Genetic and Genomic studies towards understanding Quantitative Resistance to Blackleg Infection in Canola

By

Hanna Beksissa Dandena

A Thesis Submitted to the Faculty of Graduate studies of The University of Manitoba

in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Plant Science

University of Manitoba

Winnipeg

Abstract

Brassica napus is a globally important oilseed crop. *Leptosphaeria maculans* is the causative agent of blackleg disease in *Brassica napus*. Two types of resistance mechanisms are known namely qualitative (vertical or R - mediated) and quantitative (horizontal) resistance. R-mediated resistance is well-studied and used extensively to manage *L. maculans* infection in *B. napus*. However, the genetic basis of quantitative resistance (QR) in this pathosystem is not fully understood but is apparently due to several multiple minor effect or a few major effect loci. Unlike minor effect loci that are difficult to dissect, major effect loci could be Mendelized to study the causative gene(s) controlling the QR trait in *B. napus*. Nevertheless, no QR genes have been cloned in this pathosystem, hence, the underlying mechanism is unknown. The work presented in this thesis focuses on the analysis of the major effect *BLMR2* locus, which was originally derived from the highly resistant *B. napus* cultivar Surpass 400. The segregating population and sets of near-isogenic lines (NILs) used in this study were developed *via* crossing and subsequent molecular marker assisted backcrossing to the recurrent, universally susceptible Westar parent. Cotyledon inoculation was employed for all the indoor (growth chamber and greenhouse) studies. In chapter three, the non-race specificity and intermediate resistance phenotype of the *BLMR2* locus to multiple *L. maculans* isolates at seedling and adult plant stages was described. Chapter four focuses on the narrowing down of the *BLMR2* region originally mapped on *B. napus* chromosome N10 using fine-mapping approaches. The alignment of the genetic and the physical map, based on the reference genomes of *B. napus* and *Arabidopsis thaliana*, facilitated the identification of the candidate disease resistance related cytochrome P450 gene. In chapter five, RNA sequencing (RNA-Seq) was employed to understand the mechanism underlying QR in *B. napus* and to confirm the differential expression of the identified candidate gene. By analyzing the global changes in gene expression, we indicated that the mechanism behind the R and QR strategies in *B. napus – L. maculans* pathosystem is comparable compared to the susceptible Westar cultivar. The findings in this thesis benefit the canola industry and the broader scientific community involved in canola, blackleg and resistance breeding by providing valuable information about a major effect horizontal resistance locus; the associated markers may be used in marker-assisted selection (MAS) to improve the canola crop.

Acknowledgements

I would like to thank my advisor Dr. Genyi Li for the opportunity to do my Ph.D. under his guidance. Working in his lab provided me exposure to various molecular techniques and genomic tools. I would also thank former lab members Dr. Arvind H. Hirani, Dr. Zheng Liu and Dr. Yaping Wang for the technical support, particularly at the beginning of my studies. I am grateful to my committee members Dr. Mark Belmonte and Dr. Dilantha Fernando for their support throughout my PhD.

My heartfelt appreciation goes to my family and friends. I am grateful to my parents Beksissa Dandena and Ribka Mekonnen, for their unconditional love, prayers, and sacrifices. I would also like to express gratitude to my siblings; Miheret, Natnael and Brook for believing in me. I thank my beloved husband, Dr. Tesfaye Kebede Gurmessa, for his encouragement and patience in this long journey. I truly could not have done it without your support! My wonderful kids, Yadet and Matty are very much appreciated for always bringing joy and laughter in my life even in difficult times. My special gratitude goes to my cousins Fikirte, Daniel, Lydia, Sophia, Stifanos and Anyo for making our move to Canada smooth. I am truly touched by your warmth and hospitality that my family and I had during our stay with you! My close friends, Genet and Dr. Eshetu Beshada, Birtukan and Dr. Getahun Legesse, Bemnet and Dr. Bereket Assefa, Ribka and Gebeyehu, Hibret and Dr. Getnet Muluye are very much appreciated for their kindness and best wishes. Also, I am grateful to my colleagues and friends, Drs. Jemanesh Haile, Aida Kebede, Genet Mengistu and Kidist Kibret for their encouragement and advice. Special thanks to my fellow graduate students, Laila and Keval for their friendship. I would also like to express my gratitude to the Living Gospel Church's family for the great fellowship.

Above all, I thank my heavenly Father for granting me His countless blessings and grace throughout my studies.

Table of contents

List of Tables

List of Figures

List of Appendices

Preface

This thesis is prepared in manuscript style, following the formatting of the Faculty of Graduate Studies and the Department of Plant Science. It begins with a general introduction and literature review followed by the three main chapters (3-5) of the PhD work and a general discussion and conclusion. Chapter 3 entitled “Analysis of quantitative adult plant resistance to blackleg in *Brassica napus*” is published in the journal molecular breeding (Dandena et al 2019). Chapter 4 entitle “Genetic analysis of a horizontal resistant locus *BLMR2* in canola” has been published in the journal frontiers in plant science. Chapter 5 is intended to be submitted to the journal BMC Genomics.

Contributions of Authors

Chapter 3 – HD and GL designed the experiment and wrote the manuscript. HD did most of the experiment. QZ developed the materials and molecular markers used in the experiment. AH and ZL offered technical assistance in some of the experiments. TZ, DF and RD edited the manuscript.

Chapter 4 – QZ, HD and GL designed the experiment and wrote the manuscript. QZ and HD finished most experiments. MM, HL, ZL and WX collected data of some experiments.

Chapter 5 – HD and GL designed the experiment and wrote the manuscript. HD did most of the experiment. TZ provided some of the data used in the analysis. KS and WX contributed to the bioinformatics.

1. General Introduction

Rapeseed / canola (*Brassica napus*) is an economically important oilseed crop with many uses and applications. While *Brassica* species has a long history as a source of edible oil, fuel for burning lamps and lubricants for engines (Gupta 2016), there has been unprecedented increase in rapeseed production (~8-fold) and acreage (~4-fold), making it the second most produced oilseed crop after soybean globally in the last four decades (www.fao.org). Some of the major factors that contributed to its success include, reputation as a healthy vegetable oil, adaptability of the different ecotypes (winter, semi-winter, and spring) to various climatic conditions across wider geographical regions; and its versatility as food, feed and biodiesel and other industrial applications (Friedt and Snowden, 2009). Although Canada, China, India, and the European Union (EU) are among the top rapeseed producers in the world; Canada is the leading exporter of rapeseed followed by Australia (www.fao.org).

Canadian canola production has increased steadily (~ 5-fold) over the last four decades. Canada became the number one producer of rapeseed surpassing China in the last decade (www.fao.org). Canola contributes an estimated $26.7 billion annually to the Canadian economy through the various sectors in the value chain. About 90% of the canola produced in Canada is destined for international market as seed, oil and feedstock (www.canolacouncil.org). As the production of rapeseed is increasing and seeds are being transported across the globe, disease epidemic becomes inevitable, threatening this multibillion-dollar industry with economic losses and, most recently, causing trade barriers (Fernando et al. 2016).

Diseases and pests pose serious threats to sustainable agricultural production. Historically, massive famine and migration were associated with plant disease outbreaks such as the “Irish famine” that happened in the 1840s due to the potato late blight disease caused by the pathogen *Phytophthora infestans* (Zadoks 2008). Also, significant economic losses were reported recently in crops such as rapeseed due to the blackleg disease caused by the pathogen *Leptosphaeria maculans* in Europe and Australia (Fitt et al. 2006). The use of fungicides may protect crops from devastating losses due to disease outbreaks (Zhang and Fernando, 2017), but in the long term, relying heavily on fungicides causes serious environmental problems, such as the development of resistance to chemicals by pathogens (Hahn 2014), and could also be costly. Thus, the use of chemicals should be considered as a last resort.

Major diseases of economic importance in rapeseed include blackleg, sclerotinia and clubroot. The clubroot pathogen, *Plasmodiophora brassicae* Woronin, is one of the world's oldest recognized pathogens associated with the Brassicaceae (Dixon 2009). Currently, clubroot is potentially the most serious disease in the Brassica vegetables and oil crops in China (Chai et al. 2014). On the other hand, the recent discovery of *P. brassicae* in canola fields in Alberta, Canada (Tewari et al. 2005) coupled with the spread of the disease through contaminated soils, seeds, and farm equipment poses a significant threat to the large-scale canola production in western Canada. *Sclerotinia sclerotiorum* (Lib.) de Bary, on the other hand, has been responsible for sclerotinia stem rot in canola since the mid 1970's, where serious infestation was reported in Alberta, Canada (Thomson and Stelfox 1983). However, it occurs sporadically depending on environmental conditions, mainly moisture. Blackleg disease is presumably the most significant disease of canola in Canada, Australia, and Europe with impacts including severe yield loss, quality reduction and trade disputes (Fitt et al. 2008; Fernando et al. 2016).

Blackleg caused by *Leptosphaeria maculans* anamorph Phoma lingam, has been known for more than a century, earlier associated with Brassica vegetables in *Brassica oleracea* (Henderson 1918) and later, more importantly, with rapeseed (*B. napus*). In Canada, *L. maculans* was first detected in Saskatchewan in 1975 and had spread to all the canola fields in the prairies by the 1980s (Gugel and Petrie 1992; Juska et al 1997; Zhang and Fernando, 2017). Although devastating yield losses as reported in Europe and Australia did not occur in Canada, significant yield losses of up to more than 50% were recorded in heavily infested fields in Saskatchewan (Gugel and Petrie 1992). Blackleg has been relatively controlled since the 1990s, largely due to the availability of resistant cultivars (Kutcher et al. 2013).

Despite the efficient use of resistant cultivars, disease outbreaks have been reported (Zhang et al. 2016) partly due to the widespread use of cultivars harbouring a few major resistance genes in large-scale commercial canola production in the prairies and the tight crop rotation practices due to the promising return from growing canola in western Canada (Kutcher et al. 2013). Also, the biology of the pathogen prompts its fast evolution (Rouxel 2017). There has been recorded discoveries of new *L. maculans* pathotypes in the past (Chen and Fernando 2006; Kutcher et al. 2007), which overwhelmed the previously used pathogenicity grouping system (Mengistu et al. 1991). On the other hand, pathogen isolates characterized by known *Avr* (avirulence) genes

facilitate the systematic study of the structure and dynamics of *L. maculans* strains at the population level. A recent study identified 55 *L. maculans* races in western Canada, although two races are dominant (Liban et al. 2016). The complexity in *Avr* gene composition among the provinces in western Canada and the most diverse races in Manitoba were observed. In a subsequent long-term study conducted in Manitoba, 170 races were identified, albeit one dominant race was pinpointed, confirming the selection pressure exerted by single R-genes (Fernando et al. 2018).

Two types of resistance have been known in *B. napus – L. maculans* pathosystem, qualitative and quantitative (Raman et al 2013). Qualitative resistance (race-specific) is based on "gene-for-gene" hypothesis (Flor 1942), whereas quantitative resistance (non-race specific) is controlled by many genes, each contributing towards the resistance response. Although the genetic basis of Canadian cultivars are not fully known, recent report indicated that most of the varieties harbor the same resistance gene *Rlm3*, which raises a concern about the longevity of the existing resistant cultivars, and the need for better management strategies (Zhang et al. 2016). Zhang et al. (2016) suggested that the quantitative resistance loci (QRLs) observed in the background of these cultivars probably contributed to the less dramatic resistance breakdown in Canada.

In the past, the use of bi-parental population and major R genes in the background limited the mapping of QRLs. Recent advances in genomics, sequencing technologies, bioinformatics and statistical tools offer unprecedented opportunities in the genome-wide identification of candidate resistance genes in the complex *B. napus* genome (Larkan et al. 2016a; Raman et al. 2016a; Fu et al. 2019; Batley 2020). Nevertheless, the function of the identified candidate genes needs further validation in resistant cultivars to be used in breeding programs. The focus of this PhD thesis is on the genetic and molecular studies of the blackleg resistant *BLMR2* locus displaying intermediate seedling resistance phenotype in *B. napus*. The objectives of the thesis include:

- To analyse the *BLMR2* locus at the seedling and adult plant stages (Chapter 3). We hypothesized that non-hypersensitive seedling resistance correlates with adult plant resistance (APR). The *BLMR2* is a major effect QRL showing horizontal or intermediate resistance phenotype when infected with multiple *L. maculans* isolates. Molecular markers linked to this locus were used to develop the segregating advanced backcross (BC) population and NILs which are used in the greenhouse and field studies.

- To identify candidate gene(s) in the blackleg resistance *BLMR2* locus (Chapter 4). We hypothesized that the candidate disease resistance gene in the *BLMR2* locus is probably not a typical resistance gene, but it showed resistance to all isolates tested. Screening of a large, advanced BC mapping population using flanking molecular markers facilitated in the fine-mapping of the locus to 56.3 kb physical region.
- To understand the molecular mechanism underlying the *BLMR2*-mediated QR (Chapter 5). We hypothesized that the molecular mechanism underlying QR is comparable to R-mediated resistance in *B. napus – L. maculans* pathosystem. RNA-Seq was employed to identify pathogen induced upregulated genes in the NIL containing *BLMR2* (representing horizontal or QR), transgenic line containing *LepR3* (representing R-mediated resistance) and the susceptible Westar cultivar at 4 days after inoculation.

2. Literature Review

2.1 The Brassicacaea Family

Brassicaceae is a diverse family of wild, cultivated, ornamental and crop species (Al-Shehbaz et al. 2006). Other names for this family include the Cruciferae, cabbage or mustard family. There are approximately 3,709 species classified into 338 genera that constitute this family including the model plant, Arabidopsis (Warwick et al. 2006). *Arabidopsis thaliana* has one of the smallest genomes (157Mb, n=5), which attributes an attraction to genomic studies. This model plant has been well characterized and laid the foundation for studies on comparative genomics, functional genomics and others in many flowering plants (The Arabidopsis Genome Initiative 2000).

In the Brassicaceae family, several species such as the *Brassica* species, have highly duplicated and reshuffled genomes with similar gene families, differ in copy numbers and a wide variety of segmental arrangement (Lagercrantz and Lydiate 1996). With the basic hypothetical ancestral chromosome number, n=8 (Lysak et al. 2007), an ancestral karyotype (AK) with 24 ancestral blocks was proposed for this family (Schranz et al. 2006). The center of origin of the Brassicaceae is probably the Irano-Turanian region, where it had undergone three whole genome duplication (WGD, palaeopolyploidization) events followed by species diversification (Franzke et al. 2011). It was estimated that the most recent duplication in Brassicaceae occurred ~34 million years ago (MYA) after it had been isolated from its sister Cleomaceae family (Schranz and Mitchell-Olds 2006). Systematic studies, based on morphology, genetic, and cytogenetic studies, classified the family into 25 tribes that were phylogenetically divided into at least three lineages (Schranz et al. 2006). The lineage containing the Brassiceae tribe probably diverged from the lineage containing Arabidopsis ~12-19 MYA (The Arabidopsis Genome Initiative 2000). The Brassiceae tribe contains 240 species in 52 genera, including the economically important species such as the *Brassica*, *Sinapis*, and *Raphanus* that are relatively well-studied and thought to be monophyletic (Al-Shehbaz et al. 2006; Lysak et al. 2007). The lineage containing the Brassiceae probably evolved at the junction of Irano-Turanian and Saharo-Sindian region, and later diversified in the Mediterranean region (Al-Shehbaz et al. 2006; Arias et al. 2014), and had undergone additional lineage specific whole genome triplication (mesopolyploidization) event; thus a hypothetical common hexaploid ancestor was proposed for the diploid *Brassica* species (Lagercrantz and Lydiate 1996; Parkin 2005; Wang et al. 2011;

Parkin et al. 2014). The tetraploid, *B. napus* species, has undergone a recent polyploidization event (neopolyploidization) with no major rearrangement in the A and C sub-genomes (Parkin 2005). The main difference between the ancient and the new polyploids is that the former is followed by diploidization (Schranz and Mitchell-Olds 2006).

2.2 Evolutionary relationship of the Brassica Species

The relationship among the diploids and the polyploids in the genus *Brassica* was described as the U triangle (U 1935, Figure 2.1), and was also confirmed using synthetic hybrids (Parkin et al. 1995). The three diploid species including *B. rapa* (AA, 485Mb, n=10), *B. oleracea* (CC, 648Mb, n=9), and *B. nigra* (BB, 558Mb, n=8) are the building blocks for the three tetraploid species, namely, *B. napus* (AACC, 1132Mb, n=19), *B. juncea* (AABB, 920Mb, n=18) and *B. carinata* (BBCC, n=17). Though the gene content in all the three genomes are equivalent, the A and C genomes are more comparable than the B genome (Lagercrantz and Lydiate 1996; Arias et al. 2014). Orthologous relationship between Ar (*rapa*) and Co (*oleracea*) corresponds to the homoeologous relationship between An (*napus*) and Cn (*napus*) sub-genomes. Comparative genetic mapping with Arabidopsis revealed conserved homology between An and Cn, along with additional duplicated segments in some chromosomes (Parkin 2005). On the other hand, comparing the An and Cn with their progenitor counterparts, Ar and Co, respectively, indicated substantial but unequal gene loss in the Cn than An sub-genomes (Allender and King 2010; Chalhoub et al. 2014).

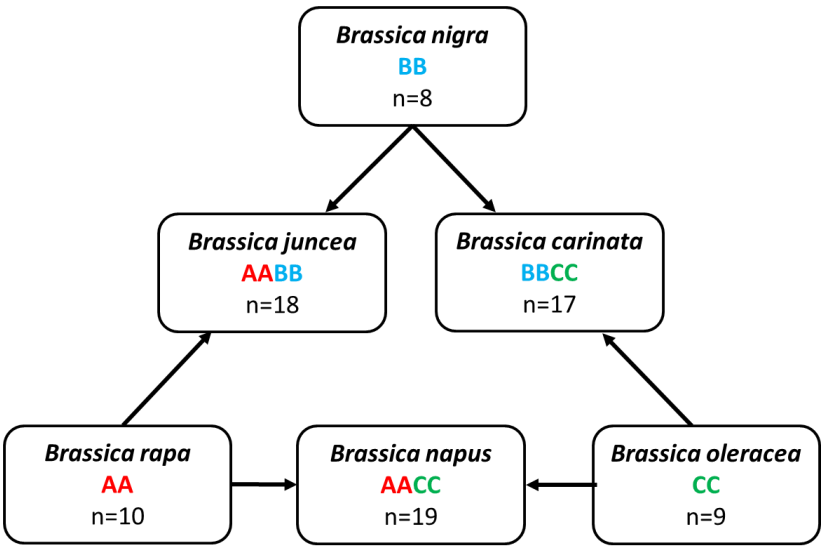


Figure 2. 1 The relationship among the six Brassica *species as proposed by U (1935)*

2.2.1 Brassica napus

Brassica napus is an allotetraploid (amphidiploid) species derived from the two diploid progenitors, *B. rapa* and *B. oleracea* (Parkin et al. 1995). *Brassica napus* is believed to be the result of spontaneous interspecific hybridization of the two progenitors (Snowdon et al. 2007). Although *B. napus* is considered to be a recent polyploid (Allender and King 2010), it is difficult to estimate the precise time of origin, as no wild relatives of this species are found. However, this species is believed to have originated during the Roman era, in the Mediteranian region where both the progenitors co-existed (Snowdon et al. 2007). A recent whole genome re-sequencing study suggested that the original ancestor of *B. napus* may have evolved from *B. rapa* subsp *rapa* (European turnip) and a common ancestor of the different *B. oleracea* subspecies, approximately 1910 - 7180 years ago (Lu et al. 2019). There was speculation in the past about *B. oleracea* or *B. montana* (a close relative of *B. oleracea*) being candidates of the maternal parent (Song and Osborn 1992), but it was ruled out. Instead, *B. rapa* (broccoletto) appears to be the best maternal candidate (Allender and King 2010). They also corroborated that *B. napus* could have multiple centers of origin.

2.3 Domestication of Rapeseed

Brassica rapa is perhaps one of the ancient *Brassica* species domesticated in India around 4000BC as vegetables, condiment, and oil for various purposes, and later introduced to other parts of Asia, particularly China and Japan (Snowdon et al. 2007; Qi et al. 2017). Conversely, in Europe, *B. rapa* has been known since the Roman times. However, it had limited use as vegetable oil in some Northern European regions in the 13th - 14th century, probably because of the availability of alternative oil sources such as olives, in Southern Europe (Gupta 2016; Friedt and Snowdon 2009)

Large scale cultivation of oilseed rape, probably *B. rapa*, began commercially in Europe in the 16th Century, primarily as a fuel source for burning lamps (Friedt and Snowdon 2009). In the late 19th century, its potential as a lubricant for ship steam engines was realized due to its excellent slippery property (Snowdon et al. 2007). Rapeseed was introduced to Canada during World War II due to shortage of edible oil or margarine (Eskin and Przybylski 2003). The spring type was adaptable and yielded better than other oilseeds such as flax and sunflower (Stefansson and Hougen 1964). However, rapeseed was considered unhealthy in the 1960s, as a high level of

erucic acid in conventional rapeseed cultivars was correlated with the risk of heart disease (Gupta 2016; Friedt and Snowdon 2009).

In the 1970s, Canada took the lead in the improvement of rapeseed for edible purposes using conventional breeding methods; the success story began with low erucic acid and later with double low *viz* low erucic acid and low glucosinolate rapeseed. The first cultivar, Tower, developed from Liho and Bronowski, was released in 1974 (Stefansson and Kondra 1975). Some of the early low erucic acid cultivars include Golden, Oro, and Echo (Eskin and Przybylski 2003). Large-scale production in western Canada dated back to the 1970s, first *B. rapa*, later surpassed by *B. napus* in the 1990s. Significant increase in rapeseed production has shifted from Asia to Canada and Europe in the past four decades (Gupta 2016).

2.4 Canola vs Rapeseed

Rapeseed refers to members of the *Brassica* species primarily used for oil. Other names include oilseed rape or oil rape. The term canola has been known since the 1970s, after the conversion of traditional rapeseed to double low rapeseed (Mcvetty et al. 2016). Canola is the high-quality version of rapeseed, in terms of food and animal feed (Steffanson and Hougen 1964). Although *B. napus* is the first and dominant species to be converted into double low rapeseed, canola quality *B. rapa* and *B. juncea* were also developed (Eskin and Przybylski 2003; Potts et al 2003). The name "Canola" is an acronym for Canadian oil low erucic acid, first coined by the Western Canadian Oilseed Crushers Association, and it is currently trademarked by Canola Council of Canada (Eskin and Przybylski 2003). The term "Canola" is commonly used in North America and Australia while "rapeseed" or "oilseed rape" is primarily used in Europe. On the other hand, high erucic acid, low glucosinolate rapeseed (HEAR), with >50% erucic acid and <20µmole glucosinotates, has specialized industrial uses (Mcvetty et al. 2016).

2.5 Biology of Rapeseed

Different morphotypes including leaf, tuber, and oil (oleiferous) exist in both *B. rapa* and *B. napus*, though the oil type seems to be the most important, diverse, and well-studied (Gupta 2016). *Brassica rapa* (*B. campestris*) is also commonly known as turnip rape or polish rape; and *B. napus* is known as swede rape or argentine rape (Gupta 2016). *Brassica napus* is self-compatible and high yielding than its progenitors. On the other hand, the diploid *Brassica* species, *B. rapa* and *B. oleracea*, are self-incompatible but with few exceptions (Snowdon et al.

2007; Mcvetty et al. 2016). *Brassica oleracea* also has various morphotypes of the inflorescence, mainly used as vegetables (Parkin et al. 2014). Moreover, three forms or ecotypes, namely winter, semi-winter and spring, are known in *B. napus* (Mcvetty et al. 2016). The winter types are the most productive and require a longer vernalization period than the semi-winter types (Lu et al. 2019). They are commonly cultivated in Europe and Asia, whereas spring types are grown in Canada, Australia, Northern Europe and Northern China (Snowdon et al. 2007; Mcvetty et al. 2016). Generally, *B. napus* is less diverse than its progenitors (Allender and King 2010), probably due to factors such as the narrow genetic base in the canola improvement program, as modern cultivars are mostly derived from the two early cultivars, Liho and Bronowski, and geographical or climatic limitations (Friedt and Snowdon 2009). Nevertheless, the potential inbreeding caused the narrowing of the genetic background in *B. napus,* so other relatives in the Brassicaceae family can be exploited to broaden the gene pool or introgress agronomically important traits in plant breeding, such as blackleg resistance in *B. napus* (Crouch et al. 1994; Friedt and Snowdon, 2009; Roy 1984).

2.6 The Leptosphaeria maculans Pathogen

2.6.1 Biology of Leptosphaeria

Leptosphaeria maculans is a hemibiotrophic fungal pathogen of *B. napus* with alternating biotrophic and necrotrophic lifestyles (Haddadi et al. 2016). The pathogen overwinters as a saprophyte on dead plant tissues on the soil surface (Fernando *et al.* 2007). Two species, *L. maculans* and *L. biglobosa,* in Canada and other rapeseed production areas, differ in the magnitude of virulence (West et al. 2001). *L.eptosphaeria maculans* is the most aggressive while *L. biglobosa* only shows superficial symptoms with no significant yield loss (Fitt et al. 2006). Sexual reproduction probably contributes to the dynamic population structure in *L. maculans* (Howlett 2004).

2.6.2 Life cycle and epidemiology

Leptosphaeria maculans may be a polycyclic pathogen (Li et al. 2007; Zhang et al. 2016) with resting spores or structures bridging consecutive infection cycles in the off-season (Huang et al. 2007). Primary infection occurs when the spores released from the wind-borne ascospores produced on the overwintering structure, pseudothecia, are transported to the infection sites, cotyledons or leaves (Travadon et al. 2007). Once the inoculum enters the host through openings

(stomates or wounds), fungal hyphae grow systematically in the apoplast until the first symptom appears on the leaves or cotyledons. Pycnidia, the fruiting body of the pathogen is borne on the surface of the infected tissue, and upon maturation, disperses to the surrounding plants through rain splash to cause multiple cycles of secondary infection (West et al. 2001; Travadon et al. 2007; Zhang et al 2016).

Severe symptoms may result in seedling death if the infection occurs at the early stage. However, the most common symptoms are leaf and stem lesions. Stem lesions that appear on the upper stem are called "phoma stem lesion", "stem canker" or "crown canker", which restricts the movement of water and nutrients, causing premature ripening (West et al. 2001; Van de Wouw et al 2016). The term "blackleg" describes the blackish lesion at the base of the hypocotyl at the seedling stage that causes the death of premature seedling or stem of mature plants (West et al. 2001; Fernando et al. 2007). Also, severe crown canker may cause mature plant to lodge. In western Canada, the release of ascospore in *L. maculans* occurs in the summer months, coinciding with growing season of spring canola (Zhang et al 2016). Guo and Fernando (2004) suggested seasonal patterns of ascospore and pycnidiospore release from July to early August. After harvesting, the pathogen colonizes the stem residue and develops a winter survival strategy. Pycnidia and pseudothecia are overwintering structures, which may survive on stubble for up to four years (Kutcher et al 2013).

2.6 Host resistance

Blackleg is mainly controlled by natural plant resistance that can broadly be classified into two: a complete, strong resistance effective from the seedling to adult plant stages (Raman et al 2013 Raman et al. 2012a, b), and a partial, weak/intermediate seedling resistance that allows colonization of cotyledons/leaves, but effectively reduce stem canker at the adult plant stage (Delourme et al. 2004; Li et al. 2004; Delourme et al. 2006; Huang et al. 2009; Raman et al. 2012b; Huang et al. 2014; Larkan et al. 2016a). The first type of resistance is assumed to be mediated by single dominant R genes and isolate specific (Delourme et al. 2004, 2006; Raman et al 2013), while the latter is probably mediated by multiple QTL and possibly isolate non-specific (Delourme et al. 2006, 2008b; Kumar et al. 2018; Raman et al. 2018, 2020). Other factors including genetic background of the host, temperature, inoculum concentration, genotype of isolates and growth stage of the plant upon first infection could influence both dominant R-gene

and QR-mediated resistance (Hammond et al. 1985; Pilet et al. 2001; Huang et al. 2006, 2009; Delourme et al. 2008a).

2.6.1 Genetic Studies of Host Resistance

Classical genetic approaches are basically used to identify the existence of genetic variability and the mode of inheritance of blackleg resistance in *B. napus* (Cargeeg and Thurling 1980; Mithen and Lewis 1988; Pang and Halloran 1996a). However, such analyses solely based on phenotype were inadequate to elucidate the genetic basis of blackleg resistance, such as understanding of the underlying causative genes. Comparisons among genes/loci identified in different experiments as heterogenous plant population, mixtures of field isolates, different screening methods (greenhouse vs field; seedling vs APR) and environmental conditions, contribute to the inconsistencies obtained in outputs (Cargeeg and Thurling 1980; Pang and Halloran 1996b; Rouxel et al. 2003; Larkan et al. 2016b; Neik et al. 2017). With the development of molecular markers, genotypes and phenotypes can be linked to study the genetics behind host resistance and develop molecular markers for marker assisted selection (MAS) in plant breeding (Mayerhofer et al. 1997).

Molecular markers are allelic DNA sequence variations that serve as landmarks in a genome (Nadeem et al. 2018). Molecular markers are applied in the construction of detailed genetic map, QTL mapping, association mapping / genome-wide association studies (GWAS), genotyping, and others. Different marker detection methods include restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), sequence related amplified polymorphism (SRAP), and single nucleotide polymorphism (SNP). Although each method has its own strengths and weaknesses, the marker density and efficiency culminate when all SNPs in a genome can be detected (Nadeem et al. 2018). Besides molecular markers, the use of well-characterized isolates / races and multi-environment trials facilitate in the accurate elucidation of the genetic basis of host resistance, environmental variations and genotype-by-environment (G*E) interaction (Rouxel et al. 2003; Raman et al. 2012b; Huang et al. 2016).

2.6.2 Molecular Markers, Genetic Maps and QTL Mapping

Genetic maps are used to identify molecular markers linked to a gene or locus in the genome, as well as the relative distance among these markers, even without prior knowledge of the gene

(Nadeem et al. 2018). Doubled haploid (DH) populations along with molecular markers were useful in the construction of genetic map in *B. napus*. DH lines are homozygous at all loci, which is particularly useful when dominant molecular markers are utilized (Foisset et al. 1996). Nevertheless, segregation distortion is often detected due to tissue culture procedures or wide crossing of parental lines (Foisset et al. 1996; Nurhasanah and Ecke 2016).

Restriction fragment length polymorphism is the first hybridization-based molecular marker detection method employed for the construction of genetic map of *B. napus* using cDNA probes/library with prior knowledge of whole genome sequence of the Brassicas or the model plant, Arabidopsis (Ferreira et al. 1994). Parkin et al (1995) developed a genetic map of *B. napus* using 1317 RFLP markers with a genome coverage of 1,656 cM, arranged into 19 linkage groups (LG) corresponding to the ten and nine chromosomes of the A and C subgenomes of *B. napus*. This map enabled us to understand the remarkable collinearity between the A and C genomes and served as a reference genetic map in previous QTL mapping (Rimmer 2006). An ideal genetic map has uniformly distributed markers which facilitate the tagging of genes or loci in QTL mapping.

Saturated genetic maps are a valuable tool for QTL mapping, especially in the absence of information of the physical map (Sun et al. 2007). Earlier QTL mapping efforts identified a smaller number of polymorphic loci since the RFLP based genetic maps had gaps and relatively low genome coverage (Ferreira et al. 1994, 1995; Dion et al. 1995; Parkin et al. 1995). Development of a consensus map derived from multiple segregating populations is a good strategy to bridge the gaps observed in various genetic maps. Lombard and Delourme (2001) constructed a consensus map using three populations, and the resulting consensus map has a total genetic distance of 2,429 cM with relatively high genome coverage. On the other hand, Foisset et al (1996) constructed an integrated genetic map using several types of molecular markers to enrich the map, as well as to carry out comparative mapping studies. Though the marker distribution in the consensus or integrated genetic maps may not be uniform, these maps are used as references or to validate linkages, especially in the absence of a whole genome sequence.

In *B. napus*, two blackleg resistance loci *LmFr* and *LEM1* from the resistance donor parents Cresor and Major, respectively, were mapped using RFLP markers (Dion et al. 1995; Ferreira et al. 1995). Mayerhofer et al. (1997) mapped the blackleg resistant gene *LmR1* using RAPD

marker and suggested possible relationship with the previously mapped *LEM1* gene (Ferreira et al. 1995), by comparing the map position on LG6, using polymorphic RFLP markers from the published genetic map of *B. napus* (Ferreira et al. 1994). In another study, Delourme et al (2004) used common RAPD markers derived from the reference consensus map (Lombard and Delourme 2001) to map *B. napus Rlm 1, 3, 4, 7 , 9* as gene clusters on chromosome N7 and *Rlm2* on chromosome N10 using a relatively well-characterized *L. maculans* isolates. They also suggested that the clustered genes on N7 correspond to the previously mapped genes on the LG6 (Parkin et al. 1995). With the availability of new markers and genetic maps, construction of consensus and integrated genetic maps became a routine, mainly to saturate the gaps in individual linkage groups (Lowe et al. 2004; Piquemal et al. 2005; Raman et al. 2012a).

Microsatellite (SSR) markers are based on highly polymorphic microsatellite repeats that are prevalent in the genome. Lowe et al. (2004) demonstrated SSR as potential molecular markers for bridging the gaps in the pre-existing genetic map (Parkin et al. 1995). On the other hand, Piguemal et al (2005) constructed a consensus genetic map using 240 SSR markers combined with SCAR markers to be used for QTL mapping, albeit some gaps still existed. Raman (2012a & b) associated SSR markers linked to the blackleg resistance genes, *Rlm1* and *Rlm4*, which are amenable to high-throughput genotyping, unlike the previous RAPD markers (Delourme et al. 2004). In another study, Yu et al (2005, 2008, 2013) mapped a novel set of blackleg R-genes *LepR1-4* derived from *B. rapa* subsp *Sylvestris* using SSR markers. With the availability of Brassica whole genome sequence, designing SSR primers has become easier compared to the previous SSR markers which were developed from genomic DNA clone libraries. One SSR motif appears at every 4 kb and are mostly co-dominant, viz differentiate between heterozygotes and homozygotes, which is important in MAS (Cheng et al. 2009).

Yet another PCR-based molecular marker, sequence related amplified polymorphism (SRAP) was developed (Li and Quiros 2001), which targets sequences in the open reading frame (ORF). The SRAP marker system was used to construct the ultra-dense *B. napus* genetic map using 13,500 SRAP markers with a genome coverage of 1,604.8cM (Sun et al 2007). SRAP coupled with other genome specific markers were used to map blackleg resistance *BLMR1* and *BLMR2* loci in *B. napus* (Long et al 2011). While PCR-based markers such as SSR and SRAP could be used for medium to high-throughput genotyping and map based cloning (Rahman et al. 2008;

Long et al. 2011), SNP is the ultimate choice of molecular marker in terms of efficiency, especially with decreasing cost of sequencing and availability of high throughput genotyping (Clarke et al 2016).

Currently, SNPs are suitable for high-throughput genotyping (Nadeem et al. 2018). Raman et al (2014) constructed an integrated map from SNP and non-SNP markers and identified a set of SNP markers completely linked to blackleg resistance *Rlm4* gene. Genetic maps constructed with SNPs could easily be aligned with any of the Brassica physical maps (*B. rapa* and *B.oleracea* and *B. napus*) (Raman et al 2016b). Currently, SNP detection methods such as Illumina 60K Infinium bead array and GBS have become a routine in *B. napus* (Raman et al. 2013; Fu et al. 2020). These high throughput genotyping methods allow parallel scoring of thousands of individuals in the whole genome of *B. napus* (Kumar et al. 2018; Clarke et al 2016). Illumina Infinium bead arrays were developed from data of re-sequencing of multiple genetic sources to retain mostly single-locus informative SNPs. The 60K array is a hybridization based genotyping tool, requiring less computational ability than GBS, which can also be used for *B. rapa* and *B. oleracea*. On the other hand, GBS requires no prior SNP information, but the computation tool is highly demanded (Clarke et al 2016).

2.6.3 Genetic map vs Association Mapping

QTL mapping is performed using two strategies: genetic map and genome wide association studies (GWAS) based on linkage disequilibrium (LD). Majority of the QTL mapping studies in the past were conducted using less diverse bi-parental populations with limited polymorphisms (Pilet et al. 2001). On the other hand, GWAS is a sort of high-resolution mapping that utilizes a collection of diverse germplasms to identify multiallelic polymorphisms and haplotypes, potentially employed across breeding programs in different continents (Rahman et al. 2016; Raman et al. 2016a; Kumar et al. 2018). Genome wide association studies is used to verify the usefulness of a marker identified using conventional genetic mapping, for MAS or vice versa (Jestin et al. 2011; Raman et al. 2016a). Jestin et al. (2011) not only identified novel genomic regions but also validated the known QTL in the *B. napus* cultivar Darmor using association analysis. With the high throughput SNP detection, the potential for GWAS in exploring the allelic diversity, identifying new loci and keeping track of introgressions could be realized (Rahman et al. 2016; Clarke et al. 2016). Unlike classical QTL mapping, which is based on

biallelic polymorphism, GWAS looks for shared haplotypes across germplasms (Kumar et al. 2018). Nevertheless, the loci identified in genetic mapping studies or GWAS could be confounded with other phenological traits such as plant maturity and flowering time (Pilet et al. 1998). Raman et al. (2020) suggested that these QTL could be more efficient in genomic selection (GS). Genome wide association studies is suitable for the high-throughput genomic selection to facilitate genetic gain in complex traits by introgressing multiple alleles using prediction models (Raman et al. 2016a; Nadeem et al. 2018).

2.6.4 QTL mapping of quantitative resistance (QR)

Although the genetic control of QR is not well understood, some authors argue that blackleg resistance is a polygenic trait that is highly affected by the environment (Huang et al. 2016; Kumar et al. 2018). Whole genome mapping approaches indicated that QR loci (QTLs) are apparently abundant than qualitative resistance (Raman et al. 2012b, 2016a) and are located in homoeologous regions (Fomeju et al. 2015). However, one should be cautious in mapping authentic and stable QR loci. Raman et al. (2012b) identified about 14 genomic regions associated with race-specific (*Rlm4*) and non-race-specific blackleg resistance using DH populations derived from bi-parental crosses. QTL mapping of multiple loci with small effect may be deficient in explaining a greater proportion of the phenotypic variation due to factors such as environmental conditions, lack of polymorphism in the mapping population, and less marker density in genetic map (Raman et al. 2016a). To improve the efficiency of QTL mapping, a multi-environment (year/location) QTL study along with the use of populations derived from different parental crosses and a denser genetic map enabled the identification and validation of the already identified QTLs (Huang et al. 2016; Raman et al. 2018). Recently, Kumar et al. (2018) carried out a detailed multi-environment QTL study using a panel of genotypes with less interference of major R genes in their background and taking into consideration the already identified QTL along with the power of SNP genotyping (60K). They emphasized that the QR identified in their study is apparently controlled by QTLs of small effect.

2.6.5 Map-based cloning and Identification of candidate genes

Traditional map-based cloning is based on a fine-mapping strategy to identify tightly linked molecular markers to the candidate gene, and to eventually clone it. Fine mapping uses a high-resolution mapping population to identify recombinants that assist in selecting molecular

markers closely linked to the probable causative gene (Martin 1995). Map-based cloning is thought to be difficult in *B. napus* due to the complex nature of the Brassica genome compared to the Arabidopsis genome, including massive duplications and minor rearrangements (Mayerhofer et al. 2005). In the absence of a physical map, saturated genetic maps and (flanking) molecular marker assisted development of near iso-genic lines (NILs) *via* backcrossing with the recurrent parent could improve the efficiency of fine-mapping (Martin 1995) .

Having a dense genetic / consensus map is instrumental in map-based cloning of R genes in *B. napus*, so that the molecular markers linked to the trait are precisely anchored on the genetic map. Once anchored, additional genome-specific markers were developed by exploiting the collinearity with the Arabidopsis genome and screening the BAC library of *B. rapa*, in the absence of the genome sequence of the Brassicas (Long et al. 2011; Larkan et al. 2013). Although such strategy is considered time consuming and labour intensive (Martin 1995), it has been effectively used in the past to clone blackleg resistance genes (Long et al. 2011; Larkan et al. 2013, 2014, 2015).

Candidate gene prediction is supposedly much harder in QR than qualitative resistance. With the advent of Illumina sequencing and the availability of genomic resources, including the whole genome sequence of the Brassicas and high throughput genotyping platforms, the success rate and efficiency of identifying QTL on physical map, developing SNP markers, as well as predicting candidate genes, has been improved (Larkan et al. 2016a, 2016b; Kumar et al. 2018; Raman et al. 2018). Larkan et al. (2016a) associated a cluster of cysteine rich kinases (CRKs) with adult plant resistance (APR) QTL on A01. They also suggested that some genes underlying the identified QTLs could be because of allelic differences or altered transcription of R gene interacting proteins. Thus, whole transcriptome studies could help us understand the transcriptional reprograming, especially in QR (Becker et al. 2017; Zhou et al. 2019). Once the candidate gene is identified and the sequence is known, gene specific markers such as SNP or SCAR could be developed for MAS, suited to high-throughput genotyping (Rahman et al. 2008). MAS is vital in speeding-up backcrossing in fine-mapping, gene pyramiding and keeping track of introgression of exotic genes in cultivars, however, candidate markers need to be validated for MAS.

2.6.6 Molecular basis of disease resistance

Plant defense system is multilayered that includes PTI and ETI (Niks et al. 2015). Pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) is activated by the interaction of PAMPs and pattern recognition receptors (PRRs), while effector-triggered immunity (ETI) is mediated by the interaction of pathogen effectors and R genes in the host. PRRs are surface-localized RLKs or RLPs while R refers to intracellular pathosystem specific immune receptors (Macho and Zipfel 2014).

Models are used to describe host-pathogen interaction at a molecular level. The Zigzag Model is basically an upgrade of the gene-for-gene model, which explains the specific interaction of specific *R* genes in the host and the corresponding *Avr* alleles in the pathogen (Jones and Dangl 2006; Van de Wouw and Howlett 2020). In this system, first pathogens induce the broad-spectrum PTI to perceive the general elicitors such as flagellin and chitin. But effective pathogens may weaken the PTI by secreting effectors. Then, plants launch the second layer of defense called ETI through specific immune receptors (*R* genes) that recognize the specific effectors. The pathogen, in turn, could breakdown the *R* genes, which explains the host - pathogen coevolution. Molecular events that lead to PTI and ETI are associated with weak and strong defense responses, respectively (Jones and Dangl 2006). However, this model has limitations such as the assumption that effectors can only be detected by specific receptors and the system also falls short in explaining the situation in pathosystems other than obligate biotrophs.

The alternative Invasion Model was proposed with the hypothesis that PTI and ETI may not be very distinct (Cook et al. 2015). This model is more generalized in the sense that it covers a wide range of response inducers including microbe associated molecular patterns (MAMPs), effectors, and damage associated molecular patterns (DAMPs) or modified self; and immune systems including biotrophs, necrotrophs, endophytes, and others. Perception of diverse ligands called invasion patterns (IPs) by IP receptors triggers IP-triggered response (IPTR). This implies the combined action of diverse invaders - receptors interaction causes a spectrum of response (Cook et al. 2015).

Regardless of the system, the common signalling pathway in plant defense can be divided into three; the upstream perception and the downstream signalling cascades and activation of defense

genes (Niks et al. 2015). Major R gene mediated resistance is associated with receptors while candidate genes underlying QR are thought to be structurally diverse (Cook et al 2015). Studies indicated that genes underlying receptor mediated resistance are prone to breakdown due to coevolution of the pathogen and the host (Van de Wouw and Howlett 2020; Zhang et al 2016). Identification of QR genes will enable us to understand the molecular mechanisms underlying QR. This will facilitate in breeding durable resistance cultivars in plant breeding.

2.6.7 Application of RNA sequencing (RNA-seq) technologies for the analysis of disease resistance

RNA-seq is currently the method of choice for transcriptome profiling, as well as quicker identification or validation of candidate genes and identification of SNPs based on the reference genome coding sequence alignment (Fu et al, 2019). RNA-seq is a relatively new and high throughput technology with applications such as understanding the mechanism underlying disease resistance, sterility, and seed weight in *B. napus* (Zhou et al 2019; Teng et al 2017; Geng et al 2018). Unlike whole-genome analysis, RNA-seq attempts to sequence only the transcribed regions at the genome-wide scale, as well as provide information about the variation in expression levels of genes. Illumina sequencing is commonly used in RNA-seq (Bräutigam and Gowik, 2010). Illumina technology enables the production of tens of millions of sequenced reads in a sample. The key steps in RNA-seq are the preparation of cDNA libraries and highly parallel sequencing. The starting material in this method is the total RNA extracted from the tissue of interest under specific conditions. The poly-adenylated (poly-A) tail of the mRNA is used to separate mRNA from rRNA in the preparation of cDNA libraries for detecting expressed genes. The adaptors are ligated to cDNA for high throughput sequencing. Once the sequencing is complete, the raw data are converted to sequencing reads using algorithms in a Fastq format for downstream data analysis. The massively sequenced short reads are further analyzed using bio-informatic tools such as BOWTIE to align the reads into the reference genome. The mapped reads are quantified for further statistical analysis using the data analysis pipelines or software such as the Bio-conductor packages in R software (Chen et al 2016; Wang and Ma 2016). The resulting differentially expressed genes (DEGs) are classified into functional groups or metabolic pathways for using software such as MapMan and Blast2Go. These programs allow to predict protein functions and convert the data into a more meaningful biological function.

The high sensitivity of RNA-seq along with the availability of reference genome sequences for both *B. napus* and *L. maculans* enabled the simultaneous transcriptome analysis of the biological processes in this pathosystem (Hadaddai et al 2016; Becker et al 2017; Zhou et al 2019). Gervais et al. (2016) attempted to explain the hemibiotrophic lifestyle of the *L. maculans* pathogen by the different sets of effector genes expressed at early / biotrophic and late / necrotrophic infection stages. They also identified novel late candidate effector genes for further functional studies. On the host side, most RNA-seq studies emphasized on the importance of the *B. napus* genes that are activated at the early stages to determine the final output (Becker et al 2017; Zhou et al 2019). Differential expression of host receptor genes and early effector genes confirm the possible host-pathogen (effector – receptor) interaction at the initial infection stage (Hadaddai et al 2015). Beside receptors, pathogen induced differential expression of other defense-related biosynthetic pathways such as hormones and secondary metabolites were implicated at genome-wide scale.

3. Analysis of quantitative adult plant resistance to blackleg in *Brassica napus*

Hanna B. Dandena, Qiang Zhang, Tengsheng Zhou, Arvind H. Hirani, Zhen Liu, Dilantha W. G. Fernando, Robert W. Duncan, Genyi Li

3.1 Abstract

Leptosphaeria maculans L. causing blackleg is a highly evolved fungal pathogen that damages the rapeseed industry in Canada, Australia and Europe. Advanced backcross populations segregating for intermediate resistance (*BLMR2*) and susceptible (*blmr2*) alleles were planted and inoculated using cotyledons. near-isogenic lines (NILs) containing the intermediate blackleg resistance locus along with the parent lines and other controls were planted in a blackleg nursery at the University of Manitoba. ANOVA indicated a correlated significant difference between the genotypes with *BLMR2* and *blmr2* alleles at the seedling and adult plant stages. Heterozygous plants with both *BLMR2* and *blmr2* alleles showed relatively lower disease severity index (DSI) while other plants with only the *blmr2* allele induced a higher DSI at seedling and adult plant stages, respectively, on a scale of 0-9. In the field, the NILs with the *BLMR2* alleles consistently showed lower stem canker severity index similar to the resistant parent Surpass 400 in three consecutive years. In comparison, lines with known *R* genes (*BLMR1* and *Rlm2*) showed relatively higher DSI in the field test. This demonstrated that the intermediate resistance locus performed well under severe blackleg disease pressure in the field while the NIL lines with single dominant *R* genes were ineffective. Because a group of isolates carrying various *Avr/avr* alleles were used in the field evaluation, our results suggest that the intermediate resistance locus confers horizontal resistance and has excellent potential in blackleg management in western Canadian canola production regions.

Keywords: *Brassica napus*, *Leptosphaeria maculans*, *BLMR2*, intermediate resistance, near-isogenic lines, NILs, SSR, SCAR

3.2 Introduction

Brassica napus L. (rapeseed or oilseed rape) is an economically important global oilseed crop. *Leptosphaeria maculans*, belonging to the Dothideomycetes, causes blackleg disease in major rapeseed growing areas including Canada, Australia and Europe (West et al. 2001). The pathogen infects various plant parts including the cotyledons, leaves, pods, stems and roots resulting in yield and quality losses (Gugel and Petrie 1992; Salisbury et al., 1995). Wind-borne ascospores and rain-splashed pycnidiospores, the potential sources of inoculum (Salisbury et al., 1995; West et al., 2001), penetrate stomatal openings to initiate infection (Li et al., 2004). After successful penetration, symptoms in the plant are exhibited in two phases. First initial lesions develop on the leaves, which is then followed by stem canker near the bottom of stems (Hammond et al., 1985; Hammond and Lewis 1987a, b; West et al., 2001; Huang et al., 2006). Absence of stem canker is an indicator of blackleg resistance in rapeseed since severe stem canker is associated with potential yield losses worldwide. However, very early infection of cotyledons and young leaves initiated by ascospores also leads to significant yield losses in Australia (Salisbury et al., 1995; West et al., 2001; Li et al., 2004; Delourme et al., 2008a).

Genetic resistance in the rapeseed and blackleg pathosystem is classified as qualitative resistance conferred by major and dominant R genes and quantitative resistance (QR) by minor polygenes or quantitative trait loci (QTLs). Resistance in cotyledons and true leaves severely restricts hyphal growth beyond penetration sites (Hammond and Lewis 1987a, b; Li et al., 2004, 2008b; Huang et al., 2006). This type of resistance is mainly associated with race-specific R genes. While at later growth stages, reduced stem canker severity is due to limited pathogen growth and multiplication in the stem, which is most likely mediated by polygenes (Hammond and Lewis 1987a, b; Li et al., 2004; Yu et al., 2005; Huang et al., 2009; Huang et al., 2014; Travadon et al., 2009). Other commonly used terms used for these types of resistance include seedling or cotyledon resistance at the early stages and adult plant resistance (APR) at the later plant growth stages (Delourme et al., 2006). However, seedling resistance and APR can be correlated in some cases (Hammond et al., 1985; Huang et al., 2006). Seedling resistance is often assessed in controlled environmental conditions using cotyledon or leaf inoculation assays, while APR is usually evaluated under field conditions where a mixture of natural inoculum or application of mixed inoculum is involved, so the term ‘field resistance’ is commonly used. The mechanism of APR is thought to be mediated by QTLs as compared to qualitative resistance mediated by

typical R genes. The R genes evaluated at the seedling stage in controlled environmental conditions are relatively simple and reliable as compared to the APR at the adult plant stage in the field (Bansal et al., 1994). In addition, identification of stable QTLs for APR requires multi-year and multi-location field trials. Some studies suggest evaluation of quantitative resistance at the seedling stage in controlled environmental conditions such as petiole and hypocotyl inoculation methods using field collected ascospores (Travadon et al. 2009; Huang et al. 2014).

Genetic resistance controlled by major and minor genes (Dion et al., 1995; Delourme et al., 2004; Rimmer 2006; Raman et al., 2012b, 2013) in combination with other management practices such as crop rotation or sanitation have kept blackleg disease under control (Salisbury et al. 1995; West et al. 2001). However, serious breakdown of resistance in cultivars with single *R* genes has been reported in France and Australia (Li et al. 2003; Rouxel et al. 2003; Sprague et al. 2006), mainly due to the use of a sole *R* gene in a tight crop rotation practices. A recent study indicated the possible loss of effectiveness of the resistant gene *Rlm3*, inferred from the lack of the complementary avirulent allele (*AvrLm3*) in the western-Canadian *L. maculans* population (Zhang et al. 2016). They also noted the predominance of *Rlm3* gene in the cultivars grown in western Canada since the early 1990's. The occurrence of a single *R* gene in most canola cultivars (Zhang et al. 2016) and the wide-range of cultivation of these cultivars with the same *R* gene (Rouxel et al. 2003; Sprague et al. 2006; Van de Wouw et al. 2014) exert evolutionary pressure on the prevalent *L. maculans* races. This had contributed to the disappearance of complementary *Avr* genes and the increased frequency of virulent alleles in Canada, France and Australia. *Rlm3* had been effective for a relatively long period of time in Canada, while sudden losses of effectiveness of *Rlm1* and *LepR3* occurred in France and Australia, respectively (Li et al. 2003; Rouxel et al. 2003). This could be due to the *Rlm3* introgression in a APR background, in a significant proportion of Canadian cultivars (Zhang et al. 2016). APR is generally mediated by polygenes that potentially exert less selection pressure on the prevalent *L. maculans* race mixture. Jet Neuf is a good example of a cultivar with quantitative resistance that remained effective for decades (Hammond and Lewis 1987a, b; Dilmaghani et al., 2009). A combination of typical *R* genes and quantitative resistance (QR) was proposed to increase the durability of the *R* genes in *B. napus* cultivars (Brun et al. 2010). Recently, the use of the more detailed labels for major *R* gene rotation has been suggested, especially in regions with tighter crop rotation practices, to ensure a better resistance stewardship (www.canolacouncil.org).

Surpass 400 is a rapeseed cultivar developed by introducing blackleg resistance from the wild *B. rapa* subsp *sylvestris* that displayed a very high level of resistance (Crouch et al. 1994). It was first released by Pacific Seeds in 2000 as a cultivar with a high level of APR in Australia (Easton, 2001). Other hybrids and open-pollinated cultivars with the *sylvestris* derived resistance were developed and released in Australia and Canada in 2001 (Easton, 2001). However, the breakdown of resistance in Surpass 400 was reported in just three years after Surpass 400 was released (Li et al. 2003). Initially, the high APR in Surpass 400 was thought to be mediated by a single dominant gene (Easton 2001; Li and Cowling 2003) and Yu et al. (2008) mapped this gene (*LepR3*) on chromosome A10 (Larkan et al. 2013). However, Van De Wouw et al. (2009) reported that at least two dominant genes (*Rlm1* and *RlmS*) that interact with *AvrLm1* and *AvrLmS*, respectively, are responsible for the APR in Surpass 400 (Marcroft et al. 2012; Larkan et al. 2016b). In another study, Long et al. (2011) identified two independent loci (*BLMR1* and *BLMR2*) in this cultivar and mapped both on chromosome A10 approximately 20 cM apart; not so distant from the previously mapped *LepR3* locus (Yu et al. 2008). *LepR3*, which has already been cloned is the same as *BLMR1* (NCBI accession No. JQ979409). The major A10 genes *LepR3* and *Rlm2* apparently are allelic (Larkan et al. 2013; Larkan et al. 2014; Larkan et al. 2015) and function as receptor-like protein while the second resistance locus, *BLMR2* that displayed intermediate cotyledon resistance phenotypes (Long et al. 2011) remained cryptic. The aim of the current study is to perform a thorough phenotypic analysis of the *BLMR2* locus. We hypothesize that *BLMR2* is a QR locus with partial seedling resistance that corresponds to APR.

3.3 Materials and methods

3.3.1 Molecular marker development and detection

According to the previous mapping data (Long et al. 2011), *BLMR2* was located in a region close to a sequence characterized amplified region (SCAR) marker 12D09. Using the genomic sequence of chromosome A10 in *B. rapa*, 35 simple sequence repeats (SSR) markers were designed and tested. Four SSR markers were identified to be closely linked to the *BLMR2* locus (Table 3.1), and together with the previous SCAR marker 12D09, all the five molecular markers were used to develop near isogenic lines through molecular marker assisted backcrossing.

DNA was extracted from leaf tissues using the CTAB method as described previously (Long et al. 2011). Also, four SSR and one SCAR molecular markers were detected following the same procedure as described by Long et al. (2011). Two unlabelled primers and the M13 primer labelled with four fluorescent dyes '6-FAM', 'VIC', 'NED' and 'PET' were used to produce PCR products which were separated with the ABI Genetic Analyzer 3130xl (ABI, California, USA). PCR was run at 94^0C, 3 min; 94^0C, 1 min; 60^0C with -1.0^0C each cycle 1 min and 72^0C, 1 min for 6 cycles; 94^0C, 1 min; 55^0C, 1 min and 72^0C, 1 min for 25 cycles.

3.3.2 Brassica napus genotypes and populations

Genotypes containing *BLMR2* were derived from recombinants in the BC_1F_3 of a cross between Westar (susceptible and recurrent parent) and Surpass 400 (resistance donor parent) as described by Long et al. (2011). Advanced backcrossed (BC_5F_3) *B. napus* population segregating for heterozygous *BLMR2*/*blmr2* and *blmr2* alleles (Table S3.1) were developed by successively backcrossing the BC_1F_3-derived recombinant lines (Long et al. 2011) to the susceptible recurrent parent Westar. Homozygous near isogenic lines (NILs) with *BLMR2* (37-7-5-4, 37-7-5-3 – BC2F4, NJ13b – BC3F4, Hn7 and Hn10 – BC5F4) were developed by selfing a series of recombinants in the backcrossed generations. These recombinant derived NILs are presumed to harbour a range of segments based on genotypic data (Table 3.2) in the *BLMR2* region. Control checks used in the field experiment include 15-92-11 (*BLMR1* NIL derived from the cross between Westar and Surpass 400, Long et al., 2011) and G4A36-1 (*Rlm2* NIL derived from the cross between Westar and Glacier), which were developed using similar procedures (backcrossing and selfing) in our lab. Surpass 400 and Westar were also used as resistant and susceptible controls, respectively.

3.3.3 Leptosphaeria maculans isolates and inoculum

Five *L. maculans* isolates representing different pathogenicity groups (PGs) (Mengistu et al., 1991), namely 87-41 (PG2), 3-42-6 (PG3), 09 Stonewall (PGT), Pl03-02-01 (PG3) and PG4-1-M (PG4) were used to inoculate individual plants. Four of them (87-41, 3-42-6, 09 Stonewall and PG4-1-M) were tested for the *Avr* gene patterns (*AvrLm1*, *AvrLm2*, *AvrLm4-7*, *AvrLm6*, *AvrLm11* and *AvrlmJ1*) by PCR (Table S3.2). The *L. maculans* isolates were prepared using pycnidiospores collected from dried inoculated cotyledons of Westar on V8 medium plates after surface sterilization under continuous light at 24 ℃. After seven days, pycnidiospores were

subcultured into fresh V8 agar media. Proliferated pycnidiospores were collected from the medium surface by scraping using glass slides and double distilled water. The suspension was spinned down and the supernatant was discarded. Distilled water was added, and the spores were re-suspended to estimate the concentration using a hemocytometer under a microscope. The final inoculum was adjusted to a concentration of 2 X 10^7 spores/ml (Mengistu et al., 1991; Long et al., 2011).

Table 3. 1 Molecular markers used for genotyping backcross populations and near isogenic lines

Marker name	Primer name	Sequence (5'-3')	Marker type*	Physical position**
N10-56	n10-56	m13TAATACTGGTTAATTATGCT	SSR	12.00
	Rn10-56	ACAGTACATTCACGTTCTAG		
N10-45	n10-45	m13CAGAAGAAGAAGGATATGGT	SSR	12.06
	Rn10-45	TCCAGTTAACCAATGCTGGT		
N10-40	n10-40	m13CACAATTTCTGGTATACAGATTG	SSR	12.13
	Rn10-40	CTTTGGAGCGAATTGTTGAAG		
N10-37	n10-37	m13CAGTCCTGACTTTGCCATCA	SSR	12.60
	n10-37	ACAGGCGAGAGGTTTGAAGA		
12D09	12D09A	m13TCCGATCACACGAGTGTTGA	SCAR	14.36
	12D09B	CAACACAGTACACACAAGCA		
	m13	CACGACGTTGTAAAACGAC		

*SSR, simple sequence repeats; SCAR, sequence characterized amplified region.

**Physical position was obtained by BLAST analysis using the genome sequence data (http://brassicadb.org/brad/blastPage.php)

3.3.4 Phenotyping and Genotyping

A total of 896 segregating BC_5F_3 (Table S3.1-experiment 1) and 128 self-pollinated (37-7-5-3, NJ13b, Hn7 and Hn10 and Westar - experiment 2) plants were cotyledon inoculated with a set of *L. maculans* isolates (87-41, 3-42-6, 09 Stonewall, Pl03-02-01, PG4-1-M) (Table S3.2), except isolate Pl03-02-01 was not used in the NIL testing (experiment 2). About 80 to 96 plants were individually cotyledons inoculated per isolate per segregating population in experiment 1 (Table S3.1) while four plants were inoculated per isolate per line in experiment 2. At the seedling stage, phenotypic data was collected by observing the cotyledon lesion development on a scale of 0-9 as described by Williams & Delwiche (1979), after 14-day inoculation (dai). At the adult plant stage, stem canker severity symptoms were scored based on physiological maturity at the ripening stage after all the pods were formed but the stems were still green, by cutting at the base of the stem and evaluating the level of blackening using a 0 - 5 scale as recommended by the

Canola Council of Canada; 0 – no disease, 1 – up to 25% of the cross-section, 2 - 26-50% of the cross-section, 3 - 51-75% of the cross-section, 4 - >75 of the cross-section with some seed production, 5 - 100% of the cross-section with no seed production, tissue dry and brittle and plant dead. Stem crown canker data were converted to a standard 0-9 scale (Delourme et al. 2008b) and the mean disease severity indices were calculated as described by Pilet et al., (1998). DSI = [(N0*0) + (N1*1) + (N2*3) + (N3*5) + (N4*7) + (N5*9)]/N_t, where N0,1,…5 is equal to the total number of scored plants for each classification and N_t is the total number of plants assessed. Genotypic information was collected using co-dominant SRAP and SSR molecular markers flanking the *BLMR2* locus (Long et al. 2011). The sub-populations with heterozygous *BLMR2/blmr2* alleles showed heterozygous dominant and those with only *blmr2* allele showed homozygous susceptible genotypes; the homozygous *BLMR2* NILs showed a homozygous resistant genotype (Table 3.2).

Table 3. 2 Genotypes of near isogenic lines and their R gene donor parent Surpass 400 and recurrent parent Westar

		Molecular marker				
Materials	Generation	12D09a	N10-56	N10-45	N10-40	N10-37
Surpass 400	Donor parent	A	A	A	A	A
37-7-5-3	BC_2F_4	a	A	A	A	A
37-7-5-4	BC_2F_4	a	A	A	A	A
NJ13b	BC_3F_4	a	A	A	A	a
HN7	BC_5F_4	a	a	A	a	a
HN10	BC_5F_4	a	a	A	a	a
Westar	Recurrent parent	a	a	a	a	a

3.3.5 Experimental setup in controlled growth chamber and greenhouse conditions

Seeds were planted in 96-well trays filled with premix soil and kept in controlled growth chamber (14 hr light at 20^{o}C and 10 hr dark at 18^{o}C). The soil filled trays were watered thoroughly before planting and every other day after emergence. A water soluble 20-20-20 (N-P-K) fertilizer was applied before planting and two to three times after planting at seedling and rosette stages. In about 7 days after planting, plants were pierced using forceps, one on each cotyledon, two wounds per plant, and inoculated with 10 µl of spore suspension (Long et al., 2011). The plants were kept at room temperature overnight and put back in the growth chamber (14 hr light at 20^{o}C and 10 hr dark at 18^{o}C). The first two true leaves were trimmed to facilitate

the progress of infection into the stems. The inoculated seedlings were later transplanted to pots at rosette stage and kept in greenhouses until physiological maturity (Marcroft et al. 2012).

The experiment was arranged in a split-plot design with a set of isolates (87-41, 3-42-6, 09 Stonewall, Pl03-02-01, PG4-1-M) (Table S3.2) as main-plot and a series of BC segregating populations (Table S3.1) were randomly allocated to the sub-plots within each of the main-plots. The population is sub-divided into heterozygous resistant (*BLMR2/blmr2* alleles) and susceptible (*blmr2* allele) groups, based on flanking molecular markers (Long et al. 2011). The experiment was repeated twice (spring 2015 – GH-1 and fall 2015 – GH-2) (Table S3.1) and each experiment was considered as complete blocks.

Four homozygous *BLMR2* NILs (37-7-5-3, NJ13b, Hn7 and Hn10) and Westar were also tested in two sets of experiment (Winter 2016 – GH-3 and Winter 2017 – GH-4) to validate the data collected from the backcrossing populations. Data was collected from four plants per each experimental unit (sub-plot) at the seedling and adult plant stages as mentioned above. The experiment was conducted in split-plot as above but only four isolates (87-41, 3-42-6, 09 Stonewall, PG4-1-M) (Table S3.2) were considered as main-plot and the five genotypes as sub-plots.

3.3.6 Experimental setup and evaluation of blackleg resistance under field conditions

A total of seven genotypes; three *BLMR2* NILs, one *BLMR1* NIL, one *Rlm2* NIL, Surpass 400 and Westar were evaluated in the blackleg nursery at Carman, Manitoba for three years (2014-2016). The nursery was infested with blackleg pathogen from previous season's stubble which comprised of well characterized isolates to their *Avr/avr* profile that represent the most important races from commercial fields. These isolates carry the virulent genes *avrLm*1, 2, 3, 4, 6, 7 and 9 in various combinations (from Dr. Fernando's lab). Each genotype was grown in a single one-meter length row and about 0.5 grams of seeds were sown in each row. Distance between adjacent rows was 0.25m and between plots (incomplete blocks) is 0.5m. The experiments were arranged in an alpha-lattice design in two replicates. Each replicate consisted of six incomplete blocks and 4 (2014), 6 (2015) and 7 (2016) rows per block. The susceptible check was arranged at each corner rows and the test genotypes were put in the middle rows. In all testing rows, 15 to

20 plants were uprooted to score crown canker severity at the physiological maturity on a scale of 0-5 as mentioned above.

3.3.7 Statistical analysis

The controlled environment experiments were analyzed as split-plot with isolates as main-plot, genotype as sub-plot and two experiments as complete blocks. A combined variance analysis was also conducted on repeated randomized complete block (RCB) experiments (GH-1 and GH-2 in the controlled experiments and three-year field experiment) with isolates nested within an experiment. Experiment could be environment or year, and we assume that genotype is fixed and experiments and blocks are random effects. Furthermore, to study the correlation of seedling and adult plant resistances, repeated measures analysis of a RCB was conducted with isolates as blocks; assuming genotype is fixed and blocks are random effects. Least significant (Ls) means were used to estimate the differences among genotypes. All the data were analyzed using the proc mixed in SAS program (University Edition).

3.4 Results

3.4.1 Seedling and adult plant resistance phenotypes in BLMR2/blmr2 alleles

The number of plants evaluated at BC_5F_3 generation in two experiments is summarized in (Table S3.1). *BLMR2* allele displayed an intermediate resistance reaction at seedling stage when inoculated with a set of *L. maculans* isolates as compared to their *blmr2* allele counterparts (Figure 3.1). Combined ANOVA (Analysis of variance) of the two experiments conducted in the spring 2015 (GH-1) and fall 2015 (GH-2) predicted nonsignificant difference between the two experiments (environment) at seedling and adult plant stages (P=0.6211 and P=0.4068, respectively) (Table S3.3). As expected, significant differences were observed between the two genotypes with *BLMR2* and *blmr2* alleles at both seedling and adult plant stages (P=0.0001 and P<.0001, respectively) (Table S3.3). When comparing the two genotypes, *BLMR2* containing genotypes exhibited higher disease resistance phenotypes at the seedling (mean DSI=4.6$\pm$0.4) and adult (mean DSI=3.4$\pm$0.5) stages than genotypes with the *blmr2* allele (mean DSI=7.2$\pm$0.4 and 7.4$\pm$0.5, respectively) (Table 3.3) when inoculated with five *L. maculans* isolates (87-41, 3-

42-6, 09 Stonewall, Pl03-02-01 and PG4-1-M) (Table S3.2). On the other hand, non-significant differences were observed among the isolates (P=0.1075 and P=0.3261) (Table S3.4) at seedling and adult stages, respectively. Significant differences were observed between genotypes (seedling and adult stages) and genotype by isolate interaction (adult stage, P=0.0465) (Table S3.4). Repeated measure analysis indicated non-significant differences (P=0.3520) between the two stages (seedling vs adult) but difference (P<.0001) between the two genotypes (*BLMR2* vs *blmr2*) (Table S3.5). These results demonstrated that there was positive relationship between intermediate cotyledon resistance and adult plant resistance of genotypes with the *BLMR2* alleles under controlled conditions.

Table 3. 3 Ls mean Disease Severity Index (DSI, 0-9) estimates of heterozygous BLMR2/blmr2 and susceptible blmr2 when inoculated with five isolates.

Label	Df	Seedling (Mean±SE)	Adult (Mean±SE)
BLMR2/blmr2	8	4.6±0.4	3.4±0.5
blmr2	8	7.2±0.4	7.4±0.5

The five isolates include: 1=PG4-1-M, 2=Pl03-02-01, 3=09Stonewall, 4=3-42-6, 5=87-41 (Table S3.2).

Figure 3. 1 Comparison of cotyledon disease phenotypes of BLMR2 and BLMR1 containing lines when inoculated with a set of isolates. The parents Westar (susceptible) and Surpass 400 (resistant) were also included as controls

3.4.2 Disease response phenotype of homozygous NILs containing BLMR2 at seedling and adult stages

To further confirm the resistant disease response phenotype of *BLMR2* allele, four homozygous NILs were compared with the susceptible recurrent parent Westar (*blmr2*) in two experiments (GH-3 and GH-4). Combined ANOVA indicated non-significant differences (P=0.7981 and P=0.2608) between the experiments (environment) but difference among genotypes (P<.0001 and P<.0001) at seedling and adult stages, respectively (Table S3.6). The *BLMR2* containing NILs as a group induced significantly (P<.0001) low DSI, with estimated mean DSI of 3.0±0.3, as compared to Westar (*blmr2*, mean DSI=6.4±0.4) at seedling stage (Table 3.4). Similarly, differences (P<.0001) were observed between the NILs (*BLMR2*) and Westar (*blmr2*) at the adult plant stage with the mean stem canker severity index of 1.9±0.5 and 8.4±0.6, respectively (Table 3.4).

Table 3. 4 Ls mean Disease Severity Index (DSI, 0-9) estimates of homozygous BLMR2 NILs and Westar (blmr2) when inoculated with four isolates.

Label	Df	Seedling Mean±SE	Adult Mean±SE
BLMR2	24	3.0±0.3	1.9±0.5
blmr2	24	6.4±0.4	8.4±0.6

The mean *BLMR2* data was pooled from the four homozygous NILs (37-7-5-3, NJ13b, Hn7 and Hn10). The four isolates include: 1=PG4-1-M, 3=09Stonewall, 4=3-42-6, 5=87-41 (Table S3.2).

3.4.3 Adult plant resistance verification of homozygous NILs containing BLMR2 under field conditions

Adult plant resistance of three homozygous NILs containing *BLMR2* and controls including the two parents (Surpass 400 and Westar), and NILs containing *BLMR1* and *RLM2* were evaluated for three years (2014-2016) under field conditions. Combined ANOVA indicated significant variations in mean DSI among genotypes (P<.0001) (Table S3.7). Moreover, significant variations were also observed for year (P=0.0208) and genotype by year interaction (P=0.0020) (Table S3.7). Pair-wise comparisons of the three years on average revealed that DSI in 2016 were significantly different from 2014 and 2015 (P=0.0123 and P=0.0316, respectively) (Table S3.7). However, no significant difference (P=0.2069) (Table S3.7) was observed between 2014 and 2015. It was possible that late planting and environmental conditions in 2016 might have contributed to the reduction in disease severity estimates as compared to the previous two years. Combined Ls mean estimates of the first two years indicated that the genotypes with the *BLMR2* allele could be categorized as resistant (R) or intermediate resistant (MR) phenotypes, whereas the lines 15C-92 and G4A14-2, with known resistant genes *BLMR1* and *RLM2*, respectively, were surprisingly categorized as susceptible (S) together with the susceptible parent Westar (Table 3.5). This demonstrated the high APR potential of *BLMR2* allele under field conditions infested with the most dominant races of the pathogen in western Canada. Looking at the year 2016, all the *BLMR2* containing lines were classified as R and the lines with *BLMR1* (15C-92) and *Rlm2* (G4A36-1) were classified as MR and MS, respectively (5).

Overall, the analysis of APR in the field in the three years showed similar trend of reduced stem crown canker severity with genotypes carrying *BLMR2* allele. The other two genotypes with the

known R genes (*BLMR1* and *Rlm2*) performed relatively poor, with higher DSI in 2014 and 2015; and lower DSI values in 2016, where the mean disease severity index was relatively lower on Westar compared to the previous two years probably due to the late planting and/or environmental conditions.

Table 3. 5 Lsmean estimates for Disease Severity Index (DSI, 0-9) of B. napus homozygous NILs and controls evaluated at adult plant stage in Carman blackleg nursery

Entry	Type	Resistant allele	(2014 and 2015)		2016	
			Estimate (mean±SE)	Phenotypic classes	Estimate (mean±SE)	Phenotypic classes
Westar	Check	-	7.5±0.5	S	6.5±0.7	S
15-92-11	NIL	BLMR1	7.7±0.5	S	2.6±0.7	MR
G4A36-1	NIL	RLM2	6.7±0.5	S	3.9±0.7	MS
NJ13b	NIL	BLMR2	2.4±0.5	MR	0.7±0.7	R
37-7-5-4	NIL	BLMR2	2.0±0.5	R	0.8±0.7	R
37-7-5-3	NIL	BLMR2	1.2±0.5	R	1.3±0.7	R
Surpass	Check	BLMR1/2	0.6±0.5	R	0.3±0.7	R

Glacier and Surpass 400 are the resistance donor parents for the NIL G4A36-1 and all the other NILs, respectively. Westar was used as a susceptible (recurrent) parent in all crosses. The NILs with *BLMR2* include 37-7-5-3 and 37-7-5-4 (BC2F4), and NJ13b (BC4F4). Blackleg disease severity ratings could be categorized into four phenotypic classes in comparison to Westar; 0-29.9% of Westar (Resistant-R), 30-49.9% of Westar (Intermediate Resistant – MR), 50-69.9% of Westar (Intermediate Susceptible -MS) and >70% of Westar (Susceptible – S).

3.4 Discussion

3.4.1 BLMR2 consistently displayed intermediate resistance to multiple isolates

The current genetic studies in the advanced BC generation (BC_5F_3) as confirmed by molecular markers (Long et al. 2011) showed consistent 1:1 segregation pattern of heterozygous resistant (*BLMR2*/*blmr2*) and susceptible (*blmr2*), confirming the stability of this locus. Moreover, we investigated the intermediate cotyledon disease reaction phenotype (Figure 3.1) of the *BLMR2* containing genotype (Hn7) when cotyledon inoculated with a range of *L. maculans* isolates (Table S3.2) in comparison with the *BLMR1* containing genotype and the resistant and susceptible parents as controls. The cotyledon disease reaction phenotype of *BLMR2* containing genotypes was intermediate; although minor differences were observed in the degree of lesion

development, suggesting partial effectiveness against all isolates tested (Delourme et al. 2006, 2008b). The genotypes containing *BLMR1* induced a typical hypersensitive response to isolate 87-41 (Long et al. 2011; Larkan et al. 2013), but intermediate phenotypic reaction was also observed with the isolate 3-42-1, and plants exhibiting a susceptible phenotype similar to Westar were observed when challenged with the virulent isolates 03-15-03, 09Stonewall and PG4-1-M (Table S3.2). The susceptible cultivar Westar displayed complete tissue collapse with prolific pycnidiospores, whereas cultivar Surpass 400 showed a range of high disease reaction (necrotic) phenotypes to all the five isolates tested, possibly due to the presence of at least the two resistance loci, *LepR3* (Larkan et al., 2013) and *BLMR2*.

3.4.2 Intermediate resistance at the cotyledon stage correspond to adult plant resistance

Numerous studies suggested the reliability of the cotyledon inoculation method as a means to predict APR after achieving significant correlation between seedling and adult plant resistances in the greenhouse and field conditions (McNabb et al. 1993; Bansal et al. 1994; Li and Cowling 2003; Delourme et al. 2004). However, others argued that seedling and APR are not necessarily correlated and distinct genetic factors control these mechanisms (Crouch et al. 1994; Pang and Halloran 1996a; Mayerhofer et al. 1997; Delourme et al. 2006; Rimmer 2006; Raman et al. 2012b, 2016a; Elliott et al. 2016). Generally, correlation of controlled and field experiments is dependent on the frequency of the *L. maculans* race structure in a disease nursery (Rouxel et al. 2003; Larkan et al. 2016a), which could be complex (various *Avr* genes in multiple combinations) and location-specific (Li et al. 2005; Sprague et al. 2006; Liban et al. 2016; Zhang et al. 2016). For instance, Australian field isolates were reported to be more diverse and virulent than probably anywhere in the world (Li et al. 2005; Sprague et al. 2006; Delourme et al. 2008a; Raman et al. 2012b, 2016a). In our study, the plants in the advanced BC generations were individually inoculated with a set of *L. maculans* isolates but the data were analyzed together to give a better insight on the potential of the genotypes with *BLMR2* alleles. Upon cotyledon inoculation with a number of contrasting isolates, the genotypes with *BLMR2* alleles resulted in an intermediate DSI score, which was expected since the lines with APR are supposed to be partially effective against all isolates (Delourme et al. 2008b). Interestingly, this correlated with partially resistant stem canker severity. Bansal et al. (1994) also observed correlated but

relatively lower disease severity percentages at adult plant stage than at the cotyledon stage. Our results were also verified in the field.

Partial resistance is generally a characteristic of quantitative resistance (QR) controlled by polygenes, often associated with APR (Pilet et al. 1998; Delourme et al. 2004, 2006, 2008b; Rimmer 2006; Huang et al. 2014; Larkan et al. 2016a), which implies that there could be tightly linked minor genes in this locus with a cumulative resistance effect. Polygenic resistance may not induce strong hypersensitive reaction at seedling stage (Li et al. 2008b), but significantly reduces stem canker severity at adult plant stage (Delourme et al. 2004; Li et al. 2004; Travadon et al. 2009). In our study, cotyledon inoculation of cultivar Surpass 400 with the isolate 87-41 (*AvrLm1*) displayed a seemingly hypersensitive response with a small necrotic lesion at the wound site while inoculations with isolates such as 03-15-03 or PG4-1-M (non-*AvrLm1*) showed a larger necrotic lesion. This is consistent with the finding by Dilmaghani et al. (2009), where Jet Neuf showed similar phenotypes with isolates containing *AvrLm4* and non-*ArLm4* alleles, respectively. Similarly, Li et al. (2004) observed an atypical reaction using a cytological study, where significantly reduced but not totally arrested hyphal growth were evident in the cotyledon of Surpass 400 as compared to a susceptible cultivar. Overall, the limited hyphal growth in Surpass 400 (Li et al. 2004) and the consistently larger necrotic lesion reactions in Surpass 400 and Jet Neuf (Dilmaghani et al. 2009), respectively, could be associated with non-specific quantitative resistance in the studied pathosystems. Surpass 400 perhaps has some APR background as proposed in other studies (Li et al. 2004, 2005; Sprague et al. 2006). If so, Surpass 400 has both single dominant gene *LepR3* (Larkan et al. 2013) and polygenic (*BLMR2*) resistance, and *BLMR2* partly explains the atypical interactions in Surpass 400. *BLMR2* introgression into a susceptible cultivar is potentially a good method to initiate research to understand mechanisms behind QR without interference of dominant R genes or to determine the genes underlying APR in Surpass 400. Our finding supports the hypothesis that the hypersensitive response is not the only mechanism explaining the interaction between Surpass 400 and *L. maculans* isolates (Li et al. 2008b).

3.4.3 Potential of BLMR2 locus in western Canada

This research demonstrates that *BLMR2* may have potential in western Canada to be used solely or in combination with other resistant genes. Li et al. (2005) proposed the use of cultivars with only polygenic resistance could potentially provide an adequate level of resistance compared to defeated major resistance genes. The observed difference in the derived series of NILs in the *BLMR2* region may attribute to the difference in the introgressed segments within this locus (unpublished) and may facilitate other research that may focus on understanding the molecular basis underlying the *BLMR2* locus.

In this study, *BLMR2* contributed to APR in canola against the blackleg disease. One could argue why then Surpass 400 is defeated in some parts of Australia. Our explanation agrees with what is described by Van de Wouw et al. (2014) who explained that the *L. maculans* population in isolated areas in Southern Australia led to the ineffectiveness of resistance in a cultivar due to the probable shift in pathogen population structure when the cultivar was grown on a large-scale. This situation exerted pressure on pathogen population to evolve. Furthermore, cultivars in Australia are grown throughout the entire year with milder winter conditions. This provides a longer period of time for the pathogen to systematically grow from leaves to stems (Travadon et al. 2009). This is followed by warm temperatures in spring/summer that favours pathogen multiplication and sexual recombination (West et al. 2001; Huang et al. 2006; Sprague et al. 2006). Also, Australian isolates are considered to be extremely diverse, so it is possible that several isolates could even break the resistance in cultivars with only polygenic resistance (Li et al. 2005, 2008a). Li et al. (2008a) observed an array of resistance expression in cultivars with only polygenic resistance because of the different *L. maculans* races prevalent across the test sites in that study. Thus, polygenic resistance could operate in a non-*Avr* manner, but the degree of expression could vary across different locations and possibly increases the durability of cultivars.

3.4.3 Resistance breakdown and durability

In agreement with the predictions by Kutcher et al. (2010) and Liban et al. (2016) that a change in population structure has occurred in western Canada. Isolates belonging to PG2 were the most dominant ones in western Canada until the early 2000s, then the more aggressive PG3, PGT

(Chen and Fernando, 2006; Kutcher et al. 2007) and PG4 isolates started to emerge in most parts of western Canada though the frequency was not high (Chen and Fernando 2006; Rimmer 2006). Conversely, there was scarce information on the identity of R genes utilized in western Canada. A very recent study revealed the prevalence of *Rlm3* in most Canadian cultivars and also reported the defeat of *Rlm3* due to the lack of *AvrLm3* in the local *L. maculans* population (Zhang et al. 2016). Our study specifically compared *BLMR1*, *BLMR2* and *Rlm2* genes solely introgressed into a common Westar background. Surprisingly, *Rlm2* was one of the potential R genes predicted to still be effective by inferring the frequency of the corresponding *AvrLm2* in the recent past (Liban et al. 2016; Zhang et al. 2016). However, the current study suggests the possible defeat of *Rlm2* or the potential to be defeated in growers' field.

The breakdown of *Rlm3* was less dramatic as compared to the situation reported in other continents, where the cultivars were rendered ineffective in a few years after their commercially release (Li et al. 2003; Rouxel et al. 2003). *Rlm3* containing cultivars were believed to be integral part of the Canadian cultivars at least since the early 1990's (Zhang et al. 2016) and hypothesized that the longevity of *Rlm3* is probably associated with its introgression into a cultivar with APR background. Similarly, Brun et al. (2010) suggested the prominent role of QR background to increase the durability of dominant R genes. Kutcher et al. (2010) reported about 97% of Canadian isolates were *Avr* (avirulent) on Surpass 400. In our study, Surpass 400 showed a high level of stem canker resistance along with most *BLMR2* containing NILs for three consecutive years (2014-2016) in a blackleg nursery in Carman, Manitoba. The effectiveness of Surpass 400 could be explained by the stable performance of *BLMR2* locus regardless of the breakdown of *BLMR1* in western Canada.

In this chapter, the phenotypic performance of the B. napus resistance BLMR2 locus containing NILs along with the susceptible Westar and other controls were evaluated at seedling and adult plant stages. We verified the potential of this locus to confer horizontal resistance (HR) under field conditions. The next chapter deals with the identification of a candidate gene(s) underlying HR phenotype using fine-mapping and genomic approaches.

4. Genetic Analysis of a Horizontal Resistance Locus *BLMR2* in *Brassica napus*

Qiang Zhang†, Hanna Dandena†, Madison McCausland, Huizhi Liu, Zheng Liu, Wen Xu, and Genyi Li*

Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada

* Correspondence:
Genyi Li
genyi.li@ad.umanitoba.ca
†These authors have contributed equally to this work and share first authorship

4.1 Abstract

Leptosphaeria maculans causes blackleg disease in *Brassica napus*. The blackleg disease is mainly controlled by resistance genes in *B. napus*. Previous studies have shown that the blackleg resistant *BLMR2* locus that conferred horizontal resistance (HR) under field conditions, is located on chromosome A10 of *B. napus*. The purpose of this study is to fine map this locus and hence identify a candidate gene underlying HR. The spectrum of resistance to *L. maculans* isolates of the resistance locus *BLMR2* was analyzed using near isogenic lines, resistant and susceptible cultivars. The results showed that this locus was horizontally resistant to all the isolates tested. Sequence characterized amplified polymorphism (SCAR) and simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers were developed in the chromosome region of *BLMR2* and a fine genetic map was constructed. Two molecular markers narrowed *BLMR2* in a 53.37 kb region where seven genes were annotated. Among the seven annotated genes, *BnaA10g11280D / BnaA10g11290D* encoding a cytochrome P450 protein were predicted as the candidate of *BLMR2*. Based on the profiling of pathogen induced transcriptomes, three of the seven annotated genes were expressed while only cytochrome *P450* showed upregulation. The candidate corresponds to the gene involved in the indole glucosinolate biosynthetic pathway and plant basal defense in the *Arabidopsis thaliana*. The molecular markers identified in this study will allow the quick incorporation of the *BLMR2* allele in rapeseed cultivars to enhance blackleg resistance.

Keywords: *Arabidopsis thaliana, Brassica napus, Leptosphaeria maculans, BLMR2,* fine mapping, cytochrome P450, glucosinolate

4.2 Introduction

Brassica napus (rapeseed / canola) is an important crop used for edible oil production worldwide. Blackleg, caused by *Leptosphaeria maculans*, is one of the most devastating diseases in rapeseed production. There are both qualitative and quantitative types of resistance to fungal pathogens like *L. maculans* (Raman et al., 2012b, 2013). Qualitative resistance is race-specific and depends on the presence of a single resistance (R) gene in plant interacting with matching avirulence (*Avr*) genes in pathogen (Zhang and Fernando 2017). In contrast, quantitative (QR) or horizontal resistance is race nonspecific, which may be mediated by several genes, expressed from the seedling to adult plant stages, conferring only partial resistance to all races of the pathogen (Delourme et al., 2006; González et al., 2012). Identification and incorporation of resistance genes, in Brassica species, to produce resistant cultivars is an efficient approach to combat blackleg disease in *B. napus* (Hayward et al. 2012).

To date, over a dozen loci including *Rlm1-10*, *LepR1* to *LepR4*, *BLMR1* and *BLMR2* conferring resistance to *L. maculans* have been mapped in the cultivated Brassica species (Delourme et al. 2006; Rimmer 2006; Yu et al. 2005, 2008, 2013; Long et al. 2011; Larkan et al., 2013). However, the effectiveness of some of these resistance genes is decreased after they are used in production for three years, and the genes need to be replaced with other novel genes (Kutcher et al. 2007; Li et al.2003; Rouxel et al. 2003). For example, *LepR3* in Surpass 400 that provides race-specific resistance to the fungal pathogen *L. maculans* suffered a major defeat in Australia in 2004 (Li et al. 2003; Sprague et al. 2006). On the other hand, quantitative resistance likely remains effective over time than qualitative resistance (Zhang et al. 2016). Therefore, it is important and necessary to identify and combine qualitative and quantitative genes for durable blackleg resistance (Huang et al. 2018).

It is very difficult to identify the genes controlling quantitative trait loci in the complex genome of *B. napus* because little information is available on the genetic control and mechanism underlying quantitative resistance to *L. maculans*. Unlike minor effect loci, major effect loci could be Mendelized to fine-map and eventually clone the gene underlying QR. In the previous study, the blackleg quantitative resistance gene *BLMR2,* originally derived from the cross between Surpass 400 and Westar showed horizontal resistance under field conditions (Dandena et al., 2019). A few blackleg resistance genes including *Rlm2*, *LepR3*, *BLMR2* and *LepR2* have

been mapped on *B. napus* chromosome A10. Among these the highly resistant *Rlm2* and *LepR3* are allelic and function as receptors (Larkan et al., 2013, 2014, 2015). Characterization of the *BLMR2* and *LepR2* need further studies although these genes are likely different from the other R genes based on the map position and phenotype (Long et al., 2011; Larkan et al., 2016b). *BLMR2* segregated as a single dominant allele and has a distinctly intermediate phenotype when cotyledon inoculated with the *L. maculans* isolate 87-41 (Long et al., 2011). This allowed the development of NILs (W+BLMR2) using molecular marker assisted backcrossing along with progeny testing of recombinants.

In this study, *BLMR2* was fine mapped in *B. napus* with the use of sequence characterized amplified polymorphism (SCAR) and simple sequence repeats (SSR) markers. Furthermore, inoculation assay with individual isolates showed that *BLMR2* has race non-specific resistance to all the *L. maculans* isolates tested. These results lay a foundation for utilizing the blackleg resistance *BLMR2* allele in developing resistant *B. napus* cultivars that effectively control *L. maculans*.

4.3 Materials and Methods

4.3.1 Materials for testing horizontal resistance

Three near isogenic lines containing resistance *BLMR1*, *BLMR2* and *Rlm2* alleles were developed using the two resistant cultivars Surpass 400 and Glacier, and a susceptible cultivar Westar. *BLMR1* and *BLMR2* were derived from the cross between Surpass 400 and Westar (Long et al. 2011) while *Rlm2* was derived from the cross between Glacier and Westar. *BLMR1* and *BLMR2* were separated at BC1F3 based on differential phenotypic interaction with the *L. maculans* isolate 87-41 and molecular marker data (Long et al., 2011). From crosses of Surpass 400 x Westar and Glacier x Westar, F3 and F1 progenies, respectively, were backcrossed to Westar four to five times and molecular marker assisted selection (MAS) was implemented during backcrossing (Dandena et al 2019). Individuals containing homozygous *BLMR1*, *BLMR2* or *Rlm2* alleles were selected based on molecular markers and inoculation assays to obtain three isogenic lines in Westar background, named as W+BLMR1 (BC_4F_4), W+BLMR2 (BC_4F_4) and W+Rlm2 (BC_4F_2), respectively. The near isogenic lines, together with two resistant cultivars Glacier and Quinta, and the susceptible Westar were used to perform interaction analysis.

4.3.2 Mapping population

The BC_1F_3 plants carrying *BLMR2/blmr2* alleles from the cross of *B. napus* cultivar Surpass 400 and Westar were backcrossed to Westar *(blmr2/blmr2)* to produce BC_2, BC_3 and BC_4 populations and the BC_4 was selfed to obtain BC_4F_4. All plants used in backcrossing and selfing were phenotyped through inoculation and genotyped using the flanking molecular markers. The BC_4F_3 and BC_4F_4 were inoculated with *L. maculans* isolate 87-41 to test cotyledon resistance. Segregation ratios of resistant to susceptible individuals in the F_4 and BC_4 were analyzed with $\chi 2$ test of goodness of fit. A total of 5,952 BC_3 individuals were used to fine map the resistance locus.

4.3.3 Pathological Assay

To test the horizontal and race-non-specific resistance of *BLMR2*, 24 *L. maculans* isolates were used in cotyledon assays. These pathogen isolates were selected from the collection at the University of Manitoba. The inoculum of all isolates was prepared, and cotyledons were wounded and inoculated as described previously (Long et al. 2011). Disease reactions were rated at 12–16 days after inoculation according to the classification of 0–9 (Chen and Fernando 2006).

4.3.5 DNA extraction and genome specific marker development

A modified CTAB method as described by Li and Quiros (2001) was used to extract DNA. The *B. rapa* genomic sequence (http://brassicadb.org/brad/) was used to identify SSR loci and primers covering SSR were designed to amplify the specific loci in *B. napus.* Sequence characterized amplified region (SCAR) loci were developed using a similar procedure as SSR markers except specific targets were sequenced from the two parental lines (Surpass 400 and Westar). The A genome-specific primers were used to amplify PCR templates to identify insertions/deletions of the targets which were used to develop SCAR markers.

4.3.6 Detection of SCAR and SSR

A set of five fluorescent dye 6-FAM, VIC, NED, PET and LIZ was used to detect signals with an ABI 3100xl Genetic Analyzer (ThermoFisher Scientific, Toronto, Canada). The LIZ color was the standard and the other four were used to label primers. For SSR and SCAR detection, the genome-specific primers were used to obtain PCR products containing SSR or deletion/insertion positions. A 10 µl PCR mixture was prepared as follows: two genome-specific primers and one

labeled M13 primer, 50 ng of genomic DNA, 0.375 mM dNTP, 1X PCR buffer, 1.5 mM $MgCl_2$ and 1 U of Taq polymerase. The PCR running program was 94℃, 3 min; 94℃, 1 min; 58℃ with -0.8℃ each cycle 1 min and 72℃, 1 min for 5 cycles; 94℃, 1 min; 57℃, 1 min and 72℃, 1 min for 25 cycles. The amplified PCR products were separated in the ABI 3100 Genetic analyzer. The data was analyzed with ABI GenScan and Genographer software.

4.3.7 RNA extraction and sequencing of the candidate gene

Fully expanded cotyledons were inoculated using *L. maculans* isolate 87-41 (inoculated) or water (mock). Four days after inoculation, cotyledon samples from eight individual plants per sample were pooled and ground in liquid nitrogen. Total RNA was extracted using fresh cotyledon tissues and Trizol reagent (ThermoFisher Scientific, Toronto, Canada). RNA quality was determined using 1% agarose gel and Nanodrop (Thermo Fisher Scientific, Toronto, Canada). cDNA was synthesized from the total RNA using the SuperScript™ III kit following manufacturer's protocol (ThermoFisher Scientific, Toronto, Canada). Then, primers (Table S4.1) were used to amply the full-length cDNA of the candidate gene of *BLMR2* and cloned into the TA cloning vector with the TOPO® TA Cloning® Kit (ThermoFisher Scientific, Toronto, Canada). Positive clones were selected to extract plasmid DNA using the standard mini preparation protocol. The plasmid DNA was sequenced using the BigDye™ terminator v3.1 cycle sequencing kit (ThermoFisher Scientific, Toronto, Canada). Full length cDNA sequence was assembled using SeqMan software.

4.3.8 Quantitative analysis of candidate gene expression

Total RNA was extracted from cotyledon samples of W+BLMR2 and Westar inoculated with *L. maculans* isolate 87-41 and mock-inoculated at 4 days after inoculation (dai). RNA quality was determined using 1% agarose gel and Nanodrop (Thermo Fisher Scientific, Toronto, Canada). cDNA was synthesized using the SuperScript IV First-Strand Synthesis kit (Thermo Fisher Scientific, Toronto, Canada). Quantitative real-time PCR (qRT-PCR) was conducted using SYBR Green Master Mix (Bio-Rad) according to manufacturer's instruction (Bio-Rad). The P450 gene specific primers were used to amplify the gene (Table S4.1). The actin gene (BnaA01g19850D) was used as a control for normalization. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

4.4 Results

4.4.1 Resistance to L. maculans isolates

Three near isogenic lines W+BLMR1, W+BLMR2 and W+Rlm2, along with Wester, Glacier and Quinta were inoculated with 24 isolates. The results showed that only W+BLMR2 was resistant to all tested isolates, indicating that the resistance of *BLMR2* was horizontal. *BLMR1* and *Rlm2* showed differential interactions with the tested isolates and the two resistant cultivars Glacier and Quinta also showed different resistant spectra while Westar was susceptible to all the isolates (Table 4.1).

4.4.2 Segregation of BLMR2 in the mapping populations

In the mapping populations of *BLMR2*, 656 F_2 individuals were used to confirm the segregation ratio of *BLMR2*. There were 513 resistant plants and 143 susceptible plants showing a 3:1 segregation ratio ($\chi 2$ test, $p>0.05$). In the BC_1 mapping population of 831 individuals, there were 438 resistant plants and 393 susceptible plants showing a 1:1 segregation ratio ($\chi 2$ test, $p>0.05$), suggesting that one dominant resistance allele is responsible for the *BLMR2* resistance to blackleg (Figure 4.1).

*Table 4. 1 Testing of resistance spectra of three near isogenic lines and three cultivars in Brassica napus using 24 isolates of Leptosphaeria maculans**

Isolates	W+BLMR2	W+BLMR1	W+Rlm2	Glacier	Quinta	Westar
87-1	R	S	S	S	S	S
M4-1	R	S	S	S	S	S
M4-2	R	S	S	S	S	S
10Aridries-dk	R	S	S	S	R	S
3-12-01	R	S	R	S	R	S
08-01-05	R	S	R	S	R	S
9STONEWALL	R	S	R	S	R	S
3-15-03	R	S	R	R	R	S
6NBW-01	R	S	R	R	R	S
89-3	R	S	R	R	R	S
ND04-05-01	R	S	R	R	R	S
86-12	R	S	R	R	R	S
4-09-109	R	R	S	R	S	S
7-02-01	R	R	S	R	S	S
53-31	R	R	S	R	S	S
Lifolle	R	R	S	R	S	S
PL03-02-01	R	R	S	R	S	S
PL03-42-06	R	R	S	R	S	S
3-54-01	R	R	S	R	R	S
4-09-01	R	R	S	R	R	S
3-01-Roland	R	R	S	R	R	S
4-09-107	R	R	R	R	R	S
87-41	R	R	R	R	R	S
Lifolle 5	R	R	R	S	R	S

*All the isolates are from the collection at the University of Manitoba; three near isogenic lines W+BLMR1, W+BLMR2, W+Rlm2, two resistant cultivars Glacier and Quinta, and susceptible cultivar Westar; R represents 0-4 ratings, S represents 5-9 ratings.

4.4.3 Fine mapping of the resistance gene

Sequence characterized amplified region (SCAR) and simple sequence repeats (SSR) markers were used to map *BLMR2* on chromosome A10. Using 1,632 BC_4 plants, six recombinant individuals were obtained. Their selfed progeny was inoculated with *L. maculans* isolate 87-41 to confirm the phenotype of recombinants. Then, more sequence-based SCAR and SSR were developed to fine map the *BLMR2* locus. Another 4,320 BC_4 individuals were inoculated with *L. maculans* isolate 87-41 to identify another 14 recombinants. Using all 20 recombinants, *BLMR2* was narrowed in a small region between molecular markers N10-47 and N10-43 after their phenotypes and genotypes were analyzed (Table 4.2, Figure 4.3). Selfed progeny of all recombinants was also tested to confirm the recombination events in the BC_4F_4.

*Figure 4. 1 Cotyledons of Surpass 400, near isogenic line W+BLMR2 and Westar, 14 days after inoculation (dai) of the pycnidiospore suspension (2 x 10^7 spores/ml) of the Leptosphaeria maculans isolate 87-41 (AvrLm1, AvrLm2, ...). Surpass 400 showing high cotyledon resistance **(A)**; W+BLMR2 showing intermediate resistance **(B)**; and Westar with the susceptible phenotype **(C)**.*

*Table 4. 2 Phenotypes and genotypes of 20 recombinants in the BC_3 population of Brassica napus**

Recombinant	Phenotype	N10a	N10-56	N10-47	N10b	N10-45	N10-43	N10-39	N10-34	N10-37	N10-38	N10-40
RC01	R	A	A	AB	AB	AB	AB	AB	AB	AB	AB	AB
RC02	R	A	A	AB	AB	AB	AB	AB	AB	AB	AB	AB
RC03	R	A	A	AB	AB	AB	AB	AB	AB	AB	AB	AB
RC04	R	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
RC05	R	AB	AB	AB	AB	AB	AB	AB	AB	A	A	A
RC06	R	AB	AB	AB	AB	AB	AB	AB	AB	A	A	A
RC07	R	AB	AB	AB	AB	AB	AB	AB	AB	A	A	A
RC08	R	AB	AB	AB	AB	AB	AB	AB	AB	A	A	A
RC09	S	A	A	A	A	A	AB	AB	AB	AB	AB	AB
RC10	S	A	A	A	A	A	A	AB	AB	AB	AB	AB
RC11	S	A	A	A	A	A	A	A	AB	AB	AB	AB
RC12	S	A	A	A	A	A	A	A	AB	AB	AB	AB
RC13	S	A	A	A	A	A	A	A	AB	AB	AB	AB
RC14	S	A	A	A	A	A	A	A	A	AB	AB	AB
RC15	S	A	A	A	A	A	A	A	A	AB	AB	AB
RC16	S	A	A	A	A	A	A	A	A	AB	AB	AB
RC17	S	A	A	A	A	A	A	A	A	AB	AB	AB
RC18	S	A	A	A	A	A	A	A	A	A	AB	AB
RC19	S	AB	AB	AB	A	A	A	A	A	A	A	A
RC20	S	AB	AB	AB	A	A	A	A	A	A	A	A

*R, resistant; S, susceptible phenotype; A, genotype of Westar; B, genotype of *BLMR2* in Surpass 400; AB, heterozygous genotypes.

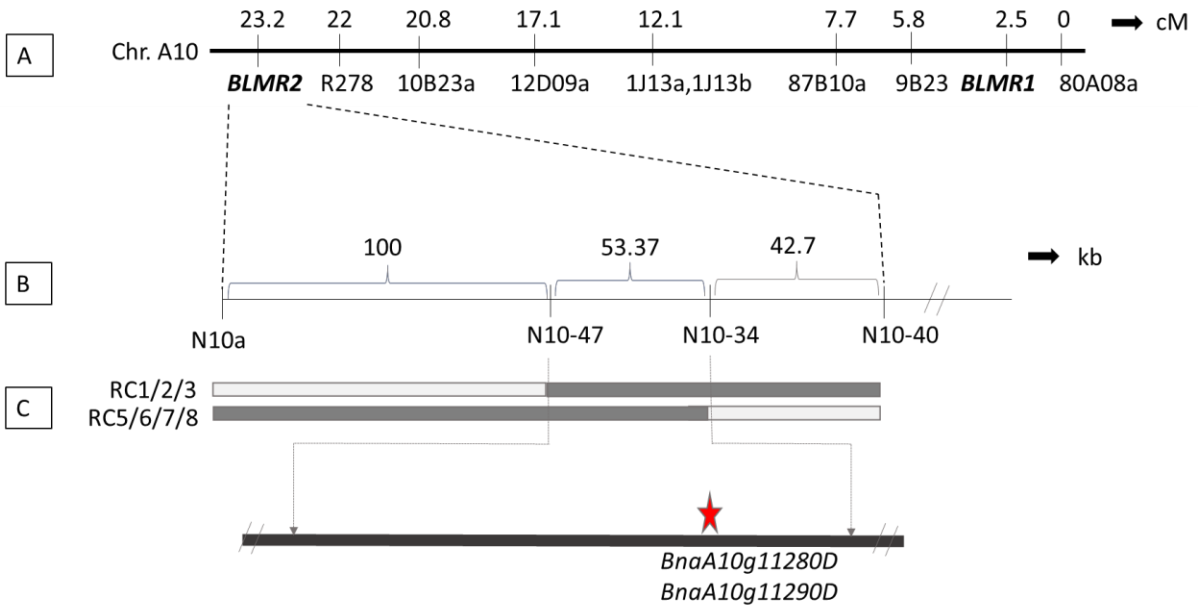


Figure 4. 3 Genetic and physical map of BLMR2 locus on chromosome A10 of Brassica napus. Genetic map showing BLMR1 and BLMR2 (Long et al. 2011, A). The flanking SSR and SCAR markers used in the fine mapping of BLMR2 (B). Molecular marker data of the recombinants with resistant phenotype showing overlapping BLMR2 region corresponding to 53.37kb physical interval. The candidate BLMR2 gene is indicated (C)

4.4.2 The CYP81F2 predicted as a candidate gene

Taking together all the twenty recombinants with the resistant and susceptible phenotypes into consideration, the *BLMR2* locus is located between the flanking molecular markers N10-47 and N10-43, corresponding to the chromosome region, spanning ~53.37 kb, from 9494993 to 9548367 in the reference genome sequence of *B. napus* A10 (www.genoscope.cns.fr),. In the reference sequence, seven genes are annotated. Among the seven genes, only five of them showed collinearity with the genes in the homologous *Arabidopsis thaliana* (Table 4.3). RNA-seq data was analyzed to compare the expression of the gene sets in the fine mapped region of *BLMR2* and the results showed that three of the six genes were expressed while only the candidate gene *CYP450* was upregulated (Chapter 5).

Table 4. 3 Gene annotation in the fine mapped BLMR2 locus of chromosome A10 of B. napus.

Chromosome Location	Brassica napus gene	Arabidopsis gene homologue	Description
9502886-9505822	BnaA10g11240D	AT5G57200	Putative clathrin assembly protein At5g57200
9511632-9512150	BnaA10g11250D	-	-
9518045-9521092	BnaA10g11260D	AT5G57210	Ypt/Rab-GAP domain of gyp1p superfamily protein
9522169-9522706	BnaA10g11270D	-	-
9535710-9537490	BnaA10g11280D	AT5G57220	cytochrome P450, family 81, subfamily F, polypeptide 2
9537760-9539609	BnaA10g11290D	AT5G57220	cytochrome P450, family 81, subfamily F, polypeptide 2
9543736-9545286	BnaA10g11300D	AT5G57230	Thioredoxin superfamily protein

4.4.3 cDNA sequencing and analysis of B. napus CYP81F2 gene structure

Full length cDNA of the candidate gene was sequenced from both the near isogenic line W+BLMR2 and Westar (Figure 4.2, Table S4.2). Aligning sequences of the two genes *BnaA10g11280D* and *BnaA10g11290D* from the reference sequence (www.genoscope.cns.fr) with the cDNA sequence, allowed us to re-annotate these genes into one *Bncyp81F2* gene homologous to *Atcyp81F2* (Figures S4.2, S4.3). The two genes seemed to be split into two during the assembly of the whole reference genome. The annotated *BnaA10g11280D* gene is truncated and the annotated *BnaA10g11290D* gene lacks the upstream sequence. Also, comparison of the cDNA sequences from the resistant (*BLMR2*) and susceptible (*blmr2*) alleles showed that there are six polymorphisms in the first and third exons, however, only one of these six nucleotide changes in the first exon is associated with a difference of amino acid (AA) (Figure 4.2; Figure S4.1).

>Westar

ATGGATTACATTTTGCTCTTATTGCCACTCGTATTGTTTCTACTAGCTTACAAATTCTTATTCTCATCTA
AGAGTTTCAATCTTCCACCAGGACCAACTCCCTTTCCCATCGTCGGCAACCTCCACCTCGTGAAACCAC
CGGTGCACCGTCTCTTCCGTCGTTTCGCGGACAAGTACGGTGACATCTTCTCCCTCCGTTACGGCTCTC
GCCAAGTCGTCGTGATCTCTTCCTTGCCCCTCGTCAGAGAATGCTTTACTGGTCAGAACGACGTTATTT
TAACGAACCGACCGCATTTTCTGACCGCAAAGTACGTTGCTTACGACTACACCACGGTTGGAACCGCC
GCATATGGCGACCACTGGCGTAATCTCCGCCGTATTTGCTCTCTTGAGATCCTTTCCTCTAACCGTCTC
ACTGGATTCCTCTCCGTTCGTAAAGACGAGATCCGACGGTTGCTCACGAAACTCTCACGTGACTATAAT
GGCCAAGTCGTTGAGCTTGAGCCTCTTCTTGCAGATTTGACGTTCAATAATATTGTCCGTATGGTCACT
GGGAGACGTTACTACGGAGACCAGGTTCACAACAAGGAAGAAGCGAACCTATTCAAGAAGCTAGTGA
CGCAGATCAACGACAATAGTGGTGCGAGCCATCCAGGAGATTATTTACCAATTCTCAAAGTTTTCGGA
CACGGCTACGAGAAGAAAGTGAAAGCACTCGGCGAAGCCATGGACACTTTCTTGCAGCGACTGCTCG
ACGATTGCCGTAGAGATGGAGAGAGCAACACAATGCTTAGTCATCTGTTGTCTTTACAAGTAGACCAA
CCCAAGTATTACAGTGACGTCATCATCAAAGGCCTCATGCTCAGTATGATGCTTGCGGGGACGGATAC
TGCAGCCGTGACACTAGAATGGGCGATGGCGAGTTTGTTGAAAAGTCCTGAAGTGTTGAAGAAGGCG
AAAGCCGAGATAGATGATAAGATTGGACATGAACGTTTGGTCGACGAACCGGACATTTTGAATCTCCC
TTATCTCCAAAACATAGTTTCTGAGACCTTCCGACTGTGTCCAGCCGCACCACTCCTTGTACCACGTTC
TCCTTCTGAAGACCTCAAGATTGGCGGATACGACATACCGCGTGGCACCATCGTACTAGTGAATTCTT
GGGCCATCCATAGAGATCCAAGGCTTTGGGATGAGCCTGAGAGGTTCATGCCAGAGCGGTTTGAGGAC
AAAGAAGCTGCCAATAATAATAAGCTTATGATGTTTGGGAACGGACGAAGGACGTGTCCCGGTGCGG
CTTTGGGTCAAAGGATGGTGTCGTTGGCTTTAGGATCGTTGATTCAATGCTTTGACTGGGAAAAAGTCA
ACGGTGAGGAAATTGATATGACCGAAAATCCTGGAATGGCTATGCGCAAGCTCGTGCCGTTACGAGCC
GTTTGCCATCAGCGTCCCATTATGACTAATCTTTTGGCTTAA

>W+BLMR2

ATGGATTACATTTTGCTCTTATTGCCACTCGTATTGTTTCTACTAGCTTACAAATTCTTATTCTCATCTA
AGAGTTTCAATCTTCCACCAGGACCAACTCCCTTTCCCATCGTCGGCAACCTCCACCTCGTGAAACCAC
CGGTGCACCGTCTCTTCCGTCGTTTCGCGGAGAAGTACGGTGACATCTTCTCCCTCCGTTACGGCTCTC
GCCAAGTCGTCGTGATCTCTTCCTTGCCCCTCGTCAGAGAATGCTTTACTGGTCAGAACGACGTTATTT
TAACGAACCGACCGCATTTTCTGACCGCAAAGTACGTTGCTTACGACTACACCACGGTTGGAACCGCC
GCATATGGCGACCACTGGCGTAATCTCCGCCGTATTTGCTCTCTTGAGATCCTTTCCTCTAACCGTCTC
ACTGGATTCCTCTCCGTTCGTAAAGACGAGATCCGACGGTTGCTCACGAAACTCTCACGTGACTATAAT
GGCCAAGTCGTTGAGCTTGAGCCTCTTCTTGCAGATTTGACGTTCAATAATATTGTCCGTATGGTCACT
GGGAGACGTTACTACGGAGACCAGGTTCACAACAAGGAAGAAGCGAACCTATTCAAGAAGCTAGTGA
CGCAGATCAACGACAATAGTGGTGCGAGCCATCCAGGAGATTATTTACCAATTCTCAAAGTTTTCGGA
CACGGCTACGAGAAGAAAGTGAAAGCACTCGGCGAAGCCATGGACACTTTCTTGCAGCGACTGCTCG
ACGATTGCCGTAGAGATGGAGAGAGCAACACAATGCTTAGTCATCTGTTGTCTTTACAAGTAGACCAA
CCCAAGTATTACAGTGACGTCATCATCAAAGGCCTCATGCTCAGTATGATGCTTGCGGGGACGGATAC
TGCAGCCGTGACACTAGAATGGGCGATGGCGAGTTTGTTGAAAAGTCCTGAAGTGTTGAAGAAGGCG
AAAGCCGAGATAGATGATAAGATTGGACATGAACGTTTGGTCGACGAACCAGACATTTTGAATCTCCC
TTATCTCCAAAACATAGTTTCCGAGACCTTCCGACTGTGTCCAGCCGCACCACTCCTTGTCCCACGTTC
TCCTTCTGAAGACCTCAAGATTGGCGGATACGACATACCGCGTGGCACCATCGTACTAGTGAATTCTT
GGGCCATCCATAGAGATCCAAGGCTTTGGGATGAGCCTGAGAGGTTCATGCCAGAGCGGTTTGAGGAC
AAAGAAGCTGCCAATAATAATAAGCTTATGATGTTTGGGAACGGACGAAGGACGTGTCCCGGTGCGG
CTTTGGGTCAGAGGATGGTGTCGTTGGCTTTAGGATCGTTGATTCAATGCTTTGACTGGGAAAAAGTCA
ACGGTGAGGAAATTGATATGACCGAAAATCCTGGAATGGCTATGCGTAAGCTCGTGCCGTTACGAGCC
GTTTGCCATCAGCGTCCCATTATGACTAATCTTTTGGCTTAA

Figure 4. 2 cDNA sequences of the resistant W+BLMR2 and susceptible Westar (576 bases – exon1; 288 bases – exon2; 612 bases – exon3); sequence variations in exons 1 and 3 (red)

4.4.4 Analysis of expression of the candidate gene by qPCR.

Quantitative RT-PCR was used to determine the relative gene expression of the candidate gene in the resistant W+BLMR2 and susceptible Westar. The relative fold change in the accumulation of *Brassica napus CYP450* transcript was significantly higher in the pathogen (*L. maculans* isolate 87-41) inoculated resistant W+BLMR2 at 4dai compared to pathogen inoculated Westar or mock checks (Figure 4.4). Our results suggest that *CYP450* possibly plays a role in *L. maculans* induced defense response in *B. napus*.

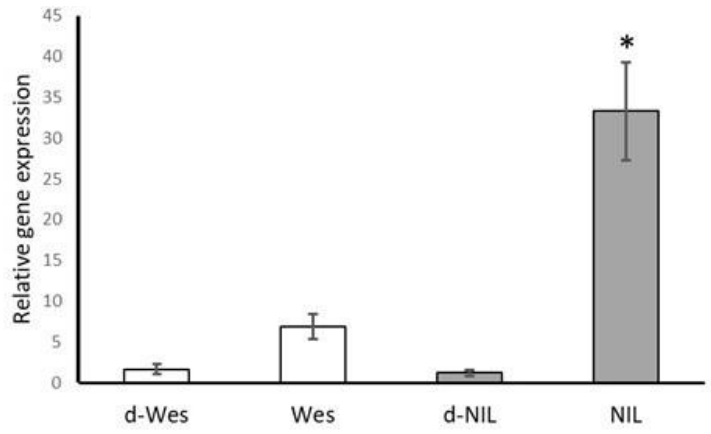


Figure 4. 4 Validation of the expression of Brassica napus CYP81F2 gene in the resistant W+BLMR2 (NIL) and susceptible Westar using qRT-PCR at 4 days after inoculation (dai) with the Leptosphaeria maculans (Lm) isolate 87-41. Samples d-Wes and d-NIL are mock and Wes and NIL are Lm inoculated. The relative gene expression value is normalized using the Actin gene (BnaA01g19850D). Error bar shows standard deviation of the mean based on three biological replicates. The asterisk (*) represents significant difference from all the other samples based on Tukey test, P<0.05.

4.5 Discussion

It is hypothesized that quantitative resistance genes work by a complex interaction of many response genes to pathogens, and any individual gene does not show strong effect, therefore there is less selective pressure on the fungus (Zhu et al. 1993; Delourme et al. 2008). However, a quantitative resistance locus with major effect, at adult plant stage, may be established as a major gene; and may be fine mapped and eventually cloned. Identification of such genes will facilitate the transfer and pyramiding of multiple resistance genes with different resistance spectra through molecular marker- assisted selection in *B. napus* for durable blackleg resistance. In this paper, the cotyledon assays of *BLMR2* to a range of isolates of *L. maculans* were analyzed. The results showed that *BLMR2* is a horizontal resistance locus to all the isolates of the blackleg pathogen, compared to typical R genes and resistant cultivars. In the previous study, the results in field trial showed that *BLMR2* conferred resistance to blackleg under field conditions, suggesting that this resistance gene is very useful to the breeding of resistant cultivars in *B. napus* (Dandena et al 2019).

In this study, *BLMR2* was fine mapped in a 56.37 kb region where nine genes were annotated. Of these nine genes, two genes that are reannotated into a single gene were identified as the candidate gene of *BLMR2* based on the analysis of gene expression. The candidate is homologous to *CYP81F2* in *Arabidopsis thaliana*. Several studies showed that *CYP81F2* catalyzes the modification of indole glucosinolate (IGS) which involves the accumulation of defensive secondary metabolite (Mithen et al. 1987; Abdel-farid et al. 2010; Wiesner et al. 2013). In Arabidopsis, the hydrolytic products of IGS mediated by myrosinases (*PEN2* and *PEN3*) are involved in innate immune response to pathogens (Bednarek et al. 2009; Clay et al. 2009). The *CYP81F2* and *PEN2* dependent hydrolytic products were also associated with callose deposition in *FLG22*-triggered basal immunity (Bednarek et al. 2009; Clay et al. 2009). While it remains elusive whether genes involved in the glucosinolate biosynthesis are linked to defense response induced by *L. maculans* in *B. napus*, a complex pattern of IGS accumulation in *B. rapa – L. maculans* interaction was observed (Abdel-farid et al. 2010). Furthermore, Robin et al. (2017) associated upregulation of *CYP81F2 (Bol026044)* gene in the moderate blackleg resistant cabbage cultivar with increased indole glucosinolate accumulation at the seedling stage. Recent global transcriptomic studies identified multiple genes involved in the IGS biosynthesis in *B. napus – L. maculans* incompatible interaction (Becker et al. 2017; Zhou et al. 2019). However,

the mechanism underlying horizontal resistance is rarely reported, and hence the finding in this study suggests that secondary metabolic pathways such as the biosynthetic pathway of IGS play a role in plant resistance to various diseases. However, how the change of amino acid in the DNA sequence confers the horizontal resistance, in this study, need further investigation.

Plant disease resistance is classified in many ways, vertical vs horizontal, qualitative vs quantitative, race-specific vs race-nonspecific, a high vs an intermediate level of resistance. Typical R genes such as NBS-LRR, receptor like proteins (RLP) and receptor like kinases (RLK) have been cloned since their phenotypes can be easily observed (Larkan et al. 2013). For some diseases such as sclerotinia in rapeseed, sunflower and soybean and fusarium head blight in cereal crops, no vertical, qualitative or race-specific resistance has been identified, so no typical R gene in these pathosystems has been cloned (Behla et al. 2017; Mesterhazy 2020). The focus of this study is investigation of the horizontal resistance*BLMR2* since it conferred resistance under field conditions (Dandena et al. 2019). Several characteristics of the resistance of the *BLMR2* locus were addressed. First, its resistance is race-nonspecific and intermediate while the resistance is dominant and resistant in all heterozygous genotypes tested. Second, single allele of this locus confers relatively strong resistance, so the phenotypes of this locus were relatively easy to be scored. The accurate scores of phenotypes made it possible to perform fine mapping and narrow the locus into a small chromosome region. Third, this kind of resistance is assumed to be controlled by polygenes and very difficult to be transferred from cultivar to cultivar while the *BLMR2* locus is genetically qualitative though the phenotype is measured in quantitative terms. *BLMR2* can be easily transferred using molecular markers targeting the six mutations in the DNA sequence. Finally, unlike all previous reports where horizontal resistance is hypothesized to have a minor effect of one locus in resistance controlled by multiple loci (Tian et al. 2006; Skowrońska et al. 2020), the resistance locus *BLMR2* confers a relative strong effect so the near isogenic lines showed the level of resistance which can meet the standard of blackleg resistance in Canada field trials (Canola Council of Canada).

Conclusion

The availability of accurate phenotypes and a large population aided in the precise mapping of the *BLMR2* locus. With a combination of *BLMR2* fine-mapping, molecular marker assisted development of NILs, and comparative physical mapping, the candidate gene for *BLMR2* was

identified as a homolog of *CYP81F2 (At5g57220)* in Arabidopsis. Markers identified in this study can be used to transfer this horizontal resistance from cultivar to cultivar using MAS or gene pyramiding for resistance durability.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

QZ, HD and GL designed the experiment and wrote the manuscript. QZ and HD finished most experiments. MM, HL, ZL and WX collected data of some experiments.

Funding

This research is supported by the Natural Sciences and Engineering Research Council (NSERC) CRD project (CRDPJ 523840 – 18).

Acknowledgments

We would like to acknowledge Dr. Dilantha Fernando who offered most isolates used in this study.

In Chapter 4, molecular markers assisted in narrowing down the BLMR2 region, hence we predicted a candidate gene(s). In chapter 5, RNA-seq technology was used to further validate the quantitative resistance of BLMR2 allele compared to the susceptible blmr2 and typical qualitative resistance (BLMR1/RLM1).

5. Identification of upregulated genes and pathways in the *Brassica napus BLMR2*-mediated horizontal resistance to blackleg infection

Hanna Dandena, Keval Shah, Tengsheng Zhou, Wen Xu, and Genyi Li

5.1 Abstract

Brassica napus is a host plant to the hemibiotrophic fungal pathogen, *Leptosphaeria maculans*. In this study, the horizontal resistance (HR) W+BLMR2 and the susceptible Westar were sequenced (RNA-Seq) at four and eight/ten days after inoculation (dai). The aim was to identify the key genes and pathways that underpin HR in *B. napus* to *L. maculans* infection. Results showed that upregulated genes at 4 dai are involved in biotic stress, signalling and regulation of transcription pathways while at 8/10dai, there were more downregulated genes in the photosynthetic, protein, cell, and amino acid metabolic pathways. Putative gene families involved in biotic stress pathway such as genes that belong to the family chitinases were fully activated while RLKs and WRKYs were partially activated in the resistant W+BLMR2 at 4 dai. On the other hand, the genes involved in the photosynthetic pathway such as genes in the PSI, PSII and Rubisco were downregulated at the whole-genome scale in the susceptible Westar at 8/10 dai. Moreover, comparison of the transcriptome profiles of a typical R-mediated resistance and HR showed equivalent but lower induction of key genes in the HR, compared to the susceptible Westar. Together, we conclude that *BLMR2* can activate the defense pathway to *L. maculans* and can be combined with R genes to maximize *L. maculans* resistance in canola breeding. However, the dramatic change of gene expression in the susceptible Westar at the late stage do not correspond to resistance, rather to the susceptibility in infected phenotypes.

Keywords: *Brassica napus*, *BLMR2*, differentially expressed genes (DEGs), horizontal resistance, *Leptosphaeria maculans*, RNA-Seq, transcriptome

5.2 Introduction

Canola (*B. napus*) is the second largest oilseed crop after soybean, cultivated in various places throughout the world (Carré and Pouzet 2014). The hemibiotrophic fungal pathogen *L. maculans* attacks *B. napus,* leading to blackleg infection, which can be very devastating in terms of yield loss (Huang et al. 2016) and quality reduction; contaminated seeds also pose a threat to trade (Zhang and Fernando 2017; Fernando et al. 2016). Fortunately, there are resistance mechanisms in Brassica crops to this devastating fungal pathogen including the well-studied R-mediated resistance and horizontal resistance. Several major resistance (R) genes associated with high level of resistance have been mapped and three of them were cloned (Larkan et al. 2013, 2015; Raman et al. 2013, 2016a). The R genes function as extracellular receptors (RLP) or membrane bound WAKL (Larkan et al. 2013, 2015,2020). On the other hand, there is little information on the genetic control and mechanism underlying horizontal resistance (HR). The phenotype of HR cannot be classified into discrete classes of high and low levels of resistance at seedling stage. It rather displays a spectrum of partial resistance to all races of a pathogen (Corwin and Kliebenstein, 2017). Singe resistance genes with incomplete resistance at adult plant stage have been characterized in rice, wheat, and barley pathosystems (Niks et al. 2015). Candidate genes underlying HR were associated with downstream signalling and defense genes. A recent field study suggested the potential of the *B. napus BLMR2* locus controlling HR to blackleg infection (Dandena et al. 2019). Combining R and HR was suggested as an approach to ensure durable resistance (Huang et al. 2018). Nevertheless, no single gene controlling HR has been functionally characterized in *B. napus-Lm pathosystem*. Near isogenic lines (NILs) containing *BLMR2* in a susceptible Westar background could assist in the study of the genetic and molecular basis of HR in *B. napus*.

Plants activate their defense systems with the plethora of pattern recognition receptors (PRRs), including receptor-like kinases and receptor-like proteins (Li et al. 2016). As the first line of defense, plants use receptors (PRRs) against the more generalized and highly conserved PAMPs, which is called PTI or basal resistance. If this basal resistance is compromised plants launch the second line of defense called effector triggered immunity (ETI) or R-mediated resistance. In ETI, R proteins are activated upon interaction with specific Avr (avirulent) proteins or pathogen

effectors, which often is accompanied by a hypersensitive reaction at the infection site. This signifies the limitation of further infection by the pathogen (Becker et al. 2019). However, R-mediated resistance could easily be eroded by loss-of-function mutations of the pathogen (Li et al. 2016; Noman and Aqeel 2019). Stotz et al (2014) coined another term for R-mediated resistance against apoplastic pathogens called effector triggered defense (ETD). ETD is slower than ETI due to the time lapse between the endophytic and biotrophic phases of the pathogen (Becker et al. 2019).

As an apoplastic fungus, *L. maculans* resistance in *B. napus* is mediated by the extracellular R proteins (receptors), that interact with coreceptors, for downstream signalling and defense responses (Stotz et al. 2014). Defense response is a costly affair as plants divert energy that is needed for growth and development into defending pathogens (Couto and Zipfel 2016). Also, some biosynthetic genes involved in defense pathways such as phytohormones play a role in other physiological processes (Nolan et al 2020). Thus, plants need to undertake a sophisticated regulatory mechanism to balance the different physiological processes (Lu and Yao 2018). Global transcriptome (RNA-seq) profiling is a method of choice, to recognize the differential expression of genes, predict protein functions and regulatory networks in *B. napus* and *L. maculans* pathosystems (Haddadi et al. 2016, 2019; Becker et al. 2017,2019; Zhou et al. 2019). However, most of the transcriptome studies in the past are on R-mediated resistance, comparing compatible and incompatible interactions; and studies on HR is lagging. In the previous chapter, BLMR2 is fine-mapped in ~56.4kb region. Seven genes homologous to the collinear *Arabidopsis thaliana* genes are annotated in this region. The W+BLMR2 is a NIL containing *BLMR2* in a Westar background. In this study, we combined fine-mapping and RNA-seq to validate the upregulated gene in the *BLMR2* region. Also, we explored the overall transcriptomic profiles of *BLMR2*, representing HR and *LepR3*, representing R-mediated resistance. The knowledge is useful to better utilize R and HR mediated resistance in plant breeding.

5.2 Materials and Methods

5.2.1 Plant and fungal material

The horizontal resistant near isogenic line (NIL) W+BLMR2 and the susceptible recurrent parent Westar were used in this study. W+BLMR2 contains *BLMR2* in an otherwise Westar genetic background (chapter 4).

Inoculation tests were preformed using the *L. maculans* isolate 87-41 (Yu et al. 2008). The pycnidiospores of *L. maculans* were maintained in Westar inoculated cotyledons. Inoculum is prepared by plating infected cotyledons in V8 containing agar media for about a week at room temperature and the fully grown pycnidiospores were collected and kept as stock at -20^{o}C. A final concentration of 10 µl of $2*10^{7}$ spores /ml suspension was used for inoculation (Long et al. 2011).

Plants were grown in flats filled with a premix soil and kept moist in the growth chamber at 21^{o}C for 16 hr and 18^{o}C for 8 hr, light and dark cycles, respectively. Cotyledons of seven-day old seedlings were punctured for inoculation tests using *L maculans* or mock treatments.

5.2.2 Sampling, RNA isolation, cDNA preparation and Illumina sequencing

Tissue samples were collected at 4, 8 and 10 days after inoculation (dai) by combining whole cotyledons from 8 seedlings per replication and kept frozen in liquid nitrogen and/or at -80^{o}C freezer, until processed. Three replications per treatment group (4 and 8/10) were considered for downstream analysis except mock_8/10 (Table S5.1). Total RNA was extracted from frozen plant samples using the TRIZOL reagent. RNA quality was checked by bioanalyzer 2100 for RNA integrity number (>6.5). Library construction using the Illumina TruSeq mRNA stranded Library Preparation and Illumina HiSeq v4-Paired End 125 bp sequencing was performed at Genome Quebec Innovation Center, McGill University.

5.2.3 Transcriptome data mapping, gene expression analysis and functional annotation

RNA-Seq samples were trimmed with Trimmomatic version - 0.36 to remove adapters (Bolger et al., 2014). HISAT2 software was used to map reads to *B. napus* (Brassica_napus_v4.1.chromosomes.fa.gz) with default parameters. The mapped reads were quantified by bedtools (2.30.0 version) with default parameters.

The count data was transformed into regularized log and variance stabilizing datasets for PCA and cluster analysis. Euclidean distance was used to classify the samples into clusters.

The differential expression (DE) analysis was performed using the edge R-quasi pipeline with the threshold Log2FC≥1 and FDR≤0.05 (Chen et al. 2016). Raw reads (Count data) were converted to counts per million (CPM) to adjust for library size differences. Also, normalization by TMM was performed to adjust for gene composition. Genes with low count reads based on expressed genes were filtered out from the analysis. About 30% of the 101 041 genes were considered for downstream analysis at early and late stages of infection.

Differential expression (DE) analysis and heatmaps were generated using the Bioconductor packages of the R software (R version 3.5.2).

5.2.4 Functional classification of DEGs by MAPMAN and gene family

The differentially expressed genes (DEGs) were assigned to MapMan functional bins (Brassica_napus.annotation-V5.cds.fa.gz). The assigned functional bins were matched with the bioinformatically annotated *B. napus* mapping file in Dr. Li. Lab for analysis of the DEGs by gene family.

5.3 Results

5.3.1 Phenotypic assessment of L. maculans infection

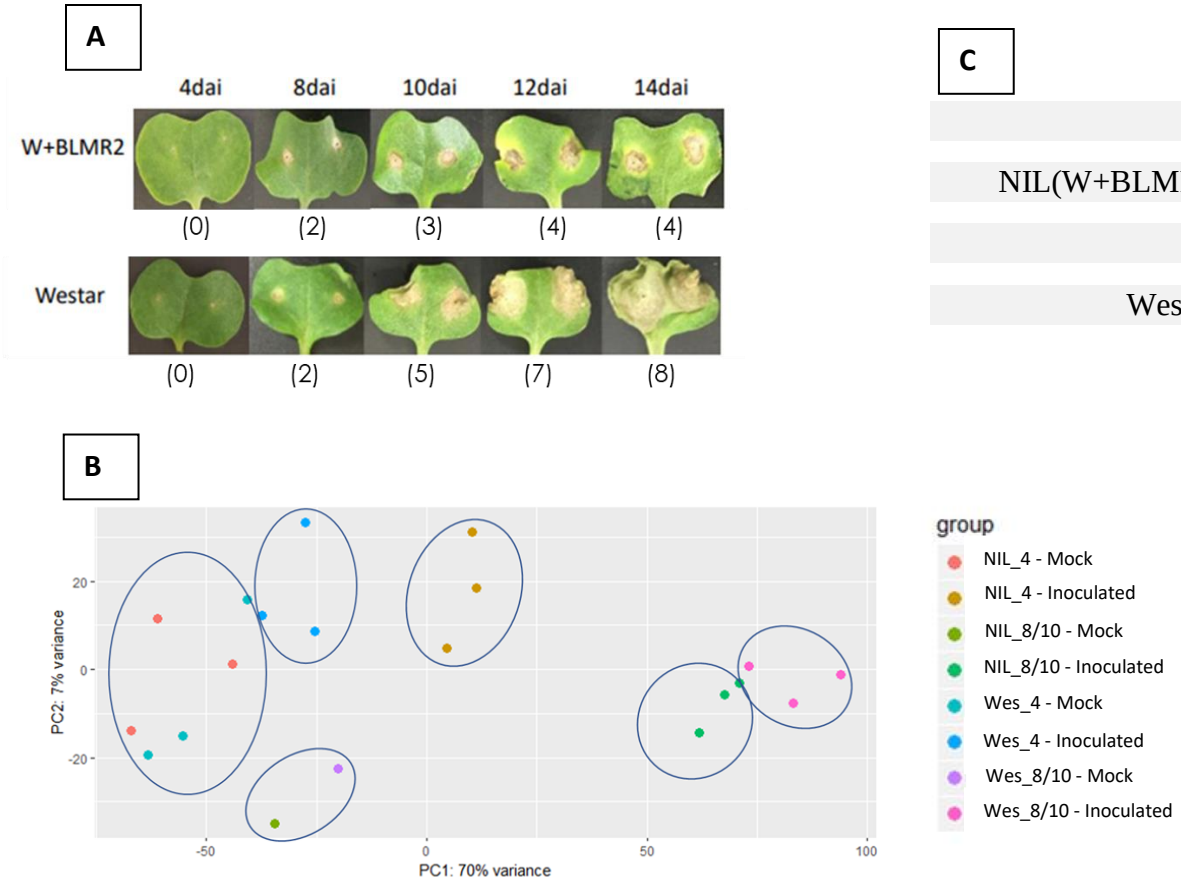
The phenotypic response to *L. maculans* infection in the *B. napus* W+BLMR2 showed horizontal resistance at seedling stage compared to Westar and other control genotypes (Chapter 4). In this study, the *L. maculans* isolate 87-41 *(AvrLm1, AvrLm2, AvrLm4-7, AvrLm6, AvrLm11, AvrLmJ1)* was selected as it appears to be less virulent on Westar (Dandena et al. 2019; Zhou et al. 2019). As shown in the figure below, no visible symptoms were observed in both W+BLMR2 and Westar at 4dai. Lesions started to appear at 8 dai and continued to progress rapidly in Westar. The disease progress was much slower in W+BLMR2 compared to Westar (Figure 5.1a).

5.3.2 Gene expression at the early and late stages of infection

Two rounds of RNA-Seq were performed. Initially, a few inoculated and mock samples were collected at 4, 8 and 10dai from W+BLMR2 and Westar to determine the ideal time of sampling (Table S5.1). Preliminary data analysis suggested that the transcriptomic profiles of samples collected at 8 and 10 dai, especially in Westar, were significantly distorted. Therefore, the second

round of RNA-seq was performed using three biological replications of inoculated and mock samples in W+BLMR2 and Westar at 4 dai. The samples collected at 8/10 dai were included in the analysis for comparison purposes Together, 20 samples were subjected to PCA and hierarchical clustering to observe the relationship among samples and replicates (Figure 5.1b; Figure S5.1). The PCA shows that samples clustered by treatment except Westar-inoculated at 4dai, which clustered close by mock samples. The inoculated samples also clustered by time-point. Since the late stage (8 and10 dai) clustered together, the sample groups, NIL_8/10 and Wes_8/10 were pooled by genotype (Figure 5.1b; Figure S5.1).

Analysis of the relative change in gene expression was based on *L. maculans* inoculated vs mock samples. In total, 1,811 and 5,316 genes were induced in the resistant W+BLMR2 at early and late stages, while 254 and 12,664 genes were induced at early and late stages in Westar (Figures 5.1c, 5.2a, 5.3a).



C

	Early-4dai	Late-8/10dai
NIL(W+BLMR2)	1,811	5,316
Westar	254	12,664

Figure 5. 1 Phenotypic and transcriptomic evaluation of Leptosphaeria maculans inoculated Brassica napus lines. Phenotypic disease development in L. maculans isolate 87-41 (AvrLm1, AvrLm2 ...) infected cotyledons of W+BLMR2 and Westar at 4,8,10,12 and 14dai. The scores, in bracket, show disease severity index (DSI) on a scale of 0-9 ***(A),*** *PCA shows groupings of inoculated or mock samples (NIL = W+BLMR2, Westar) at early (4dai) and late (8/10dai) stages of infection* ***(B),*** *Differentially expressed genes in the resistant W+BLMR2 and susceptible Westar at 4 and 8/12dai based on log2FC≥/1/ and FDR≤0.05* ***(C).***

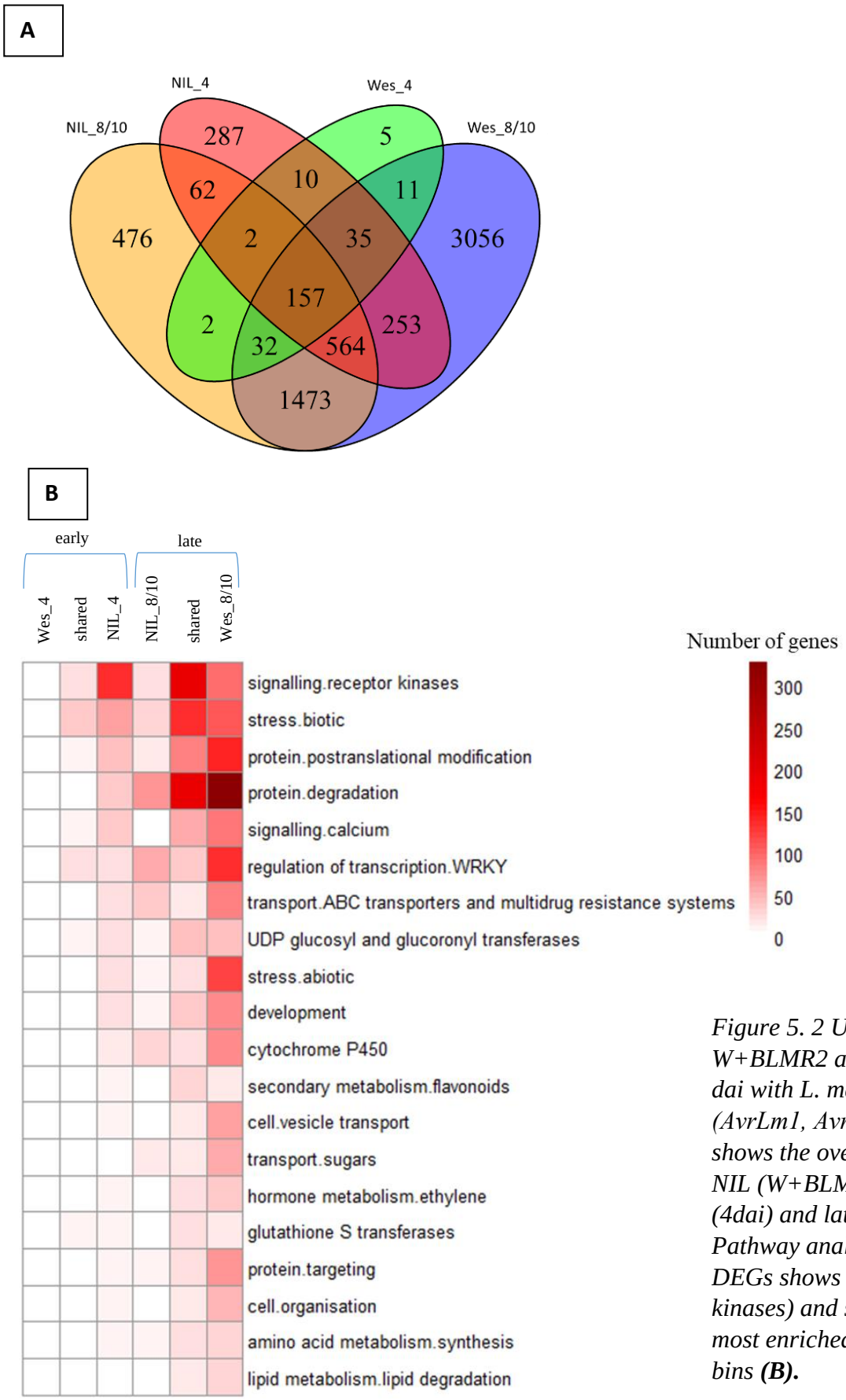


Figure 5. 2 Upregulated DEGs in W+BLMR2 and Westar at 4 and 8/10 dai with L. maculans isolate 87-41 (AvrLm1, AvrLm2…). Venn diagram shows the overlapping genes in the NIL (W+BLMR2) and Westar at early (4dai) and late (8/10dai) stages ***(A),*** *Pathway analysis of upregulated DEGs shows signalling (receptor kinases) and stress (biotic) as the most enriched MAPMAN functional bins* ***(B).***

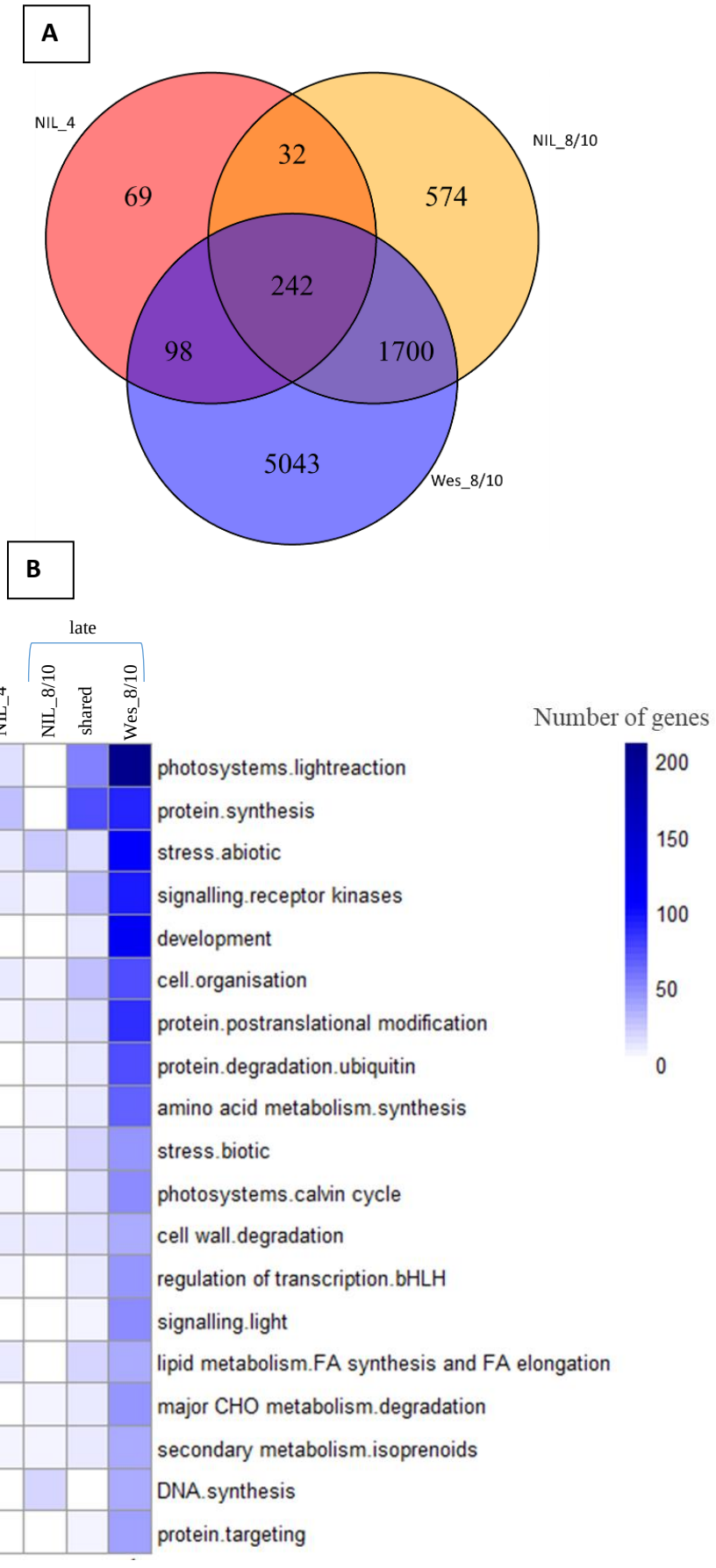


Figure 5. 3 Downregulated DEGs in W+BLMR2 at 4 and 8/10 dai and Westar at 8/10 dai of L. maculans isolate 87-41 (AvrLm1, AvrLm2…). Venn diagram shows the overlapping genes in the NIL (W+BLMR2) and Westar at early (4dai) and late (8/10dai) stages ***(A),*** *Pathway analysis of downregulated DEGs shows Photosystems (light reaction) and protein (synthesis) as the most enriched MAPMAN functional bins* ***(B).***

5.3.5 Functional classification by MAPMAN

The up- and downregulated DEGs at early and late stages of infection were subjected to MAPMAN functional classification. Of the total 34 MAPMAN functional bins, those comprising 50 or more genes were selected. Among the top 20 functional bins from upregulated genes, signalling (receptor kinases), stress (biotic), protein (degradation and post-translational modification), signalling (calcium), transport (ABC transporters and multidrug resistance systems), and RNA regulation of transcription (WRKY) are the ones with a higher number of genes (Figure 5.2b). On the other hand, for the functional bins of the downregulated genes, photosystems (light reaction), protein (synthesis), stress (abiotic), development and cell (organization) are among the ones with greater number of genes (Figure 5.3b).

5.3.6 Genome-wide expression profile analysis by gene family

A total of 16 putative gene families involved in biotic stress pathway and two gene families in photosynthetic pathway were selected for further analysis by gene family.

Upstream resistance genes can be grouped into NLRs (nucleotide binding site - NBS, Leucine rich repeat - LRR), RLPs (receptor like proteins) and RLKs (receptor like kinases) based on their structural motifs (Sekhwal et al. 2015). Tirnaz et al (2020) identified 621 NLRs, 1493 RLKs and 273 RLPs resistance gene analogs (RGAs) in Darmor-bzh V4.1 using the genome-wide prediction method. Using the same RGAugury pipeline, Dolatabadian et al (2020) identified 503 NLRs,1098 RLKs and 148 RLPs RGAs in *B. napus* pangenomes of 50 accessions including Darmor-bzh V8.1. In this study, 967 NLR, 1765 RLK and 262 RLPs were predicted bioinformatically (Table 5.1).

Intracellular disease resistant proteins, NLRs, can further divided into subfamilies including coiled-coil (CC)-NLR, Toll/Interleukin-1 receptor (TIR)-NLR and others based on the conserved domain (Dolatabadian et al. 2020). Of the 967 predicted NLRs, only 15 genes were upregulated in W+BLMR2, which were annotated as 7 TIR-NLR, 2 LRR and 6 others (disease resistant protein like).

Cell surface receptors including RLPs (*LepR3* and *Rlm2*) and the interacting RLKs (*SOBIR1*) were implicated in *B. napus* resistance to blackleg disease (Larkan et al. 2013, 2015; Ma and Borhan 2015). RLPs contain extracellular LRR (eLRR), transmembrane domain and a short

cytoplasmic tail. Of the 262 RLPs identified genome-wide, 30 were upregulated in the resistant W+BLMR2 (Table 5.1, Figure 5.4a).

Unlike RLPs, RLKs have both extracellular receptor and intracellular protein domains that function in ligand perception (*FLS2*) and signalling (*BR1*) (Shiu and Bleecker 2020). RLKs belong to the PK (protein kinase) superfamily, which can be subdivided into various gene families such as CDPK (calcium dependent PK), MAPK (mitogen associated PK), and RLK (transmembrane and cytoplasmic) based on the kinase domain. Transmembrane receptor kinases can further be divided into subfamilies based on the extracellular domain including LRR-RLKs, cysteine-rich (CRKs), and wall-associated kinases (WAKs) (Shiu and Bleecker 2020). In our study, we identified 720 LRR-RLKs, 225 CRKs, and 112 WAKs, of which 54 LRR-RLKs, 26 CRKs, 9 WAKs were upregulated in W+BLMR2 (Table 5.1, Figure 5.4a).

WRKYs belong to a superfamily of plant-specific transcription regulators with conserved DNA-binding motif or W-box. About 100 WRKYs were identified in Arabidopsis, which were assigned to three major subgroups based on the sequence of the WRKY domain (Eulgem 2000). Of the identified 327 WRKYs in this study, 40 were upregulated in W+BLMR2 (Table 5.1).

Pathogenesis related proteins (PRs) belong to diverse family and can be classified by their function (Sels et al. 2008). For example, PR1 is associated with antifungal property with unknown target while PR4 is a chitinase. The plant chitinases catalyze the hydrolysis of chitin (fungal cell wall) and can be considered as a subgroup of PRs. Chen et al (2018) identified 33 chitinases in *B. rapa*. In our study, we identified 61 chitinases, of which 15 were expressed in W+BLMR2. The peroxidases are another group of PRs with antifungal roles. They also involve in cell wall lignification and detoxification of peroxides (Tognolli et al. 2002). Of the 40 peroxidases identified, 6 were expressed in W+BLMR2 (Table 5.1, Figure 5.4a).

The Glutathione S transferases (GSTs) and cytochrome P450 (CYP450s) are multifunctional enzymes with roles including detoxification of xenobiotics and biosynthesis of secondary metabolites. In plants, they are expressed during biotic and abiotic stress or in response to phytohormones (Jun et al. 2015; Vaish et al. 2020). In this study, 18 GSTs and 21 CYP450s were expressed in W+BLMR2 (Table 5.1, Figure 5.4a).

Chloroplasts contribute to plant immunity against fungal pathogens as the hub for the production of key defense response compounds such as ROS, JA/ET, SA, in addition to photosynthesis (Lu and Yao 2018; Kretschmer et al. 2020). Photosynthesis can be divided into two: light reactions (photosystems - PSI and PSII – dependent) and carbon fixation reactions, catalyzed by ribulose biphosphate carboxylase/oxygenase (Rubisco). In our study, all the gene sets were downregulated in Westar at 8/10 dai (Table 5.2, Figure 5.4b).

Table 5. 1 Summary of the bioinformatically predicted gene sets in the biotic stress pathway and their expression in the resistant NIL and susceptible Westar inoculated with L. maculans.

Gene family	Predicted subtotal	Upregulated DEGs	
		NIL_4	Wes_4
GST	240	18	7
PR	140	8	4
Peroxidase	40	6	4
P450	756	21	2
ABC	418	24	0
Chitinase	61	15	7
WRKY	327	40	17
WAK	112	9	1
CRK	225	26	2
PK	1218	37	5
CDPK	181	3	0
MAPk	246	6	0
RLK	820	57	6
LRR-K	720	54	5
RLP	262	30	4
NLR	967	15	4

Upregulated DEGs is based on *L. maculans* inoculated vs mock at 4dai; log2FC$\geq$1 and FDR$\leq$0.05

Table 5. 2 Summary of the bioinformatically predicted gene sets in the photosynthetic pathway and their expression in the resistant NIL and susceptible Westar inoculated with L. maculans.

Gene family	Predicted subtotal	Downregulated DEGs	
		NIL_8/10	Wes_8/10
PS	119	68	119
Rubisco	40	11	14

Downregulated DEGs is based on *L. maculans* inoculated vs mock at 8/10 daiLog2FC$\leq$1 and FDR$\leq$0.05


A
Wes_4
NIL_4
NIL_8/10
Wes_8/10
BnaA07g31330D receptor like protein 15
BnaC04g43070D receptor like protein 39
BnaA09g09270D receptor like protein 6
BnaC04g43190D receptor like protein 40
BnaA04g18920D receptor like protein 22
BnaC04g43150D receptor like protein 22
BnaA10g20720D receptor like protein 19
BnaC06g35080D receptor like protein 13
BnaC04g43230D receptor like protein 25
BnaC03g08330D receptor like protein 56
BnaC03g41540D receptor like protein 34
BnaA04g10630D Protein kinase superfamily protein
BnaA04g13420D Serine/threonine-protein kinase SRK2J
BnaC06g27840D Protein kinase family protein
BnaC04g35560D Serine/threonine-protein kinase SRK2J
BnaA10g26620D Protein kinase superfamily protein
BnaAnng29100D Protein kinase superfamily protein
BnaC05g50050D Probable serine/threonine-protein kinase At1g18390
BnaA04g08480D Protein kinase family protein
BnaA03g34520D Protein kinase superfamily protein
BnaA02g12660D Protein kinase superfamily protein
BnaA07g04380D Protein kinase superfamily protein
BnaC02g16920D Protein kinase family protein
BnaC03g56180D serine/threonine-protein kinase HT1-like
BnaA10g11810D Protein kinase superfamily protein
BnaA03g25460D Cysteine-rich receptor-like protein kinase 40
BnaC01g14390D Cysteine-rich receptor-like protein kinase 11
BnaA03g45810D Cysteine-rich receptor-like protein kinase 5
BnaC01g14620D Cysteine-rich receptor-like protein kinase 10
BnaC01g14860D Putative cysteine-rich receptor-like protein kinase 23
BnaA08g10600D Cysteine-rich receptor-like protein kinase 5
BnaA02g14960D Cysteine-rich receptor-like protein kinase 2
BnaA02g20280D Cysteine-rich receptor-like protein kinase 41
BnaC09g18180D Cysteine-rich receptor-like protein kinase 41
BnaA08g10520D Cysteine-rich receptor-like protein kinase 11
BnaA03g56430D Chitinase family protein
BnaA03g20290D chitinase putative
BnaC03g19370D Chitinase family protein
BnaC05g01140D chitinase 10-like
BnaA05g03420D chitinase putative
BnaC04g53030D chitinase putative
BnaA09g00340D Chitinase family protein
BnaC01g37990D ABC transporter C family member 6
BnaA05g00840D ABC transporter B family member 4
BnaC03g57180D ABC transporter A family member 2
BnaC06g01910D ABC transporter C family member 4
BnaA01g30060D ABC transporter C family member 3
BnaAnng19200D Putative ABC transporter C family member 15
BnaC01g37940D ABC transporter C family member 6
BnaA01g30040D ABC transporter C family member 6
BnaC06g17690D Putative ABC transporter C family member 15
BnaC05g23300D ABC transporter C family member 11
BnaA09g26120D ABC transporter C family member 11
BnaA06g00960D ABC transporter G family member 24
BnaA03g32690D ABC transporter C family member 3
BnaA06g09870D ABC transporter G family member 35
BnaC07g23400D Cytochrome P450 71B20
BnaC07g46480D cytochrome P450 family 81 subfamily D polypeptide 8
BnaA03g53940D cytochrome P450 family 81 subfamily D polypeptide 8
BnaA10g24610D cytochrome P450 71A1-like
BnaA04g00950D Cytochrome P450 superfamily protein
BnaC07g37460D cytochrome P450 family 706 subfamily A polypeptide 1
BnaA10g11280D cytochrome P450 family 81 subfamily F polypeptide 2
BnaA10g11290D cytochrome P450 family 81 subfamily F polypeptide 2
BnaC09g32980D cytochrome P450 family 81 subfamily F polypeptide 2
BnaA05g04920D cytochrome P450 family 704 subfamily A polypeptide 2
BnaA03g58610D cytochrome P450 family 706 subfamily A polypeptide 1
8
6
4
2
0
Wes_4
NIL_4
NIL_8/10
Wes_8/10
BnaA06g13430D Pathogenesis-related thaumatin superfamily protein
BnaA06g13410D Pathogenesis-related thaumatin superfamily protein
BnaAnng00470D Glutathione S-transferase L1
BnaAnng01980D Probable WRKY transcription factor 26
BnaA09g05000D Probable WRKY transcription factor 30
BnaC03g67380D WRKY transcription factor 18
BnaA09g13370D WRKY transcription factor 6
BnaA09g00350D Probable WRKY transcription factor 47
BnaC07g43320D Probable WRKY transcription factor 11
BnaC07g30300D Probable WRKY transcription factor 30
BnaA06g15010D Wall-associated receptor kinase 1
BnaC05g16490D Wall-associated receptor kinase 1
BnaA06g15000D Wall-associated receptor kinase 4
BnaC06g19690D Wall-associated receptor kinase-like 9
BnaC07g45520D Calcium-dependent protein kinase 5
BnaA05g15300D Mitogen-activated protein kinase kinase 5
BnaA08g00700D Mitogen-activated protein kinase kinase kinase 1
BnaC06g08920D Mitogen-activated protein kinase kinase 4
BnaA04g27940D L-type lectin-domain containing receptor kinase IV.3
BnaC09g09840D G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130
BnaA03g39800D receptor-like protein kinase 1
BnaA10g13110D L-type lectin-domain containing receptor kinase I.8
BnaA03g09330D Receptor-like protein kinase At5g59670
BnaA02g23230D L-type lectin-domain containing receptor kinase IV.1
BnaA01g08410D receptor-like protein kinase 5
BnaC04g07660D L-type lectin-domain containing receptor kinase IV.1
BnaC09g35920D L-type lectin-domain containing receptor kinase I.8
BnaA02g16030D Putative receptor-like protein kinase At1g72540
BnaC06g06260D receptor protein kinase-related
BnaC01g09930D Receptor-like protein kinase 5
BnaC02g44410D Receptor-like protein kinase At5g59670
BnaA02g36540D L-type lectin-domain containing receptor kinase IV.4
BnaC03g72660D Receptor-like serine/threonine-protein kinase SD1-6
BnaA01g08400D Receptor-like protein kinase 5
BnaA01g08380D Receptor-like protein kinase 5
BnaA04g22430D Inactive receptor-like serine/threonine-protein kinase At2g40270
BnaC01g09950D receptor-like protein kinase 5
BnaC04g31230D Probable receptor-like protein kinase At5g39030
BnaA04g08980D Probable receptor-like protein kinase At5g38990
BnaA03g24080D G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
BnaC04g46930D Inactive receptor-like serine/threonine-protein kinase At2g40270
BnaA07g31480D Probable LRR receptor-like serine/threonine-protein kinase At1g74360
BnaA06g30250D Probably inactive leucine-rich repeat receptor-like protein kinase At5g48380
BnaC08g14590D Probable LRR receptor-like serine/threonine-protein kinase At1g51820
BnaA07g07730D Probable LRR receptor-like serine/threonine-protein kinase At1g29720
BnaA03g09340D Leucine-rich repeat protein kinase family protein
BnaA08g06400D leucine-rich repeat transmembrane protein kinase family protein
BnaA05g15170D Probable LRR receptor-like serine/threonine-protein kinase At1g51860
BnaAnng04540D Probable leucine-rich repeat receptor-like protein kinase At1g35710
BnaAnng04550D probable LRR receptor-like serine/threonine-protein kinase At4g08850
BnaC05g27580D probable LRR receptor-like serine/threonine-protein kinase At4g08850
BnaC06g35270D Probable LRR receptor-like serine/threonine-protein kinase At1g74360
BnaA08g26050D Leucine-rich receptor-like protein kinase family protein
BnaC03g11750D Probable LRR receptor-like serine/threonine-protein kinase At5g59680
BnaC05g27570D Probable leucine-rich repeat receptor-like protein kinase At1g35710
BnaC05g27560D Probable leucine-rich repeat receptor-like protein kinase At1g35710
BnaA06g32280D Probable LRR receptor-like serine/threonine-protein kinase At1g29720
BnaC08g06900D leucine-rich repeat transmembrane protein kinase family protein
BnaA06g31500D Leucine-rich repeat protein kinase family protein
BnaA02g30100D Probably inactive leucine-rich repeat receptor-like protein kinase At5g48380
BnaA02g31820D Protein kinase family protein with leucine-rich repeat domain
BnaA05g35900D Leucine-rich repeat protein kinase family protein
BnaC02g31270D LRR receptor-like serine/threonine-protein kinase EFR
BnaA05g15180D Probable LRR receptor-like serine/threonine-protein kinase At1g51860
BnaC07g28960D Protein kinase family protein with leucine-rich repeat domain
BnaC07g26450D Probably inactive leucine-rich repeat receptor-like protein kinase At5g48380
BnaA04g18600D Leucine-rich repeat receptor-like serine/threonine/tyrosine-protein kinase SOBIR1

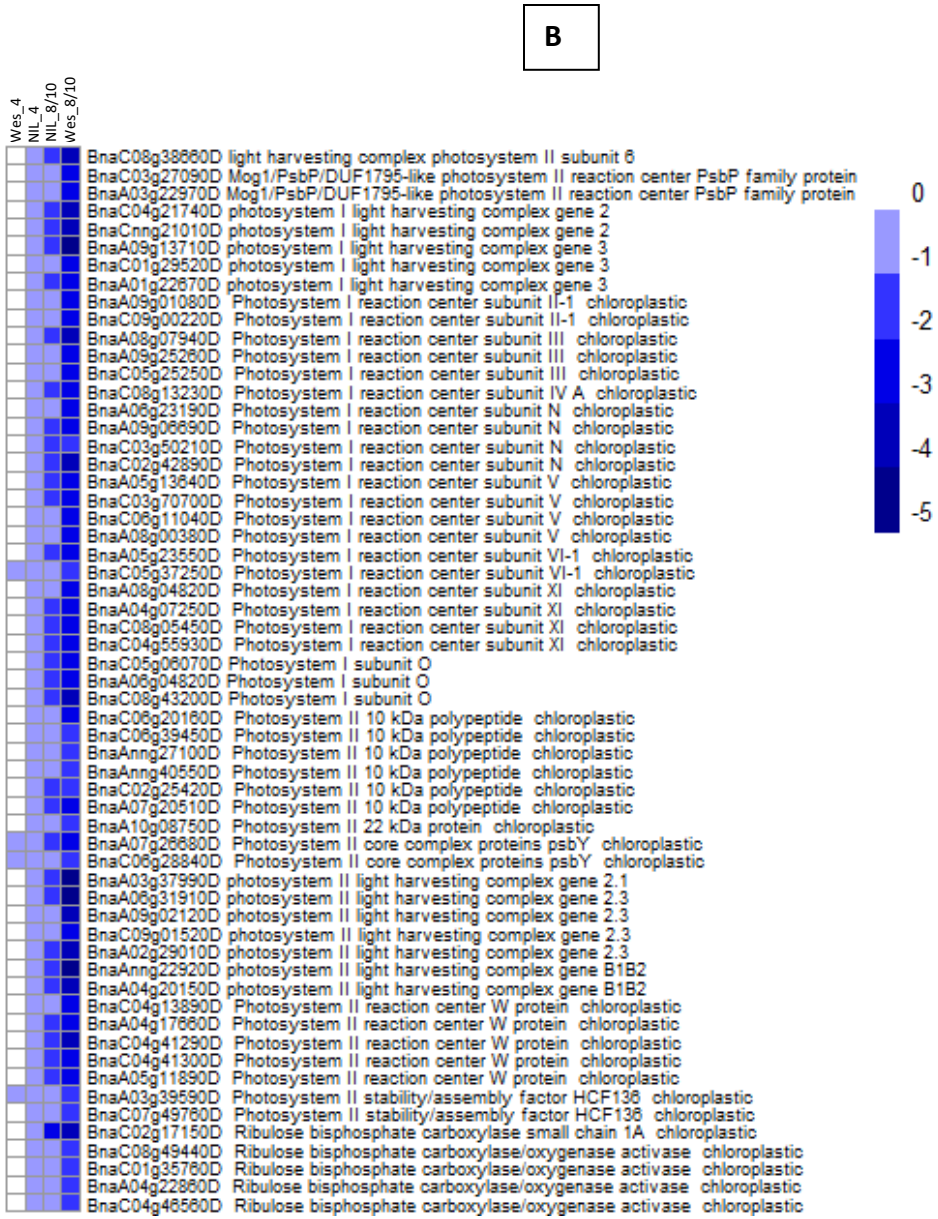


Figure 5. 4 Transcriptomic profiles of selected gene family in the resistant NIL (W+BLMR2) and Westar. Heatmap shows the expression patterns of significantly upregulated gene sets representing the family RLP, RLK, chitinase, ABC transporter, CYP450, PR, WRKY, WAK, GST in the NIL_4 compared to Wes_4 at early stage (4dai). The expression of these genes peaked at later stage in Westar_8/10 ***(A)****, Heatmap shows the expression patterns of significantly downregulated gene sets representing the family Photosystems I, II and Ribulose bisphosphate carboxylase/oxygenase in Westar_8/10 compared to NIL_8/10 at late stage (8/10dai)* ***(B)****. The gene expression is calculated based on inoculated vs mock samples, log2FC≥/1/ and FDR≤0.05. The color denotes red/blue (high) and white (low).*

5.3.3 Validation of the expression of candidate resistance gene in the BLMR2 locus

The *BLMR2* locus was fine-mapped in a 53.37 kb interval in *B. napus* (Chapter 4). Here, the gene expression of the seven genes in the *BLMR2* locus was analyzed. Interestingly, no typical R gene was identified in the delineated region. However, the *CYP450* (*BnaA10g11280D* / *BnaA10g11290D*) was predicted as a candidate resistance gene as it plays a role in innate immunity through the pathogen induced indole glucosinolate biosynthetic pathway (Burow and Halkier 2017). Among the seven annotated *B. napus* genes based on collinearity with *A. thaliana*, the two *CYP450* gene homologs were the only ones upregulated (Table 5.3)

Table 5. 3 Transcriptomic expression of the gene sets in the BLMR2 locus upon infection with L. maculans.

		Log2FC			
Gene name	Annotation	Wes4	NIL4	Wes8/10	NIL8/10
BnaA10g11240D	Putative clathrin assembly protein At5g57200	0	0	0	0
BnaA10g11250D	-	0	0	0	0
BnaA10g11260D	Ypt/Rab-GAP domain of gyp1p superfamily protein	-0.45	-0.29	0.50	0.21
BnaA10g11270D	-	0	0	0	0
BnaA10g11280D	Cytochrome P450, family 81F2	2.33	**6.26**	**4.23**	**5.27**
BnaA10g11290D	Cytochrome P450, family 81F2	2.05	**5.86**	**4.32**	**5.46**
BnaA10g11300D	Thioredoxin superfamily protein	0.57	-1.16	-0.51	-0.57

DEGs is calculated by contrasting inoculated vs mock samples; significance **(bold)** is based on log2FC>/1/ and FDR<0.05**.**

5.3.3 Comparison of R (T+LepR3) and HR (W+BLMR2)

In this sub-section, the defense systems in QR and R-mediated resistance strategies, are compared. A thorough investigation of the dynamics in the transcriptomic profiles of the compatible and incompatible interaction using transgenic lines containing major R genes in Westar background was reported previously (Zhou et al 2019). In the present study, the transcriptomic profiles of the transgenic line T+LepR3 incompatible interaction – representing typical vertical resistance (R) and the NIL W+BLMR1 – representing atypical /horizontal resistance along with the susceptible Westar were analyzed. RNA-seq data from a total of 18 samples (3 lines, 3 replications and 2 treatments – inoculated and mock) were analyzed together (Figure 5.5a). RNA-seq data for the T+LepR3 inoculated with isolate 87-41 and mock were retrieved from the NCBI (BioProject accession number PRJNA378851). Our transcriptomic analysis focused on the key genes upregulated only in incompatible interaction in the earlier study (Zhou et al. 2019). Comparison of the change in gene expression of these genes showed equivalent gene expression profiles in the W+BLMR2 and T-LepR3 compared to Westar (Figure 5.5b), suggesting significant commonality in HR and R-mediated resistance mechanisms.

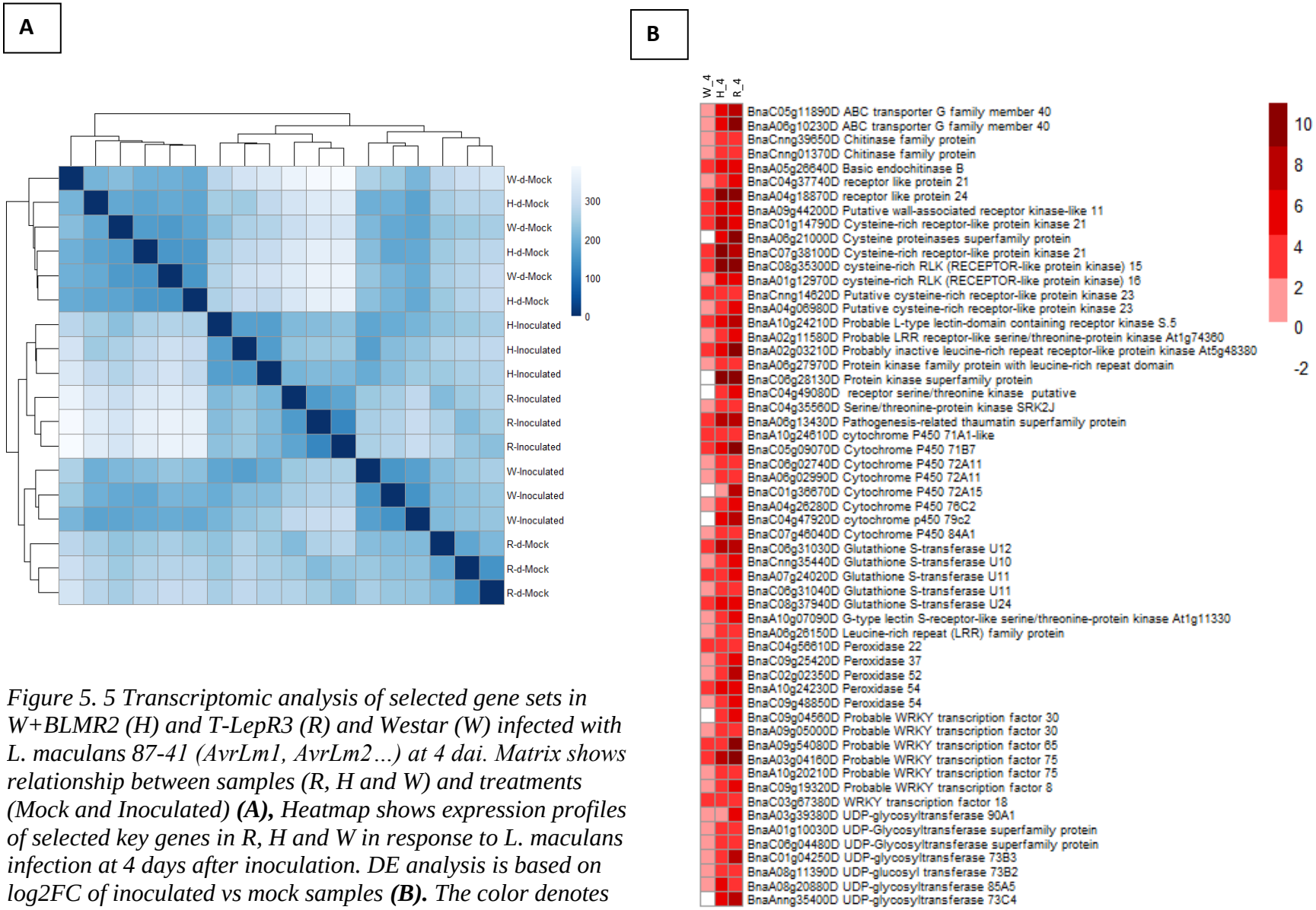


Figure 5. 5 Transcriptomic analysis of selected gene sets in W+BLMR2 (H) and T-LepR3 (R) and Westar (W) infected with L. maculans 87-41 (AvrLm1, AvrLm2…) at 4 dai. Matrix shows relationship between samples (R, H and W) and treatments (Mock and Inoculated) ***(A),*** *Heatmap shows expression profiles of selected key genes in R, H and W in response to L. maculans infection at 4 days after inoculation. DE analysis is based on log2FC of inoculated vs mock samples* ***(B).*** *The color denotes blue/red (high) to white (low).*

5.4 Discussion

5.4.1 Relationship of R and HR at the early stage

The focus of our study was to understand the molecular mechanism underlying the horizontal resistant NIL in *B. napus* using transcriptomic approaches. In the previous study, Zhou et al (2019) demonstrated significant overlap in the expression profiles of compatible and incompatible interaction compared to the basal resistance at early stage, in terms of defense related genes such as ERF, L3 ligase and WRKYs. In contrast, the expression patterns of genes such as RLPs, cytochrome P450 and peroxidases was significantly higher in the incompatible interaction than compatible and basal, suggesting roles in determining the final output. We indicated higher expression of these key genes in the resistant W+BLMR2 compared to Westar at early stage, which suggests equivalent quantitative resistance strategy in the two resistance mechanisms except minor differences in magnitude.

Haddadi et al (2019) reported similar patterns of gene expression for the lines with different R genes. The line with the intermediate resistant *LepR2* locus, generally, showed lower gene expression values than typical R genes (*LepR1/Rlm2*) in the susceptible Topas background. On the other hand, it displayed less intense expression profiles in Westar compared to Topas background (Haddadi et al. 2019). Similarly, Zhou et al (2019) compared *LepR3/Rlm2* -mediated compatible and incompatible interaction in Westar background as well as susceptible interaction and showed the gradual decrease in gene expression of key genes from incompatible to compatible interaction and the susceptible Westar had significantly lower basal resistance. The *BLMR2* locus in our study was introgressed in the susceptible Westar background with presumed weak basal resistance. The locus showed intermediate resistance phenotype when cotyledon inoculated with several *L. maculans* isolates (Dandena et al. 2019). Taken together, this locus could induce less intense expression of defense response compared to typical R gene-mediated incompatible resistance, nevertheless more intense basal or horizontal resistance than R -mediated compatible interaction.

Generally, PTI upon recognition of PAMPs and ETI against effectors are associated with slow and fast responses, respectively (Jones and Dangl 2006). However, ETD against extracellular pathogens could be slower compared to ETI triggered by intracellular pathogens (Stotz et al. 2014). Zhou et al. (2019) demonstrated that *B. napus* R genes, in the absence of the

corresponding *L. maculans Avr* genes, were able to induce expression of defense-related genes at the early stage, irrespective of the outcome, which suggested other pathogen components as elicitors of the immune response. For example, chitin hydrolysis products were demonstrated to enhance PTI (Chen et al. 2018). The *BLMR2*, as *Avr*-independent locus, may interact with such pathogen induced targets to prompt defense response (Li et al. 2016; Kimura et al. 2017). If R-mediated resistance implies ETD, horizontal resistance as demonstrated by *BLMR2* in this study, could correspond to PTI or more closely related mechanism in *B. napus*. Pathogen associated molecular pattern (PAMP) triggered immunity, in general, renders immunity against a broad range of stresses, and the elicitors encompass PAMPs and damage associated molecular pattern (DAMPs), albeit the later is not extensively studied in plants. DAMP is a recently discovered host-derived danger signal in relation to wound / injury due to tissue disruption (O'Donnell et al 2003).

5.4.2 Comparison of early and late transcriptomic profiles

Key defense related genes induced at the early stage are prominent for biotrophic interaction. The expressed genes in NIL_4 and Wes_4 overlapped significantly (50-70%) with NIL_8/10 and Wes_8/10 indicating the continued expression of most of the genes in NIL_8/10 and the much-delayed expression in Wes_8/10. Unlike typical R-mediated resistance with strong hypersensitive reaction, pathogen growth is not halted but significantly reduced in W+BLMR2 compared to the susceptible Westar (Dandena et al. 2019; Stotz et al. 2014).

Although the resistant NIL continued some resistance gene activity from early to late stages, NIL_8/10 also shared ~50% of its upregulated genes with Wes_8/10, probably due to the pathogen's change in lifestyle to necrotrophy or age-dependent gene activity (Haddadi et al. 2016). As a hemi-biotrophic pathogen, *L. maculans* has alternating lifestyles between biotrophic and necrotrophic phases (Sonah et al. 2016). Haddadai et al (2016) carried out genomic studies from both the pathogen and the plant side and observed pathogens use effectors (*Avr*) to intensify the infection process at the early stages of cotyledon colonization that coincided with the biotrophic phase and then switch to necrotrophy (>6dai) involving other pathogen derived elicitors such as necrotrophic effectors and toxins. In our study, the genes induced more at the early stage in the NIL_4 are presumably associated with biotrophic interaction and the genes expressed more in the NIL_8/10 and Wes_8/10 were linked to the necrotrophic phase. Genes that

were expressed similarly at the early and late stages in the NIL could be associated with genes that continued expressing regardless of the pathogen's lifestyle. However, genes that were not significantly expressed or showed lower expression at the late stage in the resistant NIL could indicate the gradual slow-down of activities carried out by those genes induced at the early biotrophic phase.

Numerous RNA-seq studies (Haddadi et al. 2016; Becker et al. 2017) reported significantly higher number of DEGs at late stages in the susceptible interaction than resistant interaction. This is most likely due to the extensive cell or tissue damage in the susceptible interaction. In our study, we observed the induction of NLR genes associated with programmed cell death (PCD), in Westar at late stage. This is consistent with Stotz et al (2018) that NLR was induced more at the late stage in *B. napus* - *L. maculans* compatible interaction. Also, the complex interplay between photosynthesis and defense strategy are emphasized in recent studies (Pfannschmidt et al. 2009; Lu and Yao 2018; Hu et al. 2020). Although activation of reactive oxygen species (ROS) and inhibition of PS genes are associated with active defense strategy, excess production of ROS could damage cells. This is reflected in our study that there are more downregulated genes involved in PS, metabolism of FA, lipids and amino acids in the susceptible Westar at late stage. Taken together, the differential expression of more genes at late stage is due to extensive PCD or pathogen induced tissue damage as well as age-dependent leaf senescence.

If the plant surveillance system (PRR or R) recognizes the pathogen at the early stage, defense response could be launched faster, which results in the limitation of pathogen growth (Stotz et al. 2014). In our study, the number as well as expression of genes in the family RLPs, RLKs and chitinases were higher at the early stage in the NIL_4 compared to Wes_4. In contrast, the expression of these genes peaked at the late stage in Wes_8/10 compared to NIL_8/10, although the output is susceptible phenotype. Transition to necrotrophy probably prompted more genes to be activated in the susceptible Westar than the resistant W+BLMR2 at late stage (Stotz et al. 2018).

Plants undertake an intricate gene regulation even in specific gene families to protect themselves with minimal disruption of other metabolic processes. Several WRKY transcription factor genes were upregulated at the early and late stages. Extensive studies in *A. thaliana* demonstrated the multifaceted roles WRKYs play as positive or negative regulators of defense response, crosstalk

among various hormonal signalling pathways, plant development and other physiological processes (Ülker et al. 2007; Lu 2009; Li and Palva 2012). In our study, we pinpointed prominent defense-related hormone signalling WRKYs, such as *WRKY 18 /70* and *WRKY 11* that modulate the generally antagonistic SA and JA signalling pathways, respectively. Also, *WRKY 70* plays a role in leaf senescence and repression of JA signalling (Ülker et al. 2007). Two *WRKY 70* genes were expressed at early biotrophic phase. Also, three *WRKY 18* genes were upregulated at the early stage and five *WRKY 11* genes were expressed at the late stage, coinciding with biotrophic and necrotrophic phases of the pathogen. Functional redundancy and co-operation among the *WRKYs* such as *18* and *60*; *38* and *62* and *70* and *54* (Li and Palva 2012; Phukan et al. 2016) could be reflected in our study as these genes were expressed more at the early stage in the NIL_4 and late in Wes_8/12. Other SA markers expressed in the NIL at early stage include *PR1*, *EDS1* and *PAD4*. The upstream regulation of these genes may be required for SA accumulation (Lu 2009). In contrast, JA marker genes such as *MAPK4* and *MKS1* were expressed more at the late stage (Phukan et al. 2016). In our study, JA signalling genes such as *MKS1*, *MPK4* were expressed more at the later stages coinciding with the necrotrophic phase while SA marker genes such as *PR1, EDS1* and *PAD4* were expressed at the early biotrophic stage and continue to express at the late stages.

In conclusion, the detailed transcriptomic analysis gives insight in the mechanism underlying horizontal resistance. Comparison of change in gene expression in the RGAs and other gene families involved in the resistance mechanism contribute to understand the quantitative nature of defense response as well as functional redundancy at cellular level in the resistant W+BLMR2 compared to the susceptible Westar.

Author Contributions

HD and GL designed the experiment and wrote the manuscript. HD finished most experiments. TZ collected data of some experiments. KS and WX contributed to the bioinformatics.

Funding

This research is supported by the Natural Sciences and Engineering Research Council (NSERC) CRD project (CRDPJ 523840 – 18) and MITACS.

5. General Discussion and Conclusion

Leptosphaeria maculans is a critical fungal pathogen of *B. napus* causing a worldwide economic losses of about a billion USD per season (Fitt et al 2008). Controlling blackleg disease caused by *L. maculans* contributes significantly to reduce losses. Breeding for resistance is one of the major strategies used in the canola industry to manage blackleg disease since the 1990s (West et al. 2001; Kutcher et al. 2011).

Plant genetic resistance mechanisms are classified into qualitative and quantitative. Qualitative /vertical resistance (R) is mediated by major genes and are race specific. Major genes are easy to identify, manipulate, and confer complete resistance; however, they are short-lived in case epidemic occurs (Zhang et al. 2016). In contrast, quantitative /horizontal resistance (QR) is a cost-effective plant disease control strategy as it is believed to provide a more durable resistance. However, the genetic basis of QR is not well understood and studying QR is challenging.

A commonly used technique to tackle this challenge is to eliminate the vertical resistance in a cultivar (Maramorosch and Loebenstein 2009). The horizontal resistant NILs containing a small (*BLMR2*) segment from the resistance donor parent (Surpass-400) in an otherwise susceptible Westar background were used in our research. The materials were developed using both conventional and molecular marker assisted backcross breeding approaches.

Quantitative resistance is generally complex because of the inconsistent results obtained using seedling (cotyledon or leaf) and stem assays coupled with environmental variations, which include weather conditions and pathogen population (Larkan et al 2016a; Huang et al 2019; Raman et al 2020). Controlled testing is valuable to minimize environmental variation as well as accelerate crop breeding by evaluating test materials at early stage. Because *BLMR2* interacts with *L. maculans* at seedling stage, cotyledon assay assisted in the study of QR in controlled environment. The three main chapters in the thesis describe the phenotypic and molecular studies of the *BLMR2* locus.

Chapter 3 describes the non-race specificity of the *BLMR2* locus, seedling to adult stages, in controlled conditions. This locus was previously Mendelized and is a major-effect QTLs. We also reported the horizontal resistance response of this locus against 27 *L. maculans* isolates using cotyledon inoculation. Evaluating QR in controlled environment is important to avoid

weather related environmental effect (Huang et al 2019). In addition, more than one generation (up to three) can be tested in the greenhouse from seedling to adult stages (Dandena et al 2019). Our results were supported by molecular marker data that co-segregated with the phenotype, which is useful in MAS. Furthermore, field studies conducted for three years, confirmed the stability of this locus compared to lines containing defeated R genes and the susceptible control, bred into the same Westar cultivar. *BLMR2* is the first major effect QRL or atypical resistance locus in *B. napus – L. maculans* pathosystem that is thoroughly investigated at seedling and adult stages against well-characterized isolates / races.

In chapter 4, we described the high-resolution mapping of *BLMR2* locus in the 56.3 kb physical region of the *B. napus* genome. Map-based cloning is the standard procedure to identify the causative gene underlying a trait, although time-consuming and difficult in the complex *B. napus* genome (Mayerhofer et al. 2005). To address the issue of efficiency and genome complexity in mapping, genome-specific molecular markers along with NILs and high throughput genotyping were employed. In our study, NILs were developed *via* molecular marker assisted backcrossing to narrow-down the *BLMR2* locus in an otherwise Westar genetic background. In addition, the use of genome-specific, polymorphic and codominant molecular markers assisted in the screening of a large segregating BC population. Flanking markers used in the high-throughput genotyping need to be clear / clean to effectively detect possible recombination events. For this task, SSR and SCAR molecular markers were employed. As discussed previously, SSR motifs are ubiquitous in the genome, co-dominant and easy to develop because of the availability of the *B. rapa* genome sequence at the time of marker development in our project. A SCAR marker system is also valuable in map-based cloning, especially to bridge the gaps in the genetic map. SCAR is developed based on sequence information of PCR amplified products and can be co-dominant. Informative molecular markers along with high-throughput genotyping could reduce the cost and time of genotyping (Rahman et al 2008). In our study, MAS was employed to develop the mapping population, by phenotyping only the recombinants progenies. We were able to screen (genotype) ~10,000 individuals using a medium throughput method by collecting tissue samples from the cotyledons of a 7-day old seedling, to minimize the time needed for sampling from the first true leaves. To demonstrate how this plan works, some 400 samples were collected in a day and kept at 4 degrees overnight. The next day, DNA was extracted and let it air dry overnight. The third day, the DNA was amplified using PCR and run ABI analyzer for marker

detection. Accordingly, at least 400 plants were genotyped in 10 days, from planting to detection, which translate into 10,000 plants genotyped in 250 days or ~8 months. In our study, two flanking markers were used at a time to genotype the mapping population, and only the potential recombinants were selfed to confirm the phenotype.

During fine-mapping, lack of meiotic cross-over on one side of the locus (N10-47) was the challenge to further narrow-down the region. This could be because of lack of polymorphism between the parents using the marker system employed in our study—amplification of more than one region in the genome or other reasons related to the chromosome region where the locus is located. To tackle this challenge, candidate-gene prediction approach in the 100 kb region were performed by blasting the marker sequence on the reference genome. This allowed us to predict potential disease related candidate gene. The lack of a putative R gene in the region may be fitting to the atypical reaction of this locus. Sequencing the cDNA allowed us to reannotate the two genes based on the reference Darmor-bzh into a single *B. napus* gene homologous to Arabidopsis *CYP450*. Unfortunately, cloning of the candidate *CYP450* gene has not been easy due to the limited time and resources, although attempts were made using complementation, overexpression, RNAi and CRISPR-CAS assays. Nevertheless, the materials developed in our study could be used for further functional studies. In contrast, an alternative strategy using RNA-Seq was proposed to examine the differential expression of the genes in the *BLMR2* region, which led to the last project.

Chapter 5 focuses on understanding the mechanism behind horizontal resistance in *BLMR2* using RNA-Seq. Colocalization of R and QRL, in previous QTL mapping studies, created uncertainty about the relationship between the residual effect of the supposedly ineffective R genes and QR loci. A recent RNA-seq study from our lab and the current study, corroborate equivalent QR mechanism in R and QR, only differing in magnitude. The transcriptomic data generated in this work could be used in network analysis of the key genes or analysis of individual biosynthetic pathway such as the indole-glucosinolate biosynthetic pathway, which could help to understand QR better in *B. napus*. In the past, majority of whole-genome or transcriptome studies emphasized on NBS-LRR as R gene homologs. In order to utilize QR effectively as well as bridge the knowledge gap between the two resistance mechanisms, future studies should focus on other classes of R gene homologs or gene families that contribute to host basal immunity.

Naming of R genes mapped in canola to blackleg infection

Several naming systems have been used over the years to identify the R genes mapped in *B. napus* to *L. maculans* infection. To date, there are >15 R loci mapped, including "*Rlm1-10*", "*LepR1-4*" and "*BLMR1-2*", mainly on chromosomes A07 and A10. The prefix "Rlm" with the corresponding "Avrlm" in the pathogen is mainly used for most of the R loci in *B. napus*. However, the prefix "LepR" was used to refer to R loci introgressed from the *B. rapa* subsp *sylvestris*. Moreover, "*BLMR1*" and "*BLMR2*" refer to the R loci derived from Surpass 400, which is a *sylvestris* – derived *B. napus* cultivar. These two loci were mapped in the same map region as *LepR3*, but the mapping of two loci with a high and intermediate level of resistance phenotype compelled a new naming system to differentiate it from *LepR3*. Subsequent studies suggested that *LepR3* and *BLMR1* are the same. To conclude, uniform naming system could benefit to minimize confusion and to account for the total number of R genes so far identified in *B. napus* – *L. maculans* pathosystem.

6. References

Abdel-farid, I.B., Jahangir, M., Mustafa, N.R., van Dam, N.M., van den Hondel, C.A.M.J.J., Kyong, H., Hae, Y., and Verpoorte, R. 2010. Glucosinolate profiling of *Brassica rapa* cultivars after infection by *Leptosphaeria maculans* and *Fusarium oxysporum.* Biochem. Syst. Ecol. **38**: 612–620. doi:10.1016/j.bse.2010.07.008.

Al-Shehbaz, I.A., Beilstein, M.A., and Kellogg, E.A. 2006. Systematics and phylogeny of the Brassicaceae (Cruciferae): an overview. Plant Syst. Evol. **259**: 89–120. doi:10.1007/s00606-006-0415-z.

Allender, C.J., and King, G.J. 2010. Origins of the amphiploid species *Brassica napus* L. investigated by chloroplast and nuclear molecular markers. BMC Plant Biol. **10**:54. doi:10.1186/1471-2229-10-54.

Arias, T., Beilstein, M.A., Tang, M., McKain, M.R., and Pires, J.C. 2014. Diversification times among *Brassica* (Brassicaceae) crops suggest hybrid formation after 20 million years of divergence. Am. J. Bot. **101**: 86–91. doi:10.3732/ajb.1300312.

Bansal, V., Kharbanda, P., Stringam, G., Thiagarajah, M., and Tewari, J. 1994. Comaprison of green house and field identification of blackleg resistance in doubled haploid lines of *Brassica napus*. Pl. Dis. **78**:276-281.

Batley, J., Bayer, P.E., Tirnaz, S., Hurgobin, B., and Edwards, D. 2020. Characterization of disease resistance genes in the *Brassica napus* pangenome reveals significant structural variation. **18**: 969–982. doi:10.1111/pbi.13262.

Becker, M.G., Haddadi, P., Wan, J., Adam, L., Walker, P., Larkan, N.J., Daayf, F., Hossein Borhan, M., and Belmonte, M.F. 2019. Transcriptome analysis of *Rlm2*-mediated host immunity in the *brassica napus–leptosphaeria maculans* pathosystem. Mol. Plant-Microbe Interact. **32**: 1001–1012. doi:10.1094/mpmi-01-19-0028-r.

Becker, M.G., Zhang, X., Walker, P.L., Wan, J.C., Millar, J.L., Khan, D., Granger, M.J., Cavers, J.D., Chan, A.C., Fernando, D.W.G., and Belmonte, M.F. 2017. Transcriptome analysis of the *Brassica napus – Leptosphaeria maculans* pathosystem identifies receptor, signaling and structural genes underlying plant resistance. T. Pl. J. **90**: 573–586.

doi:10.1111/tpj.13514.

Bednarek, P., Piślewska-Bednarek, M., Svatoš, A., Schneider, B., Doubský, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina, A., and Schulze-Lefert, P. 2009. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Plant Cell **323**: 101–106. doi: 10.1126/science.1163732.

Behla, R., Hirani, A.H., Zelmer, C.D., Yu, F., Fernando, W.G.D., McVetty, P., and Li, G. 2017. Identification of common QTL for resistance to *Sclerotinia sclerotiorum* in three doubled haploid populations of *Brassica napus* (L.). Euphytica **213**: 1–16. doi:10.1007/s10681-017-2047-5.

Bolger, A.M., Marc, L., and Bjoern, U. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics **30**:2114–2120.

Bräutigam, A., and Gowik, U. 2010. What can next generation sequencing do for you? Next generation sequencing as a valuable tool in plant research. Plant Biol. **12**: 831–841. doi:10.1111/j.1438-8677.2010.00373.x.

Brun, H., Chèvre, A.M., Fitt, B.D., Powers, S., Besnard, A.L., Ermel, M., Huteau, V., Marquer, B., Eber, F., Renard, M., and Andrivon, D. 2010. Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. New Phytol. **185**: 285–299. doi:10.1111/j.1469-8137.2009.03049.x.

Burow, M., and Halkier, B.A. 2017. How does a plant orchestrate defense in time and space? Using glucosinolates in *Arabidopsis* as case study. Curr. Opin. Plant Biol. **38**: 142–147. doi:10.1016/j.pbi.2017.04.009.

Cargeeg, L.A., and Thurling, N. 1980. Seedling and adult-plant resistance to blackleg (*Leptosphaeria maculans* (Desm.) Ces. et de Not.) in spring rape (*Brassica napus* L). Aust. J. Agric. Res. **31**: 37–46. doi:10.1071/ar9800037.

Carré, P., and Pouzet, A. 2014. Rapeseed market, worldwide and in Europe. OCL **21**: 1–12. doi:10.1051/ocl/2013054.

Chai, A.L., Xie, X.W., Shi, Y.X., and Li, B.J. 2014. Research status of clubroot *(Plasmodiophora brassicae)* on cruciferous crops in China. Can. J. Pl. Path. **36**:142-153.

doi:10.1080/07060661.2013.868829.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wang, X., Chiquet, J., Belcram, H., Tong, C., Samans, B., Corréa, M., Silva, C. Da, Just, J., Falentin, C., Koh, C.S., Clainche, I. Le, Bernard, M., Bento, P., Noel, B., Labadie, K., Alberti, A., Charles, M., Arnaud, D., and Guo, H. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science **345**:950-953. doi:10.1126/science.1253435.

Chen, J., Piao, Y., Liu, Y., Li, X., and Piao, Z. 2018. Genome-wide identification and expression analysis of chitinase gene family in *Brassica rapa* reveals its role in clubroot resistance. Plant Sci. **270**: 257–267. doi:10.1016/j.plantsci.2018.02.017.

Chen, Y., and Fernando, W.G.D. 2006. Prevalence of pathogenicity groups of *Leptosphaeria maculans* in western Canada and North Dakota, USA. Can. J. Plant Pathol. **28**: 533–539. doi:10.1080/07060660609507331.

Chen, Y., Lun, A.T.L., and Smyth, G.K. 2016. From reads to genes to pathways : differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline [version 2; referees 5: approved] F1000 Research **5**: 1438. doi:10.12688/f1000research.8987.1.

Cheng, X., Xu, J., Xia, S., Gu, J., Yang, Y., Fu, J., Qian, X., Zhang, S., Wu, J., and Liu, K. 2009. Development and genetic mapping of microsatellite markers from genome survey sequences in *Brassica napus.* Theor. Appl. Genet. **118**: 1121–1131. doi:10.1007/s00122-009-0967-8.

Clarke, W.E., Higgins, E.E., Plieske, J., Wieseke, R., Sidebottom, C., Khedikar, Y., Batley, J., Edwards, D., Meng, J., Li, R., Lawley, C.T., Pauquet, J., Laga, B., Cheung, W., Iniguez-Luy, F., Dyrszka, E., Rae, S., Stich, B., Snowdon, R.J., Sharpe, A.G., Ganal, M.W., and Parkin, I.A.P. 2016. A high-density SNP genotyping array for *Brassica napus* and its ancestral diploid species based on optimised selection of single-locus markers in the allotetraploid genome. Theor. Appl. Genet. **129**:1887–1899. doi:10.1007/s00122-016-2746-7.

Clay, N.K., Adio, A.M., Denoux, C., Jander, G., and Ausubel, F.M. 2009. Innate immune

response. **51**: 95–101. doi:10.1126/science.1164627.

Cook, D.E., Mesarich, C.H., and Thomma, B.P.H.J. 2015. Understanding Plant Immunity as a Surveillance System to Detect Invasion. Annu. Rev. Phytopathol. **53**:541–63. doi:10.1146/annurev-phyto-080614-120114.

Corwin, J.A., and Kliebenstein, D.J. 2017. Quantitative resistance: More Than Just Perception of a Pathogen. The Pl. Cell **29**: 655–665. doi:10.1105/tpc.16.00915.

Couto, D., and Zipfel, C. 2016. Regulation of pattern recognition receptor signalling in plants. Nat. Rev. **16**:537-552. doi:10.1038/nri.2016.77.

Crouch, J.H., Lewis, B.G., and Mithen, R.F. 1994. The Effect of A genome substitution on the resistance of *Brassica napus* to infection by *Leptosphaeria maculans.* Pl. Breed. **112**: 265–278. doi:10.1111/j.1439-0523.1994.tb00684.x.

Dandena, H.B., Zhang, Q., Zhou, T., Hirani, A.H., and Liu, Z. Fernando, D.W.G., Duncan, R.W., and Li, G. 2019. Analysis of quantitative adult plant resistance to blackleg in *Brassica napus.* Mol. Breed. **39**: 124. doi:10.1007/s11032-019-1035-y

Delourme, R., Brun, H., Ermel, M., Lucas, M.O., Vallee, P., Domin, C., Walton, G., Li, H., Sivasithamparam, K., and Barbetti, M.J. 2008a. Expression of resistance to *Leptosphaeria maculans* in *Brassica napus* double haploid lines in France and Australia is influenced by location. Ann. Appl. Biol. **153**: 259–269. doi:10.1111/j.1744-7348.2008.00258.x.

Delourme, R., Chèvre, A.M., Brun, H., Rouxel, T., Balesdent, M.H., Dias, J.S., Salisbury, P., Renard, M., and Rimmer, S.R. 2006. Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape *(Brassica napus).* Eur. J. Plant Pathol. **114**: 41–52. doi:10.1007/s10658-005-2108-9.

Delourme, R., Piel, N., Horvais, R., Pouilly, N., Domin, C., Vallée, P., Falentin, C., Manzanares-Dauleux, M.J., and Renard, M. 2008b. Molecular and phenotypic characterization of near isogenic lines at QTL for quantitative resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). Theor. Appl. Genet. **117**: 1055–1067. doi:10.1007/s00122-008-0844-x.

Delourme, R., Pilet-Nayel, M.L., Archipiano, M., Horvais, R., Tanguy, X., Rouxel, T., Brun, H., Renard, M., and Balesdent, M.H. 2004. A Cluster of major specific resistance genes to

Leptosphaeria maculans in *Brassica napus*. Phytopathology **94**: 578–583. doi:10.1094/phyto.2004.94.6.578.

Dilmaghani, A., Balesdent, M.H., Didier, J.P., Wu, C., Davey, J., Barbetti, M.J., Li, H., Moreno-Rico, O., Phillips, D., Despeghel, J.P., Vincenot, L., Gout, L., and Rouxel, T. 2009. The *Leptosphaeria maculans* - *Leptosphaeria biglobosa* species complex in the American continent. Plant Pathol. **58**: 1044–1058. doi:10.1111/j.1365-3059.2009.02149.x.

Dion, Y., Gugel, R.K., Rakow, G.F.W., Séguin-Swartz, G., and Landry, B.S. 1995. RFLP mapping of resistance to the blackleg disease [causal agent, *Leptosphaeria maculans* (Desm.) Ces. et de Not.] in canola (*Brassica napus* L.). Theor. Appl. Genet. **91**: 1190–1194. doi:10.1007/bf00220928.

Dixon, G.R. 2009. The occurrence and economic impact of *Plasmodiophora brassicae* and clubroot disease. J. Pl. Growth Regul. **28**:194–202. doi:10.1007/s00344-009-9090-y

Dolatabadian, A., Bayer, P.E., Tirnaz, S., Hurgobin, B., Edwards, D., and Batley, J. 2020. Characterization of disease resistance genes in the *Brassica napus* pangenome reveals significant structural variation. Pl. Biotech. J. **18**: 969–982. doi: 10.1111/pbi.13262.

Easton, A. 2001. Blackleg tested 9. Where does it come from and what does it mean. In: Marcroft SJ (ed) Proceedings of the 12th Australian Research Assembly on *Brassicas*, Geelong, Victoria, 2–5 October 2001, pp 47–49

Elliott, V.L., Marcroft, S.J., Howlett, B.J., and Van de Wouw, A.P. 2016. Gene-for-gene resistance is expressed in cotyledons, leaves and pods, but not during late stages of stem colonization in the *Leptosphaeria maculans-Brassica napus* pathosystem. Plant Breed. **135**: 200–207. doi:10.1111/pbr.12343.

Eskin, N.A.M., and Przybylski, R. "Rape seed oil/canola." In *Encyclopedia of food sciences and nutrition (second edition)*, edited by Luiz Trugo and Paul M. Finglas, 4911-4916. USA: Elsevier, 2003. doi:10.1016/B0-12-227055-X/01349-3

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. 2000. The WRKY superfamily of plant transcription factors. Trends in Pl. Sci. **5**: 199-206.

Fernando, W.G.D., Chen, Y.U., and Ghanbarnia, K. 2007. Breeding for Blackleg Resistance: the

biology and epidemiology. Adv. in Bot. Res. **45**:272-311. doi:10.1016/S0065-2296(07)45010-8.

Fernando, D.W.G., Zhang, X., and Amarasinghe, C.C. 2016. Detection of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* causing blackleg disease in canola from Canadian canola seed lots and dockage. Plants **5**: 3–15. doi:10.3390/plants5010012.

Fernando, W.G.D., Zhang, X., Selin, C., Zou, Z., Liban, S.H., McLaren, D.L., Kubinec, A., Parks, P.S., Harunur Rashid, M., Padmathilake, K.R.E., Rong, L., Yang, C., Gnanesh, B.N., and Huang, S. 2018. A six-year investigation of the dynamics of avirulence allele profiles, blackleg incidence, and mating type alleles of *Leptosphaeria maculans* populations associated with canola crops in Manitoba, Canada. Plant Dis. **102**: 790–798. doi:10.1094/Pdis-05-17-0630-re.

Ferreira, M.E., Rimmer, S.R., Williams, P.H., and Osborn, T.C. 1995. Mapping loci controlling *Brassica napus* resistance to *Leptosphaeria maculans* under different screening conditions. Phytopath. **85**:213-217. doi:10.1094/Phyto-85-213.

Ferreira, M.E., Williams, P.H., and Osborn, T.C. 1994. RFLP mapping of *Brassica napus* using doubled haploid lines. Theor. Appl. Genet. **89**: 615–621. doi:10.1007/BF00222456.

Fitt, B.D.L., Brun, H., Barbetti, M.J., and Rimmer, S.R. 2006. World-wide importance of phoma stem canker *(Leptosphaeria maculans and L. biglobosa)* on oilseed Rape *(Brassica napus).* Eur. J. Plant Pathol. **114**: 3–15. doi:10.1007/s10658-005-2233-5.

Fitt, B.D.L., Hu, B.C., Li, Z.Q., Liu, S.Y., Lange, R.M., Kharbanda, P.D., Butterworth, M.H., and White, R.P. 2008. Strategies to prevent spread of *Leptosphaeria maculans* (phoma stem canker) onto oilseed rape crops in China; costs and benefits. Plant Pathol. **57**: 652–664. doi:10.1111/j.1365-3059.2008.01841.x.

Flor, H.H. 1942. Inheritance of pathogenicity in *Melampsora lini.* Phytopathol. **32**:653-669

Foisset, N., Delourme, R., Barret, P., Hubert, N., Landry, B.S., and Renard, M. 1996. Molecular-mapping analysis in *Brassica napus* using isozyme, RAPD and RFLF markers on a doubled-haploid progeny. Theor. Appl. Genet. **93**: 1017–1025. doi:10.1007/BF00230119.

Fomeju, B.F., Falentin, C., Lassalle, G., Manzanares-Dauleux, M.J., and Delourme, R. 2015.

Comparative genomic analysis of duplicated homoeologous regions involved in the resistance of *Brassica napus* to stem canker. Front. Plant Sci. **15**:498 doi:10.3389/fpls.2015.00772.

Franzke, A., Lysak, M.A., Al-Shehbaz, I.A., Koch, M.A., and Mummenhoff, K. 2011. Cabbage family affairs: The evolutionary history of Brassicaceae. Trends Plant Sci. **16**: 108–116. doi:10.1016/j.tplants.2010.11.005.

Friedt, W., and Snowdon, R. "Oilseed rape." In *Handbook of Plant Breeding. Oil Crops, vol. 4.*, edited by Vollmann J., Rajcan I., 91-126. New York: Springer-Verlag, 2009.

Fu, F., Liu, X., Wang, R., Zhai, C., Peng, G., Yu, F., and Fernando, W.G.D. 2019. Fine mapping of *Brassica napus* blackleg resistance gene *Rlm1* through bulked segregant RNA sequencing. Sci. Rep. **9**: 1–10. doi:10.1038/s41598-019-51191-z.

Fu, F., Zhang, X., Liu, F., Peng, G., Yu, F., and Fernando, D. 2020. Identification of resistance loci in Chinese and Canadian canola/rapeseed varieties against *Leptosphaeria maculans* based on genome-wide association studies. BMC Genomics **21**: 1–11. BMC Genomics. doi:10.1186/s12864-020-06893-4.

Geng, X., Dong, N., Wang, Y., Li, Q., Wang, L., Guo, X., Li, J., Wen, Z., and Wei, W. 2018. RNA-seq transcriptome analysis of the immature seeds of two *Brassica napus* lines with extremely different thousand-seed weight to identify the candidate genes related to seed weight. PLoS ONE **13**:1–17. doi:10.1371/journal.pone.0101297.

Gervais, J., Plissonneau, C., Linglin, J., Meyer, M., Labadie, K., Cruaud, C., Fudal, I., Rouxel, T., and Balesdent, M.H. 2016. Different waves of effector genes with contrasted genomic location are expressed by Leptosphaeria maculans during cotyledon and stem colonization of oilseed rape. Mol. Plant Pathol. **8**: 1113–1126. doi:10.1111/mpp.12464.

González, A.M., Marcel, T.C., and Niks, R.E. 2012. Evidence for a minor gene-for-minor gene interaction explaining nonhypersensitive polygenic partial disease resistance. Phytopathology **102**: 1086–1093. doi:10.1094/phyto-03-12-0056-r.

Gugel, R.K., and Petrie, G.A. 1992. History, occurrence, impact, and control of blackleg of rapeseed. Can. J. Plant Pathol. **14**: 36–45. doi:10.1080/07060669209500904.

Guo, X.W., and Fernando, W.G.D. 2004. Seasonal and diurnal patterns of spore dispersal by *Leptosphaeria maculans* from canola stubble in relation to environmental conditions. Pl. Dis. **89**:97-104

Gupta, S.K. "Brassicas." In *Breeding Oilseed Crops for Sustainable Production*, edited by Surinder Kumar Gupta, 33-54. India: Elsevier, 2016. doi:10.1016/B978-0-12-801309-0.00003-3

Haddadi, P., Larkan, N.J., and Borhan, M.H. 2019. Dissecting R gene and host genetic background effect on the *Brassica napus* defense response to *Leptosphaeria maculans.* Sci. Rep. **9**: 6947. doi:10.1038/s41598-019-43419-9.

Haddadi, P., Ma, L., Wang, H., and Borhan, M.H. 2016. Genome-wide transcriptomic analyses provide insights into the lifestyle transition and effector repertoire of *Leptosphaeria maculans* during the colonization of *Brassica napus* seedlings. Mol. Plant Pathol. **17**: 1196–1210. doi:10.1111/mpp.12356.

Hahn, M. 2014. The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. J. Chem. Biol. **7**: 133–141. doi:10.1007/s12154-014-0113-1.

Hammond, K.E., and Lewis, B.G. 1987a. Differential responses of oilseed rape leaves to *Leptosphaeria maculans.* Trans. Br. Mycol. Soc. **88**: 329–333. doi:10.1016/S0007-1536(87)80006-2.

Hammond, K.E., and Lewis, B.G. 1987b. The establishment of systemic infection in leaves of oilseed rape by *Leptosphaeria maculans*. Plant Pathol. **36**: 135–147. doi:10.1111/j.1365-3059.1987.tb02213.x.

Hammond, K.E., Lewis, B.G., and Musa, T.M. 1985. A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. Plant Pathol. **34**: 557–565. doi:10.1111/j.1365-3059.1985.tb01407.x.

Hayward, A., Mclanders, J., Campbell, E., Edwards, D., and Batley, J. 2012. Genomic advances will herald new insights into the *Brassica: Leptosphaeria maculans* pathosystem. Plant Biol. **14**: 1–10. doi:10.1111/j.1438-8677.2011.00481.x.

Henderson, M.P. 1918. The blackleg disease of cabbage caused by Phoma lingam (Tode)

Desmaz. Phytopathol. **8**:378-431.

Howlett, B.J. 2004. Current knowledge of the interaction between *Brassica napus* and *Leptosphaeria maculans.* Can. J. Pl. Pathol. **26**: 245–252. doi:10.1080/07060660409507141.

Hu, Y., Zhong, S., Zhang, M., Liang, Y., Gong, G., Chang, X., Tan, F., Yang, H., Qiu, X., Luo, L., and Luo, P. 2020. Potential role of photosynthesis in the regulation of reactive oxygen species and defence responses to *Blumeria graminis* f. Sp. tritici in wheat. Int. J. Mol. Sci. **21**: 1–26. doi:10.3390/ijms21165767.

Huang, Y.J., Evans, N., Li, Z.Q., Eckert, M., Chèvre, A.M., Renard, M., and Fitt, B.D.L. 2006. Temperature and leaf wetness duration affect phenotypic expression of *Rlm6*-mediated resistance to *Leptosphaeria maculans* in *Brassica napus.* New Phytol. **170**: 129–141. doi:10.1111/j.1469-8137.2005.01651.x.

Huang, Y.J., Jestin, C., Welham, S.J., King, G.J., Manzanares-Dauleux, M.J., Fitt, B.D.L., and Delourme, R. 2016. Identification of environmentally stable QTL for resistance against *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). Theor. Appl. Genet. **129**: 169–180. doi:10.1007/s00122-015-2620-z.

Huang, Y.J., Liu, Z., West, J.S., Todd, A.D., Hall, A.M. and Fitt, B.D.L. 2007. Effects of temperature and rainfall on date of release of ascospores of *Leptosphaeria maculans* (phoma stem canker) from winter oilseed rape (*Brassica napus*) debris in the UK. Annals of Applied Biology **151**: 99-111. doi:10.1111/j.1744-7348.2007.00157.x. Huang, Y.J., Mitrousia, G.K., Sidique, S.N.M., Qi, A., and Fitt, B.D.L. 2018. Combining R gene and quantitative resistance increases effectiveness of cultivar resistance against *Leptosphaeria maculans* in *Brassica napus* in different environments. PLoS One **13**: 1–22. doi:10.1371/journal.pone.0197752.

Huang, Y.J., Paillard, S., Kumar, V. King, G.J., Fitt, B.D.L., and Delourme, R. 2019. Oilseed rape (*Brassica napus*) resistance to growth of *Leptosphaeria maculans* in leaves of young plants contributes to quantitative resistance in stems of adult plants. PLoS ONE **14**:1–20. doi:10.1371/journal.pone.0222540.

Huang, Y.J., Pirie, E.J., Evans, N., Delourme, R., King, G.J., and Fitt, B.D.L. 2009. Quantitative resistance to symptomless growth of *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape). Plant Pathol. **58**: 314–323. doi:10.1111/j.1365-3059.2008.01957.x.

Huang, Y.J., Qi, A., King, G.J., and Fitt, B.D.L. 2014. Assessing quantitative resistance against *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) in young plants. PLoS One **9**: 1–10. doi:10.1371/journal.pone.0084924.

Jestin, C., Lodé, M., Vallée, P., Domin, C., Falentin, C., Horvais, R., Coedel, S., Manzanares-Dauleux, M.J., and Delourme, R. 2011. Association mapping of quantitative resistance for *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). Mol. Breed. **27**: 271–287. doi:10.1007/s11032-010-9429-x.

Jones, J.D.G., and Dangl, J.L. 2006. The plant immune system. **444**: 323–329. doi:10.1038/nature05286.

Jun, X.U., Xin-yu, W., and Wang-zhen, G.U.O. 2015. The cytochrome P450 superfamily : key players in plant development and defense. J. Integr. Agric. **14**: 1673–1686. doi:10.1016/S2095-3119(14)60980-1.

Juska, A., Busch, L., and Tanaka, K. 1997. The blackleg epidemic in Canadian rapeseed as a "normal agricultural accident". Ecol. Applic. **7**:1350–1356.

Kimura, S., Waszczak, C., Hunter, K., and Wrzaczek, M. 2017. Bound by fate: the role of reactive oxygen species in receptor-like kinase signaling. Plant Cell **29**: 638–654. doi:10.1105/tpc.16.00947.

Kretschmer, M., Damoo, D., Djamei, A., and Kronstad, J. 2020. Chloroplasts and plant immunity: where are the fungal effectors? Pathogens **9**: 1–16. doi:10.3390/pathogens9010019.

Kumar, V., Paillard, S., Fopa-Fomeju, B., Falentin, C., Deniot, G., Baron, C., Vallée, P., Manzanares-Dauleux, M.J., and Delourme, R. 2018. Multi-year linkage and association mapping confirm the high number of genomic regions involved in oilseed rape quantitative resistance to blackleg. Theor. and Appl, Genet. **131**:1627–1643 doi:10.1007/s00122-018-

3103-9.

Kutcher, H.R., Balesdent, M.H., Rimmer, S.R., Rouxel, T., Chèvre, A.M., Delourme, R., and Brun, H. 2010. Frequency of avirulence genes in *Leptosphaeria maculans* in western Canada. Can. J. Plant Pathol. **32**: 77–85. doi:10.1080/07060661003594109.

Kutcher, H.R., Brandt, S.A., Smith, E.G., Ulrich, D., and Malhi, S.S. 2013. Blackleg disease of canola mitigated by resistant cultivars and four-year crop rotations in western Canada. Can. J. of Pl. Path. **35**: 209-221. doi:10.1080/07060661.2013.775600.

Kutcher, H.R., Keri, M., McLaren, D.L., and Rimmer, S.R. 2007. Pathogenic variability of *Leptosphaeria maculans* in western Canada. Can. J. Pl. Pathol. **29**: 388–393. doi:10.1080/07060660709507484.

Kutcher, H.R., Turkington, T.K., and Mclaren, D.L. 2011. Best management practices for blackleg disease of canola western Canadian blackleg management tactics. Prairie Soils Crop. J. **4**: 122–134.

Lagercrantz, U., and Lydiate, D.J. 1996. Comparative genome mapping in *Brassica*. Genetics **144**: 1903–1910.

Larkan, N.J., Lydiate, D.J., Parkin, I.A.P., Nelson, M.N., Epp, D.J., Cowling, W.A., Rimmer, S.R., and Borhan, M.H. 2013. The *Brassica napus* blackleg resistance gene *LepR3* encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector *AvrLm1*. New Phytol. **197**: 595–605. doi:10.1111/nph.12043.

Larkan, N.J., Lydiate, D.J., Yu, F., Rimmer, S.R., and Borhan, M.H. 2014. Co-localisation of the blackleg resistance genes *Rlm2* and *LepR3* on *Brassica napus* chromosome A10. BMC Plant Biol. **14**: 387. doi:10.1186/s12870-014-0387-z.

Larkan, N.J., Ma, L., and Borhan, M.H. 2015. The *Brassica napus* receptor-like protein *Rlm2* is encoded by a second allele of the *LepR3/Rlm2* blackleg resistance locus. Plant Biotechnol. J. **13**: 983–992. doi:10.1111/pbi.12341.

Larkan NJ, Ma L, Haddadi P, Buchwaldt, M., Parkin, I.A.P., Djavaheri, M., and Borhan, M.H. 2020. The *Brassica napus* Wall-Associated Kinase-Like (WAKL) gene *Rlm9* provides race-specific blackleg resistance. The Plant J. **104**:892–900. doi:10.1111/tpj.14966.

Larkan, N.J., Raman, H., Lydiate, D.J., Robinson, S.J., Yu, F., Barbulescu, D.M., Raman, R., Luckett, D.J., Burton, W., Wratten, N., Salisbury, P.A., Rimmer, S.R., and Borhan, M.H. 2016a. Multi-environment QTL studies suggest a role for cysteine-rich protein kinase genes in quantitative resistance to blackleg disease in *Brassica napus.* BMC Plant Biol. **16**: 183. BMC Plant Biology. doi:10.1186/s12870-016-0877-2.

Larkan, N.J., Yu, F., Lydiate, D.J., Rimmer, S.R., and Borhan, M.H. 2016b. Single R gene introgression lines for accurate dissection of the *Brassica* - *Leptosphaeria* pathosystem. Front. Plant Sci. **7**:1771. doi:10.3389/fpls.2016.01771.

Li, C.X., and Cowling, W.A. 2003. Identification of a single dominant allele for resistance to blackleg in *Brassica napus* "Surpass 400." Plant Breed. **122**: 485–488. doi:10.1111/j.1439-0523.2003.00863.x.

Li, C.X., Wratten, N., Salisbury, P.A., Burton, W.A., Potter, T.D., Walton, G., Li, H., Sivasithamparam, K., Banga, S.S., Banga, S., Singh, D., Liu, S.Y., Fu, T.D., and Barbetti, M.J. 2008a. Response of *Brassica napus* and *B. juncea* germplasm from Australia, China and India to Australian populations of *Leptosphaeria maculans*. Australas. Plant Pathol. **37**: 162–170. doi:10.1071/AP08005.

Li, G., and Quiros, C.F. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. Theor. Appl. Genet. **103**: 455–461. doi:10.1007/s001220100570.

Li, H., Barbetti, M.J., and Sivasithamparam, K. 2003. Field isolates of *Leptosphaeria maculans* from western Australia overcome a single dominant resistance gene in *Brassica napus* cv. Surpass 400. Thirteen. Bienn. Aust. Res. Assem. Brassicas. Proc. a Conf. Tamworth, New South Wales, Aust. 8-12 Sept. 2003: 134–135.

Li, H., Barbetti, M.J., and Sivasithamparam, K. 2005. Hazard from reliance on cruciferous hosts as sources of major gene-based resistance for managing blackleg (*Leptosphaeria maculans*) disease. F. Crop. Res. **91**: 185–198. doi:10.1016/j.fcr.2004.06.006.

Li, H., Kuo, J., Barbetti, M.J., and Sivasithamparam, K. 2007. Differences in the responses of stem tissues of spring-type *Brassica napus* cultivars with polygenic resistance and single

dominant gene-based resistance to inoculation with *Leptosphaeria maculans*. Can. J. Botany **85**: 191–203. doi:10.1139/B06-159.

Li, H., Sivasithamparam, K., Barbetti, M.J., and Kuo, J. 2004. Germination and invasion by ascospores and pycnidiospores of *Leptosphaeria maculans* on spring-type *Brassica napus* canola varieties with varying susceptibility to blackleg. J. Gen. Plant Pathol. **70**: 261–269. doi:10.1007/s10327-004-0125-8.

Li, H., Sivasithamparam, K., Barbetti, M.J., Wylie, S.J., and Kuo, J. 2008b. Cytological responses in the hypersensitive reaction in cotyledon and stem tissues of *Brassica napus* after infection by *Leptosphaeria maculans.* J. Gen. Plant Pathol. **74**: 120–124. doi:10.1007/s10327-007-0067-z.

Li, J., and Palva, E.T. 2012. WRKY54 and WRKY70 co-operate as negative regulators of leaf senescence in *Arabidopsis thaliana*. **63**: 2667–2679. doi:10.1093/jxb/err450.

Li, L., Yu, Y., Zhou, Z., and Zhou, J.M. 2016. Plant pattern-recognition receptors controlling innate immunity. Sci. China Life Sci. **59**: 878–888. doi:10.1007/s11427-016-0115-2.

Liban, S.H., Cross, D.J., Kutcher, H.R., Peng, G., and Fernando, W.G.D. 2016. Race structure and frequency of avirulence genes in the western Canadian *Leptosphaeria maculans* pathogen population, the causal agent of blackleg in *Brassica* species. Plant Pathol. **65**: 1161–1169. doi:10.1111/ppa.12489.

Lombard, V., and Delourme, R. 2001. A consensus linkage map for rapeseed (*Brassica napus* L.): construction and integration of three individual maps from DH populations. Theor. Appl. Genet. **103**: 491–507. doi:10.1007/s001220100560.

Long, Y., Wang, Z., Sun, Z., Fernando, D.W.G., McVetty, P.B.E., and Li, G. 2011. Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola cultivar "Surpass 400." Theor. Appl. Genet. **122**: 1223–1231. doi:10.1007/s00122-010-1526-z.

Lowe, A.J., Moule, C., Trick, M., and Edwards, K.J. 2004. Efficient large-scale development of microsatellites for marker and mapping applications in *Brassica* crop species. Theor. Appl. Genet. **108**: 1103–1112. doi:10.1007/s00122-003-1522-7.

Lu, H. 2009. Dissection of salicylic acid-mediated defense signaling networks. Pl. Signal. & Behav. **4**: 713–717.

Lu, K., Wei, L., Li, X., Wang, Y., Wu, J., Liu, M., Zhang, C., Chen, Z., Xiao, Z., Jian, H., Cheng, F., Zhang, K., Du, H., Cheng, X., Qu, C., Qian, W., Liu, L., Wang, R., Zou, Q., Ying, J., Xu, X., Mei, J., Liang, Y., Chai, Y.R., Tang, Z., Wan, H., Ni, Y., He, Y., Lin, N., Fan, Y., Sun, W., Li, N.N., Zhou, G., Zheng, H., Wang, X., Paterson, A.H., and Li, J. 2019. Whole-genome resequencing reveals *Brassica napus* origin and genetic loci involved in its improvement. Nat. Commun. **10**: 1–12. doi:10.1038/s41467-019-09134-9.

Lu, Y., and Yao, J. 2018. Chloroplasts at the crossroad of photosynthesis, pathogen infection and plant defense. Int. J. Mol. Sci. **19**: 1–37. doi:10.3390/ijms19123900.

Lysak, M.A., Cheung, K., Kitschke, M., and Bureš, P. 2007. Ancestral chromosomal blocks are triplicated in *Brassiceae* species with varying chromosome number and genome size. Plant Physiol. **145**: 402–410. doi:10.1104/pp.107.104380.

Ma, L., and Borhan, M.H. 2015. The receptor-like kinase *SOBIR1* interacts with *Brassica napus LepR3* and is required for *Leptosphaeria maculans AvrLm1*-triggered immunity. Front. Plant Sci. **6**: 933. doi:10.3389/fpls.2015.00933.

Macho, A.P., and Zipfel, C. 2014. Plant PRRs and the Activation of Innate Immune Signaling. Mol. Cell **54**:263-272. doi:10.1016/j.molcel.2014.03.028

Maramorosch, K., and Loebenstein, G. 2009. Plant disease resistance: natural, non-host innate or inducible. Encycl. Microbiol.: 589–596. doi:10.1016/B978-012373944-5.00341-2.

Marcroft, S.J., Elliott, V.L., Cozijnsen, A.J., Salisbury, P.A., Howlett, B.J., and Van De Wouw, A.P. 2012. Identifying resistance genes to *Leptosphaeria maculans* in Australian *Brassica napus* cultivars based on reactions to isolates with known avirulence genotypes. Crop Pasture Sci. **63**: 338–350. doi:10.1071/cp11341.

Martin, G.B. 1995. Chromosome landing : a paradigm for map-based gene cloning in plants with large genomes. **11**:63-68.

Mayerhofer, R., Bansal, V.K., Thiagarajah, M.R., Stringam, G.R., and Good, A.G. 1997. Molecular mapping of resistance to *Leptosphaeria maculans* in Australian cultivars of

Brassica napus. Genome **40**: 294–301. doi:10.1139/g97-041.

Mayerhofer, R., Wilde, K., Mayerhofer, M., Lydiate, D., Bansal, V.K., Good, A.G., and Parkin, I.A.P. 2005. Complexities of chromosome landing in a highly duplicated genome: toward map-based cloning of a gene controlling blackleg resistance in *Brassica napus*. Genetics **171**: 1977–1988. doi:10.1534/genetics.105.049098.

McNabb, W.M., Berg, C.G.J. van den, and Rimmer, S.R. 1993. Comparison of inoculation methods for selection of plants resistant to *Leptosphaeria maculans* in *Brassica napus*. Can. J. Plant Sci. **73**: 1199–1207.

Mcvetty, P.B.E., Mietkiewska, E., Omonov, T., Curtis, J., Taylor, D.C., and Weselake, R.J. 2016. "Brassica spp . Oils." In *Industrial Oil Crops,* edited by Thomas A. McKeon, Douglas G. Hayes, David F. Hildebrand, Randall J. Weselake, 113-156. AOCS Press: Elsevier Inc, 2016. doi:10.1016/B978-1-893997-98-1.00005-1

Mengistu, A., Rimmer, S.R., Koch, E., and Williams, P.H. 1991. Pathogenicity grouping of Isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid-cycling Brassicas. Pl. Dis. **75**: 1279-1282

Mesterhazy, A. 2020. Updating the breeding philosophy of wheat to fusarium head blight (FHB): resistance components, QTL identification, and phenotyping—a review. Plants **9**: 1702. doi:10.3390/plants9121702.

Mithen, R.F., and Lewis, B.G. 1988. Resistance to *Leptosphaeria maculans* in hybrids of *Brassica oleracea* and *Brassica insularis*. J. Phytopathol. **123**: 253–258. doi:10.1111/j.1439-0434.1988.tb04475.x.

Mithen, R.F., Lewis, B.G., Heaney, R.K., and Fenwick, G.R. 1987. Glucosinolates of wild and cultivated *Brassica* species. Phytochemistry **26**: 1969–1973. doi:10.1016/S0031-9422(00)81740-9.

Nadeem, M.A., Nawaz, M.A., Shahid, M.Q., Doğan, Y., Comertpay, G., Yıldız, M., Hatipoğlu, R., Ahmad, F., Alsaleh, A., Labhane, N., Özkan, H., Chung, G., and Baloch, F.S. 2018. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. Biotechnol. Biotechnol. Equip. **32**: 261–285.

doi:10.1080/13102818.2017.1400401.

Neik, T.X., Barbetti, M.J., and Batley, J. 2017. Current status and challenges in identifying disease resistance genes in *Brassica napus.* Front. Plant Sci. **8**: 1788. doi:10.3389/fpls.2017.01788.

Niks, R.E., Qi, X., and Marcel, T.C. 2015. Quantitative Resistance to Biotrophic Filamentous Plant Pathogens: Concepts, Misconceptions, and Mechanisms. Annu. Rev. Phytopathol. **53**:445–70. doi:10.1146/annurev-phyto-080614-115928.

Nolan, T.M., Vukasinović, N., Liu, D., Russinova, E., and Yin, Y. 2020. Brassinosteroids: multidimensional regulators of plant growth, development, and stress responses. Plant Cell **32**: 298–318. doi:10.1105/tpc.19.00335.

Noman, A., and Aqeel, M. 2019. PRRs and NB-LRRs : from signal perception to activation of plant innate immunity. Int. J. Mol. Sci. **20**: 1882; doi:10.3390/ijms20081882

Nurhasanah, and Ecke, W. 2016. Construction of a high fidelity genetic linkage map using AFLP and SSR markers in rapeseed (*Brassica napus* L.). Sabrao J. Breed. Genet. **48**: 189–199.

O'Donnell, P.J., Schmelz, E.A., Moussatche, P., Lund, S.T., Jones, J.B., and Klee, H.J. 2003. Susceptible to intolerance - a range of hormonal actions in a susceptible *Arabidopsis* pathogen response. Plant J. **33**: 245–257. doi:10.1046/j.1365-313X.2003.01619.x.

Pang, E.C.K., and Halloran, G.M. 1996a. The genetics of blackleg *Leptosphaeria maculans* (Desm) Ces et De Not resistance in rapeseed (*Brassica napus* L) .1. Adult-plant resistance in F-2 and first-backcross populations. Theor. Appl. Genet. **93**: 932–940. doi:10.1007/bf00224096.

Pang, E.C.K., and Halloran, G.M. 1996b. The genetics of blackleg *Leptosphaeria maculans* (Desm) Ces et De Not resistance in rapeseed (*Brassica napus* L) .2. Seedling and adult plant resistance as quantitative traits. Theor. Appl. Genet. **93**: 941–949. doi:10.1007/bf00224097.

Parkin, I.A., Sharpe, A.G., Keith, D.J., and Lydiate, D.J. 1995. Identification of the A and C genomes ofamphidiploid *Brassica napus* (oilseed rape). Genome **38**: 1122–1131. doi:10.1139/g95-149.

Parkin, I.A.P. 2005. Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana.* Genetics **171**: 765–781. doi:10.1534/genetics.105.042093.

Parkin, I.A.P., Koh, C., Tang, H., Robinson, S.J., Kagale, S., Clarke, W.E., Town, C.D., Nixon, J., Krishnakumar, V., Bidwell, S.L., Denoeud, F., Belcram, H., Links, M.G., Just, J., Clarke, C., Bender, T., Huebert, T., Mason, A.S., Chris Pires, J., Barker, G., Moore, J., Walley, P.G., Manoli, S., Batley, J., Edwards, D., Nelson, M.N., Wang, X., Paterson, A.H., King, G., Bancroft, I., Chalhoub, B., and Sharpe, A.G. 2014. Transcriptome and methylome profiling reveals relics of genome dominance in the mesopolyploid *Brassica oleracea.* Genome Biol. **15**: 1–18. doi:10.1186/gb-2014-15-6-r77.

Pfannschmidt, T., Bräutigam, K., Wagner, R., Dietzel, L., Schröter, Y., Steiner, S., and Nykytenko, A. 2009. Potential regulation of gene expression in photosynthetic cells by redox and energy state: approaches towards better understanding. Ann. Bot. **103**: 599–607. doi:10.1093/aob/mcn081.

Phukan, U.J., Jeena, G.S., and Shukla, R.K. 2016. WRKY transcription factors : molecular regulation and stress responses in plants. **7**: 1–14. doi:10.3389/fpls.2016.00760.

Pilet, M.L., Delourme, R., Foisset, N., and Renard, M. 1998. Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculans* (Desm.) Ces. et de Not., in Winter rapeseed (*Brassica napus* L.). Theor. Appl. Genet. **96**: 23–30. doi:10.1007/s001220050704.

Pilet, M.L., Duplan, G., Archipiano, M., Barret, P., Baron, C., Horvais, R., Tanguy, X., Lucas, M.O., Renard, M., and Delourme, R. 2001. Stability of QTL for field resistance to blackleg across two genetic backgrounds in oilseed rape. Crop Sci. **41**: 197–205. doi:10.2135/cropsci2001.411197x.

Piquemal, J., Cinquin, E., Couton, F., Rondeau, C., Seignoret, E., Doucet, I., Perret, D., Villeger, M.J., Vincourt, P., and Blanchard, P. 2005. Construction of an oilseed rape (*Brassica napus* L.) genetic map with SSR markers. Theor. Appl. Genet. **111**: 1514–1523. doi:10.1007/s00122-005-0080-6.

Potts, D.A., Rakow, G.W., Males, D.R., Woods, D.L. 2003. The development of canola-quality *Brassica juncea.* Can. J. Plant Sci. **83**:117-118.

Qi, X., An, H., Ragsdale, A.P., Hall, T.E., Gutenkunst, R.N., Chris Pires, J., and Barker, M.S. 2017. Genomic inferences of domestication events are corroborated by written records in *Brassica rapa.* Mol. Ecol. **26**: 3373–3388. doi:10.1111/mec.14131.

Rahman, M., Mamidi, S., del Rio, L., Ross, A., Kadir, M.M., Rahaman, M.M., and Arifuzzaman, M. 2016. Association mapping in *Brassica napus* (L.) accessions identifies a major QTL for blackleg disease resistance on chromosome A01. Mol. Breed. **36**: 1–15. Springer Netherlands. doi:10.1007/s11032-016-0513-8.

Rahman, M., Sun, Z., McVetty, P.B.E., and Li, G. 2008. High throughput genome-specific and gene-specific molecular markers for erucic acid genes in *Brassica napus* (L.) for marker-assisted selection in plant breeding. Theor. Appl. Genet. **117**: 895–904. doi:10.1007/s00122-008-0829-9.

Raman, H., Dalton-Morgan, J., Diffey, S., Raman, R., Alamery, S., Edwards, D., and Batley, J. 2014. SNP markers-based map construction and genome-wide linkage analysis in *Brassica napus*. Plant Biotech. J. **12:**851–860 doi: 10.1111/pbi.12186

Raman, H., Raman, R., Coombes, N., Song, J., Diffey, S., Kilian, A., Lindbeck, K., Barbulescu, D.M., Batley, J., Edwards, D., Salisbury, P.A., and Marcroft, S. 2016a. Genome-wide association study identifies new loci for resistance to *Leptosphaeria maculans* in Canola. Front. Plant Sci. **7**: 1–16. doi:10.3389/fpls.2016.01513.

Raman, H., Raman, R., Coombes, N., Song, J., Prangnell, R., Bandaranayake, C., Tahira, R., Sundaramoorthi, V., Killian, A., Meng, J., Dennis, E.S., and Balasubramanian, S. 2016b. Genome-wide association analyses reveal complex genetic architecture underlying natural variation for flowering time in canola. Plant Cell Environ. **39**: 1228–1239. doi:10.1111/pce.12644.

Raman, H., Raman, R., Diffey, S., Qiu, Y., Mcvittie, B., Barbulescu, D.M., Salisbury, P.A., Marcroft, S., and Delourme, R. 2018. Stable quantitative resistance loci to blackleg disease in Canola (*Brassica napus* L .) over continents. Front. Pl. Sci. **9**:1622.

doi:10.3389/fpls.2018.01622.

Raman, H., Raman, R., and Larkan, N. 2013. Genetic dissection of blackleg resistance loci in rapeseed (*Brassica napus* L .). Pl. Breed. from Lab. to Fields. 85-120. doi:10.5772/53611

Raman, H., Raman, R., Nelson, M.N., Aslam, M.N., Rajasekaran, R., Wratten, N., Cowling, W.A., Kilian, A., Sharpe, A.G., and Schondelmaier, J. 2012a. Diversity array technology markers: Genetic diversity analyses and linkage map construction in rapeseed (*Brassica napus* L.). DNA Res. **19**: 51–65. doi:10.1093/dnares/dsr041.

Raman, R., Diffey, S., Barbulescu, D.M., Coombes, N., Luckett, D., Salisbury, P., Cowley, R., Marcroft, S., and Raman, H. 2020. Genetic and physical mapping of loci for resistance to blackleg disease in canola (*Brassica napus* L.). Sci. Rep. **10**: 1–12. doi:10.1038/s41598-020-61211-y.

Raman, R., Taylor, B., Marcroft, S., Stiller, J., Eckermann, P., Coombes, N., Rehman, A., Lindbeck, K., Luckett, D., Wratten, N., Batley, J., Edwards, D., Wang, X., and Raman, H. 2012b. Molecular mapping of qualitative and quantitative loci for resistance to *Leptosphaeria maculans* causing blackleg disease in canola (*Brassica napus* L.). Theor. Appl. Genet. **125**: 405–418. doi:10.1007/s00122-012-1842-6.

Rimmer, S.R. 2006. Resistance genes to *Leptosphaeria maculans* in *Brassica napus*. Can. J. Plant Pathol. **297**: 288–297.

Robin, A.H.K., Yi, G.-E., Laila, R., Hossain, M.R., Park, J.-I., Kim, H.R., and Nou, I.-S. 2017. *Leptosphaeria maculans* alters glucosinolate profiles in blackleg disease–resistant and -susceptible cabbage lines. Front. Plant Sci. **8**: 1769. doi:10.3389/fpls.2017.01769.

Rouxel, T. 2017. Life , death and rebirth of avirulence effectors in a fungal pathogen of *Brassica* crops, *Leptosphaeria maculans*. New Phyto. **214**: 526–532. doi:10.1111/nph.14411.

Rouxel, T., Penaud, A., Pinochet, X., Brun, H., Gout, L., Delourme, R., Schmit, J., and Balesdent, M.H. 2003. A 10-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the *Rlm1* resistance gene of oilseed rape. Eur. J. Plant Pathol. **109**: 871–881. doi:10.1023/A:1026189225466.

Roy, N.N. 1984. Interspecific transfer of *Brassica juncea*-type high blackleg resistance to

Brassica napus. Euphytica **33**:295-303

Sekhwal, M.K., Li, P., Lam, I., Wang, X., Cloutier, S. and You, F.M. 2015. Disease resistance gene analogs (RGAs) in plants. Int. J. Mol. Sci. **16**: 19248– 19290.

Salisbury, P.A., Ballinger, D.J., Wratten, N., Plummer, K.M., and Howlett, B.J. 1995. Blackleg disease on oilseed *Brassica* in Australia: a review. Aust. J. of Exp. Agri. **35**: 665 - 672 doi:10.1071/ea9950665.

Schranz, M.E., Lysak, M.A., and Mitchell-Olds, T. 2006. The ABC's of comparative genomics in the Brassicaceae: building blocks of crucifer genomes. Trends Plant Sci. **11**: 535–542. doi:10.1016/j.tplants.2006.09.002.

Schranz, M.E., and Mitchell-Olds, T. 2006. Independent ancient polyploidy events in the sister families Brassicaceae and Cleomaceae. Plant Cell **18**: 1152–1165. doi:10.1105/tpc.106.041111.

Sels, J., Mathys, J., De Coninck, B.M.A., Cammue, B.P.A., and De Bolle, M.F.C. 2008. Plant pathogenesis-related (PR) proteins: a focus on PR peptides. Plant Physiol. Biochem. **46**: 941–950. doi:10.1016/j.plaphy.2008.06.011.

Shiu, S., and Bleecker, A.B. 2020. Expansion of the Receptor-Like Kinase / Pelle gene family and Receptor-Like Proteins in *Arabidopsis*. **132**: 530–543. doi:10.1104/pp.103.021964.tochaud.

Skowrońska, R., Mariańska, M., Ulaszewski, W., Tomkowiak, A., Nawracała, J., and Kwiatek, M.T. 2020. Development of triticale × wheat prebreeding germplasm with loci for slow-rusting resistance. Front. Plant Sci. **11**:447. doi:10.3389/fpls.2020.00447.

Snowdon, R., Lühs, W., Friedt, W. "Oilseed Rape." In *Genome Mapping and Molecular Breeding in Plants, Volume 2 Oilseeds* edited by C. Kole, 55-114. Berlin Heidelberg: Springer-Verlag, 2007.

Sonah, H., Zhang, X., Deshmukh, R.K., and Borhan, M.H. 2016. Comparative transcriptomic analysis of virulence factors in *Leptosphaeria maculans* during compatible and incompatible interactions with canola. Front. Plant Sci. **7**:1784.

doi: 10.3389/fpls.2016.01784.

Song, K., and Osborn, T.C. 1992. Ploy phyletic origin of *Brassica napus*: new evidence based on organelle and nuclear RFLP analyses. Genome **35**:992-1001.

Stefansson, B.R., and Hougen, F.W. 1964. Selection of Rape plants *(Brassica napus)* with seed oil practically free from erucic acid. Can. J. Pl. Sci. **44**:359-364.

Stefansson, B.R., and Kondra, Z.P. 1975. Tower summer rape. Can. J. Plant Sci. **55**:343-344

Sprague, S.J., Balesdent, M.H., Brun, H., Hayden, H.L., Marcroft, S.J., Pinochet, X., Rouxel, T., and Howlett, B.J. 2006. Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. Eur. J. Plant Pathol. **114**: 33–40. doi:10.1007/s10658-005-3683-5.

Stotz, H.U., Harvey, P.J., Haddadi, P., Mashanova, A., Kukol, A., Larkan, N.J., Hossein Borhan, M., and Fitt, B.D.L. 2018. Genomic evidence for genes encoding leucine-rich repeat receptors linked to resistance against the eukaryotic extra- and intracellular *Brassica napus* pathogens *Leptosphaeria maculans* and *Plasmodiophora brassicae.* PLoS One **13**: 1–17. doi:10.1371/journal.pone.0198201.

Stotz, H.U., Mitrousia, G.K., de Wit, P.J.G.M., and Fitt, B.D.L. 2014. Effector-triggered defence against apoplastic fungal pathogens. Trends Plant Sci. **19**: 491–500. doi:10.1016/j.tplants.2014.04.009.

Sun, Z., Wang, Z., Tu, J., Zhang, J., Yu, F., McVetty, P.B.E., and Li, G. 2007. An ultradense genetic recombination map for *Brassica napus,* consisting of 13551 SRAP markers. Theor. Appl. Genet. **114**: 1305–1317. doi:10.1007/s00122-006-0483-z.

Tewari, J.P., Strelkov, S.E., Orchard, D., Hartman, M., Lange, R.M., and Turkington, T.K. 2005. Identification of clubroot of crucifers on canola (*Brassica napus*) in Alberta. **144**: 143–144.

The Arabidopsis Genome Initiative 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana.* Nature **408**: 796–815. doi:10.1038/35048692.

Thomson, J.R., and Stelfox, D. 1983. Sclerotinia contamination of Alberta-produced rapeseed, from 1976-1981. Canadian Plant Disease Survey **63**: 19–22.

Teng, C., Du, D., Xiao, L., Yu, Q., Shang, G., and Zhao, Z. 2017. Mapping and identifying a candidate gene *(Bnmfs)* for female-male sterility through whole-genome resequencing and RNA-Seq in Rapeseed (*Brassica napus* L .). Front. Plant Sci. **8**:2086. doi:10.3389/fpls.2017.02086

Tian, Z.D., Liu, J., Wang, B.L., and Xie, C.H. 2006. Screening and expression analysis of *Phytophthora infestans* induced genes in potato leaves with horizontal resistance. Pl. Cell Rep. **25**: 1094–1103. doi:10.1007/s00299-006-0169-7.

Tirnaz, S., Bayer, P.E., Inturrisi, F., Zhang, F., Yang, H., Dolatabadian, A., Neik, T.X., Severn-Ellis, A., Patel, D.A., Ibrahim, M.I., Pradhan, A., Edwards, D., and Batley, J. 2020. Resistance gene analogs in the Brassicaceae : Identification, Characterization, Distribution, and Evolution. Pl. Physiol. **184**: 909–922. doi:10.1104/pp.20.00835.

Tognolli, M., Penel, C., Greppin, H., and Simon, P. 2002. Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. Gene **288**: 129–138. doi:10.1016/S0378-1119(02)00465-1.

Travadon, R., Bousset, L., Saint-jean, S., Brun, H., Sache, I. 2007. Splash dispersal of *Leptosphaeria maculans* pycnidiospores and the spread of blackleg on oilseed rape. Pl. Pathol. **56**: 595–603. doi:10.1111/j.1365-3059.2007.01572.x.

Travadon, R., Marquer, B., Ribulé, A., Sache, I., Masson, J.P., Brun, H., Delourme, R., and Bousset, L. 2009. Systemic growth of *Leptosphaeria maculans* from cotyledons to hypocotyls in oilseed rape: influence of number of infection sites, competitive growth and host polygenic resistance. Pl. Pathol. **58**: 461–469. doi:10.1111/j.1365-3059.2008.02014.x.

Ülker, B., Mukhtar, M.S., and Somssich, I.E. 2007. The WRKY70 transcription factor of *Arabidopsis* influences both the plant senescence and defense signaling pathways. Planta **226**: 125–137. doi:10.1007/s00425-006-0474-y.

Vaish, S., Gupta, D., Mehrotra, R., Mehrotra, S., and Basantani, M.K. 2020. Glutathione S-transferase: a versatile protein family. 3 Biotech **10**: 1–19. Springer International Publishing. doi:10.1007/s13205-020-02312-3.

Wang, X., Wang, H., Wang, J., Sun, R., Wu, J., Liu, S., Bai, Y., Mun, J.H., Bancroft, I., Cheng,

F., Huang, S., Li, X., Hua, W., Wang, J., Wang, X., Freeling, M., Pires, J.C., Paterson, A.H., Chalhoub, B., Wang, B., Hayward, A., Sharpe, A.G., Park, B.S., Weisshaar, B., Liu, B., Li, B., Liu, B., Tong, C., Song, C., Duran, C., Peng, C., Geng, C., Koh, C., Lin, C., Edwards, D., Mu, D., Shen, D., Soumpourou, E., Li, F., Fraser, F., Conant, G., Lassalle, G., King, G.J., Bonnema, G., Tang, H., Wang, H., Belcram, H., Zhou, H., Hirakawa, H., Abe, H., Guo, H., Wang, H., Jin, H., Parkin, I.A.P., Batley, J., Kim, J.S., Just, J., Li, J., Xu, J., Deng, J., Kim, J.A., Li, J., Yu, J., Meng, J., Wang, J., Min, J., Poulain, J., Hatakeyama, K., Wu, K., Wang, L., Fang, L., Trick, M., Links, M.G., Zhao, M., Jin, M., Ramchiary, N., Drou, N., Berkman, P.J., Cai, Q., Huang, Q., Li, R., Tabata, S., Cheng, S., Zhang, S., Zhang, S., Huang, S., Sato, S., Sun, S., Kwon, S.J., Choi, S.R., Lee, T.H., Fan, W., Zhao, X., Tan, X., Xu, X., Wang, Y., Qiu, Y., Yin, Y., Li, Y., Du, Y., Liao, Y., Lim, Y., Narusaka, Y., Wang, Y., Wang, Z., Li, Z., Wang, Z., Xiong, Z., and Zhang, Z. 2011. The genome of the mesopolyploid crop species *Brassica rapa.* Nat. Genet. **43**: 1035–1040. doi:10.1038/ng.919.

Warwick, S.I., Francis, A., and Al-Shehbaz, I.A. 2006. Brassicaceae: Species checklist and database on CD-Rom. Plant Syst. Evol. **259**: 249–258. doi:10.1007/s00606-006-0422-0.

West, J.S., Kharbanda, P.D., Barbetti, M.J., and Fitt, B.D.L. 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. Plant Pathol. **50**: 10–27. doi:10.1046/j.1365-3059.2001.00546.x.

Wiesner, M., Hanschen, F.S., Schreiner, M., Glatt, H., and Zrenner, R. 2013. Induced production of 1-methoxy-indol-3-ylmethyl glucosinolate by jasmonic acid and methyl jasmonate in sprouts and leaves of pak choi (*Brassica rapa* ssp. chinensis). Int. J. Mol. Sci. **14**: 14996–15016. doi:10.3390/ijms140714996.

Williams, P.H., and Delwiche, P.A. 1979. Screening for resistance to blackleg of crucifers in the seedling stage.Eucarpia Cruciferae, Conference Proceedings. Wageningen, The Netherlands 1-3 Oct. 1979, pp 164-170

Van De Wouw, A.P., Marcroft, S.J., Barbetti, M.J., Hua, L., Salisbury, P.A., Gout, L., Rouxel, T., Howlett, B.J., and Balesdent, M.H. 2009. Dual control of avirulence in *Leptosphaeria maculans* towards a *Brassica napus* cultivar with “sylvestris-derived” resistance suggests

involvement of two resistance genes. Plant Pathol. **58**: 305–313. doi:10.1111/j.1365-3059.2008.01982.x.

Van de Wouw, P., Marcroft, S.J., Howlett, B.J. 2016. Blackleg disease of canola in Australia. Crop & Pasture Science **67**:273–283. doi:10.1071/CP15221.

Van de Wouw, A.P., Marcroft, S.J., Ware, A., Lindbeck, K., Khangura, R., and Howlett, B.J. 2014. Breakdown of resistance to the fungal disease, blackleg, is averted in commercial canola *(Brassica napus)* crops in Australia. F. Crop. Res. **166**: 144–151. doi:10.1016/j.fcr.2014.06.023.

Wang, Z., and Ma'ayan, A. 2016. An open RNA-Seq data analysis pipeline tutorial with an example of reprocessing data from a recent Zika virus study [version 1; referees: 3 approved] F1000Research **5**: 1574. doi:5 10.12688/f1000research.9110.1.

Yu, F., Gugel, R.K., Kutcher, H.R., Peng, G., and Rimmer, S.R. 2013. Identification and mapping of a novel blackleg resistance locus *LepR4* in the progenies from *Brassica napus* × *B. rapa* subsp. sylvestris. Theor. Appl. Genet. **126**: 307–315. doi:10.1007/s00122-012-1919-2

Yu, F., Lydiate, D.J., and Rimmer, S.R. 2005. Identification of two novel genes for blackleg resistance in *Brassica napus*. Theor. Appl. Genet. **110**: 969–979. doi:10.1007/s00122-004-1919-y.

Yu, F., Lydiate, D.J., and Rimmer, S.R. 2008. Identification and mapping of a third blackleg resistance locus in *Brassica napus* derived from *B. rapa* subsp. sylvestris. Genome **51**: 64–72. doi:10.1139/g07-103.

Zadoks, J.C. 2008. The potato murrain on the European continent and the revolutions of 1848. Potato Res. **51**: 5-45. doi:10.1007/s11540-008-9091-4.

Zhang X., and Fernando, W.G.D. 2017. Insights into fighting against blackleg disease of *Brassica napus* in Canada. Cr. & Pas. Sci. **69**: 40-47. doi.org/10.1071/CP16401

Zhang, X., Peng, G., Kutcher, H.R., Balesdent, M.H., Delourme, R., and Fernando, W.G.D. 2016. Breakdown of *Rlm3* resistance in the *Brassica napus–Leptosphaeria maculans* pathosystem in western Canada. Eur. J. Pl. Pathol. **145**: 659–674. doi:10.1007/s10658-015-

0819-0.

Zhou, T., Xu, W., Hirani, A.H., Liu, Z., Tuan, P.A., and Elliott, C. 2019. Transcriptional insight into *Brassica napus* resistance genes *LepR3* and *Rlm2*-mediated defense response against the *Leptosphaeria maculans* infection. Front. Pl. Sci. **10**: 1–18. doi:10.3389/fpls.2019.00823.

Zhu, J.S., Struss, D., and Robbelen, G. 1993. Studies on Resistance to Phoma lingam in *Brassica napus* - *Brassica nigra*. Plant Breeding **111**: 192—197.

7. Appendices

Appendix I. Supplemental tables and figures

Table S3. 1 Segregation pattern of BLMR2 locus in BC_5F_3 population. The expected ratio was 1:1 (BLMR2/blmr2 allele) in the population.

Replicate	Total # of plants	BLMR2	blmr2	df	Expected ratio	χ^2	P > χ^2	Yates corrected χ^2	P > Yates corrected χ^2
1	95	48	47	1	1:01	0.0105	0.9183	0	1
2	96	46	50	1	1:01	0.1667	0.6831	0.09375	0.75946
3	96	50	46	1	1:01	0.1667	0.6831	0.09375	0.75946
4	93	43	50	1	1:01	0.5269	0.4679	0.5269	0.4679
5	94	44	50	1	1:01	0.383	0.536	0.26596	0.6061
Total (**GH-1**)				5		1.2538		0.98036	
Pooled	**472**	**231**	**243**	**1**	**1:01**	**0.3038**	**0.5815**	**0.25527**	**0.61339**
Heterogeneity				4	1:01	0.95	0.86589	0.72509	0.9482
1	84	46	38	1	1:01	0.7619	0.3827	0.58333	0.44501
2	83	33	50	1	1:01	3.4819	0.062	3.08434	0.079049
3	86	49	37	1	1:01	1.6744	0.1957	1.40698	0.23556
4	87	41	46	1	1:01	0.2874	0.5919	0.18391	0.66804
5	84	48	36	1	1:01	1.7143	0.1904	1.44048	0.23006
Total (**GH-2**)				5		7.9199		6.69904	
Pooled	**424**	**217**	**207**	**1**	**1:01**	**0.2358**	**0.6272**	**0.19104**	**0.66205**
Heterogeneity				4	1:01	7.6841	0.10386	6.508	0.16429

Replicate indicates BC_5F_3 sub-populations that were cotyledon inoculated with each of the 5 isolates (PG4-1-M, Pl03-02-01, 09Stonewall, 3-42-6, 87-41, respectively, Table S3.2). The sub-populations were classified into the resistant (*BLMR2*) and the susceptible (*blmr2*) alleles based on molecular marker (Long et al 2011). Yates correction for continuity was used because we only have 1 df; and Yates corrected χ^2 test indicated that all the replicates followed a 1:1 segregation ratio. The test of heterogeneity indicated that all the ten replicates were homogeneous. Thus, the replicates could be pooled and the segregation of the population followed 1:1 pattern.

Table S3. 2 Avr/avr profiles of L. maculans isolates (Zhou et al 2019) pathogenicity grouping and interaction phenotype with specific resistance locus or genotypes.

L. maculans isolates	Pathogenicity Groupings	Interaction Phenotype (IP)*			
		BLMR1	BLMR2	Surpass 400	Westar
PG4-1-M *(avrLm1,avrLm2,avrLm4-7,AvrLm6,AvrLm11,AvrLmJ1)*	PG4	S	MS	R	S
Pl03-02-01*(n/d) – but virulent on BLMR1 and Rlm2 (non-AvrLm1, non-AvrLm2)*	PG3	S	MR	R	S
09 Stonewall *(avrLm1,AvrLm2, AvrLm6, AvrLm11, and AvrLmJ1)*	PGT	S	MR	R	S
3-42-6 *(AvrLm1,avrLm2,AvrLm4-7,AvrLm6,AvrLm11,AvrLmJ1)*	PG3	MR	MR	R	S
87-41 *(AvrLm1,AvrLm2,AvrLm4-7,AvrLm6,AvrLm11,AvrLmJ1)*	PG2	R	MR	R	MS
03-15-03 *(avrLm1,AvrLm2,avrLm4-7,AvrLm6,AvrLm11,AvrLmJ1)*	PG2	S	MR	R	S

* IP at 14dai (when Westar is totally susceptible) classified into phenotypic classes: R=resistant, MR=Intermediate resistance, MS=Intermediate susceptible, S=Susceptible. Cotyledon disease reaction phenotype is also shown in Figure 3.1.

Table S3. 3 Combined analysis of randomized complete block experiments using BC_5F_3 population conducted in spring 2015 (GH-1) and fall 2015 (GH-2).

Effect	Num df	Den df	*Seedling* F value	*Adult* F value
environment	1	8	0.26	0.77
genotype	1	8	48.82*	141.3*
genotype*environment	1	8	0.97	0.89

Environment represents the two experiments that were combined to draw wider inferences. Isolate is nested in environment (Result S1.1 - seedling, Result S1.2 - adult). The five isolates include: 1=PG4-1-M, 2=Pl03-02-01, 3=09Stonewall, 4=3-42-6, 5=87-41 (Table S3.2). At $\alpha<0.05$, significant variation (*) was observed in genotypes but ns differences in genotype*environment interaction, which indicate the relative response of genotypes within each experiment (environment) would be the same.

Table S3. 4 Variance analysis of a split-plot experiment assuming repeated experiments as blocks.

Effect	Num df	Den df	Seedling F value	Adult F value
Isolate	4	4	3.91	1.62
genotype	1	5	70*	422.31*
genotype*isolate	4	5	1.97	5.39

At $\alpha<0.05$, genotype*isolate is significant at adult stage, which indicated that the relative response of genotype(s) is not the same when inoculated with different isolates. Thus, the genotype*isolate interaction means need to be explored (Result S2.1-seedling and Result S2.2-adult, Dandena et al 2019). The five isolates include: 1=PG4-1-M, 2=Pl03-02-01, 3=09Stonewall, 4=3-42-6, 5=87-41 (Table S3.2). In general, isolate 1 (PG4-1-M) is relatively less aggressive at adult stage. However, the resistant BLMR2/PG4-1-M and the susceptible blmr2/PG4-1-M still showed significant difference (P=0.0004). Isolate 4 (3-42-6), on the other hand, induced relatively more aggressive at adult stage, but BLMR2/3-42-6 and blmr2/3-42-6 showed significant difference (P=0.0006). Thus, we can conclude that the relatively low DSI towards the susceptible genotype in blmr2/PG4-1-M may have contributed to the underestimation of the DSI estimate of blmr2. the relatively high DSI towards the resistant genotype in BLMR2/3-42-6 interaction may have contributed to the overestimation of the DSI estimate of BLMR2.

Table S3. 5 Analysis of split-plot-in-time experiment, two stages (seedling and adult) were compared; the two experiments were considered as blocks.

Effect	Num df	Den df	F value
stages	1	18	0.91
genotype	1	9	209.42*
genotype*stages	1	18	1.87

At $\alpha<0.05$, only genotype showed significant difference and genotype*stages is non-significant, thus the relative response of genotypes (*BLMR2* and *blmr2* alleles) at each stage is the same.

Table S3. 6 Combined analysis of randomized complete block experiments conducted in winter 2016 (GH-3) and spring 2016 (GH-4) using homozygous NILs and Westar.

Effect	Num df	Den df	Seedling F value	Adult F value
environment	1	6	0.07	1.54
genotype	4	24	32.32*	42.53*
genotype*environment	4	24	1.11	0.46

Environment represents the two experiments that were combined to draw wider inferences. Isolate is nested in environment. The four isolates include: 1=PG4-1-M, 3=09Stonewall, 4=3-42-6, 5=87-41 (Table S3.2). At $\alpha<0.05$, significant variation was observed in genotypes but ns differences in genotype*environment interaction, which indicate the relative response of genotypes within each experiment (environment) would be the same.

Table S3. 7 Combined analysis of randomized complete block experiments conducted in three years (2014, 2015 and 2016).

Effect	Num df	Den df	F value
year	2	3	18.31*
genotype	6	18	41.24*
genotype*year	12	18	4.56*
2014 vs 2015	1	3	2.57
2014 vs 2016	1	3	29.40*
2015 vs 2016	1	3	14.57*

At $\alpha<0.05$, the effects year and genotype*year is significant, which indicated that the relative response of genotypes is not the same in the three years. Pair-wise comparisons of the effect year indicated that 2016 is different from the previous two years (2014 and 2015).

Table S4. 1 Primers used in all applications

Primer names	Sequence (5'-3')	Applications
N10-56	TAATACTGGTTAATTATGCT	Mapping
RN10-56	ACAGTACATTCACGTTCTAG	Mapping
N10-47	ACTGGCCTATGGATGACGTT	Mapping
RN10-47	AATCCAGCAGTAGACCCCAT	Mapping
N10BA	CGAAAGTAAGAAGAGCAAGA	Mapping
N10BB	GATACTCTAGTTGTTGACAA	Mapping
N10-45	CAGAAGAAGAAGGATATGGT	Mapping
RN10-45	TCCAGTTAACCAATGCTGGT	Mapping
N10-43	CTAAGAAATTTCCTATGACAC	Mapping
RN10-43	TTGTCAATGTCTCATGCTAA	Mapping
N10-39	GGCTGCGTTGTTTCATACCT	Mapping
N10-39	ATGTGGGAGCTGAGGTTGTC	Mapping
RN10-39	GTCCTTAGTTGGTCCACTGT	Mapping
N10-34	CGAGCAGCAAATCCATATCC	Mapping
RN10-34	CAATTTTGTATTTTCTTATGGAAACTG	Mapping
N10-38	TTCAACATTTCTCCGCGATA	Mapping
RN10-38	TTTCCATCTGCTTCCACCTAA	Mapping
N10-37	CAGTCCTGACTTTGCCATCA	Mapping
N10-37	ACAGGCGAGAGGTTTGAAGA	Mapping
N10-40	CACAATTTCTGGTATACAGATTG	Mapping
RN10-40	CTTTGGAGCGAATTGTTGAAG	Mapping
10BM1	TGCAGGCAATTATTTCAGTGG	MAS
10BM2	AGCTTATGTTAGGTGGAAG	MAS
HN62F	ATGGATTACATTTTGC TC T TATTG	TA cloning
HN65R	TTAAGCCAAAAGATTAGTCATA	TA cloning
ACTIN-1F	CGATGGTGAGGACATTCAGC	RT-PCR
ACTIN-1R	AGAGAGAAAGAACAGCCTGGAT	RT-PCR
MM1F	ACAAGTAGACCAACCCAA	RT-PCR
MM1R	CCACAAACTCGCCATCGC	RT-PCR

```
Wes       MDYILLLLPLVLFLLAYKFLFSSKSFNLPPGPTPFPIVGNLHLVKPPVHRLFRRFADKYG        60
NIL       MDYILLLLPLVLFLLAYKFLFSSKSFNLPPGPTPFPIVGNLHLVKPPVHRLFRRFAEKYG        60
          ************************************************************:***

Wes       DIFSLRYGSRQVVVISSLPLVRECFTGQNDVILTNRPHFLTAKYVAYDYTTVGTAAYGDH        120
NIL       DIFSLRYGSRQVVVISSLPLVRECFTGQNDVILTNRPHFLTAKYVAYDYTTVGTAAYGDH        120
          ************************************************************

Wes       WRNLRRICSLEILSSNRLTGFLSVRKDEIRRLLTKLSRDYNGQVVELEPLLADLTFNNIV        180
NIL       WRNLRRICSLEILSSNRLTGFLSVRKDEIRRLLTKLSRDYNGQVVELEPLLADLTFNNIV        180
          ************************************************************

Wes       RMVTGRRYYGDQVHNKEEANLFKKLVTQINDNSGASHPGDYLPILKVFGHGYEKKVKALG        240
NIL       RMVTGRRYYGDQVHNKEEANLFKKLVTQINDNSGASHPGDYLPILKVFGHGYEKKVKALG        240
          ************************************************************

Wes       EAMDTFLQRLLDDCRRDGESNTMLSHLLSLQVDQPKYYSDVIIKGLMLSMMLAGTDTAAV        300
NIL       EAMDTFLQRLLDDCRRDGESNTMLSHLLSLQVDQPKYYSDVIIKGLMLSMMLAGTDTAAV        300
          ************************************************************

Wes       TLEWAMASLLKSPEVLKKAKAEIDDKIGHERLVDEPDILNLPYLQNIVSETFRLCPAAPL        360
NIL       TLEWAMASLLKSPEVLKKAKAEIDDKIGHERLVDEPDILNLPYLQNIVSETFRLCPAAPL        360
          ************************************************************

Wes       LVPRSPSEDLKIGGYDIPRGTIVLVNSWAIHRDPRLWDEPERFMPERFEDKEAANNNKLM        420
NIL       LVPRSPSEDLKIGGYDIPRGTIVLVNSWAIHRDPRLWDEPERFMPERFEDKEAANNNKLM        420
          ************************************************************

Wes       MFGNGRRTCPGAALGQRMVSLALGSLIQCFDWEKVNGEEIDMTENPGMAMRKLVPLRAVC        480
NIL       MFGNGRRTCPGAALGQRMVSLALGSLIQCFDWEKVNGEEIDMTENPGMAMRKLVPLRAVC        480
          ************************************************************

Wes       HQRPIMTNLLA      491
NIL       HQRPIMTNLLA      491
          ***********
```

Figure S4. 1 Alignment of translated protein sequences. The translated protein indicated variation in a single amino acid between the NIL and Westar.

Table S4. 2 Alignment of cDNA sequences from the NIL and Westar with B. napus Darmor-bzh (GENOSCOPE).

Query	Chromosome	score	Identity	Strand	Length
Wes	A10	1468	99.8%	+	3900
	C09	1410	97.9%	-	2212
	A02	1179	90.2%	-	1783
	C02	1179	90.2%	-	1766
	C03	1169	90.5%	-	2286
	A03	1168	90.5%	-	2294
NIL	A10	1456	99.4 %	+	3900
	C09	1414	98.0%	-	2212
	C02	1185	90.4%	-	1766
	A02	1185	90.4%	-	1783
	C03	1171	90.6%	-	2286
	A03	1170	90.6%	-	2294

atcacatggattacattttgctcttattgccactcgtattgtttctactagcttacaaattcttattctcatctaagagtttcaatcttccaccaggaccaactcccttt
cccatcgtcggcaacctccacctcgtgaaaccaccggtgcaccgtctcttccgtcgtttcgcggacaagtacggtgacatcttctccctccgttacggctct
cgccaagtcgtcgtgatctcttccttgcccctcgtcagagaatgctttactggtcagaacgacgttattttaacgaaccgaccgcattttctgaccgcaaagta
cgttgcttacgactacaccacggttggaaccgccgcatatggcgaccactggcgtaatctccgccgtatttgctctcttgagatcctttcctctaaccgtctca
ctggattcctctccgttcgtaaagacgagatccgacggttgctcacgaaactctcacgtgactataatggccaagtcgttgagcttgagcctcttcttgcaga
tttgacgttcaataatattgtccgtatggtcactgggagacgttactacggagaccaggttcgtttcatttaaataattacataataatgattgatacgttgaaatc
actaatcttttactattctaatgttcttgtacaagatattaaatattaatacctaacatggacatgttctgtattgtgtttactacttctcatgcatgaaaagtcattcga
tgtggaatattttggctaattaaaatgaattatttcttaaaggttcacaacaaggaagaagcgaacctattcaagaagctagtgacgcagatcaacgacaata
gtggtgcgagccatccaggagattatttaccaattctcaaagttttcggacacggctacgagaagaaagtgaaagcactcggcgaagccatggacacttt
cttgcagcgactgctcgacgattgccgtagagatggagagagcaacacaatgcttagtcatctgttgtctttacaagtagaccaacccaagtattacagtga
cgtcatcatcaaaggcctcatgctcgtaagttctctcatccgtatgtataaacctacaaaatcatataaattatcactctttgtgtttcttagtttgcagaatgcgtt
tacaagttcgaataaatatttttcgcatctaacattaatatatatttcatcccgaagataaattttctcgaaaaaattgtttggaaaataatatttttgccttttctgatg
aatcttttattagctaataataataaactataaacttctaagttaattgtactatttgaagttttactagtttagaattattggaaacaaagaagtatataaaataatat
atttataatcaaaatttaattatttgttattaatatgtgtgaaaactctcagaatatttttttttgaaacatagaatgagtatgtttcaattacaatttgattattaatgcta
cataaaaaattgaaaaaacatagatggtttatgttttactatctgatttatgtttacataatatgtatagactaacattttaaatgttatgtagagtatgatgcttgcg
ggacggatactgcagccgtgacactagaatgggcgatggcgagtttgttgaaaagtcctgaagtgttgaagaaggcgaaagccgagatagatgataa
gattggacatgaacgtttggtcgacgaaccggacattttgaatctcccttatctccaaaacatagtttcacagattcttattctcatctaagagtttcaatcttcca
ccaggaccaactcccttccatcgtcggcaacctccacctcgtgaaaccaccggtgcaccgtctcttccgtcgtttcgcggacaagtacggtgacctctt
ctcccttcgttacggctctcgccaagtcgtcgtgatctcttccttgcccctcgtcagagaatgctttactggtcagaacgacgttattttaacgaatcgaccgc
attttctgaccgcaaagtacgttgcttacgactacaccacggtcggaaccgccgcttatggcgaccactggcgtaacctccgccgtatttgctctcttgagat
cctttcctctaaccgtctcactggattcctctccgttcgtaaagacgagatccgacggttgctcacgaaactctcacgtgactataatggccaagtcgttgag
cttgagcctcttcttgcagatttgacgttcaataatattgtccgtatggtcactgggagacgttactacggagaccaggttcgtttcatttaaataattatataata
atgattgatacgtttgaattcactaatcttttactattctaatgttcttgtacaagatattaaatattaatacctaacatggacatgttctgtattgtgtttactacttctc
atgcatgaaaagtcattcgatgtggaatattttggctaattaaaatgaattatttcttaaaggttcacaacaaggaagaagcgaacctattcaagaagctagtg
acgcagatcaacgacaatagtggtgcgagccatccaggagattatttaccaattctcaaagttttcggacacggctacgagaagaaagtgaaagcactcg
gcgaagccatggacactttcttgcagcgactgctcgacgattgccgtagagatggagagagcaacacaatgcttagtcatctgttgtctttacaagtagac
caacccaagtattacagtgacgtcatcatcaaaggcctcatgctcgtaagttctctcatccgtatgtataaacctacaaaatcatataaattatcactctttgtgt
ttcttagtttgcagaatgcgtttacaagttcgaataaatatttttcgcatctaacattaatatatatttcatcccgaagataaattttctcgaaaaaattgtttggaaa
ataatatttttgccttttctgatgaatctttattagctaataataataaactataaacttctaagttaattgtactatttgaagttttactagtttagaattattggaaaca
aagaagtatataaaataatatatttataatcaaaatttaattatttgttattaatatgtgtgaaaactctcagaatatttttttttgaaacatagaatgagtatgtttcaa
ttacaatttgattattaatgctacataaaaaattgaaaaaacatagatggtttatgttttactatctgatttatgtttacataatatgtatagactaacattttaaatgtt
atgtagagtatgatgcttgcggggacggatactgcagccgtgacactagaatgggcgatggcgagtttgttgaaaagtcctgaagtgttgaagaaggcg
aaagccgagatagatgataagattggacatgaacgtttggtcgacgaaccggacattttgaatctcccttatctccaaaacatagtttctgagaccttccgac
tgtgtccagccgcaccactccttgtaccacgttctccttctgaagacctcaagattggcggatacgacataccgcgtggcaccatcgtactagtgaattcttg
ggccatccatagagatccaaggctttgggatgagcctgagaggttcatgccagagcgttttgaggacaaaaaagctgccaataataataagcttatgatgt
ttgggaatggacgaaggacgtgtcccggtgcggctttgggtcaaaggatggtgtcgttggctttaggatcgttgattcaatgctttgactgggaaaaagtca
acggtgaggaaattgatatgaccgaaaatcctggaatggctatgcgcaagctcgtgccgttacgagccgtttgccatcagcgtcccattatgactaatctttt
ggcttaaaattacttggttcactctttcatgattactggttcgtgtttttcggatttcttttataatttgtttgatttttgggtgtgactattataaagataatgtaagatt
cgatgaagaatgttgcaatgttgtgaataagattcgatcacgaaagaaccaaagagattgctgatttgatgaagaatgttgcaatgtctaaaaaagcatgaa
agagaaacatgtaagaaagtctcctctataaaatctgtcggtagatgcatcaaaaaaaattctaatataaggtagtgtaatataaacatattttgaaataacaa
atcatatcagaaaaatgtattaatctattatcataaatgctaaaatatataatattataatttttgttccaagattaaatattgtaagtttcatttctaatttgaaaattta
tatgattttaaaattttattgctacttttaattaaactatctactaagactatatataatggtttacaaattcaacatccaattttcaaca

Figure S4. 2 B. napus Darmor-bzh genomic sequence covering 'BnaA10g11280D' (exons underlined in red) and 'BnaA10g11290D' (exons underlined in green). 'BnaA10g11280D'lack the downstream sequence (part of exon3) and 'BnaA10g11290D' lack the upstream sequence (part of exon 1).

```
BnaA10..80D      MDYILLLLPLVLFLLAYKFLFSSKSFNLPPGPTPFPIVGNLHLVKPPVHRLFRRFADKYG
BnaA10..90D      ------------------------------------------------------------
Wes              MDYILLLLPLVLFLLAYKFLFSSKSFNLPPGPTPFPIVGNLHLVKPPVHRLFRRFADKYG
NIL              MDYILLLLPLVLFLLAYKFLFSSKSFNLPPGPTPFPIVGNLHLVKPPVHRLFRRFAEKYG


BnaA10..80D      DIFSLRYGSRQVVVISSLPLVRECFTGQNDVILTNRPHFLTAKYVAYDYTTVGTAAYGDH
BnaA10..90D      ------------------------------------------------------------
Wes              DIFSLRYGSRQVVVISSLPLVRECFTGQNDVILTNRPHFLTAKYVAYDYTTVGTAAYGDH
NIL              DIFSLRYGSRQVVVISSLPLVRECFTGQNDVILTNRPHFLTAKYVAYDYTTVGTAAYGDH


BnaA10..80D      WRNLRRICSLEILSSNRLTGFLSVRKDEIRRLLTKLSRDYNGQVVELEPLLADLTFNNIV
BnaA10..90D      ------------------------------------------------------------
Wes              WRNLRRICSLEILSSNRLTGFLSVRKDEIRRLLTKLSRDYNGQVVELEPLLADLTFNNIV
NIL              WRNLRRICSLEILSSNRLTGFLSVRKDEIRRLLTKLSRDYNGQVVELEPLLADLTFNNIV


BnaA10..80D      RMVTGRRYYGDQVHNKEEANLFKKLVTQINDNSGASHPGDYLPILKVFGHGYEKKVKALG
BnaA10..90D      -MVTGRRYYGDQVHNKEEANLFKKLVTQINDNSGASHPGDYLPILKVFGHGYEKKVKALG
Wes              RMVTGRRYYGDQVHNKEEANLFKKLVTQINDNSGASHPGDYLPILKVFGHGYEKKVKALG
NIL              RMVTGRRYYGDQVHNKEEANLFKKLVTQINDNSGASHPGDYLPILKVFGHGYEKKVKALG
                  ************************************************************

BnaA10..80D      EAMDTFLQRLLDDCRRDGESNTMLSHLLSLQVDQPKYYSDVIIKGLMLSMMLAGTDTAAV
BnaA10..90D      EAMDTFLQRLLDDCRRDGESNTMLSHLLSLQVDQPKYYSDVIIKGLMLSMMLAGTDTAAV
Wes              EAMDTFLQRLLDDCRRDGESNTMLSHLLSLQVDQPKYYSDVIIKGLMLSMMLAGTDTAAV
NIL              EAMDTFLQRLLDDCRRDGESNTMLSHLLSLQVDQPKYYSDVIIKGLMLSMMLAGTDTAAV
                 ************************************************************

BnaA10..80D      TLEWAMASLLKSPEVLKKAKAEIDDKIGHERLVDEPDILNLPYLQNIVSQILILI*----
BnaA10..90D      TLEWAMASLLKSPEVLKKAKAEIDDKIGHERLVDEPDILNLPYLQNIVSETFRLCPAAPL
        179
Wes              TLEWAMASLLKSPEVLKKAKAEIDDKIGHERLVDEPDILNLPYLQNIVSETFRLCPAAPL
NIL              TLEWAMASLLKSPEVLKKAKAEIDDKIGHERLVDEPDILNLPYLQNIVSETFRLCPAAPL
                 ***********************************************: : *

BnaA10..80D      ------------------------------------------------------------
BnaA10..90D      LVPRSPSEDLKIGGYDIPRGTIVLVNSWAIHRDPRLWDEPERFMPERFEDKKAANNNKLM
Wes              LVPRSPSEDLKIGGYDIPRGTIVLVNSWAIHRDPRLWDEPERFMPERFEDKEAANNNKLM
NIL              LVPRSPSEDLKIGGYDIPRGTIVLVNSWAIHRDPRLWDEPERFMPERFEDKEAANNNKLM


BnaA10..80D      ------------------------------------------------------------
BnaA10..90D      MFGNGRRTCPGAALGQRMVSLALGSLIQCFDWEKVNGEEIDMTENPGMAMRKLVPLRAVC
Wes              MFGNGRRTCPGAALGQRMVSLALGSLIQCFDWEKVNGEEIDMTENPGMAMRKLVPLRAVC
NIL              MFGNGRRTCPGAALGQRMVSLALGSLIQCFDWEKVNGEEIDMTENPGMAMRKLVPLRAVC


BnaA10..80D      ------------
BnaA10..90D      HQRPIMTNLLA*
Wes              HQRPIMTNLLA-
NIL              HQRPIMTNLLA-
```

Figure S4. 3 Alignment of translated protein sequences from the NIL, Westar and the two B. napus genes.

Table S5. 1 List of samples used in RNA-seq

Sample name	Treatment	Phenotype	Time(dai)	Mapped reads	Sample groups
H1	I	MR	4	17456577	NIL_4
H1-d	M	MR	4	19780944	NIL_4
H2	I	MR	4	17294309	NIL_4
H2-d	M	MR	4	18298922	NIL_4
H3	I	MR	4	17433352	NIL_4
H3-d	M	MR	4	18571180	NIL_4
W1	I	S	4	21719200	Wes_4
W1-d	M	S	4	22199090	Wes_4
W2	I	S	4	18044907	Wes_4
W2-d	M	S	4	16525727	Wes_4
W-3	I	S	4	17978187	Wes_4
W3-d	M	S	4	22199090	Wes_4
WW-d	M	S	8	80116914	Wes_8/10
HH-d	M	MR	8	83360966	NIL_8/10
HH1	I	MR	8	73949156	NIL_8/10
HHH1	I	MR	10	82524312	NIL_8/10
WW	I	S	8	82901966	Wes_8/10
HHH2	I	MR	10	71055347	NIL_8/10
WWW1	I	S	10	79680223	Wes_8/10
WWW2	I	S	10	52132258	Wes_8/10
Total				833,222,627	

I=Pathogen isolate (*L. maculans* 87-41) inoculated; M=Mock (water-inoculated); MR=Intermediate resistant; S=Susceptible; 4 and 8/10 days after inoculation (dai)

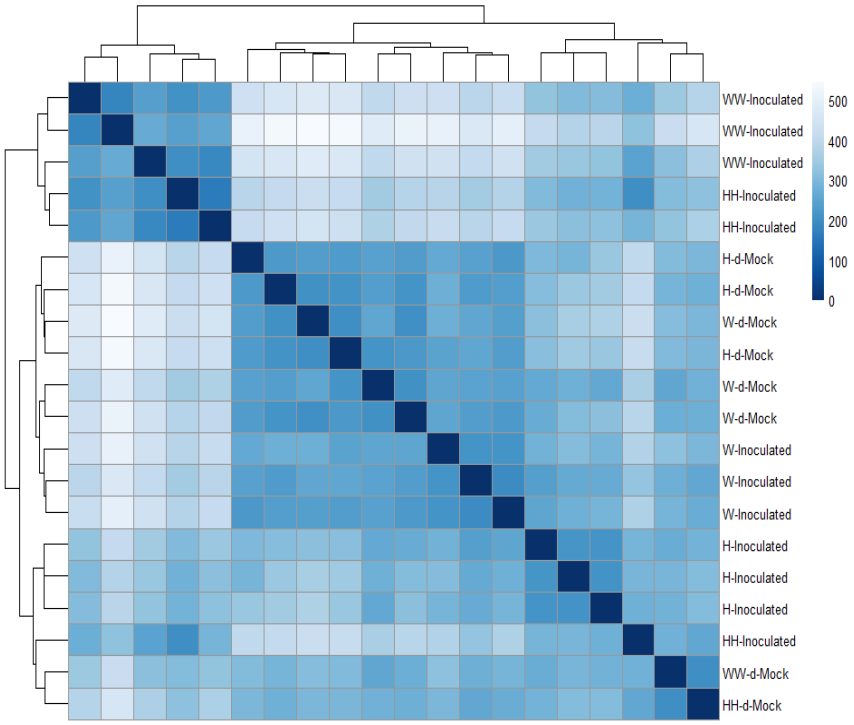


Figure S5. 1 Matrix showing similarity between samples. Samples include H and HH – W+BLMR2 at 4 and 8/12dai, W and WW – Westar at 4 and 8/12dai, respectively. The color denotes blue (high) and white (low)

Appendix II. Abbreviations

AFLP	Amplified fragment length polymorphisms
ANOVA	Analysis of variance
APR	Adult-plant resistance
Avr	Avirulent
BAC	Bacterial artificial chromosome
BC	Back cross
CC	Coiled coil
CDPK	Calcium dependent PK
cM	Centimorgan
CPM	Counts per million
CRKs	Cysteine-rich
CTAB	Cetyl methylammonium bromide
CYP450	Cytochrome P450
DAI	Days after inoculation
DE	Differential expression
DEGs	Differentially expressed genes
DH	Doubled haploid
DSI	Disease severity index
ET	Ethylene
ETD	Effector-triggered defense
ETI	Effector-triggered immunity
GBS	Genotyping-by-sequencing

G*E	Genotype-by-environment
GS	Genomic selection
GSTs	Glutathione S transferases
GWAS	Genome-wide association studies
HEAR	High erucic acid rapeseed
HR	Horizontal resistance
IGS	Indole glucosinolates
JA	Jasmonic acid
kB	kilobase
LD	Linkage disequilibrium
LG	Linkage groups
MAPKs	Mitogen-activated protein kinases
MAS	Marker-Assisted Selection
MR	Moderately (intermediate) resistant
MS	Moderately (intermediate) susceptible
MYA	Million years ago
NBS-LRR	Nucleotide binding site-leucine-rich repeats
NGS	Next-generation sequencing
NILs	Near-isogenic lines
NLRs	Nucleotide binding site, leucine rich repeats
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death

PCR	Polymerase chain reaction
PG	Pathogenicity group
PRs	Pathogenesis related proteins
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
PS	Photosystems
QR	Quantitative resistance
QRL	Quantitative resistance loci
qRT-PCR	Quantitative real-time PCR
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
RAPD	Random amplified polymorphic DNA
R	Resistant
RGA	Resistance gene analog
RLPs	Receptor-like proteins
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
Rubisco	Ribulose biphosphate carboxylase/oxygenase
SCAR	Sequence characterized amplified region
SRAP	Sequence related amplified polymorphism
SSR	Simple sequence repeats
SNP	Single nucleotide polymorphism
S	Susceptible

SA	Salicylic acid
TIR	Toll/Interleukin-1 receptor
WAKs	Wall-associated kinases
WGD	Whole genome duplication