistance. A total of eight known *R* genes (*Rlm1, Rlm2, Rlm3, Rlm4, Rlm9, RlmS, LepR1 and LepR2*) were detected (Fig. 3.1, Table 3.4, Appendix VI). However, 16 accessions were susceptible to all *L. maculans* differential isolates and therefore no *R* gene was detected for these accessions. Some of the accessions carried uncharacterized resistance genes that could not be deduced using the 22 differential isolates (Fig. 3.1, Table 3.4). This type of resistance was considered as unknown resistance in this study. The presence of unknown *R* gene resistance might be due to the effect of a novel *R* gene, other known *R* genes that were not tested in this study, or a combination of a few *R* genes. Among the *R* genes detected, *Rlm3* was present in 59 accessions, followed by *Rlm1* in 5 accessions, and *Rlm2* in 5 accessions. Both *Rlm9* and *LepR1* were detected in three accessions, while *Rlm4* was present in two accessions, and *RlmS* in two accessions as well. *LepR2* appeared to be present in only one accession. In addition, some of the other *R* genes such as *Rlm5* and *Rlm8* might be present in some accessions, however further confirmation is required. Although we do not have access to variety names of the majority of the 104 accessions tested in this study, variety names of 6 accessions developed and provided by the University of Alberta, and 11 accessions developed by the University of Manitoba were available and were described in Table 3.3. Although *R* genes in Q2, Quantum, Conquest, and Hi-Q have been previously characterized (Kutcher, Personal communication), this study confirmed their *R* genes and the results were consistent with the previous study. All six varieties (Conquest, Hi-Q, Q2, Quantum, Cougar CL, Peace) developed by the University of

Alberta were released during 1995-2001 and carried *Rlm3*. Of 11 varieties developed by the University of Manitoba, one released in 1995, two released in 2008 and 2012, respectively, carried *Rlm3,* four carried unknown resistance, and four did not carry any *R* gene. The results indicated that *Rlm3* was available in commercial varieties in the early 1990s.

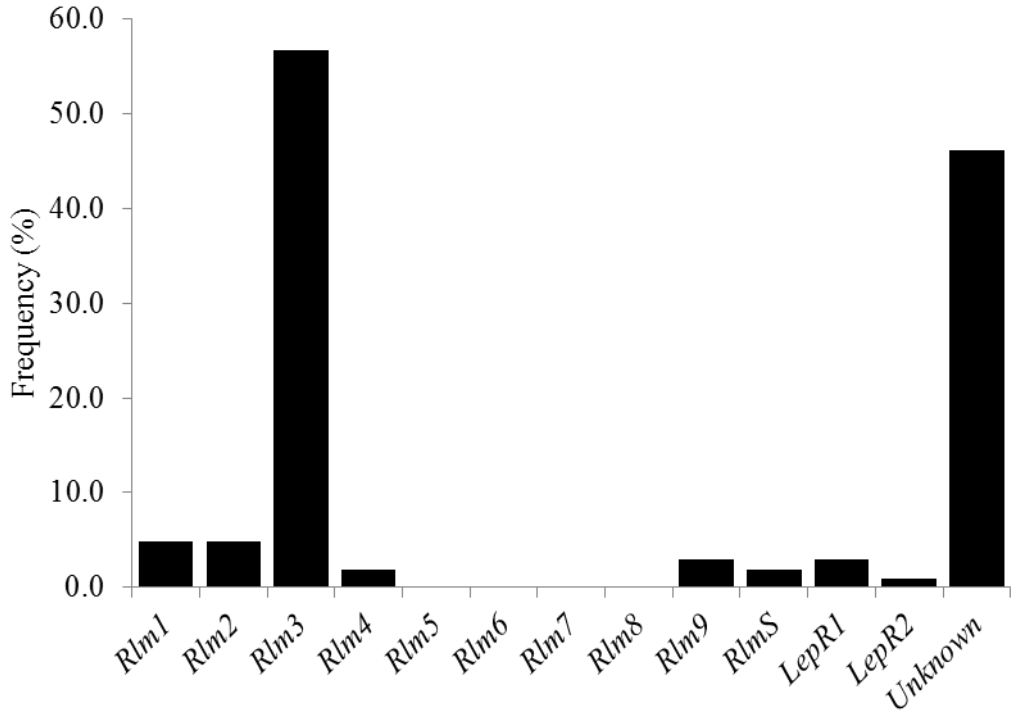

Fig. 3.1 Percentage of *R* genes in 104 Canadian *Brassica napus* accessions. Unknown means the resistance genotype could not be determined in this study, accessions that carried unknown *R* genes might be due to the presence of a new *R* gene, other known *R* genes that were not tested in this study, or combinations of several *R* genes. *Rlm5* and *Rlm8* might be present in some accessions, but further confirmation is required. *Rlm6* and *Rlm7* were not detected in the accessions tested.

Table 3.3 *R* genes and adult plant resistance of 17 Canadian *B. napus* accessions with known variety names.

Accession	Variety	Year released	*R* genes[a]	APR[b]
DF-1	Stellar	1987	None	MS
DF-2	Apollo	1990	None	R
DF-3	Allons	1995	*Rlm3* (H)	S
DF-4	Reward	1991	Unknown	S
DF-5	Sentry	1996	Unknown	S
DF-6	Hero	1989	None	MS
DF-7	MillenniUM 03	2000	None	MR
DF-8	Red River 1826	2006	Unknown (H)	MR
DF-9	Red River 1852	2006	Unknown (H)	MS
DF-10	Red River 1997	2008	*Rlm3*	MR
DF-11	Red River 1861	2012	*Rlm2* (H), *Rlm3*, unknown (H)	R
DF-12	Conquest	2000	*Rlm3*	S
DF-13	Hi-Q	1999	*Rlm3*	MR
DF-14	Q2	1998	*Rlm3*	R
DF-15	Quantum	1995	*Rlm3*	R
DF-16	Cougar CL	2003	*Rlm3*, unknown (H)	R
DF-17	Peace	2001	*Rlm3*	S

DF1-11 were developed by the University of Manitoba, DF12-17 were developed and provided by the University of Alberta.

[a] None refers to the absence of *R* gene resistance. Unknown means the resistance genotype could not be determined in this study, accessions that carried unknown *R* genes might be due to the presence of a new *R* gene, other known *R* genes that were not tested in this study, or combinations of several *R* genes. (H) refers to accessions with heterogeneous seeds whereby *R* gene was detected in 50-80% of the plants.

[b]APR refers to adult plant resistance, R-resistant, MR-moderately resistant, MS-moderately susceptible, S-susceptible.

Table 3.4 Summary of *R* genes and adult plant resistance in 104 Canadian *B. napus* accessions.

Resistance type	Resistance[a]	No. of accessions	Percentage (%)
R gene resistance	*Rlm1*	1	1.0
	Rlm3	32	30.8
	Rlm4	1	1.0
	LepR2	1	1.0
	Rlm3, Unknown	18	17.3
	Rlm3, *LepR1*	1	1.0
	Rlm1, *Rlm2*, *Rlm3*	3	2.9
	Rlm1, *Rlm3*, *Rlm9*	1	1.0
	Rlm2, *Rlm3*, Unknown	1	1.0
	Rlm2, *Rlm3*, *Rlm4*	1	1.0
	Rlm3, *Rlm9*, *RlmS*, *LepR1*	2	1.9
	Unknown	26	25.0
	None	16	15.4
Adult plant resistance (APR)	Resistant (R)	41	39.4
	Moderately resistant (MR)	17	16.3
	Moderately susceptible (MS)	17	16.3
	Susceptible (S)	29	27.9

[a] Unknown means the resistance genotype could not be determined in this study, accessions that carried unknown *R* genes might be due to the presence of a new *R* gene, other known *R* genes that were not tested in this study, or combinations of several *R* genes. None refers to the absence of *R* gene resistance.

Among 104 accessions, a total of 35 accessions carried a single *R* gene, including *Rlm1* in one accession, *Rlm3* in 32 accessions, *Rlm4* in one accession, and *LepR2* in one accession. A total of 19 accessions carried two resistance genes, and/or a combination of a known resistance gene and an unknown resistance gene or genes, such as *Rlm3* and *LepR1* or *Rlm3* and an unknown *R* gene. Eight *B. napus* accessions carried three or more *R* genes. A total of 26 *B. napus* accessions carried only unknown *R* genes. The rest 16 accessions were susceptible to all 22 differential isolates and did not carry any *R* gene (Table 3.4).

In Experiment II, *R* gene was present in 58% of seed samples. Only three *R* genes (*Rlm1*,

Rlm2, and *Rlm3*) were detected. Among the 102 seed samples, 50 carried *Rlm3*, three carried *Rlm2*, and two carried *Rlm1* (Appendix VII). An unknown *R* gene or genes were detected in 9 seed samples. Most seed samples showing seedling resistance carried single *Rlm3*. Seed samples from only three fields carried more than one *R* gene: BR1: *Rlm2, Rlm3*; BR5: *Rlm2*, *Rlm3*; BR21: *Rlm1*, *Rlm2*, *Rlm3*. Surprisingly, 43 seed samples carried none of the 12 *R* genes that could be detected using the *L. maculans* differential isolates in this study.

For the subset of 35 seed samples, 14 carried single *Rlm3*, one carried single *Rlm1*. Two seed samples each carried two *R* genes: *Rlm2* and *Rlm3*. Unknown *R* gene resistance was identified in 4 seed samples. *R* gene was not detected in 14 seed samples.

Our results clearly indicated that *Rlm3* was the major *R* gene prevalent in Canadian canola varieties and germplasm.

3.4.2 Adult plant resistance evaluation under controlled environment

In this study, the resistance observed in the APR evaluation of 104 *B. napus* accessions was assumed to be mediated by adult plant resistance genes since none of the varieties/lines tested showed seedling resistance after infection. Disease severity of 104 accessions ranged from 0 to 4.8 (0-5 scale). More than 50% of *B. napus* accessions showed disease severity of lower than 2. Five accessions showed disease severity of higher than 4 (Fig. 3.2). Significant differences of the relative disease severity among accessions were observed ($F=8.30$, $p<0.0001$). APR evaluation indicated that 58 accessions were either resistant or moderately resistant to blackleg, while the rest of the accessions were susceptible or moderately susceptible at the adult plant stage (Table 3.4). Among 58 accessions that exhibited APR, 50

also had *R* gene resistance at seedling stage, and eight exhibited only APR. Of 46 susceptible or moderately susceptible accessions, 8 were susceptible to all differential isolates at the seedling stage, and all others carried seedling resistance. In summary, a large proportion of *B. napus* accessions had both adult plant resistance and seedling resistance due to an *R* gene (most commonly *Rlm3*).

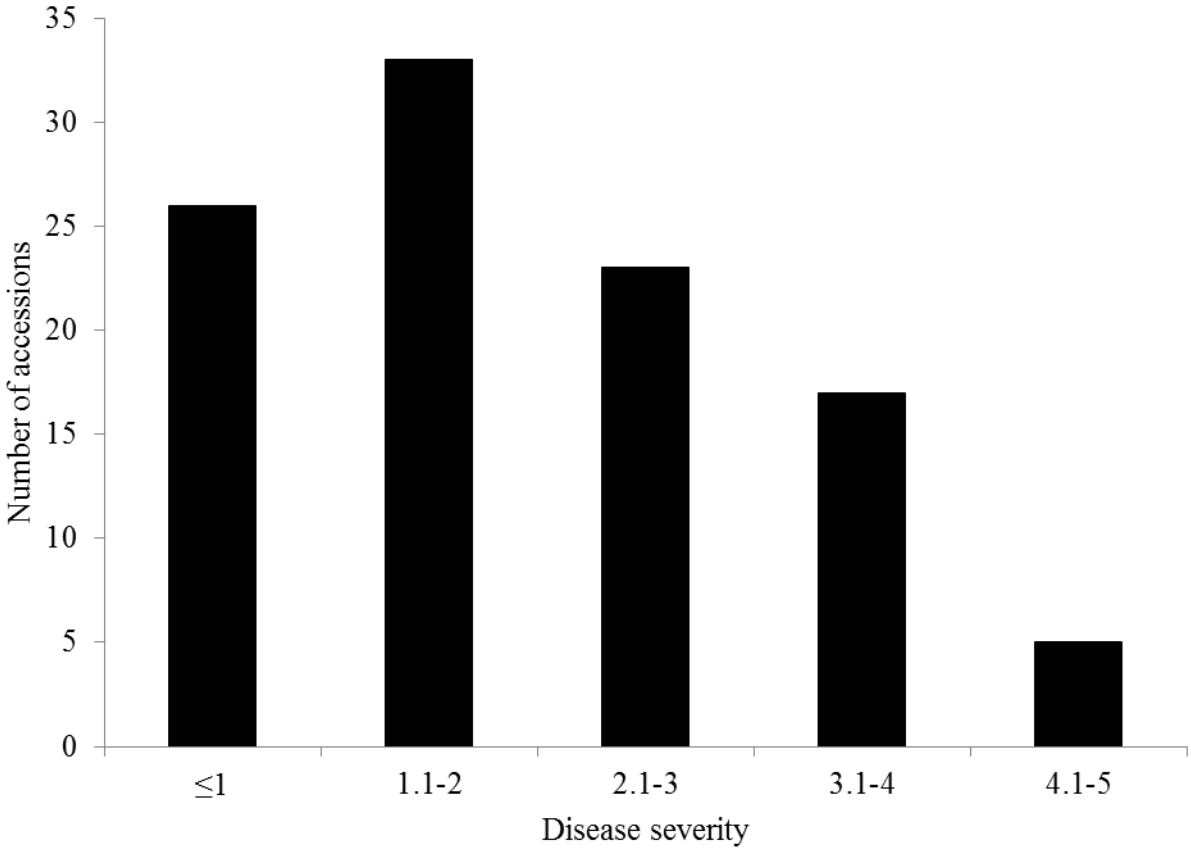

Fig. 3.2 Frequency distribution of disease severity for adult plant resistance of 104 *B. napus* accessions evaluated under controlled conditions.

3.4.3 Avirulence genotype characterization of *Leptosphaeria maculans* isolates

The avirulence genes of all isolates used as differentials in this study are listed in Table 3.2. The frequency of 12 avirulence alleles in 300 *L. maculans* isolates collected from fields were identified (Fig. 3.3). Among the 12 avirulence alleles, the frequency of *AvrLm3* was the

lowest (2.7%) and was detected in only 8 isolates. Five of the *AvrLm3*-carrying isolates were from Brandon, two were from Morden, and one from Morris. The avirulence alleles of *AvrLm9* and *AvrLepR2* were detected in 3.3% and 10.7% of the isolates collection, respectively. A few avirulence genes were detected in higher frequency: *AvrLm1*, 22.0%; *AvrLepR1*, 39.1%; *AvrLm2*, 64.3%; *AvrLm11*, 65.3% and *AvrLm6,* 66.0%. The proportion of avirulence alleles *AvrLm4*, *AvrLm5* and *AvrLm7* were the highest, which accounted for 77.1%, 80.7%, and 89.2% of the isolates collection, respectively. Only one differential variety, Surpass 400 (*RlmS, LepR3*) could be used to identify *AvrLmS*, and *LepR3* in Surpass 400 interacts with *AvrLm1*. Therefore, we were not able to identify *AvrLmS* in 73 isolates due to the presence of *AvrLm1*. The frequency of *AvrLmS* accounted for 34.4% of 227 isolates. The number of avirulence genes per isolate ranged from 2 to 9. The majority of the isolates (226) carried 5 or more avirulence alleles.

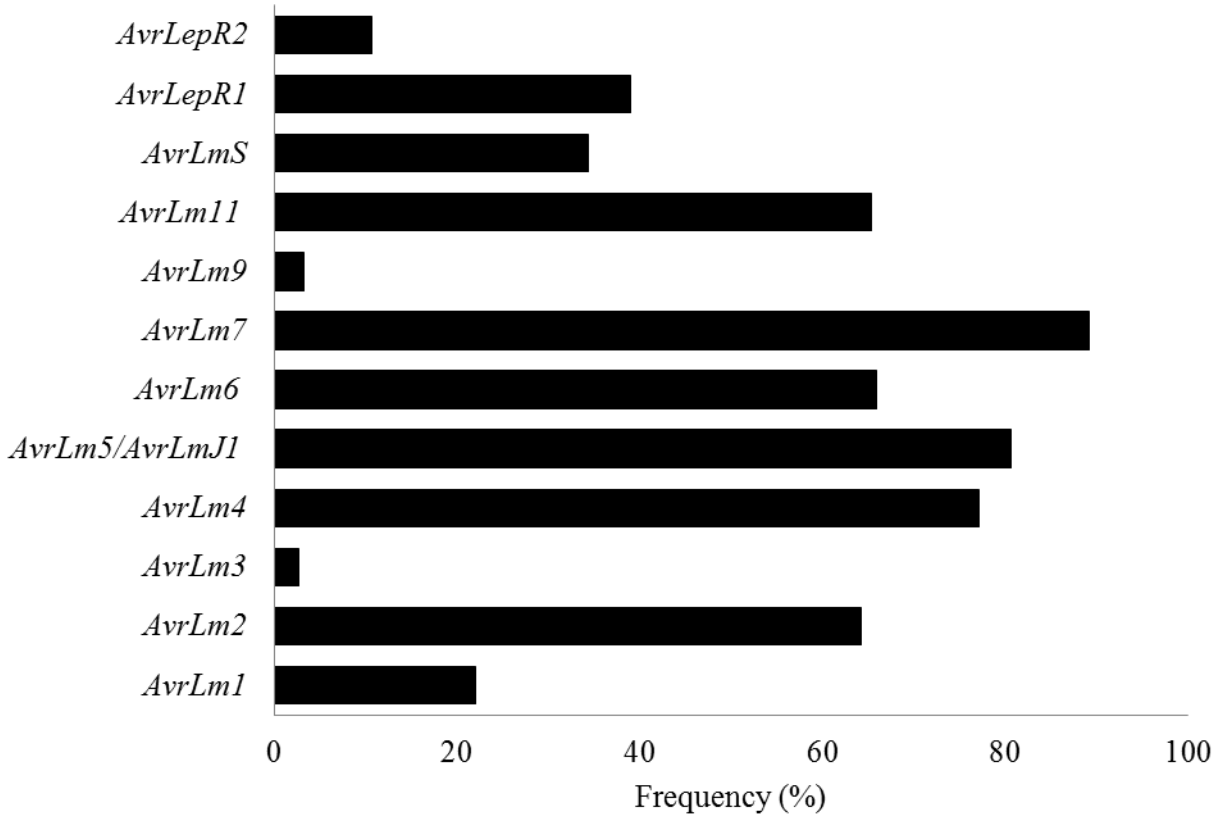

Fig. 3.3 Frequency of avirulence alleles in a collection of 300 *Leptosphaeria maculans* isolates collected in Manitoba in 2012. *AvrLmS* was assessed in 227 *L. maculans* isolates.

The race structure of *L. maculans* was assessed following the nomenclature of Balesdent et al. (2005). Combinations of 12 avirulence/avirulence alleles can produce 2^{12} (4096) races. In this study, a total of 150 races were identified and the number of isolates per race ranged from 1 to 21. A total of 43 races were comprised of more than two isolates per race (Fig. 3.4), while 107 races were represented by a single isolate. The three most frequent races were: Av 2-4-5-6-7-11, Av 1-4-5-6-7-11-(S) and Av 2-4-5-6-7-11-LepR1, which accounted for 17% of all isolates. All *AvrLm3*-carrying isolates appeared to belong to 8 different races; race structures of the 5 isolates from Brandon were: Av 1-2-3-4-6-(S)-LepR2, Av 1-3-4-9-(S)-LepR2, Av 2-3-5-LepR1-(S), Av 3-4-5-6-7-11-(S), Av 2-3-4-5-6-7-(LepR2), the

two isolates from Morden were: Av 2-3-4-7-LepR1-LepR2-(S), Av 2-3-7-LepR1-(S), and the isolate from Morris was: Av 2-3-9-LepR2.

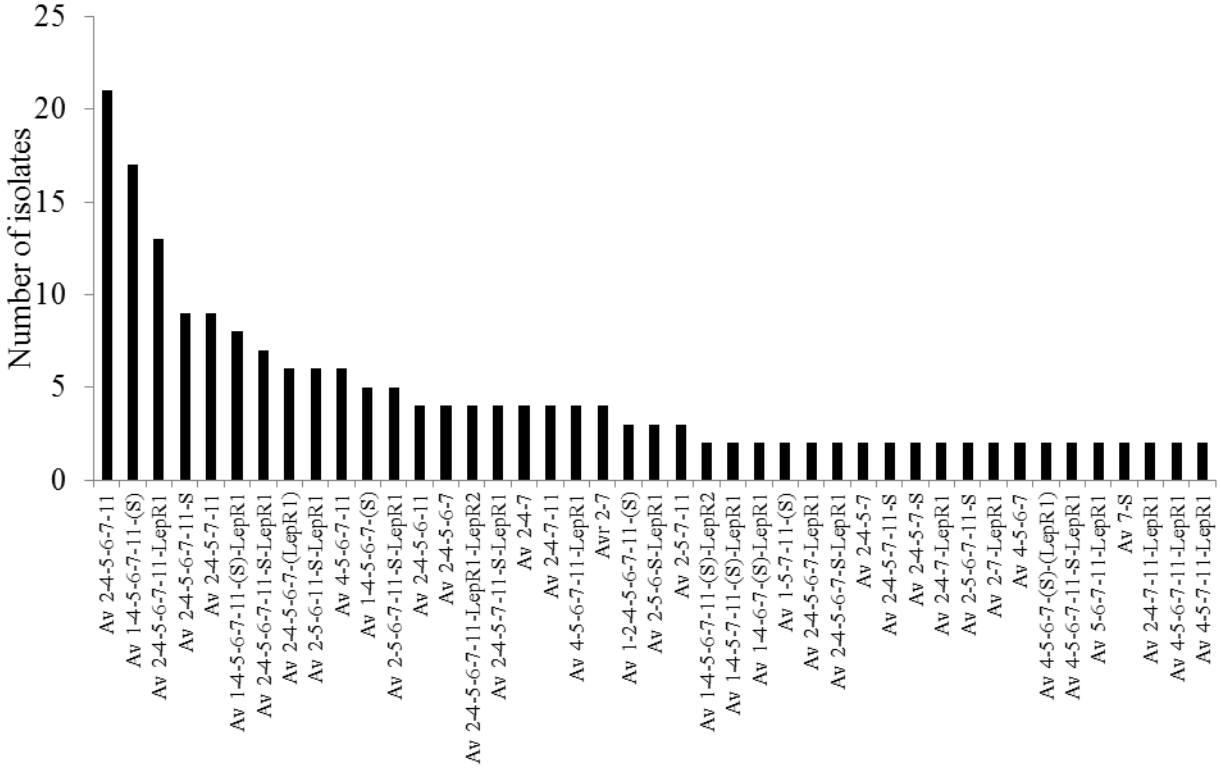

Fig. 3.4 Major races of *L. maculans* identified from 300 isolates collected in Manitoba in 2012. Race structur was identified based on 12 avirulence alleles.

The richness of the population appeared to be very high, as indicated by the Margalef index value of 26.1. Moreover, the Simpson diversity index value of 0.98 revealed that the population was quite diverse.

Frequency of Avr alleles in *L. maculans* isolates from 37 canola fields were compared (Table 3.5). Variations between fields were observed for some avirulence alleles. The frequency of *AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, and *AvrLm11* were high in the majority of fields, whereas variations between fields were observed in the frequency of *AvrLm1*, *AvrLm3*, *AvrLm9*, *AvrLmS*, *AvrLepR1*, and *AvrLepR2*. *AvrLm3* was only detected in

five fields. Among these 5 fields, seed samples from three fields did not carry any *R* gene, seed sample from one field carried unknown resistance, whereas seed samples from one field carried *AvrLm3*.

Overall, the *L. maculans* population within canola fields in Manitoba were highly variable. The *AvrLm3* allele was not present in the majority of *L. maculans* isolates. Although *Rlm3* is present in the majority of Canadian canola varieties/lines, the low frequency of the *AvrLm3* allele in the pathogen population was indicative of a breakdown of *Rlm3* resistance.

Table 3.5 *R* genes in seed samples and frequency of avirulence alleles in *L. maculans* isolates collected from 37 canola fields in Manitoba.

Fields	*R* genes [a]	No. of isolates	No. of races	*AvrLm1*	*AvrLm2*	*AvrLm3*	*AvrLm4*	*AvrLm7*	*AvrLm9*	*AvrLepR1*	*AvrLepR2*
BR1	*Rlm2*(H), *Rlm3*(H)	17	15	17.6	82.4	0.0	94.1	100.0	0.0	35.3	0.0
BR2	None	6	5	0.0	50.0	0.0	33.3	100.0	0.0	16.7	0.0
BR3	*Rlm3* (H)	5	3	0.0	80.0	0.0	80.0	80.0	0.0	20.0	0.0
BR4	*Rlm3* (H)	4	3	0.0	75.0	0.0	100.0	100.0	0.0	50.0	0.0
BR5	*Rlm2*, *Rlm3*	12	11	16.7	75.0	0.0	75.0	66.7	8.3	0.0	33.3
BR6	*Rlm3*	5	5	0.0	40.0	0.0	100.0	100.0	0.0	20.0	0.0
BR7	Unknown	8	7	37.5	62.5	0.0	75.0	75.0	0.0	25.0	0.0
BR8	None	6	6	0.0	66.7	16.7	16.7	33.3	0.0	83.3	0.0
BR9	None	4	3	0.0	50.0	0.0	100.0	100.0	0.0	75.0	0.0
BR10	N/A	3	2	0.0	66.7	0.0	100.0	100.0	0.0	100.0	0.0
BR11	None	3	3	0.0	66.7	66.7	66.7	100.0	0.0	33.3	0.0
BR17	Unknown	3	3	0.0	66.7	0.0	33.3	66.7	0.0	66.7	33.3
BR20	None	9	8	0.0	88.9	0.0	55.6	88.9	0.0	66.7	0.0
BR22	*Rlm3*(H)	10	10	30.0	60.0	0.0	70.0	90.0	0.0	60.0	0.0
BR23	*Rlm3*	10	10	40.0	50.0	20.0	40.0	70.0	20.0	30.0	70.0
MD1	None	2	2	- [b]	-	-	-	-	-	-	-
MD2	None	1	1	-	-	-	-	-	-	-	-
MD4	None	11	11	9.1	72.7	0.0	72.7	81.8	9.1	27.3	27.3
MD6	None	7	6	28.6	57.1	0.0	71.4	100.0	0.0	85.7	0.0
MD7	Unknown	9	7	22.2	55.6	0.0	88.9	100.0	0.0	44.4	0.0
MD8	None	5	5	0.0	80.0	40.0	60.0	100.0	0.0	40.0	20.0
MD9	*Rlm3*	2	2	- [b]	-	-	-	-	-	-	-
MD11	none	4	3	0.0	75.0	0.0	50.0	100.0	0.0	50.0	0.0
MD14	*Rlm3*	13	9	0.0	76.9	0.0	53.8	100.0	7.7	61.5	15.4
MD15	*Rlm3*	8	6	0.0	87.5	0.0	12.5	50.0	0.0	62.5	0.0
MO5	*Rlm3*(H)	15	11	26.7	66.7	0.0	100.0	93.3	6.7	33.3	20.0
MP1	*Rlm3*(H)	12	8	25.0	50.0	0.0	100.0	100.0	0.0	0.0	0.0
MP3	*Rlm3*	12	11	16.7	66.7	0.0	91.7	91.7	16.7	8.3	0.0
PC2	*Rlm3*	16	10	25.0	75.0	0.0	87.5	100.0	0.0	62.5	6.3
PC4	Unknown	7	7	28.6	71.4	14.3	71.4	28.6	28.6	14.3	28.6
SB1	*Rlm1*	9	6	0.0	77.8	0.0	77.8	33.3	0.0	11.1	0.0
SB2	None	9	8	44.4	66.7	0	88.9	100	0	11.1	11.1
SB3	*Rlm3*(H)	14	12	35.7	64.3	0.0	78.6	100.0	0.0	50.0	0.0
SB4	N/A	10	9	40.0	70.0	0.0	90.0	100.0	0.0	40.0	10.0
SB5	None	14	14	50.0	35.7	0.0	92.9	100.0	0.0	28.6	35.7
SB7	*Rlm3*(H)	5	4	40.0	20.0	0.0	80.0	100.0	0.0	20.0	20.0
SW20	None	10	8	60.0	30.0	0.0	100.0	100.0	0.0	30.0	0.0

Table 3.5 continued

Frequency was calculated as the percentage of isolates carrying a given avirulence allele. [a](H) refers to accessions with heterogeneous seeds whereby *R* gene was detected in 50-80% of the plants. N/A refers to seed samples were not collected in these fields. Unknown means the resistance genotype could not be determined in this study, accessions that carried unknown *R* genes might be due to the presence of a new *R* gene, other known *R* genes that were not tested in this study, or combinations of several *R* genes. None refers to the absence of *R* gene resistance. [b]Frequency of avirulence alleles in isolates collected from the field was not calculated due to small sample size.

3.5 Discussion

To the best of our knowledge, this is the first thorough report on the *R* genes present in Canadian *B. napus* germplasm. A total of eight known *R* genes were detected in 104 *B. napus* accessions with *Rlm3* being clearly the predominent *R* gene identified within the collection. The presence of *Rlm3* within Canadian *B. napus* accessions, and the deficiency of the corresponding *AvrLm3* in the *L. maculans* population clearly indicated the breakdown of *Rlm3* resistance in western Canada. The high frequency of *Rlm3* in Canadian canola accessions is likely due to the use of a single source of resistance in breeding programs since the first report of this disease in Canada in the 1970s (Gugel and Petrie 1992). When pathogenicity groups (PGs) were used to describe *L. maculans* populations, the predominant PG in western Canada was PG2 (Chen and Fernando 2006; Kutcher et al. 2007, 2010b). It can be hypothesized that breeding for blackleg resistance was conducted against a pathogen population of limited variability, only PG2 isolates (virulent on *B. napus* cv. Westar, avirulent on Glacier and Quinta). The corresponding *R* genes in differential varieties to PG2 isolates, are *Rlm2* and *Rlm3* in Glacier (Balesdent et al. 2002), and *Rlm1* and *Rlm3* in Quinta (Kutcher et al. 2010b). Due to the presence of *Rlm3* in both Glacier and Quinta, the probability of introducing *Rlm3* into canola varieties was extremely high. Our findings support this theory

as *Rlm1*, *Rlm2*, and *Rlm3* were the top three *R* genes present in the *B. napus* accessions. Furthermore, Canadian blackleg resistant canola varieties were released in the early 1990s (Kutcher et al. 2011), and this study clearly indicated that *Rlm3* was present in Canadian canola varieties released in the 1990s, such as Quantum (1995), Allons (1995), Q2 (1998), and Hi-Q (1999).

The breakdown of *Rlm3* resistance demonstrates the high evolutionary potential of *L. maculans* populations in western Canada where PG2 isolates were dominant for a very long time (Chen and Fernando 2006). More specifically, between 1984 and 1998, only PG1 (*Leptosphaeria biglobosa*) and PG2 isolates were observed, but other PGs (PG3, PG4, PGT) were identified by 1998 (Keri et al. 2001; Chen and Fernando 2006). Balesdent et al. (2005) reported that *AvrLm3* was present in 69.2% of Canadian *L. maculans* isolates collected during 1985-1992. The frequency of *AvrLm3* in *L. maculans* isolates collected between 1997 and 2005 in western Canada was 17.7%, much lower than the frequency of other avirulence alleles (Kutcher et al. 2010b). Dilmaghani et al. (2009) reported the *AvrLm3* allele was present in about 60% of *L. maculans* isolates collected in western Canada between 2005 and 2006, but variations between locations were observed. In 2010 and 2011, 8.7% of *L. maculans* isolates collected in western Canada carried the *AvrLm3* allele (Liban et al. 2016). By 2012, our results demonstrate that the frequency of *AvrLm3* in *L. maculans* isolates collected in Manitoba had dropped to 2.7%. The frequency of *AvrLm3* isolates varied between locations and years in Canada, which could be indicative of a transitory situation towards *Rlm3*. In addition to changes in the frequency of Avr alleles, disease incidence and severity of blackleg on canola has fluctuated (Canadian disease survey,

http://phytopath.ca/publication/cpds). The increase in disease severity correlates with the declining frequency of *AvrLm3* observed from 2005 to 2012, and the increasing use of resistant varieties with *Rlm3* resistance over the years. In addition, the effect of intensive production of canola from the common practice of one canola crop every four years on a field to the very common practice of one canola crop every two years has likely played a role in increased frequency and severity of blackleg (Kutcher et al. 2013). This finding strongly supports the observation of increased disease incidence as a result of the shift from *AvrLm3* to *avrLm3*, mainly due to the repeated use of *Rlm3* (Kutcher et al. 2010b; Liban et al. 2016).

Previous studies highlighted the 'boom and bust' nature of the disease of blackleg (Marcroft et al. 2012b). In Australia, breakdown of 'sylvestris' resistance on the lower Eyre Peninsula was observed in 2003, three years after the commercial release of varieties harbouring 'sylvestris' resistance (Sprague et al. 2006). However, the frequency of *L. maculans* isolates avirulent on these varieties had increased by 2005 when alternative varieties were made available (Marcroft et al. 2012b; Van de Wouw et al. 2014b). Similarly, although the frequency of the *AvrLm3* allele is currently very low in field fungal populations, reduced production of *Rlm3* varieties may prevent further breakdown and perhaps result in a gain of *AvrLm3* in fungal populations in the coming years.

Knowledge on Avr alleles of *L. maculans* isolates has major implications in deploying *R* genes in management of diseases. For example, studies on avirulence alleles in field populations (2002, 2003) of *L. maculans* suggested potential effectiveness of *Rlm6* and *Rlm7* in Europe (Stachowiak et al. 2006). Results from our study indicated very high frequency of *AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, and *AvrLm11*, while very low frequency of

AvrLm3 and *AvrLm9* in *L. maculans* populations in Manitoba (2012 collection). According to our knowledge on Avr alleles in *L. maculans* populations and *R* genes in *B. napus* germplasm, *Rlm2* and *Rlm4* are probably very useful in current Canadian *R* gene deployment. In addition, other useful *R* genes such as *Rlm5*, *Rlm6*, *Rlm7*, and *Rlm11* can be introduced into Canadian canola varieties. In contrast, *Rlm3* and *Rlm9* were overcome and *Rlm1*, *LepR1*, and *LepR2* are in the process of being overcome. However, except for *Rlm3*, there is no evidence that *Rlm1*, *LepR1*, and *LepR2* are widely used in Canada. We also do not know whether *Rlm9* was used in blackleg control in Canada as *Rlm9* was not detected in this study. In Canada, the frequency of both *AvrLm3* and *AvrLm9* in blackleg populations decreased with time (Kutcher et al. 2010b; Liban et al. 2016; Fernando et al. unpublished). This phenomenon suggested intensive use of *Rlm3* in Canada may have resulted in decrease of both *AvrLm3* and *AvrLm9* in isolates as they are in the same gene cluster. In contrast, with the decrease of *AvrLm3*, the frequency of *AvrLm7* in Canadian *L. maculans* populations increased with time (Dilmaghani et al. 2009; Liban et al. 2016). Moreover, only a small number of *L. maculans* isolates have been found to carry both *AvrLm3* and *AvrLm7* in previous studies (Balesdent et al. 2006; Kutcher et al. 2010b; Dilmaghani et al. 2009). Our study's findings corroborate this as further evidence of this phenomenon suggesting the co-existence of these two genes in *L. maculans* to be uncommon. Recently, Plissonneau et al. (2016) found *AvrLm3* was only expressed if the isolate did not carry *AvrLm7*, and illustrated the 'hide-and-seek' relationship between *AvrLm3* and *AvrLm7*. These phenomena can be at least partially explained by the fact that *AvrLm3*, *AvrLm7* and *AvrLm9* are part of the *AvrLm3-4-7-9-LepR1* genetic cluster (Balesdent et al. 2002, 2005; Ghanbarnia et al. 2012), and can further provide guidance to blackleg

management through appropriate R-gene rotations.

Marcroft et al. (2012b) demonstrated that rotation of *R* genes can minimize disease pressure by manipulating fungal populations. However, rotation of *R* genes to manage blackleg in Canada is a challenge at present due to limited *R* gene availability in our canola varieties other than *Rlm3*. Although unknown resistance was detected at the seedling stage in several canola accessions, further investigation is required to better understand the reactions by these accessions before using them as "new" resistance sources in breeding programs. Previous studies have shown that the durability and effectiveness of *R* genes varied in different circumstances (mainly different fungal population structures). For example, in Australia, research by Marcroft et al. (2012a) indicated that *Rlm3* and *Rlm4* were less effective than other seedling resistance genes most likely due to the low frequency of *AvrLm3* and *AvrLm4* in *L. maculans* populations (Dilmaghani et al. 2009); however, in Canada the durability or effectiveness of *R* genes was difficult to predict due to the lack of knowledge of *R* genes in commercial canola varieties. We are currently investigating the durability of several *R* genes under field conditions, which will be useful in terms of strategies to manage resistance breakdown (Fernando et al. unpublished).

Durability of resistance is particularly important in blackleg control (Pietravalle et al. 2006). One effective strategy to improve the durability of blackleg resistance is through the development of canola varieties with a combination of *R* genes and APR (Kiyosawa 1982; Pietravalle et al. 2006; Brun et al. 2010; Delourme et al. 2014). Canola accessions that have both APR and *R* gene resistance can increase the durability of *R* gene resistance (Brun et al. 2010; Marcroft et al. 2012b). In our study, about half of the Canadian canola accessions had

both APR and *R* gene resistance. Although APR was evaluated under controlled conditions in this study, APR identified under controlled conditions can improve the process of blackleg resistance breeding (Huang et al. 2014), and it may be considered a preliminary evaluation that can be further confirmed by field evaluations. Although APR can be isolate-specific in some cases, as described by Marcroft et al. (2012a), APR is usually considered as race non-specific (Delourme et al. 2006).

Unlike the rapid breakdown of 'sylvestris' resistance in Australia and *Rlm1* in Europe (Rouxel et al. 2003a; Sprague et al. 2006; Van de Wouw et al. 2010) that occurred within a few years, the breakdown of *Rlm3* resistance in western Canada appears to have been much slower. Although *Rlm3*-carrying varieties were released in 1990s, *Rlm3* appeared to be still very effective in 2005 because the *AvrLm3* allele was present in about 60% of *L. maculans* isolates collected in western Canada between 2005 and 2006 (Dilmaghani et al. 2009). This could be due to the fact that *Rlm3* resistance was deployed in different canola varieties with different genetic backgrounds (with different levels of APR). Although other *R* genes were rarely detected in Canadian canola accessions, they still have a role to play in blackleg control. In addition, a combination of *Rlm3* and APR, or the combination of *Rlm3* with other *R* genes in some commercial varieties may have reduced the speed of the breakdown of *Rlm3* observed. Although both ascospores (sexual) and pycnidiospores (asexual) can infect oilseed rape, ascospores have many advantages over pycnidiospores during disease epidemiology and therefore contributed more in generating variations at avirulence loci to overcome *R* genes (Dilmaghani et al. 2013). In western Canada, pycnidiospores are a major source of primary inoculum (Ghanbarnia et al. 2011; Guo et al. 2005) and this is another reason that

may explain the slowdown of breakdown as it limits genetic variability of the pathogen, along with a very limited spread in space of pycnidiosopres compared to ascospores. In addition, high clonal fractions in *L. maculans* populations in western Canada further confirmed a lack of ascospore-mediated infection and of sexual reproduction (Dilmaghani et al. 2009, 2013). Furthermore, crop rotation, and short cultivation season (3 months) of canola in Canada are not in favour of sexual mating. With this in mind, canola breeders might use the less common *R* genes to develop new varieties, to increase the number of varieties to facilitate *R* gene rotation.

Using genetic resistance is very effective to control the disease of blackleg. Characterization of *R* genes in commercial varieties and advanced breeding lines is essential for blackleg resistance breeding (Marcroft et al. 2012a). In Australia, diversification of blackleg resistance in canola varieties resulting from changes in their breeding programs (Marcroft et al. 2012a) provides alternative disease management strategies such as rotation of *R* genes (Marcroft et al. 2012b). The work presented here will be the starting point of the foundation of canola breeding programs in Canada that will combine APR with diversified and efficient *R* genes.

IDENTIFYING SEEDLING AND ADULT PLANT RESISTANCE OF CHINESE *BRASSICA NAPUS* GERMPLASM TO *LEPTOSPHAERIA MACULANS*

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4 IDENTIFYING SEEDLING AND ADULT PLANT RESISTANCE OF CHINESE *BRASSICA NAPUS* GERMPLASM TO *LEPTOSPHAERIA MACULANS*

4.1 Abstract

Blackleg disease of canola/rapeseed (*Brassica napus*), caused by the devastating fungal pathogen *Leptosphaeria maculans*, can significantly influence *B. napus* production worldwide, except for China, where only the less aggressive *L. biglobosa* has been found associated with the disease. The aim of this study was to characterize both seedling resistance (major gene resistance, *R* gene resistance) and adult plant resistance (APR) from a collection of Chinese *B. napus* varieties/lines (accessions) to *L. maculans*. Seedling resistance evaluation was carried out under controlled environment, using 11 well-characterized *L. maculans* isolates as differentials. The identification of APR was performed under multiple field environments in western Canada. *R* genes were detected in more than 40% of the accessions tested. Four specific *R* genes, *Rlm1, Rlm2, Rlm3,* and *Rlm4* were identified, with *Rlm3* and *Rlm4* being the most common genes while *Rlm1* and *Rlm2* being detected only occasionally. Field evaluation results indicated significant variations among field locations as well as accessions; a large portion of the *B. napus* accessions, regardless of the resistance level observed at the seedling stage, showed high to moderate levels of APR under all environments tested. This study highlights that both *R* gene resistance and APR are present in Chinese *B. napus* germplasm and could be potential sources of resistance against blackleg caused by *L. maculans* if the pathogen ever becomes established in China.

4.2 Introduction

Blackleg (phoma stem canker) is one of the major diseases on canola/oilseed rape (*Brassica napus*) in Australia, North America, and Europe (Fitt et al. 2006). Two closely related *Leptosphaeria* species can be associated with the disease, the more aggressive *Leptosphaeria maculans* and the less aggressive *L. biglobosa* (West et al. 2002a). These two species often co-exist as a species complex in most blackleg infected areas, with *L. maculans* being the major concern as it can cause more severe economic losses (West et al. 2001; Fitt et al. 2006). Both species can spread via airborne ascospores (sexual) that can travel a longer distance and pycnidiospores (asexual) that disperse with rain splashing in a relatively short distance (Travadon et al. 2007). Infected crop debris is the major source of *L. maculans* inoculum (West et al. 2001). Infected stem and pod debris (dockage) and seeds can also carry a trace amount of inoculum, although insignificant for disease epidemics in infected areas (Van de Wouw et al. 2015; Fernando et al. 2016). Historically, *L. maculans* has been able to spread into areas where only *L. biglobosa* had been found previously, such as Canada and Poland (Fitt et al. 2008), making *it an* invasive species.

China is one of the major oilseed rape producing countries, with considerable production of both winter oilseed rape in east-central China and spring oilseed rape in northern and northwestern China (Liu et al. 2014). Although oilseed production in China accounted for about 30% of the total world yield, the high demand in consumption has required the importation of oilseed rape seeds, meal and oil from other countries such as Canada and Australia (Liu et al. 2014). China is one of the major importers of Canadian canola seeds since 1994. However, in 2009, due to the concern of introducing *L. maculans*

into domestic fields via infected seeds, China announced a transitional period of restriction on importing canola seeds from countries including Canada where *L. maculans* is present (Zhang et al. 2014).

In China, blackleg caused by *L. biglobosa* has been reported since 1999 (West et al. 2000), and since then disease surveys have been conducted there to determine the causal agent of this disease. So far, only the less aggressive species, *L. biglobosa* has been found to be associated with blackleg in China and resulted in yield losses in some areas (West et al. 2000; Fitt et al. 2006; Liu et al. 2014; Zhang et al. 2014). To mitigate the risk of introducing *L. maculans* into China, it is important to minimize *L. maculans* inoculum carried with imported canola seeds (Fernando et al. 2016). Furthermore, identification of blackleg resistance in Chinese *B. napus* germplasm against *L. maculans* is prudent and proactive for risk mitigation that can assist future blackleg resistance breeding programs in China if the pathogen ever becomes established there.

In *Brassica* species, two types of resistance have been reported against blackleg caused by *L. maculans*, seedling resistance that is conferred by major (*R*) genes (qualitative) and adult plant resistance (APR) conferred by multiple minor/quantitative genes (Pongam et al. 1998; Jestin et al. 2015). *R* gene resistance is effective when the corresponding avirulence gene widely exists in the pathogen population, but continuing use of a single *R* gene can cause the breakdown of resistance (Rouxel et al. 2003; Sprague et al. 2006; Van de Wouw et al. 2010; Marcroft et al. 2012a; Zhang et al. 2016). The recent report on the breakdown of *Rlm3* in western Canada illustrated the requirement for more diversified *R* genes along with quantitative resistance in *B. napus* germplasm (Zhang et al. 2016). To date, at least 18 *R*

genes have been identified in *Brassica* species (Delourme et al. 2006; Raman et al. 2013). APR, on the other hand, is more durable due to less selection pressure on the pathogen population (Brun et al. 2010). The combination of *R* gene and APR can be an effective strategy for durable blackleg resistance (Brun et al. 2010; Marcroft et al. 2012b). A few earlier studies have evaluated field level blackleg resistance (APR) of Chinese *B. napus* cultivar/lines in countries where *L. maculans* was well-established such as Australia, UK, France, and Poland (Li et al. 2008; Zhang et al. 2014). Although a few *B. napus* genotypes showed at least a low level of resistance (Li et al. 2008), some Chinese *B. napus* cultivar/lines tested have been very susceptible to *L. maculans.* Only a limited number of oilseed varieties were included in these studies. For example, 20 *B. napus* genotypes were tested in Li et al. (2008), and 36 varieties were evaluated in France (Zhang et al. 2014). Moreover, none of these studies have characterized *R* genes in the Chinese *B. napus* germplasm and the resistance of Chinese *B. napus* varieties to the Canadian population of *L. maculans* was unknown until now. The objective of the current study was to conduct a comprehensive assessment of blackleg resistance in a collection of Chinese *B. napus* varieties/lines with a relatively diverse genetic background. This includes identification of *R* genes under controlled conditions and evaluation of APR under field conditions in western Canada. The information will lay important genetic foundations for blackleg disease management worldwide that can be useful to canola breeders in preserving valuable resistance sources for mitigating the risk of blackleg in China.

4.3 Materials and methods

4.3.1 Plant and fungal materials

A collection of Chinese *B. napus* germplasm consisting of 150 varieties or advanced breeding lines (accessions) provided by Anhui Academy of Agricultural Sciences, Sichuan Academy of Agricultural Sciences, and Sichuan Agricultural University was used in this study. The plant material from Anhui Province are winter type and plant material from Sichuan Province are semi-winter type *B. napus*. These Chinese *B. napus* accessions were derived from parental materials (both winter type and spring type *B. napus* germplasm from major canola growing regions in China) with high level of genetic diversity (Shumin Hou, Anhui Academy of Agricultural Sciences, personal communication). All of the 150 accessions were subjected to seedling resistance identification, and a sub-collection (136 accessions provided by Anhui Academy of Agricultural Sciences) of the 150 accessions was selected for APR identification under field conditions. A set of 11 well characterized *L. maculans* isolates (D3, D4, D5, D7, D10, ICBN14, PHW1223, R2, AD746, JN3 and J3) provided by Dr. Van de Wouw (University of Melbourne, Australia) and Dr. Balesdent (INRA, France) as well as the collection of Dr. Fernando's lab were employed for *R* gene characterization at seedling stage (Table 4.1). These 11 *L. maculans* isolates constituted the fungal differential set and can be referred to Zhang et al. 2016. Avirulence phenotypes of a few isolates can also be referred to Leflon et al. 2007 (R2), Balesdent et al. 2005 (ICBN14, PHW1223), and Balesdent et al. 2013 (JN3/v23.1.3). These isolates together can differentiate 13 known *R* genes, including *Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, Rlm11, RlmS, LepR1,* and *LepR2*. The presence of *Rlm10* was not able to be determined since we do not have access to

Rlm10-carrying plant material Thirteen *B. napus* varieties/lines with known *R* genes constituted the plant control set. The set comprised Westar (no *R* gene; Balesdent et al. 2002), 02-22-2-1 (*Rlm3*; Gout et al. 2006), Jet Neuf (*Rlm4*; Gout et al. 2006), 01-23-2-1 (*Rlm7*; Dilmaghani et al. 2009), Goéland (*Rlm9*; Balesdent et al. 2006), Quinta (*Rlm1*, *Rlm3*; Kutcher et al. 2010), Cooper (*Rlm1*, *Rlm4*; Dilmaghani et al. 2009), MT29 (*Rlm1*, *Rlm9*; Deloume et al. 2008), Bristol (*Rlm2*, *Rlm9*; Balesdent et al. 2005), Verona (*Rlm2*, *Rlm4*; Kutcher et al. 2010), Surpass 400 (*LepR3*, *RlmS*; Larkan et al. 2013), 1065 (*LepR1*; Zhang et al. 2016), and 1135 (*LepR2*; Zhang et al. 2016) .

Table 4.1 Avirulence genotypes of *Leptosphaeria maculans* isolates used for *R* gene identification and field inoculation.

Isolates	Avirulence genotypes[d]											
	AvrLm1	*AvrLm2*	*AvrLm3*	*AvrLm4*	*AvrLm5*	*AvrLm6*	*AvrLm7*	*AvrLm8*	*AvrLm9*	*AvrLmS*	*AvrLepR1*	*AvrLepR2*
D3[a]	-	-	-	-	+	-	-	-	-	-	+	-
D4[a]	-	-	-	+	+	+	+	+	-	-	+	+
D5[a]	+	+	-	+	-	-	+	-	-	+	+	+
D7[a]	+	-	+	-	+	+	-	+	-	nd[b]	+	-
D10[a]	-	-	-	-	+	+	-	+	+	+	-	-
ICBN14[a]	-	-	-	-	+	+	-	-	-	nd	+	-
PHW1223[a]	-	-	-	-	+	+	-	+	+	nd	-	-
R2[a]	-	-	-	-	+	-	+	nd	-	nd	+	-
AD746[a]	-	-	+	-	-	+	-	nd	-	nd	+	-
JN3[a]	+	-	-	+	+	+	+	+	-	nd	-	-
J3[a]	-	+	+	-	+	+	-	nd	-	+	-	-
03-12-01[b]	+	+	-	+	nd	+	+	nd	+	nd	+	-
89-3[b]	+	+	+	-	nd	+	-	nd	+	nd	+	-
03-17-09[b]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
DM65[c]	-	+	-	-	nd	+	+	nd	-	nd	-	-
DM77[c]	-	+	-	-	nd	+	+	nd	-	nd	-	-
DM78[c]	+	-	-	+	nd	+	+	nd	-	nd	-	-
DM79[c]	-	+	-	-	nd	+	+	nd	-	+	-	-
DM81[c]	-	-	-	+	nd	+	+	nd	-	+	-	-
DM85[c]	-	+	-	-	nd	+	+	nd	-	+	-	-
DM96[c]	-	+	+	-	nd	+	-	nd	-	+	-	-
DM118[c]	-	+	+	-	nd	-	-	nd	-	+	-	-
1-1[c]	-	+	-	+	nd	+	+	nd	-	nd	+	-
3-1[c]	+	-	-	+	nd	+	+	nd	-	nd	-	-
5-1[c]	-	+	-	+	nd	+	+	nd	-	nd	-	-
8-1[c]	+	-	-	+	nd	+	+	nd	-	nd	+	-
17-1[c]	-	+	-	+	-	+	+	-	-	-	-	-
21-2[c]	-	+	-	+	-	+	+	-	-	-	-	-
25-1[c]	-	+	-	+	-	+	+	-	-	-	-	-
41-2[c]	-	+	-	+	-	+	+	-	-	-	-	-

[a]*Leptosphaeria maculans* isolates used for *R* gene identification.

[b]*Leptosphaeria maculans* isolates used for artificial inoculation in the environment CA2011 (Carman, 2011).

[c]*Leptosphaeria maculans* isolates used for artificial inoculation in the environment CA2013 (Carman, 2013).

[d]"+/-" indicates the presence/absence of a specific avirulence gene, "nd" indicates avirulence genotype of the isolate was not determined.

4.3.2 Inoculum preparation

The fungal isolates, stored as pycnidiospores at -20 ℃ on filter paper discs, were grown on V8® agar medium (200 mL of V8 juice®, 0.75 g of $CaCO_3$, 15 g of agar, 800 mL of distilled H_2O, 10 mL of 0.35% (w/v) streptomycin sulfate) for 10 to 14 days under continuous fluorescent lamp light at room temperature. Sterile distilled water was added to sporulating cultures and spores were dispersed into water with a glass rod. The suspension was filtered through one layer of sterilized Miracloth and centrifuged at 9,000 rpm for 15 minutes. The supernatant was removed and spores were resuspended with 1 mL of sterile distilled water and transferred to a 1.5 mL sterilized centrifuge tube, then stored at -20 ℃ until use. Before inoculation, the inoculum was brought to room temperature and vortexed before diluting. The spore concentration was estimated using a hemacytometer, and the suspension was diluted with sterile distilled water to a final concentration of 2×10^7 spores mL^{-1}.

4.3.3 Greenhouse assay

All 150 *B. napus* accessions were screened for seedling resistance in a growth chamber at 21 ℃ (day) and 16 ℃ (night) with a 16-h photoperiod. The cotyledon inoculation test was performed using the method modified from Williams & Delwiche (1979) and described in Zhang et al. (2016). At least 12 plants per accession were inoculated with each *L. maculans* isolate. The plant control set, including the susceptible check cv. Westar, was included as controls. Disease severities on seedlings were rated 14 days post inoculation (dpi) using the rating scale of 0-9 (Williams & Delwiche, 1979). Average rating score (ARS) was calculated from 48 inoculation sites (12 individual plants, 4 sites on four lobes per plant).

Interaction phenotype (IP) was evaluated as follows: Susceptible reaction (S), ARS=6.1-9.0; intermediate reaction (I), ARS=4.6-6.0; and resistant reaction (R), ARS≦4.5 (Zhang et al. 2016).

4.3.4 Field trials

A sub-collection of 136 accessions was evaluated for blackleg resistance at three locations across the Prairies: Melfort, Saskatchewan (2012, 2013, and 2014); Carman, Manitoba (2011 and 2013); Vegreville, Alberta (2012 and 2013). Each combination of year and location was considered one environment. Therefore, there were a total of seven environments in this study: SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2012 (Alberta, 2012), SAB2013 (Alberta, 2013), SME2012 (Melfort, 2012), SME2013 (Melfort, 2013), and SME2014 (Melfort, 2014). Precipitation accumulation during the growing season at each environment was described in Table 4.2. Westar was included as the susceptible check. Two more Canadian *B. napus* varieties, AC Excel and Defender were also included in Carman as checks. These two varieties showed some level of blackleg resistance compared to Westar. Only 45 accessions were evaluated in 2012 at Vegreville, Alberta.

Table 4.2 The description of field-trial environments for blackleg (*Leptosphaeria maculans*) resistance evaluation. Precipitation accumulation during the growing season (May 1st to Sep 1st) was calculated.

Environment	Location	Year	Inoculum	Precipitation accumulation (mm)[a]
SCA2011[b]	Carman, Manitoba	2011	Natural and artificial inoculum	188.4
SCA2013[c]	Carman, Manitoba	2013	Natural and artificial inoculum	275.0
SAB2012	Vegreville, Alberta	2012	Natural inoculum	334.5
SAB2013	Vegreville, Alberta	2013	Natural inoculum	42.7
SME2012	Melfort, Saskatchewan	2012	Natural inoculum	339.1
SME2013	Melfort, Saskatchewan	2013	Natural inoculum	225.5
SME2014	Melfort, Saskatchewan	2014	Natural inoculum	288.3

[a] Data was obtained from the weather network: http://www.theweathernetwork.com/weather/historical-weather/list/ca/c.
[b] Mixture of 3 *Leptosphaeria maculans* isolates was used as inoculum: 03-12-01, 89-3, and 03-17-09
[c] Mixture of 16 *Leptosphaeria maculans* isolates was used as inoculum. DM65, DM77, DM78, DM79, DM81, DM85, DM96, DM118, 1-1, 3-1, 5-1, 8-1, 17-1, 21-2, 25-1, and 41-2.

In all locations, plant were sown in May and plants were rated during late August or late September, depending on the year or location. For the plot layout, a 2.5-meter or 3-meter single row was used, with two or three replicates seeded in a completely randomized design. Each replicate had 15 to 40 plants depending on the germination rate. Field tests were conducted in disease nurseries across the prairie where natural *L. maculans* inoculum hasestablished well. In the nurseries located in Melfort and Vegreville, the inoculation relied on the natural inoculum. In Carman, both natural inoculum and artificial inoculum were applied.In the environment SCA2011 (Carman, 2011), three Canadian *L. maculans* isolates collected during 2003 and 2005 were used as inoculum and spread at the 2-4 leaf stage of the plants. In the environment SCA2013 (Carman, 2013), a mixture of 16 Canadian *L. maculans* isolates collected during 2010 and 2011 was used for field inoculation (Table 4.1; Table 4.2).

The isolates selected for field inoculation were isolated from *L. maculans* infected stubble collected in commercial canola fields in Manitoba. Disease severity was scored for basal internal infection with the rating scale of 0-5 (West et al. 2002b). Data was presented as relative disease severity (RDS), percentage of disease severity relative to the disease severity of the susceptible check Westar (Marcroft et al. 2012a). The APR category was evaluated as follows: resistant (R), RDS ≤35%; moderately resistant (MR), RDS =36-50%; moderately susceptible (MS), RDS=51-65%; and susceptible (S), RDS ≥66% (Zhang et al. 2016).

4.3.5 Statistical analysis

SAS software (version 9.3; SAS Institute) was employed for analysis of variance (ANOVA) using PROC GLM model. RDS was approximated to normal frequency distribution by means of root square transformation before ANOVA. For field trials, only 45 of the accessions were evaluated in the environment SAB2012, and data for 51 accessions was missing from each replicate in the environment SME2014 due to poor plant establishment. Therefore, we performed ANOVA using the data obtained from the remaining five environments for the analysis of the 136 accessions. We further conducted separate ANOVA using the data of 45 accessions from all seven environments. To determine the stability of resistance across the environments, data of 41 *B. napus* accessions tested in all seven environments were subjected to genotype and genotype × environment (GGE) biplot analysis using SAS code modified from Burgueño et al. (2003).

4.4 Results

4.4.1 *R* genes in Chinese *Brassica napus* germplasm

Based on the assessment of cotyledon inoculation, several known *R* genes were present in 64 out of 150 Chinese *B. napus* accessions, which accounted for 43% of the germplasm tested (Appendix VIII). In particular, four well known *R* genes, i.e. *Rlm1*, *Rlm2, Rlm3* and *Rlm4* were found in these accessions, while other known *R* genes that could be postulated were not identified (Fig. 4.1). The *R* gene found most frequently in these accessions was *Rlm3*, which was present in 30 accessions, followed by *Rlm4* in 15 accessions. *Rlm1* and *Rlm2* were detected in 4 and 5 accessions, respectively. In addition, 22 accessions were resistant to at least one isolate but the resistance gene carried by these accessions were not determined. Therefore, these accessions were described as carrying unknown *R* gene(s) in this study. The presence of unknown *R* gene resistance may be due to the effect of a novel *R* gene, other known *R* genes that were not tested in this study, or a combination of a few *R* genes (Marcroft et al. 2012a; Zhang et al. 2016).

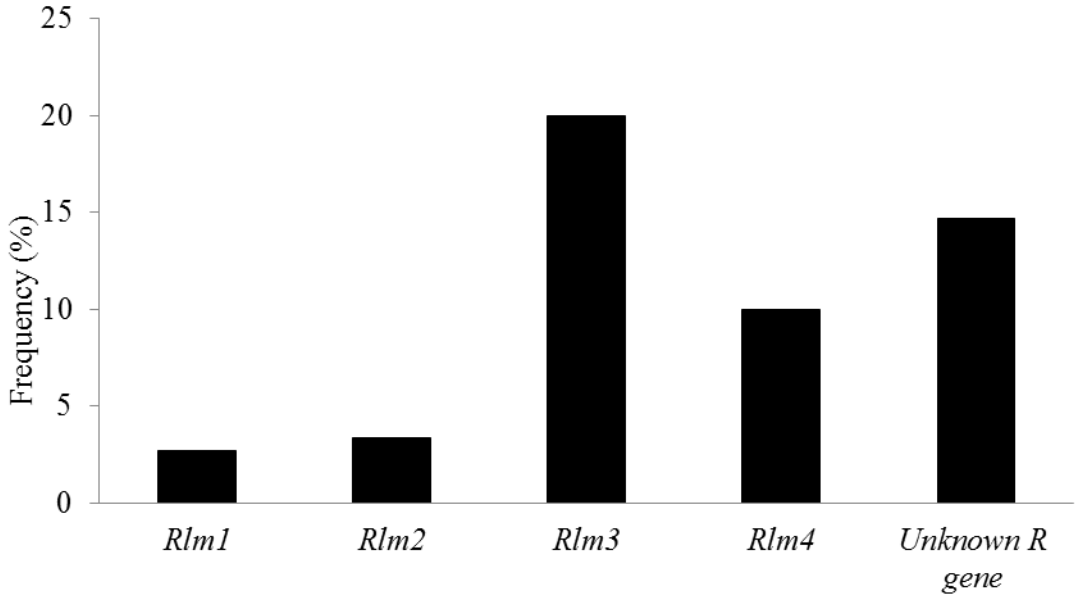

Fig. 4.1 Frequency of *R* genes identified against blackleg (*Leptosphaeria maculans*) in the Chinese *Brassica napus* germplasm. Apart from *Rlm1*, *Rlm2*, *Rlm3* and *Rlm4*, the other nine known resistance genes were not found in the germplasm collection. Some accessions were described as carrying unknown *R* gene(s) in this study. The presence of unknown *R* gene resistance might be due to the effect of a novel *R* gene, other known *R* genes that were not tested in this study, or a combination of a few *R* genes.

Most of the accessions showing seedling resistance carried a single *R* gene, and only a limited number of resistant accessions carried multiple *R* genes, either known or unknown. A total of 11 accessions carried more than one *R* genes, including 3 accessions carrying *Rlm2* and unknown resistance, 6 carrying *Rlm3* and unknown resistance, and one carrying more than two *R* genes. Another 12 accessions exhibited only unknown *R* genes, and the remaining 86 accessions were susceptible to all *L. maculans* isolate used.

4.4.2 Identification of field disease resistance

Among 136 accessions, the frequency distribution of RDS in different environments suggested low disease severity of the majority of *B. napus* germplasm in all environments, and only a few accessions were highly susceptible to the *L. maculans* population (Fig. 4.2; Appendix IX). The subsequent ANOVA showed significant differences (P<0.0001) between accessions and between environments. However, there was no significant genotype × environment interaction (Table 4.3). A summary of RDS in 5 environments were presented in appendix IX.

Table 4.3 Analysis of variance on relative disease severity (RDS) of blackleg (*Leptosphaeria maculans*) for 136 Chinese *B. napus* accessions in 5 environments and 45 accessions in 7 environments (G: Genotype; E: environment; G×E: term of the genotype by environment interaction; DF: degree of freedom; MS: mean square).

Samples	Source of variation	DF	MS	F value[c]
136 accessions in 5 environments[a]	E	4	743.0534	**250.80*****
	G	135	12.2134	**4.12*****
	G×E	531	2.8130	**0.95**
45 accessions in 7 environments[b]	E	6	327.27	**104.71*****
	G	44	24.13	**7.72*****
	G×E	254	3.46	**1.11**

[a]Data from five environments was used for the analysis: SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2013 (Alberta, 2013), SME2012 (Melfort, 2012), and SME2013 (Melfort, 2013). Data from SAB2012 (Alberta, 2012) was not used due to only 45 accessions were evaluated. Data from SME2014 (Melfort, 2014) was not used due to a large portion of data was missing.

[b]Data from all seven environments was used for the analysis: SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2012 (Alberta, 2012), SAB2013 (Alberta, 2013), SME2012 (Melfort, 2012), SME2013 (Melfort, 2013), and SME2014 (Melfort, 2014)

[c]***Significant at 0.0001 level of probability.

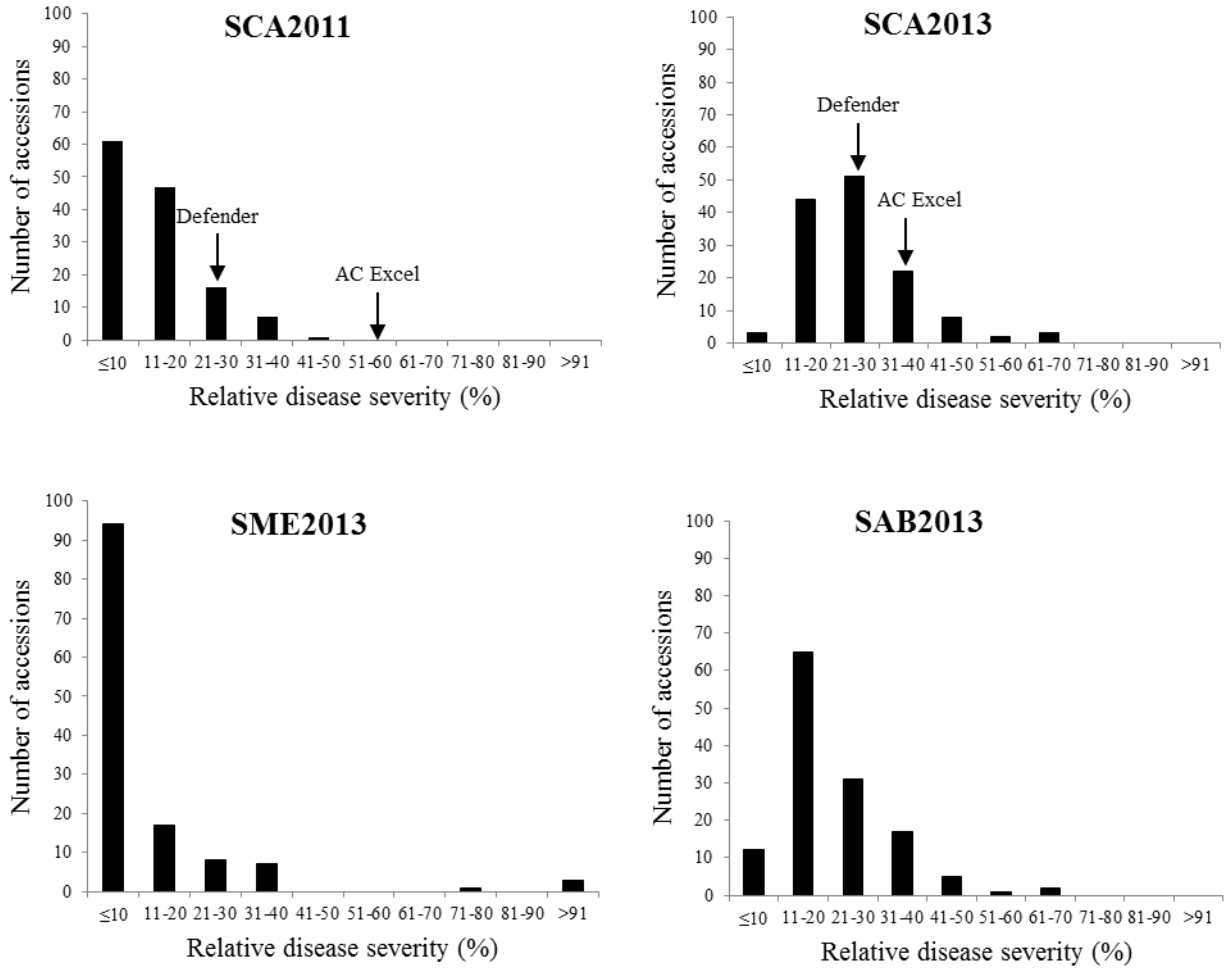

Fig. 4.2 Frequency distribution for the relative disease severities for blackleg (Leptosphaeria maculans) of 136 Chinese Brassica napus accessions in 4 Canadian field environments. RDS was calculated based on the percentage of average disease severity (0-5 rating scale) of accessions tested relative to that of the susceptible cultivar Westar. Four environments presented here were SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2013 (Alberta, 2013), and SME2012 (Melfort, 2012). Environment SME2014 (Melfort, 2014) was not shown in the figure. Position of two varieties with some level of blackleg resistance, Defender and AC Excel is shown in the figure.

In 2011 at Carman, Manitoba (SCA2011), RDS of 136 *B. napus* accessions ranged from 2.0% to 41.2%, and a total of 61 accessions showed high level of resistance with RDS lower than 10%. Only 6 accessions were considered susceptible to blackleg with RDS higher than 35%. In 2013 at Carman, Manitoba (SCA2013), the RDS ranged from 8.9% to 68.3%,

with only three accessions showing a RDS less than 10% and 19 showing higher than 35%. In 2012 at Melfort, Saskatchewan (SME2012), RDS ranged from 0 to 68.5%, with 90 accessions showing RDS less than 10% and 4 showing RDS higher than 35%. In 2013 at Melfort, Saskatchewan (SME2013), RDS ranged from 0 to 119.9%. A total of 94 accessions were highly resistant and exhibited RDS of less than 10%. In 2013 at Vegreville, Alberta (SAB2013), RDS ranged from 5.4% to 66.3%, with 12 accessions showing less than 10% but 15 showing RDS of higher than 35%.

According to the field evaluation results, a large portion of Chinese accessions showed adult plant resistance against *L. maculans* in nearly all of the environments in western Canada, relative to the susceptible control Westar. Overall, maximum RDS (among 5 environments) of 107 accessions was less than 35%, maximum RDS of 20 accessions ranged from 36% to 50%, while maximum RDS of 9 accessions were higher than 50%. Therefore, 93% of the 136 accessions tested under field conditions were considered exhibiting APR. Several accessions showed strong blackleg resistance consistently in all environments, including the lines 1051, 1058, 1068, 8010, and 8021 (Appendix IX).

A total of 41 out of 45 accessions listed in Table 4.4 were used for GGE biplot analysis. Four accessions, 1005, 1055, CC07 and CC08 were not included in the analysis due to missing data. The first two principle components (PCs) of the GGE model explained 73.36% (58.10+15.26) of variance in G+GE (Fig. 3). The polygon shows that the vertex accession CC09 contributed most to the interaction, i.e. showing highest or lowest infection (highest infection in this study) caused by the pathogen (Fig. 3; Table 4.4). The environments SME2013 and SME2014 were statistically highly discriminating in comparison with the

other environments, whereas the other five environments were more similar to each other.

Table 4.4 Relative blackleg disease (*Leptosphaeria maculans*) severity of 45 Chinese *Brassica napus* accessions in 7 environments.

Accessions[a]	Environments[b]							RDS mean	SE[c]	Rank
	SCA2011	SCA2013	SAB2012	SAB2013	SME2012	SME2013	SME2014			
8010	5.02	12.22	16.04	10.53	0	0	0	6.26	2.53	1
1001	2.01	26.11	16.29	7.95	1.94	0	0	7.76	3.77	2
8041	10.04	21.11	21.8	9.1	0	0	0	8.87	3.63	3
CC10	9.04	8.89	19.7	15.02	6.41	0	4.23	9.04	2.49	4
8016	9.04	21.67	17.91	12.59	0	1.19	0.97	9.05	3.31	5
CC01	4.02	16.67	23.58	11.75	0	2.67	6.98	9.38	3.19	6
8015	13.05	13.33	20.9	10.91	2.98	5.66	0	9.55	2.7	7
HP43	4.02	14.44	15.52	18.03	5.4	1.22	11.22	9.98	2.44	8
8037	20.08	15	23.58	13.85	2.14	0	0	10.66	3.73	9
HC1023	12.05	15.56	37.61	10.91	9.06	0	0	12.17	4.8	10
1056	22.09	30	20	9.21	8.68	0	0	12.85	4.34	11
HP46	4.02	14.44	32.84	11.73	13.92	11.32	2.33	12.94	3.77	12
HC1007	7.03	16.67	36.42	18.05	13.02	5.08	0	13.75	4.5	13
1055	11.04	-[d]	29.85	-	15.28	-	0	14.04	6.18	14
HP24	6.02	22.78	36.42	18.05	6.17	0	11.79	14.46	4.68	15
HC1021	10.04	20.56	41.16	15.53	6.17	5.24	4.39	14.73	4.94	16
HP36	14.06	16.67	40	13.85	7.81	11.68	3.1	15.31	4.45	17
HP08	5.02	20	37.31	21.82	17.32	3.88	4.82	15.74	4.62	18
HP48	8.03	23.89	45.18	14.27	6.58	8.25	5.29	15.93	5.44	19
HP49	14.06	15.56	30.45	17.63	10.19	22.87	1.41	16.02	3.48	20
1005	12.05	-[a]	41.11	-	11.57	-	0	16.18	8.76	21
HC810	14.06	23.41	38.3	20.14	10.19	2.31	6.3	16.39	4.6	22
HP06	14.06	29.44	46.98	18.88	4.81	0	2.04	16.6	6.41	23
HC702	15.06	23.89	49.25	14.27	0	9.77	4.05	16.61	6.19	24
HP22	9.04	28.33	39.1	18.5	11.03	1.83	8.58	16.63	4.92	25
8017	22.09	16.11	40	16.37	22.27	0	0	16.69	5.26	26
HP23	19.08	23.89	37.61	24.34	2.5	0	13.53	17.28	4.98	27
HC809	4.02	19.44	33.43	24.76	0	15.17	25.49	17.47	4.55	28
HP21	10.04	18.33	45.37	23.08	2.78	8.41	15.52	17.65	5.27	29
8011	6.02	28.29	33.16	18.05	15.57	11.57	14.41	18.15	3.58	30
HP27	11.04	23.25	41.19	26.44	8.85	9.77	15.41	19.42	4.44	31
HP39	31.12	18.89	34.03	24.92	9.87	11.47	10.69	20.14	3.8	32
HP15	17.07	40	53.43	18.47	1.16	11.31	4.97	20.91	7.2	33
HP26	13.05	23.89	36.87	18	15.63	39.68	0	21.02	5.24	34
CC07	4.02	-	58.21	-	2.6	-	-	21.61	18.3	35
HP18	29.11	27.78	44.48	33.57	9.23	27.99	0	24.6	5.69	36
HC1004	21.08	66.11	46.87	27.7	11.84	0	3.88	25.35	9.04	37
HC701	34.13	41.11	48.96	23.5	16.03	34.72	6.98	29.35	5.53	38
HP25	28.11	32.78	54.03	46.1	11.19	23.99	32.7	32.7	5.33	39
HP16	29.11	33.33	63.88	37.77	60.63	16.8	8.54	35.72	7.81	40
8034	40.16	33.61	65.07	20.95	68.45	9.16	62.07	42.78	8.76	41
8026	25.1	55.54	64.18	65.32	2.14	36.85	77.59	46.67	10.04	42
HP19	18.07	47.36	77.01	36.93	25.73	91.83	55.18	50.3	10.11	43
CC08	31.12	68.21	85.07	53.01	19.4	119.92	0	53.82	15.55	44
CC09	41.16	68.33	80.3	66.31	55.9	39.73	40.55	56.04	6.12	45
RDS Mean	15.3	27.07	40.45	22.34	11.83	14.32	10.57			
SE[c]	1.52	2.29	2.52	2.12	2.23	3.69	2.63			

[a]*Brassica napus* accessions were winter type varieties or advanced breeding lines provided by

Anhui Academy of Agricultural Sciences, China.

[b]Seven environments were: SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2012 (Alberta, 2012), SAB2013 (Alberta, 2013), SME2012 (Melfort, 2012), SME2013 (Melfort, 2013), and SME2014 (Melfort, 2014).

[c]Standard error of the mean.

[d]Data was missing.

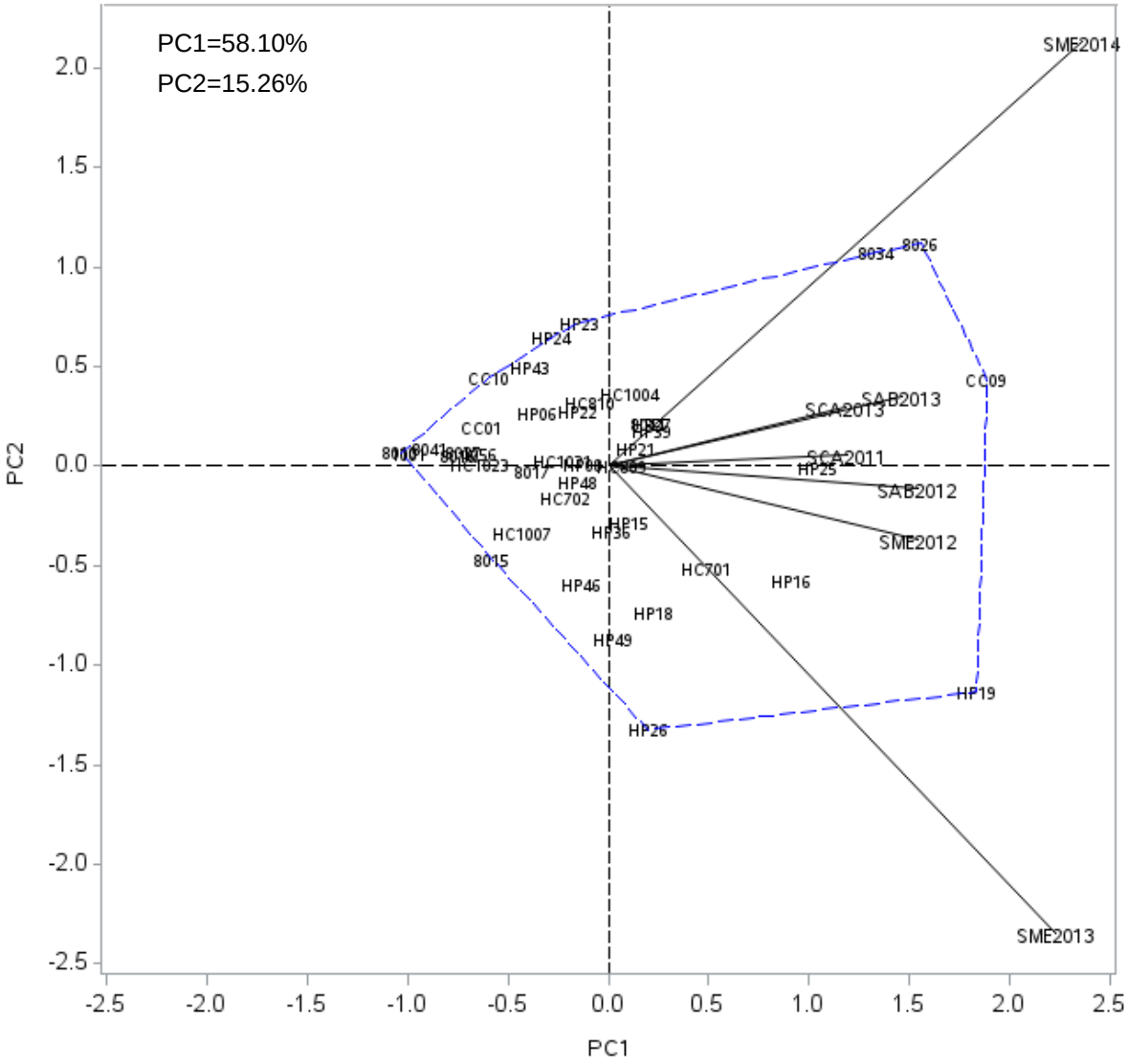

Fig. 4.3 Biplot genotype and genotype×environment (GGE) of the first and the second principle components (PC1 and PC2) based on the disease severity of 41 *Brassica napus* accessions in 7 environments. Vectors of all seven environments portrayed as solid lines. The percentage of GGE variation explained by PC1 (58.10%) and PC2 (15.26%) is shown on the top left of the biplot. Seven environments were: SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2012 (Alberta, 2012), SAB2013 (Alberta, 2013), SME2012 (Melfort, 2012), SME2013 (Melfort, 2013), and SME2014 (Melfort, 2014).

The biplot analysis also showed the accessions can be divided into different groups based on the disease severity. Accessions in the first group showed low disease severity consistently across all environments, including the accessions 8010, 1001, 8041, 1056, 8037, 8016 and HC1023. The second group consisted of accessions more susceptible to *L. maculans* as compared with accessions in the first group (i.e. 8026, 8034, CC09, HP16, HP19 and HP25). The third group included accessions with moderate level of disease severity (i.e. HC702, HP48, HP22 and HC810).

4.4.3 Comparison of *R* gene resistance and field resistance

According to *R* gene and APR identification, a large portion of the 136 Chinese *B. napus* accessions showed both *R* gene resistance and APR against blackleg. This was further illustrated by comparing the combination of *R* genes and maximum RDS (the highest RDS in all five environments) of each accession. Overall, 56 accessions possessing *R* genes and 71 accessions without *R* genes exhibited APR, only 2 accessions carrying *R* genes and 7 accessions without *R* genes were susceptible under field conditions (Fig. 4.4). All *Rlm4*-carrying accessions showed blackleg resistance under field conditions, while one of the *Rlm3*-carrying accessions, HC1004 was susceptible under at least one environment. Several of these accessions, including 8022, CC08 and HP19, were highly susceptible under both greenhouse and field conditions. One *Rlm3*-carrying accession (1068) showed strong blackleg resistance (RDS≤10%) consistently in all environments and was considered one of the most resistant *B. napus* accessions among the 136 Chinese *B. napus* accessions tested. Another two accessions, HP46 and 8015 carrying the single *Rlm4* also showed strong

blackleg resistance (RDS≤15%) consistently under all environments.

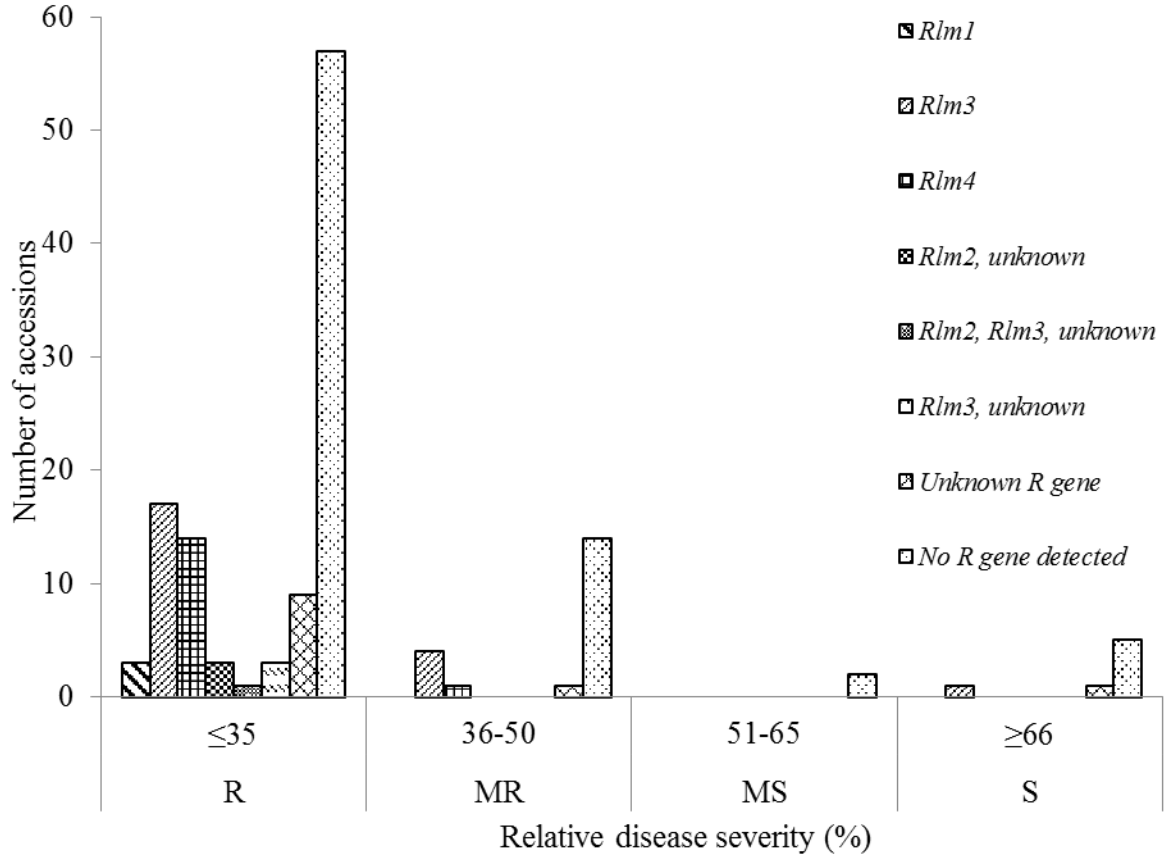

Fig. 4.4 Distribution of 136 Chinese *Brassica napus* accessions in four APR categories (resistant (R); moderately resistant (MR); moderately susceptible (MS); and susceptible (S)) based on their *R* genes against blackleg (*Leptosphaeria maculans*). Each accession was assigned to different APR categories according to its maximum relative disease severity (RDS), the highest RDS of the accession among all five environments. Five environments were: SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2013 (Alberta, 2013), SME2012 (Melfort, 2012), and SME2014 (Melfort, 2014).

4.5 Discussion

This study highlights the existence of genetic resistance in Chinese *B. napus* germplasm that can be utilized in both China and Canada against blackleg disease. Both seedling resistance and APR against blackleg were demonstrated in the Chinese *B. napus*

germplasm tested. Four *R* genes, i.e. *Rlm1*, *Rlm2*, *Rlm3* and *Rlm4* were identified. To our knowledge, this is the first thorough report of these *R* genes from a well-represented pool of Chinese *B. napus* germplasm. Field studies indicated that the majority of the accessions were highly or moderately resistant to blackleg in western Canada with RDS at 0-35%, while only a limited number of accessions were moderately or highly susceptible (RDS≥50%). In Carman, we included two blackleg resistance canola varieties, AC Excel and Defender as resistant checks. RDS of AC Excel and Defender were 59.84% and 29.72% respectively in 2011, and 32.25% and 30.25% respectively in 2013. This data further indicated better blackleg resistance in Chinese *B. napus* germplasm tested. Although some Chinese *B. napus* varieties were very susceptible to *L. maculans* as described in earlier studies (Li et al. 2008), the number of varieties selected in these studies were somewhat restricted more towards commercial varieties available at those timesvarieties. With a much broader collection of germplasm and varieties, this study demonstrated that 46% of Chinese *B. napus* accessions tested carried at least one *R* gene and 93% of the 136 accessions showed at least some level of field resistance under Canadian field conditions. Several Chinese *B. napus* genotypes showed very strong disease resistance across all field-trial environments and therefore could be used as donor germplasm for blackleg resistance in breeding programs. The difference in blackleg resistance of Chinese *B. napus* germplasm between current and previous studies is likely due to a combination of the difference in the number of accessions evaluated, sources of materials, pathogen races used/present, and the field condition under which the entries were tested. For example, the conclusion drawn by Li et al. (2008) was based on phenotypic evaluation of only 20 Chinese *B. napus* genotypes. In addition, none of the previous studies

reported *R* genes involved in the seedling resistance in Chinese *B. napus* germplasm, today a critical factor in blackleg resistance assessment.

Leptosphaeria maculans and *L. biglobosa* often share similar climatic/ecological niches and *L. biglobosa* has been detected in many rapeseed production regions in China (Zhang et al. 2014). These regions will almost certainly also be suitable for the establishment of *L. maculans*. Although there is still no evidence that *L. maculans* is present in China, the potential risk for this to happen remains based on the prior experience in other jurisdictions (Fitt et al. 2008; Zhang et al. 2014). Dockage is one of the major sources associated with introducing *L. maculans* into new areas (Van de Wouw et al. 2015; Fernando et al. 2016), and canola seeds with low level of blackleg infection, and free of or with low level of infected dockage will help reduce the risk of introduction via importation. At the same time, it is critical to be proactive in identifying useful blackleg resistance sources in the Chinese *B. napus* varieties to deal with the inevitable introduction of *L. maculans* into China.

Rlm1, *Rlm2*, *Rlm3* and *Rlm4* found in Chinese *B. napus* germplasm were also found in Canadian and Australian *B. napus* cultivars (Marcroft et al. 2012a; Zhang et al. 2016). The relatively higher frequencies of *Rlm4* in the Chinese *B. napus* accessions are of great value in dealing with the risk of blackleg associated with the canola imports from Canada, Australia and Europe. Based on recent studies, the avirulence allele of *AvrLm4* is very common in the Canadian *L. maculans* populations (Liban et al. 2016), and varieties carrying *Rlm4* should be highly resistant there. This was shown by the consistent resistance of all *Rlm4*-carrying accessions under field conditions of the current study. Although *Rlm4* was rare in Canadian canola varieties (Zhang et al. 2016), this *R* gene is also likely effective for blackleg control in

Canada against the present profile of the pathogen races (Liban et al. 2016) and could be used to develop varieties with *Rlm4* should alternative *Rlm1* resistance be needed in the future for Canada. The 11 *L. maculans* isolates used for cotyledon inoculation in this study allowed the postulation of 13 *R* genes, and a total of 10 accessions were found to carry more than one *R* genes. Those carrying more than just the *Rlm1* may be of particular significance to breeding programs in China for developing blackleg resistant varieties ready for future use of if *L. maculans* is introduced into China. Twelve accessions, however, carried at least one *R* gene unidentifiable with the set of 11 *L. maculans* isolates used; they may include *Rlm10* because the *L. maculans* differential set was not able to identify *Rlm10* or even novel genes and further studies are required to confirm this.

It is generally believed that a combination of *R* genes and APR can provide more effective and durable resistance against blackleg (Pietravalle et al. 2006; Brun et al. 2010; Marcroft et al. 2012b). *R*-gene mediated resistance is usually expressed at the seedling stage, while APR is controlled by multiple genes with quantitative effects expressed more as reduction on pathogen development after initial infection. Under field conditions, APR combined with specific *R*-gene resistance may provide double protection to the host to achieve stronger and more durable resistance than by a single mechanism alone and may offer the best approach to deal with the complexity of field environments that make the disease pressure both in commercial situations and in field trials variable across years and locations. For example, temperature and humidity under field conditions can affect the level of blackleg severity (Li et al. 2008). Such factors have likely contributed to the variations in disease levels observed between years and locations in the current study. In spite of these

variations, expression of blackleg resistance was relatively consistent for Chinese *B. napus* accessions in terms of both seedling resistance and APR traits across the field-trial environments. This indicates that, the resistance to blackleg with many of the Chinese *B. napus* accessions is robust and not substantially affected by field conditions, making it highly valuable for breeding programs.

Although the field evaluation can identify APR from those accessions carrying no specific *R* genes, in reality APR compliments major-gene resistance (Van de Wouw et al. 2014). In evaluating blackleg disease resistance, it is useful to differentiate seedling resistance from APR both for the purposes of blackleg resistance breeding and how blackleg resistance is strategically deployed to manage pathogen virulence changes in the pathogen population over time (Huang et al. 2014). In this study, all *Rlm4*-carrying accessions showed field resistance, as expected with a high frequency of *AvrLm4* in the Canadian *L. maculans* population and this could have masked APR in these accessions. Similarly, since *AvrLm2* is also common in Canadian *L. maculans* populations, any APR associated with the field resistance for the five *Rlm2*-carrying accessions could also have been masked. On the other hand, *AvrLm3* is generally at a very low frequency in western Canada and the accessions carrying only a single *Rlm3* likely did not exhibit field resistance if there was no APR involved at test locations (Zhang et al. 2016). Therefore, it is likely that the three *Rlm3*-carrying accessions that were susceptible to blackleg under field conditions lacked APR. However, the remaining 19 *Rlm3*-carrying accessions showed field resistance that most likely resulted from APR. For the 65 accessions carrying no *R* gene, the field resistance exhibited are also likely due to APR mediated by minor genes. Of the tested 111 Chinese *B. napus*

accessions showing field resistance to blackleg, at least 84 of them were mediated through APR (Jestin et al. 2015).

GGE biplot analysis allowed the identification of the accessions with lowest disease severity and those most consistent in expression of resistance across all environmental conditions of the different test sites (Rubiales et al. 2014). This consistency for some particular accessions is noteworthy because it suggests that expression of resistance in these varieties is little-affected by the environment. Such "environmentally-stable" resistance sources will likely display in commercial crops across multiple regions. These stable resistance sources may be more ideal for overall strong resistance performances in multiple regions. For example, the Environments SME2013 (Melfort, 2013) and SME2014 (Melfort, 2014) were very different as suggested by the wide angle between their vectors (Fig. 3). However, several accessions showed consistent blackleg resistance under all environments and could be considered as ideal sources of blackleg resistance in breeding programs. This underlines the importance and benefits of stability in the expression of disease resistance under different environments.

Overall, this study identified for the first time both seedling resistance and APR present in the Chinese *B. napus* germplasm for use by the *B. napus* oilseed industry in China. This is encouraging, especially given the prior did not identify high level ofblackleg resistance among Chinese rapeseed varieties (Fitt et al. 2008; Li et al. 2008). It is noteworthy that both *Rlm2* and *Rlm4* were together present in some accessions evaluated; as these *R* genes should also be highly effective against the current *L. maculans* population in western Canada where there is ahigh frequency of *AvrLm2* and *AvrLm4* in the pathogen population

(Liban et al. 2016). The current study also demonstrates the importance of international collaboration in addressing the risk from blackleg disease to China. Additional resistant sources and *R* genes could likely be identified across wider and more diverse sets of Chinese rapeseed germplasm particularly spring type *B. napus* cultviars which were not evaluated in this study.. The potential high risk of an introduction of *L. maculans* into China demands breeding programs to be prepared. Timely development of *B. napus* varieties in China with effective blackleg resistance prior to introduction of blackleg into China is essential to deal with such incursions. Although short-term strategies such as tests on imported canola seeds and blackleg disease surveys at national level have been adopted by local *B. napus* breeders and plant pathologists in China (Zhang et al. 2014), the only effective long-term strategy is breeding for blackleg resistance (both seedling resistance and APR prior to *L. maculans* incursions into China).

GLOBAL RNA SEQUENCING ANALYSIS REVEALS UNIQUE GENES INVOLVED IN *BRASSICA NAPUS* RESISTANCE TO *LEPTOSPHAERIA MACULANS*

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All RNA sequencing data is available from the Gene Expression Omnibus (GEO) data repository (accession GSE77723).

Author contributions: Xuehua Zhang conducted this work as part of her PhD thesis. Xuehua Zhang designed the experiment, carried out the experiment, analyzed the data and co-wrote the manuscript. Michael G Becker contributed to the experiment, data analysis and co-wrote the manuscript. Matthew J Granger, Jenna L Millar, Deirdre Khan, Jacob D Cavers and Ainsley C Chan contributed to histological analysis and LMD sample collection of the experiment. Mark F Belmonte and W G Dilantha Fernando designed and supervised the experiment.

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5 GLOBAL RNA SEQUENCING ANALYSIS REVEALS UNIQUE GENES INVOLVED IN *BRASSICA NAPUS* RESISTANCE TO *LEPTOSPHAERIA MACULANS*

5.1 Abstract

The hemibiotrophic fungal pathogen *Leptosphaeria maculans* is the causal agent of blackleg disease in *Brassica napus* (canola, oilseed rape) and causes significant yield loss worldwide. While genetic resistance has been used to mitigate the disease using traditional breeding strategies, there is little knowledge about the genes facilitating blackleg resistance. RNA sequencing and a streamlined bioinformatics pipeline identified unique genes and plant defense pathways specific to plant resistance in the *B. napus*-*L. maculans LepR1-AvrLepR1* interaction. We complemented our temporal analyses by monitoring gene activity directly at the site of infection using laser microdissection coupled to qPCR. Finally, we characterized unique genes involved in plant resistance to blackleg in the Arabidopsis-*L. maculans* model pathosystem. Data reveal an accelerated activation of the plant transcriptome in resistant host cotyledons associated with transcripts coding for extracellular receptors and phytohormone signaling molecules. Functional characterization provides direct support for transcriptome data and positively identifies novel resistance regulators in the *Brassicaceae*. Spatial gradients of gene activity were identified in response to *L. maculans* proximal to the site of infection. This dataset provides unprecedented spatial and temporal resolution of the plant defense response to *L. maculans* and serves as a valuable resource to those interested in host pathogen interactions.

5.2 Introduction

Brassica napus ranks second largest in production among oilseed crops worldwide and is under constant threat of blackleg disease caused by the fungal pathogen, *Leptosphaeria maculans* (Fitt et al. 2006). Resistance to blackleg mediated by race-specific resistance (*R*) genes relies on their interaction with a corresponding pathogen avirulence (*Avr*) gene (Larkan et al. 2015). Successful interaction between the products of *R* and *Avr* results in an incompatible host-pathogen interaction and inhibits pathogen colonization of the host plant conferring resistance. However, absence of either the *R*- or *Avr*- gene results in a compatible host-pathogen interaction and successful pathogen infection. Each interaction is likely governed by large sets of genes activated over time and under the control of cellular receptors and signal transduction cascades leading to either plant immunity or defeat. Despite the identification of *R* genes conferring blackleg resistance (Marcroft et al. 2012), there is little information on the mechanisms by which these genes effectively inhibit *L. maculans* colonization on canola. Previous transcriptome studies on this pathosystem focused heavily on pathogen virulence and effectors and limited analyses to a susceptible cultivar late in the infection process (Lowe et al. 2014). Thus, there is a critical need to understand the genes and regulatory pathways facilitating host resistance against fungal pathogens and how these pathways are controlled in both space and time.

Plant defense response mechanisms are commonly subdivided into two immune pathways: pattern triggered immunity (PTI) and effector triggered immunity (ETI) (Jones and Dangl, 2006). PTI is characterized by the detection of pathogen associated molecular patterns (PAMPs) via extracellular membrane receptors such as receptor-like proteins (RLPs) and

receptor-like kinases (RLKs) while ETI is characterized by the detection of pathogen effectors or their perturbation of host molecules by intracellular nucleotide binding-leucine rich repeat (NB-LRR) receptors (Tsuda and Katagiri, 2010; Dangl et al. 2013). Both of these bioprocesses utilize shared cellular machinery to elicit a defense response; however, PTI is generally associated with non-host resistance, and ETI (in conjunction with PTI) with host gene-for-gene incompatibility (Bigeard et al. 2015). However, this ETI/PTI dichotomy cannot be effectively applied to the Arabidopsis- or *B. napus-L. maculans* pathosystems. Not only are effector-triggered NB-LRR receptors required for Arabidopsis non-host resistance to *L. maculans* (Staal et al. 2006), but the recently cloned *B. napus R*-gene, *LepR3,* was identified as a transmembrane receptor-like protein (RLP) (Larkan et al. 2013). Thus, effector triggered defense (ETD) was proposed by Stotz et al. (2014) and refers specifically to RLP-triggered incompatible interactions. Unlike the rapid cell death observed in ETI, ETD is often associated with a delayed onset of cell death, as observed in *B. napus-L. maculans* incompatible interactions (Stotz et al. 2014). As *L. maculans* grows apoplastically, the ability of *R*-gene products to detect pathogens in the extracellular space is logical and supports the ETD paradigm.

Following the recognition of hemibiotrophic pathogens, early defense responses such as calcium influx, the production of reactive oxygen species (ROS), and the activation of mitogen-activated protein kinases (MAPKs) are triggered within the cell (Meng and Zhang, 2013). Large-scale transcriptional reprogramming contributes to the regulation of phytohormone signaling pathways during host-pathogen interactions (Denancé et al. 2013). Disease resistance mediated by *R* genes can trigger a localized hypersensitive response (HR),

thereby arresting pathogen growth. Salicylic acid (SA) signaling has been shown to contribute to the HR during *R*-gene-mediated resistance against biotrophic pathogens, whereas jasmonic acid (JA) and ethylene (ET) signaling pathways are often associated with response to necrotrophs (Tsuda et al. 2009; Vlot et al. 2009). Kaliff et al. (2007) showed JA, ABA, and the cellular oxidative burst all play a role in non-host resistance to *L. maculans* in Arabidopsis. In the *B. napus-L. maculans* pathosystem, JA, ET and SA signaling pathways are thought to be activated during the host-incompatible interaction (Sašek et al. 2012).

Downstream plant defense response pathways in hemibiotrophic pathosystems may involve the deposition of callose (Ellinger et al. 2013). Callose deposition is typically triggered by PAMPs, and PAMP-induced callose deposition has been used as a marker for PTI activity in Arabidopsis (Luna et al. 2011). Furthermore, secondary metabolites such as glucosinolates have been shown to play a role in the regulation of callose deposition (Clay et al. 2009). In Arabidopsis, resistance to hemibiotrophic fungi can often be dependent on the production of indole glucosinolates (Hiruma et al. 2013) or callose deposition (Staal et al. 2006; Kaliff et al. 2007), however their role in *Brassica napus-L. maculans* pathosystem remains unclear.

This current study we explore the activation of resistance pathways using RNA sequencing of *B. napus* cotyledons infected with *L. maculans* across a two-week infection period. We further investigate transcriptional programs in resistant hosts that are activated directly at the site of infection using laser microdissection (LMD) coupled with qPCR. Data reveal transcript gradients associated with the plant defense response that are amplified in resistant cotyledons that may contribute to pathogen arrest. Mutant analysis and detailed

anatomical examination of the infection process at the light and electron levels provide biological validations into the cellular processes that are likely responsible for disease resistance. Taken together, our data reveal *B. napus* resistance to *L. maculans* is controlled through the early activation of a suite of defense pathways that are controlled in both space and time directly at the site of infection and throughout the infection process.

5.3 Materials and methods

5.3.1 Plant and fungal materials

Universally susceptible *B. napus* cultivar Westar and *B. napus* line DF78 (*Rlm3, LepR1*) were inoculated with *L. maculans* isolate D3 (*AvrLm5, AvrLepR1*; Dr. A. Van de Wouw, University of Melbourne). Canola seedlings were grown in controlled environments with a 16-h photoperiod (16ºC dark, 21 ºC light). Plants were grown in Sunshine mix #4 (SunGro Horticulture, www.sungro.com). Fungal inoculum was prepared according to Zhang et al. (2016). Fungal isolates used in this study were described in Zhang et al. (2016) and can be found in Table 3.2 of this thesis. Seven day old seedlings of were point-inoculated with 10 µL of D3 pycnidiospore suspension (2×10^7 pycnidiospores mL^{-1}) or sterilized distilled water (mock).

5.3.2 Light microscopy, lignin and callose deposition

Cotyledons were processed for light microscopy exactly as reported in Chan and Belmonte (2013) using the Leica Historesin embedding procedure (Leica Microsystems). Sections cut 3 µm thick were stained with periodic acid-Schiff's (PAS) and counterstained with toluidine blue O (TBO) for general structure. For trypan blue/aniline blue staining of fungal hyphae,

fresh canola cotyledons were cleared in acetic acid: ethanol (1: 3, v/v) and stained with 0.01% trypan blue or 0.05 % aniline blue in lactoglycerol (lactic acid: glycerol: dH_2O = 1: 1: 1, v/v/v). To visualize plant lignified materials, canola cotyledons were cleared in 95% ethanol and stained in phloroglucinol-HCl (a saturated solution of Phloroglucinol in 20% HCl). Callose deposition was visualized using aniline blue staining. Cotyledons were incubated in K_2HPO_4 buffer for 30 mins and incubated in 0.05% aniline blue using fluorescence microscopy (near UV, 395 nm). All sections and tissues were visualized on a Zeiss Axio Imager Z1.

5.3.3 Scanning Electron microscopy

Scanning electron micrographs were visualized using the Hitachi, T-1000 to examine fungal infection on the surface of freshly collected canola cotyledons without tissue fixation.

5.3.4 Transmission electron microscopy

Cotyledons were infected as described above and were processed following the methods of Chan and Belmonte (2013). Cotyledons were fixed overnight in 3 % glutaraldehyde in 0.025M cacodylate buffer supplemented with 5 mM calcium chloride (pH 7.0). Plant material was rinsed with cacodylate buffer and post-fixed with 2 % osmium tetroxide in 0.8 % $KFe(CN)_6$. Cotyledons were rinsed with distilled water and stained overnight with a 0.5 % aqueous uranyl acetate solution. Processed cotyledons were then rinsed in distilled water and dehydrated in a graded ethanol series. Tissues were then dehydrated in 1:1 absolute ethanol to propylene oxide (v/v) and then in 100 % propylene oxide. Finally, dehydrated cotyledons were gradually infiltrated and embedded in Spurr’s epoxy resin at 70ºC. Sections of in 90 nm

thick were cut with a Diatome diamond knife using a Reichert–Jung Ultracut ultramicrotome and mounted on copper grids. Sections were visualized using a Hitachi H-7000 transmission electron microscope at 75 kV and images captured using the AMT Image Capture Engine version 601.384.

5.3.5 Construction of RNA sequencing libraries

RNA was collected from three biological replicates of infected and two of mock inoculated *B. napus* cotyledons at 0, 3, 7, and 11 dpi. Total RNA was isolated by using PureLink® Plant RNA Reagent (Ambion) and treated with TURBO DNA-free™ Kit (Ambion) according to the manufacturer's instructions. RNA quality and integrity was measured using the 2100 Bioanalyzer (Agilent Technologies) with the Agilent 2100 PicoChip. RNA-sequencing libraries were prepared according to alternative HTR protocol (C2) developed by Kumar et al. (2012) with the exception of a library PCR enrichment of 11 PCR cycles. RNA sequencing libraries were validated using the Agilent Bioanalyzer DNA chips (Agilent Technologies) and quantified using the Quant-iT dsDNA Assay kit (ThermoFisher Scientific). 50 bp single-end RNA-sequencing was carried out at the UC Davis genomics core facility (Davis, CA) on the Illumina HiSeq 2500 platform in high throughput mode. All data has been deposited in the Gene Expression Omnibus (GEO) data repository (accession GSE77723).

5.3.6 Data analysis

Barcode adaptors from the RNA sequence reads were clipped and low quality reads were removed (read quality < 30) using the Trimmomatic software (Bolger et al. 2014). Quality control of each sample was performed with FastQC reports

(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). RNA sequence reads passing quality filter were aligned to the *B. napus* genome (v4.1, Chalhoub et al. 2014) using Tophat2 of the Trapnell cufflinks package (Trapnell et al. 2012) allowing no more than two mismatches, in high sensitivity mode, used *B. napus* reference annotation v5.0 as a guide (Chalhoub et al. 2014), and otherwise used default settings. Identification of novel transcripts was performed using cufflinks v2.2.1 and CuffMerge (Trapnell et al. 2012) and transcript sequences were extracted from this annotation using BedTools. Open reading frames (ORFs) were identified using TransDecoder (http://transdecoder.github.io) and subsequently identified ORFs were putatively identified with alignment against *Arabidopsis* TAIR10 using ncbi-BLAST (Altschul et al. 1990). Cuffquant, CuffNorm and Cuffdiff were used to generate normalized counts in FPKM (also known as RPKM in single-ended sequencing (Mortazavi et al. 2008; Trapnell et al. 2012)) and to identify differentially expressed genes (pooled dispersion method/standard settings). Genes were considered significantly differentially expressed with a corrected p-value of < 0.05 (false discovery rate = 0.05). Clustering was performed on raw counts as part of the DESeq software package (Anders and Huber, 2010) with default settings.

5.3.7 Gene Ontology (GO) term enrichment

GO term enrichment was performed according to the methods of Orlando et al. 2009. A hypergeometric distribution test was used to identify statistically enriched GO terms overrepresented in lists of differential or co-expressed gene sets and assigned a p-value. GO terms were considered statistically enriched at $p < 0.001$. GO attributes were assigned to *B.*

napus genes by transferring GO attributes of their closest putative Arabidopsis homolog (TAIR10; www.arabidopsis.org).

5.3.8 Laser microdissection, RNA isolation, cDNA synthesis and qPCR

Inoculated cotyledons were collected and processed for LMD according to the methods of Belmonte et al. (2013). Briefly, infection sites were cut parallel to the cotyledon petiole-like structure on either side of the lesion between 11:00AM-2:00PM to minimize time of day effect. A minimum of 16 infection sites were collected from the four treatments were fixed in 3:1 (v/v) ethanol : acetic acid and fixed overnight at 4°C. Tissues were then rinsed and dehydrated in a graded ethanol series (75%, 85%, 95%, 100%, 100%) followed by xylene infiltration (3:1, 1:1, 1:3 ethanol : xylene (v/v), 100% xylenes, 100% xylenes) at 4°C overnight. Tissues were washed with 100% xylene and paraffin chips were added to the xylene infiltrated tissue and kept at 4°C overnight. Paraffin chips and tissue in xylenes were then allowed to come to room temperature and incubated at 42°C for 30 minutes followed by 60°C for 1 hour. Three changes of 100% paraffin were made every hour before embedding.

Cotyledon tissues were sectioned using a Leica RM2125RT rotary microtome at 10 µm under RNAse-free conditions and mounted on Leica PEN Membrane slides before being deparaffinized in xylene two times for 30 sec per wash. Histological sections 0-200, 200-400 and 400-600 µm from the edge of the infection site were collected into 60 µl of lysis buffer (Ambion, Origin). RNA was isolated from sections totalling at least 9000000 μm^2 (ranging from 115 to 200 microdissected sections) from at least 7 plant individuals exactly as reported in Belmonte et al. (2013). RNA quality and yield was assessed using microcapillary

electrophoresis (Agilent 2100 Bioanalyzer using an RNA 6000 pico chip) before being synthesized to cDNA.

One microgram of isolated RNA was converted to cDNA using the Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc.). Directed real-time quantitative polymerase chain reaction (qPCR) was carried out using a Bio-Rad CFX Connect^{TM} Real-Time System with SYBR® Green Supermix (Bio-Rad, USA) as per manufacturer's instructions in a 10 µl reaction volume. Conditions for the reaction were as follows: 39 cycles of 95 °C for 3 min, 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s. Melt curves (0.5 °C increments in a 55-95 °C range) for each gene were performed to assess the sample for non-specific targets, splice variants, as well as primer dimers. A list of the primer sequences used in these experiments is found in Appendix X. The ΔΔCt method was used to analyze relative transcript abundance, normalizing to the endogenous housekeeping gene *Actin* and using Westar inoculated with H_2O as a reference sample.

The ΔΔCt method was used to analyze relative mRNA abundance (Rieu and Powers, 2009). The results are based on three repeats in three independent experiments. The ΔΔCts of the replicates for each sample and distance contained tissue from at least 7 individuals. Actin (GenBank accession number: AF111812.1) was used as the internal control to normalize the expression of the target gene. Levels of gene expression were normalized relative to that in Westar (0-200 µm) control.

One-way ANOVA with Ducan's multiple range test ($p<0.05$) was performed on each gene over the three distances to test for significant fold changes between treatments ($p<0.05$).

5.3.9 Arabidopsis susceptibility screening

We screened 49 loss-of-function Col-0 background Arabidopsis mutants for susceptibility to *L. maculans* (Appendix XI). PCR was performed to confirm homozygous insertion of the mutants. Col-0 plants were used as a resistant control line and mock water-inoculated controls were performed for all lines. Plant growth and fungal inoculation procedures were similar as described in *B. napus* plant growth and fungal inoculation, with some modifications. Seeds were plated in MS medium in sterile conditions, then cold-treated for three days at 4°C, incubated in controlled environment for 14 days, and transplanted into growth tray with growth mix. Inoculation of two similarly-sized young leaves per plant was performed one week after transplantation, and after inoculation a transparent plastic cover was placed over the plants to maintain high humidity. At least 30 plants from each treatment group were evaluated for blackleg resistance at 18-24 days post inoculation and scored for disease severity.

5.4 Results

5.4.1 Phenotypic and cellular characterization of *B. napus* cotyledons in response to *L. maculans* infection

We first examined the phenotypic characteristics of resistant (DF78; LepR1) and susceptible (Westar) *B. napus* hosts infected with *L. maculans* (Fig. 5.1a). Lesions spread rapidly in susceptible cotyledons at 7 days post-inoculation (dpi) whereas lesion size in resistant hosts only appreciably increased towards the end of the 14-day infection period (Fig. 5.1b). Scanning electron and light microscopy of resistant hosts revealed minimal cellular

breakdown adjacent to the site of infection at 3 and 7 dpi (Fig. 5.1c-d, i-j) despite visible fungal hyphae within the site of infection (Fig. 5.1e), with marginal degradation proximal to the inoculation site at 11 dpi (Fig. 5.1k). In susceptible hosts, cells adjacent to the site of infection were intact at 3 dpi (Fig. 5.1f) and dramatically deteriorated at 11 dpi (Fig. 5.1g, n) with fungal fruiting bodies clearly visible (Fig. 5.1h, m).

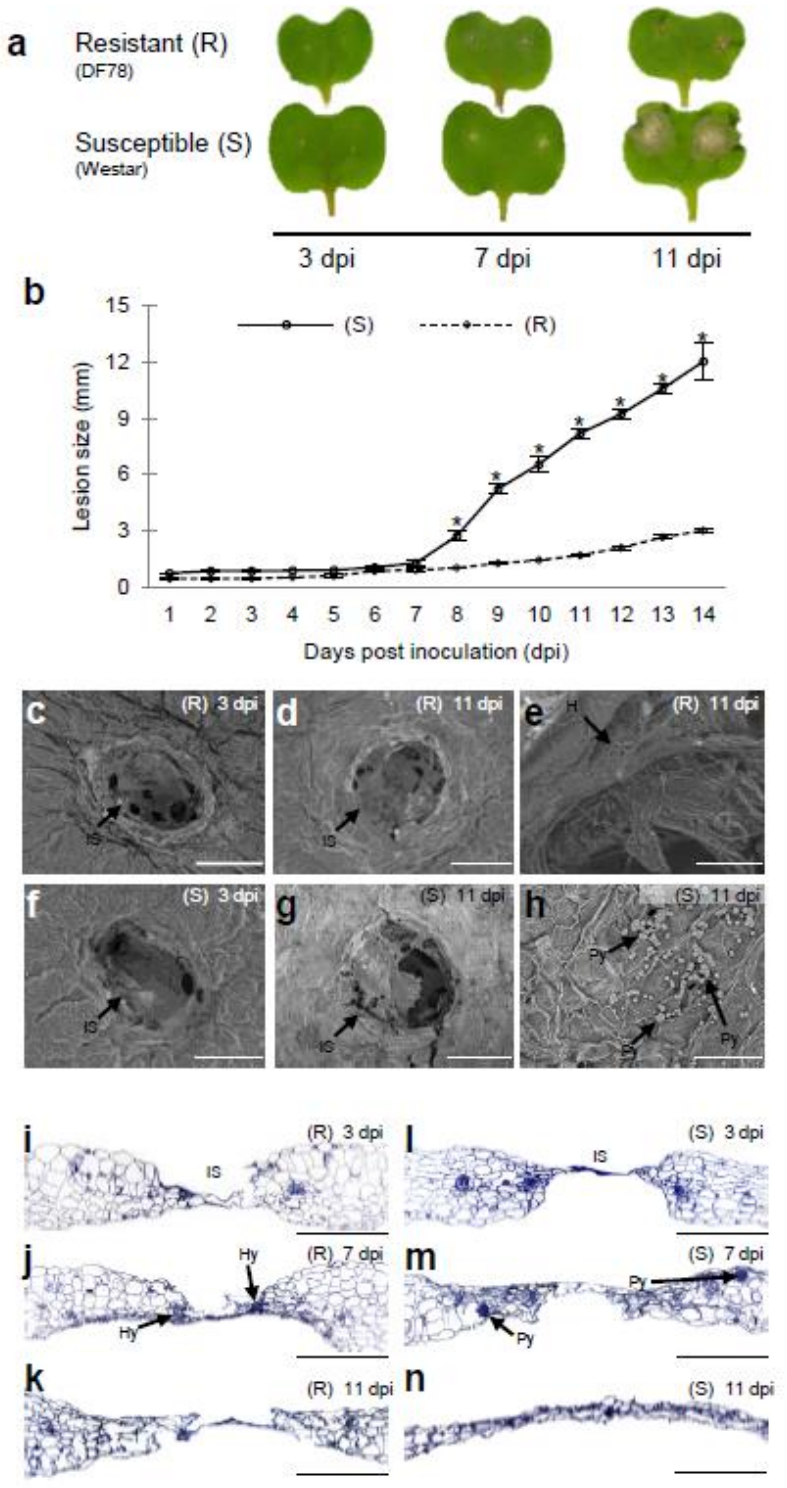

Fig. 5.1 Disease symptoms in *Brassica napus* cotyledons in response to *L. maculans* infection. (a) Disease symptoms in resistant (R) and susceptible (S) cotyledons at 3, 7 and 11 days post inoculation (dpi). (b) Lesion size over time. Asterisks ($p<0.01$, student's t test). Scanning electron micrograph (SEM) of R at 3 dpi (c) and 11 dpi (d) at the infection site (black arrow), scale = 1 mm. (e) Fungal hyphae (H) at infection site, scale = 50 µM in R at 11 dpi. (F) SEM of S at 3 dpi (f) and 11 dpi (g) at the infection site (IS), scale = 1 mm. (h) SEM of pycnidia (Py) on S cotyledons at 11 dpi, scale = 200 µM. (i-n) Light micrographs of R at 3 dpi (i), 7 dpi (j), 11 dpi (k) and S at 3 dpi (l), 7 dpi (m) and 11 dpi (n). Scale bars = 500 µM.

5.4.2 The *LepR1-AvrLepR1* interaction is responsible for the resistance phenotype

To confirm that the resistance phenotype of resistant cotyledons was a *LepR1-AvrLepR1* incompatible interaction, we performed cotyledon inoculation assays based on the gene-for-gene model developed by Flor (1971) frequently applied in the characterization of *R* genes in *B. napus* (Rouxel et al. 2003; Marcroft et al. 2012). A total of 34 characterized *L. maculans* isolates were tested against both the susceptible and resistant host and the interaction phenotype is summarized in Appendix VII (Zhang et al. 2016). Our results show DF78 is resistant to all isolates carrying either *AvrLepR1* or *AvrLm3*. As *L. maculans* isolate D3 does not carry *AvrLm3* (Appendix VII), the host resistance of DF78 cotyledons must be the result of a *LepR1–AvrLepR1* gene interaction. These results were confirmed using *B. napus* varieties/lines (Q2, 1065) previously characterized as carrying *Rlm3* (Van de Wouw et al. 2010) and *LepR1* (Kutcher et al. 2010b) respectively as controls. When Westar was challenged with all 34 isolates, no resistance was observed, confirming previous reports that Westar is universally susceptible to *L. maculans* (Appendix VII).

5.4.3 Global comparison of gene activity in the *B. napus–L. maculans* pathosystem

To identify genes responsible for *B. napus* resistance to *L. maculans*, we profiled the transcriptome of resistant and susceptible cotyledons using next generation RNA sequencing across a two-week infection period. First, hierarchical clustering analysis (Fig. 5.2a) revealed relationships between genotypes and in response to *L. maculans* infection at the global transcript level. Early in the infection process at 3 dpi, transcript populations clustered based on genotype, with susceptible and resistant hosts each forming distinct clades. By 11 dpi,

samples clustered together regardless of genotype based on exposure to *L. maculans*, suggesting similar transcript populations may accumulate in both genotypes towards the end of the infection period (Fig. 5.2a).

Fig. 5.2b summarizes transcript populations in both genotypes and across treatments. Transcript abundance was measured as Fragments Per Kilobase of gene per Million mapped reads (FPKM) where a gene was scored as 'expressed' when FPKM ≥ 1 (Mortazavi et al. 2008; Trapnell et al. 2012; Bhardwaj et al. 2015). Regardless of genotype or treatment, the number of active genes was similar, with an average number of 41,110 expressed genes (41% of the *B. napus* gene models). Transcript abundance was scored as low (FPKM ≥1, <5), moderate (FPKM ≥5, <25), or high (FPKM ≥ 25), with the vast majority of transcripts detected at low (53%) or moderate (36%) levels. Cumulatively, 57,654 transcripts were detected across all 12 treatments with an FPKM of at least 1 (Fig. 5.2b).

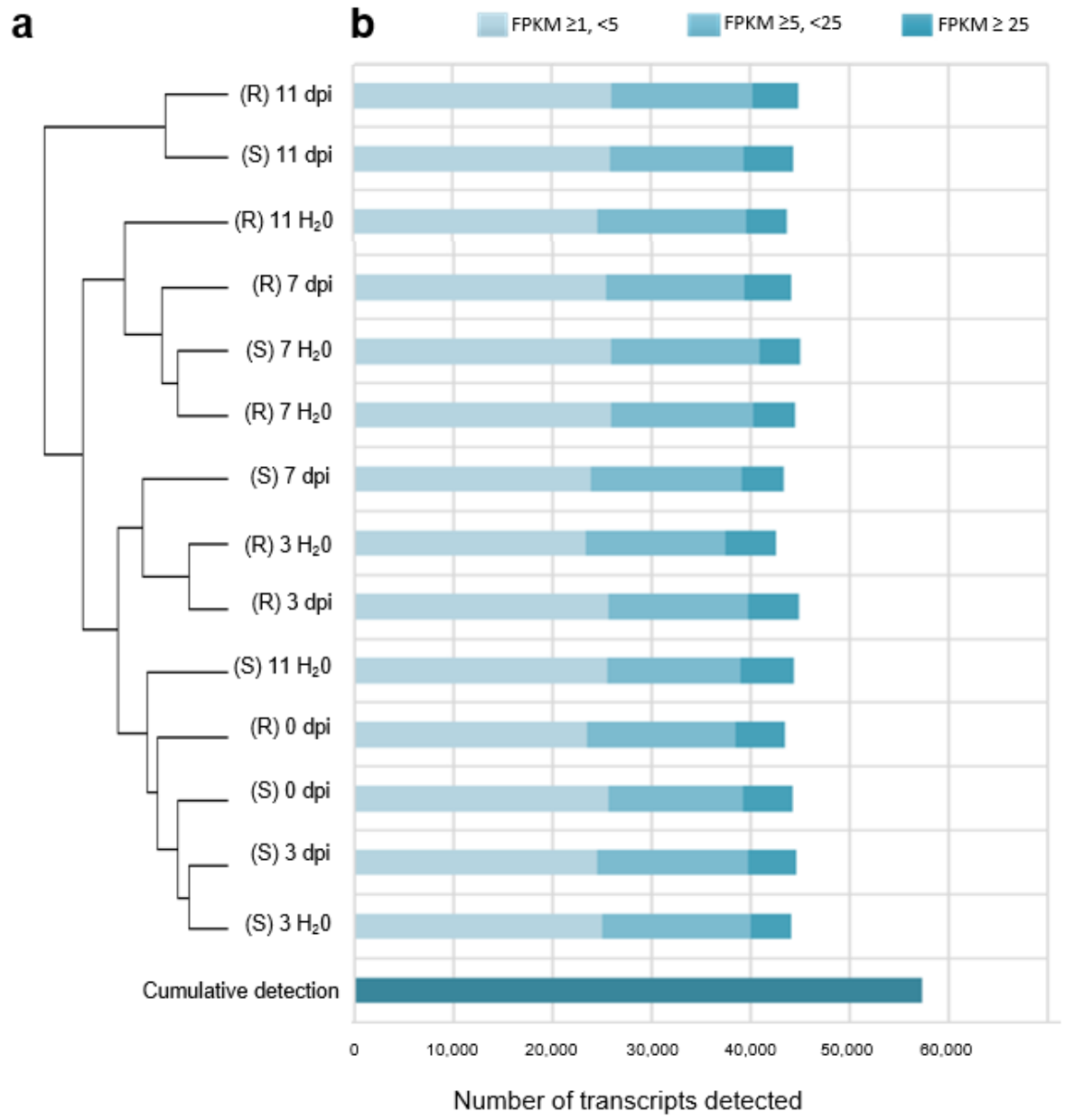

Figure 5.2 Hierarchicalclustering and global gene activity in the *B. napus-L. maculans* pathosystem. (a) Hierarchical clustering of resistant (R) and susceptible (S) hosts inoculated with *L. maculans* or water. (b) Number of transcripts detected in both genotypes across all treatments. Transcripts with an FPKM [Fragments Per Kilobase of transcript per Million mapped reads] > 1 are considered to be detected. Detected transcripts are subdivided into low (FPKM ≥1, <5), moderate (FPKM ≥5, <25), or high (FPKM ≥ 25) detection levels.

5.4.4 Thousands of genes are activated in resistant *B. napus* in response to *L. maculans*.

To identify genes contributing to plant resistance, we compared transcript abundance at all stages of the 11-day infection process in both resistant and susceptible hosts. At 3, 7, and 11 dpi, a total of 1992, 3234, and 4173 transcripts accumulated specifically in the resistant- and 571, 3873, and 8489 transcripts in the susceptible hosts, respectively (Fig. 5.3a-c). The number of shared accumulating transcripts between the two genotypes increased over time, suggesting a partially conserved response. At 3 dpi, only 102 (3.8%) of accumulated transcripts were shared between resistant and susceptible cotyledons, suggesting early and unique responses to *L. maculans* shortly after inoculation. This number increased to 2860 (28.7%) at 7 dpi and 6644 (34.4%) at 11 dpi.

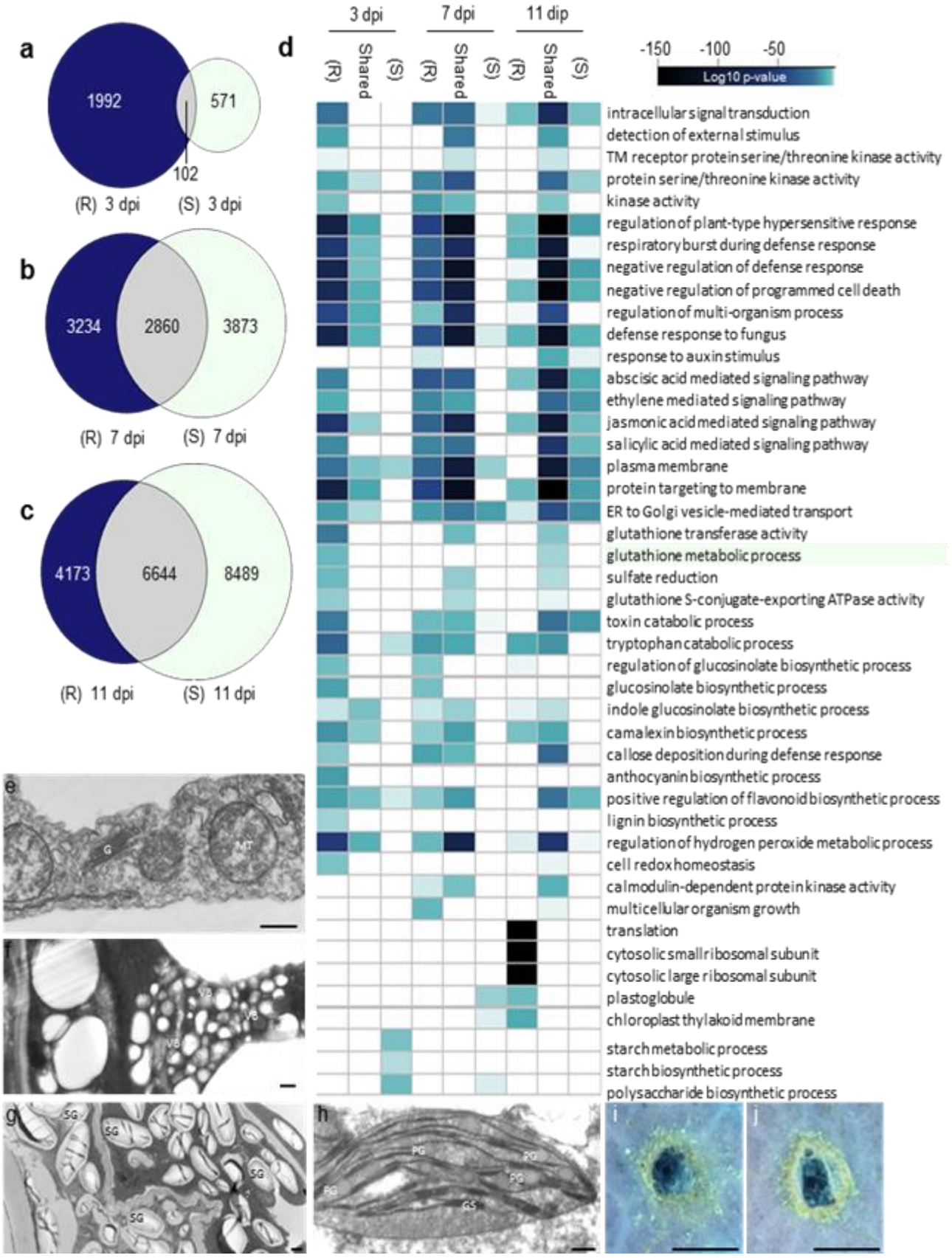

Fig. 5.3 Differential gene activity in resistant (R) and susceptible (S) *B. napus* cotyledons inoculated with *L. maculans*. (a-c) Venn diagram showing activated genes at 3 days post

inoculation (dpi; a), 7 dpi (b), and 11 dpi (c) in response to *L. maculans* in R (left), S (right) or shared between both genotypes (intersect). (d) Heatmap of enriched Gene Ontology (GO) terms. Terms are considered enriched at $P < 0.001$. Darker blue color represents a greater statistical enrichment. (e) Transmission electron micrograph of mesophyll in S host cotyledons at 7 dpi showing golgi [G] and mitochondria [MT], scale = 500 nM. (f) (R), 7 dpi, showing vesicular bodies [VB], Scale = 500 nM. (g) (S), 11 dpi, showing deposited starch granules [SG] in infected foliar tissues, scale = 2 mM. (h) (R), 11 dpi, chloroplast containing a number of plastoglobuli [PG], scale = 500 nM. (i-j) Aniline blue callose staining of S (i) and R (j) *B. napus* cotyledons inoculated with *L. maculans*, scale = 1 mm.

5.4.5 Host resistance is associated with pathogen recognition, cell signaling, and vesicular trafficking in resistant plants

To identify the biological processes, molecular functions, and cellular components contributing to resistance against *L. maculans*, we performed Gene Ontology (GO) term enrichment on differentially expressed gene sets (Fig. 5.3d). Our analysis identified transcripts contributing to kinase activity (P = 1.05E-13), signal transduction (P = 1.5E-04), and plasma membrane (P = 2.85E-30) all enriched by 3 dpi in resistant cotyledons, and included transcripts coding for wall-associated kinases (WAKs), RLKs, RLPs, LRR-NBS receptors, and transducers of signaling such as mitogen-activated protein kinases (MAPK) and MAPK kinases (MKK). Specifically, we identified two putative homologs of *RLP30* (*BnaA06g12200D*, *BnaA06g12220D*), receptor complex regulator *SUPPRESSOR OF BIR1 1* (*SOBIR1*, *BnaA03g14760D*, *BnaCnng39490D*), and homologs of signal transducer MKK9 (*BnaA02g35860D*, *BnaC02g22230D*) that were activated specifically in resistant cotyledons at 3 dpi (Table 5.1).

Genes associated with ER to Golgi vesicle-mediated transport were enriched at 7 dpi (P = 9.36E-15) in resistant cotyledons and include homologs of *MEMBRIN 12*

(*BnaC04g08330D*), *SNARE-LIKE SUPERFAMILY GENE* (*BnaC01g38970D*), *SEC23/SEC24 TRANSPORT FAMILY GENE* (*BnaA08g17130D*), and *PENETRATION 1* (*BnaC03g73490D*) (Table 5.1). Further, transmission electron micrographs clearly showed vesicle trafficking in resistant cotyledons at 7 dpi (Fig. 5.3f) that were not observed in the susceptible host (Fig. 5.3e).

Table 5.1 Accumulation of transcripts during *L. maculans* infection in resistant (DF78) and susceptible (Westar) *B. napus* cotyledons. Significant ($P < 0.05$) differences in transcript abundance compared to mock controls are in bold.

***B. napus* locus**	**Transcript Name**	**Fold Change vs. Mock Control**					
		R	**R**	**R**	**S**	**S**	**S**
		3 dpi	**7 dpi**	**11 dpi**	**3 dpi**	**7 dpi**	**11 dpi**
BnaA03g46200D	*PUTATIVE NBS-LRR RECEPTOR*	**2.16**	**6.03**	**3.02**	0.85	**10.46**	**26.07**
BnaC04g12970D	*PUTATIVE NBS-LRR RECEPTOR*	**2.12**	**3.55**	1.40	0.54	**2.40**	**12.61**
BnaA03g14760D	*SUPRESSOR OF BIR1 1*	**2.10**	**5.12**	**2.13**	1.43	**2.80**	**21.13**
BnaCnng39490D	*SUPRESSOR OF BIR1 1*	**2.99**	**3.86**	**3.19**	1.36	**3.74**	**7.21**
BnaC04g43230D	*RECEPTOR-LIKE PROTEIN 30*	**4.60**	**12.75**	**3.12**	0.70	**4.92**	**37.27**
BnaA06g12200D	*RECEPTOR-LIKE PROTEIN 30*	**2.97**	**5.90**	1.28	1.41	1.54	**12.65**
BnaA04g06980D	*CRK10*	**5.12**	**3.29**	**14.21**	0.42	0.82	**17.56**
BnaA02g21140D	*CRK39*	**5.20**	**41.07**	**10.07**	1.12	**27.26**	**205.9**
BnaA02g35860D	*MAP KINASE KINASE 9*	**2.00**	**2.86**	**2.30**	0.64	**1.72**	**12.94**
BnaC02g22230D	*MAP KINASE KINASE 9*	**5.39**	**5.27**	**2.41**	**0.40**	**4.90**	**25.43**
BnaA08g17130D	*SEC23/24 TRANSPORT GENE*	0.99	**2.40**	0.80	1.41	0.82	**2.20**
BnaC03g73490D	*SYNTAXIN OF PLANTS 121*	1.03	**1.71**	**1.86**	1.85	1.05	**7.90**
BnaA07g30760D	*KUNITZ TRYPSIN INHIBITOR 1*	**2.69**	**3.51**	**9.31**	0.59	**0.03**	**0.14**
BnaC09g20030D	*BAX INHIBITOR 1*	**1.82**	**3.08**	**4.53**	1.39	**11.54**	**38.20**
BnaC03g58590D	*NECROTIC SPOTTED LESIONS 1*	**1.70**	**1.98**	**1.70**	1.31	1.70	**19.29**
BnaC03g22580D	*NUDIX HYDROXYLASE H7*	**5.53**	**17.96**	**11.44**	1.54	**39.82**	**27.34**
BnaC01g41070D	*BOTRYTIS SUSCEPTIBLE 1*	**1.66**	1.08	1.18	0.64	0.69	**6.87**
BnaC06g13910D	*DEFENDER AGAINST DEATH 1*	**1.81**	**1.83**	**1.74**	1.28	0.55	**45.89**
BnaA07g15670D	*DEVELOPMENT AND CELL*	**2.73**	1.30	**2.20**	0.99	1.00	**28.99**
BnaC09g50680D	*SULFITE REDUCTASE 1*	**1.77**	**2.62**	0.97	0.69	1.29	**1.05**
BnaA03g38670D	*APK1*	**2.65**	**5.89**	**6.69**	1.27	0.81	**3.16**
BnaA01g34620D	*APK1*	**3.37**	**4.87**	**25.01**	0.59	0.83	2.15
BnaA09g20370D	*APS REDUCTASE 1*	**2.85**	**2.40**	**1.79**	1.14	**5.60**	**6.53**
BnaC09g22760D	*APS REDUCTASE 1*	**2.27**	1.19	1.32	1.24	**12.51**	**5.02**
BnaA06g28850D	*GLUTATHIONE SYNTHETASE 2*	**1.55**	**2.01**	**1.94**	0.99	1.64	**1.87**
BnaC07g27830D	*GLUTATHIONE SYNTHETASE 2*	**1.87**	**1.81**	**1.85**	1.03	0.84	**1.78**
BnaC09g40740D	*GLUTATHIONE S-TRANSFERASE*	**10.46**	0.44	**0.25**	**0.20**	**10.13**	**0.09**
BnaA07g24870D	*LIPOXYGENASE 2*	1.00	**19.09**	**13.06**	**0.00**	**0.00**	**0.05**
BnaA07g24880D	*LIPOXYGENASE 2*	**1.89**	**18.74**	**23.19**	**0.21**	**0.00**	**0.04**
BnaA04g17560D	*CINNAMATE-4-HYDROXYLASE*	**27.64**	**15.61**	1.48	1.50	1.61	**90.95**
BnaC04g41120D	*CINNAMATE-4-HYDROXYLASE*	**18.56**	**3.00**	1.61	0.77	1.53	**40.45**
BnaA07g32800D	*CINNAMOYL-COA REDUCTASE*	**21.61**	**45.49**	**32.21**	1.29	**116.69**	**206.3**

BnaA08g16100D	*CYP79B2*	**1.68**	**13.03**	**9.54**	1.38	1.70	**1.99**
BnaA08g04520D	*CYP83B1*	**1.78**	**2.07**	**3.70**	0.86	0.64	0.78
BnaC04g01210D	*WRKY46*	**2.43**	**3.07**	**2.18**	1.07	**11.31**	**11.3**
BnaA04g23480D	*WRKY54*	**2.49**	**6.85**	**3.24**	1.17	**4.65**	**8.72**
BnaA09g35840D	*WRKY70*	**3.32**	**12.87**	**23.49**	1.47	**27.31**	**24.71**
BnaC06g05910D	*ANAC019*	**3.09**	**2.76**	**1.95**	**0.29**	**0.20**	**191.8**
BnaA07g28000D	*ANAC019*	**4.11**	**5.69**	**2.33**	**0.16**	1.36	**1369.3**
BnaC08g18090D	*MYB51*	**1.55**	**6.58**	**5.16**	1.03	**8.40**	**13.42**

5.4.6 SA and JA signaling are strongly affected by the *LepR1-AvrLepR1* gene interaction

RNA sequencing and GO term enrichment revealed transcripts associated with salicylic acid-mediated signaling pathway (*P* = 6.70E-18), ethylene-mediated signaling pathway (*P* = 6.57E-12), and jasmonic acid-mediated signaling pathway (*P* = 2.48E-65) that accumulated specifically in resistant cotyledons by 3 dpi (Fig. 5.3d). To further characterize the temporal regulation of hormone production and signaling in response to *L. maculans*, we examined transcript levels of hormone biosynthetic genes and markers for SA, ET, JA, ABA, and auxin across the infection process in both genotypes (Appendix VIII). Further, in response to *L. maculans* levels of the SA biosynthetic gene *ISOCHORISMATE SYNTHASE 1* homologs in addition to the SA marker *PR1* increased an average of 500.8% at 3 dpi in resistant host cotyledons compared to 25.6% in their susceptible counterparts.

Data show ET biosynthesis and signaling genes are activated by 3 dpi in resistant cotyledons. *ACC OXIDASE 2* (*BnaA09g13300D, BnaC09g13570D*) and ET-JA marker *PDF1.2* (*BnaA07g32130D, BnaC02g23620D*) continue to increase until 11 dpi. Remarkably, JA biosynthetic gene in the susceptible reaction is repressed in response to *L. maculans*, with over 4-fold decrease in transcript levels of *LOX2* (*BnaA07g24870D, BnaA07g24880D*), *AOS* (*BnaC02g29610D*), and *AOC3* (*BnaC09g52550D*) as compared to mock controls at 7 and 11

dpi (Appendix VIII). Finally, activation of auxin (*NITRILASE 2, BnaA06g38980D, BnaC02g07040D, BnaC03g54910D, BnaCnng75490D*) and ABA (*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3, BnaA01g29390D, BnaC01g36910D, BnaC05g39200D*) biosynthetic genes were emphasized in susceptible cotyledons at 11 dpi and suggest possible contributions of these hormones in defense regulation at latter stages of the infection process.

5.4.7 Regulation of cell death is associated with resistance against *L. maculans*

A suite of transcripts associated with negative regulation of programmed cell death (*P* = 4.76E-76) accumulated specifically in resistant hosts at 3 dpi (Table 5.1), including putative homologs of *BAX INHIBITOR 1* (*BnaC09g20030D*), *BOTRYTIS SUSCEPTIBLE 1 INTERACTOR* (*BnaC01g41070D*), *DEVELOPMENT AND CELL DEATH 1* (*BnaA07g15670D*), *NUDIX HYDROXYLASE HOMOLOG 7* (*BnaC03g22580D*), *METACASPASE 2* (*BnaA01g14460D*), and *NECROTIC SPOTTED LESIONS 1* (*BnaC03g58590D).*

5.4.8 Rapid activation of genes associated with sulfur assimilation and metabolism in resistant host cotyledons

Accumulation of transcripts associated with sulfate reduction (*P* = 1.51E-07), sulfate assimilation (*P* = 1.14E-11), and glutathione metabolic process (*P* = 8.64E-08) were observed specifically in resistant cotyledons at 3 dpi (Figure 5.3d), and included sulfur assimilators *APS REDUCTASE* (*APR1*, *BnaA09g20370D*, *BnaC09g22760D*), *APR2* (*BnaC04g19270D*), *APR3 (BnaC01g13420D, BnaC07g37060D)*, and *SULFITE REDUCTASE* (*BnaC09g50680D*),

and sulfate activators *ADENOSINE 5'-PHOSPHOSULFATE KINASE 1* (*APK1, BnaA03g38670D*) and *APK2* (*BnaA01g34620D, BnaC01g00790D, BnaC07g51290D*). Additionally, homologs of *GLUTATHIONE SYNTHETASE 2* (*BnaA06g28850D, BnaC07g27830D*) were activated specifically in resistant hosts at 3 dpi (Table 5.1). In addition to its role as a redox regulator, glutathione is a key intermediary in sulfur metabolism and the largest reservoir of non-protein reduced sulfur in the cell. It also directly serves a role in toxin neutralization through the activity of glutathione-S-transferases (GST). Genes associated with glutathione s-transferase (GST) activity (P = 2.77E-21) were identified specifically in resistant hosts at 3 dpi, including *GST PHI 2 (GSTF2*, BnaA03g26140D), *GSTF6* (*BnaC05g01540D*), *GSTF12* (*BnaC09g40740D*), *EARLY RESPONSE TO DEHYDRATION 9* (*ERD9, BnaA06g06160D*), *ERD13* (*BnaA03g14150D*), as well as 26 other GSTs.

5.4.9 Lignin accumulation in resistant host cotyledons

Genes coding for the formation of monolignols, *CINNAMATE-4-HYDROXYLASE* (*BnaA04g17560D, BnaC04g41120D*), *CINNAMOYL-ALCOHOL DEHEHYDROGENASE 8/ELICITOR-ACTIVATED GENE 3* (*BnaC03g61120D*), and *CINNAMOYL-COA REDUCTASE* (*BnaA07g32800D*) had a combined average 17.6-fold increase in transcript abundance in response to *L. maculans* infection resistant hosts at 3 dpi with no appreciable increase in the susceptible genotype (Table 5.1). This is supported by histochemical analyses of lignin deposition at the inoculation sites of both genotypes (Appendix XIV). Infected cotyledons of the resistant host showed prominent and coordinated lignin deposition proximal

to the site of infection and surrounding vasculature whereas lignin deposition was diffuse and uncoordinated in the susceptible host and provides histochemical validation of the RNA sequencing data.

5.4.10 Activation of indole glucosinolate biosynthetic and callose deposition genes

We identified transcripts associated with indole glucosinolate (IGS) biosynthetic process enriched in resistant cotyledons (P = 5.38E-05) by 3 dpi. Each step of the IGS biosynthetic pathway significantly accumulated earlier and to higher levels in resistant hosts, whereas levels of transcripts essential to IGS production, such as *CYP79B2* and *CYP83B1* (Table 5.1), declined in response to *L. maculans* in the susceptible genotype (Appendix XIV). We then investigated transcripts associated with callose deposition during the defense response (P = 1.98E-05) identified in resistant cotyledons at 3 dpi, which largely overlapped with the IGS biosynthetic genes and regulators described above. To visualize callose deposition, we stained infected and non-infected cotyledons with aniline blue. Lignin deposition was diffuse in susceptible hosts (Figure 3i) while deposition of callose in resistant hosts was found directly adjacent to the site of infection (Figure 3j).

5.4.11 NAC and WRKY transcription factors are associated with the accelerated defence response in resistant hosts

To identify transcription factors (TFs) associated with the accelerated defense response of resistant hosts, we identified TFs from the enriched GO terms regulation of plant-type hypersensitive response (P = 1.05E-95), intracellular signal transduction (P = 1.54E-23), and defense response to fungus (P = 3.03E-93) (Appendix XV). Of the 36 TF-coding transcripts,

19.4% and 30.5% coded for members of the NAC and WRKY TF families, respectively. We also identified IGS-promoting *MYB51*, JA-responsive JAZ TFs, and *BZIP60* and *HSF-A4A* associated with the cellular heat-shock response. Interestingly, although specifically activated in resistant hosts early at 3 dpi, 94.6% of these transcripts accumulate in susceptible cotyledons to levels exceeding all other treatments by 11 dpi (Appendix XVI).

5.4.12 Identification of genes specifically activated by the *LepR1-AvrLepR1* gene interaction

To identify resistant-specific genes responding to *L. maculans*, we compared both the susceptible and resistant host transcriptomes across the infection process. We first identified 1221 shared transcripts at 3, 7 and 11 dpi in resistant host cotyledons (Fig. 5.4a). Of these 1221 transcripts, 54 accumulated only in the resistant host when compared to its susceptible counterpart (Fig. 5.4b). These 54 resistant-specific transcripts included genes involved in signal transduction and gene regulation, such as *RLP30* (*BnaA06g12220D*), *CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASE 11* (*CRK11, BnaA01g12650D*), *CRK21* (*BnaAnng25570D*), *NON-INDUCIBLE IMMUNITY-INTERACTING GENE 1* (*NIMIN-1*, *BnaC07g23070D*), and *ERF-1* (*BnaAnng21280D*). Further, this list contains two genes associated with sulfur assimilation, *SULFATE TRANSPORTER 4.1* (*BnaA03g04410D*) and *APS-KINASE 2* (*APK2, BnaC07g51290D*), and multiple enzymes catalyzing steps in IGS biosynthesis (Fig. 5.4c). The complete list of the 54 genes resistant-specific genes can be found in Appendix XVII.

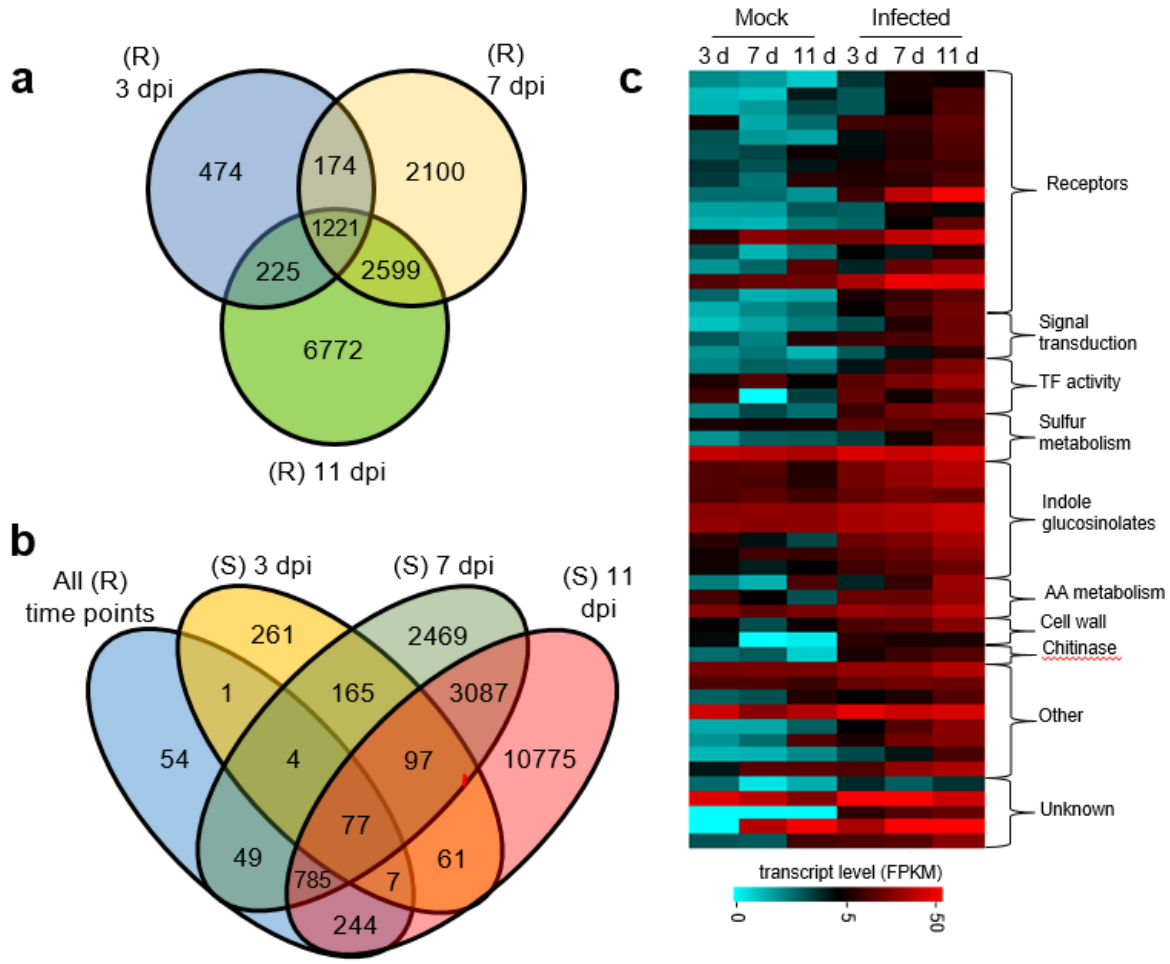

Fig. 5.4 Identification of transcripts accumulating in resistant (R) cotyledons inoculated with *L. maculans*. (a) Venn diagram showing transcripts accumulating in R hosts at 3, 7, and 11 days post inoculation (dpi). (b) Identification of R-specific transcripts unique to R hosts (c) Expression profiles of 54 transcripts specific to R hosts. Transcript levels are measured in FPKM (Fragments Per Kilobase of transcript per Million mapped reads).

Bohman et al. (2004) established the Arabidopsis-*L. maculans* pathosystem as a model for *L. maculans* infection of the *Brassicaceae*. While a non-host to *L. maculans*, Arabidopsis plants will become susceptible to this pathogen if compromised in their ability to detect and/or respond appropriately. To functionally characterize the resistant-specific genes identified during our analyses, we challenged 49 corresponding loss-of-function Arabidopsis mutants with *L. maculans*. Six mutants displayed a breakdown of Arabidopsis non-host

resistance to *L. maculans* by 20 dpi (Fig. 5.5). This included *apk2* mutants, deficient in production of activated sulfur required for biosynthesis of sulfur-containing secondary compounds including IGS and camalexin (Mugford et al. 2009). Additionally, *gstf6* plants, which are also compromised in camalexin production (Su et al. 2011), were susceptible to *L. maculans* infection. Mutants of cell death repressor *KUNITZ TRYPSIN INHIBITOR 1* (*KTI1*) were sensitive to infection and production of pycnidia were clearly visible by 20 dpi. Finally, two mutant receptors (*at4g18250, at3g53490)*, and receptor partner *lysm-interacting kinase 1* (*lik1*), also failed to prevent *L. maculans* colonization of host tissues. LIK1, a phosphorylation target of the main chitin receptor CERK1, is associated with activation of JA-ET signaling and the repression of SA immune responses (Le et al. 2014). The complete list of screened mutants is found in Appendix XVII.

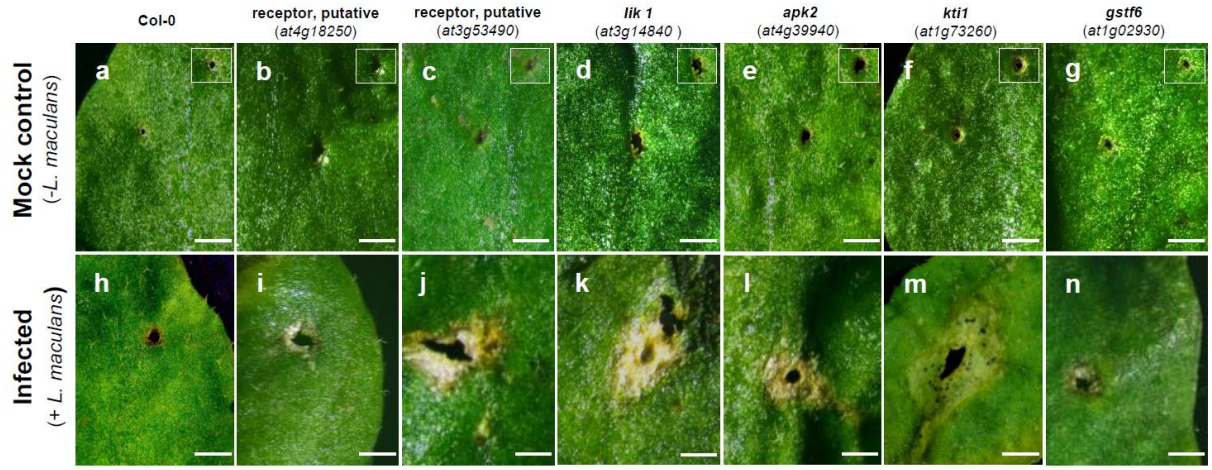

Fig. 5.5 Disease symptoms in Arabidopsis following *L. maculans* infection. (a-g) Mock inoculated controls at 20 days post inoculation (dpi). (h-n) *L. maculans* lesion in mutants following D3 inoculation at 20 dpi. (a,h) Wild-type Col-0 (b,i) *at4g18250.1,* putative receptor (c,j) *at3g53490.1,* putative receptor (d,k) *at3g14840.1, lysm interacting kinase 1* (e,l) *at4g39940.1, aps kinase 2* (f,m) *at1g73260.1, kunitz trypsin inhibitor 1,* (g,n) *at1g02930.2, glutathione s-transferase 6.* Scale bar = 1 mm.

5.4.13 Laser microdissection and gene activity directly at the site of infection

We used LMD coupled with qPCR to better understand how resistant-specific genes and other important defense regulators are spatially partitioned within the cotyledon directly at the site of infection (Fig. 5.6). We focused our attention on cotyledons at 7 dpi; a critical time point observed between the two genotypes in response to *L. maculans* (Figure 1b). In response to *L. maculans*, the levels of all nine genes that were tested *(LIK1*, *PR1*, *WRKY25*, *PDF1.2*, *APK2*, *RBOHF*, *CYP79B2*, *BnaA03g43720D* and *BnaC04g27200D),* were significantly elevated in the resistant hosts as compared to every other treatment at one or more of the distances tested, suggesting a highly controlled immune response in this genotype proximal to the site of inoculation.

When resistant host cotyledons were challenged with *L. maculans*, levels of *APK2*, *RBOHF*, *WRKY25*, *PR1*, *BnaA03g43720D*, and *BnaC04g27200D* transcripts were concentrated within tissues 0-200 µm from the site of infection. Levels of *LIK1* and *CYP79B2* accumulated at greatest levels 200-400 µm from the site of infection. Marker of JA-ET signaling, *PDF1.2*, was the only transcript to accumulate highest in tissues taken distally (400-600 µm) from the site of infection in resistant hosts, with detection of 5.7-fold higher than at 0-200 µm. These data show spatial coordination of gene activity in tissues directly at the site of infection in response to *L. maculans* attack.

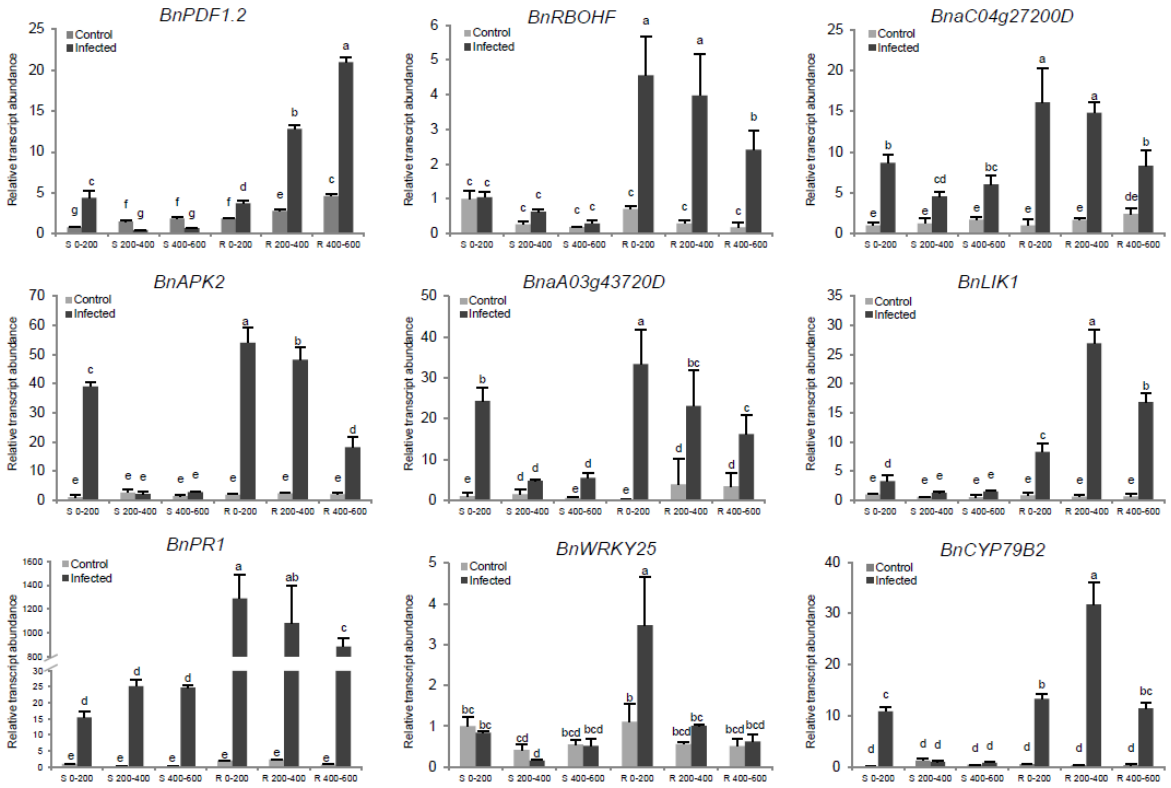

Fig. 5.6 *B. napus* following inoculation with *Leptosphaeria maculans*. Relative transcript abundance of *BnPDF1.2*, *BnRBOHF*, *BnaC04g27200D*, *BnAPK2*, *BnaA03g43720D*, *BnLIK1*, *BnPR1*, *BnWRKY25* and *BnCYP79B2* in susceptible (S) and resistant (R) cotyledons as measured 0-200, 200-400, and 400-600 µm from the inoculation site. Actin (GenBank accession number: AF111812.1) was used as the internal control and to normalize expression data. Relative transcript abundance is normalized relative to S mock (0-200 µm) treatment. Error bars represent standard deviation of the mean. For each gene, different lowercase letters indicate significant differences among mean values (one-way ANOVA with Ducan's multiple range test (p<0.05)). The results are based on three replicates in three independent experiments.

5.5 Discussion

Global RNA profiling canola cotyledons reveals genes uniquely associated with plant resistance to the hemibiotrophic fungus, *L. maculans*. An accelerated defense response in resistant host tissues coincides with the deposition of lignin and callose that likely prevents *L. maculans* colonization and reproduction in apoplastic spaces in canola cotyledons. Transcripts associated with resistance accumulated as gradients away from the infection site providing unprecedented spatial resolution into the *B. napus-L. maculans* pathosystem.

Accelerated defense gene activity observed in resistant host cotyledons is associated with the rapid activation of RLPs, RLKs, TIR-NBS receptors, and receptor partner proteins. Of these, 17 receptors were specific to the resistant line and 12 were uncharacterized with no previously described host-pathogen annotation in *B. napus, A. thaliana* or any other plant pathosystem. As ETD pathways are mediated through extracellular RLPs and their associated partner proteins (Stotz et al. 2014), activation of these receptors may produce a positive feedback loop amplifying the plant immune response and improving pathogen detection. Arabidopsis mutants of two homologous receptors (*at4g18250* and *at3g53490*) were susceptible to *L. maculans* infection, suggesting they have a conserved defensive role in the *Brassicaceae* during defense against *L. maculans*.

R-gene efficacy is often independent from the host CD response (Schiffer et al. 1997; Cawly et al. 2005), suggesting that CD may not always be responsible for host resistance, but rather a by-product of runaway immune response or cell damage due to infection. Indeed, many necrotrophic or facultatively necrotrophic pathogens will induce host cell death mechanisms to facilitate infection (Lorang et al. 2007; Kabbage et al. 2013). We identified specific activation of phytopathogen-induced CD repressor *KUNITZ TRYPSIN INHIBITOR 1* (*KTI1*) in resistant hosts. When challenged with *L. maculans*, lesions spread rapidly in *kti* Arabidopsis plants after 18 dpi and is similar to the phenotype of *accelerated cell death 2* plants described by Bohman et al. (2004). As a hemibiotrophic fungus, *L. maculans* has the ability to switch to a necrotrophic form of growth and can survive within dead or dying plant tissues, and has been defined as primarily necrotrophic (Staal et al. 2008). Thus the recognition of *L. maculans* and activation of CD regulators early in the infection process are

likely contributing factors to host resistance. The comparative lack of these regulators early in susceptible hosts may explain its rapid lesion formation following the biotrophic-necrotrophic transition of *L. maculans.*

Susceptible cotyledons show a notable lag in JA response through diminished expression of integral JA biosynthetic enzymes *LOX2*, *AOS*, and *AOC*, at the time of rapid lesion spread. This is consistent with the specific accumulation of *LIK1* in resistant host cotyledons and the observed susceptibility of *lik1* Arabidopsis plants to *L. maculans*. LIK1, a direct phosphorylation target of CHITIN ELICTIOR RECEPTOR KINASE 1, acts as a positive regulator of ET-JA signaling pathways and has been implicated in defense against necrotrophic fungal pathogens (Le et al. 2014). The activity of NAC TFs early in resistant host cotyledons may directly promote JA production, as *NAC019* and *NAC055* are linked to JA-induced transcription of *LOX2* (Bu et al. 2008), and *anac019anac055* double mutants are susceptible to fungal necrotrophic pathogens (Bu et al. 2008).

The activation of genes associated with IGS biosynthesis provides another potential mechanism contributing to *B. napus* resistance against *L. maculans*. IGS have been shown to play a role in the HR against some hemibiotrophic fungi (Hiruma et al. 2013), and *in vitro* studies have shown S-glycosides from *B. napus*, predominantly those derived from the glucosinolate sinigrin, are toxic to *L. maculans* (Mithen et al. 1986). Our data show the complete IGS biosynthetic pathway was activated in resistant cotyledons. The production of IGS is linked to sulfur metabolism as all indole-derived phytoalexins in the Brassicas have one or more sulfur atoms (Pedras et al. 2011). Thus, the coinciding activation of genes associated with sulfur assimilation during the *LepR1-AvrLepR1* interaction supports the

production of IGS. Mugford et al. (2009) directly linked sulfur activator APK2 activity to IGS production in Arabidopsis. Although we have shown that *apk2* Arabidopsis plants are susceptible to *L. maculans*, the mechanism by which susceptibility in conferred is unclear. Other members of the IGS biosynthetic pathway that were challenged, including *cyp79b2*, *cyp79b3*, *cyp83b1*, and *cypb5c* had no discernable phenotype; however, *gstf6* plants, required for production of IGS and camalexin (Su et al. 2011), showed discernable lesions following *L. maculans* infection. The lack of a phenotype in IGS-compromised Arabidopsis plants may be due to complementation by the antifungal indole alkaloid camalexin also shown to be effective against *L. maculans* (Bohman et al. 2004). As *B. napus* lacks the ability to produce camalexin, IGS-derived phytoalexins may play a more important role in defense against *L. maculans*.

We gained further insight into the spatiotemporal control of blackleg resistance in canola using a combination of LMD and qPCR. LMD has successfully been applied to the Arabidopsis-*Golovinomyces orontii* (powdery mildew) pathosystem (Chandran et al. 2010) and the soybean incompatible response to *Heterodera glycines* (Klink et al. 2007). In both studies, LMD was able to increase the resolution of pre-existing data and identify novel defense regulators undetected in gross tissue collection. In the current study, we applied LMD to investigate gene activity at the onset of lesion formation directly at the site of infection, focusing on essential genes identified through mutant analysis. Our data show targeted activity of receptors consistent with their role in pathogen detection and signal transduction and suggest the plant defense response is separated in both space and time through transcriptional gradients of defense gene expression.

While hormone levels are known to flux over time during plant defense, there is little data on the development of spatial hormone gradients in the Brassicas. Interestingly, we show an antagonistic spatial relationship between SA and JA signalling pathways that is established specifically in resistant hosts. This is in agreement with the antagonistic temporal shifts in SA and JA signaling characteristic of the *B. napus-L. maculans* pathosystem (Sašek et al. 2012; Lowe et al. 2014).

Levels of *CYP79B2*, a marker for IGS biosynthesis, was greatest 200-400 µm away from the site of infection, in an area of combined SA and JA-ET gene activity. Frerigmann and Gigolashvili (2014) found the expression of the main IGS-inducing TF *MYB51* was greatest with joint application of SA and JA, and is consistent with our dataset. Thus, deposition of antifungal IGS-derived phytoalexins most likely does not occur in areas of direct pathogen contact, but rather upstream of invading *L. maculans* and is potentially guided by hormone gradients formed during defense.

Although the phenotypic response to *L. maculans* is different in susceptible and resistant host cotyledons, the majority of defense regulators including transcription factors are shared between both genotypes late in the infection process. The explanation for this phenomenon may come from early gene activation in resistant hosts triggering deposition of anti-fungal metabolites and callose preceding fungal invasion. The ability of resistant hosts to direct gene activity to the host-pathogen interface is likely another contributing factor, as is shown with the spatial expression profile of *WRKY25*, which acts synergistically with WRKY33 to regulate ET biosynthesis and heat-shock activation (Li et al. 2011). By concentrating the defense response to areas of direct fungal contact the energetic cost of

defense is reduced and damage to the host organism is mitigated.

Although this study mainly focused on genes and regulatory pathways in disease resistance response, modifying plant genes that critically facilitate host-pathogen compatibility could provide alternative strategies in disese control. When mutated, such susceptibility (*S*) genes can cause pathogen-specific resistance due to impaired prepenetration or postpenetration requirements, thus have the potential to be used in disease resistance breeding (Van Schie and Takken 2014). A good example of *S* gene is *Mlo* gene in barley, of which a recessive mutant provided non-race-specific and potential durable resistance to powdery mildew (Jorgensen 1992). Callose deposition at the site of infection is an induced defense response that is critical in resistant host. In this study, enhanced callose deposition was observed in resistant host. In *Arabidopsis*, overexpression of a callose synthase, *PMR4*, leads to complete resistance to powdery mildew through enlarged callose deposits. However, *pmr4* loss-of-function plants with less callose also showed reduced susceptibility to podwer mildew (Ellinger et al. 2013).Some defense-suppressing WRKYs were found in *Arabidopsis*, rice, and pepper (Van Schie and Takken 2014). Interestingly, in this study, a large number of TFs accumulate in susceptible cotyledons to levels exceeding all other treatments by 11 dpi. It is possible that some of these TFs are potential *S* genes in the *B. napus-L. maculans* pathosystem.

Our data represent a valuable resource to support studies on host susceptible and resistant interactions in oilseed crops. For resistant cotyledons, R protein-Avr protein recognition may occur at the cell surface at early infection stage, then receptors trigger a set of downstream defense pathways including hormone signaling (JA, SA, ET), IGS. Morover,

structural barriers such as callose deposition and lignification would prevent fungal colonization. For susceptible cotyledons, there is no recognition and defense pathways are triggered by basal immunity. These defense pathways eventually suppressed the accumulation of JA and other defense molecules, and eventually strong cellular barriers observed in resistant cotyledons do not occur. We have demonstrated that comparative bioinformatics analyses provide the necessary platform to successfully probe RNA sequence data for genes and gene products responsible for specifying plant resistance. The identification of genes responsible for mitigating plant disease through these methods further demonstrates the utility of the dataset. Furthermore, our data provides valuable insight into the spatial regulation of defense-related genes activated in response to the hemibiotrophic pathogen *L. maculans* and provides a preliminary framework in support of a transcriptional gradient responsible for disease mitigation in *B. napus*. While much of the underlying molecular mechanisms responsible for host resistance remain unresolved, access to technologies that are able to dissect cells and tissues immediately at and distal to the site of infection should provide clues for directed crop improvement.

6 GENERAL DISCUSSION ANDFUTURE DIRECTIONS

This thesis provided the foundation towards a better understanding of disease resistance genes and the underlying disease defense mechanisms in the *B. napus-L. maculans* pathosystem. Characterization of *R* genes in Canadian *B. napus* germplasm can facilitate the development of more efficient breeding strategies and a sustainable blackleg management strategy. The identification of both *R* genes and APR in a collection of Chinese *B. napus* germplasm will provide guidance in future disease resistance breeding programs in China while helping the Canadian canola seed to be exported without major restrictions being imposed. A genome-wide transcriptional comparison of defense mechanisms between compatible and incompatible interactions has highlighted key genes and pathways responsible for successful disease resistance against *L. maculans* infection.

6.1 Characterization of blackleg resistance in *B. napus* germplasm

Breeding for blackleg resistance is fundamental to successful disease management (Li and McVetty 2013). In Canada, although canola varieties labeled as resistant to blackleg have been used for the past 30 years in the field, disease incidence of blackleg has increased in recent years (Canadian plant disease survey, http://phytopath.ca/publication/cpds). The finding that *Rlm3* was overcome by the evolution of fungal populations further highlighted the high evolutional potential of the pathogen. While the predominant *Rlm3* is not effective at present, some other genes such as *Rlm2* and *Rlm4* will be very effective according to avirulence alleles in field fungal populations (Liban et al. 2016). Marcroft et al. (2012b) demonstrated that rotation of *R* genes can minimize disease pressure by manipulating fungal

populations. Although the diversity of *R* genes in current canola varieties is quite limited, *R* gene rotation strategies can still be a good choice to the Canadian canola industry. This strategy can be made according to results from this study and other related studies on *Avr* gene profiles of field fungal populations. For instance, *Rlm2* and *Rlm4* can be categorized into two different groups to reduce the selection pressure on fungal populations. *Rlm3* and *Rlm9* should be considered as belonging to one resistance group as *AvrLm3* and *AvrLm9* are in one gene cluster in the fungal genome (Balesdent et al. 2002). Some potential novel genes were identified in this study, and these genes should be further identified by mapping and gene cloning.

In Chinese *B. napus* germplasm, several known *R* genes (*Rlm1*, *Rlm2*, *Rlm3*, and *Rlm4*) and some unknown resistance genes were identified. It is evident that a combination of major gene resistance and APR can provide more effective and durable resistance against blackleg (Kiyosawa 1982; Pietravalle et al. 2006; Brun et al. 2010; Marcroft et al. 2012b). The presence of these four major genes in Chinese *B. napus* germplasm is of great importance as part of the strategy to address the risk of blackleg associated with introduction of *L. maculans* from canola imports. Most of the Chinese *B. napus* germplasm showed strong field resistance, and this can facilitate good resistance towards diverse fungal populations. Furthermore, the combination of APR and seedling resistance in these materials tested highlighted that Chinese *B. napus* industry has the genetic potential to fight against *L. maculans* infection.

6.2 Comparison of defense mechanisms in compatible and incompatible interactions in the *B. napus*-*L. maculans* pathosystem

The involvement of thousands of genes in disease defense responses highlighted the complexity of host defense mechanisms against the fungal pathogen, *L. maculans*. A time-course study uncovered the crucial role of early activation of a set of transcripts in incompatible interaction, including genes involved in pathogen recognition, callose and lignin deposition, hormone biosynthesis and signaling, and vesicular trafficking. Light microscopy further supported the evidence of stronger and more coordinated accumulation of callose and lignin around the site of infection. High levels of vesicular trafficking in the line DF78 was proven by TEM examination.

Hormone signaling pathways including JA, SA and ET are very important in both compatible and incompatible interactions (Donnell et al. 2003; Robert-Seilaniantz et al. 2011). In this study, early specific accumulations of SA-marker genes in DF78 were observed. This is in agreement with the role of SA signaling in *Rlm1*-mediated resistance in *B. napus* (Šašek et al. 2012). Results also suggested JA-responsive JN1/MYC2 as a putative regulator of SA-mediated pathways in incompatible interaction. A clear response lag in JA production was observed in susceptible cv. Westar at the time of rapid lesion spread. Additionally, a suite of WRKY TFs-targeting genes associated with regulation of SA biosynthesis was predicted to be activated in resistant line DF78 by 3 dpi. LMD data suggests localized SA signaling in areas directly adjacent to sites of infection as indicated via marker *PR1*, and elevated JA-ET signaling with migration from the site of infection as indicated by marker *PDF1.2*. Taken

together, early activation of hormone signaling pathways, especially SA and JA seem to play a crucial role in resistance response against *L. maculans* infection.

In this study, earlier (as early as 3 dpi) and higher levels of transcripts associated with each step of IGS biosynthesis were observed in resistant line DF78, compared with susceptible cv. Westar. Moreover, levels of transcripts essential to IGS production, declined in response to *L. maculans* in susceptible cv. Westar. *CYP79B2*, a marker for IGS biosynthesis, is essential in the initial step in IGS biosynthesis (Frerigmann and Gigolashvili 2014). This gene plays a crucial role in disease resistance of Arabidopsis to pathogens including *Phytophthora brassicae* and *Verticillium longisporum* (Zhao et al. 2002; Schlaeppi et al. 2010; Iven et al. 2012). We further studied site-specific expression of *CYP79B2*, and the results indicated that the expression level of *CYP79B2* was highest at 200-400 µm from the site of infection in resistant line DF78, in an area of high SA and JA-ET marker activity. Moreover, the involvement of IGS in callose deposition during disease defense has been addressed in many pathosystems (Clay et al. 2009). Callose deposition in earlier studies has been found to be involved in the *B. napus-L. maculans* resistance interaction (Kaliff et al. 2007).

In the *A. thaliana-L. maculans* interaction, callose depositions has been found to be crucial in *R* gene-mediated resistance against *L. maculans* infection (Staal et al. 2006; Kaliff et al. 2007). In this study, transcripts associated with callose deposition during the defense response were also identified in line DF78 at 3 dpi, and largely overlapped with the IGS biosynthetic genes and regulators. Histological analysis indicated the coordinated deposition of callose around the site of infection in resistant line DF78, whereas a diffuse pattern of

callose accumulation around the site of infection was observed in susceptible cv. Westar. These results highlight the important roles of IGS biosynthesis related genes in regulating site-specific and global defense responses, perhaps in the form of facilitating callose deposition, during the *LepR1-AvrLepR1* interaction. In addition, some TFs accumulated in susceptible cotyledons at 11 dpi could be potential *S* genes that can be used in gene editing.

RNA-Seq is a powerful tool in understanding global disease defense mechanisms in plants. However, plant defense response is very complicated, involving contributions of regulatory networks of thousands of genes, transcripts and downstream regulators. In this study, a set of 54 incompatible-specific genes were identified through global transcriptome comparison, where functions of these genes included signal transduction, gene regulation, sulfur assimilation, and receptor coding. Significant roles of some of these genes in the defense responses in the incompatible interaction were confirmed in this study. Although some Arabidopsis loss-of-function mutants tested in this study showed breakdown of resistance to *L. maculans* infection, their roles in disease defense remain unresolved.

6.3 Contribution to knowledge

To the best of our knowledge, this is the first study characterizing *R* genes in Canadian canola germplasm. The key finding on the breakdown of *Rlm3* resistance in Canada can benefit the canola industry as a whole. Primarily, this finding can guide canola breeders to make appropriate plans in breeding for blackleg resistance and furthermore, this finding can also guide canola growers to grow canola in a better way to reduce the incidence of blackleg in canola fields. This is also the first study characterizing *R* genes in Chinese *B. napus*

germplasm. Results obtained from this study will not only provide us with a better understanding on the risk of introducing *L. maculans* into China, but also provide us guidance in order to make proper decisions in blackleg resistance breeding programs in China. In the *B. napus-L. maculans* pathosystem, this is the first transcriptome-wide time-course study in compatible and incompatible host-pathogen interactions. This study identified a set of key genes and pathways involved in this pathosystem. These genes and pathways can encourage future studies aiming at the fight against *L. maculans* infection.

6.4 Future directions

To facilitate a proper and effective rotation of *R* genes in disease control, it is important to identify more *R* genes in *Brassica* species that confer disease resistance (Raman et al. 2013). In Canadian canola germplasm, unknown resistance including some potential novel *R* genes was shown. These findings highlighted the importance of *R* gene characterization in discovering novel *R* genes. To further identify these potential *R* genes, future studies such as association mapping and linkage mapping can be performed. Perfect molecular markers that can assist blackleg resistance breeding are in high demand to achieve a more successful disease management strategy. However, among 16 known *R* genes in *Brassica* species, only two have been cloned (Larkan et al. 2013, 2015). Therefore, *R* gene cloning is of essence in future studies. Due to the complexity of *B. napus* genome, cloning of *R* genes requires much more effort. With the rapid development of NGS technology and the availability of a *B. napus* reference genome, *R* gene cloning will become more efficient in the future.

This thesis reported the presence of several *R* genes in Chinese *B. napus* germplasm.

Chinese *B. napus* varieties were considered to be very susceptible to blackleg according to previous studies (Li et al. 2008; Zhang et al. 2014). However, in this study, we identified a large number of *B. napus* lines with both *R* gene resistance and APR. The differences in this study and previous studies could be mainly due to differences in sample size of the plant material. In previous studies, only a small number of varieties (less than 30) were used, whereas 150 accessions were used in this study. This finding will benefit the canola industry as a whole, especially towards of mitigating the risk of blackleg caused by *L. maculans* in China.

Although quantitative resistance is not the major target of this thesis, quantitative resistance has been proven to be valuable in disease control by increasing the durability of resistance (Brun et al. 2010). However, our knowledge on defense mechanisms underlying quantitative resistance remains somewhat elusive. Future studies targeting defense mechanisms associated with quantitative resistance are of great importance in achieving a more durable blackleg resistance.

Transcriptome studies highlighted the key roles of early activation of a set of genes and regulatory pathways in the *LepR1-AvrLepR1* interaction. Function validation of these key genes identified from this study is of essence in validating the initial findings. Similar studies of other *R* genes may identify additional resistance mechanisms, which may help design new strategies for *R* gene rotation based on modes of action, as well as the Avr gene profile in the pathogen population. Genes conferring disease susceptibility have been identified in some host plant species, editing these genes could provide alternative strategies in disease control.

Identification of *S* genes in *B. napus* can further expand our knowledge and support strategies to defeat *L. mauclans*.

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APPENDICES

Appendix I. Disease symptoms caused by blackleg (*Leptosphaeria maculans*) on canola cotelydons, leaves, stems and stem base.

Appendix II . Lodging and plant death of susceptible canola (*Brassica napus*) caused by blackleg (*Leptosphaeria maculans*).

Appendix III. Canola stubbles damaged by blackleg (*Leptosphaerica maculans*).

Appendix IV. Blackleg seedling disease resistance rating scale. Interaction phenotypes (IP) on 14 day post inoculation (dpi) cotyledons were scored on a 0 to 9 scale. One cotyledon is shown for each score. 0 - no disease, 1 - dark necrotic lesions around wound, sporulation absent, lesion diameter = 0.5–1.5 mm, 3 - dark necrotic lesions around wound, sporulation absent, lesion diameter = 1.5–3.0 mm, 5 - dark necrotic lesions around wound, sporulation absent, lesion diameter = 3.0–6.0 mm, lesion size may increase during later infection stage, 7 - tissue collapse, lesions with diffuse margins, sporulation present, 9 - rapid tissue collapse, lesions with diffuse margins, profuse sporulation.

Appendix V. Blackleg adult plant disease rating scale. Disease symptoms on cross-section of the crown (stem base) were scored on a 0 to 5 scale. 0 - no noticeable infection, 1 - diseased tissue occupies ≤ 25% of the cross-section, 2 - diseased tissue occupies 25–50% of cross-section, 3 - between 50–75% of the cross-section infected, 4 - more than 75% of the cross-section infected, 5 - 100% of cross-section were diseased, plant dead.

Appendix VI. *R* genes and adult plant resistance (APR) of Canadian *Brassica napus* varieties/lines.

Accession	Reaction to each isolate																						Putative *R* genes	APR[d]
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D13	D14	S7	ICBN14	PHW1223	R2	AD746	JN2	JN3	J3	J20	Q12		
DF-1	S[a]	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	MS
DF-2	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	R
DF-3	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3* (H)[b]	S
DF-4	S	S	S	S	S	S	R[a]	S	S	S	S	R	R	S	S	S	R	S	S	R	S	S	Unknown[c]	S
DF-5	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	Unknown	S
DF-6	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	MS
DF-7	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	MR
DF-8	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	S	Unknown (H)	MR
DF-9	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S	R	S	S	S	S	S	S	Unknown (H)	MS
DF-10	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MR
DF-11	R	S	R	S	R	S	R	S	S	S	S	R	R	R	S	S	R	S	S	R	R	S	*Rlm2* (H), *Rlm3*, unknown (H)	R
DF-12	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	*Rlm3*	S
DF-13	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MR
DF-14	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	R
DF-15	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	*Rlm3*	R
DF-16	S	S	S	S	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown (H)	R
DF-17	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	S
DF-18	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	Unknown	MS
DF-19	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	Unknown	S
DF-20	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MS
DF-21	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MR
DF-22	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MS
DF-23	S	S	R	S	S	S	R	S	S	S	S	S	R	S	S	R	R	S	S	R	R	S	*Rlm3*	R
DF-24	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MS
DF-25	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	S
DF-26	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	*Rlm3*	S
DF-27	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	S

DF-28	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	S
DF-29	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	S
DF-30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	R
DF-31	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	Unknown	S
DF-32	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	R
DF-33	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	S
DF-34	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MR
DF-35	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	S
DF-36	S	S	S	S	R	R	R	S	S	S	S	R	R	S	S	S	S	S	R	S	S	S	*Rlm1*	R
DF-37	S	S	S	S	R	R	R	S	S	S	S	R	R	S	S	S	R	S	R	R	R	S	*Rlm1*, *Rlm2* (H), *Rlm3*	R
DF-38	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	R	S	*Rlm3*	S
DF-39	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	S
DF-40	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	R	R	S	*Rlm3*, unknown	S
DF-41	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown	S
DF-42	S	S	S	S	R	R	R	S	S	S	S	R	R	S	S	S	R	S	R	R	R	R	*Rlm1*, *Rlm2 (H)*, *Rlm3*	R
DF-43	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S	S	R	S	S	S	R	S	*Rlm3*, unknown	MS
DF-44	S	S	S	S	R	R	R	S	S	S	S	R	R	S	S	S	R	S	R	R	R	S	*Rlm1*, *Rlm2* (H), *Rlm3*	R
DF-45	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	S
DF-46	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown	MS
DF-47	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MR
DF-48	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*	MR
DF-49	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	S	R	S	*Rlm3* (H)	S
DF-50	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S	S	Unknown	S
DF-51	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	Unknown	R
DF-52	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	R
DF-53	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	R
DF-54	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown	R
DF-55	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	Unknown	R
DF-56	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown	R
DF-57	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	*Rlm3*	R
DF-58	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	S

DF-59	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Unknown	MR
DF-60	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	Unknown	S
DF-61	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	Unknown	S
DF-62	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	MR
DF-63	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	S
DF-64	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	MS
DF-65	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown	R
DF-66	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	S	S	Unknown	MS
DF-67	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	S
DF-68	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	Unknown	MS
DF-69	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	Unknown	MR
DF-70	S	R	R	S	S	R	S	S	S	R	S	R	R	S	R	R	R	S	R	R	R	S	*Rlm1, Rlm3, Rlm9*	R
DF-71	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	Unknown	MR
DF-72	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	MR
DF-73	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown	S
DF-74	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rl*m3, unknown	MS
DF-75	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	Unknown	MR
DF-76	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	Unknown	MS
DF-77	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	Unknown	MR
DF-78	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	*Rlm3, LepR1*	R
DF-79	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	*Rlm3, Rlm9, RlmS, LepR1*	R
DF-80	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	*Rlm3, Rlm9, RlmS, LepR1*	MR
DF-81	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	Unknown	R
DF-82	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown (H)	R
DF-83	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	R
DF-84	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*(H)	R
DF-85	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown (H)	R
DF-86	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	R
DF-87	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	*Rlm3*(H)	R
DF-88	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None	R
DF-89	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	Unknown	R

DF-90	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*,unknown	MR
DF-91	R	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Unknown	R
DF-92	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown (H)	R
DF-93	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	Unknown	R
DF-94	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	*Rlm3*	R
DF-95	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	Unknown (H)	R
DF-96	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	R
DF-97	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	R
DF-98	S	S	S	S	R	S	R	S	S	S	R	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown	R
DF-99	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	Unknown (H)	R
DF-100	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*	S
DF-101	S	S	S	R	R	S	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	*Rlm4*	MS
DF-102	R	S	S	R	R	S	R	S	S	S	R	S	S	S	S	S	R	S	R	R	R	S	*Rlm2, Rlm3, Rlm4*	MS
DF-103	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	S	*Rlm3*, unknown	S
DF-104	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	*LepR2*	MS

[a] R/S indicates resistant or susceptible reaction to each *Leptosphaeria maculans* isolate.

[b] (H) refers to accessions with heterogeneous seeds whereby *R* gene was detected in 50-80% of the plants.

[c] Unknown means the resistance genotype cannot be determined in this study, accessions showed unknown resistance might be due to the presence of a new *R* gene, other known *R* gene or combination of several *R* genes.

[d] APR refers to adult plant resistance, R-resistant, MR-moderately resistant, MS-moderately susceptible, S-susceptible.

Appendix VII. *R* genes of seed samples collected from growers' fields based on their interactions with genetically characterized *Leptosphaeria maculans* isolates.

Names	Reaction to isolates											Putative *R* genes
	D3	D4	D5	D7	D10	ICBN14	PHW1223	R2	AD746	JN3	J3	
MP1	S[a]	S	S	R[a]	S	S	S	S	R	S	R	*Rlm3* (H)[b]
MP2	S	S	S	S	S	S	S	S	S	S	S	None
MP3	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MP4	S	S	S	S	S	S	S	S	S	S	S	None
MP5	S	S	S	S	S	S	S	S	S	S	S	None
MP6	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*(H)
MP7	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*(H)
BR1	S	S	R	R	S	S	S	S	R	S	R	*Rlm2* (H), *Rlm3*(H)
BR2	S	S	S	S	S	S	S	S	S	S	S	None
BR3	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
BR4	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
BR5	S	S	R	R	S	S	S	S	R	S	R	*Rlm2*,*Rlm3*
BR6	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
BR7	S	S	I	I	S	S	S	S	R	S	S	Unknown[c]
BR8	S	S	S	S	S	S	S	S	S	S	S	None
BR9	S	S	S	S	S	S	S	S	S	S	S	None
BR11	S	S	S	S	S	S	S	S	S	S	S	None
BR12	S	S	S	S	S	S	S	S	R	S	S	Unknown
BR13	S	S	S	S	S	S	S	S	S	S	S	None
BR14	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
BR15	S	S	S	S	S	S	S	S	S	S	S	None
BR16	S	S	S	S	S	S	S	S	S	S	S	None
BR17	R	S	S	S	S	S	S	S	S	S	S	Unknown
BR18	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
BR19	S	S	S	S	S	S	S	S	R	S	S	Unknown
BR20	S	S	S	S	S	S	S	S	S	S	S	None
BR21	R	S	R	R	S	S	S	S	R	R	R	*Rlm1, Rlm2, Rlm3*
BR22	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*(H)
BR23	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
BR24	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
PC1	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
PC2	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
PC3	S	S	S	S	S	S	S	S	S	S	S	None
PC4	S	S	S	S	S	S	S	S	R	S	S	Unknown
PC5	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
PC6	S	S	S	S	S	S	S	S	S	S	S	None
PC8	S	S	S	S	S	S	S	S	S	S	S	None
PC10	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
PC11	S	S	S	S	S	S	S	S	S	S	S	None

SB1	S	S	R	R	S	S	S	S	S	S	S	*Rlm1*
SB2	S	S	S	S	S	S	S	S	S	S	S	None
SB3	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*(H)
SB5	S	S	S	S	S	S	S	S	S	S	S	None
SB6	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*(H)
SB7	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*(H)
SB8	S	S	S	S	S	S	S	S	S	S	S	None
SB9	S	S	S	S	S	S	S	S	R	S	R	Unknown
SB10	S	S	S	S	S	S	S	S	S	S	S	None
SB11	S	S	S	S	S	S	S	S	S	S	S	None
SB12	S	S	S	S	S	S	S	S	S	S	S	None
MO2	S	S	S	S	S	S	S	S	S	S	S	None
MO3	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MO4	S	S	S	S	S	S	S	S	S	S	S	None
MO5	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*(H)
MO6	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*(H)
MO7	S	S	S	S	S	S	S	S	S	S	S	None
MO8	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MO9	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MO10	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MO11	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
MO12	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MD1	S	S	S	S	S	S	S	S	S	S	S	None
MD2	S	S	S	S	S	S	S	S	S	S	S	None
MD3	S	S	S	S	S	S	S	S	S	S	S	None
MD4	S	S	S	S	S	S	S	S	S	S	S	None
MD5	S	S	S	S	S	S	S	S	S	S	S	None
MD6	S	S	S	S	S	S	S	S	S	S	S	None
MD7	S	S	S	S	S	S	S	S	R	S	R	Unknown
MD8	S	S	S	S	S	S	S	S	S	S	S	None
MD9	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MD10	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
MD11	S	S	S	S	S	S	S	S	S	S	S	None
MD12	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MD13	S	S	S	S	S	S	S	S	S	S	S	None
MD14	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
MD15	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
SW1	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
SW2	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
SW3	S	S	S	S	S	S	S	S	S	S	S	None
SW4	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
SW5	S	S	S	S	S	S	S	S	S	S	S	None
SW6	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
SW7	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
SW8	S	S	S	S	S	S	S	S	S	S	S	None
SW9	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*

SW10	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
SW11	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
SW12	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
SW13	S	S	S	S	S	S	S	S	R	S	R	Unknown
SW14	S	S	S	S	S	S	S	S	R	S	R	Unknown
SW15	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
SW16	S	S	S	S	S	S	S	S	S	S	S	None
SW17	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
SW18	S	S	S	R	S	S	S	S	R	S	R	*Rlm3* (H)
SW19	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
SW20	S	S	S	S	S	S	S	S	S	S	S	None
GR2	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
GR3	S	S	S	S	S	S	S	S	S	S	S	None
GR4	S	S	S	S	S	S	S	S	S	S	S	None
GR5	S	S	S	S	S	S	S	S	S	S	S	None
GR6	S	S	S	S	S	S	S	S	S	S	S	None
GR7	S	S	S	S	S	S	S	S	S	S	S	None

[a] R/S indicates resistant or susceptible reaction to each Leptosphaeria maculans isolate.
[b] (+), (H) refers to accessions with heterogeneous seeds whereby *R* gene was detected in 50-80% of the plants.
[c] Unknown means the resistance genotype cannot be determined in this study, accessions showing unknown resistance might be due to the presence of a new *R* gene, other known *R* gene or combination of several *R* genes.

Appendix VIII. *R* genes against blackleg (*Leptosphaeria maculans*) where cotyledons were inoculated at 7 days after seeding and assessed for disease severity at 14 days post inoculation identified in Chinese *Brassica napus* accessions.

Accession	Reaction to isolates											Putative *R* genes
	D3	D4	D5	D7	D10	ICBN14	PHW1223	R2	AD746	JN3	J3	
1001	S[a]	S	R[a]	S	S	S	S	S	S	S	S	Unknown[b]
1005	S	S	S	S	S	S	S	S	S	S	R	Unknown
1017	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
1021	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
1036	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
1037	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
1051	S	S	S	S	S	S	S	S	S	S	S	None
1055	R	S	R	S	S	S	S	S	R	S	R	*Rlm2*, unknown
1056	R	S	R	S	S	S	S	S	R	S	R	*Rlm2*, unknown
1058	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
1068	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
1075	S	S	S	S	S	S	S	S	S	S	S	none
8010	S	S	S	R	S	S	S	R	R	S	R	*Rlm3*, unknown
8011	S	S	S	S	S	S	S	S	R	S	R	Unknown
8012	S	S	S	S	S	S	S	S	S	S	S	None
8013	S	S	S	S	S	S	S	S	S	S	S	None
8014	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
8015	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
8016	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
8017	S	S	S	S	S	S	S	S	S	S	S	None
8021	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
8022	S	S	S	S	S	S	S	S	S	S	S	None
8024	S	S	S	S	S	S	S	S	S	S	S	None
8025	S	S	S	S	S	S	S	S	S	S	S	None
8026	S	S	S	S	S	S	S	S	S	S	S	None
8027	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
8030	S	S	S	S	S	S	S	S	S	S	S	None
8034	R	S	R	S	S	S	S	S	S	S	S	Unknown
8037	S	S	R	R	S	S	S	S	R	R	R	*Rlm2*,*Rlm3*,unknown
8041	R	S	R	S	S	S	S	S	R	S	R	*Rlm2*, unknown
8048	S	S	S	S	S	S	S	S	S	S	S	None
CC01	S	S	S	R	S	S	S	R	R	S	R	*Rlm3*, unknown
CC02	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
CC03	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
CC04	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
CC05	S	S	S	S	S	S	S	S	S	S	S	None
CC06	S	S	S	S	S	S	S	S	S	S	S	None
CC07	S	S	S	S	S	S	S	S	S	S	S	None

CC08	S	S	S	S	S	S	S	S	S	S	S	None
CC09	S	S	S	S	S	S	S	S	S	S	S	None
CC10	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HC1001	S	S	S	S	S	S	S	S	S	S	S	None
HC1002	S	S	S	S	S	S	S	S	S	S	S	None
HC1003	S	S	S	S	S	S	S	S	S	S	S	None
HC1004	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
HC1005	S	S	S	S	S	S	S	S	S	S	S	None
HC1006	S	S	R	S	S	S	S	S	S	S	S	Unknown
HC1007	S	S	S	S	S	S	S	S	S	S	S	None
HC1008	S	S	S	S	S	S	S	S	S	S	S	None
HC1009	S	S	R	S	S	S	S	S	S	S	S	Unknown
HC1010	S	S	S	S	S	S	S	S	S	S	S	None
HC1011	S	S	S	S	S	S	S	S	S	S	S	None
HC1012	S	S	S	S	S	S	S	S	S	S	S	None
HC1013	S	S	S	S	S	S	S	S	S	S	S	None
HC1014	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
HC1015	S	S	S	S	S	S	S	S	S	S	S	None
HC1016	S	S	S	S	S	S	S	S	S	S	S	None
HC1017	S	S	S	S	S	S	S	S	S	S	S	None
HC1018	S	S	S	S	S	S	S	S	S	S	S	None
HC1019	S	S	S	S	S	S	S	S	S	S	S	None
HC1020	S	S	S	S	S	S	S	S	S	S	S	None
HC1021	S	S	S	S	S	S	S	S	R	S	S	Unknown
HC1022	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HC1023	S	S	S	S	S	S	S	S	S	S	S	None
HC1024	S	S	S	S	S	S	S	S	S	S	S	None
HC1025	S	S	S	S	S	S	S	S	S	S	S	None
HC1026	S	S	S	S	S	S	S	S	S	S	S	None
HC1027	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
HC1028	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HC701	S	S	S	S	S	S	S	S	S	S	S	None
HC702	S	S	S	S	S	S	S	S	S	S	S	None
HC703	S	S	S	S	S	S	S	S	S	S	S	None
HC801	S	S	S	R	S	S	S	S	S	S	R	*Rlm3*
HC802	S	S	S	S	S	S	S	S	S	S	S	None
HC803	S	S	S	S	S	S	S	S	S	S	S	None
HC804	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
HC805	S	S	S	S	S	S	S	S	S	S	S	None
HC806	S	S	S	S	S	S	S	S	S	S	S	None
HC807	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
HC808	S	S	S	S	S	S	S	S	S	S	S	None
HC809	S	S	S	S	S	S	S	S	S	S	S	None
HC810	S	S	S	S	S	S	S	S	R	S	S	Unknown

HC811	S	S	S	S	S	S	S	S	S	S	S	None
HC812	S	S	S	S	S	S	S	S	S	S	S	None
HC813	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
HC814	S	S	S	S	S	S	S	S	S	S	S	None
HC815	S	S	S	S	S	S	S	S	S	S	S	None
HP01	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
HP02	S	S	S	S	S	S	S	S	S	S	S	None
HP03	S	S	S	S	S	S	S	S	S	S	S	None
HP04	S	S	S	S	S	S	S	S	S	S	S	None
HP05	S	S	S	S	S	S	S	S	S	S	S	None
HP06	S	S	S	S	S	S	S	S	S	S	S	None
HP07	S	S	S	S	S	S	S	S	S	S	S	None
HP08	7.3	R	R	S	S	S	S	S	S	R	9.0	*Rlm4*
HP09	S	S	R	R	S	S	S	S	S	R	9.0	*Rlm1*
HP10	S	S	S	R	S	S	S	S	S	S	R	*Rlm3*
HP11	S	S	S	S	S	S	S	S	S	S	S	None
HP12	S	S	S	S	S	S	S	S	S	S	S	None
HP13	S	S	S	S	S	S	S	S	S	S	S	None
HP14	S	S	S	S	S	S	S	S	S	S	S	None
HP15	S	S	S	S	S	S	S	S	R	S	S	Unknown
HP16	S	S	S	S	S	S	S	S	S	S	S	None
HP17	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP18	S	S	S	S	S	S	S	S	S	S	S	None
HP19	S	S	S	S	S	S	S	S	S	S	S	None
HP20	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP21	S	S	R	R	S	S	S	S	R	S	R	*Rlm3*, unknown
HP22	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP23	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP24	S	S	S	S	S	S	S	S	S	S	R	Unknown
HP25	S	S	S	S	S	S	S	S	S	S	S	None
HP26	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP27	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP28	S	S	R	R	S	S	S	S	S	R	S	*Rlm1*
HP29	S	S	S	S	S	S	S	S	S	S	S	None
HP30	S	S	S	S	S	S	S	S	S	S	S	None
HP31	S	S	S	S	S	S	S	S	S	S	S	None
HP32	S	S	S	S	S	S	S	S	S	S	S	None
HP33	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
HP34	S	S	S	S	S	S	S	S	S	S	S	None
HP35	S	S	S	S	S	S	S	S	S	S	S	None
HP36	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP37	S	S	R	R	S	S	S	S	S	R	S	*Rlm1*
HP38	S	S	S	S	S	S	S	S	S	S	S	None
HP39	S	S	S	S	S	S	S	S	S	S	S	None

HP40	S	S	S	S	S	S	S	S	S	S	S	None
HP41	S	S	S	S	S	S	S	S	S	S	S	None
HP42	S	S	S	S	S	S	S	S	S	S	S	None
HP43	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP44	S	S	S	S	S	S	S	S	S	S	S	None
HP45	S	S	S	S	S	S	S	S	S	S	S	None
HP46	S	R	R	S	S	S	S	S	S	R	S	*Rlm4*
HP47	S	S	S	S	S	S	S	S	S	S	S	None
HP48	S	S	S	S	S	S	S	S	S	S	S	None
HP49	S	S	R	S	S	S	S	S	S	S	S	Unknown
C1	S	S	S	S	S	S	S	S	S	S	S	None
C2	S	S	S	S	S	S	S	S	S	S	S	None
C3	S	S	S	S	S	S	S	S	S	S	S	None
C4	S	S	S	R	S	S	S	S	R	S	R	*Rlm3*
C5	S	S	S	S	S	S	S	S	S	S	S	None
C6	S	S	S	S	S	S	S	S	S	S	S	None
C9	S	S	S	S	S	S	S	S	S	S	S	None
C12	S	S	R	R	S	S	S	S	S	R	R	*Rlm1*,*Rlm2*
C13	S	S	R	R	S	S	S	S	R	S	R	*Rlm3*, unknown
C14	S	S	R	R	S	S	S	S	R	S	R	*Rlm3*, unknown
C15	S	S	R	R	S	S	S	S	R	S	R	*Rlm3*, unknown
C16	S	S	R	S	S	S	S	S	S	S	S	Unknown
C17	S	S	S	S	S	S	S	S	S	S	S	None
C18	S	S	S	S	S	S	S	S	S	S	S	None

[a] R/S indicates resistant or susceptible reaction to each Leptosphaeria maculans isolate.
[b] Unknown means the resistance genotype cannot be determined in this study, accessions showing unknown resistance might be due to the presence of a new R gene, other known R gene or combination of several R genes.

Appendix IX. Relative blackleg (*Leptosphaeria maculans*) disease severity of 136 *Brassica napus* accessions in 5 field environments. Five environments were: SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2013 (Alberta, 2013), SME2012 (Melfort, 2012), and SME2014 (Melfort, 2014).

Accessions[a]	Environment[b]					RDS Mean	SE[c]	Rank
	SCA2011	SCA2013	SAB2013	SME2012	SME2013			
CC07	4.02	-[d]	-	2.60	-	3.31	0.71	1
1058	2.01	12.78	6.29	0.00	0.00	4.22	2.43	2
1068	7.03	10.00	5.38	0.00	0.00	4.48	1.97	3
8010	5.02	12.22	10.53	0.00	0.00	5.55	2.56	4
8021	6.02	11.11	12.59	0.00	0.00	5.94	2.66	5
8013	10.04	11.11	10.91	0.00	0.00	6.41	2.62	6
1051	6.02	10.00	17.33	0.00	0.00	6.67	3.27	7
CC01	4.02	16.67	11.75	0.00	2.67	7.02	3.10	8
CC02	-	11.11	16.79	2.25	0.00	7.54	3.91	9
1001	2.01	26.11	7.95	1.94	0.00	7.60	4.82	10
8012	6.02	14.44	14.27	3.33	0.00	7.61	2.91	11
HC804	6.02	18.89	10.07	0.00	3.38	7.67	3.25	12
HC1011	11.04	13.89	12.59	1.10	0.00	7.72	2.97	13
8014	4.02	21.67	7.97	5.41	0.00	7.81	3.70	14
CC10	9.04	8.89	15.02	6.41	0.00	7.87	2.42	15
8041	10.04	21.11	9.10	0.00	0.00	8.05	3.91	16
HP20	6.02	17.22	6.08	0.00	11.00	8.06	2.88	17
HC1009	2.01	18.89	8.39	10.00	2.26	8.31	3.09	18
HP43	4.02	14.44	18.03	5.40	1.22	8.62	3.23	19
CC04	6.02	20.00	16.79	0.00	0.92	8.75	4.10	20
HC812	10.04	16.11	11.33	6.94	0.00	8.88	2.67	21
8016	9.04	21.67	12.59	0.00	1.19	8.90	3.97	22
1017	6.02	15.56	19.72	3.79	0.00	9.02	3.71	23
8015	13.05	13.33	10.91	2.98	5.66	9.19	2.07	24
HC803	6.02	23.63	16.37	0.00	0.00	9.20	4.69	25
8025	10.04	20.00	15.21	1.30	0.00	9.31	3.88	26
HC801	9.04	18.33	13.01	4.17	2.20	9.35	2.93	27
1036	6.02	13.61	19.44	3.47	4.89	9.49	3.05	28
HC1023	12.05	15.56	10.91	9.06	0.00	9.52	2.60	29
HP47	11.04	17.22	11.75	0.00	9.68	9.94	2.80	30
HC1010	6.02	16.67	13.01	5.34	9.77	10.16	2.13	31
8037	20.08	15.00	13.85	2.14	0.00	10.21	3.89	32
CC06	4.02	21.67	21.82	4.25	0.00	10.35	4.71	33
CC05	9.04	16.67	20.56	6.24	0.00	10.50	3.67	34
HP32	3.01	22.78	15.53	7.44	3.88	10.53	3.78	35
HP24	6.02	22.78	18.05	6.17	0.00	10.60	4.22	36
HC807	12.05	13.33	18.05	5.00	5.03	10.69	2.52	37

HP09	7.03	17.78	20.14	6.37	3.67	11.00	3.32	38
HP46	4.02	14.44	11.73	13.92	11.32	11.09	1.87	39
HC813	6.02	26.67	14.69	2.53	5.86	11.15	4.37	40
HC1021	10.04	20.56	15.53	6.17	5.24	11.51	2.90	41
HC1022	5.02	17.22	24.34	0.00	12.41	11.80	4.31	42
1005	12.05	-	-	11.57	-	11.81	0.24	43
HC1019	18.07	26.11	6.71	0.00	8.17	14.76	4.60	44
HC1007	7.03	16.67	18.05	13.02	5.08	11.97	2.57	45
HC1026	6.02	31.11	18.73	4.50	0.00	12.07	5.69	46
HP04	9.04	30.00	9.49	11.92	0.00	12.09	4.91	47
HC1008	13.05	30.00	17.63	0.00	0.00	15.17	5.68	48
HP48	8.03	23.89	14.27	6.58	8.25	12.20	3.21	49
CC03	7.03	20.00	25.18	9.80	0.00	12.40	4.53	50
HP30	22.09	27.78	12.17	0.00	0.00	12.41	5.65	51
HC1012	10.04	31.11	10.49	10.91	0.00	12.51	5.08	52
HC806	12.05	32.78	14.27	3.51	0.00	12.52	5.71	53
HP21	10.04	18.33	23.08	2.78	8.41	12.53	3.63	54
HC702	15.06	23.89	14.27	0.00	9.77	12.60	3.89	55
HC809	4.02	19.44	24.76	0.00	15.17	12.68	4.66	56
1037	19.08	26.67	13.35	2.31	2.31	12.74	4.75	57
HP36	14.06	16.67	13.85	7.81	11.68	12.81	1.48	58
HC1017	18.07	20.00	17.63	3.79	5.00	12.90	3.50	59
HP28	10.04	31.11	13.43	6.94	3.22	12.95	4.84	60
1055	11.04	-	-	15.28	-	13.16	2.12	61
HP37	12.05	18.89	26.44	4.04	4.89	13.26	4.25	62
HP06	14.06	29.44	18.88	4.81	0.00	13.44	5.20	63
HP02	10.04	33.89	12.59	10.82	0.00	16.84	5.56	64
HP08	5.02	20.00	21.82	17.32	3.88	13.61	3.81	65
HP34	8.03	26.11	24.35	8.40	1.83	13.74	4.84	66
HP22	9.04	28.33	18.50	11.03	1.83	13.75	4.51	67
HC1015	14.06	22.22	30.22	2.53	0.00	13.81	5.74	68
8030	13.05	13.33	17.61	16.67	8.51	13.83	1.60	69
HP42	19.08	20.00	16.06	11.95	2.44	13.91	3.19	70
HC1006	14.06	23.33	25.60	6.67	0.00	13.93	4.85	71
HP23	19.08	23.89	24.34	2.50	0.00	13.96	5.29	72
1056	22.09	30.00	9.21	8.68	0.00	14.00	5.33	73
HC810	14.06	23.41	20.14	10.19	2.31	14.02	3.72	74
HP07	4.02	31.67	23.92	3.85	7.00	14.09	5.75	75
HC1024	6.02	33.89	15.95	3.29	12.77	14.38	5.38	76
HC1025	6.02	25.56	31.89	1.74	6.77	14.40	6.00	77
HP31	12.05	20.56	18.47	19.23	4.55	14.97	2.99	78
8017	22.09	16.11	16.37	22.27	0.00	15.37	4.07	79
HP35	23.09	26.11	18.88	5.00	5.53	15.72	4.42	80
HP41	10.04	26.11	25.60	10.06	7.54	15.87	4.10	81

HP27	11.04	23.25	26.44	8.85	9.77	15.87	3.71	82
HP01	11.04	22.78	27.76	1.34	16.49	15.88	4.60	83
8011	6.02	28.29	18.05	15.57	11.57	15.90	3.70	84
HP33	22.09	27.78	18.88	1.54	9.65	15.99	4.66	85
1021	18.07	26.11	28.35	3.79	3.82	16.03	5.27	86
HC1027	17.07	36.11	18.47	8.52	0.00	16.03	6.01	87
HP49	14.06	15.56	17.63	10.19	22.87	16.06	2.09	88
HC1005	9.04	31.67	25.79	14.64	0.00	16.23	5.69	89
HC1028	11.04	18.33	34.83	8.33	9.02	16.31	4.96	90
HP38	10.04	26.11	15.11	22.65	9.16	16.61	3.37	91
HP17	18.07	23.89	18.05	20.46	3.03	16.70	3.58	92
HC815	16.06	21.11	12.59	32.74	4.40	17.38	4.71	93
HP45	9.04	28.33	29.38	15.87	4.71	17.47	4.98	94
8024	20.08	27.22	15.11	16.11	9.06	17.52	3.00	95
HP15	17.07	40.00	18.47	1.16	11.31	17.60	6.37	96
HP14	8.03	34.44	33.57	1.49	10.70	17.65	6.85	97
HC1016	11.04	31.11	22.24	15.83	8.46	17.74	4.08	98
HP11	11.04	18.89	35.67	10.64	13.20	17.89	4.68	99
1075	14.06	28.89	39.03	1.89	5.86	17.95	7.01	100
HC1018	9.04	34.44	13.01	8.10	27.96	18.51	5.35	101
HP03	7.03	20.00	29.80	26.85	10.15	18.77	4.48	102
HC1014	13.05	50.00	26.44	1.02	3.67	18.84	8.97	103
HP39	31.12	18.89	24.92	9.87	11.47	19.25	4.01	104
HC805	12.05	30.56	41.13	9.03	4.84	19.52	6.96	105
HP12	14.06	29.44	15.11	14.10	25.13	19.57	3.23	106
HC802	29.11	39.44	24.34	5.52	2.93	20.27	7.00	107
HC811	23.09	25.56	28.16	8.74	20.23	21.16	3.37	108
HP29	16.06	22.22	18.15	19.51	34.21	22.03	3.20	109
HP26	13.05	23.89	18.00	15.63	39.68	22.05	4.76	110
HC1002	16.06	37.22	38.32	19.35	0.00	22.19	7.16	111
HP10	37.15	41.11	26.44	3.97	2.44	22.22	8.13	112
HC1001	18.07	29.44	31.06	20.10	13.95	22.52	3.32	113
8027	7.03	40.00	31.73	2.19	32.25	22.64	7.54	114
HC1020	25.10	25.00	31.06	17.58	15.61	22.87	2.80	115
HP40	31.12	26.67	31.89	1.67	27.86	23.84	5.63	116
HC1013	30.12	25.00	17.63	21.70	25.13	23.92	2.07	117
HP44	23.09	38.89	22.66	22.29	12.87	23.96	4.19	118
HC703	14.06	26.11	36.23	34.96	9.08	24.09	5.46	119
8048	23.09	47.78	23.61	26.22	0.00	24.14	7.57	120
HC814	32.13	27.22	43.96	0.00	21.99	25.06	7.24	121
HP05	9.04	34.44	33.15	8.62	40.37	25.12	6.76	122
HC1004	21.08	66.11	27.70	11.84	0.00	25.35	11.20	123
HP18	29.11	27.78	33.57	9.23	27.99	25.54	4.21	124
HC808	16.06	42.22	43.65	1.81	29.32	26.61	7.96	125

HP25	28.11	32.78	46.10	11.19	23.99	29.55	5.69	126
HC701	34.13	41.11	23.50	16.03	34.72	29.90	4.47	127
HP13	31.12	46.55	38.61	15.53	28.57	32.08	5.19	128
8034	40.16	33.61	20.95	68.45	9.16	34.47	10.03	129
HP16	29.11	33.33	37.77	60.63	16.80	35.53	7.18	130
HC1003	9.04	50.56	30.98	20.83	71.18	36.52	11.02	131
8026	25.10	55.54	65.32	2.14	36.85	36.99	11.19	132
HP19	18.07	47.36	36.93	25.73	91.83	43.98	12.95	133
8022	11.04	41.67	48.26	38.64	91.44	46.21	12.97	134
CC09	41.16	68.33	66.31	55.90	39.73	54.29	6.04	135
CC08	31.12	68.21	53.01	19.40	119.92	58.33	17.57	136
RDS Mean	13.56	26.07	21.70	9.51	10.64			
SE[c]	0.73	0.98	0.96	0.96	1.56			

[a]*Brassica napus* accessions were winter type cultivars or advanced breeding lines provided by Anhui Academy of Agricultural Sciences, China.
[b]Five environments were: SCA2011 (Carman, 2011), SCA2013 (Carman, 2013), SAB2013 (Alberta, 2013), SME2012 (Melfort, 2012), and SME2013 (Melfort, 2013).
[c]Standard error of the mean.
[d]Data was missing.

Appendix X. qPCR primers used in this study.

B. napus LMD Primers	Sequence (5' --> 3')
qBnaPR1.F	TCTCGTTGACCCAAAGGTTC
qBnaPR1.R	CAGCCTTCGCTCAAAGCTAC
qBnaPDF1.2.F	GCTGCTTTTGAAGCACCAAC
qBnaPDF1.2.R	GTTGCAAGATCCATGTCGTG
qBnaCYP79B2.F	TCAACGCGTGTCTCATTCTC
qBnaCYP79B2.R	TACCGGGAAAAGAGGTTGTG
qBnaC04g27200D.F	TCGTCTAGGCCAAGTTCGTC
qBnaC04g27200D.R	AAAGAAGAAGCGGCAACAAG
qBnaLIK1.F	TTGGCACTTCCCCACTTAAC
qBnaLIK1.R	GCGTATCTTGGACCGATCAC
qBnaAPSK2.F	GTTGGGAGCCTTAGGAAACC
qBnaAPSK2.R	ACCGTCCATCATCTGCTCTC
qBnaA03g43720D.F	TAGGCTGTGACGGGACTACC
qBnaA03g43720D.R	TCCGGCTTCATAGAATGTCC
BnWRKY25.F	TTC ACC GAC CTC CTT GCT TC
BnWRKY25.R	GAA GCT GCT GCG AGA AGA TTG CG
BoRBOHF.F	CTT GGC ATT GGT GCA ACT CC
BoRBOHF.R	TCC GAG ARC GAA TCC GCT TG

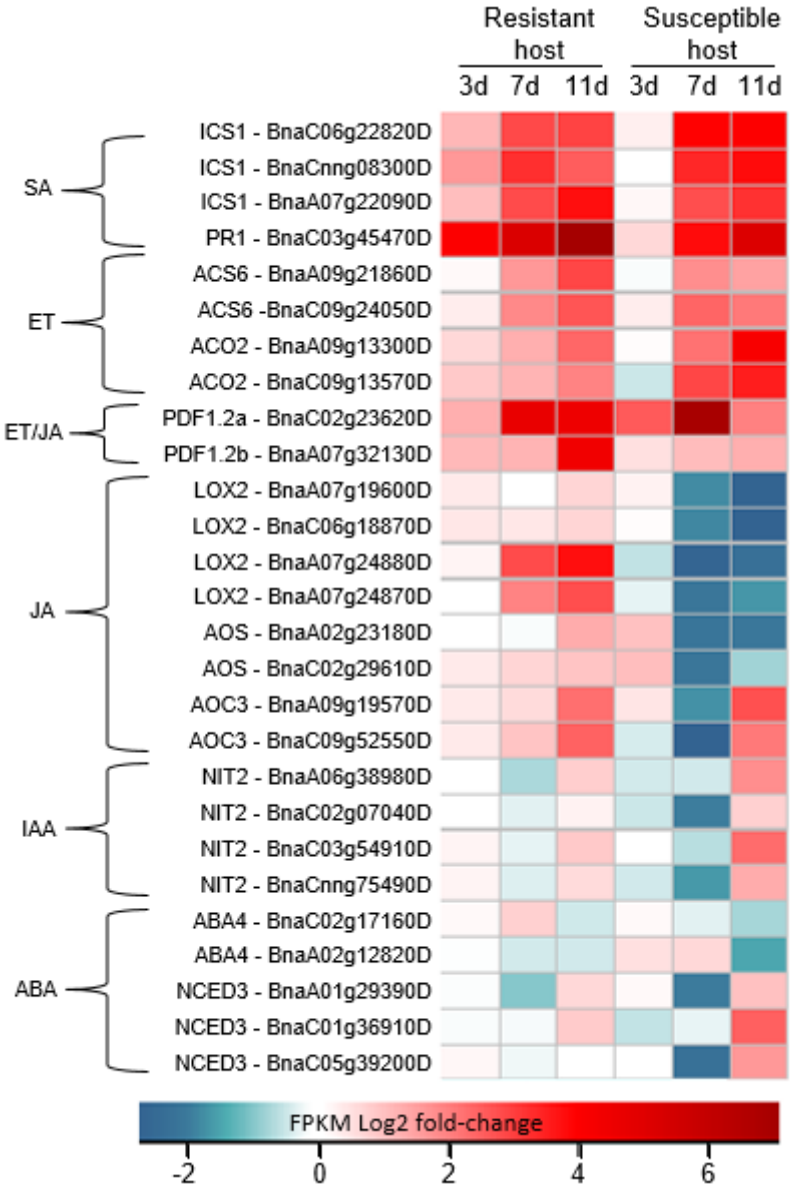
Appendix XI. Results of Arabidopsis mutant susceptibility screening for blackleg disease. A total of 49 loss-of-function Col-0 background Arabidopsis mutants were screened for blackleg disease susceptibility Blackleg resistance evaluation: R, resistant, infected plants showed small lesions with clear black borders; IR, intermediately resistant, infected plants showed breakdown of non-host resistance, and larger lesions with or without clear black borders (compared with WT).

Mutant	T-DNA insertion line	Gene name	Insertion site	Blackleg resistance
N/A	Col-0	N/A	N/A	R
at1g02930	SALK_026398C	GSTF6	Intron	IR
at3g53490	SALK_036238	u/c	Promoter	IR
at3g14840	SALK_030855C	LIK1	Exon	IR
at4g18250	SALK_043853C	u/c	Intron	IR
at1g73260	SALK_131716C	KTI1	Promoter	IR
at4g39940	SALK_025296C	APK2	Exon	IR
at4g21120	SALK_087921C	AAT1	Exon	R
at4g21120	SALK_059873C	AAT1	Intron	R
at1g33950	SALK_000761C	u/c	Intron	R
at1g02930	SALK_065940C	GSTF6	Exon	R
at4g17500	SALK_036267	ERF-1	Promoter	R
at4g04540	SALK_098187C	CRK39	Exon	R
at3g60420	SALK_057524C	u/c	promoter	R
at3g60420	SALK_059036C	u/c	promoter	R
at3g61640	SALK_092212C	AGP20	promoter	R
at3g05360	SALK_008911C	RLP30	Exon	R
at3g05360	SALK_145342C	RLP30	Exon	R
at4g23290	SALK_022512C	CRK21	Exon	R
at4g23290	SALK_035263C	CRK21	Exon	R
at4g22880	SALK_120680C	LDOX	Promoter	R
at4g22880	SALK_073183	LDOX	Exon	R
at4g04540	SALK_036225C	CRK39	Exon	R
at4g11850	SALK_089968	LPLDGAMMA1	Promoter	R
at4g39940	SALK_060023C	APK2	Promoter	R
at3g53490	SALK_645697C	u/c	5' UTR	R
at3g14840	SALK_056862	LIK1	Promoter	R
at4g18250	SALK_072295C	u/c	Promoter	R
at5g14930	SALK_022911C	SAG101	Exon	R
at5g01750	SALK_089519C	u/c	Promoter	R
at5g01750	CS372146	u/c	Promoter	R
at4g23190	SALK_054888	CRK11	Exon	R
at4g23190	SALK_054880	CRK11	Exon	R
at5g53110	SALK_136256	u/c	Exon	R
at5g53110	SALK_004123	u/c	Intron	R

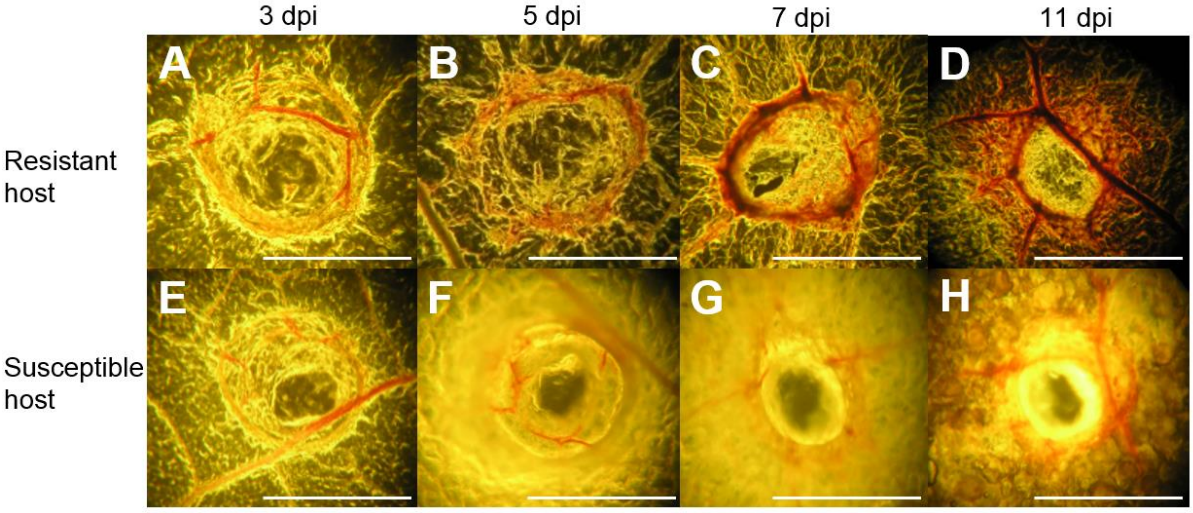
at3g25882	SALK_148447C	NIMI-2	Exon	R
at3g25882	SALK_06674C	NIMI-2	Promoter	R
at2g30860	SALK_148672C	GSTF9	Promoter	R
at2g30860	SALK_001519C	GSTF9	Exon	R
at1g66880	SALK_034755	u/c	Exon	R
at1g66880	SALK_137021	u/c	Exon	R
at5g17220	SALK_105779C	u/c	Intron	R
at5g17220	SALK_113805C	u/c	Promoter	R
at5g41020	SALK_108569C	u/c	Promoter	R
at1g74650	CS2104374	MYB31	Promoter	R
at4g39950	SALK_113348C	CYP79B2	Exon	R
at4g31500	SALK_102615	CYP83B1	Promoter	R
at1g26420	SALK_079007	u/c	Promoter	R
at2g46650	SALK_027748C	CYTB5-C	Exon	R
at1g11330	SALK_076543C	u/c	Promoter	R

Appendix XII. Characterization of *R*-genes carried in line DF78 and cv. Westar. A total of 34 characterized L maculans isolates were tested against cv DF78 and cv Westar and interaction phenotype was recorded as resistant [R] or susceptible [S] The genotype of Avr genes enclosed in ( ) are not determined.

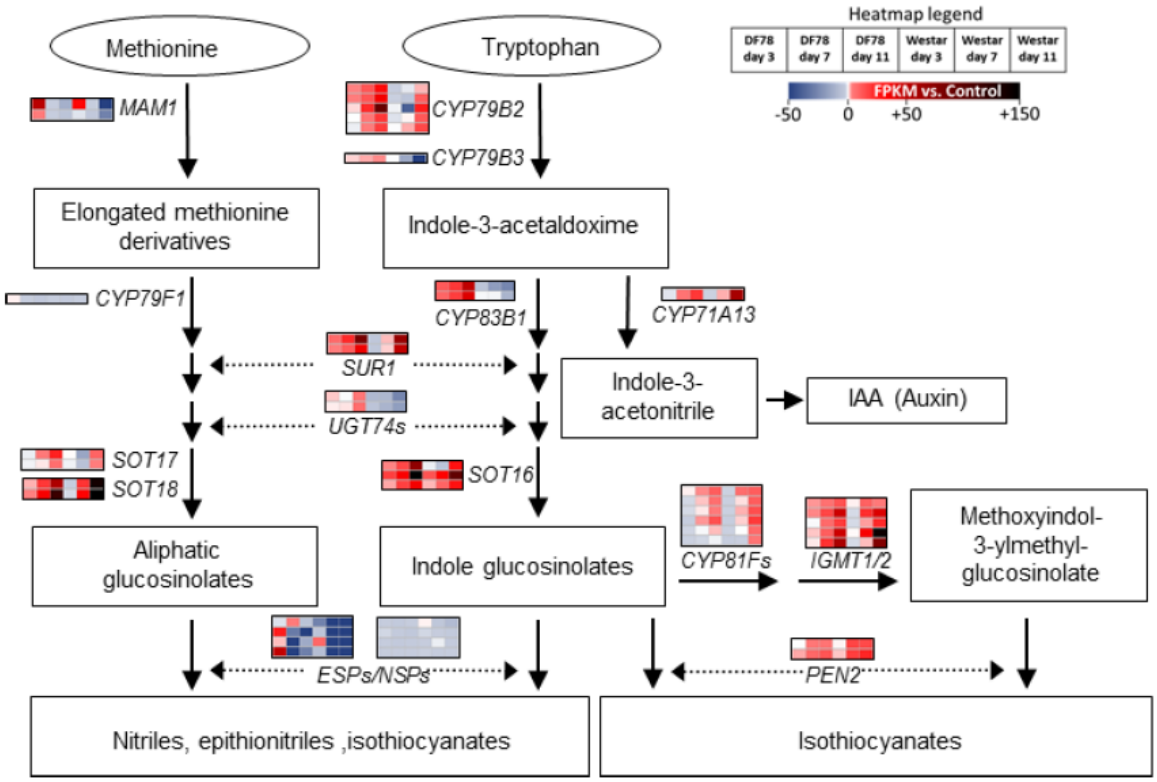
Isolates	Avirulence genotype	Interaction with DF78	Interaction with Westar
D1	*AvrLm2,5,6,9,(10),S,AvrLepR1, 2*	R	S
D2	*AvrLm5,6,8,(10),11,S,AvrLepR1*	R	S
D3	*AvrLm5,(10),11,AvrLepR1*	R	S
D4	*AvrLm4, 5,6,7,8,(10),11,AvrLepR1,2*	R	S
D5	*AvrLm1,2,4,7,(10),11,S,AvrLepR1,2*	R	S
D6	*AvrLm1,5,6,8,(10),11,S*	R	S
D7	*AvrLm1,3,5,6,8,(10),11,(S),AvrLepR1*	R	S
D8	*AvrLm5,7,(8,10),11, AvrLepR1*	R	S
D9	*AvrLm5,6, 7,(8, 10),11, AvrLepR1*	R	S
D10	*AvrLm5,6,8,9,(10),11,S*	R	S
D13	*AvrLm4,6,7,(8,10),11*	**S**	S
D14	*AvrLm1,7,(5,8,10),11,S, AvrLepR1*	R	S
S7	*AvrLm1,5,6,7,(8), 11,AvrLepR1*	R	S
ICBN14	*AvrLm5,6,10,AvrLepR1*	R	S
PHW1223	*AvrLm5,6,8,9,11*	R	S
R2	*AvrLm5,7,10,(8), AvrLepR1*	R	S
AD746	*AvrLm3,6,(8), AvrLepR1*	R	S
JN2	*AvrLm5,6,7,8, 11,AvrLepR1*	R	S
JN3	*AvrLm1,4,5,6,7,8,11*	R	S
J3	*AvrLm2,3,5,6,(8,10),11,S*	R	S
J20	*AvrLm2,3,6,(8,10),11,S,AvrLepR1*	R	S
Q12	*AvrLm2, 4,5,7,(8,10), 11,AvrLepR1*	R	S
L-MD7-14	*AvrLm4,5,6,7,(8,10),11*	S	S
L-PC4-1	*AvrLm2,4,(8,10),11*	S	S
L-MP1-8	*AvrLm2,4,5,6,7,(8,10),11*	S	S
L-Sb1	*AvrLm2,3,5,6,7, (8,10),S,11*	R	S
L-MP1-6	*AvrLm4,5,6,7,(8,10),11*	S	S
L-Sb7-6	*AvrLm4,5,6,7,(8,10),11, LepR1*	R	S
L-Br17-1	*AvrLm5,6,7,(4,8,10),11,LepR1*	R	S
L-Mo5-1	*AvrLm2,4,5,6,7, (8,10),11, LepR2*	S	S
L-Br1-16	*AvrLm1,4,5,6,7,(8,10, S),11*	S	S
RL25	*AvrLm5,6,7,(8,10),11,S*	S	S
DS103	*AvrLm5,9,(8,10),11*	S	S
CV8-7	*AvrLm2,4,5,6,7,(5,8,10),11,S*	S	S



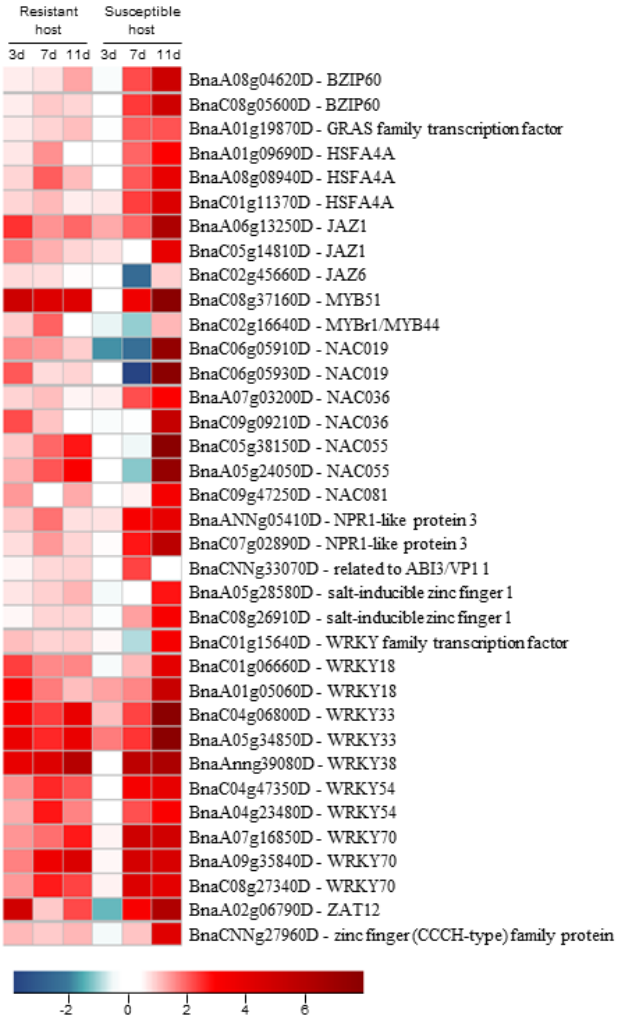
Appendix XIII. Expression levels of hormone biosynthesis genes and hormone signaling markers in response to *L. maculans*. Heatmap of Log2 transcript level fold-change vs. mock controls in resistant (R) and susceptible (R) cotyledons at 3, 7 and 11 days post inoculation.



Appendix XIV. Deposition of lignified plant material at the site of infection in ressitant DF78 and susceptible Westar. Cotyledons are infected with L. maculans and stained with phloroglucinol-HCl. Lignified plant material appear dark orange/red. Scales=1 mm.



Appendix XV. Activation of glucosinolate and indole glucosinolate biosynthetic genes in *B. napus* cotyledons infected with *L. maculans*. Changes in expression of glucosinolate- and indole glucosinolate- biosynthetic gene homologs are shown across their respective biosynthetic pathways. Fluctuations in gene expression are recorded as FPKM [Fragments Per Kilobase of transcript per Million mapped reads] deviation from mock controls. A more intense red color reflects gene activation, a more intense blue color represents gene repression.



Appendix XVI. Expression levels of transcription factors activated in response to *L. maculans*. Heatmap of Log2 transcript level fold-change vs. mock controls in resistant (R) and susceptible (S) cotyledons at 3, 7, and 11 days post *L. maculans* inoculation.

Appendix XVII. List of genes specifically activated in resistant hosts in response to *L. maculans*. Complete list of 54 genes with significantly ($P < 0.05$) elevated expression in response to *L. maculans* at every time point specifically in resistant host, and their putative Arabidopsis homolog and annotation. Genes with no identifiable Arabidopsis homolog from nucleotide or protein BLAST searches are marked as 'no hit' and are of unknown function.

B. napus locus	Putative Arabidopsis homolog	Putative Annotation
BnaC05g38740D	AT3G14840	LYSM RLK1-interacting kinase 1
BnaA01g12650D	AT4G23190	cysteine-rich RLK (RECEPTOR-like protein kinase) 11
BnaAnng25570D	AT4G23290	cysteine-rich RLK (RECEPTOR-like protein kinase) 21
BnaCnng49020D	AT4G04540	cysteine-rich RLK (RECEPTOR-like protein kinase) 39
BnaA03g25470D	AT4G04540	cysteine-rich RLK (RECEPTOR-like protein kinase) 39
BnaC07g06130D	AT2G17120	lysm domain GPI-anchored protein 2 precursor
BnaC03g71330D	AT5G01950	Leucine-rich repeat protein kinase family protein
BnaA02g12640D	AT1G66880	Protein kinase superfamily protein
BnaA03g36540D	AT4G11850	phospholipase D gamma 1
BnaA07g22750D	AT1G73260	Receptor-like protein kinase, serine/threonine
BnaA06g12220D	AT3G05360	receptor like protein 30
BnaA03g43720D	AT4G18250	receptor serine/threonine kinase, putative
BnaA07g30760D	AT1G73260	Receptor-like protein kinase, serine/threonine
BnaA10g07090D	AT1G11330	S-locus lectin protein kinase family protein
BnaCnng55880D	AT1G11330	S-locus lectin protein kinase family protein
BnaAnng21280D	AT4G17500	ethylene responsive element binding factor 1
BnaA02g25110D	AT5G47220	ethylene responsive element binding factor 2
BnaA09g50010D	AT1G06160	octadecanoid-responsive Arabidopsis AP2/ERF 59
BnaC07g23070D	AT3G25882	NIM1-interacting 2
BnaA03g04410D	AT5G13550	sulfate transporter 4.1
BnaC07g51290D	AT4G39940	APS-kinase 2
BnaCnng04780D	AT1G25220	anthranilate synthase beta subunit 1
BnaA01g34610D	AT4G39950	cytochrome P450, family 79, subfamily B, polypeptide 2
BnaC01g00800D	AT4G39950	cytochrome P450, family 79, subfamily B, polypeptide 2
BnaA04g12790D	AT2G22330	cytochrome P450, family 79, subfamily B, polypeptide 3
BnaA08g04520D	AT4G31500	cytochrome P450, family 83, subfamily B, polypeptide 1
BnaC08g05690D	AT4G31500	cytochrome P450, family 83, subfamily B, polypeptide 1
BnaA04g27110D	AT2G46650	cytochrome B5 isoform C
BnaC04g50950D	AT2G46650	cytochrome B5 isoform C
BnaC06g21620D	AT1G76790	Indole Glucosinolate O-methyltransferase 5
BnaA03g58530D	AT4G21120	amino acid transporter 1
BnaA07g23890D	AT1G70260	Usually multiple acids movie in and out transporter 36
BnaA06g31460D	AT3G28480	Oxoglutarate/iron-dependent oxygenase
BnaC03g62400D	AT4G35630	phosphoserine aminotransferase
BnaAnng33720D	AT1G20160	Response secreted protease

BnaC01g41020D	AT4G19810	Chitinase C
BnaAnng42000D	AT4G29700	Alkaline-phosphatase-like family protein
CUFF.2933.3	AT5G14930	senescence-associated gene 101
BnaA05g29820D	AT3G14040	Pectin lyase-like superfamily protein
BnaA06g37630D	AT4G04775	zinc ion binding
BnaA04g17910D	AT2G30860	glutathione S-transferase PHI 9
BnaA09g28900D	AT1G26420	FAD-binding Berberine family protein
BnaA05g07460D	AT2G36970	UDP-Glycosyltransferase superfamily protein
BnaC07g47720D	AT4G38540	FAD/NAD(P)-binding oxidoreductase family protein
BnaC06g18710D	AT1G21310	extensin 3
BnaC04g55140D	AT3G60420	Phosphoglycerate mutase family protein
BnaC04g21680D	AT3G61640	arabinogalactan protein 20
BnaC09g52960D	AT5G53110	RING/U-box superfamily protein
BnaA09g19740D	AT5G01750	Protein of unknown function (DUF567)
BnaC04g27200D	AT3G53490	Protein of unknown function
BnaC06g28720D	no hit	N/A
BnaC02g31360D	no hit	N/A
BnaC06g41090D	no hit	N/A
BnaA03g08620D	no hit	N/A

Appendix XVIII. List of abbreviations.

ABA	Abiscisic acid
ANOVA	Analysis of variance
APR	Adult-plant resistance
ARS	Average rating score
CAGE	Cap analysis of gene expression
CD	Cell death
DPI	Days post inoculation
ET	Ethylene
ETD	Effector-triggered defense
ETI	Effector-triggered immunity
FPKM	Fragments Per Kilobase of gene per Million mapped reads
GC	Base composition
HR	Hypersensitive response
ICBN	International Blackleg of Crucifers Network
IGS	Indole glucosinolates
IR	Intermediate resistant
JA	Jasmonic acid
LMD	Laser microdissection
LTR	Long terminal repeat
MAPKs	Mitogen-activated protein kinases
MBC	Methyl Benzimidazole Carbamate
MDS	Mean disease severity
MKK	MAPK kinases
MPSS	Massively parallel signature sequencing
MR	Moderately resistant
MS	Moderately susceptible
NBS-LRR	Nucleotide binding site-leucine-rich repeats
NGS	Next-generation sequencing

ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PG	Pathogenicity group
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
QTL	Quantitative trait loci
R	Resistant
RDS	Relative disease severity
RIP	Repeat-induced point mutation
RLPs	Receptor-like proteins
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
S	Susceptible
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SBS	Sequencing by synthesis
SOLiD	Sequencing by Oligo Ligation Detection
TE	Transposable elements
WAKs	Wall-associated kinases